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EFFECT OF PARITY AND DAYS IN MILK ON MILK UREA CONCENTRATION AND MILK COMPONENTS IN HOLSTEIN DAIRY COWS

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ABSTRACT

The objective of this study was to assess the effects of parity and of days in milk (DIM) on milk urea (MU) and other milk components in Holstein dairy cows. Milk yield was lower ($P < 0.05$) in primiparous cows compared with multiparous cows and tended to increase until the third parity and then decrease. The MU concentration was lower ($P < 0.05$) at the first lactation than at the other lactations. Milk fat and protein contents were higher ($P < 0.05$) in the primiparous cows than that in multiparous cows and were reduced until the third parity and then increased. Lower ($P < 0.05$) milk lactose content was found in primiparous cows and tended to increase until the third parity and then decreased. Primiparous cows had lower ($P < 0.05$) daily protein and fat yields compared with multiparous cows. The lowest value of milk yield was recorded during the first lactation with a peak of lactation between 61—90 DIM and a persistence of lactation of 3 %. The highest milk yield was confirmed at the third and fourth lactations with a peak of lactation between 61—90 DIM and a persistence of lactation of 7 % and 6 %. The effect of DIM on MU confirmed a significant ($P < 0.01$) positive relationships in primiparous and multiparous cows. The lowest level of MU concentration was found between 0—30 DIM. The content of MU increased and the maximum was achieved between 271—305 DIM. It is suggested that although MU for nutritional management and measures of production or reproduction are used, non-nutritional factors should be considered.

Key words: milk yield; non-nutritional factors; urea in milk

INTRODUCTION

The content of lactose in milk and the quantity of milk are affected by the composition of the feed ration through the starch content in total mixed ration (TMR) and its rumen fermentation to produce propionic acid, which in gluconeogenesis in the liver affects blood glucose levels [25]. Precursors for milk protein synthesis in the mammary glands are metabolizable amino acids that are transported from the small intestine into the mammary glands and amino acids mobilized from the body reserves [17]. The synthesis of fat in the mammary glands are supported

by two sources. The first source is the synthesis of short and medium chain fatty acids *de novo* in the mammary glands of acetic acid and beta hydroxybutyric acid after the microbial fermentation of carbohydrates in the rumen. A second source are long chain fatty acids circulating in the blood reserves [4].

Urea as a part of the non-protein fraction of nitrogen in milk represents the final product of protein metabolism in the rumen of ruminants [9]. In dairy cows, urea can be found as a component in blood, milk, saliva and urine. The ammonia appears as a product of protein or non-protein nitrogen components digestion by the microorganisms in the rumen. The rumen microflora uses ammonia and energy for microbial proteins production. The excess of ammonia in the rumen appears when microorganisms are not supplied with a sufficient quantity of energy or when the protein component in the diet is in surplus. The excess of ammonia enters into the bloodstream and is transported into the liver, where it is transferred to urea by metabolic processes [5]. Urea is transported by the bloodstream to the kidneys and excreted from the organism or is returned to the rumen or is transported to the mammary glands, where it can easily pass through the cell membrane and increase the urea concentration in milk [16]. M a r e n j a k et al. [18] reported that the normal concentration of MU in cow's milk ranges between 10 to 30 $mg.dl^{-1}$ and P y t l e w s k i et al. [22] reported it to be 25 $mg.dl^{-1}$.

The concentration of MU depends on various genetic, productive, nutritional and non-nutritional factors. Only a few researchers have investigated the effects of non-nutritional factors on milk and MU concentration [1, 23]. Non-nutritional factors explained 13.3% of the variation in MU [2] and production and environmental factors explained 37% of the MU variation in cows [11].

The aim of our study was to determine the effects of non-nutritional factors, such as parity and DIM on milk yield, milk components and MU in the milk dairy cows.

MATERIALS AND METHODS

Data collection

This study included 5838 Holstein cows from a Slovakian dairy farm from January 2018 to December 2018. Cows were selected according to parity: primiparous cows (lactation number = 1) and multiparous cows (lactation number > 1, range 2—5+). According to DIM, cows were grouped into 30 days (d) increments (0—30, 31—60, 61—90, 91—120, 121—150, 151—180, 181—210, 211—240, 241—270, 271—305 d).

The cows were maintained in free-stall housing and fed with a TMR, based on corn and alfalfa silage, supplemented with different carbohydrate's feeds (cereal grains and cereal grain by-products) and protein supplements (rapeseed meal) provided *ad libitum*. The TMR was formed monthly depending on the nutrient requirements according to the milk yield and capacity of dry matter intake (DMI) for primiparous and multiparous cows. The samples of the TMR were taken from the feed manger on the control day and were analysed for dry matter (DM), which consistent of crude protein (CP), fat, acid and neutral detergent fibre (ADF, NDF) and starch analysed by conventional methods [8]. The DM was determined by weight upon drying the sample at 105 °C under the prescribed conditions. The CP content was determined by the Kjeldahl method using a 2300 Kjeltec Analyzer Unit (Foss Tecator AB, Hoganas, Sweden). The fat was determined by the device Det-Gras (JP SELECTA, Spain). The ADF and NDF were determined using Dosi-Fiber Analyzer (JP SELECTA, Spain) and the content of starch was determined by polarimetry. The net energy of lactation (NEL) and non-fibre carbohydrates (NFC) were calculated using regression equations according to the National Research Council (NRC) [20].

The cows were milked twice a day and individual milk samples were analysed once per month. This took place in the Central Analytical Laboratory of Milk with accreditation under the registration number 096/5878/2015/2 in collaboration with The Breeding Services of Slovakia, using the breeding information system. Milk samples were analysed for the milk protein, fat, lactose and MU concentration by a near-infrared spectrophotometric assay using MilkoScan FT+ (Foss Electric, Hillerød, Denmark) and BENTLEY FTS (Bentley Instruments Inc., Chaska, USA). Daily yields of protein and fat were calculated by multiplying the percentage of milk components with milk yield per day [24].

Ethical considerations

Data used in this study were obtained with the consent of the farm in collaboration with The Breeding Services of Slovakia, using the Breeding Information System.

Statistical analysis

The data were processed by the IBM SPSS Statistics version 24.0 [12]. The results were expressed as the means ± standard deviations (SD). One-Way analysis of variance (ANOVA) for multiple comparison of means were conducted to evaluate the differences in parameters of milk yield, milk components and MU, where the parity was set as the main factor at a significance level of $P < 0.05$. Subsequently, the analysis of correlation between MU concentration (dependent variable) and DIM (independent variable) was performed during lactation in primiparous and multiparous cows using the correlation procedure IBM SPSS Statistics version 24.0 [12]. Differences between both groups were declared significant at $P < 0.01$.

RESULTS

The composition of the dairy herd according to parity is shown in Fig. 1. The dominated group was young dairy cows in the first lactation (38.4%), following the dairy cows in second (28.5%) and third (20.6%) lactation. Dairy cows in fourth, fifth and over lactation represent 6.8% and 5.7% of the herd.

The composition and average concentration of nutrients in TMR are presented in Table 1. The nutritional composition of TMR for the year fluctuated in the range, which was recommended by the nutrient requirement [20] for primiparous and multiparous cows. Separate formed TMR for primiparous and multiparous cows showed no significant differences in the analysed nutrients content.

Daily intake of nutrients in primiparous and multiparous cows are presented in Table 2. The daily intake of nutrients, on average for the year and in early lactation

Table 1. The composition and analysed nutrients content of TMR in primiparous and multiparous cows

	Primiparous cows	Multiparous cow
Composition of TMR [kg]		
Corn silage	7.0—39.0	7.0—39.0
Clover silage	6.0—14.0	6.0—16.0
Grass silage	2.0—6.0	2.0—6.0
Alfalfa silage	13.0—22.0	13.0—22.0
Rye silage	7.0—17.0	7.0—17.0
Oat silage	2.0—17.0	2.0—17.0
Alfalfa hay	0.7—3.0	0.8—3.0
Wheat straw	0.5—1.0	0.5—1.0
Cereal grain mixture	0.8—3.7	0.5—4.5
Rapeseed meal	0.4—6.0	0.4—6.0
Wet distillers grains	4.0	4.0
Concentrate mixture	0.3—4.2	0.3—4.5
Nutrients content of TMR [g.kg^{-1} DM]		
CP	154.0 ± 12.2	154.3 ± 11.9
Starch	222.4 ± 53.5	220.5 ± 52.2
Fat	41.4 ± 6.3	41.0 ± 5.6
NDF	379.3 ± 42.2	378.5 ± 42.9
ADF	233.5 ± 30.0	234.4 ± 29.6
NFC	351.1 ± 38.8	351.7 ± 39.2
NEL	6.4 ± 0.3	6.4 ± 0.3

DM—dry matter; CP—crude protein; NDF—neutral detergent fibre; ADF—acid detergent fibre; NFC—non-fibre carbohydrates; NEL—net energy of lactation; TMR—total mixed ration

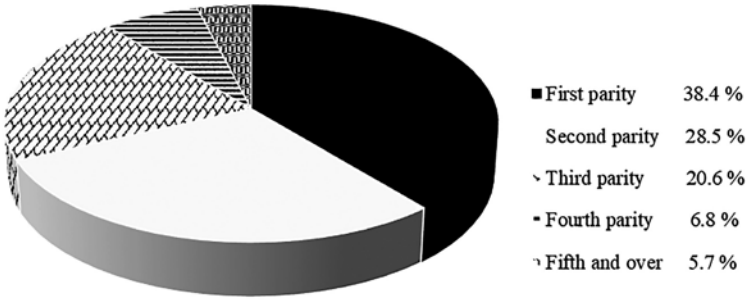

Fig. 1. Composition of dairy herd according to parity

Table 2. Daily intake of nutrients in primiparous and multiparous cows

	Daily intake of nutrients [kg.d^{-1}]			
	Primiparous cows		Multiparous cows	
	1st—3rd phase	1st phase	1st—3rd phase	1st phase
	± SD (Min—Max)		± SD (Min—Max)	
DMI	21.07 ± 1.7 17.0—24.0	22.77 ± 1.2 21.30—24.30	21.29 ± 2.1 18.00—25.30	23.68 ± 1.8 20.90—26.00
CP intake	3.20 ± 0.3 2.14—3.79	3.45 ± 0.3 2.76—3.79	3.24 ± 0.6 2.23—4.20	3.61 ± 0.4 3.00—4.08
Starch intake	4.69 ± 1.1 3.66—6.45	6.02 ± 0.4 5.30—6.72	4.71 ± 1.4 2.17—6.78	6.10 ± 0.7 4.43—6.75
NDF intake	7.72 ± 1.0 6.10—12.05	8.00 ± 0.3 7.79—8.51	7.78 ± 1.4 6.14—12.51	8.23 ± 0.6 7.79—9.11
NEL intake	132.22 ± 12.4 114.46—161.47	150.66 ± 7.1 141.24—161.47	133.67 ± 24.7 123.20—170.28	156.45 ± 10.6 138.15—170.28

DMI—dry matter intake; CP—crude protein; NDF—neutral detergent fibre; NEL—net energy of lactation

Table 3. Effect of parity on milk yield, milk components and MU

Lactation order	Milk yield [kg.d^{-1}]	MU [mg.dl^{-1}]	Fat [%]	Protein [%]	Lactose [%]	Protein yield [kg.d^{-1}]	Fat yield [mg.dl^{-1}]
1	32.5 ± 6.4^{a}	27.42 ± 6.8^{a}	3.75 ± 0.7^{a}	3.25 ± 0.3^{a}	4.75 ± 0.1^{a}	1.05 ± 0.2^{a}	1.20 ± 0.3^{a}
2	37.2 ± 9.3^{b}	27.90 ± 7.2^{b}	3.59 ± 0.7^{b}	3.23 ± 0.3^{a}	4.85 ± 0.2^{b}	1.18 ± 0.3^{b}	1.30 ±0.3^{b}
3	38.5 ± 10.3^{c}	28.30 ± 7.3^{b}	3.52 ± 0.8^{c}	3.18 ± 0.4^{b}	4.95 ± 0.2^{c}	1.20 ± 0.3^{b}	1.32 ±0.4^{b}
4	37.5 ± 10.0^{b}	28.00 ± 7.8^{b}	3.64 ± 0.7^{d}	3.20 ± 0.4^{b}	4.92 ± 0.3^{c}	1.19 ± 0.3^{b}	1.34 ±0.4^{b}
5+	34.9 ± 10.5^{d}	28.14 ± 6.8^{b}	3.73 ± 0.8^{e}	3.22 ± 0.3^{b}	4.82 ± 0.2^{d}	1.11 ± 0.3^{c}	1.28 ± 0.4^{c}

MU—milk urea; d—day; $^{a, b, c, d, e}$—Means within the same column with different superscripts are significantly different at $P < 0.05$

showed no significant differences between primiparous and multiparous cows. Differences were found between maximum and minimum nutrient intake values.

Effects of parity on milk yield, milk components and MU are presented in Table 3. Milk yield was lower ($P < 0.05$) in primiparous cows compared with multiparous cows and tended to increase until the third parity and then decrease.

The mean value of MU for the first lactation was lower ($P < 0.05$) than that of the second, third, fourth and greater parity means.

The milk fat and protein contents were higher ($P < 0.05$) in primiparous cows than that in the multiparous cows and tended to be reduced until the third parity and then increase. Lower ($P < 0.05$) milk lactose content was found in primiparous cows compared with multiparous cows and tended to increase until the third parity and then decrease.

Primiparous cows had lower ($P < 0.05$) daily protein and fat yields compared with the multiparous cows.

The dynamics of milk yield in different parities are shown in Fig. 2. Evaluation of milk yield according to parity confirmed the lowest milk yield during the first lactation (32.5 ± 6.4 kg.d^{-1}) with the lowest initial quantity of milk (29.0 ± 7.3 kg.d^{-1}) and the lowest peak of lactation (34.8 ± 6.3 kg.d^{-1}) between 61—90 DIM and persistence of lactation 3 %. The highest milk yield was confirmed in the third and fourth lactation (38.5 ± 10.3; 37.5 ± 10.0 kg.d^{-1}) with the highest initial quantity of milk

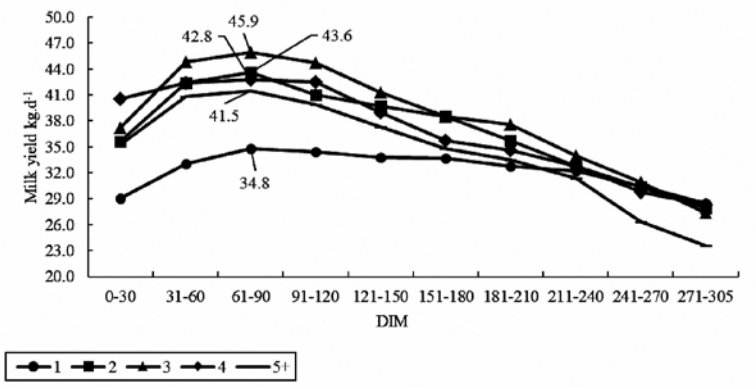

Fig. 2. Dynamics of milk yield in different parities

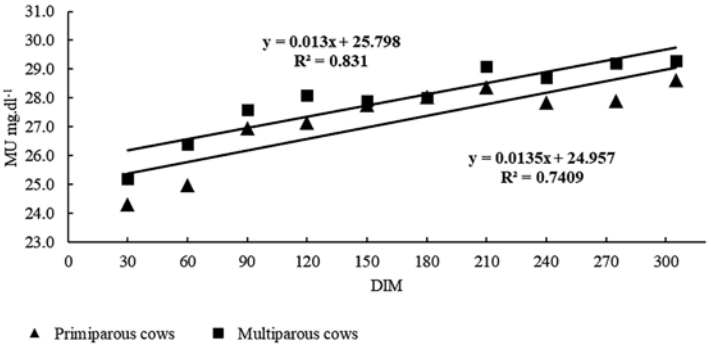

Fig. 3 Effect of DIM on MU concentration in different parities

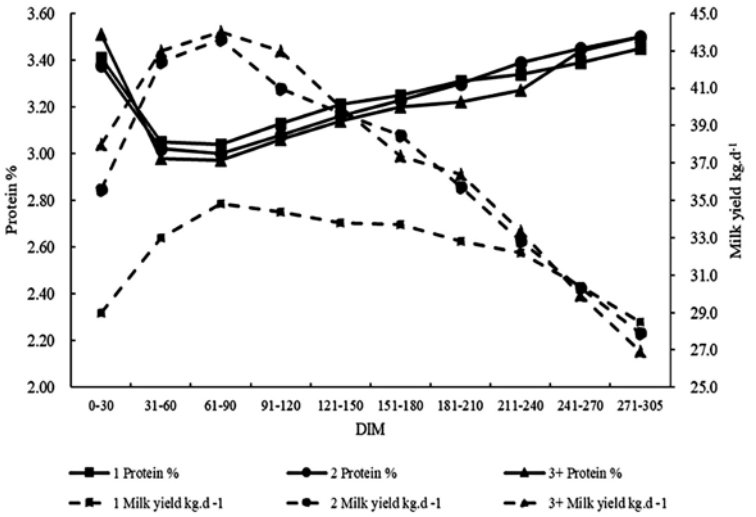

Fig. 4. Dynamics of milk yield and protein content in different parities

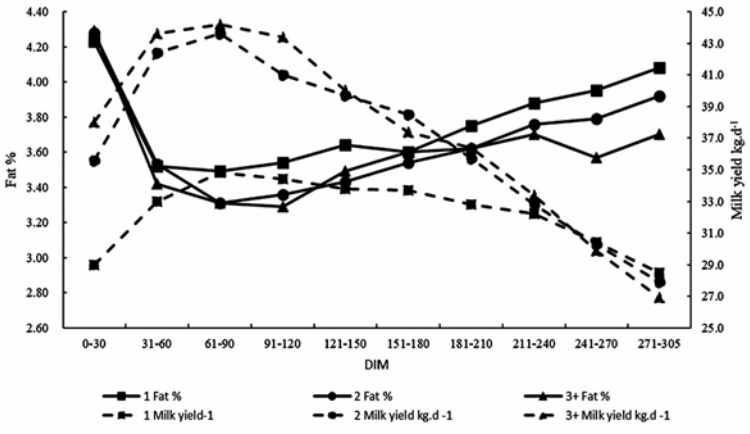

Fig. 5. Dynamics of milk yield and fat content in different parities

(37.2 ± 9.1; 40.5 ± 7.3 kg.d^{-1}) and the peak of lactation (45.9 ± 7.3; 42.8 ± 9.0 kg.d^{-1}) also between 61—90 DIM and persistence of lactation 7% and 6%. First lactation cows achieved 76% of the peak for mature cows and 79% of the peak for second lactation cows. Cows at the second lactation achieved 94% of the peak for mature cows.

The effect of the DIM on MU in different parities is shown in Fig. 3. The MU concentration was significantly ($P < 0.01$) influenced by the DIM with the coefficient of determination $R^2 = 0.74$ in primiparous cows and $R^2 = 0.83$ in multiparous cows.

The lowest levels of MU concentration (24.32 ± 7.6; 25.23 ± 9.1 mg.dl^{-1}) were found between 0—30 DIM in primiparous and multiparous cows. In the following DIM, the content of MU increased and the maximum concentration (28.63 ± 6.9; 29.13 ± 6.7 mg.dl^{-1}) was achieved between 271—305 DIM.

The dynamics of milk yield, protein and fat contents in different parities are shown in Fig. 4 and Fig. 5.

According to parities, it was confirmed that lower milk yields and higher protein and fat contents occurred in the primiparous cows, compared with the multiparous cows. According to DIM, it was confirmed that the milk yield increased proportionally with an increase of DIM into the peak of lactation and thereafter decreased. Protein and fat contents decrease into the peak of lactation and then after the peak, they tended to increase.

DISCUSSION

The lower milk yields in primiparous cows compared to multiparous cows are consistent with a previous study by Yoon et al. [27]. The lactation order is associated with the age of the cows. When the primiparous cows use nutrients from TMR for full development of tissues of the mammary gland and body frame, the milk synthesis is limited [21].

The lower MU content in primiparous cows compared to multiparous cows is conditioned by the growth of the body and higher efficiency of amino acid utilization. As a result, deamination of amino acid and urea formation in the liver are reduced [7]. While some studies [10, 16] reported that MU was lower in primiparous cows, other studies [9, 14, 15] found the highest MU concentration in primiparous cows and the lowest in multiparous cows.

Higher milk fat and protein contents in primiparous cows were connected with the lower milk yield; when the limiting milk yield associated with the growth of mammary gland and promotes a higher fat and protein contents in milk [21].

Lower milk lactose in primiparous cows compared with multiparous cows and tend to increase until the third parity and then decrease is due to increase of milk yield during the lactation, when the synthesis of lactose in the mammary gland regulates the milk secretion [19].

Increased need of nutrients for primiparous cows limits milk yield with a decrease in protein and fat yield compared to multiparous cows [21].

The confirmed results of the lowest milk yield during the first lactation with the low initial production and peak of lactation compared with cows in third and fourth lactation fully correspond to those of Baul et al. [3], who confirmed a sharper course of the lactation curve in the

group of mature cows as a consequence of a lower start of production, with a lower peak and a slower decline in milk production after the peak of lactation in primiparous cows compared to mature cows. Milk yield increases as parity proceeds because large cows produce more milk than small cows due to large body size and increased mammary gland development that comes with repeated pregnancies as well as the full development of tissues of the mammary gland. Milk yields declined in the fifth and over parity. The decline is due to the decline in body condition over the recurring pregnancies [21].

The results of the lower MU between 0—30 DIM in primiparous and multiparous cows and maximum MU concentration between 271—305 DIM correspond to those of Yoon et al. [27], who observed the highest values of MU during late lactation and the lowest during early lactation. According to Jílek et al. [14] the lower MU content in the first month of lactation is because of the inability of cows to digest a sufficient amount of feed at the beginning of the lactation. This can result in a relatively lower intake of proteins. In later lactations, as milk production declines, the protein requirement also decreases, and that MU should also decline. High of MU in the late lactations suggests the possibility that, protein is overfed in late lactations or the ration contained a different amount of rumen degradable protein than earlier in lactations [15].

The declining dynamics of milk yield during lactation is consistent with conclusions by Vijayakumar et al. [26], influenced by homeorhetic stimulation of metabolic regulation of nutrients intake for milk synthesis, as well as the synthetic capacity of mammary gland by different recovery of secretory cells at the peak and end of lactation. The increase of milk protein and fat contents during lactation is consistent with previous studies by Bondan et al. [6]. The decrease of milk yield in continuous lactation cause the protein and fat contents to remain more concentrated [13].

CONCLUSIONS

Our results confirmed the significant impact of non-nutritional factors (parity and DIM) on milk yield, milk components and MU. Results from this study show statistically significant ($P < 0.05$) impact of parity to milk yield, milk components and MU. Positive relationships ($P < 0.01$) between DIM and MU were also confirmed. These results suggest that previously mentioned non-nutritional factors should be evaluated when the relationship between MU concentration and nutritional management on dairy farms is determined.

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

REFERENCES

1. **Abdouli, H., Rekik, B., Haddad-Boubaker, A., 2008:** Non-nutritional factors associated with milk urea concentrations under Mediterranean conditions. *World J. Agric. Sci.*, 4, 2, 183—188. DOI: 10.12691/wjar-9-3-3.
2. **Arunvipas, P., Dohoo, I. R., Vanleeuwen, J. A., Keefe, G. P., 2003:** The effect of non-nutritional factors on milk urea nitrogen levels in dairy cows in Prince Edward Island, Canada. *Prev. Vet. Med.*, 59, 1—2, 83—93. DOI: 10.1016/s0167-5877(03)00061-8.
3. **Baul, S., Cziszter, L. T., Acatincai, S., Cismas, T., Gavojdian, D., Tripon, I., Erina, S., Buzamat, G., 2013:** Effect of parity on dynamics of milk yield and composition during normal lactation in dairy cows. *J. Anim. Sci. Biotechnol.,* 46, 2, 285—289.
4. **Bauman, D. E., Griinari, J. M., 2003:** Nutritional regulation of milk fat synthesis. *Annu. Rev. Nutr.*, 23, 203—227. DOI: 10.1146/annurev.nutr.23.011702.073408.
5. **Bendelja, D., Prpić, Z., Mikulec, N., Ivkić, Z., Havranek. J., Antunac, N., 2011:** Milk urea concentration in Holstein and Simmental cows. *Mljekarstvo*, 61, 1, 45—55.
6. **Bondan, C., Folchini, J. A., Noro, M., Quadros, D. L., Machado, K. M., González, F. H. D., 2018:** Milk composition of Holstein cows: A retrospective study. *Cienc. Rural*, 48, 12, 1—8. DOI: 10.1590/0103-8478cr20180123.
7. **Cao, Z., Huang, W., Wang, T., Wang, Y., Wen, W., Ma,**

M., Li, S., 2010: Effects of parity, days in milk, milk production and milk components on milk urea nitrogen in Chinese Holstein. *J. Anim. Vet. Adv.*, 9, 4, 688—695. DOI: 10.3923/java.2010.688.695.

8. **Commission Regulation (EC) No. 691/2013, 2013:** Amending Regulation (EC) No. 152/2009 as regards methods of sampling and analysis. *OJEU*, L 197/1—L197/12.
9. **Čobanović, K., Kučević, D., Plavšić, M., Bogdanović, V., 2017:** Impact of non-nutritional factors on milk urea concentration and its relationship with production and fertility traits in Vojvodina dairy herds. *Mljekarstvo*, 67, 4, 267—276. DOI: 10.15567/mljekarstvo.2017.0404.
10. **Godden, S. M., Lissemore, K. D., Kelton, D. F., Leslie, K. E., Walton, J. S., Lumsden, J. H., 2001:** Factors associated with milk urea concentrations in Ontario dairy cows. *J. Dairy Sci.*, 84, 1, 107—114. DOI: 10.3168/jds.S0022-0302 (01)74458-X.
11. **Hojman, D., Kroll, O., Adin, G., Gips, M., Hanochi, B., Ezra, E., 2004:** Relationship between milk urea and production, nutrition and fertility trails in Israeli dairy herd. *J. Dairy Sci.*, 87, 4, 1001—1011. DOI: 10.3168/jds.S0022-0302(04)73245-2.
12. **IBM Corporation Released, 2016:** *IBM SPSS Statistics for Windows*, Version 24.0. Armonk, NY, IBM Corporation.
13. **Idamokoro, E. M., Muchenje, V., Masika, P. J., 2017:** Yield and milk composition at different stages of lactation from a small herd of Nguni, Boer, and non-descript goats raised in an extensive production system. *Sustainability*, 9, 6, 1000. DOI: 10.3390/su9061000.
14. **Jílek, F., Řehák, D., Volek, J., Štípková, M., Němcová, E., Fiedlerová, M., Rajmon, R., Švestková, D., 2006:** Effect of herd, parity, stage of lactation and milk yield on urea concentration in milk. *Czech J. Anim. Sci.*, 51, 12, 510—517. DOI: 10.17221/3971-CJAS.
15. **Johnson, R. G., Young, A. J., 2003:** The association between milk urea nitrogen and DHI production variables in western commercial dairy herds. *J. Dairy Sci.*, 86, 9, 3008—3015. DOI: 10.3168/jds.S0022-0302 (03)73899-5.
16. **Konjačić, M., Kelaval, N., Ivkić, Z., Ivanković, A., Prpi, Z., Vnučec, I., Ramljak, J., Mijić, P., 2010**: Non-nutritional factors of milk urea concentration. *Mljekarstvo*, 60, 3, 166—174.
17. **Maas, J. A., France, J., McBride, B. W., 1997:** Model of milk protein synthesis. A mechanistic model of milk protein synthesis in the lactating bovine mammary gland. *J. Theor. Biol.*, 187, 3, 363—378. DOI: 10.1006/jtbi.1997.0437.
18. **Marenjak, T., Poljičak-Milas, N., Stojević, Z., 2004:** The aim of urea determination in cows' milk (In Croatian, English abstract). *Praxis Veterinaria*, 52, 3, 233—241.
19. **Miglior, F., Sewalem, A., Jamrozik, J., Lefebvre, D. M., Moore, R. K., 2006:** Analysis of milk urea nitrogen and lactose and their effect on longevity in Canadian dairy cattle. *J. Dairy Sci.*, 89, 12, 4886—4894. DOI: 10.3168/jds.S0022-0302(06)72537-1.
20. **NRC 2001:** *Nutrient Requirements of Dairy Cattle*. 7th rev. edn., 263—265.
21. **Nyamushamba, G. B., Halimani, T. E., Imbayarwo-Chikosi, V. E., Tavirimirwa, B., 2014:** Comparative evaluation of non-genetic factors affecting milk yield and composition of Red Dane and Jersey cattle in Zimbabwe. *Springer Plus*, 3, 88. DOI: 10.1186%2F2193-1801-3-88.
22. **Pytlewski, J., A-ntkowiak, I., Skrzypek, R., 2010:** Relationships between somatic cells counts and urea level in the milk of polish Holstein-Friesian cows of black-and-white and red-white varieties. *Acta Sci. Pol.*, 9, 2, 39—50.
23. **Rajala-Schultz, P. J., Saville, W. J., 2003:** Sources of variation in milk urea nitrogen in Ohio dairy herds. *J. Dairy Sci.*, 86, 5, 1653—1661. DOI: 10.3168/jds.S0022-0302 (03)737 51-5.
24. **Stoop, W. M., Bovenhuis, H., Van Arendonk, J. A. M., 2007:** Genetic parameters for milk urea nitrogen in relation to milk production traits. *J. Dairy Sci.*, 90, 4, 1981—1986. DOI: 10.3168/jds.2006-434.
25. **Tripathi, M. K., 2014:** Effect of nutrition on production, composition, fatty acids and nutraceutical properties of milk. *J. Adv. Dairy Res.*, 2, 2, 1—11. DOI: 10.4172/2329-888X. 1000115.
26. **Vijayakumar, M., Park, J. H., Ki, K. S., Lim, D. H., Kim, S. B., Park, S. M., et al., 2017:** The effect of lactation number, stage, length, and milking frequency on milk yield in Korean Holstein dairy cows using automatic milking system. Asian-Australas. *J. Anim. Sci.*, 30, 8, 1093—1098. DOI: 10. 5713/ajas.16.0882.
27. **Yoon, J. T., Lee, J. H., Kim, C. K., Chung, Y. C., Kim, C. H., 2004:** Effects of milk production, season, parity and lactation period on variations of milk urea nitrogen concentration and milk components of Holstein dairy cows. Asian-Australas. *J. Anim. Sci.*, 17, 4, 479—484. DOI: 10.5713/ajas.2004.479.

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RETROSPECTIVE OVERVIEW OF COVID-19 IN EUROPE

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ABSTRACT

A disease of unknown origin connected with severe pneumonia was identified in Wuhan (China) in December 2019. It was named coronavirus disease 2019 (COVID-19). The disease had rapidly spread all over the world, including Europe. The World Health organization (WHO) declared the disease a pandemic. The aim of this study is to summarize and to compare objectively the epidemiological situation of COVID-19 in European countries from 15 February 2020 to 31 December 2021. Due to the significant difference in the population of individual states, all data were calculated per 1 million people (parameter/1M). Cases/1M, number of death/1 M, and % of death (case fatality rate) were compared. The actual situation on 31 December 2021 was quantified by comparing the active cases/1 M in each European country. The situation in Europe has been compared also with those on the other continents of the world, respectively on 31 December 2021. In order to monitor the development of the disease spread on the national level, the European countries were compared after division into six regions: South, West, North, Middle, Balkan and East. These data were recorded daily from 15 February to 31 December 2021.

Key words: COVID-19; Coronavirus; Europe; spread of disease

INTRODUCTION

A respiratory disease of unknown origin was identified in Wuhan (China) in December 2019 [5, 22]. A novel coronavirus named "Severe acute respiratory syndrome coronavirus 2" (SARS-CoV-2) was isolated from the clustered cases. The disease caused by SARS-CoV-2 has been named Coronavirus Disease 2019 (COVID-19) [12]. The first identified case in Europe was detected by the National Reference Centre for Respiratory Viruses at the Institute Pasteur in Paris (France) on 24 January 2020 [3]. During February 2020, there were COVID-19 cases in 35 countries, with 67,120 confirmed infections and 1,526 deaths. China accounted for 99.22 % of infections and 99.87 % of

deaths [1]. After the first confirmed cases imported into Europe, model studies assumed a high risk of COVID-19 widespread for Europe, especially in countries with important international airports [10]. However, the reality exceeded the assumptions. Due to a global travelling of people the virus had rapidly spread all over the world and the outbreak is now widespread. On 11 March 2020 the disease was reported in 114 countries and the Director General of the World Health Organization (WHO) declared a pandemic [6]. Currently, the COVID-19 is present in all 48 European countries; Montenegro (17 March) and Isle of Man (19 March) were the last affected countries [15].

The attack rate or transmissibility of a virus is indicated by its reproductive number (R0), which represents the average number of people to which a single infected person will transmit the virus. The R0 is calculated by a mathematic model based on epidemiological data. WHO estimated that the transmission rate of SARS-CoV-2 virus varied between 1.4 and 2.5 [11, 14]. But according to other models the R0 can be 2.24 to 3.58 [8, 21]. Preliminary studies had estimated R0 to be between 1.4 and 3.5 [9, 11]. The measure of disease severity is represented by the case fatality rate (CFR) expressed as a percentage of death from the disease for a particular period. In March 2020, the CFR was expected to be at around 2—3 %, but it may vary by country [13]. Data dealing total and current numbers of cases or mortality can be found on various national and global sites. Uniformly processed data are accessible from the website Worldometer (https://www.worldometers.info/coronavirus/), which is a reference website that provides counters and real-time statistics for diverse topics. It is currently also dealing with the situation in COVID-19 based on data provided by individual countries from all continents of the world [20].

The aim of this study is to objectively summarize the epidemiological situation in COVID-19 in each European country from 15 February 2020 to 31 December 2021. The individual countries emphasize the information dealing the total number of infected patients, the total number of deaths and the total number of active cases, which characterizes the epidemiological situation at a given time. Given the markedly different population sizes in different countries, it is more objective from an epidemiological point of view to report prevalence values, i. e. the number of cases, the number of deaths or the number of active cases per million inhabitants [2].

COVID-19 situation in Europe and on the other continents on 31 December 2021

The European situation in comparison with that on the other continents is described in Figure 1. All data about COVID-19 cases, death, mortality rate (percentage of death) and active cases were recorded on 31 December 2021 and have been calculated per million of the population of individual continents of the world, respectively (cases/1 M, death/1 M, active cases/1 M). The number of population has been recorded based on the Worldometer population data also on 31 December 2021. Only the population of countries with registered COVID-19 occurrence has been counted.

In Europe, North America including the Caribbean and in South America, all countries are registered. In Asia, there are no available data from North Korea. In Africa, the data from Saint Helena are inaccessible and in Oceania data were registered only in 6 countries from 23 (Australia, New Zealand, French Polynesia, Papua New Guinea, Fiji and New Caledonia). It can be assumed that the number of total cases can be significantly higher worldwide, because the disease can occur with mild atypical symptoms, or, even asymptomatic [16].

From the Figure 1 it is evident that while in the number of cases and deaths per million of the population, the results were the worst in South America, the actual situation (active cases/1 M) was the hardest in North America. The CFR was the highest in South America (2.99 %). The average CFR value in the world (1.89 %). For Europe, this value has a declining trend over time, but the rate of decrease in % of death varies from country to country (details not shown). The number of all cases/1 M in Europe was highest, up to 120 thousand.

Epidemiological situation in European countries on 31 December 2022

In order to monitor the development of the disease spread on the national level, the European countries were compared after division into regions. This division does not follow exactly the political division of European countries, but is based on the geographical position of the given country. In this article Europe was divided into 6 regions:

South: Italy and Vatican City (Italy & V), Spain, Portugal, Malta, Andorra, San Marino, Gibraltar;

West: Germany, France, United Kingdom (UK), Ireland, Switzerland, Belgium, Netherlands, Luxembourg, Liechtenstein, Monaco, Channel Islands, Isle of Man;

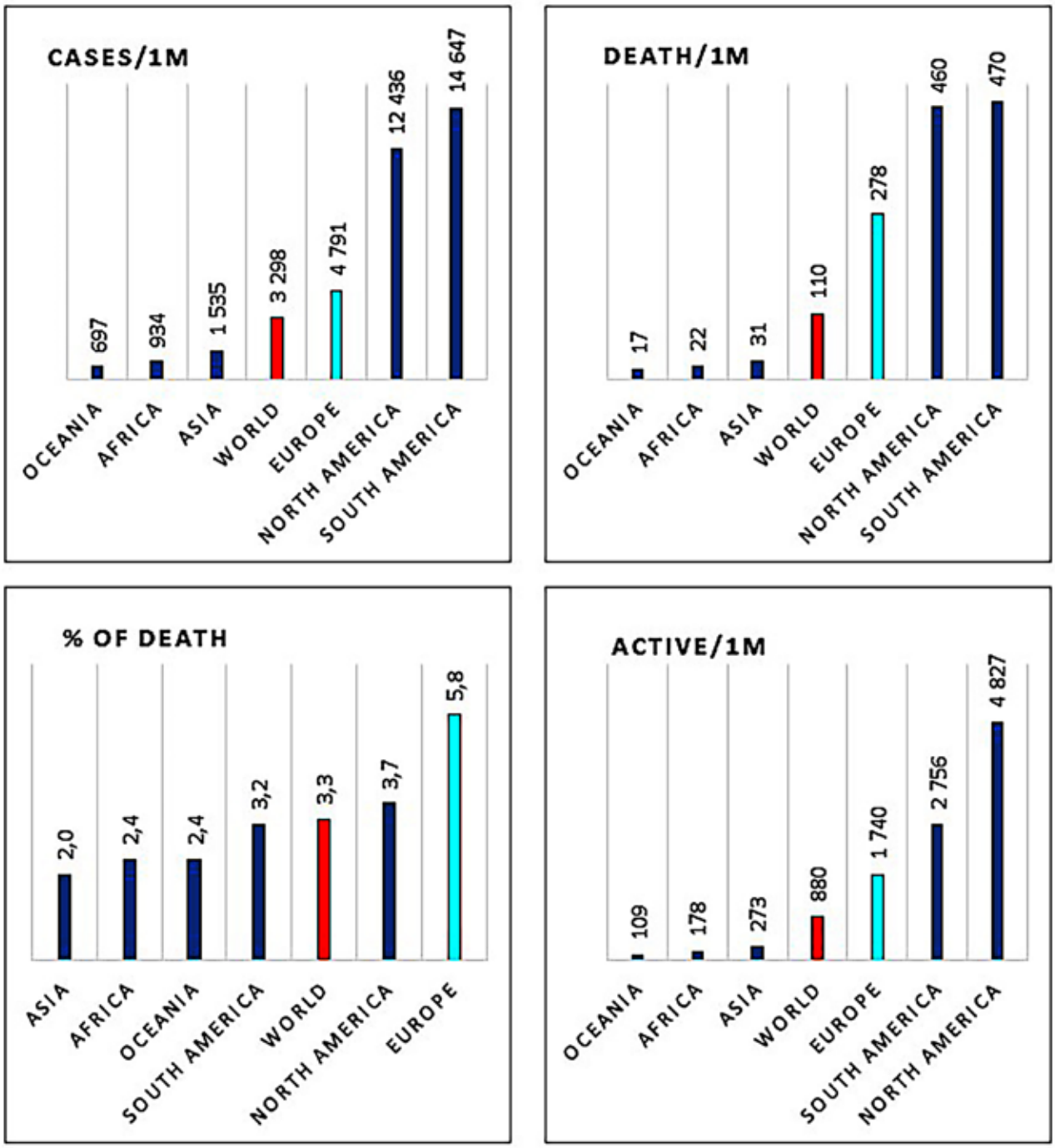

Fig. 1. Number of cases per million, number of deaths per million, % of deaths and active cases per million worldwide as of 31 December 2021. Data from Worldometer as of 31. 12. 2021

North: Sweden, Finland, Norway, Denmark, Iceland, Faroe Islands;

Middle: Austria, Czech Republic, Poland, Slovakia, Hungary, Slovenia;

Balkan: Croatia, Serbia, Bosnia and Herzegovina, Montenegro, North Macedonia, Albania, Bulgaria, Greece;

East: Russia, Belarus, Ukraine, Latvia, Lithuania, Estonia, Moldova, Romania.

Cases/1 M in individual countries

As described in Table 1, most cases/1 M were recorded especially in the southern countries. However, the prevalence of the disease is often significantly higher in the smaller countries with a smaller area and lower populations. The population of San Marino at 31 December 2021 was 33,943/240,966 cases in total, in Andorra the population was 77,286 persons/306,520 cases in total. It should

Table 1. Southern European countries

State	Active cases/1 M	Cases/1 M	Death/1 M	% of death
Italy + Vatican	14 934	101 539	2 278	2.24
Spain	26 378	136 547	1 912	1.40
Portugal	17 603	136 881	1 867	1.36
Malta	26 738	118 368	1 076	0.91
Andorra	39 212	306 520	1 808	0.59
San Marino	29 967	240 966	2 938	1.22
Gibraltar	26 013	258 374	2 969	1.15
Europe	15 145	117 115	2 041	1.74

Data from Worldometer as of 31. 12. 2021

Table 2. Western European countries

State	Active cases/1 M	Cases/1 M	Death/1 M	% of death
Germany	8 086	85 246	1 339	1.57
France	26 840	152 280	1 889	1.24
UK	36 167	189 095	2 172	1.15
Ireland	42 757	157 073	1 178	0.75
Switzerland	36 275	152 525	1 408	0,92
Belgium	29 159	179 137	2 427	1.35
Netherlands	27 398	182 225	1 217	0.67
Luxembourg	12 497	161 828	1 427	0.88
Liechtenstein	8 461	159 511	1 802	1.13
Monaco	12 431	130 289	958	0.74
Channel Islands	26 410	156 058	641	0.41
Isle of Man	3 827	159 159	782	0.49
Europe	15 145	117 115	2 041	1.74

Data from Worldometer as of 31. 12. 2021

be borne in mind that in these countries the infrastructure is being built for a given population, and a sudden increase in the number of infected people poses as serious a problem for the health service as in larger countries with larger populations.

From epidemiological data, it is known that the virus spreads most rapidly in places with high urbanization and population density [4]. Therefore, it is understandable that in the city-state of San Marino, the case/1M in COVID-19 was so high. Of the other countries, the situation was quite serious in Spain, Moldova or Sweden. The prevalence of COVID-19 as of 31 December 2021 was generally lowest in Central European countries. WHO highlighted the risks in cities and urban environments and the need to put measures in place, in particular the benefit of using masks [18, 19].

Death/1 M and the percentage of death in individual countries

Although WHO has issued guidelines for surveillance and certification of COVID-19 as a cause of death, coun-

Table 3. Northern European countries

State	Active cases/1 M	Cases/1 M	Death/1 M	% of death
Sweden	10 528	128 982	1 498	1.16
Finland	38 304	46 869	282	0.60
Norway	55 427	71 884	238	0.33
Denmark	32 097	135 223	561	0.41
Iceland	18 482	84 839	107	0.13
Faroe Islands	14 753	117 295	285	0.24
Europe	15 145	117 115	2 041	1.74

Data from Worldometer as of 31. 12. 2021

Table 4. Middle European countries

State	Active cases/1 M	Cases/1 M	Death/1 M	% of death
Austria	3 322	140 765	1 512	1.07
Czech Republic	10 479	230 543	3 367	1.46
Poland	10 085	108 727	2 569	2.36
Slovak Republic	7 876	154 062	3 045	1.98
Hungary	10 968	130 555	4 072	3.12
Slovenia	8 431	223 169	2 688	1.20
Europe	15 145	117 115	2 041	1.74

Data from Worldometer as of 31. 12. 2021

tries have not followed uniform rules for reporting the total number of deaths due to this disease [17, 18]. A distinction should be made between deaths caused directly by COVID-19 and deaths related to COVID-19 [18]. Data for this parameter (Table 1—6) are difficult to compare because we do not know the key for assigning cause of death to each condition. Overall, the highest number of deaths occurred in Hungary (4,072/1 M) and the Czech Republic (3,367/1 M), the number of deaths in Europe (2,041/1 M) was compared.

Data from some countries are interesting: e. g. in the Czech Republic 2.51 times more death/1 M has been reported compared to Germany but the % of death was higher in Germany (1.57) than in the Czech Republic (1.46). In Hungary, death/1 M was 2.7 times higher than in Austria while the % of death was 2.9 times higher. For comparison, in Slovakia the mortality rate/1 M was 1.5 times higher than in Europe, the % mortality was 1.13 times higher.

Active cases/1 M in individual countries on 31 December 2021 and the current trend

On 31 December 2021, the highest number of active cases/1M in Europe was reported from Norway (55,427 cases). The value was also high in Ireland (42,757 cases). In the other countries in the ranking, the values were at least more than 2,000/1 M lower. Unfortunately, after the summer period, the number of active cases/1 M increased rapidly in many European countries (Table 1—6). In Denmark and Hungary, the number of active cases increased more than 10-fold in one month, in the Czech Republic and Iceland more than 5-fold, and in 15 European countries the number of new cases more than doubled. In many European countries, the highest number of new cases per day since the COVID-19 outbreak was recorded after 31 August.

Table 5. Balkan countries

State	Active cases/1 M	Cases/1 M	Death/1 M	% of death
Croatia	6 775	175 850	3 083	1.75
Serbia	2 775	149 613	1 464	0.98
Bosnia & Herzegovina	26 353	89 628	4 136	4.61
Montenegro	13 912	270 675	3 838	1.42
North Macedonia	2 517	108 028	3 821	3.54
Albania	2 175	73 170	1 120	1.53
Bulgaria	15 103	108 748	4 506	4.14
Greece	17 078	117 027	2 009	1.72
Europe	15 145	117 115	2 041	1.74

Data from Worldometer as of 31. 12. 2021

Table 6. Eastern European countries

State	Active cases/1 M	Cases/1 M	Death/1 M	% of death
Russia	4 980	71 904	2 115	2.94
Belarus	132	74 022	591	0.80
Ukraine	2 374	84 625	2 213	2.61
Lithuania	9 827	194 999	2 773	1.42
Latvia	6 231	149 154	2 464	1.65
Estonia	11 893	181 799	1 455	0.80
Moldova	858	93 573	2 556	2.73
Romania	585	94 977	3 085	3.25
Europe	15 145	117 115	2 041	1.74

Data from Worldometer as of 31. 12. 2021

The COVID-19 spread in the European regions from 15 February to 31 December 2021

The first cases of COVID-19 in individual European countries were confirmed from 24 January (France, Bordeaux) to 19 March (Isle of Man) [3, 7]. However, the rate of spread varied from region to region (Table 1—6). In the southern and western countries, the virus started to spread very soon after its first outbreak (Table 1, 2). Since mid-March, there has been a rapid increase in cases almost in all southern (Table 5), western (Table 2) and northern (Table 3) countries. The spread rate was lower in the Central European countries (Table 4). In the eastern countries (Table 5), the spread has intensified about mid-April. From mid-April, the disease started to spread also in the Balkans (Table 6). The situation was similar in the Eastern European countries, with the exception of the Baltic States, where the spread of COVID-19 was smaller and slower.

In terms of deaths within the regions of the Balkan countries, Bulgaria had the highest number of deaths/1 M throughout the reporting period; its number significantly exceeded that of Bosnia and Herzegovina. Among the Western countries, the situation was worst in Belgium, the United Kingdom and France (Table 2). The data from Sweden (1,498 deaths/1 M) significantly exceeded those from the other Nordic countries (Table 3). In Central European countries, the number of deaths more or less slowed

down at the end of May, with the exception of Poland. During the whole period under review, the highest number of deaths/1 M was in Hungary (4, 072 deaths/1 M) and the Czech Republic (3,367 deaths/1 M) (Table 4).

In the Balkan countries, the highest number of deaths/1 M was in Bulgaria (4,506 deaths/1 M) and Bosnia and Herzegovina (4,136 deaths/1 M). In the other Balkan countries, a similar situation started to change in mid-July, with data from Bulgaria currently the worst (Table 5). Among the eastern countries, despite the high number of total deaths in Romania (3,085 deaths/1M), the highest figures were in Lithuania and Moldova. The number of deaths/1 M in Belarus was lower than in the Baltic States (Table 6).

In each region, there were countries with both worse and better situations, but on 31 December 2021 the number of active cases/1 M was the lowest in Eastern European region (Table 6). It is questionable how strongly they will be affected by the second wave of the epidemic. It is already clear that the position of individual countries may change completely during the next wave of COVID-19.

COVID-19 appeared in Europe on 24 January 2020 (France) and by 19 March (Isle of Man) the infection was confirmed in all European countries. The total number of people infected was naturally higher in countries with large populations, but the prevalence (number of cases per million) in smaller countries was even higher than in large countries. The ranking of countries with a high number of all cases is not the same as the ranking of countries by COVID-19 prevalence. The virus spreading is not equally fast in different European regions. The virus started to spread first in the southern and western countries, soon in the northern region and, to a lesser extent, in the countries of Central Europe.

The number of deaths/1M was generally highest in the southern and western regions of Europe as of 31 December 2021, but these figures may be influenced by the method of certifying COVID-19 as the cause of death. It can be assumed that individual European countries are not uniform in their assessment of this parameter. The mortality rate for new coronaviruses from all continents was highest in Europe, although this value is gradually decreasing over time.

The current epidemic situation characterized by the number of active cases, more objectively expressed as the number of active cases/1M, was worst in Norway (55,427 active cases/1 M) and Ireland (42,757 active cases/1 M) as of 31 December 2021. In many countries, however, the new wave of COVID-19 has started with a record number of new cases per day. According to epidemiologists, mortality rates may vary because the virus can mutate. By comparison, approximately 290,000 to 650,000 people die each year worldwide as a result of complications from seasonal influenza viruses. The R0 for the common influenza is 1.3. The mortality rate for SARS in 2002—2003 was 10 %; for MERS in 2012, the mortality rate was 34 %. Both coronavirus epidemics were considerably less widespread: 8,096 people were infected with SARS-CoV and 2,494 with MERS-CoV.

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REFERENCES

1. **Cao, Y., Cai, K., Xiong, L., 2020:** Coronavirus disease 2019: A new severe acute respiratory syndrome from Wuhan in China. *Acta Virol.*, 64, 2, 245—250. DOI: 10.4149/av_2020_201.
2. **European Centre for Disease Prevention and Control. Population-wide Testing of SARS-CoV-2, 2020:** country experiences and potential approaches in the EU/EEA and the UK Stockholm, ECDC, 2020. https://www.ecdc.europa.eu/sites/default/files/documents/covid-19-population-wide-testing-country-experiences.pdf. Accessed January 8, 2021.
3. **Gámbaro, F., Behillil, S., Baidaliuk, A., Donati, F., Albert, M., Alexandru, A., et al., 2020:** Introductions and early spread of SARS-CoV-2 in France, 24 January to 23 March. *Euro Surveill.*, 25, 26, 2001200. DOI: 10.2807/1560-7917.ES.2020.25.26.2001200.
4. **Hassell, J. M., Begon, M., Ward, M. J., Fèvre, E. M., 2017:** Urbanization and disease emergence: Dynamics at the wildlife-livestock-human interface. *Trends Ecol. Evol.*, 32, 1, 55—67. DOI: 10.1016/j.tree.2016.09.012.

5. **Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., et al., 2020:** Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet*, 395, 497—506. DOI: 10.1016/S0140-6736(20)30183-5.
6. **Kinross, P., Suetens, C., Gomes Dias, J., Alexakis, L., Wijermans, A., Colzani, E. et al., ECDC Public Health Emergency Team, 2020:** Rapidly increasing cumulative incidence of coronavirus disease (COVID-19) in the European Union/European Economic Area and the United Kingdom, 1 January to 15 March 2020. *Euro Surveill.*, 25, 11, 2000285. DOI: 10.2807/1560-7917.ES.2020.25.11.2000285.
7. **Lescure, F.X., Bouadma, L., Nguyen, D., Parisey, M., Wicky, P.H., Behillil, S., et al., 2020:** Clinical and virological data of the first cases of COVID-19 in Europe: a case series. *Lancet Infect. Dis.*, 20, 6, 697 706. DOI: 10.1016/S1473-3099(20)30200-0.
8. **Liu, Y., Gayle, A.A., Wilder-Smith, A., Rocklöv, J., 2020:** The reproductive number of COVID-19 is higher compared to SARS coronavirus. *J. Travel Med.*, 27, 2, taaa021. DOI: 10.1093/jtm/taaa021.
9. **Majumder, M.S., Mandl, K.D., 2020:** Early Transmissibility Assessment of a Novel Coronavirus in Wuhan, China. *SSRN* [Preprint], 3524675. DOI: 10.2139/ssrn.3524675.
10. **Pullano, G., Pinotti, F., Valdano, E., Boëlle, P.Y., Poletto, C., Colizza, V., 2020:** Novel coronavirus (2019-nCoV) early-stage importation risk to Europe, January 2020. *Euro Surveill.*, 25, 4, 2000057. DOI: 10.2807/1560-7917.ES.2020.25.4.2000057.
11. **Read, J.M., Bridgen, J.R.E., Cummings, D.A.T., Ho, A., Jewell, C.P., 2020:** Novel coronavirus 2019-nCoV: early estimation of epidemiological parameters and epidemic predictions. medRxiv 2020.01.23.20018549, preprint. DOI: 10.1101/2020.01.23.20018549.
12. **Spiteri, G., Fielding, J., Diercke, M., Campese, C., Enouf, V., Gaymard, A., et al., 2020:** First cases of coronavirus disease 2019 (COVID-19) in the WHO European Region, 24 January to 21 February 2020. *Euro Surveill.*, 25, 9, 2000178. DOI: 10.2807/1560-7917.ES.2020.25.9.2000178.
13. **Wang, C., Horby, P.W., Hayden, F.G., Gao, G.F., 2020:** A novel coronavirus outbreak of global health concern. *Lancet*, 395, 10223, 470—473. DOI: 10.1016/S0140-6736(20)30185-9.
14. **World Health Organization, 2019:** *Statement on the Meeting of the International Health Regulations (2005) Emergency Committee Regarding the Outbreak of Novel Coronavirus (2019-nCoV)*. Jan 2020. https://www.who.int/news/item/30-01-2020-statement-on-the-second-meeting-of-the-international-health-regulations-(2005)-emergency-committee-regarding-the-outbreak-of-novel-coronavirus-(2019-ncov)/. Accessed January 5, 2022.
15. **World Health Organization, 2020:** *Director-General's Opening Remarks at the Media Briefing on COVID-19*. 11 March 2020. https://www.who.int/director-general/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19-11-march-2020/. Accessed January 23, 2021.
16. **World Health Organization, 2020a:** *Advice on the Use of Masks in the Context of COVID-19*. Interim Guidance. 5 June 2020. https://apps.who.int/iris/handle/10665/332293/. Accessed January 5, 2022.
17. **World Health Organization, 2020b:** *Global Surveillance for COVID-19 Caused by Human Infection with COVID-19 Virus*. Interim Guidance. 20 March 2020. https://apps.who.int/iris/handle/10665/331506/. Accessed January 5, 2022.
18. **World Health Organization, 2020c:** *Medical Certification, ICD Mortality Coding, and Reporting Mortality Associated with COVID-19*. Technical note. 7 June 2020. https://apps.who.int/iris/handle/10665/332297/. Accessed January 8, 2022.
19. **World Health Organization, 2020d:** *Strengthening Preparedness for COVID-19 in Cities and Urban Settings. Interim guidance for local authorities*. 28 April 2020. https://www.who.int/publications/i/item/strengthening-preparedness-for-covid-19-in-cities-and-urban-settings/. Accessed January 5, 2022.
20. **Worldometer:** https://www.worldometers.info/coronavirus/. Accessed January 5, 2022.
21. **Zhao, S., Lin, Q., Ran, J., Musa, S.S., Yang, G., Wang, W., et al., 2020:** Preliminary estimation of the basic reproduction number of novel coronavirus (2019-nCoV) in China, from 2019 to 2020: A data-driven analysis in the early phase of the outbreak. *Int. J. Infect. Dis.*, 92, 214—217. DOI: 10.1016/j.ijid.2020.01.050.
22. **Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., et al., 2020:** A novel coronavirus from patients with pneumonia in China, 2019. *Engl. J. Med.*, 382, 8, 727—733. DOI: 10.1056/NEJMoa2001017.

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PREVALENCE OF SOLE ULCER IN DAIRY COWS EXPOSED TO HEAT STRESS

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ABSTRACT

The aim of this study was to investigate the seasonal thermal effect on the prevalence of the sole ulcer in dairy cows. The observations were performed on a Holstein-Friesian (HF) dairy farm in Eastern Slovakia. The clinical signs of heat stress were recognised in several animals during the afternoon milking on July 8, 2015. The claw examination done three months after the heat stress episode was compared with three examinations: twelve and two months prior to the heat stress and ten months afterward. The orthopaedic examinations were focused on the prevalence of the sole ulcer. Statistical analysis was performed by running a chi-squared test. The temperature-humidity index (THI) on July 8, 2015, was 88. We found 15.2 %, 18.6 %, 28.1 %, and 12.9 % cows with sole ulcer in the autumn 2014, spring 2015, autumn 2015, and spring of the following year, respectively ($P < 0.05$). In conclusion, the results of our observations showed that the heat stress in dairy cows can be associated with an increase in the prevalence of the sole ulcer.

Key words: dairy cows; heat stress; sole ulcer

INTRODUCTION

Lameness is a severe welfare problem in cattle and has detrimental effects on longevity, productivity, and reproductive performance [7]. Digital dermatitis, sole ulcers, and white line disease are the most frequently reported foot lesions related to the lameness of dairy cows [9, 15]. Consequently, the lameness belongs to the most frequent cause of the largest economic losses in the dairy industry [6]. Regardless of whether foot lesions cause lameness, they have a high impact on animal welfare and farm economics. Claw disorders that result in either lameness or no lame condition are estimated to cost on average $ 95 or $ 18 per case, respectively [3]. The painful lesions associated with the sole and toe ulcers have resulted in milk losses of 574 kg per cow per a 305-day lactation; furthermore, in dairy cows treated for digital dermatitis, milk yields increased in the months after treatment compared with the period before the treatment [1]. Sole/toe ulcers also have been associated with impaired fertility, and sole ulcers have been associated with an increased occurrence of milk fever [22]. Sole ulcers (Fig. 1), which are one of the most severe claw diseases causing lameness, are non-infectious in nature and occur when claw horn formation is dis-

Fig. 1. Sole ulcer on the outer claw of hind limb

rupted. The underlying tissue then becomes inflamed and sometimes exposed [17]. Cows are particularly vulnerable around the time of parturition; changes in hormone levels cause increased vascular permeability, increasing the risk of oedema and ischemia in the hoof [11], while concurrent weakening of connective tissue causes the pedal bone to drop and compress the corium, further disrupting claw horn formation [27].

Heat stress is defined as the sum of external forces acting on an animal that causes an increase in body temperature and evokes a physiological response [14]. The temperature-humidity index (THI) has been commonly used to estimate the effect of heat stress on production and reproduction. There is a general agreement that significant effects are observed at a mean daily THI of around 72 [29]. Documented physiological heat stress coping strategies used by dairy cows include: increased respiration rate, panting, sweating, reduced milk yield, and reproductive performance. Behavioural coping strategies include: modified drinking and feed intake (e. g., increased water intake and shifting feeding times to cooler periods during the day), increased standing time, shade seeking, decreased activity, and movement [12]. The duration of elevated temperatures has an inverse relationship with feed intake. Therefore, it is not surprising that heat stress is considered a major risk factor for lameness, but whether this association is a consequence of increased standing times or due to alterations in nutrient metabolism caused by a decrease in dry matter intake (DMI) is not known [4]. Claw horn lesions, such as sole ulcer, are believed to develop from increased pedal bone mobility induced by changes in the corium at calving [13] and potentially from nutritional insults such as subacute ruminal acidosis [25].

The main aim of this study was to evaluate the effects of heat stress on the occurrence of the sole ulcer in dairy cows.

MATERIALS AND METHODS

The observations were performed on a middle-scale Holstein-Friesian (HF) dairy farm in Eastern Slovakia. All dairy cows were milked twice daily (at 06 : 00 and 14 : 30 h) and had free access to water and were fed a total mixed rations (corn silage, corn, soybeans, minerals and vitamins), according to their lactational stage. No specific measures to protect the animals against the heat stress were taken on the farm. The clinical signs of the heat stress (increased respiration rate, panting, and sweating) were recognised in several animals during the afternoon milking on July 8, 2015. The claws of dairy cows were controlled three month (October 2015) after the heat stress event and the results were compared with results of the claw controls performed twelve and two months prior to the heat stress (October 2014, May 2015), and ten months after it (May 2016). The weather data of air temperature and relative humidity in the region on the day of the heat stress were obtained from the Slovak Hydrometeorological Institute to calculate temperature-humidity index. The THI was calculated as per the equation proposed by the National Research Council of the United States [18]:

$$THI = (1.8 \times T + 32) - (0.55 - 0.0055 \times RH) \times (1.8 \times T - 26)$$

where

T is the outdoor ambient temperature in °C, and

RH is relative humidity expressed in %

Statistical analysis of differences in sole ulcer prevalence among the controls was performed by running a chi-squared test using the statistical software StatSoft, version 8.0. P values < 0.05 were considered significant.

Ethical statement

All procedures concerning the animals were performed in compliance with the national guidelines for animal care.

RESULTS

Comparisons of the four claw health controls revealed a highest prevalence ($P < 0.05$) of sole ulcers in the examination three months after (October 2015) the animals had been challenged by heat stress (Table 1). The lowest portion of the sole ulcers was detected in May 2016 (12.7%) without significant differences to terms in May 2015 (18.619%) and October 2014 (15.1%). The local outdoor ambient temperature and humidity on July 8, 2015 were 35.6 °C and 61.3%, respectively. The calculation of THI resulted in the value of 88.

DISCUSSION

Claw lesions are commonly categorized according to their aetiology into infectious and non-infectious lesions [20]. Infectious lesions include digital dermatitis, interdigital dermatitis, heel horn erosion, and foot rot, whereas the most common non-infectious lesions are sole ulcer, toe ulcer, sole haemorrhage, and white line disease. Non-infectious lesions affect the claw horn, and their occurrence is associated with metabolic and hormonal events around calving that weaken the foot suspensory apparatus [26], lower the Body Condition Score (BCS) [10], toe overgrowth, exposure to hard flooring [24], and thickness of the digital cushion [2]. The prevalence of sole ulcers can differ according to the parity of the cows. Thus, the prevalence of sole ulcers was 4.2 and 27.8% for parity 1 and parity >1, respectively [2]. In the large scale study on 156 dairy farms in Canada, a prevalence of sole ulcer was 6.4% which was the second most prevalent foot lesion after digital dermatitis (21.8%) in the study [23].

Sole ulcer prevalence in the present study varied between 10 and 20% in the sessions which were not related to the heat stress event. The higher number of the sole ulcer detected three months after the heat stress in our study indicate a causative effect of the organ and metabolic changes triggered by the body reaction to high ambient temperature and humidity. It has been suggested that sole ulcers are a consequence of subclinical laminitis [28]. Laminitis is an important predisposing cause of claw disorders in cattle. Inflammation of the corium results in the activation of tissue matrix metallo-proteinases (MMPs) that weaken the collagen fibre bundles that make up the suspensory apparatus within the claw. Coincident with this is the release of horn growth and necrosis factors that contribute to the inflammatory process and accelerate claw horn growth [16]. The vascular disturbances associated with laminitis preclude the normal diffusion of nutrients and oxygen into the living-cell layers of the epidermis destined to become claw horn. This interrupts the normal differentiation of these cells and leads to the formation of weaker or softer claw horn. Some have suggested that "claw horn disruption" may be a better term for the condition of laminitis, because it more accurately reflects the nature of the anatomical and physiological lesions associated with laminitis [21]. The pathogenesis of laminitis is believed to be associated with a disturbance in the micro-circulation of blood within the corium which leads to weakening of the suspensory apparatus within the claw and thus permits downward displacement and rotation of the third phalanx (P3). The result is compression of the corium and supporting tissues that lie between P3 and the sole which predisposes to the development of sole ulcers [13]. Rumen acidosis is considered to be a major predisposing cause of laminitis and presumably mediates its destructive effects through various vasoactive substances (endotoxins, lactate, and possibly histamine) that are released into the blood stream in coincidence with the development of rumen acidosis. The SubAcute Ruminal Acidosis (SARA) is more common than the acute form of this disease. It is generally observed when recently calved cows are introduced to a diet considerably different than that fed during the dry period. Alternatively, it may occur when lactating cows are fed diets low in effective fibre. Major clinical manifestations would include: variable feed intake, depressed fat test, poor body condition despite sufficient energy intake, mild to moderate

Table 1. Prevalence of sole ulcers in dairy cows challenged by the heat stress in four different periods

	October 2014	May 2015	*October	May 2016	x^2
Cows total	211	210	209	212	NS
Sole ulcer (n)	32[a]	39[a]	59[b]	27[a]	$P < 0.05$

[a, b]—prevalence differs ($P < 0.05$); NS—not significant; *—Heat stress July 8, 2015

diarrhoea, and occasional cases of epistaxis (nose-bleed) or haemoptysis (the expectoration of blood from the mouth). Conditions such as laminitis or undefined lameness, abomasal disorders, and liver abscesses are generally secondary observations [8]. SARA can be triggered not just by increased carbohydrate intake but also by heat stress. The primary avenues for heat loss during periods of hot weather are sweating and panting. In severe heat, panting progresses to open-mouth breathing characterized by a lower respiratory rate and greater tidal volume. The result is respiratory alkalosis as a result of the increased loss of carbon dioxide. The cow compensates by increasing urinary output of bicarbonate (HCO_3^-). Simultaneously, the salivary HCO_3^- pool for rumen buffering is decreased by the loss of saliva from drooling in severely stressed cows. The end result is rumen acidosis because of reduced rumen buffering and an overall reduction in total buffering. The effect of ambient air temperature on rumen pH was evaluated in lactating Holstein cows fed either a high roughage or high concentrate diet in both a cool and a hot environment. Rumen pH was lower in cows exposed to the higher temperatures and those fed the higher concentrate diets [19]. In addition, behavioural changes observed and traditionally associated with heat stress are confounded by changes in locomotion score that typically occur over the late summer months. Increases in claw horn lesion development in the late summer may be associated with the increase in total standing time per day due to heat stress [4].

The environmental conditions driving heat stress in dairy cattle are presented using the temperature-humidity index (THI), a calculated index that incorporates the effects of environmental temperature with relative humidity [5]. The authors defined THI < 68 to be outside the thermal danger zone for cows. Mild signs of heat stress are observed at THI of 68 to 74, and a THI ≥ 75 will cause drastic decreases in production performance. The THI value is usually the main determinant for management decisions related to heat stress as most meteorological stations close to farms provide this data. Therefore, THI of 88 observed in the present study speaks clearly for a severe stressful event in the examined animals.

CONCLUSIONS

Results of our observations show that the heat stress in dairy cows can be associated with increase in the prevalence of the sole ulcer. The findings may be useful in stressing a need to take measures to prevent the heat stress in dairy cows.

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REFERENCES

1. **Amory, J. R., Barker, Z. E., Wright, J. L., Mason, S. A., Blowey, R. W., Green, L. E., 2008:** Associations between sole ulcer, white line disease and digital dermatitis and the milk yield of 1824 dairy cows on 30 dairy cow farms in England and Wales from February 2003—November 2004. *Prev. Vet. Med.*, 83, 381—391. DOI: 10.1016/j.prevetmed.2007.09.007.
2. **Bicalho, R. C., Machado, V. S., Caixeta, A. L. S., 2009:** Lameness in dairy cattle: A debilitating disease or a disease of debilitated cattle? A cross-sectional study of lameness prevalence and thickness of the digital cushion. *J. Dairy Sci.*, 92, 3175—3184. DOI: 10.3168/jds.2008-1827.
3. **Bruijnis, M. R, Hogeveen, N. H., Stassen, E. N., 2010:** Assessing economic consequences of foot disorders in dairy cattle using a dynamic stochastic simulation model. *J. Dairy Sci.*, 93, 2419—2432. DOI: 10.3168/jds.2009-2721.
4. **Cook, N. B., Mentink, R. L., Bennett, T. B., Burgi, K., 2007:** The effect of heat stress and lameness on time budgets of lactating dairy cows. *J. Dairy Sci.*, 2007, 90, 1674—1682. DOI: 10.3168/jds.2006-634.
5. **De Rensis, F., Garcia-Ispierto, I., Lopez-Gatius, F., 2015:** Seasonal heat stress: Clinical implications and hormone treatments for the fertility of dairy cows. *Theriogenology*, 84, 659—666. DOI: 10.1016/j.theriogenology.2015.04.021.
6. **Ettema, J. F., Ostergaard, S., 2006:** Economic decision making on prevention and control of clinical lameness in Danish dairy herds. *Livest. Sci.*, 102, 92—106. DOI: 10.1016/j.livprodsci.2005.11.021.
7. **Garbarino, E. J., Hernandez, J. A., Shearer, J. K., Risco, C. A., Thatcher, W. W., 2004:** Effect of lameness on ovarian activity in postpartum Holstein cows. *J. Dairy Sci.*, 87, 4123—4131. DOI: 10.3168/jds.S0022-0302(04)73555-9.

8. **Garrett, E. F., Pereira, M. N., Nordlund, K. V., Armentano, L. E., Goodger, W. J., Oetzel, G. R., 1999:** Diagnostic methods for the detection of subacute ruminal acidosis in dairy cows. *J. Dairy Sci.*, 82, 1170—1178. DOI: 10.3168/jds.S0022-0302(99)75340-3.
9. **Green, L. E., Hedges, V. J., Schukken, Y. H., Blowey, R. W., Packington, A. J., 2002:** The impact of clinical lameness on milk yield of dairy cows. *J. Dairy Sci.*, 85, 2250—2256. DOI: 10.3168/jds.S0022-0302(02)74304-X.
10. **Green, L. E., Huxley, J. N., Banks, C., Green, M. J., 2014:** Temporal associations between low body condition, lameness and milk yield in a UK dairy herd. *Prev. Vet. Med.*, 113, 63—71. DOI: 10.1016/j.prevetmed.2013.10.009.
11. **Knott, L., Tarlton, J., Craft, H., Webster, A. J. F., 2007:** Effects of housing, parturition and diet change on the biochemistry and biomechanics of the support structures of the hoof of dairy heifers. *Vet. J.*, 174, 277—287. DOI: 10.1016/j.tvjl.2006.09.007.
12. **Lees, A., Sejian, V., Wallage, A., Steel, C., Mader, T., Lees, J., et al., 2019:** The impact of heat load on cattle. *Animals*, 2019, 9, 322. DOI: 10.3390/ani9060322.
13. **Lischer, Ch. J., Ossent, P., Raber, M., Geyer, H., 2002:** Suspensory structures and supporting tissues of the third phalanx of cows and their relevance to the development of typical sole ulcers (Rusterholz ulcers). *Vet. Rec.*, 151, 23, 694—698.
14. **Mulling, C. K. W., Lischer, C. J., 2002:** New aspects on etiology and pathogenesis of laminitis in cattle. In *Proceedings of the XXII World Buiatrics Congress* (keynote lectures), Hannover, Germany, 236—247.
15. **Mudroň, P., 2016:** Effects of manure bedding on the rate of claw diseases in dairy cows. *Folia Veterinaria*, 60, 14—19. DOI: 10.1515/fv-2016-0032.
16. **Murray, R. D., Downham, D. Y., Clarkson, M. J., Faull, W. B., Hughes, J. W., Manson, F. J., et al., 1996:** Epidemiology of lameness in dairy cattle: description and analysis of foot lesions. *Vet. Rec.*, 138, 586—591. DOI: 10.1136/vr.138.24.586
17. **National Research Council (U.S.), 1971:** *A Guide to Environmental Research on Animals*. National Academy of Sciences, Washington DC, USA, 374 pp.
18. **Niles, M. A., Collier, R. J., Croom, W. J., 1998:** Effects of heat stress on rumen and plasma metabolite and plasma hormone concentrations in Holstein cows. *J. Anim. Sci.*, 50 (Suppl. 1), 152.
19. **Potterton, S. L, Bell, N. J., Whay, H. R., Berry, E. A., Atkinson, O. C. D., Dean, R. S., et al., 2012:** A descriptive review of the peer and non-peer reviewed literature on the treatment and prevention of foot lameness in cattle published between 2000 and 2011. *Vet. J.*, 193, 612—616. DOI: 10.1016/j.tvjl.2012.06.040.
20. **Räber, M., Scheeder, M. R., Ossent, P., Lischer, C., Geyer, H., 2006:** The content and composition of lipids in the digital cushion of the bovine claw with respect to age and location – a preliminary report. *Vet. J.*, 172, 173—177. DOI: 10.1016/j.tvjl.2005.03.009.
21. **Moragues, A. R., Carulla, P., Mínguez, C., Villagrá, A., Estellés, F., 2021:** Dairy cows activity under heat stress: A case study in Spain. *Animals*, 11, 2305.
22. **Sogstad, A. M., Osteras, O., Fjeldaas, T., 2006:** Bovine claw and limb disorders related to reproductive performance and production diseases. *J. Dairy Sci.*, 89, 2519—2528. DOI: 10.3168/jds.S0022-0302(06)72327-X.
23. **Solano, L, Barkema, H. W., Mason, S., Pajor, E. A., LeBlanc, S. J., Orsel, K., 2016:** Prevalence and distribution of foot lesions in dairy cattle in Alberta, Canada. *J. Dairy Sci.*, 99, 6828—6841. DOI: 10.3168/jds.2016-10941.
24. **Somers, K., Frankena, J. G., Noordhuizen-Stassen, E. N., Metz, J. H. M., 2003:** Prevalence of claw disorders in Dutch dairy cows exposed to several floor systems. *J. Dairy Sci.*, 86, 2082—2093. DOI: 10.3168/jds.S0022-0302(03)73797-7.
25. **Stone, W. C., 2004:** Nutritional approaches to minimize subacute ruminal acidosis and laminitis in dairy cattle. *J. Dairy Sci.*, 87, E13—E26. DOI: 10.3168/jds.S0022-0302(04) 70057-0.
26. **Tarlton, J. F., Holah, D. E., Evans, K. M., Jones, S., Pearson, G. R., Webster, A. J. F., 2002:** Biomechanical and histopathological changes in the support structures of bovine hooves around the time of first calving. *Vet. J.*, 163, 196—204. DOI: 10.1053/tvjl.2001.0651.
27. **Thoefner, M. B., Wattle, O., Pollitt, C. C., French, K. R., Nielsen, S. S., 2005:** Histopathology of oligofructose-induced acute laminitis in heifers. *J. Dairy Sci.*, 88, 2774—2782. DOI: 10.3168/jds.S0022-0302(05)72957-X.
28. **Vermunt, J. J., 2007:** One step closer to unravelling the pathophysiology of claw horn disruption: For the sake of the cows' welfare. *Vet. J.*, 174, 219—220. DOI: 10.3168/jds.2010-3177.
29. **West, J. W., 2003:** Effects of heat stress on production in dairy cattle. *J. Dairy Sci.*, 86. 2131—2144. DOI: 10.3168/jds.S0022-0302(03)73803-X.

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DOG BEHAVIOUR PREDICTION TESTING

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ABSTRACT

Dogs exhibit behavioural heterogeneity as a result of their close proximity to people as pets, working animals, or research animals. This variability stems from their natural talents as well as contextual effects. This document examines the several types of dog behavioural tests, including those that are used to evaluate dogs and others that are used to categorize individual animals. This study revealed a lack of agreement on all of these testing procedures. Individual variations in behaviour, or personality differences, may now be quantified and described in the working dog literature. The predictive association between certain dog behavioural features (if any) and crucial working results is less well-known.

Key words: behaviour prediction; dog behaviour; puppy temperament; temperament

INTRODUCTION

Studies on the development of behaviour in domestic dogs are crucial for selecting puppies for acceptable families, identifying behavioural predispositions at an early stage, and predicting their fitness for work as support dogs. However, research on the predictive validity of tests administered during the socialization phase is mixed. Furthermore, some experts utilize neonatal testing to enhance subsequent evaluations of puppy selection as working dogs, however these tests have not been verified.

There is a lot of knowledge about the dog's behavioural expressions and individuality when it comes to testing and selecting individuals for specific uses, but there isn't a thorough consensus of ideas. The materials and procedures used in the field are discussed in this article.

Dogs exhibit a wide range of behaviour as a result of coexisting near to people throughout the domestication process, which is due to the individual's natural qualities. We will concentrate on behavioural exams, which relate to the proper selection of the individual, as everyone is unique in their own way.

Individual variances in behaviour emerge from the criteria used to assess the fitness of individual canines for specific business uses, which are now characterized by considerable variability and subjectivity of the assessor of individual features.

Characteristics of behavioural testing

Animal behavioural tests are standardized experimental conditions in which stimuli are used to elicit behaviour that is compared statistically to the behaviour of other people in the same situation in order to classify the test animal [36]. Dog behaviour tests have been created and employed in a variety of domains, including: genetic and breeding assessments [35], behavioural development and cognitive ability assessments [31], and forecasting outcomes such as adoption likelihood [22]. The authors focused on the role of behavioural tests as predictors of success (i. e., required performance during/after training and in a working position) for "working dogs," which are defined as dogs who have or are chosen for a job that is associated with either assistive work, protective work, or detection work, and this specialized work is regulated and certified. With the growing recognition of working dogs' value in assisting people with physical, emotional, and developmental problems [8, 15, 43], as well as the importance of military and police working dogs in today's global political climate [21, 38]; it's more important than ever to evaluate the quality of the procedures used to predict the success of these working animals in terms of their ability to perform the tasks they've been trained and specialize in.

Despite the high financial costs of training and acquiring military, police, and assistance dogs [27], dogs perform many valuable tasks, contributing to the development and performance of multiple industries [7], and the benefits of assistance dogs are associated with significant economic savings in terms of reduced reliance on conventional support services [14, 34]. Nonetheless, it is reported that only 50 % of working canines are fully functioning on average in different industries [2, 3, 5, 25]. Another common theme in the literature on working dogs is the feature of individual dogs' performance in specific activities, with behavioural qualities, as well as sensory sensitivity or other physical distinctions, being the most likely factors behind their success [11, 33]. This has an impact not only on the monetary value of the work done, but also on the public's impression of the value of working dogs in society [32].

There are a variety of experimental behavioural tests for analysing canine behaviour, such as observation of the dog's behaviour in a novel scenario [29] and a variety of questionnaires that are filled out by the owner or handler, such as the Positive and Negative Activation Scale (PANAS) [37]. To evaluate attributes that may be essential in working dogs, various descriptions such as "character" [42], "personality," and "temperament" are utilized [46]. When examining a dog's behaviour in various contexts, a major premise of many behavioural testing methods is to watch the dog's behaviour in numerous situations:

a) subjective assessment of specific behaviour (e. g. rest) in a test situation performed by trained or known observers, e. g. on the Likert scale (e. g. 1 = not at all calm; 6 = very calm) [23], or
b) the presence or absence of specific attitudes or behaviours (e. g. bites) to quantify behavioural tendencies (e. g. aggression) [17].

There is currently no agreement on the terminologies used to describe the behavioural profiles employed in these tests or surveys. These saws can be swapped out or utilized differently in various testing. For example, the authors refer to the Canine Behavioural Evaluation and Research Questionnaire [18] as a behavioural and temperamental assessment, while it actually defines the collection of context-specific behaviour. Some experts believe that this classification is improper because it is more accurately described as a "character" or "behavioural profile" assessment. Furthermore, most authors reserve the term "personality" for instruments that are created expressly to characterize more general physiologically based features that underpin individual variations (e. g. Monash Questionnaire on dog personality [23]). The phrase "temperament," which is reserved for instruments targeted at more limited influence construction and regulation, is widely used and misunderstood, e. g. the canine Dog Impulsivity Rating Scale; (DIAS) [46]. This distinction might help to clarify what is being examined and what the most essential aspects of the issue may be.

Although questionnaires can decrease the need for time-consuming behavioural tests that can assess behaviour in a wide range of scenarios, it is not always easy to find someone who knows enough about dogs to fill in the items accurately. This is especially true for working dog evaluations, which are frequently performed at a young age by unknown [4] and known handlers [16]. Behavioural

tests have also been demonstrated to provide a more objective assessment of a dog's behaviour than relying on the handler's or owner's own memories and views, which may have a skewed view of the dog's attachment.

The reliability and validity of behavioural observation tests should be used to measure their worth [40]. It is advised that in working dogs in particular, focus be made on behavioural assessments based on three essential criteria [39]:

1. **Evaluator consistency:** the degree to which various observers describe the same person in the same way.
2. **Repeat test reliability:** how well behavioural tests detect traits that are stable over time and context; individuals' results should be generalized across time and settings.
3. **Predictive validity:** in the case of working dogs, the feature must be related to some component of performance and hence be predictive of certification and/or long-term field performance.

In order to predict behaviour, behavioural tests must be differentiated based on their reliability and validity. Procedures for testing and evaluating working dogs can not only improve the work that these dogs do (by selecting the best individuals for each specialty), but they can also decrease the time and money spent teaching failing pups. As a result, the criteria outlined by Sinn et al. [39] are critical in the development of a test battery.

Review of dog behaviour prediction tests

Several studies have been done in the past to assess puppies and predict their adult temperament. At their study in Bar Harbor, Scott and Fuller [35] conducted a significant number of experiments on puppies and young dogs from birth to one year of age. The major goal of these tests was to identify behavioural, developmental, and genetic differences among dog breeds. One of the report's most notable result was that several features show a lot of stability along the maternal line, implying that maternal impact is quite essential. The tests in the Scott and Fuller study [35] were not used to forecast how the test dogs will be utilized in the future.

They reported a series of repeating tests done on puppies aged 8 to 12 weeks in a later research to predict their future usage as guide dogs. After the first tests at the age of eight weeks, the tests were found to be acceptable predictors, indicating that retesting is not required. However, the heritability of the studied qualities was found to be low, and the impacts of the mother's surroundings were found to be significant.

In Australia, Goddard and Beilharz [12] reviewed guide dog testing and discovered strong heritability, particularly for fear. Fear, according to these authors, can be assessed as early as 12 weeks of age, making it one of the most common reasons for dog rejection. This assertion was significantly adjusted in a later publication by Goddard and Beilharz [13], and it was suggested that the predictive value be increased if dogs were tested later in life.

A variety of tests on young puppies were also carried out in order to assess the impact of various experimental circumstances. Dykman et al. [9] reported a puppy test that was used to distinguish between dogs from stable and unstable lines. Newton et al. [28] compared behavioural tests to physiological parameters in a later study, concluding that physiological tests can distinguish neurologically unstable and stable dogs with 100% confidence, while behavioural tests can do so with 95% confidence. Martinek et al. [26] found that puppies' capacity to move from a cold to a warmer surface differed at four months of age, and that this skill correlated with addiction testing. Puppy behaviour tests were also documented by Fox [10] and Campell [6], which were used to select puppies from a litter. The goal of these experiments was to see if puppy testing could be used to predict how beneficial adults will be.

The goal of puppy behavioural testing is to determine the degree of effect of the handler on puppy and adult dog behaviour. The findings of behavioural tests performed on puppies are then compared to the results of behavioural tests performed on adults, which has proven to be the most successful technique for selecting dogs for work and breeding [41, 44].

Pfaffenberger et al. [30] evaluated the temperament of border collie puppies in their initial days of life, then repeated the tests at 6 weeks with the same puppies. Temperament tests in the first week of life did not reveal a predictive value of puppy behaviour at 6 weeks in this sample of 18 border collie puppies. According to Pfaffenberger et al. [30], it is required to determine the validity and predictive usefulness of temperament tests of puppies at the age of 6 weeks for the tendentious behaviour of an adult in adulthood. These findings come from a pilot

investigation that will be followed by a study including roughly 60 border collie puppies who will be assessed at less than one week, six weeks, and eighteen months of age to examine the development of an individual's personality.

According to the findings by Kutzler's [20] investigations, the predictive validity of puppy tests is more likely to be exhibited when testing aggression and submissiveness, and less predictive when measuring other character qualities including trainability, fear, overall activity, and sociability. The author also contends that puppy socialization is critical for improved obedience and reduced fear responses, which can be accomplished through puppy classes in which puppies of similar ages socialize with one another.

The time and financial demands of questionnaire testing have sparked a new trend. For example, a research on the validation of C-BARQ in military canines was undertaken in Sweden. In their investigation, Foyer et al. [11] discovered a link between individual dogs' success and their scores in individual C-BARQ subcategories. They discovered a substantial positive association in areas like trainability and hyperactivity, as well as a large negative correlation in areas like non-social dread and fear of strangers. They also point to the handler's strong influence on a child's behaviour during the first year of life, as well as behavioural predictive research's inclination to focus on qualities that negatively affect performance [11].

Hunt et al. [19] used a questionnaire to investigate the predictive ability of behavioural tests in guide and assistance dogs. "The revised Puppy Walker Questionare, r-PWQ," which was completed by volunteer puppy carers, was used. The questionnaire is for eight-month-old dogs. R-PWQ has traits like "distraction" and "excitement," which are common reasons for dogs leaving the guide dog program. The Canine Behavioural Assessment and Research Questionnaire (C-BARQ) was used to compare the predictive validity of the r-PWQ. The findings suggested that r-PWQ can be used to predict a guide dog's prognosis and that it may be more appropriate for guide dog populations than C-BARQ [19].

Barnard et al. [4] identified two objectives for their research. First, to see if instruments commonly used to measure personality in dogs can be used to determine individual personality features of puppies as early as 2 months of age (i. e. an adjective based questionnaire and a method of coding behaviour). Second, to look at how the two methodologies compare when it comes to defining behavioural patterns. Overall, the questionnaire and behavioural analyses revealed that when puppies are exposed to both social and non-social stimuli in an open-field test at 2 months of age, they already demonstrate specific behavioural patterns that can be identified for personality traits [4]. Both techniques were found to be reliable for assessing personality traits, with excellent inter-observer reliability in both cases, corroborating prior findings [45], and strong personality trait correspondence between the two independent measures utilized [4].

The research of Alberghina et al. [1] is another study that is indirectly related to behavioural testing. The authors looked at the consistency of assessors and the factors that influenced the outcome of a basic puppy test. Three people observed the puppies' conduct, which was clearly characterized by the test evaluation categories. Except for one subtest, the individual tests were found to have a high degree of agreement. Although some protocol modifications are required for this subtest, the low variability and high Kendall's W values demonstrate the test's validity. The three observers had a modest level of variability. There are few cases of evaluators agreeing among two or more independent observers [24]. According to these research, multiple individuals could rate the same puppy using this scoring method in the same way [1]. This study supports the feasibility of developing a behavioural battery that can produce consistent findings when administered by various test administrations.

CONCLUSIONS

Inconsistencies in terminology, research parameters, and success indicators were discovered in the review, as well as a general lack of information about the reliability and validity of behaviour assessment measures. To increase the knowledge base of what attributes are predictive of optimal performance in working dog jobs, improve selection procedures, and reduce working dog redundancy, there is a need to standardize the reporting of these components of behavioural testing. We recommend that one utilize a framework that explains the test's direct or indirect link to core affect.

The approaches may be used to a variety of cynology fields. Whether it will be a different concentration of working or support dogs, where a technique is still miss-

ing. Or for use in laic cynology, such as when selecting and placing dogs from shelters, where a behavioural profile for each dog would be produced to make adoption easier. For instance, while selecting a suitable dog for the home that is tailored to the personality and temperament of the future owner.

REFERENCES

1. **Alberghina, D., Giunta, F., Gioè, M., Panzera, M., 2020:** Behaviour test for seven-week old puppies (*Canis familiaris*): Inter-rater reliability and factors associated with test performance. *PLOS ONE*, 15, 7, e0236271.
2. **Arnott, E. R., Early, J. B., Wade, C. M., McGreevy, P. D., 2014a:** Estimating the economic value of Australian stock herding dogs. *Animal Welfare*, 23, 2, 189—97. DOI: 10.7120/09627286.23.2.189.
3. **Arnott, E. R., Early, J. B., Wade, C. M., Mcgreevy, P. D., 2014b:** Environmental factors associated with success rates of Australian stock herding dogs. *PLOS ONE*, 9, 8, e104457. DOI: 10.1371/journal. pone. 0104457.
4. **Barnard, S., Marshall-Pescini, S., Passalacqua, C., Beghelli, V., Capra, A., Normando, S., Valsecchi, P., 2016:** Does subjective rating reflect behavioural coding? Personality in 2 month-old dog puppies: an open-field test and adjective-based questionnaire. *PLOS ONE*, 11, 3, e0149831.
5. **Batt, L. S., Batt, M. S., Baguley, J. A., McGreevy, P. D., 2008:** Factors associated with success in guide dog training. *J. Vet. Behav. Clin. App. Res.*, 3, 4, 143—51. DOI: 10.1016/j.jveb.2008.04.003.
6. **Campell, W. L., 1975:** A behaviour test for puppy selection. In **Campell, W. L.:** *Behaviour Problems in Dogs*. Am. Vet. Publ., Santa Barbara, 137—144.
7. **Cobb, M., Branson, N., McGreevy, P., Lill, A., Bennett, P., 2015:** The advent of canine performance science: Offering a sustainable future for working dogs. *Behav. Processes*, 110, 96–104. DOI: 10.1016/j.beproc.2014.10.012.
8. **Duffy, D. L., Serpell, J. A., 2012:** Predictive validity of a method for evaluating temperament in young guide and service dogs. *Appl. Anim. Behav. Sci.*, 138, 1—2, 99—109. DOI: 10.1016/j.applanim.2012.02.011.
9. **Dykman, R. A., Murphree, O. D., Ackerman, P. T., 1965:** Litter patterns in the offspring of nervous and stable dogs: II. Autonomic and motor conditioning. *J. Nerv. Mental Dis.*, 141, 4, 419—431.
10. **Fox, M. W., 1972:** *Understanding Your Dog*. Coward, McCann and Geoghegan. Inc., New York, NY, 74 pp.
11. **Foyer, P., Bjällerhag, N., Wilsson, E., Jensen, P., 2014:** Behaviour and experiences of dogs during the first year of life predict the outcome in a later temperament test. *Appl. Anim. Behav. Sci.*, 155, 93—100. DOI: 10.1016/j.applanim.2014.03.006.
12. **Goddard, M. E., Beilharz, R. G., 1984:** A factor analysis of fearfulness in potential guide dogs. *Appl. Anim. Behav. Sci.*, 12, 3, 253—265. DOI: 10.1016/0168-1591(84)90118-7.
13. **Goddard, M. E., Beilharz, R. G., 1986:** Early prediction of adult behaviour in potential guide dogs. *Appl. Anim. Behav. Sci.*, 15, 3, 247—260.
14. **Hall, S., Dolling, L., Bristow, K., Fuller, T., Mills, D. S., 2016:** *Companion Animal Economics: The Economic Impact of Companion Animals in the UK*. United Kingdom, 80 pp.
15. **Hall, S. S., MacMichael, J., Turner, A., Mills, D. S., 2017:** A survey of the impact of owning a service dog on quality of life for individuals with physical and hearing disability: a pilot study. *Health Qual. Life Outcomes*, 15, 1, 59. DOI: 10.1186/s12955-017-0640-x.
16. **Harvey, N. D., Craigon, P. J., Blythe, S. A., England, G. C. W., Ashe, L., 2017:** An evidence-based decision assistance model for predicting training outcome in juvenile guide dogs. *PLOS ONE*, 12, 6, e0174261. DOI: 10.1371/journal.pone.0174261.
17. **Haverbek, E. A., De Smet, A., Depiereux, E., Giffroy, J. M., Diederich, C., 2009:** Assessing undesired aggression in military working dogs. *Appl. Anim. Behav. Sci.*, 117, 1—2, 55—62. DOI: 10.1016/j.applanim.2008.12.002.
18. **Hsu, Y., Serpell, J. A., 2003:** Development and validation of a questionnaire for measuring behavior and temperament traits in pet dogs. *J. Am. Vet. Med. Assoc.*, 223, 9, 1293—300. DOI: 10.2460/javma.2003.223.1293
19. **Hunt, R. L., England, G. C., Asher, L., Whiteside, H., Harvey, N. D., 2020:** Concurrent and predictive criterion validity of a puppy behaviour questionnaire for predicting training outcome in juvenile guide dogs. *Animals*, 10, 12, 2382.
20. **Kutzler, M., 2014:** Puppy behavioor, socialization, and personality. *Adv. Small Anim. Med. Surg.*, 27, 6, 1—3.
21. **Lazarowski, L., Dorman, D. C., 2014:** Explosives detection by military working dogs: Olfactory generalization from components to mixtures. *Appl. Anim. Behav. Sci.*, 151, 84—93. DOI: 10.1016/j.applanim.2013.11.010.
22. **Ledger, R. A., Baxter, M. R., 1997:** The development of a validated test to assess the temperament of dogs in a res-

cue shelter. In *Proceedings of the First International Conference on Veterinary Behavioural Medicine,* Birmingham, UK, 87—92.

23. **Ley, J., Bennett, P., Coleman, G., 2007:** Personality dimensions that merge in companion canines. *Appl. Anim. Behav. Sci.*, 110, 3. DOI: 10.1016/j.applanim.2007.04. 016.
24. **Ley, J., Bennett, P., Coleman, G., 2008:** Personality dimensions that emerge in companion canines. *Appl. Anim. Behav. Sci.*, 110, 3—4, 305—317. DOI: 10.1016/j.applanim. 2007.04.016.
25. **Maejima, M., Inoue-Murayama, M., Tonosaki, K., Matsuura, N., Kato, S., Saito, Y., et al., 2007:** Traits and genotypes may predict the successful training of drug detection dogs. *Appl. Anim. Behav. Sci.*, 107, 3—4, 287—98. DOI: 10. 1016/j.applanim.2006.10.005.
26. **Martinek, Z., Lát, J., Pribik, V., 1978:** Individual behavioral differences in puppies during neonatal-period. *Physiologia Bohemoslovaca*, 27, 3, 260—260. Publ. house Czech Academia Science, Vodickova 40, Prague, Czech Republic.
27. **Moore, G. E., Burkman, K. D., Carter, M. N., Peterson, M. R., 2001:** Causes of death or reasons for euthanasia in military working dogs: 927 cases (1993—1996). *J. Am. Vet. Med. Assoc.*, 219, 2, 209—14. DOI: 10.2460/javma.2001. 219.209.
28. **Newton, J. E. O., Chapin, J. L., 1978:** Operant performance patterns of normal and nervous water-deprived dogs. *The Pavlovian Journal of Biological Science: Official Journal of the Pavlovian*, 13, 1, 22—28.
29. **Palestrini, C., Previde, E. P., Spiezio, C., Verga, M., 2005:** Heart rate and behavioural responses of dogs in the Ainsworth's strange situation: a pilot study. *Appl. Anim. Behav. Sci.*, 94, 1—2, 75—88. DOI: 10.1016/j.applanim. 2005.02.005.
30. **Pfaffenberger, C. J., Scott, J. P., Fuller, J. L., Ginsburg, B. E., Bielfelt, S. W., 1976:** *Guide Dogs for the Blind: Their Selection. Development, and Training*. Elsevier, Amsterdam, 23—37.
31. **Pongrácz, P., Miklósi, Á., Vida, V., Csányi, V., 2005:** The pet dogs ability for learning from a human demonstrator in a detour task is independent from the breed and age. *Appl. Anim. Behav. Sci.*, 90, 3—4, 309—23. DOI: 10.1016/j.appla nim.2004.08.004.
32. **Rayment, D. J., De Groef, B., Peters, R. A., Marston, L. C., 2015:** Applied personality assessment in domestic dogs: Limitations and caveats. *Appl. Anim. Behav. Sci.*, 163, 1—18. DOI: 10.1016/j.applanim.2014.11.020.
33. **Rooney, N. J., Gaines, S. A., Bradshaw, J. W. S., Penman, S., 2007:** Validation of a method for assessing the ability of trainee specialist search dogs. *Appl. Anim. Behav. Sci.*, 103, 1—2, 90—104. DOI: 10.1016/j.applanim.2006.03.016.
34. **Sanders, C. R., 2000:** The impact of guide dogs on the identity of people with visual impairments. *Anthrozoös*, 13, 3, 131—9. DOI: 10.2752/089279300786999815.
35. **Scott, J. P., Fuller, J. L., 1965:** *Genetics and the Social Behaviour of the Dog*. Chicago, University of Chicago Press, 506 pp.
36. **Serpel, J. A., Hsu, Y., Scott, J. P., Fuller, J. I., 2001:** Development and validation of a novel method for evaluating behavior and temperament in guide dogs. *Appl. Anim. Behav. Sci.*, 72, 4, 347—64. DOI: 10.1016/S0168-1591(00)00210-0.
37. **Sheppard, G., Mills, D., 2002:** The development of a psychometric scale for the evaluation of the emotional predispositions of pet dogs. *Int. J. Comp. Psychol.*, 15, 1, 201—22.
38. **Sherman, B. L., Gruen, M. E., Case, B. C., Foster, M. L., Fish, R. E., Lazarowski, L., et al., 2015:** A test for the evaluation of emotional reactivity in Labrador retrievers used for explosives detection. *J. Vet. Behav. Clin. Appl. Res.*, 10, 2, 94—102. DOI: 10.1016/j.jveb.2014.12.007.
39. **Sinn, D. L., Gosling, S. D., Hilliard, S., 2010:** Personality and performance in military working dogs: Reliability and predictive validity of behavioral tests. *Appl. Anim. Behav. Sci.*, 127, 1—2, 51—65. DOI: 10.1016/j.applanim.2010.08.007.
40. **Taylor, K. D., Mills, D. S., 2006:** The development and assessment of temperament tests for adult companion dogs. *J. Vet. Behav. Clin. Appl. Res.*, 1, 3, 94—108. DOI: 10.1016/j. jveb.2006.09.002.
41. **Tomkins, L. M., Thomson, P. C., McGreevy, P. D., 2011:** Behavioural and physiological predictors of guide dog success. *J. Vet. Behav. Clin. Appl. Res.*, 6, 3, 178—87. DOI: 10. 1016/j.jveb.2010.12.002.
42. **Trybocka, R., 2010:** Character assessment testing to test suitability for guide dogs. *Vet. Nurs. J.*, 25, 11, 32—3. DOI: 10. 1111/j.2045-0648.2010.tb00101.x.
43. **Walther, S., Yamamoto, M., Thigpen, A. P., Garcia, A., Willits, N. H., Hart, L. A., 2017:** Assistance dogs: historic patterns and roles of dogs placed by ADI or IGDF accredited facilities and by non-accredited U. S. facilities. *Front. Vet. Sci.*, 4, 1. DOI: 10.3389/fvets.2017.00001.
44. **Wilsson, E., Sundgren, P. E., 1997:** The use of a behaviour test for selection of dogs for service and breeding. II. Heritability for tested parameters and effect of selection based on service dog characteristics. *Appl. Anim. Behav. Sci*., 54, 2–3, 235—41. DOI: 10.1016/S0168-1591(96)01175-6.

45. **Wilsson, E., Sundgren, P. E., 1998:** Behaviour test for eight-week old puppies heritabilities of tested behaviour traits and its correspondence to later behaviour. *Appl. Anim. Behav. Sci.*, 58, 1—2, 151—162.

46. **Wright, H. F., Mills, D. S., Pollux, P. M., 2011:** Development and validation of a psychometric tool for assessing impulsivity in the domestic dog (*Canis familiaris*). *Int. J. Comp. Psychol.*, 24, 2, 210—225.

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IMMUNOHISTOCHEMICAL STUDY OF SMOOTH MUSCLE CELLS AND ELASTIN IN GOOSE LUNGS

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ABSTRACT

There are many differences (morphological, physiological and mechanical) between the lungs of birds and the lungs of mammals. Birds have a more efficient exchange of oxygen and carbon dioxide than mammals. In this article, we studied the presence of four antibodies (actin, α-smooth muscle actin, desmin and elastin) in the lungs of geese. Smooth muscle cells (SMCs) immunoreactive to actin, α-SMA and desmin were observed in the primary and secondary bronchi and arranged as a continuous layer. In the tertiary bronchus (parabronchus), immunoreactive cells on α-SMA and desmin were observed as aggregations of smooth muscle cells in the septum tips in atrial opening. A small number of α-SMA and desmin-positive cells were observed on the periphery of the parabronchi and between the air and blood capillaries. The elastic fibres were found in the large bronchi in connection with smooth muscle bands. In the parabronchi the elastic fibres form an elastic membrane lining the parabronchial lumen. In the blood vessels, the elastic fibres form the inner and outer elastic membrane. The individual elastic membranes connect neighbouring blood vessels.

Key words: elastin; goose; immunohistochemistry; lung; smooth muscle cells

INTRODUCTION

The respiratory system of birds differs significantly from that of mammals. Birds have relatively stiff lungs, lack a diaphragm, and the lungs open into 9 air sacs that act as mosses with a unidirectional airflow pattern [7, 14, 18]. The birds must be able to exchange gases at a high rate, as their oxygen consumption at rest is higher than that of all other vertebrates, including mammals, and increases many times during the flight. The volume of gas in the lungs of birds is small compared to the volume of mammals, but the lungs are connected to large air sacs by a series of tubes, making the total volume of the respirato-

ry system about twice as large as mammals of comparable size. Due to the constant one-way air flow, the lungs of birds are better ventilated than the lungs of mammals.

The smooth muscle can also apply mechanical forces to physically sculpt epithelia [21]. As a mesenchymal tissue, the smooth muscle can participate in reciprocal signalling with the epithelium to generate complex patterns of epithelial folds. Smooth muscle is now recognized as a key contributor to the morphogenesis of branched and folded organs [8].

Smooth muscle cells (SMCs) are a major structural and functional component of many organs during embryonic development and adulthood. These cells are a key component of the structure and physiology of vertebrates [3]. SMCs can be organized very differently and perform specialized functions. SMCs play an important role during organ development and morphogenesis and provide different functions in adulthood depending on their organ location and distribution. Although α-SMCs are known to play a key role mainly postnatally, their role in lung morphogenesis has been studied in mammals [11, 12]. The position and morphology of the domain branches are highly stereotyped, as is the SMCs pattern, which varies around the base of each branch [5].

SMCs are specialized cells found mainly in the walls or near hollow organs, including the circulatory and respiratory systems [6, 23]. In the lung, SMCs surround the bronchial tree and play an important role in the structure and function of the airways [24]. These cells form layers around the periphery of the bronchial tree, with differences in organization between the upper and lower airways [1]. SMCs play an integral part in the pathogenesis of chronic airway diseases, where they contribute to airway remodelling and inflammation [3].

Actin is a major component of the cytoskeleton and is present in most cell types and was localized as a stable component of SMCs. Today, smooth muscle is identified as a tissue containing cells expressing α-smooth muscle actin (α-SMA). α-SMA is a differentiation marker of smooth muscle cells and is also present in a special type of fibroblast called myofibroblast [28]. Similar to other vascular contractile cells, pulmonary arterial smooth muscle cells play central roles in physiological and pathologic vascular remodelling because of their remarkable ability to dynamically modulate their phenotype to ensure contractile and synthetic functions [4].

Desmin is a cytoskeletal protein that has been shown to contribute to the mechanical properties of skeletal, cardiac and visceral smooth muscle. Desmin influences airway compliance, lung recoil, and airway contractile responsiveness. Therefore, desmin could be well situated to participate in passive and contractile force transmission in the lungs. The desmin could also affect the axial mechanical properties of the airways [21].

Elastin is expressed in most tissues that require elastic recoil. Elastin is a critical component of the pulmonary interstitium, which provides a recoil property into the vascular, conductive airways and terminal air space of the lungs [13]. The elastin is widely distributed in compartments of the mammalian lung including pleura, septa, large vessels, and elastic cartilage. Elastin is expressed by several types of cells in the lungs, including mesothelial cells in the pleura, smooth muscle cells in the airways and blood vessels, endothelial cells, and interstitial fibroblasts [15].

The aim of this study was to describe the presence of actin, SMA and desmin in smooth muscle cells and elastin in the lungs of geese using immunohistochemical methods.

MATERIALS AND METHODS

The lungs of five adult clinically healthy geese of Slovak white goose breed were used in this study. The birds were sacrificed and their lungs were removed. Lung samples were excised and fixed in 10% buffered formalin and processed into paraffin sectioning. For histological study, 5 µm thick sections were stained with Harris haematoxylin and eosin. For immunohistochemical study the sections were pre-treated with 3% H_2O_2 in methanol and pre-incubated with 2% goat serum. The sections were incubated with the primary antibodies (Table 1) and washed in phosphate-balanced salt solution (PBS). Afterwards, the sections were incubated with biotinylated secondary antibody for 45 min, washed in PBS, and finely incubated with avidin-biotin-peroxidase complex (ABC kits, Vector Laboratories, USA). The reaction product formation was achieved by incubating for 10 minutes at room temperature, using a mixture of an equal volume of 0.02 % hydrogen peroxide and 0.1% 3,3´-diaminobenzidine tetrahydrochloride made in Tris buffer. For negative controls, the first antibody was substituted by PBS or by normal rabbit serum.

Table 1. Antibodies used in the study

Antibodies	Donor	Isotype	Dilution	Source
Actin	Rabbit	IgG2a	1 : 50	Sanbio
α-Smooth muscle actin	Mouse	IgG2a	1 : 200	Sanbio
Desmin	Mouse	IgG1	1 : 5000	Dako
Elastin	Mouse	IgG2a	1 : 100	Sigma

Anatomy of bird lungs

Morphologically, the birds' respiratory system is divided into lungs (gas exchange part) and air sacs (non-respiratory part). The avian lung airway system includes a three-layer air duct system, primary bronchus, secondary bronchus, and tertiary bronchus. Parabronchus (tertiary bronchus) occupy more than half the volume of a bird's lungs in the form of dense bundles of tubes. The wall of each parabronchus was pierced by numerous, more or less pentagonal or hexagonal openings that connected the air lumen to pocket-like compartments called atria which lead to the infundibulae. Infundibulae in turn form air capillaries that are tightly connected by a network of blood capillaries that form tissue-exchanging tissue in the lungs.

Actin and α-SMA

Goose lungs have actin and α-SMA immunoreactive smooth muscle cells organized as plates, bands, or individual cells, depending on the location of the site. Smooth muscle cells form a well-developed layer in the primary bronchi, which, like small bundles, extends into the secondary bronchi. Actin is expressed in SMCs, which form a main layer of blood vessels (Fig. 1).

The α-SMA-positive SMCs were observed as a distinct layer in the wall of the primary and secondary bronchi. In addition, positive SMCs were present in the blood vessels mainly in arteries where they form a thick layer (Fig. 2). In the tertiary bronchus (parabronchus), α-SMA-positive cells were found at the tips of the atrial septum. Here, the smooth muscle cells were observed as thickening of parabronchial wall lining the parabronchial lumen. Individual positive smooth muscle cells were observed in the interlobular space as one component of the parabronchial mantle (Fig. 2).

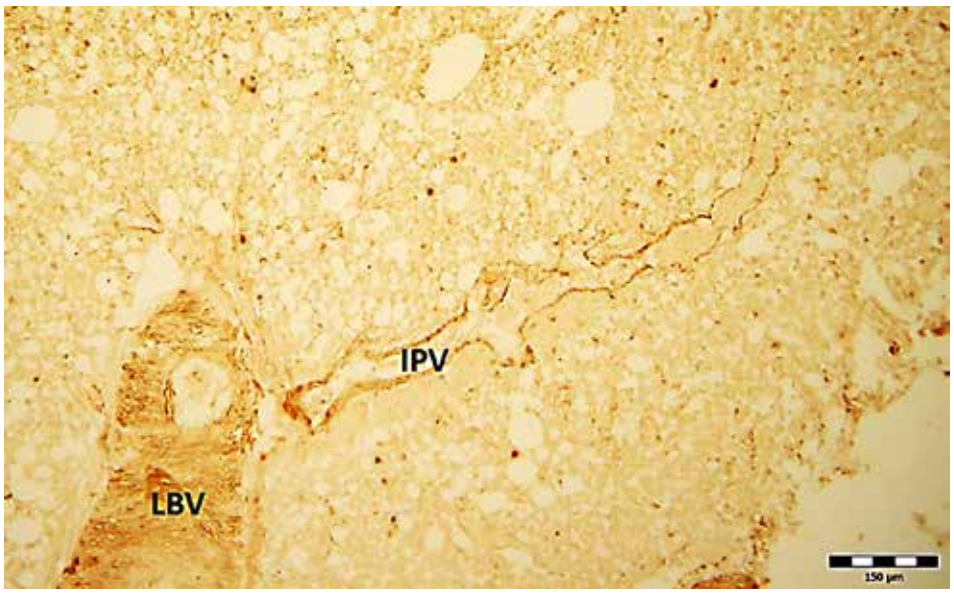

Fig. 1. Immunostaining for actin. Photomicrograph showing a large blood vessel (LBV), intraparabronchial vessel (IPV) and their branches

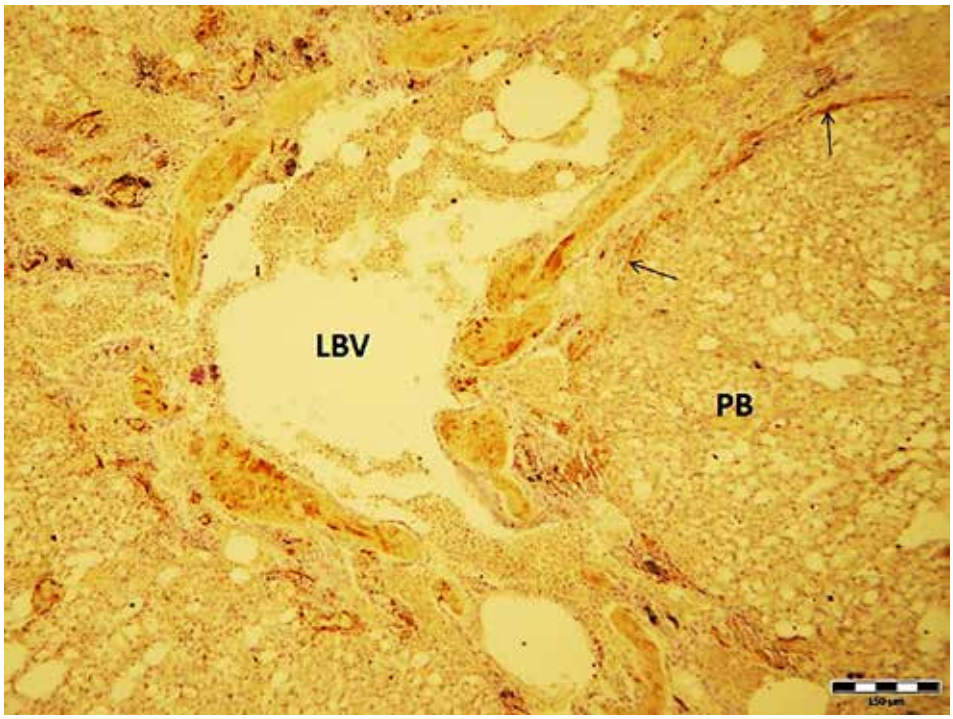

Fig. 2. Immunostaining for SMA. Photomicrograph showing a large blood vessel (LBV) with bands of desmin-positive smooth muscle cells. Small amounts of positive SMCs are on the periphery of the parabronchus (PB) (arrows)

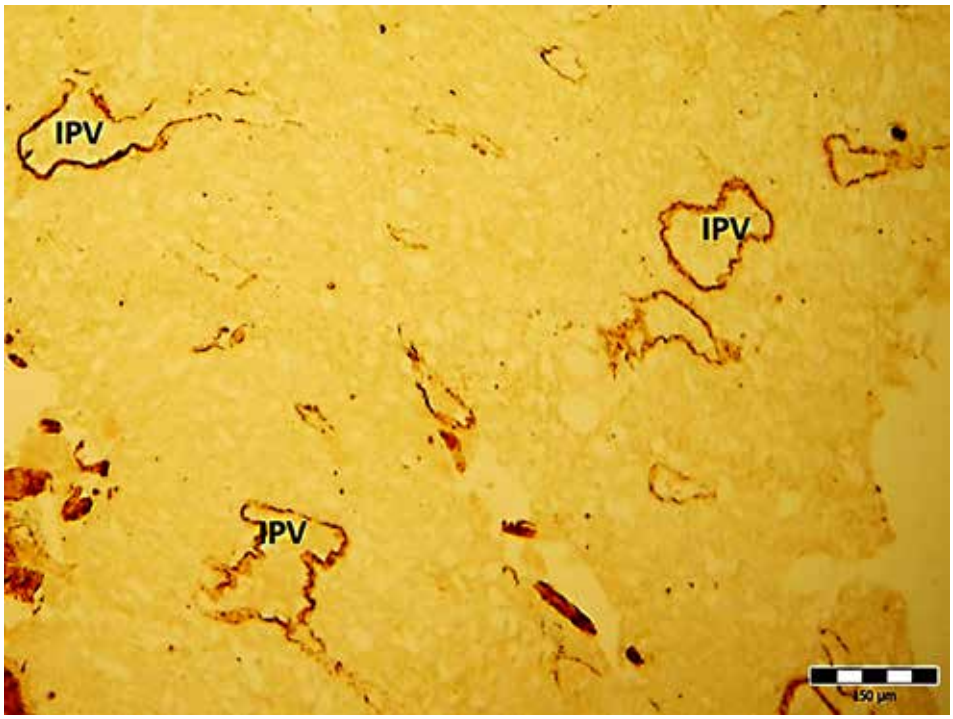

Fig. 3. Immunostaining for SMA. Photomicrograph showing intraparabronchial blood vessels (IPV) and their branches with positive SMCs

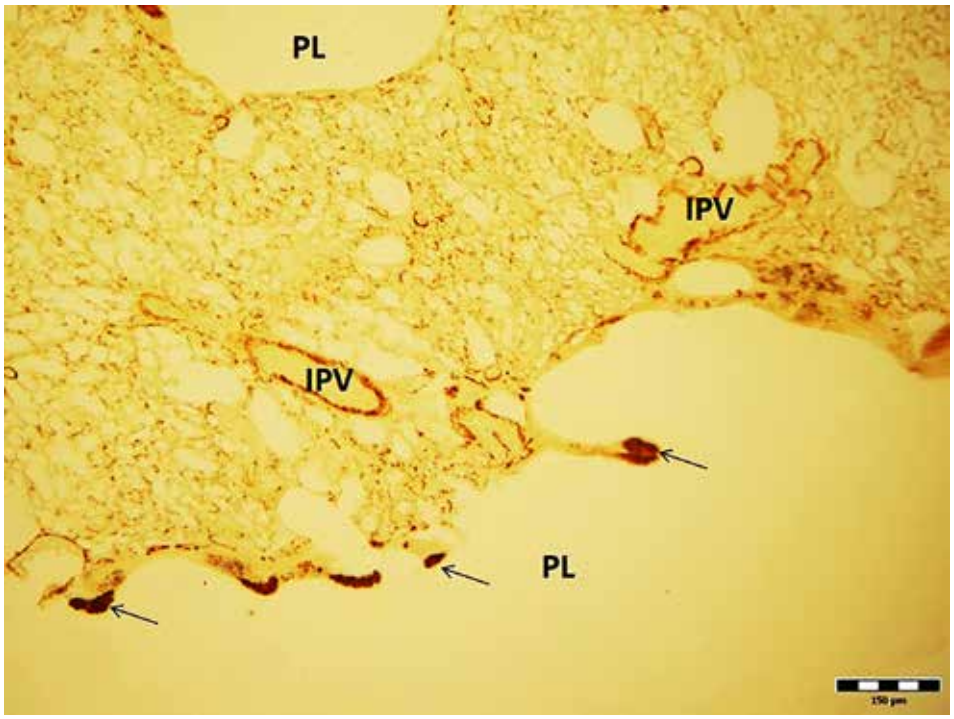

Fig. 4. Immunostaining for desmin. Photomicrograph showing a portion of the parabronchial lumen (PL) surrounded by a parenchyma. The tips of the parabronchial wall contain SMCs intensely stained for desmin (arrows). Two positive intraparabronchial blood vessels (IPV) are inside the lung parenchyma

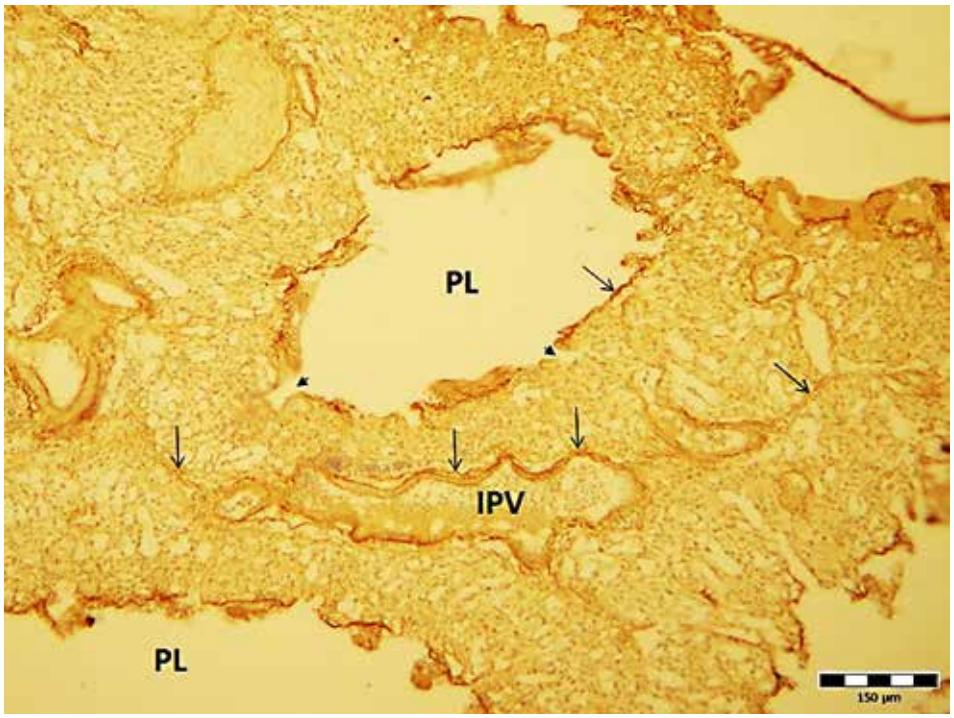

Fig. 5. Immunostaining for elastin. Photomicrograph showing intraparabronchial blood vessels (IPV) with positive elastic membranes. The parabronchial lumen (PL) is lined by a positive elastic membrane interrupted by atrial invagination (arrowheads). The individual elastic membranes connect neighbouring blood vessels (arrows)

Desmin

In goose lungs, desmin was expressed in the SMCs located in the airway walls and inside the parenchyma in relation with bronchi. In the primary and secondary intrapulmonary bronchi, they form a complete or incomplete wall. High accumulation of desmin-positive SMCs was observed at the periphery of the parabronchial cavity with coarse spikes (Fig. 4). Small amounts of positive SMCs were observed between the air capillaries and blood capillaries. Individual desmin-positive SMCs were observed in the interlobular septum.

Elastin

The elastic fibres in the lungs of geese were found mainly in the wall of the blood vessels and a thick membrane on the luminal side of the parabronchi. Elastin in the blood vessels were seen as a distinct outer elastic membrane (Fig. 5). Some elastic fibres connect neighbouring blood vessels. A small accumulation of elastic fibres was also present as one component of the fibrous tissue in the space between the parabronchi. In addition, a high accumulation of elastic fibres as a thick elastic membrane were observed lining the parabronchial cavity. His identity was disrupted in the atrial invagination (Fig. 5).

DISCUSSION

Smooth muscle cells surround the epithelium of various organs including: the intestine, blood vessels, lungs, bladder, ureter, uterus, fallopian tube, and epididymis. The fundamental role of airway smooth muscle in the lung formation is particularly evident because this tissue has no clear physiological function in adults [9, 20]. The vascular and visceral tissues of the lung smooth muscle performs a number of tasks that are critical to lung function [26]. Mouse smooth muscle cells envelop the airway epithelium and progress from a thick layer around the bronchi to thinner bundles around the small conductive airways as well as the larger airways in the lungs [26]. Interactions between the epithelium and smooth muscle are likely to be predominantly mechanical [9].

α-SMA-immunoreactive cells have already been described in respiratory bronchioles and alveolar ducts [16, 29]. Mitchel et al. [16] and Wagner et al. [27] reported that α-SMA-containing cells are found in mature alveolar interstitium. Septal interstitial cells containing α-SMA may play an important role in alveolar formation. Immunoelectron microscopy has shown that α-SMA is localized mainly in cell protrusions [28]. In rats, α-SMA-containing cells were found in primitive alveolar septa during perinatal and early postnatal days. In adult lungs, α-SMA-positive cells were found only in the alveolar ducts, but were not found in the secondary septum. In older rats, α-SMA immunoreactive cells were elevated at the tips of the septum. These results suggest that some interstitial septal cells are transiently α-SMA positive during maturation [28]. In birds, α-SMA-containing cells were concentrated in the atrial tips of adults. The increase in α-SMA immunoreactive cells in rats may correspond to the accumulation of SMCs as we observed in goose lungs.

Desmin is an intracellular load-bearing protein that influences airway compliance and lung recoil [21]. In the lungs, desmin is expressed by smooth muscle cells located in the walls of the airways [6] and alveolar ducts [2, 29]. The desmin filaments expressed in cells containing α-SMA are largely circumferentially arranged [21]. This arrangement of desmin could affect the axial mechanical properties of current cells. According to Sjuve et al. [22], the desmin fibres contribute to the stabilization of the contractile unit and are involved in the transmission of stress between contractile units and anchorage sites associated with the extracellular matrix. Myofibroblasts, which are commonly found in alveolar septa, have been shown to express desmin in some species, such as rodents and pigs [10].

More comprehensive mammalian studies have reinforced the notion that the epithelium and endothelium regulate smooth muscle contraction *in vivo* by releasing specific factors. Ruan et al. [17] considered the epithelium to be an important regulator of smooth muscle contraction in many vital organs or tissues through interaction with other cell types and the release of epithelial-derived factors. Among these prostaglandins, they have been shown to play a multifaceted role in controlling smooth muscle contraction. Endothelium is a source of molecules that either stimulate or inhibit the contraction of essential SMCs, cytokines and growth factors, as well as nitric oxide and endothelin, which affect vascular tone and SMCs contraction/relaxation. Such factors may interact with other stimuli, such as proliferation, migration, and differentiation [25]. The fundamental role for airway smooth muscle in

shaping the lung is particularly revealing because this tissue has no clear physiological function in the adult [9, 20].

The elastic fibres are important for the elasticity and extensibility of lung tissue. In developing lungs, the elastic fibres appear in greatest numbers during the process or period of alveolarization [19]. In terms of chemical properties, elastin is one of the most non-polar proteins secreted by mammalian cells. In addition, elastin is one of the longest-lived proteins secreted into the extracellular matrix [19]. In mammals, elastic fibres are present in the lung structures such as: the trachea, bronchi, airways including the alveoli, alveolar ducts, blood vessels and pleura [13]. In the geese, elastin fibres were distributed mainly in the bronchi and in the wall of blood vessels. A special organization of elastic fibres was found in the parabronchi.

Elastin is expressed by several cell types in the lungs, including pleural mesothelial cells, airway and blood vessel smooth muscle cells, endothelial cells, and interstitial lung fibroblasts [15]. The elastic fibres can be woven into many different shapes depending on the mechanical needs of the tissue. In large pulmonary vessels, elastin forms continuous layers or lamellae that separate the smooth muscle layers. Outside the vasculature, the elastic fibres form an extensive network of fibres [15]. The specific localization of elastic fibres in relation to blood vessels in geese may represent a functional difference in the oxygen demand of birds.

REFERENCES

1. **Amrani, Y., Panettieri, R. A., 2003:** Airway smooth muscle: contraction and beyond. *J. Biochem. Cell Biol.*, 35, 272—276. DOI: 10.1016/S1357-2725(02)00259-5.
2. **Costa, M. L., Escaleira, R., Cataldo, A., Oliv, F., Mermelstein, C. S., 2004:** Desmin: Molecular interactions and putative functions of the molecule intermediate filament protein. Braz. *J. Med. Biol. Res.*, 37, 1819—1830.
3. **Donadon, M., Santoro, M. M., 2021:** The origin and mechanisms of smooth muscle cell development in vertebrates. *Development*, 31, 148 (7), dev197384. DOI: 10.1242/dev.197384.
4. **Duncker, H. R., 1974:** Structure of the avian respiratory tract. *Resp. Physiol.*, 22, 1—2, 1—19.
5. **Goodwin, K., Mao, S., Guyomar, T., Miller, E., Radisky, D., Kosmrlj, A., Nelson, M., 2019:** Smooth muscle differentiation shapes domain branches during mouse lung development. *Development*, 25, 146 (22), dev181172. DOI: 10.1242/dev.181172.
6. **Halayko, A. J., Salari, H., Ma, X., Stephens, N. L., 1996:** Markers of airway smooth muscle cell phenotype. *Am. J. Physiol. Lung. Cell. Mol. Physiol.*, 270, L1040—L1051, DOI: 10.1152/ajplung.1996.270.6L.
7. **Härtle, S., Kaspers, B., 2014:** The avian respiratory immune system. In **Schat, K. A., Kaspers, B., Kaiser, P. (Eds.):** *Avian Immunology*, 2nd edn., Academic Press, New York, 456 pp.
8. **Jaslove, J. M., Nelson, C. M., 2018:** Smooth muscle: a stiff sculptor of epithelial shapes. *Phil. Trans. R. Soc.*, 373. 20170318. DOI: 10.1098/rstb.2017.0318.
9. **Jesudason, E. C., 2009:** Airway smooth muscle: an architect of the lung? *Thorax*, 64, 541—545. DOI: 10.1136/thx.2008.107094.
10. **Kapanci, Y., Ribaux, C., Chaponnier, C., Gabbiani, G., 1992:** Cytoskeletal features of alveolar myofibroblasts and pericytes in normal human and rat lung. *J. Histochem. Cytochem.*, 40, 1955—1963. DOI: 10.1177/40.12.1333502.
11. **Kim, H. Y., Pang, M. F., Varner, V. D., Kojima, L., Miller, E., Radisky, D. C., Nelson, C. M., 2015:** Localized smooth muscle differentiation is essential for epithelial bifurcation during branching morphogenesis of the mammalian lung. *Dev. Cell*, 34, 719—726. DOI: 10.1016/j.devcel.2015.08.012.
12. **Low, R. B., White, S. L., 1998:** Lung smooth muscle differentiation. *Int. J. Biochem. Cell, Biol.*, 30, 869—883. DOI: 10.1016/s1357-2725(98)00049.
13. **Mariani, T. J., Sandefur, S., Pierce, R. A., 1997:** Elastin in lung development. *Exp. Lung Res.*, 23, 131—145. DOI: 10.3109/01902149709074026.
14. **McLelland, J., 1989:** Anatomy of the lungs and air sacs. In **King, A. S., McLelland, J. (Eds.):** *Form and Function in Birds*. Vol. 4. Academic Press, Toronto, 591 pp.
15. **Mecham, R. P., 2018:** Elastin in lung development and disease pathogenesis. *Matrix Biol.*, 73, 6—20. DOI: 10.1016/j.matbio.2018.01.005.
16. **Mitchell, J. J., Reynolds, S. E., Leslie, K. O., Low, R. B., Woodcock-Mitchell, J., 1990:** Smooth muscle cell markers in developing rat lung. *Am. J. Respir. Cell. Mol. Biol.*, 3, 515—523. DOI: 10.1165/arcmb/3.6.515.
17. **Ruan, Y. Ch., Zhou, W., Chang, H., Chan, H. Ch., 2011:** Regulation of smooth muscle contraction by the epithelium: role of prostaglandins. *Physiology* (Bethesda), 26, 3, 156—170. DOI: 10.1152/physiol.00036.2010.
18. **Reemers, S. S., van Haarlem, D. A., Koerkamp, M. J.**

G., Vervelde, L., 2009: Differential gene-expression and host-response profiles against avian influenza virus within the chicken lung due to anatomy and airflow. *J. Gen. Virol.*, 90, 2134—2146.

19. **Rucker, R. B., Dubick, M. A., 1984:** Elastin metabolism and chemistry: potential roles in lung development and structure. *Environ. Health Perspect.*, 55, 179—191. DOI: 10.1289/ehp.8455179.
20. **Seow, C., Fredberg, J. J., 2001:** Historical perspective on airway smooth muscle: the saga of a frustrated cell. *J. Appl. Physiol.*, 91, 938—952. DOI: 10.1152/jappl. 2001.91.2.938.
21. **Shardonofsky, F. R., Capetanaki, Y., Boriek, A. M., 2006:** Desmin modulates lung elastic recoil and airway responsiveness. *Am. J. Physiol. Lung. Cell. Mol. Physiol.*, 290, 5, 890—896. DOI: 10.1152/ajplung.00397.2005.
22. **Sjuve, R., Arner, A., Li, Z., Mies, B., Paulin, D., Schmittner, M., Small, J. V., 1998:** Mechanical alterations in smooth muscle from mice lacking desmin. *J. Muscle Res. Cell Motil.*, 19, 415—429.
23. **Sparrow, M. P., Lamb, J. P., 2003:** Ontogeny of airway smooth muscle: structure, innervation, myogenesis and function in the foetal lung. *Respir. Physiol. Neurobiol.*, 137, 361—372. DOI: 10.1016/S1569-9048(03)00159-9.
24. **Stephens, N. L., 2001:** Airway smooth muscle. *Lung*, 179, 333—373. DOI: 10.1007/s004080000073.
25. **Stratman, A. N., Burns, M. C., Farrelly, O. M., Davis, A. E., Li, W., Pham, V. N., et al., 2020:** Chemokine mediated signalling within arteries promotes vascular smooth muscle cell recruitment. *Commun. Biol.*, 3, 1, 734. DOI: 10.1038/s42003-020-01462-7.
26. **Treuting, P. M., Dintzis, S. M., Frevert, C. W., Liggitt, H. D., Montine, K. S., 2012:** *Comparative Anatomy and Histology: A Mouse and Human Atlas*, 1st edn., Amsterdam, Elsevier/Academic Press, 552 pp.
27. **Wagner, T. E., Frevert, C. W., Herzog, E. L., Schnapp, L. M., 2003:** Expression of the integrin subunit a8 in murine lung development. *J. Histochem. Cytochem.*, 51, 1307—1315. DOI: 10.1177/002215540305101008.
28. **Yamada, M. Y., Kurihara, H., Kinoshita, K., Saka, T., 2005:** Temporal expression of alpha-smooth muscle actin and debrin in septal interstitial cells during alveolar maturation. *J. Histochem. Cytochem.*, 53, 735—744. DOI: 10.1369/jhc. 4A6483.
29. **Yamamoto., Y., Tanaka., A., Kanamaru., A., Tanaka., S., Tsubone., H., Atoji., Y., Suzuki., Y., 2003:** Morphology of aging lung in F344/N rat: Alveolar size, connective tissue, and smooth muscle. *Cell Markers. Anat. Rec.*, Part A, 272A, 538—547. DOI: 101002/ar.a.10172.

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OCCURRENCE AND ANTIBIOTIC RESISTANCE PROFILES OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) IN LAYER CHICKENS IN KEBBI, NIGERIA

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ABSTRACT

Antimicrobial resistance (AMR) is a global health threat, and antimicrobial use in animal production for growth enhancement or prophylaxis contributes to the development of AMR. A cross-sectional study was conducted to investigate the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in poultry and farm attendants in Kebbi, North-Western Nigeria. A total of 207 cloacal and nasal swabs were randomly collected from four farms comprising 50 samples from each poultry farm and only 7 samples from farm attendants. The samples were analysed using routine bacteriological culture and identification techniques. Presumptive MRSA isolates were confirmed by PCR assay and AMR profiles of the isolates were evaluated using the Kirby-Bauer disk diffusion method. Of the 207 samples examined, 37.5 % (75/200) of layer birds tested positive for MRSA and 71.4 % (5/7) of farm attendants were MRSA positive. All the isolates were susceptible to vancomycin, with an AMR index > 0.3. The findings of this study indicated colonization of layer chickens and humans by multidrug resistant MRSA, thus highlighting the potential role of poultry sources of transmission of multidrug-resistant MRSA strains to humans and vice versa.

Key words: antibiotics; chickens; Kebbi; Nigeria

INTRODUCTION

Antimicrobial resistance (AMR) is a worldwide health concern [35]. AMR is considered as one of the most serious public health threats of the century [9, 25, 36]. Over the years, research has shown a considerable empirical evidence highlighting the contribution of antimicrobial use (AMU) to the overall burden of AMR emergence [4, 19]. A major contributing factor to this emergence is the misuse of antimicrobials in livestock production. The magnitude of usage is expected to increase considerably in the future due to increased livestock farming activities [31]. Most of the knowledge and assumptions on the prevalence and evolution of AMR in animal production systems relate to

organisms that often are considered commensals in poultry such as *Escherichia coli* [23], and *Staphylococcus aureus* [25].

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been detected in several species of animals [34]. Livestock can act as a reservoir and source for the emergence of novel MRSA clones in humans [14]. Livestock acquired methicillin-resistant *Staphylococcus aureus* (LA-MRSA) in farmers constitutes a major threat to public health and the health care system [5, 34]. MRSA prevalence in humans has been reported to be strongly associated with the prevalence in animals and intensity of contact with animals positive for methicillin-resistant *Staphylococcus aureus* [24]. LA-MRSA has been reported to cause infections amongst farm workers and their relatives [14].

Researchers have implicated poultry as reservoirs for AMR bacteria that may spread to humans, with poultry farming widely believed to be a major risk factor for AMR in humans [24]. However, quantitative evidence describing the role of poultry in the emergence and transmission of AMR bacteria to human populations is lacking [21], particularly in Kebbi State, Nigeria. In the absence of routine surveillance of AMR in Kebbi State, understanding the prevalence of AMR is key to developing effective strategies targeted towards reduction in the emergence and spread of such resistance genes in the future.

This study investigates the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) and its multi-drug resistant index in poultry farms and their attendants in the Kebbi State, Nigeria.

MATERIALS AND METHODS

Ethical approval

All experiments were done in accordance with the guidelines to the use and care of animals of the Research Ethics Committee of the Faculty of Veterinary Medicine, Usmanu Danfodiyo University, Sokoto (UDUS/FAREC/03/2019).

Study area

Kebbi is in North-Western Nigeria and lies between latitude 12.45 0N and longitude 4.2 0E. Kebbi State has an agriculturally viable environment since it is endowed with high soil fertility, vast farmlands and economically viable rivers sheltered by a fine tropical climate. Agriculture has remained the major source of revenue and indeed, the backbone of the economy of the state. Major food crops in the area are: millet, guinea-corn, maize, cassava, potatoes, rice, beans, onions, and vegetables.

Sample size determination

A total of 200 cloacal swab samples were randomly collected from four different farms within the State. The sample size was determined using an estimated prevalence of 13.7% as reported by Kwoji et al. [17] in the formula described by Thrusfield [30]:

$$N = \frac{t^2 \times P_{exp}(1 - P_{exp})}{d^2}$$

where

t^2 = Standard deviation $(1.96)^2$

P_{exp} = Expected MRSA prevalence by Kwoji [17] 13.7% i.e. 0.137

d^2 = $(0.05)^2$

$$N = \frac{1.96^2 \times 0.137\,(1 - 0.137)}{0.025} = 180.16$$

Study design and sample collection

A cross-sectional study was conducted between April and June 2019. Cloacal swabs were collected from each chicken. A total of two hundred laying birds (fifty samples from each farm) were randomly sampled from four poultry farms in the study area. Also, nasal swabs were taken from apparently healthy farm attendants (whom have not used antibiotics in the last three months) after seeking their consent. Only seven (7) farm attendants volunteered to partake in the study (Farm A: 1, Farm B: 3, Farm C: 1, Farm D: 2).

Bacteriological culture and isolation

Upon arrival to the laboratory (central research laboratory, Usmanu Danfodiyo University Sokoto), cloacal swabs from poultry were pre-enriched in peptone water for 24 h at 37 ºC before inoculation on blood agar containing 5% horse blood and incubated aerobically at 37 ºC for 24 h. Presumptive colonies that characteristically appeared like Staphylococcus spp. were preliminarily identified using colony morphology and Gram staining for cellular

morphology. Colonies that appeared relatively small, circular, convex, smooth, and grayish to white were picked and streaked on a freshly prepared Mannitol Salt agar (Oxoid, Basingstoke, UK) and incubated aerobically at 37 °C for 24 h. Two to three presumptive colonies of the Staphylococcus species that appeared small, smooth, golden, shiny, convex, and with golden yellow zones were picked and sub-cultured in nutrient agar (Oxoid) slant and incubated at 37 ºC for 18—24 h. Staphylococcus isolates were further confirmed biochemically using catalase and coagulase tests as described by O c h e i, K o h l h a t k a r [26] and C h e e s b r o u g h [6].

Phenotypic characterization of MRSA

Overnight fresh cultures of presumptive *S. aureus* were inoculated onto a freshly prepared Oxacillin Resistance Screening Agar Base using appropriate supplement (ORSAB; Oxoid, Basingstoke, UK) to determine phenotypic methicillin resistance. Presumptive colonies on ORSAB that appeared deep blue in color on a colorless background were phenotypically considered as MRSA. Media preparation and interpretation of colonies were carried out as described in the manufacturer's guide.

Antimicrobial susceptibility test

Antimicrobial susceptibility testing for 7 antibiotics; erythromycin (E 15 µg), oxytetracycline (OXT 30 µg), neomycin (N 5 µg), penicillin (P 30 µg), sulfonamide (SUL 23.75 µg), gentamicin (CN 10 µg) and vancomycin (VAN 30 µg) that are commonly used in veterinary and human medicine in Kebbi State was studied using the disc diffusion method (Oxoid Ltd, Basingstoke, UK). Antibiotic discs were dispensed onto bacteria-containing agar plates and incubated for a period of 18 hours at 35 °C, according to standard protocols. Zones of inhibition were recorded and interpreted according to the recommendations of the Clinical and Laboratory Standards Institute CLSI [8].

Multiple antimicrobial resistance index (MARI) was also determined by dividing the number of antibiotics each isolate is resistant to by the total number of antibiotics included in the panel.

Genotypic characterization of MRSA

Genomic DNA extraction

Genomic DNA was extracted using the traditional boiling method as described by C h e n et al. [7] with slight modification. Briefly, a loop-full suspension of overnight grown cultures on nutrient agar plate was transferred into a 1.5 ml micro-centrifuge tube containing 100 µl of sterile distilled water. The suspension was first incubated at room temperature for 5 min, and then heat treated to 96 °C for 10 min. This was then followed by centrifugation at 12,000 x g for 5 min. The supernatant (containing the DNA) was then collected in a new 1.5 ml tube using a micropipette and kept at 4 ºC until used.

PCR detection of *mecA* gene

DNA templates of all the ORSAB positive *S. aureus* isolates were subjected to PCR assay for the detection of the 163 bp fragment of the *mecA* gene using the following primers; *MecA*1 5'-AAAATCGATGGTAAAGTTGGC-3'(forward), *MecA*2 5'AGTTCTGCAGTACCGGATTTGC-3'(reverse) as described by M e h r o t r a et al. [20]. The PCR assay was performed in a 25 µl reaction mixture containing 3 µl of nuclease free water, 5 µl (0.5 µg) of the DNA template, 1 µl (0.2 µM) of each forward and reverse primer, 2.5 µl of coral red dye and 12.5 µl (100 µM) of the master mix (Qiagen). The amplification protocol comprised of 35 cycles of amplification with an initial denaturation of 94 °C for 5 min, denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min, extension at 72 °C for 1 min and then final extension at 72 °C for 7 min. The PCR products were visualized after electrophoresis for 45 min at 90 volts in a 1 % agarose gel. The amplicons were then viewed under a UV trans-illuminator (Biorad Gel Doc XR System w/ Universal Hood II).

Data analysis

The results obtained were presented in tables. Chi-squared statistics were performed using Invivostat v 4.1 to determine possible association between MRSA infection and farms sampled.

RESULTS

***S. aureus* and MRSA in poultry and farm attendants**

Of the 200 poultry cloacal swabs examined 72.0 % (144/200) were positive for *Staphylococcus aureus* based on culture, biochemical and molecular characterization. Farm B had the highest prevalence with 42/50 (84 %), followed by Farm A with 39/50 (78 %). Farms D and C had

Table 1. Distribution of *Staphylococcus aureus* in poultry farms in Kebbi state

Grouping factor	No. of samples	+ve *S. aureus*	χ2	Df	P-value
Farm A	50	39 (78%)			
Farm B	50	42 (84%)	9.72	3	0.02
Farm C	50	29 (58%)			
Farm D	50	34 (68%)			

Df—degree of freedom

Table 2. Distribution of MRSA in Poultry farms in Kebbi State

Grouping factor	No. of samples	+ve MRSA	χ2	Df	P-value
Farm A	50	25 (50%)	7.91	3	0.04
Farm B	50	21 (42%)			
Farm C	50	17 (34%)			
Farm D	50	12 (24%)			

Df—degree of freedom

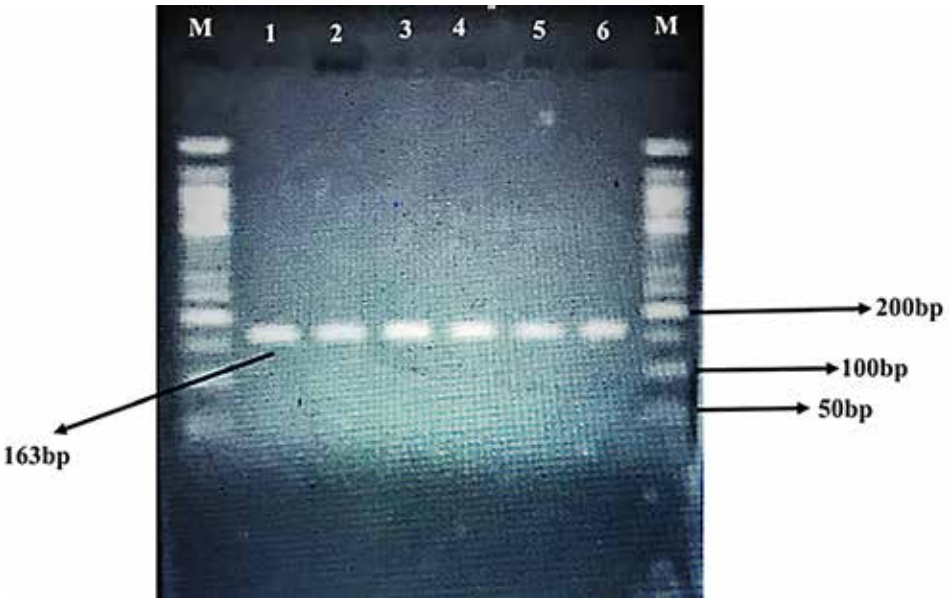

Fig. 1. Gel image of 163bp gene fragment of the *mecA* gene using 50 bp molecular weight DNA ladder

34/50 (68%) and 29/50 (58%) frequencies, respectively. There was a statistically significant association (P=0.02) between the recovery rate of *S. aureus* and Farms with farm B more likely to have *S. aureus* colonization than the other farms (Table 1).

In this study, phenotypic detection of MRSA on ORS-AB showed that 37.5% (75/200) of cloacal swabs were positive. Farm A had the highest frequency of 25/50 (50%), followed by Farm B with a frequency of 21/50 (42%), Farms C and D had 17/50 (34%) and 12/50 (24%)

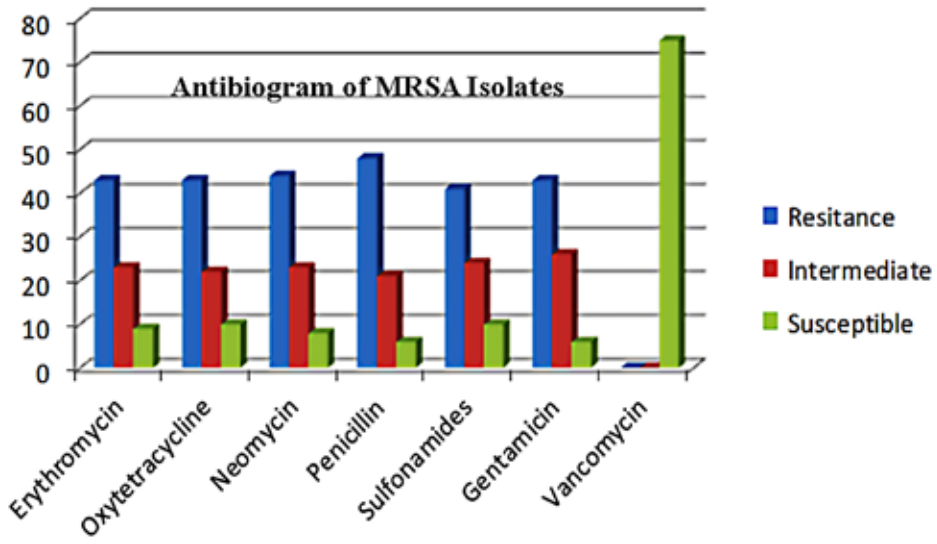

Fig. 2. Radar chat of antibiotic sensitivity profile of MRSA isolates (n = 75) from poultry farm in Kebbi State

Table 3. Antibiotic resistant pattern of MRSA isolates from poultry farms in Kebbi State

Antibiotic resistant pattern	Number of isolates	Multiple antibiotic resistance index
ERY-OXY-NEO-SUL-GEN	1	0.71
OXY-NEO-PEN-SUL-GEN	1	0.71
ERY-OXY-NEO-PEN-GEN	1	0.71
ERY-PEN-SUL-GEN	2	0.60
OXY-NEO-PEN-SUL	2	0.60
ERY-OXY-NEO-PEN-GEN	2	0.71
ERY-OXY-NEO-PEN	5	0.60
ERY-OXY-NEO-PEN-SUL	13	0.71
ERY-OXY-NEO-PEN-SUL-GEN	17	0.86

frequencies, respectively. There was a statistically significant association (P=0.04) between the recovery rate of MRSA and Farms with farm A more likely to have MRSA colonization than the other farms (Table 2). The seven farm attendants sampled and examined, showed 5/7 (71.4%) colonization rate for both *S. aureus* and MRSA. PCR amplification of all the eighty (80) positive samples on ORSAB for the detection of *mecA* gene showed 100% positivity (Figure 1). Hence, the overall molecular detection rate of MRSA from poultry farms studied was 38.6% (80/207).

Antimicrobial resistance profiles of *S. aureus* and MRSA from poultry and farm attendants

Antimicrobial susceptibility tests revealed that the ORSAB positive isolates exhibited varying level of resistance against the antimicrobials tested. Out of the 75 MRSA isolates recovered from poultry farms, 43 (57.3%) of them were resistant to erythromycin, oxytetracycline and gentamycin, 44 (58.7%) were resistant to neomycin, 41 (54.7%) and 48 (64%) were resistant to sulfonamides and penicillin respectively. All isolates were susceptible to vancomycin (Figure 2). A total of 17 isolates were found to show multidrug resistance (MDR) with ERY-OXY-NEO-PEN-SUL-GEN being the most common pattern and the multiple antibiotic resistance (MAR) index is > 0.2 (Table 3).

DISCUSSION

The emergence of MRSA does not only pose significant public health threat challenge but also threatens animal health as an emerging veterinary pathogen of great importance throughout the world. Over time, there is a steady increase in the prevalence of MRSA colonization in healthy food producing animals [10, 29].

In this study, the overall occurrence rate of MRSA from poultry farms was 38.6% with the following rates among different farms (Farm A: 50%, Farm B: 42%, Farm C: 34% and Farm D: 24%) and 2.4% in farm attendants.

The isolation rate in our study was much higher than the 8.82%, 6.3% and 7.9% recorded by Kwoji et al. [17], Bale et al. [2] and Musawa et al. [22], respectively. These variations could be attributed to difference in the sensitivity of the detection method, sample size, sample source and the indiscriminate use of antimicrobials in animal production [13]. It is noteworthy that Musawa et al. [22] conducted their research on poultry carcass rinse while Kwoji et al. [17] and Bale et al. [2] only used phenotypic characteristics to determine MRSA in their study. Contrarily, all *S. aureus* isolates from apparently healthy animals from Tunisia and China were methicillin-susceptible [15, 38]. The absence of MRSA in these studies could be attributed to the good management practices in the study areas as administration of antibiotics is regulated in animal farming. The occurrence of MRSA in poultry in this study however could be due to indiscriminate use of antibiotics in poultry production by farmers without prescription by a veterinarian [19]. MRSA is probably the best example of a prevalent and important multidrug-resistant bacterium that has successfully transitioned from an almost exclusively nosocomial setting to being widespread in the community [33].

Most of the MRSA isolates in this study were multidrug-resistant strains (exhibiting resistance to six out of seven antibiotics exposed to), a result of which agreed with the report of Gaddafi et al. [13] in healthy pigs in Kebbi State. The result showed a high resistance to penicillin, oxytetracycline, neomycin, erythromycin, gentamicin, and sulfonamides. Reports from Anueyiagu and Isiyaku [1], Jahan et al. [16] and Gaddafi et al. [12] in agreement showed a remarkably high MRSA resistance to penicillin because it is the drug of choice in *S. aureus* infection. High resistance to oxytetracycline noticed in this study could be attributed to the assertion by Olatoye [27] who opinioned that tetracycline is commonly used as a feed additive and for prophylaxis in animal production in Nigeria. Similarly, Usman et al. [32] opinioned that resistance to tetracycline is a major problem in veterinary practices in Nigeria due to availability and affordability of the medication. High resistance to erythromycin and gentamicin observed in this study could possibly reflect its frequent use in livestock in the study area. In agreement to this, Anueyiagu and Isiyaku [1], reported significant levels of resistance to erythromycin and gentamicin in Nigeria. Results of MRSA susceptibility testing from this study showed 100% susceptibility to vancomycin. This finding is unsurprising because vancomycin is rarely used in the treatment of diseases in livestock in the study area [13].

Forty-four MRSA strains isolated from this study showed multidrug resistance. The isolates were resistant to a combination of the four, five and six antibiotics tested. Bitrus et al. [3] explained that multi-drug resistance in *S. aureus* may be partly attributed to the acquisition of resistance determinants domiciled in mobile genetic elements. Lay et al. [18] also opined that the determinants of multi-drug resistance are capable of being disseminated in a region or between regions because of antibiotic selective pressure in either livestock or humans. All methicillin resistant *S. aureus* examined in this study had an MAR index of 0.6 and above. The MAR index gives an indirect indication of the probable source of an organism. An organism originates from an environment with high levels of antibiotic use when its MAR index is greater than 0.2 [11]. The findings of the current study underscore the circulation of multidrug-resistant MRSA strains among apparently healthy poultry farms and their attendants and accordingly, poultry may be considered as a reservoir for such multidrug-resistant strains which may easily pass to humans through direct contact resulting in horizontal transmission between humans, with great public health consequences. More so, in Nigeria, the indiscriminate use of antibiotics in livestock production could have also contributed immensely to the patterns and index of antibiotic resistance recorded in this study.

To date, the correlation between MRSA phenotype and genotype must be considered from the surrogate test for oxacillin and *mecA* gene existence [13]. In this study, all eighty MRSA strains examined carried the *mecA* gene, indicating that the phenotypic resistance exhibited by the strains on ORSAB was due to the possession of the gene. This finding agrees with the report of Gaddafi et al. [13] who also detected *mecA* gene in all the forty-four MRSA strains isolated from pig farms and their attendants in Zuru. Contrarily, our findings differed from the report of Musawa et al. [22] and Yakubu et al. [37] who detected the *mecA* gene in only fifteen out of thirty-seven and twelve out of twenty-two MRSA isolated from poultry carcass rinse and some retailed animal products in Sokoto, respectively. Phenotypic expression of resistance to methicillin in MRSA varies and each strain has a characteristic

profile of the proportion of bacterial cells that grow at specific concentrations of methicillin [28].

CONCLUSIONS

The antibiotic susceptibility profiles of the MRSA strains isolated from poultry showed significant levels of resistance to penicillin, erythromycin, gentamicin, and oxytetracycline. This finding is of great public health concern because these antibiotics are commonly used in Nigeria either therapeutically in human and veterinary practices or as growth promoters and for prophylaxis in poultry production. This also highlighted the roles of layer chickens and possibly eggs as potential sources of infection to humans.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest with regards to the publication of this manuscript.

REFERENCES

1. **Anueyiagu, K. N., Isiyaku, A. W., 2015:** Isolation and identification of *Staphylococcus aureus* from bovine milk and its antibiotic susceptibility. *Int. J. Livest. Product.*, 6, 6, 74—77. DOI: 10.5897/IJLP2015.0248.
2. **Bale, M. I., Babatunde, S. K., Adedayo, M. R., Ajiboye, A. E., Ajao, A. T., 2019:** Characterization of methicillin-resistant *Staphylococcus aureus* isolates from apparently healthy individuals in Malete, Kwara state, Nigeria. African *J. Clin. Exper. Microbiol.*, 20, 1, 17—24. DOI: 10.4314/ajcem.v20i1.3.
3. **Bitrus, A. A., Zunita, Z., Goni, M. D., Mshelia, I. T., 2018:** Dissemination of resistance and virulence determinants in methicillin-resistant *Staphylococcus aureus* during colonization and disease: A review. *Adv. Anim. Vet. Sci.*, 6, 1, 44—54. DOI: 10.17582/journal.aavs/2018/6.1.44.54.
4. **Bortolaia, V., Espinosa-Gongora, C. and Guardabassi, L., 2016:** Human health risks associated with antimicrobial-resistant enterococci and *Staphylococcus aureus* on poultry meat. *Clin. Microbiol. Infect.*, 22,130—40. DOI: 10.1016/j.cmi.2015.12.003.
5. **Broens, E. M., Graat, E. A., Van der Wolf, P. J., Giessen, A., De Jong, M., 2011:** Prevalence and risk factor analysis of livestock associated MRSA-positive pig herds in The Netherlands. *Prev. Vet. Med.*, 102, 1, 41—49. DOI: 10.1016/j.prevetmed.2011.06.005.
6. **Cheesbrough, M., 2000:** *District Laboratory Practice in Tropical Countries*. Part II. Cambridge University Press, Cambridge, UK. 65, 38—39.
7. **Chen, L., Mediavilla, J. R., Oliveira, D. C., Willey, B. M., De Lencastre, H., Kreiswirth, B. N., 2009:** Multiplex real-time PCR for rapid staphylococcal cassette chromosome mec typing. *J. Clin. Microbiol.*, 47, 11, 3692—3706. DOI: 10.1128/JCM.00766-09.
8. **Clinical and Laboratory Standards Institute 2018:** *Performance Standards for Antimicrobial Susceptibility Testing*. 28th edn., CLSI supplement M100. Wayne, PA. 130—134
9. **Davies, S. C., Fowler, T., Watson, J., Livermore, D. M., Walker, D., 2013:** Annual report of the Chief Medical Officer: infection and the rise of antimicrobial resistance. *Lancet Infect. Dis.*, 381, 9878, 1606—1609. DOI: 10.1016/S0140-6736(13)60604-2.
10. **El-Deeb, W., Fayez, M., Elmoslemany, A., Kandeel, M., Zidan, K., 2018:** Methicillin resistant *Staphylococcus aureus* among goat farms in Eastern province, Saudi Arabia: Prevalence and risk factors. *Prev. Vet. Med.*, 156, 84—90. DOI: 10.1016/j.prevetmed.2018.05.005.
11. **Furtula, V., Jackson, C. R., Farrell, E. G., Barrett, J. B., Hiott, L. M., Chambers, P. A., 2013:** Antimicrobial resistance in Enterococcus spp. isolated from environmental samples in an area of intensive poultry production. *Int. J. Environ. Res. Pub. Health*, 10, 3, 1020—1036. DOI: 10.3390/ijerph10031020.
12. **Gaddafi, M. S., Yakubu, Y., Garba, B., Bello, M. B., Musawa, I. A., Lawal, H., 2020:** Occurence and antimicrobial resistant patterns of methicillin resistant *Staphylococcus au-*

reus (MRSA) among practicing veterinarians in Kebbi State, Nigeria. *Folia Vet.*, 64, 55—62. DOI: 10.2478/fv-2020-0038.

13. **Gaddafi, M. S., Yakubu, Y., Junaidu, A. U., Bello, M. B., Garba, B., Bitrus, A. A., et al., 2021:** Nasal colonization of pigs and farm attendants by *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) in Kebbi, Northwestern Nigeria. *Thai J. Vet. Med.*, 51, 1, 119—124. DOI: 10.14456/tjvm.2021.16.
14. **García-Álvarez, L., Holden, M. T., Lindsay, H., Webb, C. R., Brown, D. F., Curran, M. D., et al., 2011:** Methicillin-resistant *Staphylococcus aureus* with a novel *mecA* homologue in human and bovine populations in the UK and Denmark: a descriptive study. *Lancet Infect. Dis.*, 11, 8, 595—603. DOI: 10.1016/S1473-3099(11)70126-8.
15. **Gharsa, H., Slama, K. B., Gómez-Sanz, E., Lozano, C., Zarazaga, M., Messadi, L., et al., 2015:** Molecular characterization of *Staphylococcus aureus* from nasal samples of healthy farm animals and pets in Tunisia. *Vector-Borne Zoonot. Dis.*, 15, 2, 109—115. DOI: 10.1089/vbz.2014.1655.
16. **Jahan, M., Rahman, M., Parvej, M. S., Chowdhury, S. M. Z. H., Haque, M. E., Talukder, M. A. K., et al., 2015:** Isolation and characterization of *Staphylococcus aureus* from raw cow milk in Bangladesh. *J. Adv. Vet. Anim. Res.*, 2, 1, 49—55. DOI: 10.5455/javar.2015.b47.
17. **Kwoji, I. D., Tambuwal, F. M., Abubakar, M. B., Yakubu., Y., Bitrus, A. A., Jauro, S., 2017:** Occurrence of methicillin resistant *Staphylococcus aureus* in chickens and farm personnel in Sokoto, Northwestern Nigeria. *J. Adv. Vet. Anim. Res.*, 4, 3, 255—260. DOI: 10.5455/javar.2017.d220.
18. **Lay, K. K., Torio, H. E., Bitrus, A. A., Mala, W., Nuananong, S., Chuanchuen, R., 2020:** Multidrug resistant *Escherichia coli* harboring extended-spectrum β-lactamase-encoding genes isolated from clinically-healthy pigs. *Thai J. Vet. Med.*, 51, 2, 303—310. DOI: 10.14456/tjvm.2021.38.
19. **Marshall, B. M., Levy, S. B., 2011:** Food animals and antimicrobials: Impacts on human health. *Clin. Microbiol. Rev.*, 24, 718—33. DOI:10.1128/CMR.00002-11.
20. **Mehrotra, M., Wang, G., Johnson, W. M., 2000:** Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1 and methicillin resistance. *J. Clin. Microbiol.*, 38, 3, 1032—1035. DOI: 10.1128/JCM.38.3.1032-1035.2000.
21. **Muloi, D., Kiiru, J., Ward, M. J., Hassell, J. M., Bettridge, J. M., Robinson, T. P., et al., 2019:** Epidemiology of antimicrobial-resistant *Escherichia coli* carriage in sympatric humans and livestock in a rapidly urbanizing city. *Int. J. Antimicrob. Agents*, 54, 5, 531—537. DOI: 10.1016/j.ijantimicag.2019.08.014.
22. **Musawa, I. A., Yakubu, Y., Garba, B., Ballah, F. M., Jibril, H. A., Bello, A. S., et al., 2020:** Dressed chicken as potential vehicle for spread of methicillin-resistant *Staphylococcus aureus* in Sokoto, Nigeria. *Future Sci. OA*, 6, 10, 619. DOI: 10.2144/fsoa-2020-0066.
23. **Nhung, N. T., Chansiripornchai, N., Carrique-Mas, J. J., 2017:** Antimicrobial resistance in bacterial poultry pathogens: A review. *Front. Vet. Sci.*, 4, 126. DOI: 10.3389/fvets.2017.00126.
24. **O'Neill, J., 2015:** *Agriculture and the Environment: Reducing Unnecessary Use and Waste. The Review on Antimicrobial Resistance*. Wellcome Trust and HM Government, London, UK. 1—44.
25. **O'Neill, J., 2016:** *Tackling Drug-resistant Infections Globally: Final Report and Recommendations*. Wellcome Trust and HM Government, London, UK. 1—84
26. **Ochei, J. O., Kolhatkar, A. A., 2000:** *Medical Laboratory Science: Theory and Practice*. McGraw Hill Education. 648—657.
27. **Olatoye, I. O., 2010:** The incidence and antibiotics susceptibility of *Escherichia coli* O157:H7 from beef in Ibadan Municipal, Nigeria. *Afric. J. Biotechnol.*, 9, 8, 1196—1199.
28. **Plata, K. B., Riosa, S., Singh, C. R., Rosato, R. R., Rosato, A. E., 2013:** Targeting of PBP1 by β-lactams determines recA/SOS response activation in heterogeneous MRSA clinical strains. *PLOS ONE*, 8, 4, 61083. DOI: 10.1371/journal.pone.0061083.
29. **Rahman, M. M., Amin, K. B., Rahman, S. M. M., Khair, A., Rahman, M., Hossain, A., et al., 2018:** Investigation of methicillin-resistant *Staphylococcus aureus* among clinical isolates from humans and animals by culture methods and multiplex PCR. *BMC Vet. Res.*, 14, 1, 1—6. DOI: 10.1186/s12917-018-16111-0.
30. **Thursfield, M., 2005:** *Veterinary Epidemiology*. 3rd edn., Blackwell science, Ltd., London, UK. 228—246.
31. **Tiseo, K., Huber, L., Gilbert, M., Robinson, T. P., Van Boeckel, T. P., 2020:** Global trends in antimicrobial use in food animals from 2017 to 2030. *Antibiotics*, 9, 12, 918. DOI: 10.3390/antibiotics9120918.
32. **Usman, R. Z., Mustapha, B. M., Mohammed, F. I., 2016:** Isolation and identification of methicillin resistant *Staphylococcus aureus* (MRSA) from traditionally fermented milk "nono" and yoghurt in Kaduna Metropolis, Nigeria. *Food Sci. Qual. Manag.*, 55, 42—50.

33. **Van Duin, D., Paterson, D. L., 2020:** Multidrug-resistant bacteria in the community: Trends and lessons learned. *Infect. Dis. Clin.*, 30, 2, 377—390. DOI: 10.1016/j.idc.2020.08.002.
34. **Waters, A. E., Contente-Cuomo, T., Buchhagen, J., Liu, C. M., Watson, L., Pearce, K., et al., 2011:** Multidrug-resistant *Staphylococcus aureus* in US meat and poultry. *Clin. Infect. Dis.*, 52, 10, 1227—1230. DOI: 10.1093/cid/cir181.
35. **World Health Organization. Antimicrobial Resistance: Global Report on Surveillance, 2014:** Available from: http://www.who.int/drugresistance/documents/surveillance report/en/.
36. **World Health Organization. Global action plan on antimicrobial resistance, 2015:** *WHO*, Geneva, 45 pp.
37. **Yusuf, Y., Aliyu, M. I., Bello, S. A., Fatima, B., Abdullahi, A., Aliyu, M. D., 2020:** Occurrence of methicillin resistant *Staphylococcus aureus* (MRSA) in some retailed animal products in Sokoto, Nigeria. *Alexandria J. Vet. Sci.*, 64, 1. DOI: 10.5455/ajvs.44682.
38. **Zhou, Z., Zhang, M., Li, H., Yang, H., Li, X., Song, X., et al., 2017:** Prevalence and molecular characterization of *Staphylococcus aureus* isolated from goats in Chongqing, China. *BMC Vet. Res.*, 13, 1, 1—8. DOI: 10.1186/s12917-017-1272-4.

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CONTENTS OF SELECTED ELEMENTS IN THE BLOOD SERUM OF BROILER CHICKENS AFTER SUPPLEMENTATION OF HUMIC SUBSTANCES

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ABSTRACT

This study was conducted to determine the concentrations of mineral substances, such as: calcium (Ca), magnesium (Mg), copper (Cu), and zinc (Zn); in the blood serum of broiler chicks after supplementation of humic substances. Group 1 (G1) was supplemented with 0.7 % Humac Natur Mycosorb (HNMy); G2 with 0.7 % Humac Natur Monogastric (HNM); G3 with 0.3 % HNMy; and the control group (GC) received a basal diet without any supplements. In the G2 group, the serum Ca level increased slightly compared to the other experimental groups. A statistically significant decrease ($P<0.001$) in the blood serum of Mg was detected in the broilers from group G2 (0.60 mmol.l^{-1}) and G1 ($P<0.05$; 0.68 mmol.l^{-1}) in comparison to the GC. The Zn in the blood serum of broilers from group G2 (22.05 µmol.l^{-1}) was significantly increased ($P<0.05$) in broilers from group G2 in comparison to the control group (19.47 µmol.l^{-1}) and G1 group (19.61 µmol.l^{-1}). The serum Cu (12.72 µmol.l^{-1}) was significantly increased ($P<0.001$) in broilers from group G2 in comparison to the GC (10.28 µmol.l^{-1}). In the group G1 there was a significant decrease ($P<0.05$) in the level of the Cu in the blood serum (8.69 µmol.l^{-1}) in comparison to the serum Cu in broilers from groups G3 (10.45 µmol.l^{-1}) and G2 (12.72 µmol.l^{-1}). The addition of Humac Natur Mycosorb (0.3 % and 0.7 %) in the feed caused a strong correlation in the blood serum between the Ca and Mg ($r=0.7494$; $r=0.7648$). The addition of 0.3 % Humac Natur Mycosorb in the feed caused a high negative correlation ($r=–0.7078$) in the blood serum between the Mg and Zn.

Key words: blood serum; broilers; calcium; copper; humic substances; magnesium; zinc

INTRODUCTION

Nutritionists are constantly looking for viable additives because conventional supplements have been criticized for negatively affecting the food chain. Humic substances (HS), primarily humic acids and fulvic acids, attract the deliberate attention of scientists for many reasons. The acidic nature of these compounds is explained by the pres-

ence of carboxyl groups, which allows them to be considered polycarboxylic acids. Today, however, HS are being increasingly applied in practical agriculture. This is explained by the fact that humic substances have high physiological activity, which is expressed in stimulating the growth and development of both plants and animals [5]. Humic substances have: antibacterial, antiviral, antithyroid and anti-inflammatory effects in animals, improve immunity and reduce mortality, and increase poultry growth [9], and have shown a strong affinity for binding various substances, such as heavy metals [13], minerals [8] and aflatoxins [20, 22]. Moreover, including natural additions to chickens' diet, such as humates or HS, has been found to positively affect and improve gut health [3], influences the increase in absorption of mineral components which are necessary for the organism [17]. Mineral components, e.g. calcium and phosphorus, enter the composition of tissues and body fluids, fulfilling a body-building function [18]. Stepchenko [21] proved that adding biologically active supplements of humic nature to the diets of animals: stimulated the metabolic processes and the digestibility of nutrients, promoted an increased nitrogen deposition, and activated the absorption of calcium and phosphorus, as well as some other mineral elements. Differentiated effects have been seen in both humic acids and trace elements, and especially in Cu and Zn. The Zn, Cu, and Mn are essential trace elements and play multiple biological and physiological roles in the development and health of all animals, leaving the susceptible to various nutrient and ingredient antagonisms that impair absorption. Antagonisms can occur between minerals. For example, high levels of zinc reduce the availability of copper, and the opposite is also true [18]. The influence of humates on changes in biochemical parameters has been studied by some researchers [4, 10, 11, 17].

The aim of this study was to monitor the effects of humic acid on selected mineral elements (Ca, Mg, Zn, Cu) and their concentrations in the blood serum of broiler chickens.

MATERIALS AND METHODS

Animals

One-day-old broiler chicks of hybrid ROSS 308 were randomly divided into 4 groups of 20 animals each. Dietary treatments were as follows: The broilers were fed with a commercial feed mixture BR1, diet for fattening broilers within 10 days of age, BR2 diet for growing to 30 days of age and BR3 final fed mixture (AGROCASS plus, Ltd. Čaňa, Slovak Republic) for the duration of the experiment (42 days).

Experimental design and diet

The control group (GC) was fed a basal diet without any supplement. The experimental group G1 was supplemented with 0.7% Humac Natur Mycosorb (HNMy) which represented 0.7 kg.100 kg^{-1} feed. Group G2 was supplemented with 0.7% Humac Natur Monogastric (HNM) and thus enriched with calcium formate. Group G3 was supplemented with 0.3% Humat Natur Mycosorb 0.3 kg.100 kg^{-1}) feed. The Humac Natur Mycosorb and Humac Natur Monogastric were obtained from HUMAC Ltd. Košice, Slovak Republic. During fattening, chickens had access to water and feed *ad libitum*. The supplementation of humic substances to animals in feed does not require withdrawal periods. The broiler chicks were reared on deep litter and microclimate conditions complied with the requirements for fattening of broilers. They were reared under a conventional temperature regimen at 21 °C. The relative humidity was maintained between 60—70%. After fattening, the animals were stunned and killed by cervical dislocation.

Ethical statement

The experiments were carried out in accordance with the European directive on the protection of vertebrate animals used for experimental and other scientific purposes (86/609/EU) and with the consent of the State Veterinary and Food Administration of the Slovak Republic No. 3090/13-221 on the premises for poultry housing of the University of Veterinary Medicine and Pharmacy in Košice (Slovak Republic).

Blood samples

The blood sera were obtained on day 42 of the life of broilers by sampling the blood from vena cutanea ulnaris. After proper clotting, the blood samples were centrifuged at 3500 rpm for 15 minutes for serum separation. The blood serum was diluted 1 : 1 with La_2O_3 in a 50 ml volumetric flask. This solution was analysed for the content of Ca, Mg, Zn, and Cu directly by using a flame atomic

Table 1. Composition of the broiler diets

Item [%]	BR1—Fattening [1—10 day]	BR2—Growing [11—30 day]	BR3—Finishing [31—42 day]
Maize	35.00	40.00	37.00
Wheat	35.00	35.00	36.80
Soybean meal	21.30	18.70	20.00
Dried blood	1.25	–	–
Limestone	1.00	1.05	1.12
Monocalcium phosphate	1.00	0.70	1.00
Salt	0.10	0.15	0.20
Lysine	1.20	1.15	0.98
Methionine	0.60	0.46	0.40
Premix	0.50	0.50	0.50
Composition by analysis			
ME [MJ.kg^{-1}]	12.01	12.03	12.37
N-substances [%]	22.00	19.50	19.00
Ash [%]	6.00	4.00—6.00	4.00—6.00
Fat [%]	2.50—5.00	6.00—8.00	6.00—10.00
Crude fibre [%]	Max. 4.00	Max. 4.50	Max. 4.00
Non-phytate phosphorus [%]	Min. 0.42	Min. 0.40	Min. 0.40
Ca [%]	Min. 0.90	Min. 0.85	Min. 0.85
Na [%]	Min. 0.15	Min. 0.14	Min. 0.14
Retinol [MJ.kg^{-1}]	12 500	12 500	10 000
Cholecalciferol [MJ.kg^{-1}]	3 000	3 000	2 000
Alpha-tocopherol [mg.kg^{-1}]	50.00	40.00	30.00
Propylgallata [mg.kg^{-1}]	100.00	100.00	100.00
Narazinb [mg.kg^{-1}]	70.00	–	–
Salinomycin sodiumb [mg.kg^{-1}]	–	70.00	–

ME—metabolizable energy; a—antioxidant; b—coccidiostat Ca—calcium; Na—sodium

Table 2. Composition of feed supplements (powder, particle size up to 100 µm)

Ingredient	Humac Natur Mycosorb (HNMy) (Group 2)	Humac Natur Monogastric (HNM) (Group 3)
Humic substances [%]	60	60
Fulvic acid [%]	5	5
Formic acid [%]	–	3.24
Ca [g.kg^{-1}]	42.278	51.1
Mg [g.kg^{-1}]	5.100	4.855
Fe [mg.kg^{-1}]	19.046	18.094
Cu [mg.kg^{-1}]	15.00	14.25
Zn [mg.kg^{-1}]	37.00	35.15
Mn [mg.kg^{-1}]	442.00	135.00
Co [mg.kg^{-1}]	1.24	1.18
Se [mg.kg^{-1}]	1.67	1.59
V [mg.kg^{-1}]	42.10	40.00
Mo [mg.kg^{-1}]	2.70	2.57

Ca—calcium; Mg—magnesium; Fe—iron; Cu—copper; Zn—zinc; Mn—manganese; Co—cobalt; Se—selenium; V—vanadium; Mo—molybdenum

absorption spectrometer (Unicam Solar, 939, Great Britain). The flame conditions were those recommended by the instrument manufacturer for Ca, Mg, Zn and Cu (wavelength 422.7; 285.2; 213.9; 324.8 nm, respectively, band pass 0.5 nm). Determinations were done according to the methodology specified in the List of Official Methods and Laboratory Diagnostics of Food and Feed in the Bulletin of the Ministry of Agriculture the Slovak Republic, 2004 [12].

Statistical analysis

The differences between means were determined, according to the paired t-test using GraphPad Prism 6 software. Correlations between pairs of elements in blood serum in experimental and control groups were determined by Pearson correlation analyses. Only samples with detectable mineral levels were included in the analysis. Only significant correlations with an r value > 0.3 were reported.

RESULTS AND DISCUSSION

The mean concentrations of Ca, Mg, Zn, and Cu determined in blood serum of broilers are presented in Table 3.

The level of serum Ca in broilers from the control and experimental groups were almost balanced. The mean Ca serum levels (2.05; 2.14; 2.16; 2.08 mmol.l^{-1}, respectively) were below the physiological range (2.50–3.0 mol.l^{-1}). The addition of 0.7 % HNM to feed for broilers increased the Ca level in the serum of G1, G2 and G3 groups in comparison to the control group. Similarly, Jaďuttová et al. [10] after the addition of 0.8 % and 1 % of humic substances to broiler feed found an increase in Ca (2.90 and 3.23 mmol.l^{-1}, resp.) and Mg (0.81 and 0.85 mmol.l^{-1}, resp.). Naď et al. [15] found that the supplementation of 0.7 % Humac Natur Monogastric to broilers significantly reduced ($P<0.001$) serum Ca compared to the control group (1.42 mmol.l^{-1}; 1.98 mmol.l^{-1}) and Abdel-Mageed [1] noted that birds fed a diet supplemented with humic substances at low levels showed a decrease in serum Ca and P levels. The increase in Ca can affect positively bone metabolism and structure [17]. Boguslawska-Tryk et al. [6] found that the concentration of Ca in the blood of broilers showed a gradual increase along with increasing levels of cellulose in the diet.

Serum Mg values detected in our study in groups G1, G2, G3 were slightly lower compared to the control group. A significant decrease ($P<0.001$) in serum Mg was determined (0.60 mmol.l^{-1}) in broiler chickens supplemented with 0.7 % HNM (G2) while a significant decrease ($P<0.05$; 0.68 mmol.l^{-1}) was detected in broiler chickens fed with the addition of 0.7 % HNMy (G1) in comparison to the control group. On the other hand, Kovacik et al. [11] observed that the addition of 0.5 % humic acid to the feed for pheasants increased the level of the Mg in the serum of pheasants.

In contrast, the serum Zn (22.05 µmol.l^{-1}) was significantly increased ($P<0.05$) in broilers from group G2 in comparison to the control group (19.47 µmol.l^{-1}) and G1 group (19.61 µmol.l^{-1}). In our experiment, supplementation of humic substances (0.7 % HNMy; 0.7 % HNM;

Table 3. The content of mineral elements in blood serum of broiler chickens

Group	Ca [mmol.l^{-1}]	Mg [mmol.l^{-1}]	Zn [μmol.l^{-1}]	Cu [μmol.l^{-1}]
Control GC	2.05 ± 0.20	0.74 ± 0.07	19.47 ± 0.98	10.28 ± 1.27
G1	2.14 ± 0.31	0.68 ± 0.10*	19.61 ± 2.23	8.69 ± 1.25*
G2	2.16 ± 0.30	0.60 ± 0.06***	22.05 ± 2.75*	12.72 ± 0.97***
G3	2.08 ± 0.36	0.71 ± 0.10	20.62 ± 2.96	10.45 ± 1.82

The data represent the mean of 6 samples of blood serum from each group; Control group—diet without the addition of humic substances; G1—0.7 % HNMy—diet with the addition of Humac Natur Mycosorb; G2—0.7 % HNM – diet with the addition of Humac Natur Monogastric; G3—0.3 % HNMy—diet with the addition of Humac Natur Mycosorb; *—P < 0.05 ; ***—P <0.001

0.3 % HNMy) increased the serum Zn levels (19.61; 22.05; 20.62 μmol.l^{-1}, resp.) in broilers from all three supplemented groups compared to the control group (19.47 μmol.l^{-1}). This increase can be attributed to the humac natur additive used, which binds inorganic ions and facilitates the transport of these minerals [9]. Additionally, Zn concentration in the serum slightly increased in birds supplemented with 90 mg.kg^{-1} of $ZnSO_4$ compared to non-supplemented birds [7]. O l u k o s i et al. [16] conducted experiment with supplementation of sulphate or hydroxychloride and reported that the higher Zn supplemental level released greater quantity of Zn into the blood.

The serum Cu (12.72 μmol.l^{-1}) was significantly increased ($P<0.001$) in broilers from experimental group G2 in comparison to the control group (10.28 μmol.l^{-1}). On the other hand, the addition 0.7% HNMy to the feed for broilers significantly decreased ($P<0.05$) serum Cu (8.69 μmol.l^{-1}) in G1 in comparison with serum Cu in broilers fed 0.3 % HNMy (10.45 μmol.l^{-1}) and 0.7 % HNM (12.72 μmol.l^{-1}). Copper plays an important role in the formation of red blood cells. It is required to absorb, utilize, and synthesize haemoglobin, which is necessary for red blood cells formation. The addition of humic substances to the feed for quails (360; 480; 600 mg.kg^{-1}) caused an increase in serum Zn (31.47; 35.79; 35.66 μmol.l^{-1}) and a decrease in serum Cu (31.47; 35.79; 35.66 μmol.l^{-1}) in three supplemented groups compared to the control broilers [4]. Blood and liver Cu concentrations are positively associated with the plasma ceruloplasmin concentration [2]. M i l l e r et al. [14] observed the levels of trace elements in sea ducks' blood and found that Cu concentrations differed little among species, site, or age class (adult females versus ducklings), perhaps due to Cu being closely regulated in the blood.

Table 4 shows the results of correlation analysis that revealed some relationships in the blood serum between the content of elements in the control and HNM supplemented groups.

Results obtained in our study indicated different correlations of the analysed elements in blood components as well as the effect on parameters of blood biochemical profiles. The addition of Humac Natur Mycosorb (0.3 % and 0.7 %) to feed resulted in strong correlation of blood serum levels between Ca and Mg ($r=0.7494$; $r=0.7648$). In contrast, the addition of 0.3 % Humac Natur Mycosorb in feed caused negative correlation ($r=–0.7078$) of blood serum levels between Mg and Zn. Also, the addition of 0.3 % Humac Natur Mycosorb to the feed showed a negative correlation ($r=−0.7616$) of the blood serum levels compared to the control group between the elements Cu and Ca. In the other groups mainly medium correlations were detected. For comparison, a positive correlation was observed for magnesium to zinc in the muscle of broilers fed 0.7 % Humac natur [19].

CONCLUSIONS

The results of this study demonstrate that the use of 0.7 % Humac Natur Monogastric as a feed additive significantly increased the levels of magnesium, zinc, and copper in the blood serum of broilers. The addition of Humac Natur Mycosorb (0.7 % and 0.3 %) to the broiler diet showed did not affect significantly the mineral content in the blood

Table 4. Correlation coefficients between blood serum mineral elements (Ca, Mg, Cu, Zn) in individual groups

		Control group				G1				G2				G3			
		Ca	Mg	Zn	Cu	Ca	Mg	Zn	Cu	Ca	Mg	Zn	Cu	Ca	Mg	Zn	Cu
Control	Ca	1.0	0.3296	0.0691	0.5597	−0.1012	−0.1839	0.2093	−0.2012	−0.2024	0.3114	−0.6598	−0.6078	0.1016	0.1098	−0.2741	0.1760
	Mg		1.0	0.3077	0.0352	0.3314	0.6036	0.3851	−0.3045	0.3411	0.1744	−0.6978	0.2696	0.6937	0.5386	−0.2594	0.3626
	Zn			1.0	0.0701	0.5547	0.3466	−0.3301	−0.4685	−0.0448	−0.3914	−0.4441	−0.1141	0.6499	0.1390	0.2921	−0.4050
	Cu				1.0	−0.2324	−0.3446	0.5157	−0.4902	−0.7616	−0.2058	−0.3077	−0.2084	0.1147	0.3760	−0.2142	0.3788
G1	Ca					1.0	0.7648	−0.5006	−0.292	0.4549	−0.2404	−0.5239	−0.1663	0.3969	−0.0443	0.3899	−0.0219
	Mg						1.0	−0.1162	−0.2904	0.4474	−0.2860	−0.5073	−0.1723	0.3569	−0.0412	0.3615	0.3639
	Zn							1.0	0.0286	−0.3894	−0.0128	−0.123	0.3168	0.0114	0.4797	−0.2900	0.6485
	Cu								1.0	0.3566	0.2525	0.3299	−0.1873	−0.5674	−0.2681	0.0908	−0.2245
G2	Ca									1.0	0.3310	−0.086	0.1498	0.2447	0.0767	−0.1220	−0.2322
	Mg										1.0	−0.0206	−0.0322	−0.0331	0.0765	−0.5656	−0.0784
	Zn											1.0	0.4213	−0.4382	−0.2154	0.0317	−0.3681
	Cu												1.0	0.3967	0.5037	−0.3221	0.1224
G3	Ca													1.0	0.7494	−0.3975	−0.1219
	Mg														1.0	−0.7078	0.1285
	Zn															1.0	−0.0925
	Cu																1.0

Control group—diet without the addition of humic substances; G1—0.7 % HNMy—diet with the addition of Humac Natur Mycosorb
G2—0.7 % HNM—diet with the addition of Humac Natur Monogastric; G3—0.3 % HNMy—diet with the addition of Humac Natur Mycosorb

serum. The advantages of dietary HA supplementation as a feed additive are promising. Humic acid has a favourable role in boosting productive performance due to its useful impact on nutrient utilization and absorption.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

1. **Abdel-Mageed, M.A.A., 2012:** Effect of dietary humic substances supplementation on performance and immunity of Japanese quail. *Egyptian Poult. Sci.*, 32, 3, 645—660.
2. **Acetoze, G., Kurzbard, R., Klasing, K.C., Ramsey, J.J., Rossow, H.A., 2017:** Liver mitochondrial oxygen consumption and proton leak kinetics in broilers supplemented with dietary copper or zinc following coccidiosis challenge. *J. Anim. Physiol. Anim. Nutr.*, 101, e210—15. doi: 10.1111/jpn.12591.
3. **Arif, M., Alagawany, M., Abd El-Hack, M.E., Saeed, M., Arian, M.A., Elnesr, S.S., 2019:** Humic acid as a feed additive in poultry diets: a review. *Iran. J. Vet. Res.*, 20, 167—172.
4. **Avci, M., Denek, M., Kaplan, O., 2007:** Effects of humic acid at different levels on growth performance, carcass yields and some biochemical parameters of quails. *J. Anim. Vet. Advan.*, 6, 1, 258—265.
5. **Bezuglova, O., Klimenko, A., 2022:** Application of humic substances in agricultural industry. *Agronomy*, 12, 3, 584. DOI: 10.3390/agronomy12030584.
6. **Boguslawska-Tryk, M., Szymeczko, R., Piotrowska, A., 2012:** The level of major proteins and minerals in the blood serum of chickens fed diets with pure cellulose. *Folia Biol.* (Kraków), 60, 1—2. DOI: 10.3409/fb60_1-2.65-70.
7. **Bortoluzzi, C., Vieira, B.S., Lumpkins, B., Mathis, G.F., King, W.D., Graugnard, D., et al., 2019:** Can dietary zinc diminish the impact of necrotic enteritis on growth performance of broiler chickens by modulating the intestinal immune-system and microbiota? *Poult. Sci.*, 98, 3181—93. doi: 10.3382/ps/pez045.
8. **Elfarissi, F., Pefferkorn, E., 2000:** Kaolinite/humic acid interaction in the presence of aluminium ion, *Colloids Surf. A.*, 168, 1—12. DOI: 10.1016/S0927-7757(99)00292-7.
9. **Islam, K.M.S., Schumacher A., Groop, J.M., 2005:** Humic acid substances in animal agriculture. *Pakistan J. Nutr.*, 4, 126—134. DOI: 10.1.1.1086.8724&rep=rep1&type=pdf.
10. **Jaďuttová, I., Marcinčáková, D., Bartkovský, M., Semjon, B., et al., 2019:** The effect of dietary humic substances on the fattening performance, carcass yield, blood biochemistry parameters and bone mineral of broiler chickens. *Acta Vet. Brno*, 88, 307—313. DOI: 10.2754/avb201988030307.
11. **Kovacik, A., Gasparovic, M., Tvrda, E., Tokarova, K., Kovacikova, E., Rolinec, M., et al., 2020:** Effects of humic acid diet on the serum biochemistry and oxidative status markers in pheasants. *Vet. Medicina*, 65, 258—268.
12. **List of the Official Methods and Laboratory Diagnostic of Food and Feed (In Slovak), 2004:** In *Bulletin of the Ministry of Agriculture of the Slovak Republic*. SR 2004.
13. **Madroňová, L., Kozler, J., Cezikova, J., Novák, J., János, P., 2001:** Humic acids from coal of the North-Bohemia coal field. III. Metal-binding properties of humic acids—measurements in a column arrangement. *React. Funct. Polymers*, 47, 119—123. DOI: 10.1016/S1381-5148(00)00077-8.
14. **Miller, M.W.C., Lovvorn, J.R., Matz, A.C., Taylor, R.J., Latty, C.J., Brooks, M.L., Hollmén, T.E., 2019:** Interspecific pattern of trace elements in sea ducks: Can surrogate species be used in contaminants monitoring? *Ecol. Indicators*, 98, 830—839. DOI: 10.1016/j.ecolind.2018.11.023.
15. **Naď, P., Marcin, A., Bujňák, L., Skalická, M., Gancarčíková, S., 2021:** Evaluation of the growth performance and some blood parameters in broilers with the addition of humic substance in the diet. *Acta Fytotechn. Zootechn.*, 24, 150—154. DOI: 10.15414/afz.2021.24.mi-prap.150-154.
16. **Olukosi, O.A., van Kuijk, S., Han, Y., 2018:** Copper and zinc source and levels of zinc inclusion influence growth performance, tissue trace mineral content, and carcass yield of broiler chickens. *Poult. Sci.*, 97, 11, 3891—3898. doi: 10.3382/ps/pey247.
17. **Ozturk, E., Ocak, N., Turan, A., Erener, G., Altop, A., Cankaya, S., 2012:** Performance, carcass, gastrointestinal tract and meat quality traits, and selected blood parameters of broilers fed diets supplemented with humic substances. *J. Sci. Food Agric.*, 92, 1, 59—65. DOI: 10.1002/jsfa.4541.
18. **Richards, J.D., Zhao, J., Harrell, R.J., Atwell, C.A., Dib-**

ner, J. J., 2010: Trace mineral nutrition in poultry and swine. *Asian-Australian J. Anim. Sci.*, 23, 1527—1534. DOI: 10.5713/ajas.2010.r.07.

19. **Skalická, M., Naď, P., Bujňák, L., Hudák, M., 2019:** Effect of humic substances on the mineral composition of chicken meat. *Anim. Husb. Dairy Vet. Sci.*, 3, 1—5. doi: 10.15761/ahdvs.1000168.
20. **Skalická, M., Koréneková, B., 2016:** The effects of sodium humate and aflatoxin B1 on body weight of broiler chicks. *Iran. J. Appl. Anim. Sci.*, 6, 415—421.
21. **Stepchenko, L. M., 2006:** Participation of humic preparations from peat in the control of metabolic processes in broiler chickens. In *Proceedings of the Int. Conference* (In Russian), Minsk, Belarus, May 29—June 2, 143—145.
22. **Van Rensburg, C. J., Van Rensburg, C. E. J., Van Ryssen, J. B. J., Casey, N. H., Rottinghaus, G. E. 2006:** *In vitro* and *in vivo* assessment of humic acid as an aflatoxin binder in broiler chickens. *Poult. Sci.,* 85, 1576—1583. DOI: 10.1093/ps/85.9.1576.

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EVIDENCE OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) IN PET AND STRAY DOGS WITHIN SOKOTO METROPOLIS, NIGERIA

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ABSTRACT

Methicillin Resistant *Staphylococcus aureus* (MRSA) is an important zoonotic pathogen capable of causing life threatening disease conditions in humans. A cross-sectional study was conducted to investigate the presence of MRSA in both pet and stray dogs within the Sokoto metropolis. A total of 100 oral swabs comprising 50 each from pet and stray dogs were collected and analyzed using routine bacteriological cultures and molecular identifications. Out of the 100 samples examined, 15% (15/100) were positive for MRSA with varying detection rates of 9/50 (18%) and 6/50 (12%) for the pet and stray dogs respectively. The statistical analysis showed no significant association between the occurrence of MRSA and the dogs (P = 0.401). The study revealed the presence of MRSA in dogs within the Sokoto metropolis, which presents health risks to pet dog owners, veterinarians, dog catchers and other individuals who may come into close contact with these dogs.

Key words: antibiotics; dogs; Nigeria; Sokoto

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a global public health problem, associated with considerable morbidity and mortality [29]. It is a well-known and widespread pathogen that has the ability to cause a wide spectrum of clinical diseases in both humans and other animals [7]. MRSA infections have been on the increase in the past few decades and as a result, the community acquired methicillin resistant *Staphylococcus aureus* (CA-MRSA) has been reported in many middle- and low-income countries. Although the origin of the CA-MRSA is still unclear, several clones of the pathogen have been reported to be rapidly spreading within communities and healthcare settings in many countries. Individuals having close contact with animals have been shown to be at a higher risk of contracting livestock acquired MRSA [25, 33].

Methicillin resistance is conferred by the *mecA* gene, which encodes an aberrant penicillin-binding protein (PBP2a) with decreased affinity for β-lactam antibiotics, so that PBP2a are able to catalyze the transpeptidation reaction required for peptidoglycan cross-linking thereby, ena-

bling cell wall synthesis in the presence of high concentrations of β-lactams which otherwise would inhibit the endogenous PBPs; thus, conferring resistance to all β-lactam antibiotics [3, 14].

There is a sizeable population of stray dogs in Nigeria that serve as reservoirs of many environmental pathogens and potential source of infections to pet dogs that are let outdoors unmonitored. Both dogs share the same outside environment and often come into contact with each other with or without the knowledge of the owners. Thus, there is potential interchange of pathogens between the dogs which could consequently extend to the pet owners or persons in close contact with dogs such as admirers and veterinarians.

MRSA strains carry a diverse and transmissible genetic element designated as MRSA colonization in dogs which have been extensively studied in most parts of the world with varying prevalence rates of 0—6% [23]. However, despite reports of the pathogen in humans [11] and the unethical use of antibiotics by veterinary quacks and animal owners, there is very little information about the presence of MRSA in dogs in Nigeria.

MATERIALS AND METHODS

Ethical approval

All experiments were done in accordance with the guidelines to the use and care of animals of the Research Ethics Committee of the Faculty of Veterinary Medicine, Usmanu Danfodiyo University, Sokoto, Nigeria (UDUS/FAREC/01/2019).

Study design and sample collection

A cross-sectional study was conducted between March and November, 2019. With the consent of the owners, oral swabs were collected from pet dogs across the Sokoto metropolis and from stray dogs captured and stationed at the Army barracks in Mammy Market, Sokoto. A total of one hundred samples comprising 50 each from both pet and stray dogs were randomly collected using a swab stick and transported in Brain Heart Infusion Broth (BHIB) to the Bacterial Zoonoses Laboratory, Usmanu Danfodiyo University, Sokoto, Nigeria.

Bacteriological culture and isolation

All swab samples were incubated in the transport medium (brain heart infusion broth) for 24 hours at 37ºC, before being inoculated onto blood agar (containing 5% sheep blood) and incubated aerobically at 37ºC for 24 hours. Colonies that appeared relatively small, circular, convex, smooth, and grayish to white and displayed light to golden yellow pigment were presumed to be *Staphylococcus aureus* and were sub-cultured onto Mannitol Salt agar (Oxoid, Basingstoke, UK) and incubated aerobically at 37ºC for 24 hours. Presumptive colonies of *Staphylococcus aureus* were identified as small, smooth, golden, shiny, convex, and with golden yellow zones. The colonies were then preserved by sub-culturing onto nutrient agar (Oxoid) slant and incubated at 37ºC for 12 hours. The presumptive *Staphylococcus aureus* isolates were further confirmed using a biochemical test including catalase and coagulase tests as described by Ochei, Kohlhatkar [28] and Cheesbrough [5].

Phenotypic and genotypic characterization of MRSA

To determine phenotypic methicillin resistance, overnight fresh cultures of *S. aureus* were inoculated onto a freshly prepared Oxacillin Resistance Screening Agar Base using the appropriate supplement (ORSAB; Oxoid®). Colonies that appeared intense blue on ORSAB were phenotypically presumed to be MRSA.

For genotypic characterization, the genomic DNA of the presumptive MRSA isolates were extracted using a boiling method with slight modifications as described by Chen et al. [6]. Briefly, a loop-full suspension of overnight grown cultures on nutrient agar plate was transferred into a 1.5 ml micro-centrifuge tube containing 100 µl of sterile distilled water. The suspension was first incubated at room temperature for 5 minutes, and then heat treated to 96 °C for 10 minutes. This was followed by centrifugation at 12,000 g for 5 minutes. The supernatant (containing the DNA) was then collected in a new 1.5 µl tube and kept at 4 ºC until use.

PCR detection of the *mecA* gene

The DNA templates of all the positive ORSAB *S. aureus* isolates were subjected to PCR for the detection of the 163 bp fragment of the *mecA* gene using that described by Mehrotra [24] (Table 1). The PCR assay was performed in a 25 µl reaction mixture containing 3 µl

of nuclease free water, 5 µl (0.5 µg) of the DNA template, 1 µl (0.2 µM) of each forward and reverse primer, 2.5 µl of coral red dye load and 12.5 µl (100 µM) of the Master mix (Qiagen). The amplification protocol consisted of 35 cycles of amplification with an initial denaturation of 94 °C for 5 min, denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min, an extension at 72 °C for 1 min and then final extension at 72 °C for 7 min. Confirmed *mecA* through sequencing in the lab was used as a positive control, while nuclease free water was used in place of DNA template as a negative control. The PCR products were visualized after electrophoresis for 45 min at 90 volts in 1 % agarose gel. The amplicons were then viewed under a UV trans-illuminator (Biorad Gel Doc XR System w/Universal Hood II).

Data analysis

The results presented in tables and percentages were computed using Microsoft Excel software version 2010. Chi-squared statistics were performed using SPSS v23 to determine the possible association between MRSA colonization and the dog type.

Table 1. Oligonucleotide sequences for the detection of *mecA* gene

Primer name	Primer sequence [5' to 3']	Band size [bp]
*mecA*1_forward	AAAATCGATGGTAAAGTTGGC	163
*mecA*2_reverse	AGTTCTGCAGTACCGGATTTGC	

RESULTS

Of the 100 oral swabs examined, 44 % (44/100) were positive for *Staphylococcus aureus* based on culture, and biochemical characterization. Pet dogs had the highest

Table 2. Distribution of *Staphylococcus aureus* in pet and stray dogs

Status	Pet dogs	Stray dogs	Total
Positive	26	18	44
Negative	24	32	56
Total	**50**	**50**	**100**

P = 0.796; 95 % CI: 0.117—16.444

Table 3. Distribution of methicillin resistant *Staphylococcus aureus* in pet and stray dogs

Status	Pet dogs	Stray dogs	Total
Positive	9	6	15
Negative	41	44	85
Total	**50**	**50**	**100**

χ^2 = 0.706; p = 0.401, 95 % CI: 0.203—1.899

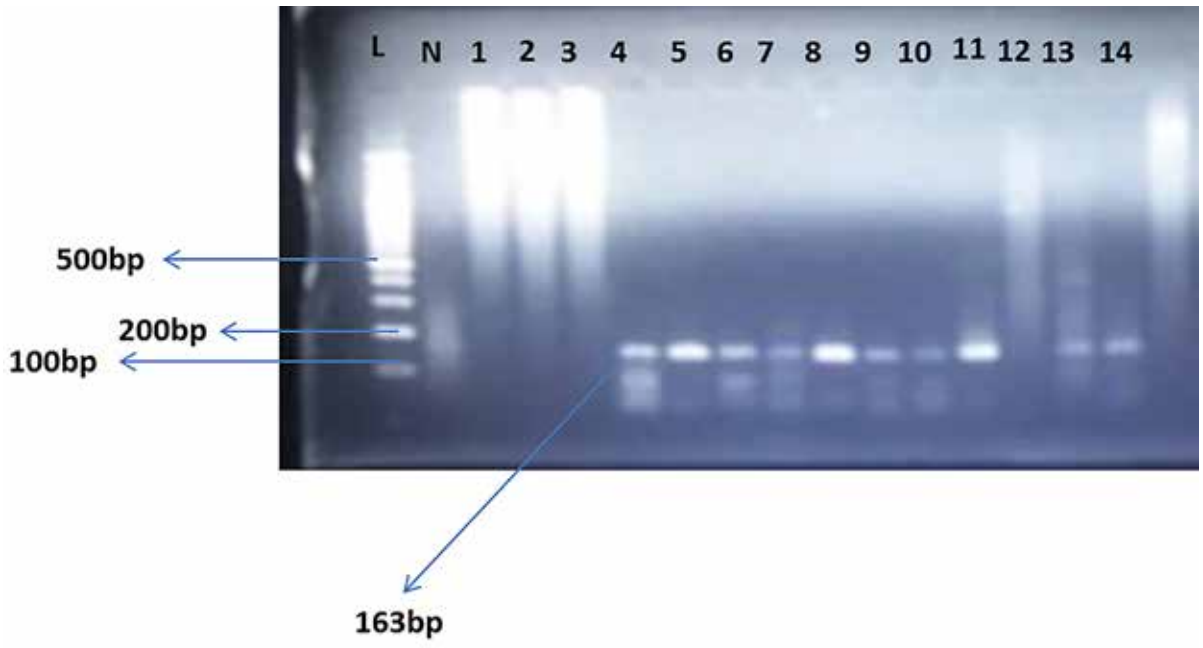

Fig. 1. Gel image of 163 bp gene fragment of the *mecA* gene using 100 bp molecular weight DNA ladder

prevalence with 52% (26/50), while stray dogs had 36% (18/50). There was no statistically significant association (P = 0.796) between the recovery rate of *S. aureus* and dog type (Table 2). The PCR detection of *mecA* gene showed that 15% (15/100) of MRSA isolates from dogs were positive (Figure 1). This comprised 18% (9/50) from pet dogs and 12% (6/50) from stray dogs. There was no statistically significant association (P = 0.40) between the recovery rate of MRSA and dog type (Table 3).

DISCUSSION

Methicillin resistance has increasingly been reported in staphylococcal isolates from canines in several countries [2, 16, 17, 36]. In the Sokoto state, the association between pet animals and humans has changed in the last few years. The number of owned dogs has dramatically increased in the society which has made dogs in closer contact with humans. This increase in acceptance of dogs as pets, partly due to the rising security challenges in the State and country at large, has increased the frequency of close contact between dogs and humans. Thus, it increases the risk of disease transmission between pets and humans. In this study, the overall oral carriage of MRSA from dogs was 15% (15/100), which was higher than the reports by Sasaki et al. [32], Abbott et al. [1], Kottler et al. [19], Penna et al. [30] Loeffler et al. [22] and Chah et al. [4] who, respectively, recorded 1.7%, 1.1%, 3.3%, 3.4%, 9.0% and 12.8% of MRSA in the dogs in the Ireland, UK, the US, and Nigeria, respectively.

This discrepancy is likely due to: differences in the breeds of dogs utilized in this study, indiscriminate antibiotic medication, which is prevalent in impoverished nations, harsher environmental difficulties often found in the study area, and malnutrition, which causes stress and impairs a dog's immune system. According to Kutdang et al. [20] findings, stray dogs and pet dogs raised in underdeveloped nations are more exposed to the outside environment and therefore, have a higher risk of catching diseases than domestic dogs. Floras et al. [10] added to the findings of this investigation by reporting on an increased rate of MRSA identification in dogs in Canada.

Contrarily, higher MRSA carriage of 67.5% and 51.1% have been reported by Vinczе et al. [35] and Iverson et al. [15], respectively. The non-significant statistical association (P = 0.401) observed between the presence of MRSA and dog type showed that both pet and stray dogs harbor the pathogen without preference associated with type of living condition. Pet dogs could acquire MRSA through contact with humans and other dogs [9]. However, the presence of the pathogen in stray dogs indicates possible contact between pet and stray dogs, which could be directly or indirectly through sharing the same environment [34]. Although, isolation of *S. aureus* is not uncommon in canine infection, MRSA isolation is of great concern as it becomes more prevalent among dogs in different regions. MRSA isolation among dogs has great public health implications because of the high risk of human infections following close contact with dogs [15]. Several studies have described potential transmission of MRSA strains between pet dogs and humans [9, 34]. However, to the best of our knowledge, this is the first study on the occurrence of MRSA in stray dogs in Nigeria. The presence of the pathogen in this type of dogs have serious public health consequences as they could serve as reservoir and sources of infection for pet dogs and other animals, including livestock that could have close contact with these dogs.

Several studies have suggested that dogs may be one of the gates through which MRSA found its way outside healthcare facilities leading to possible community transmission of such pathogen among animal and human populations [8, 21]. In agreement to this assertion, several studies in different countries have reported MRSA strains from dogs to be indistinguishable from the human strains [7, 26].

The prevalence of *S. aureus* and MRSA in the nasal cavity of dogs was extremely high. These findings show that dogs are more likely to get contaminated through their nostrils. Contaminations can also occur as a result of scavenging of death corpses from backyard poultry farms that have been exposed to antibiotics. These data, along with those of Rich, Roberts [31] and Khanna et al. [18], show that nasal sampling is a better way to detect MRSA colonization in animals. A single case of MRSA discovery from nasal swab samples of 255 dogs, rather than from the throat and skin of the same animals was confirmed by Rich and Roberts [31].

The current study's PCR investigation indicated the presence of the *mecA* gene at 163 bp in 15 of the 20 isolates that were phenotypically MRSA positive. When it comes to MRSA detection and characterization, the pres-

ence of the *mecA* gene is the gold standard [11]. According to studies, the existence of the *mecA* gene in animals and animal products supports this claim [12, 27, 37].

Some of the phenotypic MRSA positive isolates did not display *mecA* on amplification, which could imply the occurrence of *mecA* PCR negative MRSA isolates in Sokoto, according to the PCR study. G a r c i a - A l v a r e z et al. [13] discovered a novel allele of the *mecA* gene encoding an alternative penicillin binding protein that mediates methicillin resistance in bovine *S. aureus* isolates and humans in the UK, Denmark, and Germany who were Methicillin-resistant but *mecA* PCR negative in a previous study. These unique *mecA* alleles (*mecA* LGA251) exhibit a nucleotide similarity of 70 % with the archetypal *mecA* gene. Furthermore, the findings suggest that an additional *mecA* allele is circulating in the environment, which could be acquired by *S. aureus* and result in the development of a new MRSA strain.

CONCLUSIONS

This study showed the presence of MRSA in apparently healthy dogs (stray and pets) within the Sokoto metropolis of Nigeria. The findings also indicated that both pet and stray dogs harbour the pathogen with a potential risk of infecting individuals in close contact. There is the need to educate pet owners and dog catchers on the potential zoonotic transmission of MRSA from dogs and the need to take appropriate preventive measures.

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REFERENCES

1. **Abbott, Y. B., Legget, B., Rossney, A. S., Leonard, F. C., Markey, B. K., 2010:** Isolation rates of methicillin resistant *Staphylococcus aureus* in dogs, cats and horses in Ireland. *Vet. Rec.*, 166, 451—455. DOI: 10.1136/vr.b4814.
2. **Baptiste, K. E., Williams, K., Williams, N. J., Wattret, A., Clegg, P. D., Dawson, S., et al., 2005:** Methicillin resistant Staphylococci in companion animals. *Emerg. Infect. Dis.*, 11, 1942—1944. DOI: 10.3201/eid1112.050241.
3. **Berger-Bachi, B., 1995:** Factors affecting methicillin-resistant *Stapylococcus aureus*. *Int. J. Antimicrob. Agents*, 6. 13—21. DOI: 10.1016/0924-8579(95)00021-y.
4. **Chah, K. F., Soaz, E. G., Nwanta, J. A., Asadu, B., Agbo, I. F., Lozano, C., et al., 2014:** Methicillin-resistant coagulase negative Staphylococci from health dogs in Nsukka, Nigeria. *Braz. J. Microbiol.*, 45, 1, 215—220. DOI: 10.1590/S1517-83822014005000034.
5. **Cheesbrough, M., 2006:** *District Laboratory Practice in Tropical Countries*, Part 2. 2nd edn., Cambridge University Press. Cambridge, UK, 434 pp.
6. **Chen, L., Mediavilla, J. R., Oliveira, D. C., Willey, B. M., De Lencastre, H., Kreiswirth, B. N., 2009:** Multiplex real-time PCR for rapid staphylococcal cassette chromosome mec typing. *J. Clin.*, 47, 11, 3692—706. DOI: 10.1128/JCM.00766-09.
7. **David, M. Z., Daum, R. S., 2010:** Community-associated methicillin-resistant *Staphylococcus aureus*: Epidemiology and clinical consequences of an emerging epidemic. *Clin. Microbiol. Rev.*, 23, 616—687. DOI: 10.1128/CMR.00081-09.
8. **Deurenberg, R., Stobberingh, E. E., 2008:** The evolution of *Staphylococcus aureus*. *Infect. Genetic Evolut.*, 8, 747—763. DOI: 10.1016/j.meegid.2008.07.007.
9. **Ferreira, J. P., Anderson, K. L., Correa, M. T., Lyman, R., Ruffin, F., Reller, L. B., Fowler, V. G., 2011:** Transmission of MRSA between companion animals and infected human patients presenting to outpatient medical care facilities. *PLOS ONE*, 6, 11, 26978. DOI: 10.1371/journal.pone.0026978.
10. **Floras, A., Lawn, K., Slavic, D., Golding, G. R., Mulvey, M. R., Weese, J. S., 2010:** Sequence type398 methicillin-resistant *Staphylococcus aureus* infection and colonisation in dogs. *Vet. Rec.*, 166, 26, 826—827. DOI: 10.1136/vr.b4870.
11. **Gaddafi, M. S., Yakubu, Y., Garba, B., Bello, M. B., Musawa, I. A., Lawal, H., 2020:** Occurrence and antimicrobial resistant patterns of methicillin resistant *Staphylococcus aureus* (MRSA) among practicing veterinarians in Kebbi State, Nigeria. *Folia Vet.*, 64. 55—62. DOI: 10.2478/fv-2020-0038.
12. **Gaddafi, M. S., Yakubu, Y., Junaidu, A. U., Bello, M. B., Garba, B., Bitrus, A. A., et al., 2021:** Nasal colonization of pigs and farm attendants by *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) in Kebbi, Northwestern Nigeria. *Thai. J. Vet. Med.*, 51, 1, 119—124. DOI: 10.14456/tjvm.2021.16.

13. **Garcia-Alvarez, L., Holden, M.T., Lindsay, H., Webb, C. R., Brown, D.F., Curran, M. D., 2011:** Methicillin-resistant *Staphylococcus aureus* with a novel *mecA* homologue in human and bovine population in the UK and Denmark: A descriptive study. *Lancet Infect., Dis.*, 11, 595—603. DOI: 10.1016/S1473-3099(11)70126-8.
14. **Hunter, P.A., Dawson, S., French, G.L., Goossens, H., Hawkey, P.M., Kuijper, E.J., et al., 2011:** Antimicrobial-resistant pathogens in animals and man: prescribing, practices and policies. *J. Antimicr. Chem.*, 66, 3—17. DOI: 10.1093/jac/dkp433.
15. **Iverson, S.A., Brazil, A.M., Ferguson, J.M., Nelson, K., Lautenbach, E., Rankin, S.C., et al., 2015:** Anatomical patterns of colonization of pets with staphylococcal species in homes of people with methicillin-resistant *Staphylococcus aureus* (MRSA) skin or soft tissue infection (SSTI). *Vet. Microbiol.*, 176, 1—2, 202—208. DOI: 10.1016/j.vetmic.2015.01.003.
16. **Jang, Y., Bae, D., Cho, J., Bahk, G., Lim, S., Lee, Y., 2014:** Characterization of methicillin-resistant *Staphylococcus* spp. isolated from dogs in Korea. *Jap. J. Vet. Res.*, 62, 4, 163—170. DOI: 10.14943/jjvr.62.4.163.
17. **Kanako, I., Mieko, S., Natsumi, S., Yasukazu, M., Shigeki, M., Yutaka, T., 2014:** Methicillin-resistant *Staphylococcus aureus* carriage among veterinary staff and dogs in private veterinary clinics in Hokkaido, Japan. *Microbiol. Immunol.,* 58, 149—154 DOI: 10.1111/1348-0421.12128.
18. **Khanna, T., Friendship, R., Dewey, C., Weese, J.S., 2008:** Methicillin resistant *Staphylococcus aureus* colonization in pigs and pig farmers. *Vet. Microbiol.*, 128, 3—4, 298—303. DOI: 10.1016/j.vetmic.2007.10.006.
19. **Kottler, S., Middleton, J.R., Perry, J., Weese, J.S., Cohn, L.A., 2010:** Prevalence of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* carriage in three populations. *J. Vet. Int. Med.*, 24, 1, 132—139. DOI: 10.1111/j.1939-1676.2009.0424.x.
20. **Kutdang, E.T., Bukbuk, D.N., Ajayi, J.A.A., 2010:** The prevalence of intestinal helminths of dogs (*Canis familiaris*) in Jos, Plateau State, Nigeria. *Research*, 2, 8, 51—56.
21. **Lin, Y., Barker, E., Kislow, J., Kaldhone, P., Stemper, M.E., Pantrangi, M., et al., 2011:** Evidence of multiple virulence subtypes in nosocomial and community-associated MRSA genotypes in companion animals from the upper mid-western and northeastern United States. *Clin. Med. Res.*, 9, 1, 7—16. DOI: 10.3121/cmr.2010.944.
22. **Loeffler, A., Boag, A.K., Sung, J., Lindsay, J.A., Guardabassi, L., Dalsgaard, A., et al., 2005:** Prevalence of methicillin-resistant *Staphylococcus aureus* among staff and pets in a small animal referral hospital in the UK. *J. Antimicrob. Chemother.*, 56, 4, 692—697. DOI: 10.1093/jac/dki312.
23. **Manian, F.A., 2003:** Asymptomatic nasal carriage of mupirocin-resistant, methicillin-resistant *Staphylococcus aureus* (MRSA) in a pet dog associated with MRSA infection in household contacts. *Clin. Infect. Dis.*, 36, 26—28. DOI: 10.1086/344772.
24. **Mehrotra, M., Wang, G., Johnson, W.M., 2000:** Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1 and methicillin resistance. *J. Clin. Microbiol.*, 38, 3, 1032—1035. DOI: 10.1128/JCM.38.3.1032-1035.2000.
25. **Monecke, S., Skakni, L., Hassan, R., Ruppelt, A., Ghazal, S.S., Hakawi, A., et al., 2012:** Characterization of MRSA strains isolated from patients in a hospital in Riyadh, Kingdom of Saudi Arabia. *BMC Microbiol.*, 12, 146. DOI: 10.1186/1471-2180-12-146.
26. **Moodley, A., Stegger, M., Bagcigil, A., Baptiste, K.E., Loeffler, A., Lloyd, D., et al., 2006:** Spa typing methicillin-resistant *Staphylococcus aureus* isolated from domestic animals and veterinary staff in the UK and Ireland. *J. Antimicrob. Chemother.*, 58, 1118—1123. DOI: 10.1093/jac/dkl394.
27. **Musawa, I.A., Yakubu, Y., Garba, B., Ballah, F.M., Jibril, H.A., Bello, A.S., et al., 2020:** Dressed chicken as potential vehicle for spread of methicillin-resistant *Staphylococcus aureus* in Sokoto, Nigeria. *Future Sci. OA*, 6, 10, 619. DOI: 10.2144/fsoa-2020-0066.
28. **Ochei, J.O., Kolhatkar, A.A., 2000:** *Medical Laboratory Science: Theory and Practice*. McGraw Hill Education, 648—657.
29. **Pallab, R., Singh, R., 2013:** Methicillin-resistant *Staphylococcus aureus* carriage screening in intensive care. *Indian J. Crit. Care. Med.*, 17, 4, 205—06. DOI: 10.4103/0972-5229.118409.
30. **Penna, B., Marcella, B.S., André, E.R., Soares, A.T.R., Vasconcelos, M.S.R., Fabienne, A.F., et al., 2021:** Comparative genomics of MRSA strains from human and canine origins reveals similar virulence gene repertoire. *Sci. Rep.*, 11, 1, 4724. DOI: 10.1038/s41598-021-83993-5.
31. **Rich, M., Roberts, L., 2004:** Methicillin-resistant *Staphylococcus aureus* isolates from companion animals. *Vet. Rec.*, 154, 10, 310. PMID: 15053141.
32. **Sasaki, T., Kikuchi, K.., Tanaka, Y., Takahashi, N., Kamata, S., Hiramatsu, K., 2007:** Methicillin resistant *Staphylo-*

coccus pseudintermedius in a veterinary teaching hospital. *J. Clin. Microbiol.*, 45, 1118—1125. DOI: 10.1128/JCM.02193-06.

33. Stefani, S., Chung, D.R., Lindsay, J.A., Friedrich, A.W., Kearns, A.M., Westh, H., et al., 2012: Methicillin-resistant *Staphylococcus aureus* (MRSA): global epidemiology and harmonization of typing methods. *Int. J. Antimicr. Agents*, 39, 4, 273—82. DOI: 10.1016/j.ijantimicagi.2011.09.030.

34. Vincze, S., Brandenburg, A.G., Espelage, W., Stamm, I., Wieler, L.H., Kopp, P.A., et al., 2014: Risk factors for MRSA infection in companion animals: results from a case-control study within Germany. *Int. J. Med. Microbiol.*, 304, 7, 787—93. DOI: 10.1016/j.ijmm.2014.07.007.

35. Vincze, S., Stamm, I., Kopp, P.A., Hermes, J., Adlhoch, C., Semmler, T., et al., 2014: Alarming proportions of methicillin-resistant *Staphylococcus aureus* (MRSA) in wound samples from companion animals, Germany 2010—2012. *PLOS ONE*, 20, 9, 1, 85656. DOI: 10.1371/journal.pone.0085656.

36. Worthing, K.A., Schwendener, S., Perreten, V., Saputra, S., Coombs, G.W., Pang, S., et al., 2018: Characterization of Staphylococal Cassette chromosome mec elements from methicillin-resistant *Staphylococcus pseudintermedius* infection in Australian animals. *mSphere*, 3, 491—518. DOI: 10.1128/mSphere.00491-18.

37. Yusuf, Y., Aliyu, M.I., Bello, S.A., Fatima, B., Abdullahi, A., Aliyu, M.D., 2020: Occurrence of methicillin resistant *Staphylococcus aureus* (MRSA) in some retailed animal products in Sokoto, Nigeria. *Alexandria J. Vet. Sci.*, 64, 1. 135—142. DOI: 10.5455/ajvs.44682.

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EVALUATION OF EXTERIOR FAULTS IN SELECTED SLOVAK RABBIT BREEDS

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ABSTRACT

The aim of this study was to evaluate the exterior faults in selected Slovak breeds of rabbits of different colour varieties according to a current Book of Rabbit Standards. Of 336 rabbits from 9 exhibitions organized in the territory of the Slovak and Czech Republics, 2 national medium-sized breeds of Liptov Bold-Spotted Rabbit (270 pcs) and Nitra Rabbit (66 pcs) were selected. The qualities and exterior faults of typical breed traits were evaluated in seven positions including: weight, shape, type, coat, top colour (eventually markings), under-colour (eventually intermediate colour), condition and health. Our evaluations showed that the most common exterior faults in these categories were in the: positions, shape, type, coat, and top-colour. In the shape position there were found significant exterior faults, such as slightly protruding hips with slanting rump, and worse legs position with loose skin on the body. In the type position, the faults observed in all selected breeds included: narrow chest, body too lean or too long, more delicate head with ears too fine and longer. The coat was usually thick and less elastic with a lighter under-colour at the base of the skin and non-sharply defined intermediate colour. In the top colour position of evaluated breeds there were numerous deficiencies involving uneven, and incomplete colour, and significant faults in the drawing. The data obtained in this study were innovative, as they represented a new approach that may help to characterize the

Slovak breeds of rabbits included in this study and to select individuals with the best exterior traits towards improving the quality of these breeds.

Key words: assessment; coat; Liptov Bold-Spotted rabbit; Nitra rabbit, shape; standard

INTRODUCTION

Today, a wide range of giant, medium, small and dwarf rabbit breeds are bred. By breed we mean a group of animals of the same phylogenetic origin, which has the same useful traits and characteristics. The group must be large enough. It transmits its characteristics to the offspring as long as the conditions of the external environment do not change [11, 14]. By 2017, there existed at least 305 breeds of domestic rabbit in 70 countries round the world [3].

Among Slovak breeders, national breeds are represented. Among them there are: Slovak Grey Blue Rex and Dwarf Slovak Grey Blue Rex, Blue of Holic Rabbit, Nitra Rabbit (Ni), Zobor Rabbit, Zemplin Rabbit, Liptov Bold-Spotted Rabbit (LL), Zemplin Rex, Slovak Pastel Rabbit, Slovak Pastel Rex and its dwarf form (Dwarf Slovak Pastel Rex), Štrba Gepard Rabbit, Štrba Gepard Rex, Chrabrany Rabbit, and Saris Giant Rabbit waiting for recognition. These national breeds are often exhibited at most breeding exhibitions held in the Czech and Slovak Republics [9, 10].

Determining the breeding value of rabbits by assessing the exterior at various exhibitions has always been a major issue. The breeding efforts of any species of animals require certain limits that set the trend for the given field [7]. Standards of breeds serve as parameters that help the breeders to achieve the optimum appearance in order to get as close as possible to the dreamed-of ideal of the breed. It is in fact a detailed description of the exterior of individual breeds that characterizes the ideal representative of the breed. There is also description of faults—deviations from the ideal. Defects are divided according to their severity into minor and mayor faults. The purebred animal is evaluated at exhibitions by trained judges. They compare the animal′s characteristics with the standard requirements. The final evaluation serves then as the guiding information for the breeding selection of animals within the further purebred breeding [16].

A summary of all standards is given in the current Breed Books of Standards. A relevant pattern book of rabbit breeds is a specific publication which is published under the auspices of the Association of Breeders that unites the breeders in a given territory. There are prepared, regularly updated or newly published pattern books for the given areas of expertise [8, 9].

The aim of this study was to evaluate exterior faults of two Slovak breeds of rabbits presented at 9 exhibitions, organized in the territory of the Slovak and Czech Republics, based on the current Book of rabbit standards.

MATERIALS AND METHODS

Selection and characteristics of Slovak rabbit breeds

For the purpose of this study we selected two Slovak medium-sized breeds, namely Liptov Bold-Spotted Rabbit (270 pcs) and Nitra Rabbit (66 pcs), which were evaluated at 9 exhibitions in 2018 and 2019.

Liptov Bold-Spotted Rabbit (LL)

LL is one of the younger Slovak national breeds of rabbits. It is a smaller to medium breed with good productivity and fertility, which appears more and more often at exhibitions of all kinds (Fig. 1). There are several colour patterns of Liptov Bold-Spotted Rabbit: wild-coloured, grey-blue, black and blue [1]. The weight is from 3.50 to 4.25 kg. The body is stocky, cylindrical and the posture is half-high on strong, erect forelegs. The ears are firm with a length of 9.0—11.0 cm. The sign of the marking is a white coat that starts at the base of ears and runs over the forehead and nose towards the lips. The ideal width of the coat is 1–3 cm in its centre. A sharp border of the marking and its regularity along the entire length is required. Toenails are always pigmented [9].

Nitra Rabbit (Ni)

Nitra rabbit is one of the favourite Slovak breeds of rabbits, mainly for its very good utility properties. Currently, breeders in Slovakia keep 1,300—1,500 Nitra rabbits [1]. The Ni breed is the result of a combination of genes of three high-quality foreign breeds, namely French Silver, Hymalayan and California. At present, they are reared in blue and a wild-blue colour and is a favourite breed of rabbits especially due to its very good fertility [12].

Fig. 1. Liptov Bold-Spotted Rabbit and Nitra Rabbit
Photo: Mikurda (2020) and Šimek (2014)

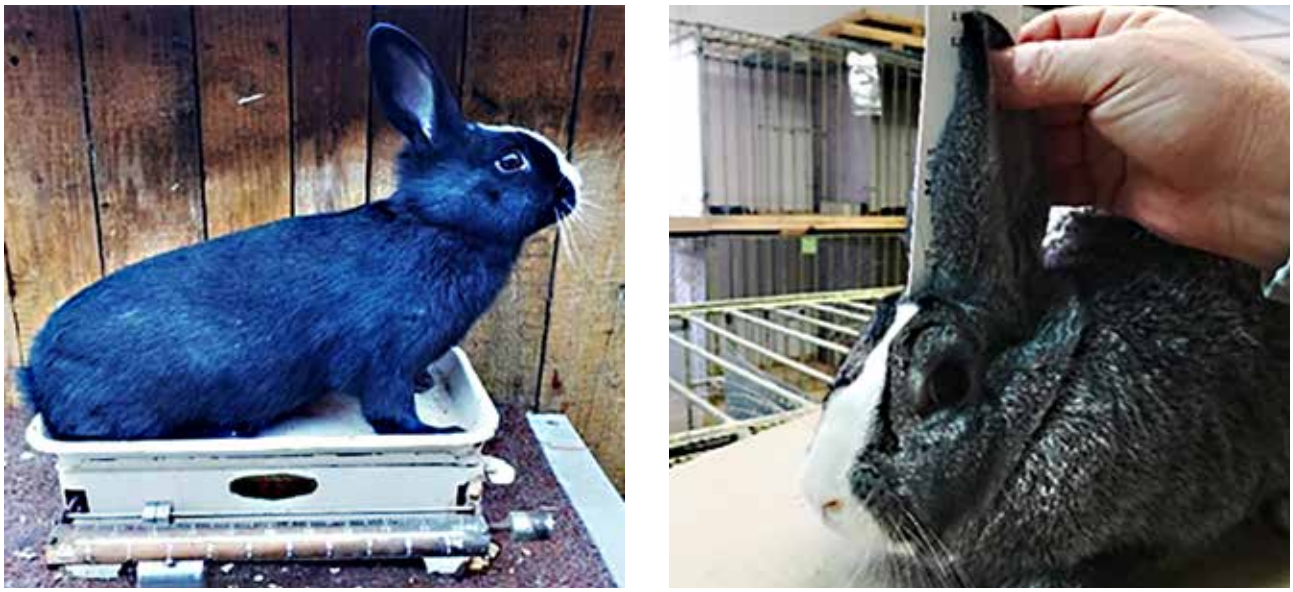

Fig. 2. Evaluation of the rabbit in individual positions
Photo: Britaňáková (2019)

Nitra rabbits weigh from 4.25 to 5.25 kg. The body is very stocky with a strong musculature of the thighs and a wide head. The limbs are shorter and stronger and the posture is semi-upright. The ears are firm and they are 11–12 cm long. The marking of the head consists of a mask and coloured ears. The mask covers the nasal part, has a regular oval shape and extends to the eye level. The marking of the ears is sharply defined at the root. The limbs are coloured to the elbow joint or to the heel. The toenails are horn-coloured and the colour of eyes are light red with a dark red pupil (Fig. 1) [8].

Assessment of rabbits and characteristics of individual positions

Exterior features were compared with the faults and strengths listed on the evaluation cards of rabbits that were assessed at 9 exhibitions in the Czech Republic and Slovakia. Observation, evaluation and subsequent awarding of prizes at exhibitions were performed by rabbit judges who have passed the relevant professional examinations. Prior to the self-assessment, the rabbit judge checks the legibility and accuracy of the tattoo. At the beginning of the judging, the rabbits are weighed and their sex is checked.

Table 1. Rabbit scoring system and distribution of points

Position number	Czech/Slovak	Czech[1]	Slovak[2]
	Characteristic of position	Standard	Standard
1	Weight	10	10
2	Shape	20	20
3	Type	20	15
4	Coat	15	20
5	Specific breed traits[4]	20	20
6	Specific breed traits[5]	10	10
7	Condition and health	5	5
Total		**100**	**100**

Points are distributed according to the relevant national Rabbit Breed Books of Standards in the [1]—Czech Republic, [2]—Slovak Republic; [4, 5]—Specific breed traits are given in each specific breed standard

Table 2. Classification scale of rabbit assessment is also expressed verbally

Point range	Classification
97.0 to 100	Excellent
95.0 to 96.5	Outstanding
93.0 to 94.5	Very good
89.0 to 92.5	Good
85.0 to 88.5	Satisfactory
84.5 and under	Not satisfactory

Overall score of rabbits is expressed as several classifications in the Slovak Republic. Source: table modified according to [10]

When evaluating, the examiners try to be as considerate as possible so as to disturb the rabbits as little as possible. If the animal is shy, they give it time to calm down and show their normal posture. Judging of a rabbit consists in comparing the exterior of a particular rabbit with the requirements of the standard for individual positions (Fig. 2). These positions are: weight, shape, type, coat, top colour (eventually markings), under colour (eventually intermediate colour), condition and health [17, 18].

These exterior positions are similar in most of the Books of Rabbit Breed Standards but its specific name, arrangement and the points maximum depend on the specific country (Tab. 1). The result of the judging is a rabbit's remarks card with assigned points and notes about individual positions, and the total score of points according to which the rabbit is classified (Tab. 2) [8, 9].

Statistical analysis

Exterior faults and points assigned to the judged breeds for individual positions were summarized and statistically compared to the rabbit show judges' remark cards. Statistical analysis was performed using software Microsoft Excel 2007. The dependence between individual positions of selected breeds was statistically analysed using the chi-squared test with the significance level $\alpha = 0.05$, critical value $\chi^2 = 3.502$, and testing value—G. Statistical independence between individual positions was confirmed when $G > \chi^2$; the independence was not statistically significant at testing values of $G < \chi^2$.

RESULTS

The identified exterior deficiencies and advantages were evaluated according to individual positions, which correspond to the prescribed standard on the rabbit's remark card. The evaluation of the weight—first position of the assessed national breeds of rabbits is shown in Figure 3. In this position, deficiencies in reduced weight were observed, especially in Ni (10.6%) that specified by the standard. In individuals with reduced weight, only points were deducted from total score without the exclusion of rabbits due to insufficient weight.

The occurrence of the most common deficiencies in the second position, which is the shape, as shown in Table 3. The most common deficiencies in this position in both analyzed national breeds was protruding pelvic bones in the

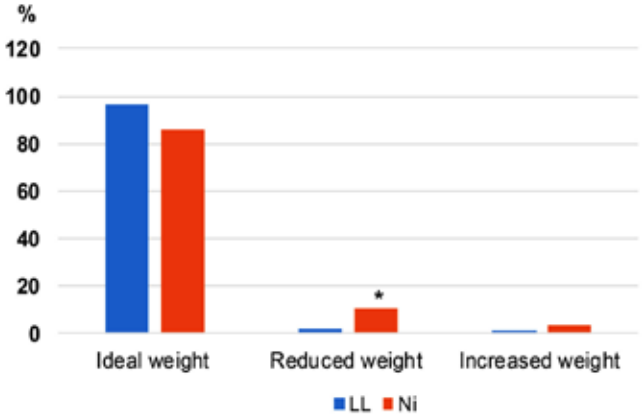

Figure 3. First position—weight assessment
*—P < 0.05, significant differences between the compared breeds of rabbits

LL breed (45.6%) and in 37.8% of Ni. The second most common faults in this position found of both breeds was dewlap, with looser skin on the body (20.4% of LL and 12.1% of Ni), turned to lobes in some individuals. This fault with the occurrence of a lobe was observed especially in females of both breeds. On the other hand, in the position shape were observed individuals in the Ni breed with very good formation of the back line (60.6%).

Table 3 shows the exterior deficiencies in the position of the type in which the frame of the body, limbs, exhibition posture, sexual expression, ears and head were evaluated. From the evaluated individuals of the Ni rabbits, up to 15.0% had a longer, narrower or shorter body and finer structure of ears (5.0%) were found. In the LL breed were found narrower or shorter body (12% of individuals) and finer structure of the ears (6.3% of individuals).

Table 3. Summary of the most common exterior faults in positions—Shape and Type

Position	Exterior faults	LL (270 pcs)		Ni (66 pcs)		P value
		pcs	%	pcs	%	
Shape	Slightly protruding hips	123	45.6	25	37.8	5.040*
	Bowed or splayed legs	15	5.6	2	3.0	1.805
	Loose skin on the body	55	20.4	8	12.1	3.570*
	Dewlap (does)	22	16.8	17	42.5	4.751
Type	Narrow chest/body too lean or too long	32	12.0	10	15.0	3.603*
	Finer structure of ears	17	6.3	5	7.6	2.704
	Irregular shape of ears	38	14.1	0	0	1.557

LL—Liptov Bold-Spotted Rabbit; Ni—Nitra Rabbit; *Chi-squared test significance level $\alpha = 0.05$; critical value $\chi^2 = 3.502$; Testing value (G) and statistical independence of individual position was confirmed when $G > \chi^2$; the independence was not statistically significant when testing value was $G < \chi^2$; NS—not significant

Table 4. Summary of the most common exterior faults in positions – Coat

Position		LL (270 pcs)		Ni (66 pcs)		P value
		pcs	%	pcs	%	
	Exterior faults					
Coat	Poor structure	5	1.9	10	15.0	3.570*
	Dense and less elastic	15	5.6	17	26.0	3.890*
	Soft or longer	2	0.7	1	1.5	1.502
	Exterior advantages					
Coat	Very good density and structure of coat	75	28.0	15	23.0	4.203*

LL—Liptov Bold-Spotted Rabbit; Ni—Nitra Rabbit; NS—not significant; *—Chi-squared test significance level $\alpha = 0.05$; critical value $\chi^2 = 3.502$; Testing value (G) and statistical independence of individual position was confirmed when $G > \chi^2$; the independence was not statistically significant when testing value was $G < \chi^2$

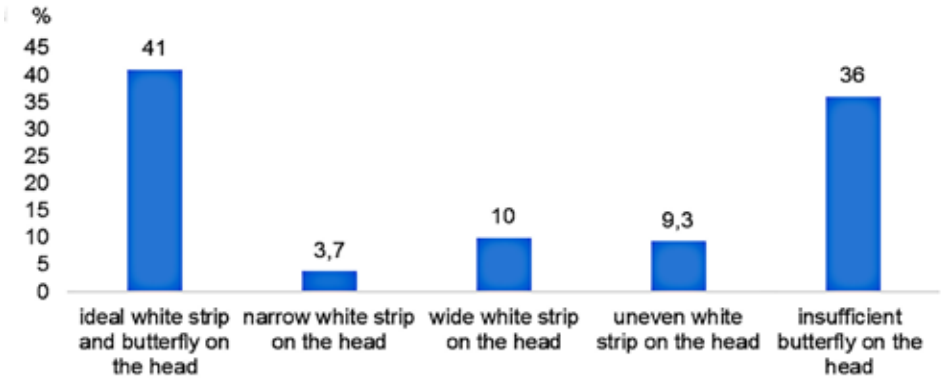

Fig. 4. The most common marking deficiencies in LL
LL—Liptov Bold-Spotted Rabbit; Ni—Nitra Rabbit

Exterior faults and advantages of position—coat is described in Table 4. The coat in both analyzed national breeds were often less flexible and dense. On the other hand, 28 % of LL, and 23 % of Ni rabbits had very good density and structure of the coat.

Breeding traits including top colour (eventually markings), under colour (eventually intermediate colour) were assessment in fifth and sixth position. More than half of the LL rabbits had marking deficiencies (59.0 %). The most common shortcomings in marking in LL and their percentage are shown in Figure 4. The most common faults in the Ni drawing were less defined drawing in 36.4 % of the rabbits, lower intensity of colouration (22.7 %) and lower or higher marking on limbs in 15.2 % of the rabbits (Figure 6).

In the sixth position, the undercoat and the intermediate colour were evaluated. Mainly, the absence of intermediate colour was a frequent error. From LL individuals with wild factor was observed a less pronounced intermediate colour (18.6 %). In the Slovak breed of the Ni, the most common fault was the yellowish colouring observed in 18.2 % of the individuals. The second common faults observed in LL rabbits were isolated white hairs in the base in 15.6 % of rabbits, followed by a slightly brownish cover colour in 13.7 % of the rabbits.

The seventh position is reserved for the evaluation of the animal's condition, pre-exhibition care and health which can affect the overall score. In total, faults in health care were detected in five rabbits of the Ni breed and seven LL rabbits, which represents 3.57 % of the total number of 336 rabbits. Most animals had untreated claws (3 pcs of Ni and 3 pcs of LL breed) and unclean genitals (2 pcs of Ni and 4 pcs of LL rabbits).

DISCUSSION

Exterior deficiencies of selected breeds of LL and Ni were classified according to individual positions on the remark card. These positions include: weight, shape, type, coat, colour and marking, under-colour (eventually intermediate colour) and condition and health.

Weight is a basic and very important feature of every breed. The Ni and LL belong to the group of medium breeds of rabbits, which are the most numerous rabbits bred and exhibited in Slovakia and the Czech Republic. This is probably due to the fact that these rabbits are not bred only for exhibitions but also for high quality meat or attractive fur. Optimum weight for each breed is specified by the relevant standard [13]. According to the current Books of Standards for rabbits, the ideal weight of Ni is in the range of 4.25—5.25 kg and of LL in the range of 3.50—4.25 kg [14]. It is sometimes problematic to reach the minimum required weigh with young exhibited individuals, which was also confirmed in our study. We observed more frequent deficiencies in the Ni breed (10.6 %) involving lower weight than that specified by the standard.

Since the Ni rabbit belongs to the meat-type rabbit breeds with a minimum weight of 4.25 at 8 months of age, the breeders should ensure an optimum nutrition throughout the period of fattening. According to Neirurerová and Fik [4] rabbits of this breed gain weight most efficiently between months 2 and 4 of age when they reach a carcass weight of about 3 kg. In the following period (after 4 months of age), the authors recorded reduced weight gains in 12 out of 21 females and in 3 out of 15 males compared to the standard, which corresponds up to 42 % of rabbits with unsatisfactory weight. With respect to the

Fig. 5. Irregular and ideal white belt on the head in LL
Photo: Britaňaková (2019)

above results, it is necessary to focus on the correct fattening regimen of rabbits and checking their weight gains before assigning them for exhibitions.

In the shape position, we assessed: the course of the dorsal line, position of the limbs, position of the tail, the skin, and the external genital organs [10]. According to our previous findings, protruding pelvic bones are one of the most common defects in most rabbit breeds [5, 18]. This was confirmed also in our study. The protruding hips refers to weak muscling of the rump that is often a problem in the meat performance traits among medium breeds of rabbits. This group of medium breeds is characterized by rapid growth and development of the skeleton during the first 6 months of life. In young individuals included in exhibitions, there is often incomplete muscularization, which is manifested with protruding pelvic bones in the LL breed (45.6 %) and in 37.8 % of the Ni. On the contrary, in the Ni breed, we recorded a very good to excellent formation of the back line in most individuals (60.6 %). Another most common fault in this position were the relatively numerous changes in the skin. The most serious faults in skin changes were found in females of the evaluated breeds of rabbits, in which looser skin under the neck turned to lobes in some individuals. This was observed mainly in females of both breeds, Ni (42.5 %) and LL (16.8 %) (Tab. 3).

The rabbit type is a very important position in which we evaluate the traits that often define the very exterior essence of the breed. The frame of: the body, limbs, exhibition posture, sexual expression, ears and head are evaluated [7]. In the group of Ni rabbits, up to 15.2 % of the animals had a longer, narrower or shorter body. Of all rabbit breeds, the highest incidence of longer neck deficiency (3.0 %) was found in the Ni.

Both breeds evaluated in our study have erect ears. The ears of these breeds should be: kept firm, as close together as possible, well furred, spoon-shaped, and of ideal length [9]. Significant deficiencies in the length of the ears, especially the ears longer than the standard length, were observed in the LL breed (14.1 %). As a result of this fault (longer ears), 14 individuals (2.6 %) of this breed were excluded.

In the position of coat, Zhang et al. [15] and Rogers et al. [6] reported five basic parameters that are evaluated in this position in all rabbit breeds. These are: length, balance, density, flexibility, and structure. The exterior hair deficiencies observed in the evaluated medium breeds of rabbits included mainly dense, less elastic hair.

In both selected breeds, the coat was most often less elastic (31.6 %) and dense. In the position of the coat, Liptov Bold-Spotted Rabbit did not deviate significantly from the average; most often the coat was dense and less elastic (5.6 %). Ni rabbit had more significant coat faults compared to LL, as in up to 26.0 % of cases a dense and less elastic coat was recorded (Tab. 4).

Breeding of rabbits with marking is very popular, but achieving a drawing that meets the standard is challenging [3]. The Slovak national breed LL has a unique marking with very specific coat on the rabbit's head. Overall, more than half of the rabbits of this breed had marking deficiencies (59.0 %). The most common shortcomings in marking in LL and their percentage are shown in Fig. 5.

The features of the Ni rabbit marking include dark colouration of the ears, nasal mask, tail, and lower limbs [8]. The mask covers the nasal part, has a regular oval shape and extends to the eye level. The marking on ears is sharply defined at the root. The limbs are coloured up to the elbow joint, or up to the heel. The tail is also coloured.

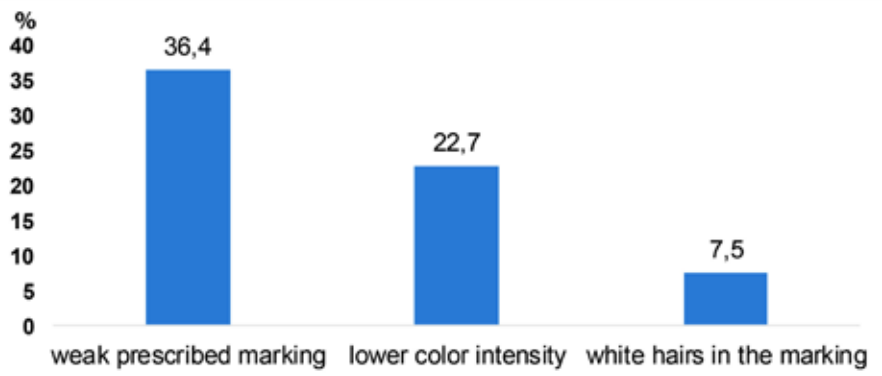

Fig. 6. The most common marking deficiencies in Ni rabbit

According to V a š í č k o v á et al. [12] low intensity of colouration and lack of compact colour attributes is frequently observed in this breed and was also confirmed in our study. The faults found in the Ni drawing involved mainly less defined drawing in 36.4% of the rabbits, lower intensity of colouration (22.7%) and lower or higher marking on limbs in 15.2% of the rabbits (Fig. 6).

In the following position were evaluated mainly the undercoat and the intermediate colour. The undercoat starts at the skin and continues upwards. We mainly evaluated the colour and integrity of the base. The intermediate colour was located between the undercoat and the cover hair. It is a coloured stripe that occurs in rabbits with a wild factor. The colour and its intensity, width, and border are evaluated for the intermediate colour [18].

According to D a l l e Z o t t e et al. [2] in practice we encounter more frequently variations in colour intensity rather than the problems with the colour itself. The most common type of intermediate colour is yellow-brown to rusty brown-red, which is characteristic of the wild coloured LL. The width is prescribed by the standard, but in practice its evaluation most often depends on the experience of the assessor. We consider the absence of intermediate colour to be an inadmissible error. In the LL breed with wild factor, the most common deficiency was a less pronounced intermediate colour (18.6%).

In the Slovak breed Ni, the colour of the white base and eyes are evaluated. Since the base colour is white, the most common fault was the yellowish colouring observed in 18.2% of the rabbits. In the LL breed, in addition to the undercoat and the intermediate colour, the colour of the base and marking and eyes and claws colour are also evaluated. The most common faults observed in the LL rabbits were isolated white hairs in the base in 15.6% of rabbits, followed by a slightly brownish cover colour in 13.7% of the rabbits. The major fault observed in 1.1% of the rabbits involved the presence of a white claw.

CONCLUSIONS

The results of this study showed that the exterior deficiencies in the positions of weight, shape, type, coat and cover colour were most frequently observed in the evaluated breeds of rabbits. Insufficient weight was detected mainly in Nitra Rabbits and excessive mainly in LL. In the shape position, there was observed looser skin on the neck of the females, which in some cases turned into a lobe. Slightly protruding pelvic limbs was another observed fault. On the other hand, in the position shape were observed individuals in the Ni breed with very good formation of the back line (60.6%). Many rabbits showed body frame deficiencies such as a narrower or shorter body and finer and longer ears were relatively common. The coat was often dense and less elastic. Many observations involved deficiencies in specific marking. In breeds with a wild factor, there was a lighter intermediate colour and faults in other colour traits involved lighter undercoat.

Breeders face a difficult task related to breeding and selection if they want to preserve a wide variety of rabbit breeds for future generations. Only the proper breeding approach can eliminate exterior deficiencies and strengthen the typical breed traits of rabbits. However, selection of animals for breeding should respect not only to exterior traits but also performance characteristics of rabbits, such as fertility, growth, etc. We cannot neglect the fact that not

only heredity but also breeding conditions, such as feeding or breeding hygiene, are involved in shaping of the rabbit exterior.

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REFERENCES

1. **Chrenek, P., Makarevič, A., Kubovičová, E., Bulla, J., Supuka, P., 2019:** *Slovak National Animal Breeds* (In Slovak), SPU Nitra and NPCC VUŽV Nitra. 100 pp. Available online: http://www.efsa.eu.int/science/ahaw/ahaw_opinions/1174/ahaw_rabbits_report2.pdf.
2. **Dalle Zotte, A., Ricci, R., Sartori, A., et al., 2013:** Body morphometric development during growth and maturity of coloured dwarf rabbits available in the Italian market. *World Rabbit Sci.*, 21, 227—233. DOI: 10.4995/wrs.2013.1386.
3. **Morton, D., et al., 2005:** The impact of the current housing and husbandry systems on the health and welfare of farmed domestic rabbits. *EFSA J.*, 267, 1—31.
4. **Neirurerová, P., Fik, M., 2018:** Analysis of selected utility properties of Nitra rabbit (In Slovak). In *Proceedings of scientific and professional works Current Directions in Rabbit Breeding*. NPPC—VÚŽV Nitra, 30–37. DOI: 10.1016/j.atg.2013.03.001.4.
5. **Pyskatý, O., Zigo, F., 2018:** Exterior changes in medium breeds of rabbits—Large light silver, Large chinchilla and rabbits of Viennese type (In Czech). In *Proceedings of scientific and professional works Current Directions in Rabbit Breeding*. Nitra, 84—89.
6. **Rogers, A.D., Lupton, C.J., Lukefahr, S.D., 2006:** Fibre production and properties in genetically furred and furless rabbits. *J. Anim. Sci.*, 84, 2566—2574. DOI: 10.2527/jas.2006-106.
7. **Šimek, V., Sedláková, M., Softić, A., Zapletal, D., 2019:** The comparison of the selected morphometric traits in three medium-sized rabbit breeds. *Acta Fytotechn. Zootechn.*, 22, 138—143. DOI: 10.15414/afz.2019.22.04.138-143.
8. **Šimek, V., et al., 2020:** *Rabbit Breed Standards Book 2020* (In Czech). Prague, Czech Republic, 508 pp.
9. **Supuka, P., et al., 2020:** *Rabbit Breed Standards Book.* Nitra, Slovak Breeders' Association, 500 pp.
10. **Supuka, P., Maženský, D., 2014:** *Atlas of Rabbit Breeds* (In Slovak). University of Veterinary Medicine and Pharmacy in Košice, Košice, Slovakia, 412 pp.
11. **Tůmová, E., Martinec, M., Chodová, D., 2011:** Analysis of Czech rabbit genetic resources. *Scientia Agriculturae Bohemica*, 42, 113—118. Available online: https://www.researchgate.net/publication/292703080_Analysis_of_Czech_rabbit_genetic_resources.
12. **Vašičková, K., Ondruška, Ľ., Baláži, A., Parkányi, V., Vašíček, D., 2016:** Genetic characterization of Nitra rabbits and Zobor rabbits. *Slovak J. Anim. Sci.*, 48, 3, 104—111. http://www.vuzv.sk/slju/16_3/2_vasickova.pdf.
13. **Zadina, J., 2003:** *Book of the Rabbit Standards* (In Czech), Brno, Print-Typia, spol. s.r.o., Czech Republic, 371 pp.
14. **Zapletal, D., Švancarová, D., Gálik, B., 2021:** Growth of suckled rabbit kits depending on litter size at birth. *Acta Fytotechn. Zootechn.*, 24, 1, 55—59. DOI: 10.15414/afz.2021.24.01.55-59.
15. **Zhang, Y., Zheng, T.Q., Wang, X. Q., Liu, H.W., 2011:** Structure structural characteristics of rabbit hair. *Trans. Tech. Publ.*, 332—334, 1073—1076. Available online: http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.855.7785&rep=rep1&type=pdf.
16. **Zigo, F., Pyskatý, O., Šimek, V., et al., 2019a:** Comparison of exterior traits in selected large breeds of rabbits. *Int. J. Avian Wildlife Biol.*, 4, 96—100. DOI: 10.15406/ijawb.2019.04.00159.
17. **Zigo, F., Pyskatý, O., Ondrašovičová, S., et al., 2019b:** Assessment and most common exterior faults in medium breeds of rabbits—part I: in position weight – type and shape. *Int. J. Avian Wildlife Biol.*, 4, 4, 129—135. https://medcraveonline.com/IJAWB/IJAWB-04-00164.pdf.
18. **Zigo, F., Pyskatý, O., Ondrašovičová, S., et al., 2020:** Assessment and most common exterior faults in medium breeds of rabbits, Part II: In position coat, topcolour and undercolour. *Int. J. Avian Wildlife Biol.*, 5 1, 15—21. https://medcraveonline.com/IJAWB/IJAWB-05-00168.pdf.

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CORRELATIONS BETWEEN SODIUM SELENITE AND VITAMIN E WITH SERUM MACRO-MINERALS IN MALE LAMBS

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ABSTRACT

The effects of selenium and vitamin E (Vit. E) were evaluated on the macro-elements and their relationships were also surveyed. Lambs were divided into 4 groups of control, Vit. E, sodium selenite and sodium selenite/Vit. E (Se/E). Sodium selenite (0.1 mg.kg^{-1}) and Vit. E (8 mg.kg^{-1}) were administered. The mean blood selenium was high in sodium selenite. Selenium only in the Se groups was continuously increased. The lowest and highest blood selenium were in Vit. E and sodium selenite, respectively. The mean serum macro-minerals were within the normal range. The trend of calcium and magnesium in the selenium groups was to increase until day 60, but phosphorus was to decline. The lowest and highest calcium were observed in the control and sodium selenite groups, for magnesium in Vit. E and control groups, and for inorganic phosphorus were in the control group, respectively. The comparison of minerals showed that selenium had a significant increase from day 14 to 90 in sodium selenite. Selenium showed the most positive relationships with calcium and inorganic phosphorus in Vit. E and sodium selenite. Overall, selenium was correlated with macro-minerals on day 90 and overall sampling times were with calcium. In conclusion, administration of selenium with Vit. E increased selenium, which was more observed in the sodium selenite group. Sodium selenite alone or with Vit. E had no effect on the macro-minerals. The least correlation was observed between selenium and macro-minerals in the Se/E administration. The correlation between macro-minerals was positive. It was concluded that the effect of sodium selenite on treatment and prevention of selenium deficiency was more effective than other groups. Vit. E per selenium did not change the selenium concentration. Selenium administration was associated with an increase in calcium level and their co-administration is recommended.

Key words: calcium; lambs; selenium; sodium selenite; vitamin E

INTRODUCTION

Selenium and vitamin E (Vit. E) are considered as the most predominant antioxidants in animals, which minimize cell damage against membranes and intracellular peroxides, thus increasing the viability and functionality of cells [14]. Selenium has a biological action close to Vit. E

at the cellular level and is one of the basic constituents of glutathione peroxidase, which is an indicator of oxidative stress and responsible for removal of hydrogen peroxide and hydroperoxide from fats [9]. Selenium components as organic and inorganic selenium in the diet of lambs have effective roles in improving the immune and nervous systems [3]. Reportedly, selenium deficiency is associated with: reproductive problems [5], production deficiency [19], reduced neonatal growth performance [17], and ultimately causes great economic losses. Vit. E is also required to prevent oxidation of cell membranes against peroxides and therefore neutralizing the oxidative stress [13—45]. It has been reported that the combination of Vit. E [23] and selenium protects cells from any oxidative stress damages [10]. The importance of selenium as a metalloenzyme in the health and production of livestock is well established [43] and its deficiency is related to immune, gastrointestinal, and respiratory disorders which are characterized by signs of: anorexia, emaciation, pica, anaemia, decreased wool production, milk, and death [33—46]. Selenium in soil and forage is variable, so organic and minerals supplements such as selenium methionine [34] and sodium selenite [41] in concentrate feed [8] will be associated with favourable results in lambs and ewes [30].

The critical roles of macro-minerals in: anatomical development, physiological functions, enzyme structure, homeostasis and acid-base balance of blood, interstitial fluid osmolarity, and finally, in body growth, production and reproduction mechanisms are clear [6]. Calcium is the most predominant and abundant macro-mineral with various physiological functions in: health, energy metabolism, muscle contraction and neuromuscular stimulation [4], growth [1], metabolic procedures, and production performance [13]. Magnesium is the third major mineral with many: anatomical, physiological, enzymatic, immune, weight gain and reproductive functions in ruminants [4—37]. Inorganic phosphorus has also vital roles in coordination with calcium in: skeletal formation, as a component of cell membranes, various physiological functions, and as a part of nucleic acids in energy storage [15—37]. The investigation of macro-minerals individually indicates: the health status, growth performance, production, and reproduction situation [6] in animals.

The level of macro-minerals could be decreased directly due to their deficiency in food or indirectly in interactions with trace minerals and cause disturbances in an animals' life [21]. One of the trace minerals is selenium, which sometimes causes adverse effects such as competition and inhibition of absorption of the macro-minerals such as calcium, magnesium or inorganic phosphorous [2]. A previous study showed the low concentrations of macro-minerals were associated with high selenium in the diet [26] while other studies have not reported the same results [7]. Such inconsistent reports led us to examine the role of selenium, in lambs which are in the fast growing stage and have a crucial need for minerals, and assess the interactions among selenium, calcium, magnesium and inorganic phosphorous. The objectives were:

1) To determine the level of selenium in the blood of animals experimentally administered sodium selenite, Vit. E and Se/E groups;
2) To identify the serum level of macro-minerals;
3) To present the relationships among minerals.

MATERIALS AND METHODS

Animals and sampling

In this study, 16 Makuie male lambs were selected and the females were arranged for a different trail. They were classified into 4 groups of 4 heads including control, Vit. E, sodium selenite and Se/E. The mean and standard error of weight of lambs in each group were 21.7 ± 1.13, 20.9 ± 0.75, 25.2 ± 1.5 and 23.4 ± 0.75 kg, respectively. The overall mean ± standard error of weight was 21.89 ± 0.59 kg. The lambs were fed twice per day from their mothers' milk in the morning and afternoon. Lambs were free to graze on the grains and legumes pasture between milk feeding. Before performing any tests and medications, the lambs were examined for vital and clinical signs such as temperature, respiration, and heart rate to ensure their health.

The lambs in the control group were administered 0.1 ml.kg^{-1} saline subcutaneously, Vit. E group received 8 ml.kg^{-1} intramuscular Vit. E (Raha pharmaceutical Co, Iran), sodium selenite group were injected with 8 ml.kg^{-1} sodium selenite subcutaneously (CAS No. 7782-82-3, EC Number 231-966-3, USA) and Se/E (Nasr Company, Iran) group were injected with the Vit. E/Se (1 ml.20 kg^{-1} body weight) according to the dose of medicine [42]. The study began on the first day with blood sampling and then medication. Then, on days 7, 14, 30, 60, 90 (overall 6 times), the 5 millilitre of the jugular blood was taken

with a long 18-gauge needle and collected in a test tube (Glass types, with EDTA for selenium detection and without additives for serum macro-minerals detection, manufactured in Iran). Whole blood was used for the selenium evaluation and blood serum was centrifuged in 3000 round for 15 minutes to isolate the serum for calcium, magnesium and inorganic phosphorus measurement.

Evaluation of samples

The blood selenium concentration was measured by the atomic absorption method using an atomic absorption device (Technicon RA-1000, USA) made by Shimatzo and selenium lamp. In this procedure, selenium (Se^{+4}) is combined with 2 and 3 diamino naphthalene and converted to a fluorescent form derived from 4 and 5 benzapiazel selenol. For this purpose, 0.25 ml of haemolysed blood, 1 ml of a mixture of 15.8 mol.l^{-1} nitric acid and 11.8 mol.l^{-1} perchloric acid were added and heated at 150 °C for 30 minutes. It was then stored for 2 hours at 190 °C and 2 hours at 210 °C. After cooling the mixture, 0.2 ml of 6 mol.l^{-1} hydrochloric acid was added and stored at 150 °C until nitric acid was no longer evaporated. Then, one ml of a solution containing 20 mmol of EDTA, 7 mol of ammonia solution and 10 mg of purple cresol bromide per litre were added to the mixture and heated at 140 °C until the solution turned into yellow. After this step, it was transferred to a cold medium and 1.5 ml of 66 mmol.l^{-1} hydrochloric acid was added to adjust the pH between one and two. Fluorescence of the solution was measured at an excitation wavelength of 366 nm and emission at 544 nm using a spectrofluorometer (Jasco, UK, London). Calcium, magnesium and inorganic phosphorus were determined by autoanalyzer (autoanalyzer, RA-1000, USA) using commercial kits made by Pars Azmoun Iran.

ETHICAL STATEMENT

Ethical clearance was obtained from the Urmia university, Urmia, Iran.

Statistical analysis

SPSS version 23.0 (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.) statistical program and case summaries, student t-test, one-way analysis of variance (ANOVA) and correlation tests (Pearson) were used for each of the parameters in the groups and sampling times by individually or for overalls. The results were drawn and interpreted as Tables and Figures in groups and daily sampling. A P value less than 0.05 was considered statistically significant.

RESULTS

The mean blood selenium concentration was the highest in the sodium selenite group (F-value = 19.4, degree of freedom = 3, $P < 0.01$) (Table 1). The blood selenium continuously increased in the selenium groups with time; such elevation was not observed in the control or Vit. E groups. The level of selenium in the Vit. E group showed a slight increase up to day 14 which was not statistically different compared to the control group (Fig. 1). The highest percentage of selenium elevation was in the sodium selenite (15.7 %) and the lowest level was observed in the control group (7.1 %). Overall, selenium was significantly high in the selenium groups. The minimum and maximum blood selenium concentrations were 180.4 and 262.8 nmol.l^{-1} in the Vit. E and sodium selenite, respectively.

Table 1. Mean ± SE of lambs blood minerals in 4 groups at 6 sampling times in 90 days (n = 24)

Group/Minerals	Selenium [nmol.l^{-1}]	Calcium [mg.dl^{-1}]	Magnesium [mg.dl^{-1}]	Phosphorus [mg.dl^{-1}]
Control	200.5 ± 1.13	8.82 ± 0.33	1.58 ± 0.16	6.72 ± 0.25
Vitamin E	198.6 ± 1.64	8.76 ± 0.22	1.34 ± 0.17	6.24 ± 0.22
Sodium selenite	226.9 ± 4.82	8.54 ± 0.29	1.20 ± 0.18	6.45 ± 0.20
Sodium selenite/E	208.4 ± 2.68	8.27 ± 0.35	1.27 ± 0.18	6.38 ± 0.22
Overall	208.6 ± 2.82	8.60 ± 0.30	1.35 ± 0.17	6.45 ± 0.22

Table 2. Mean comparison of lambs' blood minerals among sampling times in 90 days (n = 24)

Day/Minerals	Selenium [nmol.l^{-1}]	Calcium [mg.dl^{-1}]	Magnesium [mg.dl^{-1}]	Phosphorus [mg.dl^{-1}]
1	1.06	0.02	2.06	0.33
7	3.03†	1.13	0.195	0.556
14	9.75**	0.97	1.67	0.18
30	21.28**	0.26	0.51	0.87
60	48.45**	0.43	0.61	10.58**
90	17.21**	4.45*	6.51**	10.67**

**—P < 0.01; *—P < 0.05; †—P < 0.1 > 0.05

Table 3. Mean comparison of lambs' blood minerals among sampling times in 4 groups (n = 24)

Groups	Parameters	Sum Square	Mean Square	F-values
Control	1	437.6	87.52	6.00**
	2	15.18	3.04	1.27
	3	8.09	1.62	4.28**
	4	8.68	1.74	1.25
Vitamin E	1	831.4	166.3	4.54**
	2	18.84	3.77	1.85
	3	9.50	1.90	6.07**
	4	11.54	2.31	2.85**
Sodium selenite	1	11389.2	2277.9	28.20**
	2	8.03	1.60	0.76
	3	6.98	1.40	2.42**
	4	12.48	2.50	5.16**
Sodium selenite/E	1	3311.94	662.4	18.49**
	2	18.20	3.64	1.37
	3	9.41	1.88	4.17**
	4	14.83	2.97	4.41**

selenium [nmol.l^{-1}]; calcium [mg.dl^{-1}]; magnesium [mg.dl^{-1}]; inorganic phosphorus [mg.dl^{-1}];
**—P < 0.01; F-value—value of the F distribution

The overall mean blood calcium concentration did not show any significant change among the groups (F-valve = 0.60, degree of freedom = 3, P = not significant) and was within the standard range (Table 1). The calcium level varied among the groups for 30 days and showed a slight increase at the end, but a decrease was observed in selenium groups (Fig. 2). The lowest and highest calcium levels were in the control group and sodium selenite groups (3.96 and 10.5 mg.dl^{-1}, respectively).

The overall mean serum magnesium concentration in lambs was not statistically different among the groups (F-value 1.61; Degree of freedom = 3; Probability = not significant) and was within the standard range (Table 1). The level of magnesium decreased during the first 30 days in all groups but then increased by day 90 in groups although not significant, except in sodium selenite and Vit. E groups. The increasing trend of magnesium in the Se groups was less than the Vit. E and control groups; it

Table 4. Correlations between selenium and blood minerals in groups and sampling times (n = 4)

Groups	Calcium [mg.dl^{-1}]	Magnesium [mg.dl^{-1}]	Phosphorus [mg.dl^{-1}]
Selenium 1 day 7 (Control)	0.84	0.13	0.95*
Selenium day 7 (Vit. E)	0.99**	0.70	0.86
Selenium day 14 (Se/E)	0.97**	-0.47	-0.40
Selenium day 7 (Overall)	0.11	-0.70**	0.58**
Selenium day 7 (Overall)	0.76**	-0.53**	0.74**

**—P < 0.01; *—P < 0.05

decreased insignificantly at the end of the study. The minimum and maximum magnesium level were in Vit. E and control groups (0.72—2.61 mg.dl^{-1}), respectively.

The overall mean serum inorganic phosphorus concentration in all groups was not statistically different (F-value = 0.17; Degree of freedom = 3; Probability = not significant) (Table 1) and no deficiency was observed. Phosphorus changes during the study varied especially in the selenium groups until day 60, then non-significantly increased by day 90 (Fig. 4). The minimum and maximum serum inorganic phosphorus level were both in the control group (3.84 and 8.86 mg.dl^{-1}).

The mean comparison (One-way ANOVA) of selenium, calcium, magnesium and inorganic phosphorus concentrations among 6 sampling times (Table 2) showed significant differences in selenium on days 30, 60 and 90 ($P < 0.01$) which were the highest increase in sodium selenite group (Fig. 1). Inorganic phosphorus was significant on day 60 and 90 ($P < 0.01$). The mean comparison (One-way ANOVA) of selenium, calcium, magnesium and inorganic phosphorus concentrations within groups (Table 3) showed significant changes in selenium, magnesium and inorganic phosphorus in all groups ($P < 0.01$) with the exception of inorganic phosphorus in the control group which was not different. The calcium levels did not differ among the groups.

The Pearson correlation analysis of selenium with calcium, magnesium and inorganic phosphorus based on groups and sampling frequency (Table 4) showed positive and significant relationships ($P < 0.05$) between selenium/calcium on day 7 in the Vit. E group, day 14 in Se/E and day 90 in all samples. Selenium showed a significant negative relationship with magnesium on days 60 and 90 in all groups. Also, selenium showed a significant positive relationship with inorganic phosphorus on day 7 in the control group, and days 60 and 90 in all groups ($P < 0.05$). Therefore, the correlations between selenium and all minerals were significant and positive on day 90 when groups were mixed and was positive and significant with calcium when sampling days were mixed (Table 4). The relationships between calcium/magnesium (r 0.99), calcium/inorganic

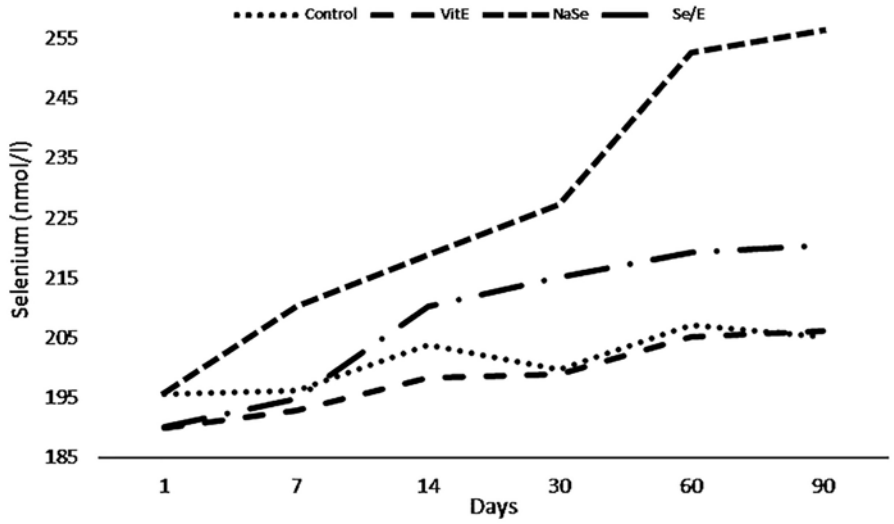

Fig. 1. Mean comparison of lambs' serum selenium among groups in 90 days

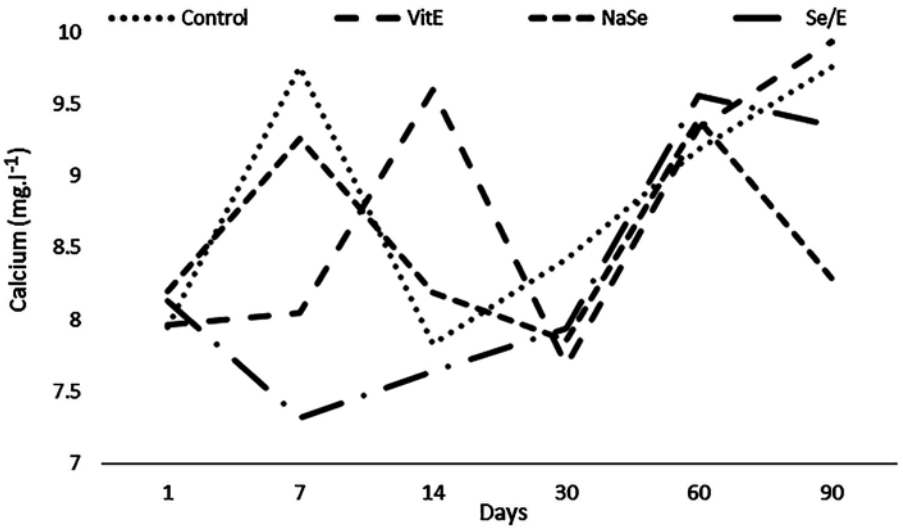

Fig. 2. Mean comparison of lambs' serum calcium among groups in 90 days

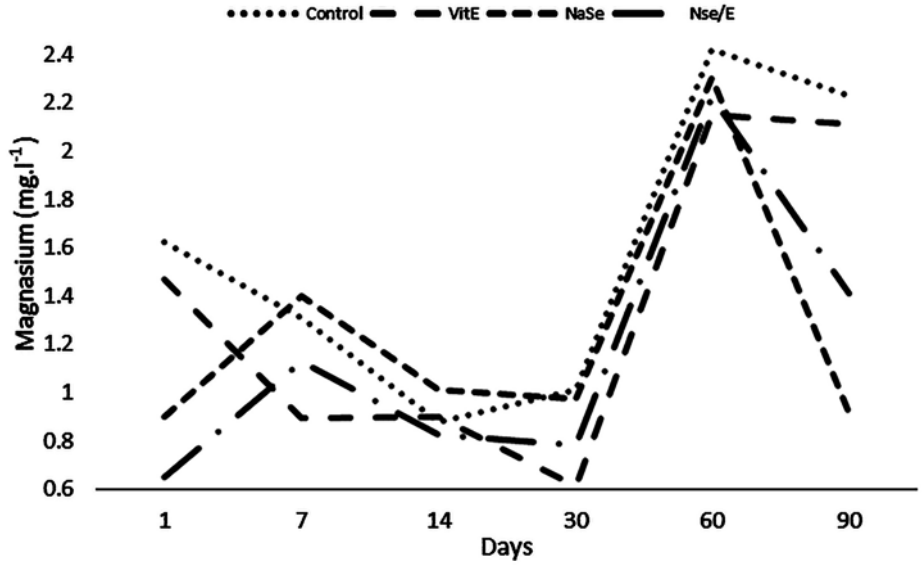

Fig. 3. Mean comparison of lambs' serum magnesium among groups in 90 days

phosphorus (r=0.98) and inorganic phosphorus/magnesium (r=0.89) were all positive and significant (P<0.05).

DISCUSSION

Selenium is considered as the prominent micronutrient in the prevention and elimination of oxidative stress in the cells of organisms [39]. A blood selenium concentration of 70—100 ng.ml^{-1} could prevent: reproductive disorders, heart myopathy and respiratory diseases [31]. Selenium is involved in the metabolic processes of: ruminants [40], and its deficiency weakens the immune system, increases the incidence of tumours [44], enhances thyroid hormone disorders [36], and causes muscular dystrophy [25]. Thus, the assessment of selenium in lambs that need trace elements such as selenium and macro-minerals such as calcium, magnesium and inorganic phosphorus indicates their co-occurring or interacting effects.

The mean concentration of selenium in the selenium groups were significantly higher compared to the other groups. K a r r e n et al. [22] showed an increase in blood

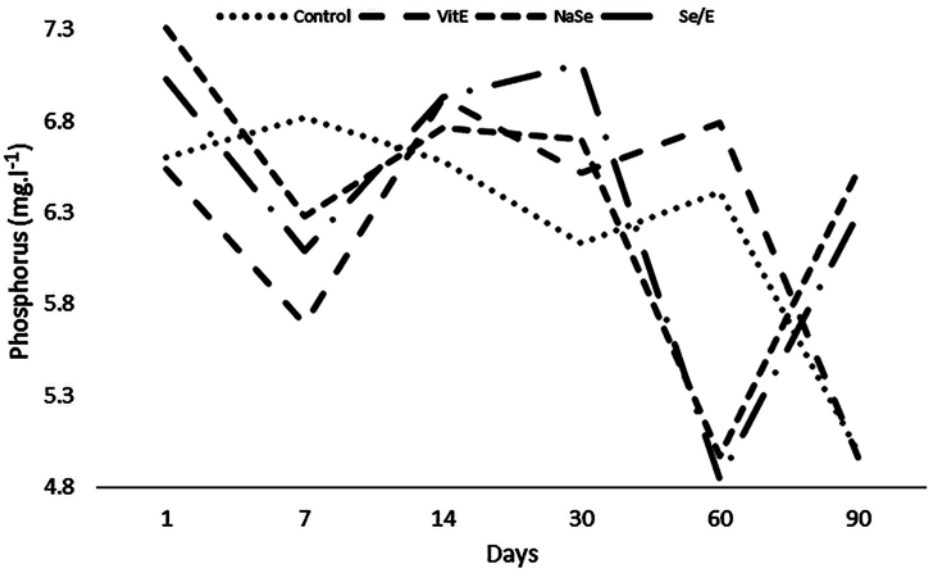

Fig. 4. Mean comparison of lambs' serum phosphorus among groups in 90 days

selenium after administration of the selenium supplements, which was consistent with the results of this study. Blood selenium was continuously increased in selenium groups for 90 days, which was 2 times higher in sodium selenite in comparison with the Se/E group, while it was not significant in the control or Vit. E groups. It means that Vit. E has no role in increasing blood selenium, although it physiologically binds to each other to remove destructive peroxides at the cellular level [14]. Similar results have been reported by Shi et al. [42] showing a significant increase in blood and tissue selenium following dietary selenium supplementation. The importance of selenium as the main antioxidants is related to the elimination of oxidative stress, which prevents cell damage against peroxides, and finally, leads to increase viability and functionality of the cells [38]. The presence of selenium components in food, in addition to the earlier mentioned effects, will result in the strengthening of the immune and nervous systems [27], and its deficiency is accompanied by reproductive system complications [40], production disorders [13] and reduction in neonatal growth performance [17]. Therefore, selenium, especially with Vit. E [23], will protect cells from any oxidative stress damages [14].

The physiological range for calcium, magnesium and inorganic phosphorus concentrations reported by Azarzar [6] in ewes were 8.4—11.2, 1.9—2.77 and 2.8—9.8 $mg.dl^{-1}$, respectively. The corresponding values in this study were at the lowest normal range mainly for calcium and magnesium, although those values were reported for adult ewes and our experimental animals were lambs, it means that no macro-mineral deficiencies were expected in these lambs. These results show that, the concentrations of lambs' macro-minerals were at its lowest level, which is not possible to be supplied through the dams' milk, and because lambs probably do not receive concentrate, and despite milk is rich in calcium, so lambs will be at the risk of deficient for a period of time, and therefore, they need mineral supplements just for calcium and magnesium while the concentration of inorganic phosphorus was favourable and was increased at the end of the study. According to current information, lambs under 2 months of age are also at risk of selenium deficiency [19] so they should be fed with calcium, and magnesium together [3]. These results show that oral administration of selenium and Vit. E supplements separately and together did not affect the concentration of the macro-elements, but up to day 60 caused a slight increase in calcium and magnesium. Therefore, the combination of selenium and macro-minerals in the forms of injection or dietary supplements could be helpful [17].

Abraheem et al. [2] emphasized that the information on the effect of selenium and Vit. E on calcium level is limited and also controversial. Juniper et al. [20], Kumar et al. [27], Shokrollahi et al. [44] and Bagnick et al. [7] reported that selenium supplements were not effective on calcium absorption in lambs. In another study, intramuscular injection of Se/E did not significantly affect the serum levels of calcium and magne-

sium [11]. In contrary to the earlier mentioned findings, Mahmoud et al. [29] found that administration of 5 mg sodium selenite and 450 mg Vit. E significantly increased serum calcium concentration in male lambs. Also, Bickhardt et al. [12] showed that the administration of selenium and calcium are effective in the treatment of ketosis and hypocalcemia in cows. While Kessler et al. [24], Garcia et al. [18], Mehdi and Dufrasne [31] showed that high calcium consumption has a negative effect on dietary selenium absorption. There is no available scientific information about the effects of selenium on magnesium and inorganic phosphorus, or vice versa, and it may indicate that these minerals are not sensitive to each other. Deficiency of macro-elements has been observed in metabolic and nutritional diseases in ewes [26], which can also be observed in lambs requiring selenium. The results of this study showed that their co-administration improve each other and is suitable for growth and production.

In this study, the highest correlation was observed between selenium/calcium, followed by inorganic phosphorus and magnesium. In the overall groups, macro-minerals were correlated on days 60 and 90 and in overall sampling times only calcium was associated. These results show that the co-administration of selenium and calcium is appropriate and ideal, and it does not interfere with each other and acts independently and can even strengthen each other too. The results of this study was contradicted with the findings of Kessler et al. [24], Garcia et al. [18] Mehdi and Dufrasne [31] Sayiner et al. [40] who mentioned a negative relationship and also Bagnick et al. [7] who reported no relationship between selenium and calcium. Naziroğlu [35] reported a link between selenium and calcium-induced nerve waves and oxidative stress in human epilepsy, in which selenium deficiency may exacerbate oxidative stress by decreasing GPX, so it causes nerve waves via a calcium factor, and this may indicate an indirect relationship between selenium and calcium. The relationships among macro-minerals in this study were consistent with the findings of others [6—28].

CONCLUSIONS

In conclusion, co-administration of selenium with Vit. E in the form of Se/E increased the blood selenium, although the selenium level was higher in sodium selenite compared to Se/E. Sodium selenite and Se/E had no effect on the values of the macro-elements. There was no correlation between selenium and macro-elements in sodium selenite and Se/E administration, but in all groups, the correlation was positive and significant for all elements. There were positive and significant relationships among the macro-elements. It was concluded that the role of sodium selenite in selenium deficiency was more favourable than Se/E. Vit. E injection alone was not effective in increasing the blood selenium. Selenium had the highest correlation with calcium, and co-administration of Se/E is helpful in field veterinary practice.

REFERENCES

1. **Abdelrahman, M. M., Alhidary, I., Adeniji, Y. A., Alobre, M. M., Albaadani, H., Aljumaah, R., 2021:** Manipulating phosphorus, calcium, and magnesium utilization by growing lambs using natural zeolite (clinoptilolite). *Sustainability*, 13, 1539. DOI: 10.3390/su13031539.
2. **Abrahem, M. S., Mohammed, W. J., Ahmead, W. K., 2019:** Effect of injecting different levels of vitamin E and selenium on some physiological characteristics of blood and milk yield and compositions in Awassi ewes. *Euphrates J. Agric. Sci.*, 11, 2, 7—13.
3. **Ataollahi, F., Friend, M., McGrath, S., Dutton, G., Peters, A., Bhanugopan, M., 2020:** Maternal supplementation of twin bearing ewes with calcium and magnesium alters immune status and weight gain of their lambs. *Vet. Anim. Sci.,* 9, 100097. DOI: 10.1016/j.vas.2020.100097.
4. **Ataollahi, F., Friend, M., McGrath, S., Dutton, G., Peters, A., Bhanugopan, M., 2018:** Effect of calcium and magnesium supplementation on minerals profile, immune responses, and energy profile of ewes and their lambs. *Livestock Sci.*, 217, 167—173. DOI: 10.1016/j.livsci.2018.10.001.
5. **Awawdeh, M. S., Eljarah, A. H., Ababneh, M. M., 2019:** Multiple injections of vitamin E and selenium improved the reproductive performance of oestrus-synchronized Awassi ewes. *Trop. Anim. Health Prod.*, 51, 1421—1426. DOI: 10.1007/s11250-019-01826-0.
6. **Azarzar, O., 2020:** *Blood Mineral Status of Suffolk Sheep Influenced by Season, Sheep Lines and Geographical Location*. Veterinary Academy, Faculty of Veterinary Medicine, Lithuanian University of Health Sciences, thesis, Kanus, 11—17.
7. **Bagnick, E., Kościuczuk, E. M., Jarczak, J., Jóźwik, A.,**

Strzałkowska, N., Słoniewska, D., Krzyżewski, J., 2017: The effect of inorganic and organic selenium added to diets on milk yield, milk chemical and mineral composition and the blood serum metabolic profile of dairy cows. *Anim. Sci. Papers and Reports*, 35, 1, 17—33.

8. **Bampidis, V., Azimonti, G., Bastos, M. L., Christensen, H., 2019:** Safety and efficacy of sodium selenate as feed additive for ruminants. *EFSA J.*, 17, 7, 5788.
9. **Barcelos, B., Gomes, V., Vidal, A. M. C., Júnior, J. F. F., Araújo, M. L. C., Alba, H. D. R., Netto, A. S., 2022:** Effect of selenium and vitamin E supplementation on the metabolic status of dairy goats and respective goat kids in the peripartum period. *Trop. Anim. Health Prod.*, 54, Article number 36. DOI: 10.1007/s11250-021-03034-1.
10. **Barwary, M. S., Alkass, J. E., Ahmed, S. J., 2016:** Studies the effect of selenium and vitamin E on some haematological and biochemical parameters in female Meriz goats. *J. Uni. Duhok Agri. Vet. Sci.*, 19, 1, 210—215.
11. **Bednarek, D., Kondracki, M., Cakala, S., 1996:** Effect of selenium and vitamin E on white cells, serum concentration of several minerals and trace elements as well as immunologic parameters in calves. *DTW Deutsche Tierarztliche Wochenschrift*, 103, 11, 457—459.
12. **Bickhardt, K., Henze, P., Ganter, M., 1998:** Clinical findings and differential diagnosis in ketosis and hypocalcaemia in sheep. *DTW Deutsche Tierarztliche Wochenschrift*, 105, 11, 413—419.
13. **Cirne, L. G. A., de Carvalho, G. G. P., Viana, P. T., Luz, V. S., de Silva, M. J., de Fidueiredo, M. P., 2020:** Impact of high-concentrate diets with cottonseed associated with calcium lignosulfonate on the metabolic, productive, and carcass characteristics of feedlot lambs. *Trop. Anim. Health Prod.*, 52, 1821—1832. DOI: 10.1007/s11250-019-02194-5.
14. **Dhanasree, G., Pillai, U. N., Deepa, C., Ambily, V. R., Shynu, M., Sunanda, C., 2020:** Evaluation of oxidative stress in caprine anaplasmosis and effect of vitamin E–selenium in monitoring oxidative stress, *Trop. Anim. Health Prod.*, 52, 2695—2698. DOI: 10.1007/s11250-019-02175-8.
15. **Dittmer, K. E., Morley, R. F., Smith, R. L., 2017:** Skeletal deformities associated with nutritional congenital rickets in newborn lambs. *NZ Vet. J.*, 65, 1, 51—55. DOI: 10.1080/00480169.2016.1241165.
16. **Fadlalla, I. M. T., Omer, S. A., Atta, M., 2020:** Determination of some serum macroelement minerals levels at different lactation stages of dairy cows and their correlations. *Sci. Afr.*, 8, e00351. DOI: 10.1016/j.sciaf.2020.e00351.
17. **Farghaly, M. M., Hassan, E. H., Abdel-Raheem, S. M., 2017:** Influence of dietary supplementation of various selenium sources on nutrient digestibility, growth performance and blood metabolites in male buffalo calves. *Egyptian J. Nutr. Feeds*, 20, 421—428. DOI: 10.21608/ejnf.2017.75226.
18. **García-Vaquero, M., Miranda, M., Benedito, J. L., Blanco-Penedo, I., López-Alonso, M. 2011:** Effect of type of muscle and Cu supplementation on trace element concentrations in cattle meat. *Food Chem. Toxicol.*, 49, 1443—1449. DOI: 10.1016/j.fct.2011.03.041.
19. **Ibrahim, E. M., 2017:** Effect of parenteral supplementation of vitamin E plus selenium on nutrient digestibility, productive performance and some serum biochemical indicators of lambs. *Egyptian J. Sheep Goats Sci.*, 12, 1—12.
20. **Juniper, D. T., Phipps, R. H., Jones, R. K., Bertin, G., 2006:** Selenium supplementation of lactating dairy cows: Effect on selenium concentration in blood, milk, urine and faeces. *J. Dairy Sci.*, 89, 9, 3544—3551. DOI: 10.3168/jds.S0022-0302(06)72394-3.
21. **Kargin, F., Seyrek, K., Bildik, A., Aypak, S., 2004:** Determination of the levels of zinc, copper, calcium, phosphorus and magnesium of Chios ewes in the Aydin region. *Turk. J. Vet. Anim. Sci.*, 28 609—612. https://journals.tubitak.gov.tr/veterinary/abstract.htm?id=7048.
22. **Karren, B. J., Thorson, J. F., Cavinder, C. A., Hammer, C. J., Coverdale, J. A., 2014:** Effect of selenium supplementation and plane of nutrition on mares and their foals: Selenium concentrations and glutathione peroxidase. *J. Anim. Sci.*, 88, 991—997. DOI: 10.2527/jas.2008-1743.
23. **Kasapidou, E., Enser, M., Wood, J. D., Richardson, I. R. I., Wilkinson, R. J., Sinclair, L. A., 2009:** Influence of vitamin E supplementation and basal diet on the vitamin E status, performance and tissue fatty acid concentration in lambs. *Animal*, 3, 4, 516—526. DOI: 10.1017/S1751731108003820.
24. **Kessler, J., 1993:** Carence en sélénium chez les ruminants: Mesures prophylactiques. *Review. Suisse Agri.*, 25, 1, 21—26.
25. **Kivanç, I., Handan, M., Nihat, M., Nesrullah, A., Dogan, S. I., Arzu, C., 2021:** Evaluation of various biochemical parameters in the serum and tissues of lambs with white muscle disease. *Indian J. Anim. Res.*, 55, 11, 1293—1297. DOI: 10.18805/ IJAR.B-1372.
26. **Kojouri, G., Sharifi, S., 2013:** Preventing effects of nanoselenium particles on serum concentration of blood urea nitrogen, creatinine, and total protein during intense exercise in donkey. *J. Equine Vet. Sci.*, 33, 597—600. DOI: 10.1016/j.jevs.2012.09.008.

27. **Kumar, N., Garg, A. K., Mudgal, V., Dass, R. S., Chaturvedi, V. K., Varshney, V. P., 2008:** Effect of different levels of selenium supplementation on growth rate, nutrient utilization, blood metabolic profile, and immune response in lambs. *Biol. Trace Element Res.*, 126, 1, S44—S56. DOI: 10.1007/s12011-008-8214-8.

28. **Li, Z., Liao, Q., Liang, Y. H., Liu, D. H., 2020:** A study of serum mineral, antioxidant capacity, and haematobiochemical parameters in horses with pica in China. *J. Vet. Behav.*, 37, 81—88. DOI: 10.1016/j.jveb.2020.04.008.

29. **Mahmoud, G. B., Abdel-Raheem, S. M., Hussein, H. A., 2013:** Effect of combination of vitamin E and selenium injections on reproductive performance and blood parameters of Ossimi rams. *Small Rum. Res.*, 113, 1 103—108. DOI: 10.1016/j.smallrumres. 2012.12.006.

30. **Maraba, K. P., Mlambo, V., Yusuf, A. O., Marume, U., Hugo, A., 2018:** Effect on growth performance, stress biomarkers, and meat quality. *Small Rumin. Res.*, 160, 31—37. DOI: 10.1016/j.smallrumres.2018.01.009.

31. **Mehdi, Y., Dufrasne, I., 2016:** Selenium in cattle: A review. *Molecules*, 21, 545. DOI: 10.3390/molecules21040545.

32. **Mohd Mutalip, S. S., Ab-Rahim, S., Rajikin, M. H., 2018:** Vitamin E as an antioxidant in female reproductive health, *Antioxidants*, 7, 2, 22. DOI: 10.3390/antiox7020022.

33. **Morsy, A. S., El-Zaiat, H. M., Saber, A. M., Anwer, M. M., Sallam, S. M., 2019:** Impact of organic selenium and vitamin E on rumen fermentation, milk production, feed digestibility, blood parameters and parasitic response of lactating goats. *J. Agri. Sci. Technol.*, 21, 1793—1806.

34. **Mousaie, A., Valizadeh, R., Chamsaz, M., 2017:** Selenium-methionine and chromium-methionine supplementation of sheep around parturition: impacts on dam and offspring performance. *Archives Anim. Nutr.*, 71, 2, 134—149. DOI: 10.1080/1745039X.2017. 1283825.

35. **Nazıroğlu, M., 2009:** Role of selenium on calcium signaling and oxidative stress-induced molecular pathways in epilepsy. *Neurochem. Res.*, 34, 12, 2181—2191. DOI: 10.1007/s11064-009-0015-8.

36. **Novoselec, J., Šperanda, M., Klir, Z., Mioč, B., 2018:** Blood biochemical indicators and concentration of thyroid hormones in heavily pregnant and lactating ewes depending on selenium supplementation. *Acta Vet. Brno*, 86, 353—363. DOI: 10.2754/avb201786040353.

37. **Radostits, O., Gay, C. C., Hinchcliff, K. H., Constable, P. D., 2010:** *Veterinary Medicine*. 8th edn., Bailliere and Tindall Publ., Ltd, London, 1450—1452.

38. **Raisbeck, M. F., 2020:** Selenosis in ruminants. Veterinary clinics. *Food Anim. Pract.*, 36, 3, 775—789. DOI: 10.1016/j.cvfa.2020.08.0.

39. **Ramadan, S. G. A., Mahboub, H. D. H., Helal, M. A. Y., Sallam, M. A., 2018:** Effect of vitamin E and selenium on performance and productivity of goats. *Int. J. Chem. Biomed. Sci.*, 4, 2, 16—22. http://www.aascit.org/journal/ijcbs.

40. **Sayiner, S., Darbazb, I., Ergene, O., Aslan, S., 2021:** Changes in antioxidant enzyme activities and metabolic parameters in dairy cows during different reproductive periods. *Theriogenology*, 159, 116—122. DOI: 10.1016/j.theriogenology. 2020.10.024.

41. **Sherief, M. G., Abdel-Raheem, B., Mahmoud, S., Waleed, M., Taymour, El-Sherry, T. M., 2019:** Influence of vitamin E and selenium supplementation on the performance, reproductive indices and metabolic status of Ossini ewes. *Slovenian Vet. Res.*, 56, 353—363.

42. **Shi, L., Xun, W., Yue, W., Zhang, C., Ren, Y., Shi, L., 2011:** Effect of sodium selenite, Se-yeast and nano-elemental selenium on growth performance, Se concentration and antioxidant status in growing male goats. *Small Rumin. Res.*, 96, 49—52. DOI: 10.1016/j.smallrumres.2010.11.005.

43. **Shi, L., Ren, Y., Zhang, C., Yue, W., Lei, F., 2018:** Effects of organic selenium (Se-enriched yeast) supplementation in gestation diet on antioxidant status, hormone profile and haemato-biochemical parameters in Taihang Black Goats. *Anim. Feed Sci. Technol.*, 238, 57—64. DOI: 10.1016/j.anifeedsci. 2018.02.004.

44. **Shokrollahi, B., Mansouri, M., Amanlou, H., 2013:** The effect of enriched milk with selenium and vitamin E on growth rate, haematology, some blood biochemical factors, and immunoglobulins of newborn goat kids. *Biol. Trace Element Res.*, 153, 184—190. DOI: 10.1007/s12011-013-9685-9.

45. **Tsiplakou, E., Mitsiopoulou, C., Karaiskou, C., Simoni, M., Pappas, A. C., Righi, F., Labrou, N. E., 2021:** Sesame meal, vitamin E and selenium influence goats' antioxidant status. *Antioxidants*, 10, 392. DOI: 10.3390/antiox10030392.

46. **Zhou, Q., Zhang, B., Chen, X., Chen, Q., Hao, L., 2021:** Association of serum selenium with anaemia-related indicators and risk of anaemia. *Food Sci. Nutr.*, 9, 6, 3039—3047. DOI: 10.1002/fsn3.2261.

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