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CONTENTS

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FOLIA VETERINARIA, 67, 1: 1—14, 2023

EFFECTS OF *IN OVO* INJECTION OF INORGANIC SALTS OF ZINC AND COPPER ON PERFORMANCE AND SERUM BIOCHEMICAL INDICES OF TWO STRAINS OF BROILER CHICKENS

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ABSTRACT

This study was composed of two experiments which investigated the response of two strains (Arbor Acre and Cobb 500, respectively) of broiler chickens to *in ovo* injection of inorganic salts of zinc, copper and their combination. A total of 300 hatching eggs [only 148 (59.20 %) and 232 (90.27 %), respectively, were fertile] each of Arbor Acre and Cobb 500 strains of broiler chickens were used in both experiments. These eggs were distributed into four treatments: control, *in ovo* inorganic Zn (80 µg.egg^{-1}), Cu (16 µg.egg^{-1}) and combined Zn and Cu (80 µg.egg^{-1} Zn and 16 µg.egg^{-1} Cu). The data obtained in both experiments were subjected to Completely Randomized Design (CRD) at the 5 % probability level. The results showed increased hatchability ($P < 0.05$) in eggs injected with the combination of inorganic salts of Zn and Cu in Experiment I and daily intake was influenced in both experiments. The carcass traits, organ development and gut morphometry were not significantly influenced by the treatment groups. The total serum protein and albumin of the birds were significantly ($P < 0.05$) increased by *in ovo* injection of inorganic salts of Zn and Cu at day 49 in the Experiment I. The study concluded that *in ovo* injection of inorganic salts of Zn at 80 µg.egg^{-1} and/or Cu at 16 µg.egg^{-1} could be adopted to increase feed intake with: attendant enhanced growth, enhanced immune response, increased albumin and total protein contents of blood serum in the strains of broiler chickens used.

Key words: Arbor Acre; Cobb 500; Cu; *in ovo* injection; Zn

INTRODUCTION

Most research on poultry are carried out post-hatch with the objectives of improving rearing or health conditions and optimisation of nutrition for an enhanced productivity. On the other hands, recent advances in technology and techniques as well as the possibilities associated with egg micro-manipulation have opened up completely new research on the birds' embryos. The research documents

the impact of various biologically important compounds including: amino acids and their derivatives, hormones, antibiotics, and inorganic salts on the development and biochemical changes of embryos, hatchability, as well as post-hatch growth performance of chicks [34, 40].

The nutritional management in young broiler chicks is essential for good performance during the growing period, because the digestive system develops mainly during the initial growth phase, followed by muscular and skeletal growth. However, the short lifespan of broiler chickens, discourages enough time for compensatory growth [6, 16, 22]. It is pertinent to stimulate the initial growth of the birds during the first days of age to achieve maximum growth rates; hence, the relevance of *in ovo* feeding. In a study [9] of *in ovo* nutrition, it was reported to improve: the digestive capacity, increase growth rate, and feed efficiency, reduce post-hatch mortality and morbidity, improve immune response to enteric antigens, reduce the incidence of developmental skeletal disorders, and increase muscle development and meat yield. The late term embryo obtains nutrients via the amnion fluid [15], and delivery of extra nutrients to this area compensated for any energy deficiencies during hatch.

The nutritional deficiencies associated with trace minerals such as Cu and Zn have pointed to the significant roles of these minerals in maintaining normal physiological processes in poultry production [17]. In recent time, biological assay methods clarified the significance and importance of mineral elements for human and animal nutrition and modern analytical techniques led to the detection of trace elements as essential nutrients and this is still an active area of current research. Zinc is essential for a variety of biochemical processes. Dietary Zn supplementation enhances the expression levels of metallothionein (MT) and copper- and Zn-containing superoxide dismutase as free radical scavengers in the tissues of broilers [23, 33]. In a study [39], involving *in ovo* injection of a solution containing Zn, improved the mechanical properties of the bones of chicks at 3 and 14 days of age. The authors [4] injected a mixture of Zn and other trace elements into the amniotic cavity of the 18 days old embryos, and found that the enrichment modulated the cell-mediated immunity of the resulting broiler chicks. On the other hands, it was posited [29] that copper is important in promoting efficient growth and health in poultry due to its roles in diverse biochemical processes with the tendency to improve nutrients transport and overall productive performance. Broiler chickens spend about 38 % of their entire lifespan inside the egg as embryos; thus embryogenesis is quite a critical period of growth and development in chickens [3, 27].

The Arbor Acre strain of broiler chickens is being steadily improved to ensure all products consistently add value to customer operations through established breed selection processes that use both traditional scientific techniques and the latest in technology (https://www.eu.aviagen.com). On the other hands, the Cobb 500 strain of broiler chicken is a flexible broiler that is arguably the world's most productive efficient line of meat chickens. It has: a very low feed conversion ratio, converts cheap low-density feed very well, has consistent performance across climates, produces optimally with the best uniformity, and it has consistent processing results including: white feather, yellow skin as carcass, and about 96 % liveability (https://www.cobb-vantress.com/en_US/products). The benefits in terms of meat yield of these products could not be undermined in meeting the protein needs of the teeming population.

The authors [39] stated that the embryonic nutrient reserves are insufficient and might be depleted in the embryonic period because of the increased metabolic rate of the embryos of today. Therefore, *in ovo* technique provides a practical means to safely introduce exogenous nutrients into developing embryos [5, 11, 20] to salvage the problem associated with nutrient depletion during embryonic development. This study investigated the response of two strains of broiler chickens to *in ovo* injection of inorganic salts of copper, zinc and their combination.

MATERIALS AND METHODS

Description of the experiments

This study comprised of two experiments which were undertaken concurrently. In Experiment I, the response of Arbor Acre strain of broiler chickens to *in ovo* injection of inorganic salts of zinc and copper and their combination was studied while the response of the Cobb 500 strain of broiler chickens to *in ovo* injection of inorganic salts of zinc and copper and their combination was studied in Experiment II.

Ethical consideration

The study protocol was approved by Animal Care and Use Review Committee (AC&URC) of the Federal University of Agriculture, Abeokuta, Ogun State, Nigeria [13].

Experimental site

The hatchery phase of the experiments was carried out at the hatchery of the College of Animal Science and Livestock Production, while the field trial was undertaken at the poultry unit of the Directorate of University Farms (DUFARMS), Federal University of Agriculture, Abeokuta, Nigeria located at Latitude 7° 15' N, Longitude 3° 26' E.

Source and preparation of test ingredients

Inorganic salts of zinc and copper used for this study were obtained from a reputable veterinary store within Ogun State. Thereafter, the compounds were dissolved in deionised water at 80 µg of inorganic salt of zinc ($ZnSO_4.7H_2O$) and 16 µg of inorganic salt of copper ($CuSO_4.5H_2O$) into 100 ml of deionised water, respectively and administered at 0.1 ml per egg.

Source and management of fertile eggs

For each of the experiments, a total of 300 hatching eggs were procured from reputable hatcheries. The eggs were fumigated using potassium tetraoxomanganate VII ($KMnO_4$) and formalin at a ratio 1 : 2. The treatment lasted for 20 minutes in a closed chamber. The eggs were set in egg trays in a Chick Master® incubator with broad ends upward to prevent rupture of the air cell. Temperature (37.5–37.8 °C) and humidity (60–65 %) were automatically regulated. Egg turning was done manually on an hourly basis to prevent developing embryos from sticking to the shell, and also to ensure uniform distribution of nutrients.

Table 1. Experimental layout

Treatment groups	*In ovo* injection
Group I	Control (no *in ovo* supplementation)
Group II	*In ovo* supplementation with 80 µg.egg^{-1} of inorganic salts of zinc (Zn 351.80 µg.100 ml^{-1} deionised water)
Group III	*In ovo* supplementation with 16 µg.egg^{-1} of inorganic salt of copper (Cu 62.87 µg.100 ml^{-1} deionised water)
Group IV	*In ovo* supplementation with 80 µg.egg^{-1} of inorganic salts of zinc and 16 µg.egg^{-1} copper

A total of 300 hatching eggs (only 148 (59.20 %) and 232 (90.27 %), respectively, were fertile) each of Arbor Acre and Cobb 500 strains of broiler chickens were used in both experiments. Each of the *in ovo* treatment group (control, inorganic salts of zinc, copper and their combination) contained 37 and 58 fertile eggs in Experiment I and II, respectively. The treatment groups are shown in Table 1.

On the 18th day of incubation, the candled eggs (*in ovo* groups) were injected with nutrients (organic salts of zinc and copper) into amnion using a 24-gauge hypodermic needle. Before injections, the site was sterilised with 10 % ethanol and the injections were done at the broad end of the egg. Following *in ovo* feeding, the injection site was sealed with sterile paraffin and the eggs transferred to the hatching compartment. The *in ovo* injection of each treatment was completed within 30 minutes of taking the eggs out from the incubator.

Post-hatch chick and management

In Experiment I, a total of 21 chicks hatched (14.19 % hatchability); 10 chicks (27.02 % hatchability) from the control; 2 chicks (5.41 % hatchability) from Zn-injected eggs; 4 chicks (10.81 % hatchability) from Cu-injected eggs and 5 chicks (13.51 % hatchability) from ZnCu-injected eggs. The resulting chicks were distributed into replicate with unequal number of replications on the basis of *in ovo* treatment groups. This thereby informed the need for the second experiment but with a different strain (Cobb 500) of broiler chickens. In Experiment II, a total of 156 chicks hatched (67.245 % hatchability); 51 chicks (87.93 % hatchability) from the control; 53 chicks (91.38 % hatchability) from Zn-injected eggs; 34 chicks (58.625 % hatchability) from Cu-injected eggs, and 18 chicks (31.03 % hatchability) from ZnCu-injected eggs. The first two treatment groups were replicated equally (10 birds per replicate of 5), Cu-injected treatment group were replicated as 10 birds per replicate of 3 and ZnCu-injected treatment groups were replicated on 6 birds per replicate of 3.

The chicks were brooded for 2 weeks and fed commercial feeds containing 23 % crude protein, 3200 kcal. kg^{-1} metabolisable energy, 0.45 % available P, 190.30 ppm Zn and 29.30 ppm Cu at the starter phase, and 20 % crude protein, 3000 kcal.kg^{-1} metabolisable energy, 0.40 % available P, 88.10 ppm Zn, and 13.80 ppm Cu at the finisher phase. They were served fresh clean water *ad libitum*. They were raised intensively in a deep litter system for 7 weeks

in Experiment I and for 5 weeks (due to COVID-19 restriction on movement) in Experiment II with all necessary medications and vaccinations strictly adhered to.

Data collection

Hatching traits

a) Egg weight was determined by weighing the hatchling eggs on a sensitive weighing scale with the average weight recorded.
b) Percentage hatchability was calculated as: % Hatchability = (Number of hatched chicks/ Number of fertile eggs) × 100.
c) Weight (g) of the chicks at hatch was determined by weighing the chicks hatched in each replicate and the average weight was calculated.
d) Chicks to egg ratio was determined as the ratio of the chick weight to egg weight.

Gut morphometry

On the 7th and 49th day of post-hatch, two birds of average weight from each replicate were slaughtered by cervical dislocation for gut development studies. Gut morphometry was done by recording the weights of gizzard, proventriculus, liver as well as the weight and length of the duodenum, jejunum, ileum and caecum which were expressed as cm per 100 g live weight.

Post-hatch growth performance parameters

Feed intake

This was measured and recorded on replicate basis at the end of every week. Left over feed was subtracted from the amount of feed offered and the average feed intake was recorded as well. Feed wastage was greatly reduced by using a feeding trough with a lip and it was ensured that the trough was one-third filled at all times. Also, the feeding trough was hung at the level of the birds' back.

The following calculations were done on replicate basis:

Feed intake [g] = Feed offered [g] – Feed left [g]

Average feed intake [g.bird^{-1}] =

(Feed intake/Number of birds)

Body weight

Body weight gain was calculated on replicate basis by subtracting the final weight from initial weight and average body weight gain was calculated.

Weight gain [g] = Final weight [g] – Initial weight [g]

Average weight gain [g.bird^{-1}] =

(Weight gain/Number of birds)

Feed conversion ratio (FCR)

This is the proportion of feed converted into flesh by the birds. FCR was calculated as:

FCR = (Feed intake/Weight gain)

Cell-mediated immune response

At the 7th day of post-hatch, 1 bird per replicate was selected in experiment I because of the number of birds hatched while 2 birds per replicate were selected in experiment II, and their cell-mediated immune response to phytohaemaglutinin type P (PHA-P) was studied [7]. About 0.1 ml (concentration 1 mg.ml^{-1}) of PHA-P was injected at 3rd and 4th inter-digital space of the right foot. The left foot served as control and was injected with 0.1 ml phosphate-buffered saline (PBS). The foot web index was calculated as a difference between the swelling in the right and left feet before and after 24 hours of injection and expressed in millimetres [7].

The foot web/pad index was calculated as follows:

Cell-mediated immune response

(CMIR) = (R2 – R1) – (L2 – L1)

Where:

R2 = Thickness of the right foot web after 24 hours of injection

R1 = Thickness of the right foot web before injection

L2 = Thickness of the left foot web after 24 hours of injection

L1 = Thickness of the left foot web before injection

Humoral immune response

The antibody response to the Sheep Red Blood Cell (SRBC) was studied at the 21st day post-hatch, wherein 1 ml of 1 % SRBC was injected intravenous into the birds. The SRBC was washed thrice and centrifuged at 704 g for 10 minutes after each washing. After 5 days of SRBC immunization, 1 bird per replicate was selected in Experiment I (because of the number of birds hatched) and 2 birds per replicate in Experiment II, and 2 ml of blood was collected from the wing vein and the antibody titre was recorded by haemaglutination (HA) titre as enunciated in previous studies [31, 37].

Collection of blood samples

On the 7th day in both experiments and at 49th and 35th day post-hatch in experiment I and II, respectively, two birds per replicate were selected for blood collection; 2 ml of the blood samples was collected through the jugular vein into the blood sample bottles for the determination of serum biochemical indices using standard procedures [18]. All blood samples were analysed using routine laboratory procedures [12].

Determination of serum parameters

Serum total protein, albumin and cholesterol were determined spectro-photometrically according to the method [35] using Randox(R) diagonistic kit manual. Alanine aminotransferase was determined spectro-photometrically according to the method [28] using a commercial Randox kit.

Statistical analysis

The data collected were subjected to one-way Analysis of Variance in a Completely Randomized Design. Significant ($P < 0.05$) difference among means was separated using Tukey test as contained in the Minitab® version 17.1.0 [26].

RESULTS

Effects of *in ovo* injection of inorganic salts of zinc, copper and their combination on hatching traits of Arbor Acre and Cobb 500 strains of broiler chickens

Figure 1 shows the effects of *in ovo* injection of in organic salts of zinc, copper and their combination on hatching traits of Arbor Acre strain of broiler chickens. The highest hatchability of 27.02 % was obtained from the eggs of the control group, while the least hatchability of 5.41 %

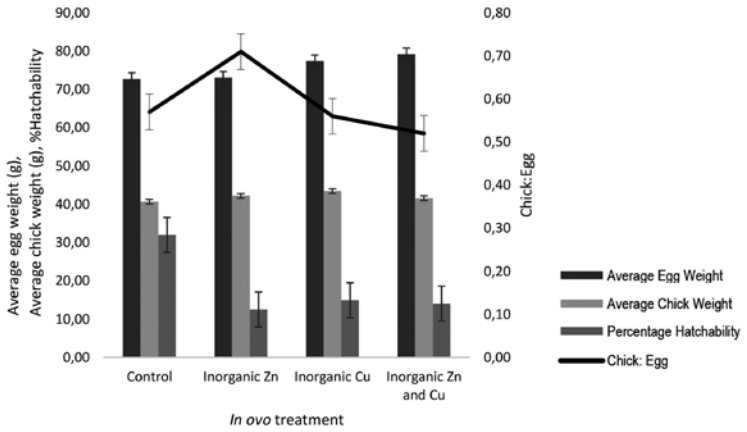

Fig. 1. Effect of *in ovo* injection of inorganic salts of Zn, Cu and their combination on hatching traits of Arbor Acre broiler chickens

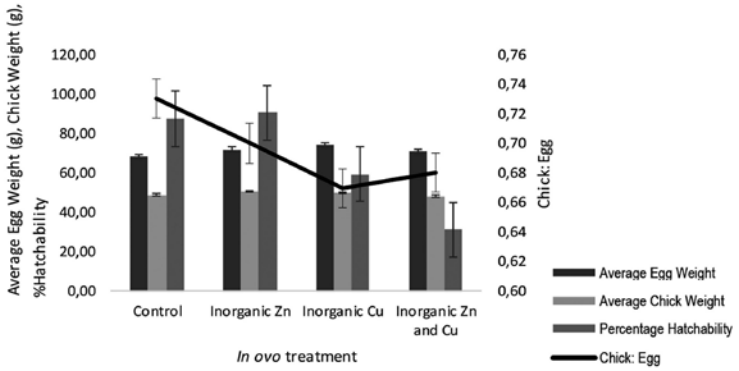

Fig. 2. Effect of *in ovo* injection of inorganic salts of Zn, Cu and their combination on hatching traits of Cobb 500 broiler chickens

was recorded in the eggs of the *in ovo* injection of inorganic salts of zinc. Hatchability of 13.51 % and 10.81 % were recorded in eggs of the *in ovo* injection of the combination of inorganic salts of zinc and copper (ZnCu) and eggs of the *in ovo* injection of salts of copper, respectively. It was observed that the highest chick weight of 41.58 g was obtained in chicks from eggs of the *in ovo* ZnCu; this was followed by chicks resulting from eggs injected with inorganic copper salts (34.77 g) and those in the control group (25.60 g). The lowest chick weight of 16.88 g was obtained in chicks from the *in ovo* injection of inorganic Zn salts. Chick to egg ratio ranged from 0.36 to 0.52, with the least values recorded in groups of the *in ovo* injection of inorganic salt of zinc while the highest chick to egg ratio was obtained in the eggs of the *in ovo* treatment injected with the combination of inorganic salts of zinc and copper.

In Figure 2, the effects of *in ovo* injection of inorganic salts of zinc, copper and their combination on hatching traits of Cobb 500 strains of broiler chickens are shown. Highest hatchability of 91.38 % was obtained in eggs from the *in ovo* injection of inorganic salt of zinc while the least hatchability of 31.03 % was recorded in eggs from the *in ovo* injection of salts of zinc and copper combined. Eggs injected with copper salt and eggs in the control, both had hatchability 58.62 % and 87.93 %, respectively. The highest chick weight of 50.52 g was obtained in birds from eggs with the *in ovo* injection of zinc salt; this was followed by birds resulting from eggs injected with inorganic copper salt (49.87 g) and those in the control group (49.33 g). The lowest chick weight of 49.06 g was obtained in birds from the *in ovo* injection of the combination of inorganic salt of zinc and copper. Chick to egg ratio ranged from 0.67 to 0.73, with least values recorded in birds from the *in ovo* injection of inorganic copper while the highest chick to egg ratio was obtained in eggs from the control group.

Effects of *in ovo* injection of inorganic salts of copper, zinc and their combination on growth performance of Arbor Acre and Cobb 500 strains of broiler chickens

Table 2 shows the effects *in ovo* injection of inorganic salts of copper, zinc and their combination on growth performance of Arbor Acre strain of broiler chickens. The effects of *in ovo* injection of inorganic salts of copper, zinc and their combination (ZnCu) showed significant ($P < 0.05$) effect on the daily feed intake of Arbor Acre strain of broiler chickens. Birds from eggs injected with in-

Table 2. Effects of *in ovo* injection of inorganic salts of copper and zinc on growth performance of Arbor Acre and Cobb 500 strains of broiler chickens

Parameters	*In ovo* injection					
	Control	Zn	Cu	ZnCu	SEM	P-value
Arbor Acre						
Initial weight [g.bird^{-1}]	44.67	ND	44.50	47.13	2.18	0.011
Final weight [g.bird^{-1}]	1440.00	ND	2100.00	1683.00	124.0	0.140
Daily weight gain [g.bird^{-1}.d^{-1}]	28.48	ND	41.95	33.39	2.53	0.140
Daily feed intake [g.bird^{-1}.d^{-1}]	76.78^{b}	ND	129.96^{a}	86.51$^{a, b}$	4.88	0.036
Feed conversion ratio	**2.71**	**ND**	**3.10**	**2.59**	**0.18**	**0.366**
Cobb 500						
Initial weight [g.bird^{-1}]	65.56	67.76	61.90	65.28	1.59	0.152
Final weight [g.bird^{-1}]	928.90	889.10	876.50	904.20	85.30	0.974
Daily weight gain [g.bird^{-1}.d^{-1}]	24.67	23.47	23.27	23.97	2.46	0.977
Daily feed intake [g.bird^{-1}.d^{-1}]	47.85^{b}	51.83a^{b}	58.56^{a}	52.90$^{a, b}$	1.88	0.020
Feed conversion ratio	**1.95**	**2.23**	**2.67**	**2.24**	**0.27**	**0.365**

$^{a, b}$ – Means on the same row having different superscripts differ significantly ($P < 0.05$); ZnCu – combination of zinc and copper; ND – No data (only 2 chicks hatched, hence no replication); SEM – Standard error of mean; P-value – Probability value

Table 3. Effects of *in ovo* injection of inorganic salts of zinc, copper and their combination on organ development and gut morphometry of Arbor Acre and Cobb 500 strains of broiler chickens at day 7

Parameters	*In ovo* injection					
	Control	Zn	Cu	ZnCu	SEM	P-value
Arbor Acre						
Live weight [g.bird^{-1}]	**83.50**	**ND**	**97.00**	**59.00**	**10.93**	**0.199**
Organs [%]						
Heart	0.83	ND	0.76	1.17	0.195	0.503
Liver	3.55	ND	2.42	4.72	1.16	0.527
Proventriculus	1.05	ND	0.73	0.58	0.95	0.575
Gizzard	7.26	ND	5.01	9.96	2.61	0.542
Gut						
Duodenum [%]	2.41	ND	1.76	2.270	0.32	0.532
Duodenum [cm.100 g^{-1} LW]	13.57	ND	9.90	22.14	4.47	0.332
Jejunum [%]	3.13	ND	1.56	3.12	0.56	0.335
Jejunum [cm.100 g^{-1} LW]	28.69	ND	22.27	40.98	5.63	0.261
Ileum [%]	4.01	ND	1.28	3.04	0.55	0.161
Ileum [cm.100 g^{-1} LW]	30.43	ND	22.99	43.98	4.13	1.333
Caecum [%]	1.30	ND	0.64	1.55	0.41	0.482
Caecum [cm.100 g^{-1} LW]	14.78	ND	9.18	18.28	1.57	0.121
Colon [%]	0.86	ND	0.31	1.01	0.15	0.175
Colon [cm.100 g^{-1} LW]	4.77	ND	3.71	6.12	1.33	0.484
Cobb 500						
Live weight [g.bird^{-1}]	**141.50**	**148.50**	**156.00**	**115.50**	**12.50**	**0.258**
Organs [%]						
Heart	1.00	0.97	0.99	0.91	0.09	0.905
Liver	4.52	4.94	4.54	5.00	0.72	0.937
Proventriculus	0.82	1.03	0.81	1.00	0.09	0.309
Gizzard	6.94	6.89	7.67	8.43	0.74	0.493
Gut						
Duodenum [%]	1.73	1.79	1.94	2.05	0.08	0.134
Duodenum [cm.100 g^{-1} LW]	11.49	10.46	9.66	12.06	0.96	0.404
Jejunum [%]	3.09	2.80	2.93	2.30	0.41	0.962
Jejunum [cm.100 g^{-1} LW]	22.83	24.94	17.68	25.77	2.99	0.348
Ileum [%]	2.68	2.660	2.21	3.16	0.42	0.533
Ileum [cm.100 g^{-1} LW]	22.85	25.73	19.31	25.74	2.24	0.278
Caecum [%]	0.818	0.68	0.61	0.88	0.14	0.546
Caecum [cm.100 g^{-1} LW]	10.74	10.10	8.32	10.63	1.83	0.776
Colon [%]	0.48	0.34	0.48	0.43	0.06	0.333
Colon [cm.100 g^{-1} LW]	2.984	2.70	2.75	2.86	0.28	0.819

ZnCu – combination of zinc and copper; LW – Live weight; SEM – Standard error of mean;
ND – No data (only 2 chicks hatched hence no replication); P-value – Probability value

Table 4. Effects of *in ovo* injection of inorganic salts of zinc, copper and their combination on organ development and gut morphometry of Arbor Acre and Cobb 500 strains of broiler chickens at days 49 and 35, respectively

Parameters	*In ovo* injection					
	Control	Zn	Cu	ZnCu	SEM	P-value
Arbor Acre						
Live weight [g.bird^{-1}]	**2063.00**	**ND**	**2100.00**	**1765.00**	**274.66**	**0.573**
Organs [%]						
Heart	0.59	ND	0.62	0.53	0.17	0.910
Liver	1.92	ND	1.91	2.06	0.20	0.795
Proventriculus	0.39	ND	0.43	0.38	0.06	0.884
Spleen	0.13	ND	0.14	0.17	0.05	0.742
Gizzard	1.82	ND	2.38	2.03	0.16	0.191
Pancreas	0.23	ND	0.24	0.29	0.06	0.526
Gut						
Duodenum [%]	0.74	ND	0.38	0.82	0.09	0.704
Duodenum [cm.100 g^{-1} LW]	1.57	ND	0.76	1.73	0.25	0.153
Jejunum [%]	1.32	ND	1.00	1.34	0.17	0.521
Jejunum [cm.100 g^{-1} LW]	3.15	ND	2.88	3.49	0.53	0.728
Ileum [%]	1.08	ND	1.05	1.17	0.22	0.921
Ileum [cm.100 g^{-1} LW]	3.29	ND	2.60	3.55	0.71	0.735
Caecum [%]	0.49	ND	0.52	0.71	0.15	0.420
Caecum [cm.100 g^{-1} LW]	1.94	ND	1.71	2.11	0.41	0.829
Colon [%]	0.15	ND	0.16	0.19	0.06	0.852
Colon [cm.100 g^{-1} LW]	0.37	ND	0.39	0.47	0.09	0.634
Cobb 500						
Live weight [g.bird^{-1}]	**1366.70**	**1333.30**	**1033.30**	**1316.70**	**84.60**	**0.077**
Organ [%]						
Heart	0.41	0.48	0.62	0.51	0.05	0.154
Liver	2.70	2.59	2.91	2.77	0.47	0.969
Proventriculus	0.42	0.62	0.07	0.59	0.07	0.127
Spleen	0.11	0.11	0.14	0.13	0.02	0.622
Gizzard	3.75	3.54	3.764	3.69	0.44	0.982
Pancreas	0.25	0.19	0.23	0.29	0.04	0.504
Gut						
Duodenum [%]	0.88	1.02	1.08	9.57	0.13	0.756
Duodenum length [cm.100 g^{-1} LW]	2.145	2.19	2.83	2.38	0.23	0.217
Jejunum [%]	2.73	8.02	2.89	2.13	0.39	0.445
Jejunum [cm.100 g^{-1} LW]	5.69	6.65	7.01	6.09	0.74	0.618
Ileum [%]	2.29	2.57	2.72	2.04	0.29	0.402
Ileum [cm.100 g^{-1} LW]	6.12	7.12	7.07	6.57	0.65	0.680
Caecum [%]	0.76	0.70	0.90	0.88	0.07	0.225
Caecum [cm.100 g^{-1} LW]	2.00	3.00	4.00	3.00	0.23	0.013
Colon [%]	0.53	0.29	0.36	0.43	0.10	0.411
Colon [cm.100 g^{-1} LW]	0.60	0.63	0.78	0.80	0.1	0.413

ZnCu – combination of zinc and copper; LW – Live weight; SEM – Standard error of mean;
ND – No data (only 2 chicks hatched hence no replication); P-value – Probability value

organic copper salt and birds from eggs injected with combined salts of ZnCu were statistically similar and followed by birds resulting from eggs of the control group (76.78 g). The same trend was observed in the Cobb 500 strain of broiler chickens. Birds from eggs injected with copper had the highest ($P < 0.05$) daily feed intake (58.56 g) followed by birds from eggs injected with ZnCu combination, zinc and the un-injected eggs.

Effects of *in ovo* injection of inorganic salts of zinc, copper and their combination on organ development and gut morphology of Arbor Acre and Cobb 500 strains of broiler chickens at day 7

In Table 3, the effects of *in ovo* injection of inorganic salts of zinc, copper and their combination on organ development and gut morphometry of Arbor Acre and Cobb 500 strains of broiler chickens at day 7 of age are shown. In both strains of broiler chickens, there were no significant ($P > 0.05$) variations in all the organ and gut morphometry parameters measured.

Effects of *in ovo* injection of inorganic salts of zinc, copper and their combination on organ development and gut morphology of Arbor Acre and Cobb 500 strains of broiler chickens at days 49 and 35, respectively

The effects of *in ovo* injection of inorganic salts of zinc, copper and their combination on organ development and gut morphometry of Arbor Acre and Cobb 500 strains of broiler chickens at days 49 and 35, respectively are shown in Table 4. In both strains of broiler chickens, there were no significant ($P > 0.05$) differences in all the parameters measured.

Effects of *in ovo* injection of inorganic zinc, copper and their combination on cell-mediated immunity and humoral immunity of Arbor Acre and Cobb 500 strains of broiler chickens

The effects of *in ovo* injection of inorganic salts of zinc, copper and their combination on cell-mediated immunity and humoral immunity (after 24 hours of injecting phytohaemagglutinin type-P (PHA-P) of Arbor Acre and Cobb 500 strains of broiler chickens are shown in Figures 3 and 4, respectively. In Arbor Acre (Figure 3), the highest cell-mediated immune response was recorded in birds on the control group and this reduced across the treatment groups. The same trend was observed in the humoral immunity but with upsurge in the humoral immunity of the birds using *in ovo* injection of the combination of inorganic salts of zinc and copper. In Cobb 500 strain (Figure 4), birds from eggs with *in ovo* injection of inorganic salt of

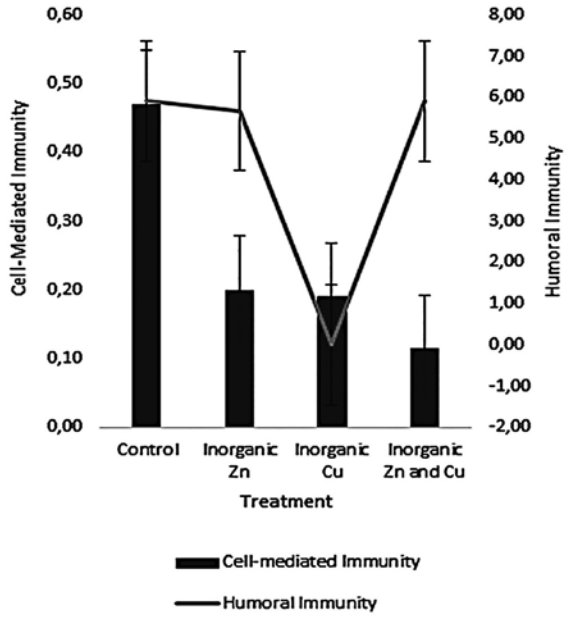

Fig. 3. Effect of *in ovo* injection of inorganic salts of Zn, Cu and their combination on cell-mediated immunity and humoral immunity of Arbor Acre broiler chickens

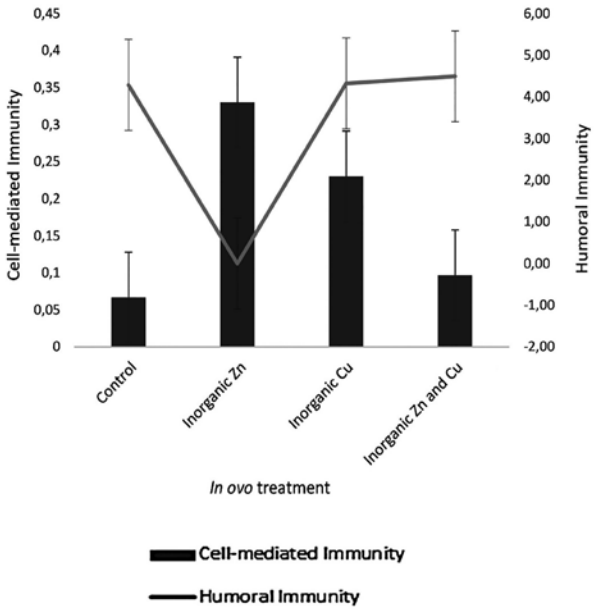

Fig. 4. Effect of *in ovo* injection of inorganic salts of Zn, Cu and their combination on cell-mediated immunity and humoral immunity of Cobb 500 broiler chickens

Table 5. Effects of *in ovo* injection of inorganic salts of zinc, copper and their combination on serum biochemical indices of Arbor Acre and Cobb 500 strains of broiler chickens at days 49 and 35, respectively

Parameters	*In ovo* injection					
	Control	Zn	Cu	ZnCu	SEM	P-value
Arbor Acre						
Total protein [g.dl^{-1}]	2.60^{b}	ND	4.60^{a}	4.00^{a}	0.11	0.012
Albumin [g.dl^{-1}]	1.50^{b}	ND	2.60^{a}	2.35$^{a, b}$	0.12	0.040
Globulin [g.dl^{-1}]	1.10	ND	2.00	1.65	0.22	0.191
Cholesterol [g.dl^{-1}]	76.10	ND	71.50	56.00	6.96	0.259
Triglyceride [g.dl^{-1}]	99.70	ND	127.90	118.1	21.63	0.698
Low density lipoprotein [g.dl^{-1}]	19.65	ND	15.60	9.40	3.61	0.274
Very low-density lipoprotein [g.dl^{-1}]	19.95	ND	25.60	23.65	4.34	0.698
High density lipoprotein [g.dl^{-1}]	36.55	ND	30.30	22.95	7.43	0.479
Aspartate aminotransferase [U.l^{-1}]	61.00	ND	80.00	60.50	4.71	0.188
Alanine aminotransferase [U.l^{-1}]	14.00	ND	14.00	16.50	3.71	0.850
Cobb 500						
Total protein [g.dl^{-1}]	4.50	4.90	5.45	5.40	0.33	0.282
Albumin [g.dl^{-1}]	2.70	3.15	3.10	3.25	0.12	0.098
Globulin [g.dl^{-1}]	1.85	1.75	2.35	2.15	0.43	0.759
Cholesterol [g.dl^{-1}]	61.2	88.9	78.6	102.5	17.0	0.461
Glucose [g.dl^{-1}]	170.1	144.0	116.9	110.7	17.4	0.203
Triglyceride [g.dl^{-1}]	74.05	70.40	69.40	72.05	2.87	0.701
Low density lipoprotein [g.dl^{-1}]	16.45	25.90	20.65	29.35	6.04	0.520
Very low-density lipoprotein [g.dl^{-1}]	14.80	14.10	13.90	14.00	0.58	0.727
High density lipoprotein [g.dl^{-1}]	29.90	48.80	44.00	58.70	11.6	0.454
Aspartate aminotransferase [U.l^{-1}]	82.00	81.50	79.50	77.50	5.76	0.939
Alanine aminotransferase [U.l^{-1}]	28.50	30.50	26.50	28.00	2.17	0.657

$^{a, b}$ – Means on the same row having different superscripts differ significantly ($P < 0.05$); ZnCu – combination of zinc and copper; ND – No data; SEM – Standard error of mean; P-value – Probability value

zinc, had the highest cellular immunity of 0.33 mm after 24 hours of injection; this was followed by birds from eggs on *in ovo* injection of inorganic salts of copper (0.23 mm) and birds from eggs on *in ovo* injection of inorganic salts of zinc and copper with 0.09 mm, respectively. The lowest cellular immunity of 0.06 mm was obtained in the birds from eggs in the control group after 24 hours of injecting PHA-P. Humoral response to sheep red blood cell (SRBC) ranged from 4.49 mm to 4.28 mm. The lowest value was recorded in birds from eggs using *in ovo* injection of inorganic salts of zinc while the highest value was recorded in birds injected with the combination of inorganic salts of zinc and copper.

Effects of *in ovo* injection of inorganic salts of zinc, copper and their combination on serum biochemical indices of Arbor Acre and Cobb 500 strains of broiler chickens at days 49 and 35, respectively

Table 5 shows the effect of *in ovo* administration of inorganic salts of zinc, copper and their combination on

serum biochemical indices of Arbor Acre and Cobb 500 strains of broiler chickens at days 49 and 35, respectively. Chicks from eggs injected with Cu had the highest ($P < 0.05$) total protein production, followed by chicks resulting from ZnCu injected group while the least total protein value was recorded in chicks resulting from the control group. The same trend was also observed in serum albumin. In Cobb 500 strain of broiler chickens, there were no significant ($P > 0.05$) differences in all the parameters measured.

DISCUSSION

The least hatchability results obtained from eggs of the *in ovo* injection of inorganic salt of zinc treatment group in Experiment I (Arbor Acre strain) contradicted the result of [32] on improved hatchability in the Zn-injected hatchings eggs. The difference may be attributed to different strains of broiler chickens used. In addition, there were many other factors that could contribute to the varying hatchability of *in ovo* treatment groups including: the source of the eggs, the age of the breeder flock, nutrition, the egg storage condition before setting, and the injection time. The highest percentage of hatchability obtained in the control group on the *in ovo* injection group was in line with the findings [2] that *in ovo* supplementation of trace minerals enriched solution did not show any significant difference in hatchability of *in ovo* injected group compared to eggs without *in ovo* supplementation. In Experiment II, the hatchability result obtained in Zn-injected eggs of Cobb 500 strain of broiler chickens correlate with the results that affirmed [32] that zinc as a crucial trace mineral for embryonic development of broiler chickens. Zinc is distributed throughout the body and plays a critical role in improving: reproduction, development of blood cells, immune system function, and bone development [1].

In Experiments I and II, *in ovo* injection of these minerals (Zn, Cu and ZnCu) did not significantly affect chick weight and this corroborated the findings [19] that *in ovo* injection particularly of nano-zinc had no influence on hatch weight. There have been varying results on growth traits with *in ovo* administration of trace minerals in broiler chickens where no effect [30] and increased body weights with *in ovo* administered groups over control group were reported [32]. These varying results can be attributable to the differences in the strains of broiler chickens and the growth-promoting effects of various substances administered *in ovo*. Similarly, birds from Cu-injected eggs recorded the highest feed intake in both Experiments I and II of this study.

These results have demonstrated the positive effect of *in ovo* injection of inorganic salt of Cu especially at minute quantities on feed intake stimulation in broiler chicken. According to one study [38], improved feed intake was observed in birds fed dietary copper at the level of 200 ppm when compared with birds fed dietary copper at 600 and 800 ppm. The authors attributed the improved intake at 200 ppm to the reduction of pathogenic microorganisms and improved activity of digestive enzymes such as protease, amylase and lipase, which ensured better digestion and utilization of the feed within the digestive tract.

The non-significant differences in digestive organs length observed in both Experiments I and II at different ages were not in line with the findings [36]. The authors indicated that *in ovo* feeding improved gastrointestinal tract development of hatchlings and functionally similar to that of conventional 2-day old chicks offered feed immediately after hatch. In addition, it was reported [14] that the development of gut occurs throughout incubation and when the embryo starts consuming amniotic fluid orally, intestinal weight increases from approximately 1 % at 17 days of incubation to 3.5 % at hatch.

The literature is limiting on the influence of *in ovo* feeding of inorganic salts on serum biochemical indices of broiler chickens. However, at day 49 of Experiment I on Arbor Acre strain of broiler chickens, the highest total protein and albumin were observed in birds from eggs with *in ovo* injection of Cu and the values were comparable to the values obtained in birds from ZnCu injected eggs. This indicated that albumin is synthesized in the right quantity for transporting insoluble substance in the blood and aids the maintenance of oncotic pressure [10]. A higher concentration denotes dehydration while lower concentration may be due to factors such as malnutrition and infection [8]. Both total protein and albumin values were within the normal range (TP 3.0–4.9 $mg.dl^{-1}$ and 1.17–2.74 $g.dl^{-1}$, respectively) reported [25] for healthy broiler chickens.

In Experiment II, improved cellular immunity was obtained in birds from eggs of the *in ovo* injection of Zn and this same trend was seen in birds on other *in ovo* treatment groups. Also, the highest humoral immunity was obtained

in birds from eggs injected with the combination of zinc and copper salt. This result was at variance with findings [32] of non-significance in the growth of immune organs and response to PHA-P or SRBC thereby suggesting that *in ovo* injection of inorganic salts of Zn, Cu and their combination might not be immunomodulatory in broiler chickens of improved genetic lines. However, the results obtained in this study is in line with the reports on *in ovo* injection of lysine [24], arginine [21] and 8 µg.egg^{-1} of inorganic Cu ($CuSO_4$), which were found to enhance the immune response of broiler chickens [14].

CONCLUSIONS

From this study, it could be concluded that:

- administration of inorganic salts Zn and Cu *in ovo* reduced percentage hatchability in Arbor Acre strain but improved percentage hatchability in Cobb 500 strain of broiler chickens;
- *in ovo* administration of the combination of inorganic salts of Zn and Cu did not significantly affect the chick weights of both strains of broiler chickens but there was a numerical increase in the final weights of the birds due to *in ovo* administration of inorganic salt of Cu in Arbor Acre broiler chicken from significantly increased daily feed intake;
- *in ovo* injection of inorganic salts of Zn and Cu did not significantly affect the gut development of both strains of broiler chickens;
- administration of inorganic salts of Zn and Cu *in ovo* had an appreciable significant influence on the cellular and humoral immunity of Cobb 500 strain of broiler chicken, thereby, indicating that the ability of the inorganic nutrients to enhance antibody production;
- the total protein and albumin of the Arbor Acre strain of broiler chickens were significantly increased by the *in ovo* administration of inorganic salts of Zn, Cu and their combination.

CONFLICT OF INTEREST

The authors hereby declare that there is no conflict of interest in the conception, design and implementation of the study.

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AMYLOLYTIC AND CELLULOLYTIC ACTIVITIES, THE DIGESTIBILITY OF DRY MATTER OF BROILER CHICKENS AFTER FEED INTAKE OF HUMIC SUBSTANCES

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ABSTRACT

This study aimed to investigate the effects of feed intake of humic substances (HS) on the cellulolytic and amylolytic activities, and the digestibility of dry matter (DM) in the chymus of the intestine and ileum of broiler chickens (Cobb 500). Four groups of birds (A, B, C, negative control; n = 120) were fed with mash diets (starter HYD1 230.20, grower HYD2 222.20, finisher HYD3 209.40 g crude protein (CP) per kg of DM) with the added HS from day 1 to 37 days as follows: A – 0.7 % Humac nature; B – 0.7 % Humac nature monogastric (HNM); C – 0.5 % HNM (Humac Ltd., Slovakia). The cellulolytic activities were increased in the intestine in B/C by 2.50 (P < 0.01)/1.65 (P < 0.05) on day 3 and 1.02 (P < 0.001)/0.3 (P < 0.05) µmol.l^{-1}.min^{-1}.g^{-1} on day 24. There were observed higher values of digestibility coefficient of DM in the intestine (P < 0.05) in C by 15.14 % and 14.85 %, and in B by 20.73 % on days 17, 24 and 31, respectively. The enhancement of ileal digestibility of DM was observed in B by 7.38 % (P < 0.05) and CP in A and B by 3.7% and 6.1% (P < 0.05). The feed intake of HS favourably influenced the cellulolytic activities and increased the DM digestibility in the intestine and the ileum, as well as the CP digestibility in the ileum.

Key words: crude protein; dry matter, humates; intestinal digestibility; poultry nutrition

INTRODUCTION

The breeding of broiler chickens is a very important sector of poultry farming which provides animal-based proteins. The importance of broiler chicken meat production stands out and poultry meat is the best alternative to red meat. It is a source of high-quality protein, minerals, and vitamins which are used to balance the human diet [24].

Various kinds of feed additives and their combinations are utilized for the improvement of production and health parameters of poultry such as: probiotics and prebiotics [3, 12], organic acids [23], enzymes [35] and plant extracts [2, 27, 28]. In addition to the feed supplements, humic substances (HS) have been attracting the attention of scientists for several years.

The humic substances (HS) are components of the organic part of the soil, geological organic deposits and soil water. Their presence was demonstrated in lake sediments, brown coals and peats. From the chemical point of view, HS are heterogenous mixtures of polydispersed compounds produced as a result of chemical reactions in the

time of transformation of plants or organic matter during humification. The substrates used in the process of humification are: polysaccharides, proteins, nucleic acids, lipids, melanin, plant lignin, cutin and char particles [19]. HS are considered as supramolecular associations of self-organizing heterogenous and relatively small molecules which resulted from the decomposition of plant residues [33].

The characteristics of HS are based of the solubility at various pH and retention on hydrophobic resins. Three main components are: humic, fulvic acids (FA) and humin (HA). Whereas, humic acids are soluble at higher pH and insoluble at pH < 2, fulvic acids are soluble under all pH values and humin is insoluble in acids and alkalis [31].

The application of HS in animal breeding is bound with its adsorptive, anti-inflammatory and antibacterial activities [25]. Many experiments testing their positive characteristics have been performed with animal species like pigs [34, 36], sheep [40], cattle [42] and laboratory micromammals [41]. Several significant positive results have been achieved in poultry farming with broiler chickens and laying hens [6, 22].

However, different results of the feed conversion ratio and digestibility of broiler chickens have been published. In the case of production parameters, the effects of 0.7 % supplementation of HS on the growth performance of broiler chickens were not so significant [18], as on the composition and quality of breast meat. On the contrary, broilers fed with HS extracted from worm compost in the drinking water, showed lower feed conversion ratio (FCR), higher energy digestibility and higher retention of dry matter (DM), ash, N and energy [15].

A scientific hypothesis was based on the positive influence of HS on the enzymatic activities in the gastrointestinal apparatus of poultry with the effect on the digestion of dry matter in the intestine.

The aim of this study was to investigate the effects of dietary intake of HS on the amylolytic and cellulolytic activities in the intestine as well as on the digestibility of DM in broiler chickens.

MATERIALS AND METHODS

Broiler chickens and feed

One-day-old broiler chickens of the hybrid Cobb 500 with an average weight of 50 g were supplied by a commercial hatchery. The chickens were randomly divided into 3 experimental groups and a control group of 30 birds. They were housed in floor pens in a shed specialized for poultry production. The area for one broiler chicken was 0.12 m^2. Access to feed and water was constant.

Starter (HYD1), grower (HYD2) and finisher (HYD3) ground feeds were fed for 37 days. Starter and grower diets contained an anticoccidial agent (salinomycin sodium), while all other diets did not contain antimicrobial agents.

HS were added to the feeds of the experimental groups (A, B, C) as components of two products. The first HN was added to the feed of group A in the amount of 0.7 %, the second HNM was added to the feeds of groups B or C in the amounts of 0.7 % and 0.5 %, respectively.

The HN or HNM products had the following parameters: Particle size up to 100 mm, maximum moisture content of 15 % and the content of

- HA min. 650/570, FA min. 50/50 $g.kg^{-1}$,
- Ca 42.28/51.1, Mg 5.11/4.86, Fe 19.05/18.09 $g.kg^{-1}$,
- Cu 15/14.25, Zn 37/35.15, Mn 142/135,
- Co 1.24/1.18, Se 1.67/1.59 and
- Mo 2.7/2.57, V 42.1/40 $mg.kg^{-1}$ of DM.

The calculated content of HA/FA in feeds used in the experimental groups was the following: A 4.55/0.35, B 3.99/0.35, C 2.85/0.25 $mg.kg^{-1}$. The measurement of zootechnical performance was performed once a week.

Ethical statement

Experiments were performed under the European Directive for the protection of vertebrate animals used for experimental and other scientific purposes (86/609/EU) and with the permission of the State Veterinary and Food Administration of the Slovak Republic No. 3090/13-221 in the poultry breeding facilities of the University of Veterinary Medicine and Pharmacy in Košice, Slovakia.

Analysis of feed

The analysis of the experimental feeds was performed according to the methods (Table 1) of the Association of Official Analytical Chemists (AOAC) [8].

The determination of dry matter (DM), crude protein (CP), crude fat (CF), starch, and ash was performed. Neutral detergent and acid detergent fibres were analysed using the methods by Van Soest et al. [39]. The atomic absorption spectrophotometry was used for the determination of the mineral composition of the feeds [38]. The

Table 1. Nutrients and ingredients in feeds

Nutrients [g.kg^{-1}]	Feed mixtures		
	Starter HYD1	Grower HYD2	Finisher HYD3
Dry mater	1000.00	1000.00	1000.00
Crude protein	230.20	222.20	209.40
Crude fat	31.30	83.80	67.70
NDF	112.60	122.10	128.80
ADF	54.60	62.50	68.10
Ash	57.30	60.60	52.10
Ash insoluble in HCl	2.10	1.80	2.40
Starch	485.60	446.80	448.60
Calcium	4.93	6.00	7.41
Phosphorus	5.73	7.93	5.13
Sodium	2.98	1.93	1.60
Magnesium	2.86	3.06	3.11
Potassium	9.03	8.83	8.49
Copper	0.0275	0.0578	0.0594
Zinc	0.0229	0.0294	0.1336
Manganese	0.0789	0.1472	0.1437
Metabolisable energy [MJ.kg^{-1}]*	13.27	14.30	13.58
Ingredients			
HYD1/HYD2/HYD3	maize, wheat, soybean meal, vegetable oil, limestone, amino acids and their salts, monocalcium phosphate, lysine, methionine, mineral-vitamin premix		
HYD1/HYD2	anticoccidial agent salinomycin sodium 70 mg.kg^{-1}		

* – [13]; NDF – neutral detergent fibre; ADF – acid detergent fibre

phosphorus was quantitated spectrophotometrically [7]. The insoluble portion of ash in HCl in the feed mixture was determined [9]. The metabolisable energy (ME) was calculated using a formula from Commission Regulation (EC) No. 152/2009 [13], which described the method of calculation and expression of energy value.

Enzymatic activities and intestinal digestibility

Both kinds of analyses were performed at 17, 24 and 31 days of age. All samples of excreta were taken directly from the cloaca into sterile glass containers.

Samples for quantification of digestive enzyme activities were prepared as follows: The fresh sample (1 g) was diluted with 49 ml of sterile TBS buffer (TRIS-hydroxymethyl aminomethane 10 mmol.l^{-1}, HCl 0.5 mol.l^{-1}, pH 7.0) and homogenised. The diluted samples were then used for the determination of the amylolytic and cellulolytic activities [26] which were analysed with the substrates starch (Fisher Slovakia Ltd.) and methyl hydroxyethylcellulose (Merck Ltd., Germany). The DM, ash and percent of insoluble ash in HCl were determined by the methods mentioned in the feed analysis. The calculation of the digestibility coefficient of the DM of chicken was done according to the method of G u g l i e m o and K a r a s o v [16], modified by M a r c i n et al. [29].

Measurement of ileal digestibility

Analysis of ileal digestibility was performed after slaughter on day 37. The samples of ileal chyme were collected in sterile glass containers. For the determination of DM, ash and ash insoluble in HCl, the above methods were used.

The calculation of the ileal digestibility was performed according to J o h n s o n et al. [21]. The comparison of nutrients in the feed in the ileal chyme referred to the content of the insoluble part of the ash and was used to determine digestibility according to the following formula:

Ileal digestibility (%) = (A/B – C/D) * B/A * 100

where,

A – nutrient content in the diet [g.kg^{-1}], B – indicator in the diet [g.kg^{-1}], C – nutrient output in the ileum [g.kg^{-1}], and D – indicator in the ileum [g kg^{-1}]

The insoluble portions of ash in HCl in feeds and in the ileal chymus were used as the indicators in the diets and ileum [9].

Statistical analysis

The data were statistically analysed using IBM SPSS Statistics, version 24. The results of treatments were compared by one-way analysis of variance and by the Tukey-Kramer multiple comparison test.

RESULTS

Zootechnical performance of broiler chickens

The use of HS as a feed additive had a positive effect on improving the performance and nutrient digestibility of poultry [11].

Table 2. Growth data of broiler chickens

Groups (n = 30)	BW [g] in days							ADWG [g.day^{-1}]
	1	7	14	21	28	35	37	1–37
A	50.0 ± 3.75	182.07^{b} ± 4.66	488.97 ± 65.44	963.07 ± 120.89	1571.48 ± 280.39	2311.92 ± 367.72	2506.67^{b} ± 160.47	66.39 ± 16.85
B	50.0 ± 2.65	188.82^{ab} ± 5.85	512.75 ± 67.64	986.37 ± 116.20	1636.63 ± 210.66	2326.56 ± 292.20	2490.25^{b} ± 166.39	65.95 ± 17.77
C	50.0 ± 3.35	192.27^{ab} ± 7.18	521.5 ± 45.87	989.33 ± 102.5	1585.17 ± 286.5	2281.93 ± 326.51	2377.75^{ab} ± 133.19	62.91 ± 18.06
Control	**50.0 ± 4.15**	**191.73^{a} ± 8.41**	**514.86 ± 54.30**	**985.57 ± 100.03**	**1633.0 ± 193.26**	**2291.79 ± 320.88**	**2319.42^{a} ± 92.55**	**61.34 ± 17.31**

Means (mean ± SD) with different superscript letters differ significantly ^{ab} – $P < 0.05$; ^{ac} – $P < 0.01$;
BW – body weight; ADWG – average daily weight gain

Table 3. Average daily feed intake and feed conversion ratio of broiler chickens

Groups (n = 30)	ADFI [g.day^{-1} per chicken] in days						FCR [kg. kg^{-1}]
	7	**14**	**21**	**28**	**35**	**37**	**1 – 37**
A	28.11	60.39	105.82	147.09	167.20	145.18	1.65 ± 0.29
B	27.29	66.54	112.17	152.38	188.36	154.71	1.64 ± 0.19
C	29.11	60.42	100.95	135.24	177.83	149.52	1.53 ± 0.26
Control	**27.94**	**63.62**	**106.12**	**140.82**	**156.10**	**146.38**	**1.51 ± 0.17**

ADFI – average daily feed intake; FCR – feed conversion ratio

Growth data of broiler chickens are shown in Table 2. The addition of HS 0.7 % to the diet of groups A and B improved the value of the average daily weight gain (ADWG) by 8.23 % and 7.52 %, respectively, compared with the control on days 1–37. A significant decrease in individual BW was observed on day 7. The value was 9.66 g lower in the A group compared to the control group ($P < 0.05$). The values of BW at the end of the experiment were increased in A and B groups by 187.25 and 170.83 g ($P < 0.05$) on day 37.

The values of the average daily feed intake (ADFI) and feed conversion ratio (FCR) are shown in Table 3. Although the improvement of ADFI was observed in the experimental groups, no significant differences in FCR were determined.

Enzymatic activities in the intestinal apparatus

The feed additive containing HS had a favourable effect on the increase of the cellulolytic activities in the intestine (Table 4) of chickens in groups B or C by 2.50 ($P < 0.01$) and 1.65 ($P < 0.05$) $\mu mol.l^{-1}.min^{-1}.g^{-1}$ on day 3 as well as by 1.02 ($P < 0.001$) and 0.3 ($P < 0.05$) $\mu mol.l^{-1}.min^{-1}.g^{-1}$ on day 24. The only decrease of this parameter was in the A or B groups on day 17 ($P < 0.05$) by 1.77 and 1.99 $\mu mol.l^{-1}.min^{-1}.g^{-1}$, respectively. In the case of the amylolytic activity, there were not observed significant differences in the experimental groups compared to the control.

Digestibility of dry matter in the intestinal apparatus

The feed intake of HS had a beneficial effect on the digestibility coefficient of DM (Table 5) in the intestine

Table 4. Digestive enzyme activities in the intestinal chyme of broiler chickens

Age [day]	Groups (n = 6)	Amylolytic (glucose) [$\mu mol.l^{-1}.min^{-1}.g^{-1}$]	Cellulolytic (glucose) [$\mu mol.l^{-1}.min^{-1}.g^{-1}$]
3	A	2.72 ± 0.604	3.98^{a} ± 0.811
	B	6.28 ± 3.440	5.33^{c} ± 0.614
	C	4.26 ± 0.485	4.48^{b} ± 0.396
	Control	**3.85 ± 0.726**	**2.83^{a} ± 0.162**
10	A	3.14 ± 0.706	3.08 ± 0.554
	B	2.59 ± 0.213	3.10 ± 0.172
	C	2.93 ± 0.117	2.52 ± 1.436
	Control	**3.08 ± 0.520**	**2.63 ± 0.459**
17	A	0.87 ± 0.315	1.37^{b} ± 0.294
	B	1.65 ± 0.409	1.15^{b} ± 0.267
	C	1.10 ± 0.120	2.05^{a} ± 0.865
	Control	**1.05 ± 0.481**	**3.14^{a} ± 0.769**
24	A	0.37 ± 0.090	0.59^{b} ± 0.016
	B	0.76 ± 0.263	1.38^{d} ± 0.198
	C	0.46 ± 0.278	0.66^{b} ± 0.048
	Control	**0.55 ± 0.418**	**0.36^{a} ± 0.085**
31	A	2.26 ± 0.229	3.01 ± 1.627
	B	1.75 ± 0.533	2.99 ± 0.262
	C	1.29 ± 0.169	2.16 ± 0.401
	Control	**1.67 ± 0.390**	**1.75 ± 0.381**

Means (mean ± SD) with different superscript letters differed significantly: ^{a,b} – $P < 0.05$; ^{a,c} – $P < 0.01$; ^{a,d} – $P < 0.001$

Table 5. Digestibility coefficient of dry matter

Groups (n = 6)	Digestibility coefficient [%] in days		
	17	24	31
A	$56.82^{a} \pm 6.057$	$56.83^{a} \pm 4.356$	$55.56^{a} \pm 8.891$
B	$54.59^{a} \pm 5.327$	$58.58^{a} \pm 4.501$	$71.29^{b} \pm 9.441$
C	$66.04^{b} \pm 2.700$	$61.14^{b} \pm 2.689$	$61.50^{a} \pm 7.715$
Control	**$50.90^{a} \pm 2.525$**	**$46.29^{a} \pm 8.368$**	**$50.56^{a} \pm 6.054$**

DM – dry matter; HS – humic substances; means (mean ± SD) with different superscript letters differed significantly: $^{a, b}$ – $P < 0.05$

Table 6. The ileal digestibility of dry matter on day 37

Groups (n = 6)	DM [%]
A	$33.27^{a} \pm 5.26$
B	$38.32^{b} \pm 0.18$
C	$23.66^{c} \pm 0.56$
Control	**$30.94^{a} \pm 2.41$**

DM – dry matter; HS – humic substances; means (mean ± SD) with different superscript letters differed significantly: $^{a, b}$ – $P < 0.05$; ^{c} – $P < 0.01$

of the experimental group. Higher values were observed after intake of 0.5 % or 0.7 % HNM in feed in the C or B groups compared to control by 15.14 %, 14.85 % and 20.73 % ($P < 0.05$) on days 17, 24 and 31.

Ileal digestibility of DM was increased at the inclusion level of HA/FA at a minimum of 3.99/0.35 g.kg^{-1} feed (Table 6) on day 37. A higher value was observed in the B group by 7.38 %.

DISCUSSION

The results of the growth data of broiler chickens indicated that the dietary intake of HS was effective in the enhancement of the zootechnical performances of broilers. A similar beneficial effect on the growth performance in broiler chickens (final body weight, daily weight gains) was observed by Gomes-Rosales and deAngeles [14] when worm leachate containing HS was mixed at levels of 20 % to 30 % in the drinking water. The results of Jaďuttová et al. [20] confirmed our findings. They observed that the addition of 10 g.kg^{-1} feed of humic substances had a positive insignificant effect on the growth parameters, breast and thigh meat yield, improved blood parameters and Ca content in bones of broiler chickens (Cobb 500). On the contrary, Hassan et al. [17] added 5 or 10 g of humate per kg of feed for the broilers (1–28 days of age). They observed a negative effect of the higher dose of the additive on the productive performance as regards to feed conversion ratio and performance index. The possible explanation for the positive action of HS on the production parameters of chickens was provided by Arif et al. [1]. The HS formed a protective film on the mucus epithelia of the gastrointestinal apparatus against infectious pathogenic agents and toxins which resulted in an increased weight gain and final weight of the animals.

The results of the average daily feed intake and the feed conversion ratio of broiler chickens are consistent with the findings reported by Bahadori et al. [5]. They demonstrated that the increase in the daily weight gains, the decrease in the feed intake and the decrease of the feed conversion ratio with the addition of the earthworm meal containing HS from 10–30 g.kg^{-1} DM. Similarly, Demeterová et al. [10] determined the improvement of the feed conversion ratio and the European efficiency index of broiler chickens on day 41 as the effect of the dietary addition of HS combined with the probiotic strain *Enterococcus faecium*. Jaďuttová et al. [20] indicated that feeding with HS at the level of 0.8 % and 1.0 % resulted in similar feed consumption in all groups but the improvement of the feed conversion ratio was especially in the group of broiler chickens with 1.0 % addition of HS. On the contrary, the growth performance and yield of breast and thighs did not show any difference between control and experimental groups of broiler chickens after feed intake of the extract of HS [11].

The explanation of the improvement of zootechnical performances can be based on the enhanced digestive enzymatic activities influenced by HS which are ensuring better utilisation of nutrients from feed. The second interpretation of the positive influence of HS on FCR, provided by Avci et al. [4] involves the fact that humic acids stabilise the intestinal microflora, therefore ensuring better utilisation of nutrients from the feed.

The data of the amylolytic and cellulolytic activities in the intestinal chymus of intestine of broilers are in

agreement with the experimental results achieved with sheep [30] when the peroral intake of HS increased the amylolytic and cellulolytic activities in the forestomach of sheep. The effect of HS on the amylolytic and cellulolytic activities can be influenced by the amount of the additive applied to the feed. T a k l i m i et al. [37] demonstrated that a feed additive based on HS can improve the digestion coefficient of nutrients as the effect of improved enzymatic activities, the increased gut length and the lessening in the passage rate of the intestinal contents.

The beneficial influence of HS on the digestibility coefficient of DM in the intestine and on the ileal digestibility of DM was confirmed in our experiments. The digestibility of nutrients present in the chymus of the intestine or ileum depends on the enzymatic activities in the intestinal apparatus which could be positively influenced by HS. G o m e z - R o s a l e s and d e A n g e l e s [14] observed the increased ileal digestibility of the quadratic response ($P < 0.05$) by increasing the dose of HA in the drinking water and the improved retention of nutrients. The possible explanation for the improved nutrient digestibility could be the increasing of villus length, then the increased area for absorption of nutrients after feed intake of HS [37]. On the other hand, HS have been reported to stabilize the intestinal microbiota when the increase in the counts of lactic acid bacteria and the decrease in enterobacteria in the intestinal content were observed [32]. It can have a positive effect on intestinal digestibility as well.

CONCLUSIONS

The feed intake of humic substances was beneficial in the enhancement of the cellulolytic activities in the intestine and positively influenced the intestinal and the ileal digestibility of DM, as well. The enhancement of the finishing body of birds resulted from the favourable physiological effects of HS in the intestinal apparatus.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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HINDLIMB SKELETAL MORPHOLOGY OF THE HELMETED GUINEA FOWL (*NUMIDA MELEAGRIDIS*)

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ABSTRACT

The Guinea fowl (*Numida meleagridis*) is a semi-domestic bird whose commercial popularity is on the increase. The gross anatomy of the hindlimbs of 8 helmeted Guinea fowls of equal sexes were studied. The pubic shafts were free, thus they did not fuse ventrally to form a symphysis. The ilium had a quadrilateral and roughly triangular pre and post acetabular wings, respectively. A conspicuous renal fossa was observed in the post acetabular wing. An incomplete concentric obturator foramen, lacking a caudal margin, was formed by the ventral border of the ischium and dorsal border of the cranial pubic shaft, while a prominent ischiopubic fissure was formed by the same bones, caudally. The incomplete concentric nature of the obturator foramen created a common space between it and the ischiopubic fissure. The femoral major trochanter was more proximal than the head, while the minor trochanter and the head were on the same horizontal level. The femur lacked a visible pneumatic foramen. The tibiotarsus had a prominent cranial cnemial crest. The fibular spine terminated at the tibia mid-shaft, leaving one interosseous space. An intertarsal sesamoid bone was identified, indicative of an adult skeleton. Crests and channels depicted the hypotarsus. Vascular foramen was identified in the distal extremity of the tibiotarsus. Digit I was oriented to the planter direction. These results, and others, were compared with those of other birds in the available literature, and some morpho-functional paradigm of the Guinea fowl hindlimb skeleton was established.

Key words: femur; Guinea fowl; *os coxae*; tarsometatarsus; tibiotarsus

INTRODUCTION

The evolution of the avian skeleton is geared towards adaptation to flight and egg production. To this end, an array of unique features most especially the pneumatic sacs of the avian skeleton, in comparison to mammalian skeleton, has been identified. Some of these include the pneumatisation of the femur and humerus, to reduce bone weight, necessary for flight, and the fusion and resultant reduction in number of bones such as the vertebrae (resulting in bones such as notarium, synsacrum and pygostyle), tibiotarsus and tarsometatarsus, to achieve maximum skeletal rigidity and minimal body weight, necessary for flight [16]. Others are the extensive keel, necessary to provide large surface area for attachment of flight muscles [16] and the absence of pubic symphysis, necessary for development and contact incubation of eggs [10]. Despite these general unique features of avian skeleton, a wide range of differences still exists in the skeletal morphology within

the different members of the avian class, including their hindlimbs. For example, the ostrich is the only extant avian specie with a pubic symphysis [8, 40]. The obturator foramen is a complete concentric foramen in the owl [32] but incomplete in the emu [8, 38]. The Digit I and IV of parrots, such as budgerigar have planter orientation, while Digits II and III have dorsal orientation, known as zygodactyly [6], while only Digit I has planter orientation in the domestic fowl, known as anisodactyly [21]. These unique features of avian skeleton sequels the need to explore the skeletal anatomy of different birds, and compare results with existing data of other birds to provide more insight on avian skeletal anatomy.

The subject of this study is the helmeted Guinea fowl. It is a member of the order Galliformes, and family Numididae. It is native to Africa, named after Guinea, a country on the sub-Saharan Africa. It is a ground feeding bird, characterized by a very wide crown, filled with grey-blue bare skin topped by a prominent horn coloured bony casque (helmet) in red brown colour, a feature which is unique in the guinea fowl [45]. The commercial popularity of the Guinea fowl is on the increase; they are used as ornamental birds [26], pest control in crop farms [36], and dietary source of high protein [26, 27]. Studies have also shown that the guinea fowl is relatively more resistant to endemic poultry diseases than the domestic fowl [17, 35].

The anatomy of some body systems of the helmeted Guinea fowl is available in the literature. These include: the digestive system [14, 31], reproductive system [2, 11], respiratory system [5, 15], integuments [29, 41, 45], nervous system [3], muscular system [9] and forelimb skeleton [1]. Generally, studies on forelimb anatomy of avian species abound, probably due to the relevance of the forelimb to flight. However, the anatomy of avian hindlimb, especially that of the helmeted Guinea fowl, is dearth in available literature. Apart from the comparative anatomy of the femur of some birds including the Guinea fowl [44], comparative anatomy of the *os coxae* of the Guinea fowl and the pigeon [20] and comparative morphometry of the tibiotarsus of the turkey and Guinea fowl [1], there is no comprehensive anatomical report on the entire bones of the hindlimb of the Guinea fowl available in the literature, hence, this research.

This study will make available a comprehensive anatomical report on the entire bones of the helmeted Guinea fowl hindlimb. Such information is necessary for comparative avian anatomy; it will serve as a reference aid for radiographic interpretation and surgical intervention of hindlimb abnormalities in the specie and a baseline for management of other pathologies of the Guinea fowl hindlimb. Consequently, the research was aimed at biological maceration of the Guinea fowl hindlimb, assembling the entire bones of the hindlimb, studying and reporting their gross anatomy, comparing the results with reports of other birds in the available literature, and establishing some morpho-functional paradigm of the Guinea fowl hindlimb skeleton.

MATERIALS AND METHODS

Eight (8) adult helmeted Guinea fowls (*Numida meleagridis*), comprising of 4 females and 4 males, with an average weight of 3.0 kg, were used for the research. They were purchased from a local market at Ilorin, Kwara State, Nigeria, and housed in the Gross Anatomy Laboratory of the Department of Veterinary Anatomy, Faculty of Veterinary Medicine, University of Ilorin, Nigeria. Prior to commencement of the study, they were fed with grains and water *ad libitum*. During the acclimatization period, they were clinically examined and ascertained to be apparently healthy and without any skeletal deformity.

The birds were sacrificed by severing the jugular vein. They were dissected using a surgical blade to remove feathers, skin, thoracic, abdominal and pelvic viscera while the muscles were teased away leaving the bones with minimal soft tissue attachments. The carcasses were placed in a bowl (after being sprinkled with water) and allowed to stay for 3 days in an open space outside of the laboratory. This was to allow flies to perch on them, and lay eggs. After 3 days, they were taken back into the laboratory and kept at room temperature for 5 months. This was to allow for larva appearance, growth and feeding on the soft tissues.

The recovered bones were disarticulated, washed, rinsed and sun-dried for 3 days. Their morphological features were noted and described. Lastly, their photographs were taken using a digital camera.

Ethical approval

Ethical approval was obtained from the Animal Ethics Committee of the University of Ilorin, Nigeria.

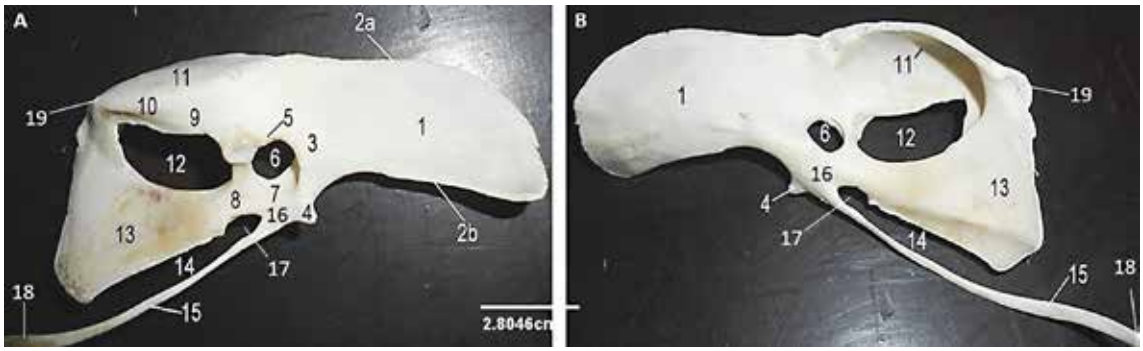

Fig. 1. Dorsolateral (A) and medial (B) surfaces of the Guinea fowl pelvic girdle

1 – Pre acetabular wing of ilium; 2a – Dorsal iliac crest; 2b – Lateral iliac crest; 3 – Body of ilium; 4 – Acetabular tubercle; 5 – Antitrochanter; 6 – Acetabular foramen; 7 – Acetabulum; 8 – Body of Ischium; 9 – Lateral surface of post-acetabular wing; 10 – Dorsolateral iliac crest; 11 – Dorsal surface of post acetabular wing; 11' – Ventral surface of post acetabular wing (renal fossa); 12 – ilioischiadic foramen; 13 – Wing of ischium; 14 – Ischiopubic fissure; 15 – Shaft of pubis; 16 – Body of pubis; 17 – Obturator foramen; 18 – Apex of pubis; 19 – Caudal iliac spine

Table 1. Morphometric data of bones of the Guinea fowl (*Numida meleagridis*) hindlimb

Bones	Range [cm]	Mean ± SEM [cm]
Ossa coxarum	1.8	10.49 ± 2.34
Femur	0.8	7.95 ± 1.88
Tibiotarsus	0.9	11.91 ± 1.78
Fibula	1.3	7.15 ± 0.87
Tarsometatarsus	0.5	7.24 ± 0.75

RESULTS

The hind limb comprised the bones of the pelvic girdle, femur, fibula, tibiotarsus, tarsometatarsus and phalanges. Table 1 presents the morphometric data obtained from the study.

The pelvic girdle comprised of the paired ilium, ischium and pubis. Each pair was fused to form the *os coxae*. The two *ossa coxarum* did not fuse ventrally; thus, there was no pubic symphysis. The pelvic girdle was located on the caudodorsal aspect of the skeleton. Each *os coxae* articulated medially with the synsacrum and laterally with the femoral head at the acetabulum.

The *os ilium* (Figure 1) presented a flat bone, the largest of the three bones of the pelvic girdle. It laid dorsal to the last 3 ribs and caudal to the caudal extremity of the scapula. It was composed of a craniolateral pre acetabular wing, a body and a dorsal post acetabular wing. The pre acetabular wing was quadrilateral in shape; it had a concave lateral and convex medial surfaces bounded by dorsal and lateral iliac crests. The dorsal iliac crest articulated with the fused spinous processes of the synsacrum. The body formed the dorsal and cranial parts of the acetabulum which was placed caudolateral to the pre acetabular wing. The acetabulum was perforated within the articular socket to form the acetabular foramen. Caudal to the body and dorsal to the acetabulum was the less prominent antitrochanter. The post acetabular wing was roughly triangular in shape; it presented three surfaces: ventral, dorsal and lateral. The lateral was separated from the dorsal by a dorsolateral iliac crest. The ventral surface was concave, forming the renal fossa, to lodge the caudal extremity of the kidney. The dorsal surface was smooth and slightly convex. It ended caudally as a small process, the caudal iliac spine. The lateral surface presented a ventral border that formed the dorsal margin of the ilioischiadic foramen.

The *os ischii* (Figure 1) presented a flat, roughly triangular bone with a cranial body, a caudal wing with lateral and ventral surfaces. The body formed the caudal margin of the acetabulum, the cranioventral margin of the ilioischiadic foramen and the craniodorsal margin of the obturator foramen. The dorsal border of the wing formed the caudoventral margin of the ilioischiadic foramen. The ilioischiadic foramen is the largest of the three foramina on the *os coxae*. It was oblong in shape.

The *os pubis* (Figure 1) presented a thin, slender bone that extended caudoventrally. It was comprised of a body, shaft and apex. The body formed the ventral margin of the acetabulum. It gave off an acetabular tubercle (pectineal process) that extended cranioventrally. The shaft extended from the body cranially, to the apex caudally, projecting beyond the level of the *os ischii*. The dorsal border of the cranial part of the shaft did not fuse with the ventral border

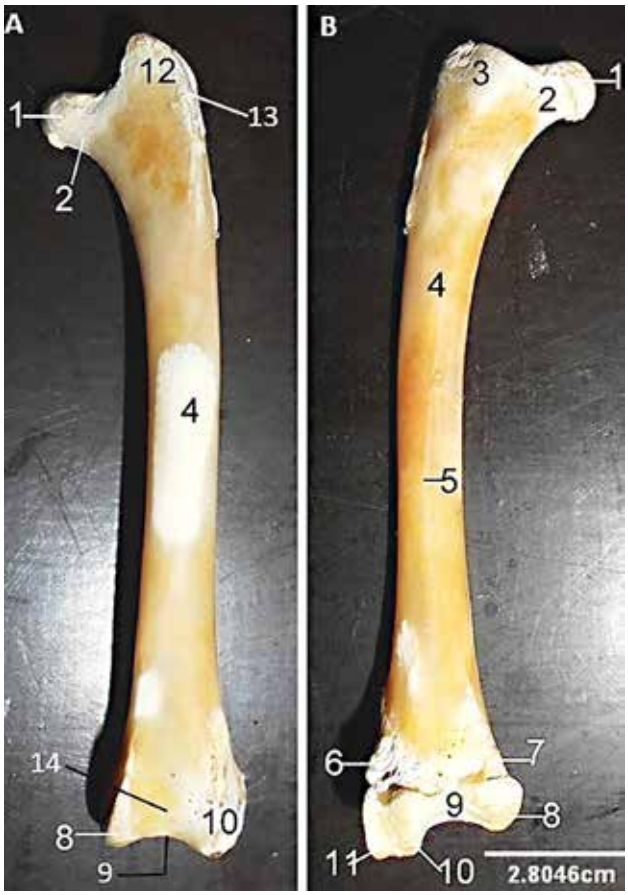

Fig. 2. Cranial (A) and caudal (B) views of the left femur of the Guinea fowl

1 – Head; 2 – Neck; 3 – Minor trochanter; 4 – Shaft; 5 – Labium; 6 – Lateral epicondyle; 7 – Medial epicondyle; 8 – Medial condyle; 9 – Intercondylar groove; 10 – Lateral condyle; 11 – Fibula trochlea; 12 – Major trochanter; 13 – Trochanteric crest; 14 – Patella groove

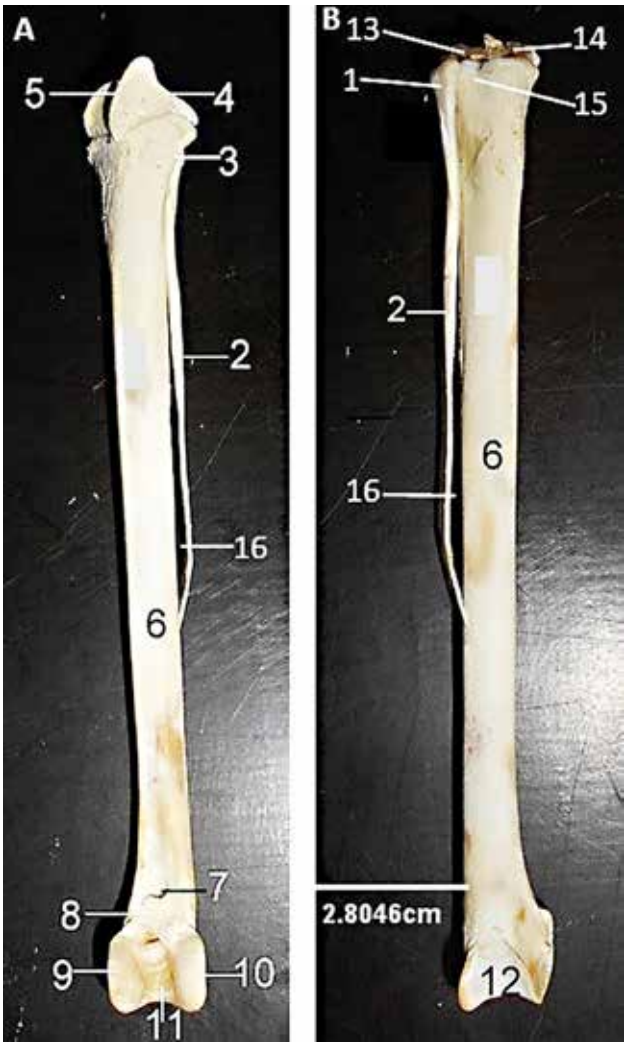

Fig. 3. Cranial (A) and caudal (B) views of the Guinea fowl left tibiotarsus and fibula

1 – Head of fibula; 2 – Spine of fibula; 3 – Lateral cnemial crest; 4 – Patella crest; 5 – Cranial cnemial crest; 6 – Shaft of tibiotarsus; 7 – Extensor groove; 8 – Supratendinal bridge; 9 – Medial condyle; 10 – Lateral condyle; 11 – Intercondylar incisure; 12 – Trochlea for tibial cartilage; 13 – Lateral articular surface; 14 – Medial articular surface; 15 – Flexor fossa; 16 – Interosseous space

of the *os ischii*. This resulted to an incomplete obturator foramen where the dorsal border of the cranial part of the pubic shaft contributed as the ventral margin of the foramen. Caudally, a wide and long ischiopubic fissure was created between the ventral border of the ischium and the dorsal border of the pubic shaft. The incomplete concentric nature of the obturator foramen created a common space between the obturator foramen and the ischiopubic fissure. The apex was the caudal end of the *os pubis*.

The femur (Figure 2) presented a somewhat cylindrical shaped bone having a shaft and two extremities, the proximal and distal. The proximal extremity presented a medially directed round head, a cranio-lateral major trochanter and a caudo-lateral minor trochanter. The major trochanter was more proximal than the head, while the minor trochanter and the head were on the same horizontal level. The head bore a small shallow notch, the *fovea capitis*, and was connected to the shaft by a distinct neck. A rough ridge, the trochanteric crest extended distally in an oblique fashion, from the major trochanter to a short distance on the cranial surface of the femoral shaft. Two muscular lines (lateral and medial labium) also extended distally on the caudal surface of the shaft from the minor trochanter and ended on the distal extremities. The distal extremity bore the medial and lateral condyles separated by an intercondylar groove. Caudally, the fibula trochlea was formed lateral to the lateral condyle creating a sulcus between them. Also on this caudal surface, the medial and lateral epicondyles were located proximal to the respective condyles with the popliteal fossa between them. The cranial surface of the intercondylar groove formed the pa-

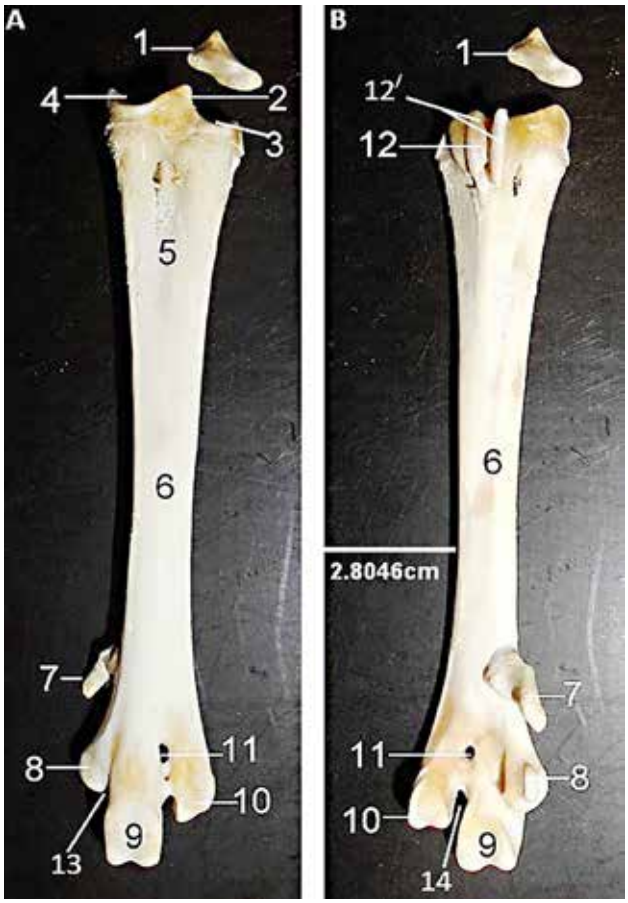

Fig. 4. Dorsal (A) and plantar (B) surfaces of Guinea fowl tarsometatarsus

1 – Intertarsal sesamoid bone; 2 – Intercondylar eminence; 3 – Lateral cotyla; 4 – Medial cotyla; 5 – Tendinous grooves; 6 – Shaft; 7 – Metatarsal I; 8 – Medial trochlea (trochlear of metatarsal II); 9 – Middle trochlea (trochlear of metatarsal III); 10 – Lateral trochlea (trochlear of metatarsal IV); 11 – Vascular foramen, 12 – Hypotarsus (channel); 12'– Hypotarsus (crests); 13 – Medial intertrochlear incisure; 14 – Lateral intertrochlear incisure

tellar groove for articulation with the patella. The patella was a large irregularly shaped sesamoid bone with straight roughened external surface and concave internal surface.

The tibiotarsus (Figure 3) presented a straight cylindrical shaft with an expanded proximal extremity, a narrow distal extremity, cranial and lateral surfaces. At its expanded proximal extremity, a small lateral and large medial articular surface for articulation with the femoral condyles were separated by an interarticular area. The cranial surface of the proximal extremity consisted of an elevated patella crest that extended transversely from the medial articular surface ending laterally and perpendicularly to form a small lateral cnemial crest. The prominent cranial cnemial crest extended from the middle summit of the transverse patella crest downwards. Between the cnemial crests was the intercnemial groove for muscular attachment. On the caudal surface of the proximal extremity, the flexor fossa was located below the articular surfaces. The distal extremity bore the medial and lateral condyles separated by the intercondylar incisure. The articular surfaces of the condyles coalesced caudally, forming the trochlea for articulation with the tibial cartilage. An extensor groove was located cranially, just proximal to the condyles and spanned or bridged by the delicate supratendinal bridge.

The fibula (Figure 3) presented a head and a slender body. The head presented a medial articular facet, for the tibiotarsus and a lateral articular facet for the lateral condyle of the femur. The thin rod-like body tapered to a pointed spine. The fibular spine terminated at the mid-shaft of

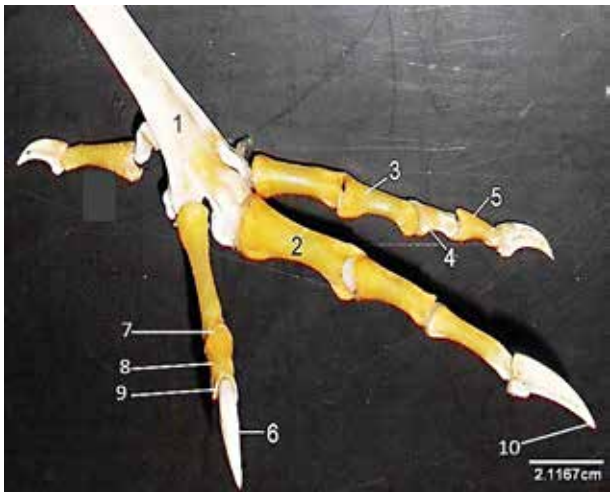

Fig. 5. Dorso-lateral view of the Guinea fowl hindlimb digits

1 – Tarsometatarsus; 2 – Proximal phalanx of digit III; 3 – First intermediate (second) phalanx of digit IV; 4 – Second intermediate (third) phalanx of digit IV; 5 – Third intermediate (fourth) phalanx of digit IV; 6 – Distal phalanx of digit II, 7 – Base of phalanx; 8 – Body of phalanx; 9 – Capitulum of phalanx; 10 – Apex of distal phalanx

the tibia, leaving only one interosseous space. The Guinea fowl presented an irregularly shaped intertarsal sesamoid bone which lodged in the trochlear of tibial cartilage at the distal extremity of the tibiotarsus.

The tarsometatarsus (Figure 4) presented a long straight somewhat flattened shaft having proximal and distal extremities, as well as planter and dorsal surfaces. The proximal extremity bore the medial and lateral condyle with articular surfaces for articulation with the respective condyles of the tibiotarsus. They were separated by the intercotylar eminence. On the plantar surface of the proximal extremity, a hypotarsus extended longitudinally as crests, separated by shallow channels. The channels were not enclosed by the crests to form canals. The shaft presented shallow tendinous grooves on its dorsal and plantar surfaces. Metatarsal I articulated on the medial surface of the distal third of the shaft. The distal extremity presented three trochleae (medial, middle and lateral) of the metatarsal II to IV, respectively. Trochlear of metatarsal III was the longest as it distended more than others. The trochleae were separated by the intertrochlear incisures. A vascular foramen was present between the middle and lateral trochlea.

The digits (Figure 5) were made up of 2, 3, 4 and 5 phalanges for digits I, II, III and IV respectively. Digit I was oriented to the planter direction, while digits II to IV were oriented dorsally. With the exception of the distal phalanx of each digit, the phalanges (proximal and intermediate) presented a base with a concave articular surface, a body, and a capitulum incorporating a trochlear articular surface. The base articulated with either the trochlear of metatarsal (for proximal phalanges) or with the trochlear of the capitulum of a preceding phalanx (for intermediate phalanges). The capitulum was distal, articulating with the base of a succeeding phalanx by its trochlear. The distal phalanx consisted of a base and a pointed apex; its base articulating with the penultimate phalanx.

DISCUSSION

The gross anatomical features of the hindlimb bones of the Guinea fowl (*Numida meleagridis*) presented similarities and differences when compared to previously studied avian species such as the domestic fowl, cattle egret, and the barn owl. The *os coxae* was formed by the fusion of the ilium, ischium and pubis. This finding is in agreement with findings reported by D y c e et al. [12] and T a h o n et al. [39] in chicken, and R e z k [28] in cattle egret. The pubic shafts were free, thus did not fuse ventrally to form a pubic symphysis. This absence of pubic symphysis has been reported in many extant birds as an evolutionary adaptation to allow for contact incubation of eggs and to evade physical constraint on egg size [10]. It is also indicated in the protection of underlying visceral organs [18, 30]. The ostrich (*Struthio camelus*) is the only extant bird with a pubic symphysis [8, 45], an adaptation that is detrimental to their egg size, but supportive to bear the weight of their large visceral organs. In our study, the dorsal iliac crest articulated with the fused spinous processes of the synsacrum. This was also reported in the same specie by L a v a n y a et al. [20], Indian-eagle owl by S a r m a et al. [32] and duck by C h u k k a t h et al. [8]. In contrast, there is no such fusion in the pigeon [20], rather a canal is left between the dorsal iliac crest and the fused spinous processes of the synsacrum.

The quadrilateral and roughly triangular shapes of the pre and post acetabular parts of the Guinea fowl ilium, respectively, as reported in our study, is consistent with those of the ostrich and emu [8, 38]. However, N i c k e l et al. [25] recorded that the post acetabular part of the ilium in the duck and turkey is quadrilateral in shape (not roughly triangular). While the dorsal iliac crest fused with the synsacrum in our study, the fusion of the synsacrum in the ostrich and emu is with both the dorsal iliac crest and the medial surface of the pre acetabular wing of the ilium [8]. This broad surface area of fusion of the *os coxae* and synsacrum in the ostrich and emu, necessary to bear the heavy weight of these birds, is not necessary in the much lighter Guinea fowl. Furthermore, the antitrochanter observed in the Guinea fowl ilium from our study was not prominent. S r i d e v i et al. [38] reported that it is very prominent in the emu, with a conspicuous facet for articulation with the trochanter major of the femur. S a t h y m o o r t h y et al., [34], R e s k [28] and S a r m a et al. [32] also reported that it is prominent in the pelicans, cattle egret and owl, respectively. Since the joint formed by the antitrochanter and the trochanter major of the femur inhibits excessive movement of the coxofemoral joint [21], it may imply that this inhibitory effect is less indicative in the Guinea fowl than in the emu, owl, pelicans and cattle egret. The dorsally placed caudal iliac spine observed in our study has been reported in the pelicans [34]. In the pigeon, it is presented

more laterally than in the Guinea fowl due to the wider lumbosacral mass wedged between the two pelvic bones [20]. Similar lateral presentation was reported in the domestic fowl by M a i e r l et al. [21] who referred to it as the dorsolateral iliac spine of the chicken. It is completely absent in the emu [38]. The conspicuous renal fossa observed in this study is also lacking in the emu [8, 18, 38], goose [33] and ostrich [8] because of the attachment of the bodies of the vertebrae to the ventral surface of the post acetabular wing in these species. V e n k a t e s a n et al. [43] had earlier reported that the kidneys of the emu reside ventral to the synsacrum, exposing them to traumatic insults. The presence of renal fossa confers better protection to the kidneys of the helmeted Guinea fowls than those of the emu, goose and ostrich.

The flat, roughly triangular shape of the ischium in our study has also been reported in the Indian-eagle owl by S a r m a et al. [32], duck and domestic fowl by C h u k k a t h et al. [8]. Contrarily, C h u k k a t h et al. [8] and S r i d e v i et al. [38] described the ischium of the emu as a long and broad rod-like bone. It is also a long, but narrow bone in the ostrich [40]. The ilium, ischium and pubis contributed in forming the acetabular foramen, but only the ilium and ischium formed the ilioischiadic foramen in the Guinea fowl from our research. This is similar with the domestic fowl [12], pigeon [20] and owl [32].

The acetabular tubercle that exited from the body of the pubis in our study is the same as in the emu [38]. It differed from the domestic fowl as M a i e r l et al. [21] reported that the tubercle exited from the ilium, thus, referred to as pre-acetabular tubercle. It is completely absent in the Japanese quail [23], pigeon [20] and owl [32]. This tubercle, also called the pectineal process, provides attachment for the pectineus muscle. The incomplete concentric obturator foramen observed in this work has been reported by L a v a n y a et al. [20] in the same Guinea fowl. It has also been reported in the ostrich by T a m i l s e l v a n et al. [40], pigeon by L a v a n y a et al. [20], emu by C h u k k a t h et al. [8] and S r i d e v i et al. [38] and the duck by C h u k k a t h et al. [8]. In fact, C h u k k a t h et al. [8] concluded that the caudal border of obturator foramen was formed by connective tissue in emu and duck, instead of the pubis. This may be the case in the Guinea fowl, although the connective tissue may have been lost during maceration, leaving an incomplete concentric obturator foramen that communicated caudally with the ischiopubic fissure. A complete concentric obturator foramen has been observed in the domestic fowl [21] and owl [32]. The obturator foramen creates passage for the obturator nerve and obturator medialis muscle [21]. The ischiopubic fissure observed in the Guinea fowl exists as a large foramen in the ostrich due to the cartilaginous joint between the caudal end of the ischium and the ipsilateral pubis in the ostrich [8, 40]. C h u k k a t h et al. [8] also demonstrated that it exists as a notch in the emu (ilioischiadic notch) because there is no such fusion between the caudal end of the ischium and the ipsilateral pubis. In an earlier study by V e n k a t e s a n et al. [43] had reported an absence of joint between the caudal end of the ischium and the pubis in the emu. The pubic shaft extended beyond the level of the ischium in our study, similar to the ostrich [40], domestic fowl [21], pigeon [20], goose [33] and owl [32]. Conversely, it did not project beyond the level of the ischium in the emu [24, 38].

The morphological features of the long cylindrical shaped femur are largely similar to findings in the cattle egret [28] and the barn owl [42]. However, the major trochanter observed to be more proximal than the femoral head in this study is absent in birds like the *Parus major*, *sittaeuropaea* and *passer domesticus*. In the turkey, the major trochanter is present, but, unlike the Guinea fowl, is on the same horizontal level with the femoral head; in the ostrich, the femoral head is more proximal than the major trochanter, unlike in the Guinea fowl [44]. Furthermore, C h o u d h a r y et al. [7] observed that the femoral head and major trochanter are on the same level in the serpent eagle and the brown wood owl, unlike in the Guinea fowl. The shallow notch of the *fovea capitis* observed in the Guinea fowl is similar to those of near-same size adult birds like the domestic fowl [8, 21] and duck [8]. In the ostrich, the *fovea capitis* is a large and deep notch surrounded by boney ridges [44], while in the emu, it is indistinct [8, 19]. The muscular lines observed on the caudal surface of the Guinea fowl femur has been observed in the same specie by V a n k a t e s a n et al. [44]. However, the authors observed that the lines are indistinct in the turkey, but well developed in the ostrich. They have also been reported in the peahen [37], serpent eagle and brown wood owl [7]. One unique feature of the Guinea fowl femur from this study is the absence of a vascular/nutrient foramen. This foramen has been reported on the caudal surface of the femur of many other birds including

the quail [13], turkey and ostrich [44], emu, duck and domestic fowl [8]. Furthermore, pneumatic foramen reported on the femur, below the greater trochanter in the crested serpent eagle by C h o u d h a r y et al. [7], in the ostrich by V e n k a t e s a n et al. [44] and emu by C h u k k a t h et al. [8], is lacking in the Guinea fowl from this study. C h o u d h a r y et al. [7] did not observe it in the brown wood owl, C h u k k a t h et al. [8] did not observe it in the domestic fowl and duck, while V e n k a t e s a n et al. [44] did not observe it in the turkey. According to C h u k k a t h et al. [8], pneumatic foramen is indicated in the femur of large birds to reduce the body weight while accommodating their large body size. This is not necessary in the Guinea fowl, domestic fowl, duck, owl and turkey, as they are smaller birds compared to ostrich and emu. The roughness of the external surface of the patella is for attachment of tendon of the ambient muscle [21].

The tibiotarsus was formed by the fusion of the tibia and the proximal row of tarsal bones. The tibiotarsus in our study, was the longest bone. This is similar to the findings in the chicken [25], cattle egret [28], barn owl [42], emu and ostrich [8], serpent eagle and brown wood owl [7]. The lateral cnemial crest observed in our study is not as extensive as that reported in the ostrich by C h u k k a t h et al. [8], who described it as a large tuberosity in the ostrich. The prominent nature of the cranial cnemial crest of the Guinea fowl is comparable to those of the peahen [37], cattle egret [28], Indian eagle-owl [32], serpent eagle and brown wood owl [7]. It serves as the point of origin of extensor muscles of the stifle joint [22]. The conspicuous intercnemial groove observed in our study serves as the point of origin for the long digital extensor muscle, which inserts on the extensor groove on the same cranial surface, just proximal to the tibial condyles; also, the flexor fossa is for origin of the long digital flexor muscle [21]. The fibular spine terminated at the mid-shaft of the tibia in this study, similar to that of the duck [8], but unlike that of the domestic fowl that extended to the distal extremity of the tibia [8]. Also, contrary to the findings in the Guinea fowl, the fibula reached up to distal third of the lateral border of the tibiotarsus in the serpent eagle and brown wood owl [7]. Only one interosseous space, without a distinct fibular crest, was observed between the tibia shaft and fibular spine in this study. Conversely, C h o u d h a r y et al. [7] observed both proximal and distal interosseous spaces, created by distinct fibular crests in both the serpent eagle and brown wood owl. Two interosseous spaces were also reported in the Indian eagle-owl by S a r m a et al. [32]. The extensor groove observed in this study was reported in the same location in the crested serpent eagle, as an extensor canal, by C h o u d h a r y et al. [7] who did not observe it in the brown wood owl. This groove guides the distal tendons of the extensors of the toes [21]. The intertarsal sesamoid bone identified in our study is indicative of an adult skeleton. It was consistent in all the birds used for the study. It is a product of ossification of the tibial cartilage, and it provides a gliding surface for tendons of the gastrocnemius and digital flexor muscles [21].

The tarsometatarsus was formed by the fusion of the middle and distal rows of tarsal bones with metatarsus II-IV, and had similar presentation to that of the chicken [39] and cattle egret [28]. The hypotarsus is a component of the distal tarsal bones. C h u k k a t h et al. [8] reported that the hypotarsus enclosed a bony canal for guiding flexor tendons in domestic fowl and duck, which were lacking in ostrich and emu. These canals were also lacking in the Guinea fowl hypotarsus, from our study, rather crests and channels depicted the Guinea fowl hypotarsus. Also, the tendinous grooves observed on the shaft of the tarsometatarsus in the Guinea fowl were shallow, but in the emu, C h u k k a t h et al. [8] reported that they were deep. The vascular foramen identified between the middle and lateral trochlea in the Guinea fowl was identified above the lateral trochlear on the same cranial surface of the tarsometatarsus in the crested serpent eagle and brown wood owl by C h o u d h a r y et al. [7].

The number and arrangement of metatarsal trochlear and digital phalanges in the Guinea fowl presented seems to be the normal presentations in most birds as similarity was noted in the chicken [4], cattle egret [28], barn owl [42], serpent eagle and brown wood owl [7]. However, C h u k k a t h et al. [8] reported that the ostrich has only two trochlear (medial and middle) and two digits (digits III and IV) while the emu has three trochlear (like the Guinea fowl), but only three digits (digits II–IV). Z h a n g et al. [46] and C h u k k a t h et al. [8] further observed that the digit IV of the ostrich is devoid of the 5th phalanx. This is unlike the digit IV of the Guinea fowl from this study, which has 5 phalanges, just like most other birds in the literature. Based on the planter orientation of only digit I, the Guinea fowl foot is anisodactyl. This is the same as most extant birds. Conversely digits I and IV of the budgerigars

orient to the planter direction, while digits II and III remain in the dorsal orientation [6]. Thus, the budgerigar foot is zygodactyl.

CONCLUSIONS

This study on the hindlimb of the Helmeted Guinea fowl (*Numida meleagridis*) has been able to elucidate the morphological features of the bones of the hindlimb while comparing their similarities with documented literature on some other birds.

CONFLICT OF INTEREST

Authors declare that no conflict of interest is associated with this work.

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DETECTION OF ANTIBIOTIC RESIDUES AND MYCOTOXINS IN MILK USING COMPETITIVE IMMUNOCHROMATOGRAPHIC TESTS

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ABSTRACT

As milk should be free from harmful substances before leaving the farm, this study provides the results of the analysis of the presence of β-lactam and tetracycline residues and Aflatoxin M1 (AFM1) in milk samples obtained within one year from Ireland and Slovakia. To evaluate the presence of β-lactam and tetracycline residues, Duplex BT Scan assay, produced by Zeulab S.L. was used. For the detection of AFM1, AflaM1 Scan (Zeulab S.L.) tests were used. Of a total of 69 raw cow's milk samples analysed, 40 samples were obtained from the farms in Slovakia and 29 samples from the farms in Ireland. Among the 69 analysed samples, 17 (24.6 %) samples were tested positive for the presence of β-lactam residues, 12 (17.4 %) samples for tetracycline residues and 59 (88.4 %) samples for AFM1. Milk samples positive for antibiotic residues and mycotoxins were not acceptable and it is important that the reputation of milk as a healthy and safe food is protected worldwide. Dairy farmers and consumers want to be confident that milk and milk products are of high quality and free of all pharmacologically active substances and toxins.

Key words: aflatoxin M1; betalactams; detection; milk; tetracyclines

INTRODUCTION

Milk is considered one of the most valuable foods in the world and therefore has a vital role in the life of animals and especially in humans. The milk production industry has steadily grown in recent years. In the last year, 544 tonnes of cow's milk were produced worldwide. The European Union has produced 145 tonnes of milk in the last year, making it the largest producer of dairy products worldwide [17].

Raw milk must come from animals that: do not show any symptoms of infectious diseases communicable to humans through milk; that are in a good general state of health, without any symptoms of disease that might result in the contamination of milk, and, in particular, are not suffering from any infection of the genital tract with discharge, enteritis with diarrhoea and fever, or a recognisable inflammation of the udder; that do not have any udder wounds likely to affect the milk; to which no unauthorised substances or products have been administered and that have not undergone illegal treatment within the meaning of the Directive 96/23/EC and in respect of which, where authorised products or substances have been administered, and the withdrawal periods prescribed for these products or substances have been observed. The official veterinarian shall verify: that the health status of the animals, the absence of the use of prohibited or un-authorised pharma-

cologically active substances, and the possible presence of residues of authorised pharmacologically active substances, such as the pesticides or contaminants does not exceed the levels laid down in Regulations (EU) No. 37/2010, (EC) No. 396/2005 or (EC) No. 1881/2006. Milk and colostrum production holdings shall undergo official controls by the competent authorities to verify that hygiene requirements laid down in Regulation (EC) No. 853/2004 are being complied with. These controls may involve inspections and the monitoring of controls carried out by professional organisations. If it is demonstrated that the hygiene is inadequate, the competent authorities shall verify that appropriate steps are taken to correct the situation [5, 6, 7, 20].

Antibiotic residues and mycotoxins are the most common contaminants of milk. Each year, around 63,151 tons of antibiotics are used in livestock worldwide, whereas antimicrobial usage in animals is double compared to human's worldwide [10]. Many antibiotics are released unaltered meaning there is release of potential antimicrobial activity into the environment. Once released, most antibiotics are persistent and biologically active [19]. On the other hand, mycotoxins occur naturally through the growth of moulds. They are primarily found on cereal grains and forage. Especially harmful are Aflatoxins. These are a type of mycotoxin which occurs naturally by the growth of *Aspergillus flavus* and *Aspergillus parasiticus*. They can grow on soil, decaying vegetation, forage and grains [2]. Specifically, Aflatoxin M1 (AFM1) that is a hydroxylated metabolite of Aflatoxin B1 found in milk and milk products from animals who have ingested contaminated feed [22]. Due to the high toxicity and carcinogenic properties of AFM1 and antibiotic residues, its presence in milk is a concern. AFM1 and some antibiotics are resistant to thermal inactivation, pasteurisation, autoclaving and other varieties of food processing procedures. Thus, to produce high quality milk, it is essential to keep feeds free from contamination [2, 19].

The residues of antimicrobials and mycotoxins must be monitored and controlled in accordance with applicable European legislation. Commission Implementing Regulation (EU) No. 2019/627 laid down on 15 March 2019 which outlines practical arrangements for carrying out official controls on products of animal origin intended for human consumption in accordance with Regulation (EU) No. 2017/625 of the European Parliament and of the Council on monitoring certain substances and residues thereof in live animals and animal products in accordance with Council Directive 96/23/EC of 29 April 1996 [4, 7].

As the presence of antibiotic residues as well as mycotoxins may pose a risk factor for public health, the aim of this study was to analyse the milk collected at the farm level for the presence of the most commonly used groups of antibiotics (β-lactam and tetracycline) and AFM1.

MATERIALS AND METHODS

Milk Samples

A total of 69 milk samples were collected from a variety of dairy farms in Ireland (29) and Slovakia (40), frozen and stored in a freezer at a temperature of –18 °C. The Irish samples were transported in freezer bags with ice packs to Slovakia to prevent thawing and protect samples. Immediately before testing, the samples were heated to a temperature of 40 °C for 20 minutes and homogenized.

Duplex BT Scan

Duplex BT Scan (Zeulab S.L., Zaragoza, Spain) is a qualitative automatic test in cassette format designed for the detection of β-lactams and tetracyclines in raw cows, sheep and goat's milk. It is a competitive immunochromatographic test with two components: a tube with freeze-dried reagents that has to be mixed with the milk sample, and a strip which is subsequently introduced into the tube. The tube contains receptors and antibodies bound to gold particles and the strips have specific antibodies. If the sample is free from β-lactams or tetracyclines, the receptors present in the tube will bind to its specific line of the strip when the liquid runs vertically, and intense red lines will appear (negative sample). If an antibiotic is present in the sample, it binds to its receptor of the tube, which is then not able to bind to the specific line in the strip, inhibiting the appearance of the red colour of the corresponding test line, or decreasing its intensity (positive sample). Limits of detection are showed in Table 1. The test was used according to the manufacturer's instructions. In short, the cassette was removed from the sterile package and inserted into the IRIS device. 100 µl of the milk samples were added into the cassette and the automatic assay was started using IRIS app. The procedure lasted 6 minutes. This assay was designed and validated in accordance with Commission Regulation (EU) No. 37/2010 of 22 December 2009

Table 1. Duplex BT Scan limits of detection [µg.l^{-1}, ppb] of β-lactams and tetracyclines in cow's milk

Antibiotic	MRL [µg.l^{-1}, ppb]	LOD [µg.l^{-1}, ppb]
Amoxicillin	4	3
Ampicillin	4	3
Cefalonium	20	4
Cefoperazon	50	5
Cefquinome	20	10
Ceftiofur	100	100
Cephalexin	100	> 600
Cephapirin	60	8
Chlortetracycline	100	40
Cloxacillin	30	5
Nafciline	30	15
Oxacillin	30	5
Oxytetracycline	100	60
Penicillin G	4	2
Tetracycline	100	40

MRL – maximum residues limit; LOD – limit of detection
Source: https://www.zeulab.com/en/landing-duplex-bt-scan-iris-eng/

on pharmacologically active substances and their classification regarding maximum residue limits (MRL) in foodstuffs of animal origin and according to internal validation ISO 13969:2003.

AflaM1 Scan

AflaM1 Scan (Zeulab S.L., Zaragoza, Spain) is a rapid test designed for quantification (30–200 ppt (ng.kg^{-1}) of AFM1 in cows, sheep and goat's raw milk. It is a competitive immunochromatographic test consisting of a tube with freeze-dried AFM1 detection compounds and a strip with specific reagents. The presence of AFM1 is inversely proportional to the intensity of the test line, and quantification requires an IRIS reader. The test was used according to the manufacturer's instructions. In short, 200 µL of milk samples were added into the tube with freeze-dried reagent, mixed well with the pipette and incubated for 3 minutes at 40 °C. Subsequently, the test strip was introduced into the tube and incubated for another 7 minutes at 40 °C. After incubation, the strip was placed in the tray and the results were read with the IRIS device using the IRIS app.

IRIS

IRIS (Zeulab S.L., Zaragoza, Spain) is an automatic device for the analysis (automatic assay) and reading (read strip) of the rapid tests from Zeulab. The device evaluates the presence of contaminants in the matrix by measuring of the absorbance, which is expressed in numerical values. The results are displayed on the device when the test is done with the keypad, in the app and in the cloud Test4all. es, when the test is done with the app.

RESULTS

Beta-lactam and tetracycline residues

Of a total of 69 cow's milk samples analysed, 17 (24.6 %) samples were tested positive for ß-lactam and tetracycline antibiotic residues. Out of the 17 samples tested positive, 12 (70.6 %) samples were tested positive for tetracycline residues and 17 (100 %) samples were tested for β-lactam residues. Out of the 40 Slovak (SK) milk samples tested, 11 (27.5 %) samples were positive for β-lactam residues and 8 (20 %) samples were positive for tetracycline residues. Out of the 29 Irish (IE) milk samples analysed, 6 (20.7 %) samples were tested positive for β-lactam residues and 4 (13.8 %) samples were positive for tetracycline residues. Numerical values of the results are shown in Table 2. A graphical comparison of the positivity rate of samples from Slovakia and Ireland is shown in Figure 1.

Aflatoxin M1

A total of 69 raw cow's milk samples were examined for the presence of AFM1. Out of all samples analysed,

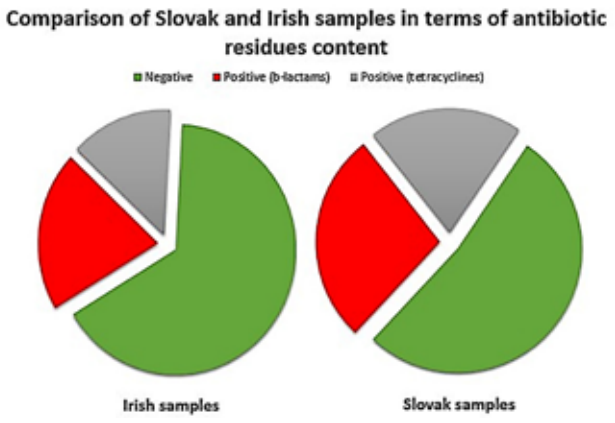

Fig. 1. Content of antibiotic residues in milk samples originated from Slovakia and Ireland

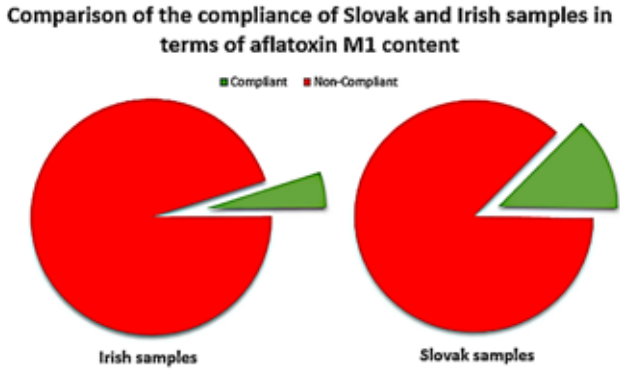

Fig. 2. Aflatoxin M1 content in milk originated from Slovakia and Ireland

59 (85.5 %) samples were tested positive (non-compliant, NON-COM) and only 10 (14.5 %) samples were tested negative (compliant, COM). Of a total of 40 Slovak samples analysed, 32 (80 %) samples were tested positive (NON-COM) and 8 (20 %) samples were tested negative (COM). Out of the 29 Irish samples analysed, 27 (93.1 %) samples were tested positive (NON-COM) and only 2 (6.9 %) samples were tested negative (COM). A comparison of the compliance of the Slovak and Irish samples is shown in Figure 2.

Of a total of 59 samples tested positive (NON-COM), up to 57 (96.6 %) samples contained AFM1 at the con-

Table 2. Content of β-lactam and tetracycline residues and Aflatoxin M1 in analysed raw cow's milk samples

Sample	Country of origin	ß-lactam residues	Value*	Tetracycline residues	Value*	AflaM1	Value**
1	SK	POS	0.6	NEG	1.3	COM	<30
2	SK	NEG	2.3	NEG	4.1	NON-COM	>200
3	SK	NEG	1.7	NEG	2.9	NON-COM	69
4	SK	NEG	5.9	NEG	2.1	NON-COM	>200
5	SK	POS	0.5	POS	0.9	NON-COM	>200
6	SK	NEG	2.1	NEG	4.4	NON-COM	>200
7	SK	NEG	2.4	NEG	4.9	COM	35
8	SK	POS	0.4	POS	0.9	COM	>200
9	SK	POS	0.9	POS	0.7	NON-COM	>200
10	SK	NEG	2.0	NEG	4.0	NON-COM	139
11	SK	NEG	1.6	NEG	6.7	NON-COM	>200
12	SK	NEG	2.8	NEG	4.9	NON-COM	69
13	SK	POS	0.3	POS	0.8	NON-COM	>200
14	SK	NEG	5.8	NEG	4.7	NON-COM	>200
15	SK	NEG	2.4	NEG	3.8	NON-COM	>200
16	SK	NEG	4.4	NEG	5.2	NON-COM	>200
17	SK	POS	0.7	NEG	3.7	COM	<30
18	SK	NEG	3.9	NEG	2.8	NON-COM	>200
18	SK	NEG	4.5	NEG	2.9	NON-COM	>200
19	SK	POS	0.3	POS	0.4	NON-COM	>200
20	SK	NEG	5.9	NEG	6.7	NON-COM	>200
21	SK	NEG	4.6	NEG	3.2	NON-COM	133
22	SK	NEG	5.8	NEG	4.1	COM	<30
23	SK	NEG	3.4	NEG	6.2	NON-COM	>200
24	SK	POS	0.2	NEG	4.5	NON-COM	>200
25	SK	NEG	4.1	NEG	4.7	NON-COM	>200
26	SK	NEG	3.8	NEG	3.6	NON-COM	>200
27	SK	NEG	5.7	NEG	5.1	NON-COM	>200

Table 2. continue

Sample	Country of origin	ß-lactam residues	Value*	Tetracycline residues	Value*	AflaM1	Value**
28	SK	POS	0.4	POS	0.9	NON-COM	>200
29	SK	NEG	4.9	NEG	2.9	COM	31
30	SK	NEG	4.8	NEG	3.4	NON-COM	>200
31	SK	NEG	2.6	NEG	3.8	NON-COM	>200
32	SK	NEG	2.9	NEG	6.5	NON-COM	>200
33	SK	POS	0.2	POS	0.9	NON-COM	>200
34	SK	NEG	2.6	NEG	4.5	NON-COM	>200
35	SK	NEG	2.9	NEG	2.3	NON-COM	>200
36	SK	NEG	4.7	NEG	5.7	NON-COM	>200
37	SK	NEG	2.5	NEG	4.9	COM	36
38	SK	NEG	3.8	NEG	3.1	NON-COM	>200
39	SK	NEG	3.2	NEG	5.5	NON-COM	>200
40	SK	POS	0.4	POS	0.8	COM	<30
41	IE	NEG	2.5	NEG	3.2	NON-COM	>200
42	IE	NEG	2.3	NEG	3.5	NON-COM	>200
43	IE	NEG	2.9	NEG	3.6	NON-COM	>200
44	IE	NEG	3.1	NEG	4.1	NON-COM	>200
45	IE	POS	0.5	POS	0.7	NON-COM	>200
46	IE	NEG	2.8	NEG	2.4	NON-COM	>200
47	IE	NEG	3.2	NEG	5.4	COM	32
48	IE	POS	0.3	NEG	6.1	NON-COM	>200
49	IE	NEG	2.8	NEG	3.7	NON-COM	>200
50	IE	NEG	2.7	NEG	3.9	NON-COM	>200
51	IE	NEG	3.4	NEG	2.2	NON-COM	>200
52	IE	NEG	3.9	NEG	3.4	NON-COM	>200
53	IE	NEG	5.5	NEG	4.0	NON-COM	>200
54	IE	POS	0.5	NEG	3.1	NON-COM	>200
55	IE	NEG	5.1	NEG	5.0	NON-COM	>200
56	IE	NEG	4.2	NEG	6.1	NON-COM	>200
57	IE	POS	0.9	POS	0.4	NON-COM	>200
58	IE	NEG	2.9	NEG	2.5	NON-COM	>200
59	IE	NEG	3.5	NEG	3.7	NON-COM	>200
60	IE	NEG	4.5	NEG	5.5	NON-COM	>200
61	IE	NEG	3.3	NEG	4.9	NON-COM	>200
62	IE	NEG	4.9	NEG	2.8	NON-COM	>200
63	IE	NEG	3.1	NEG	3.6	NON-COM	>200
64	IE	NEG	2.8	NEG	4.6	NON-COM	>200
65	IE	NEG	3.0	NEG	5.0	NON-COM	>200
66	IE	POS	0.8	POS	0.2	NON-COM	>200
67	IE	NEG	3.9	NEG	5.4	COM	<30
68	IE	POS	0.5	POS	0.6	NON-COM	>200
69	IE	NEG	5.5	NEG	6.0	NON-COM	>200

* – Absorbance directly proportional to the content of contaminants; ** – Values expressed in ppt [ng.kg^{-1}];
POS – positive sample; NEG – negative sample; COM – compliant sample; NON-COM – non-compliant sample

centration higher than the maximum detection limit of the used AflaM1 Scan assay (> 200 ppt (ng.kg^{-1}). Compliance or non-compliance with numerical expression of AFM1 content of all the samples is shown in Table 2.

DISCUSSION

The presence of mycotoxins and antibiotic residues in the feed or milk are of growing concern nowadays. These harmful residues can cause many detrimental effects to the health of both, humans and other animals.

It has been reported that antibiotic residues in milk can have a negative impact on human health, milk processing and quality, and can prevent the fermentation process in the production of yogurts and cheese [1]. It is evident that the spread of antibiotic resistance has become a global health threat in recent years. The Centres for Disease Control and Prevention in America described resistance as one of the most prominent health problems due to the number of antibiotic resistant bacteria discovered in recent studies [23].

In our study of β-lactam and tetracycline residues, they were analysed as they are widely used in therapy of bacterial infections, especially for the treatment of mastitis in cows. Any milk containing antibiotics is unfit for human consumption [5]. Out of all 69 samples analysed, 17 (24.6 %) of the samples were tested positive for ß-lactam and tetracycline residues. Positivity for the presence of ß-lactam and tetracycline residues for the Slovak samples was 27.5 % and for the Irish samples was 20.7 %. The results indicated that the excessive use of antibiotic treatment of dairy cows could be associated with the absence of isolation of milk produced by dairy cows treated with antibiotics. Therefore, contaminated milk is mixed with uncontaminated milk and milk from other farms during collection. This also indicated a lack of screening of antibiotic residues in milk. Out of all 69 milk samples analysed, 17 (24.6 %) samples were tested positive for β-lactam residues and 12 (17.4 %) samples were tested positive for tetracycline residues. The results indicated a higher prevalence of β-lactam residues compared to the tetracycline residues in cow's milk.

In other studies that were conducted in Palestine, similar results were obtained. They reported the presence of β-lactam and tetracycline residues in 22.2 % of the analysed milk samples. Surprisingly in Romania, they reported that more samples where positive for tetracycline residue (25.7 %) compared to β-lactam residues (5.7 %) [18]. In Iran, milk samples were tested using the β-star test which showed that 5.33 % of sample where tested positive for antibiotic residues [16]. In India, the Charm β-lactam Tetracycline Combo test was used and it was found that 4.2 % of the samples were tested positive for β-lactam and tetracycline residues [24]. In Benin, milk was tested using the Twin Sensor kit 020 and the result showed 83.9 % positivity for β-lactam and tetracycline residues. They believe the most common cause of the presence of antibiotic residues in milk is non-compliance with the period for antibiotic excretion from the animal body, followed by overuse of banned antibiotics [24]. Other causes may include the therapeutic or prophylactic misuse of antibiotics and violation of the withdrawal period, illegal use of antibiotics or use of antibiotics as growth promoters [11].

Overall, antibiotic residues are often present in milk and therefore of great concern to dairy farmers, milk processors, regulatory agencies and consumers [21]. Antibiotic residues in milk have strong public health significance as they can cause adverse health effects in humans such as allergic reactions, carcinogenicity, mutagenicity, teratogenicity and drug resistance from long term exposure to antibiotics, which can cause failure of antibiotic therapy in clinical situations and result as a global problem. Antibiotic residues can be responsible for hepatotoxicity, bone marrow toxicity and reproductive disorders [13].

It is important for adequate consumer protection and quality assurance of dairy products that the presence of chemical residues in milk is not detected. Residues in milk must be controlled and the level must not exceed the acceptable maximum residue level [15]. To help reduce antibiotic residues in milk, simple, rapid, sensitive tests should be used and discarded if it exceeds the MRL. The concentration of MRL should be regularly monitored nationwide. It is vital that farmers are educated and follow withdrawal periods, store drugs in the correct location and maintain good hygiene management during antibiotic administration. Milk must be withdrawn and discarded from all quarters following intra-mammary infusion of antibiotics. All cows should be easily identified and antibiotic treated cows should be separated from the others to minimize the risk. Most important farmers should be made competent about maintaining proper quality of milk as well as its assurance [3].

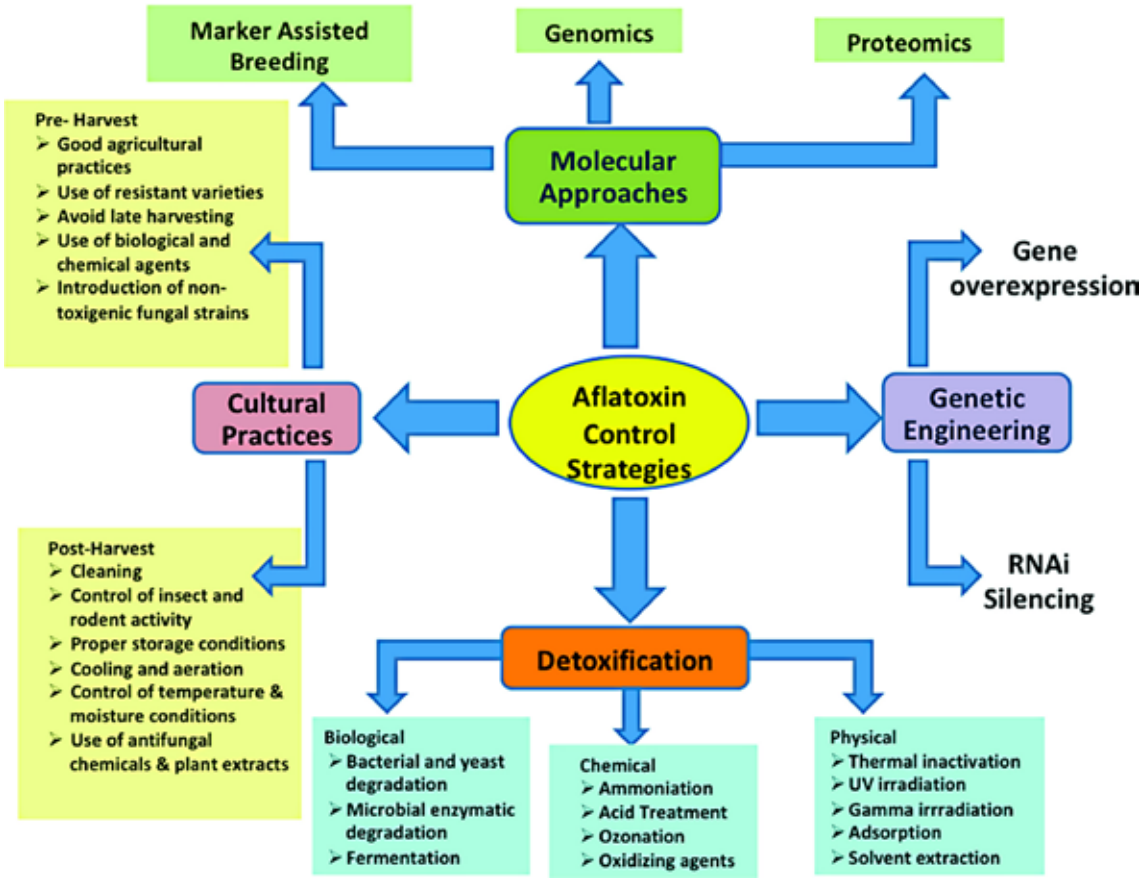

Fig. 3. Aflatoxin control strategies
Source: https://link.springer.com/chapter/10.1007/978-3-319-95354-0_11

In case of the milk samples tested for the presence of AFM1 in this study, it is important to note the high level of contamination. Out of all of the samples analysed, only 14.5 % were tested negative (compliant) for the presence of AFM1. The compliance of the Irish samples tested was 6.9 % and compliance of the Slovak samples tested was 20 %. The results indicated a huge prevalence of the problem at farm level both, in Ireland and Slovakia. The content of AFM1 in non-compliant samples varied from 69 ppt to >200 ppt.

If we compare these results to previous studies carried out worldwide, the prevalence is exceptionally high. A study [14] of raw milk in Sarab city of Iran in 2005 showed 40 % compliance. This is a sixfold increase if compared to Irish results and twice as high as Slovak results found in this study. A further study [12] by H u s a i n and A n w a r (2008) in Pakistan carried out a monthly analysis of AFM1 levels in milk. This study provides us with a more unbiased comparison than the aforementioned study. If each milk sample taken is compared to its corresponding month sample, the October levels in their study ranged from 0.27 $\mu g.l^{-1}$ to 0.45 $\mu g.l^{-1}$ with a mean value of 0.345 $\mu g.l^{-1}$. The next month for comparison was February, the mean value was 0.466 $\mu g.l^{-1}$. Out of all of the samples tested, 99.4 % of the samples exceeded the European community/Codex Alimentarius recommended limit 0.05 $\mu g.l^{-1}$ or 0.05 $\mu g.kg^{-1}$. This limit has been established following the ALARA (as low as reasonably achievable) principle. The “as low as reasonably achievable” principle was the reason that the European levels of acceptable aflatoxin contamination is so low. This is why the majority of samples exceeds these levels when tested. Further education and research into this problem are required to reduce these levels [8].

The risk of aflatoxin exposure is considered higher in regions with a tropical or subtropical climates. Extreme temperatures and droughts may be factors in the growth of *Aspergillus* spp. in crops [2]. The season of the year may also impact on the occurrence of AFM1. Levels observed are higher in the winter months. This is due to the nutrition

of housed animals. Commonly cattle are fed forage and concentrates whilst in winter accommodation. In the summer months in comparison, the cattle will be grazing fresh pastures and the risk of aflatoxin exposure is diminished [22].

There are a variety of control strategies for aflatoxins available both pre and post-harvest (Figure 3) including detoxification, cultural practices, molecular approaches, and genetic engineering.

Moulds will grow in humid or damp conditions. It is important to adequately dry and store feeds. Higher consumption of concentrates in the diet is also a factor which may lead to high levels of aflatoxins carried over into the milk. By inspecting grains regularly whether growing or stored, and checking for any signs of contamination or exposure to aflatoxins, we can reduce the risk of them occurring. Discard any that look mouldy, discoloured or shrivelled. Damage to grains should be avoided whilst drying, in storage. Damaged grains are more prone to the invasion of moulds especially aflatoxins. Farmers and food business operators should buy fresh grains where possible. Storage areas for grains and forage should be kept free of insects and not too warm. The purchase of animal feeds a long period of time before they are required should be avoided, extended storage times adds risk. To lessen the risk of mycotoxin risk on a farm, it is also recommended to provide a varied diet. By feeding a variety of grains and forages the risk of over exposure to one mould will be reduced [2, 14, 22].

The European Food Safety Authority report [9] summarises the monitoring data generated in 2020 in the frame of official control activities on the presence of residues of veterinary medicinal products and certain substances in live animals and animal products in the European Union, Iceland and Norway. A total of 620,758 samples for nearly 13 million single analytical results were reported to the European Commission by the 27 EU Member States, Iceland and Norway; of those samples, 331,789 were targeted samples and 4,259 suspect samples reported under Council Directive No. 96/23/EC, while 2,551 samples were collected at import and 282,159 samples tested in the framework of programmes developed under the national legislation. The majority of countries fulfilled the minimum requirements for sampling frequency laid down in Council Directive 96/23/EC and in Commission Decision 97/747/EC. Overall, the percentage of non-compliant samples in 2020 (0.19 %) was lower compared to 2019 (0.30 %), but also compared to the previous 11 years (0.25–0.37 %). The same overall pattern was observed for targeted samples in 2020 (0.27 %) compared to the previous 3 years (0.30–0.35 %). For the group of antibacterials (B1) including antibiotics (e.g. beta-lactams, tetracyclines, macrolides, aminoglycosides), but also sulphonamides and quinolones; the total number of analyses carried out in 2020 in targeted samples was 93,920 of which 127 (0.14 %) were non-compliant. For the group of antibacterials, there were 9,489 milk samples analysed of which 6 countries reported a total of 7 (0.074 %) non-compliant results. For mycotoxins (subgroup B3d), there were 1,568 milk samples analysed of which 4 (0.26 %) samples were reported non-compliant relating to AFM1, reported by 3 countries.

CONCLUSIONS

Currently, the existence of antibiotic residues and aflatoxins in milk is a major issue worldwide. There are many factors that can lead to the presence of antibiotic residues in milk including therapeutical use, prophylactic use, lack of maintenance of proper withdrawal time, lack of awareness of farmers about residual effects and improper cleaning of antibiotic contaminated equipment. The detection of antibiotic residues can be challenging and should be completed in a simple, fast and cost effective way for the farmer. Aflatoxicosis may cause acute clinical signs or in many cases prolonged exposure at low concentrations may cause chronic health problems. Acute toxicity will cause severe symptoms soon after exposure, whereas chronic exposure can cause illness such as cancer, immune deficiencies, digestive and reproductive signs. Due to the carcinogenic qualities of mycotoxins and in particular aflatoxins, their maximum limits must be as low as reasonably achievable (ALARA). These measures ensure that farmers are compliant and thus prevent and reduce contamination as far as possible to protect public health. The veterinarian has an important role in education and prevention. If farmers understand the health consequences for animals and humans and the potential economic losses, they may be encouraged to test their feeds and milk products for the presence of antibiotic residues and aflatoxins. Both, Duplex BT Scan and AflaM1 Scan produced by Zeulab S.L. used for the detection of antibiotic residues and AFM1 in

milk, are one of the fastest ways to analyse milk and are also very cost effective for the farmers worldwide. In addition, in conjunction with the IRIS device, it is an efficient and accurate method of detection. Analysis results show the necessity of monitoring milk samples in order to protect public health, reduce antibiotic resistance and avoid economic losses.

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TESTING OF THE EFFICACY OF BEE PROBIOTIC LACTOBACILLI UNDER *IN VIVO* CONDITIONS

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ABSTRACT

American foulbrood is amongst the most dangerous diseases of the bee-family affecting many honeybee colonies worldwide. In countries of European Union, based on veterinary legislation, the bee colonies tested positive to American foulbrood are eradicated with high economic losses. It is therefore necessary to look for effective prevention, especially by the using of natural ingredients such as probiotics. In this study, we used lactobacilli isolated from digestive tracts of adult healthy honey bees and selected based on their good probiotic properties and ability to inhibit the growth of *Paenibacillus larvae*. These isolates were identified as *Lactobacillus brevis* and *Lactobacillus plantarum*. Night cultures of both strains were used for the preparation of probiotic suspensions and pollen was selected as an appropriate carrier for application of probiotic lactobacilli to the hives. Half a litre of pollen suspension was prepared for each hive. The suspension for experimental hives contained probiotic lactobacilli in concentration of 10^7–10^8 CFU.ml^{-1}. Bees in control hives received pollen suspension without addition of probiotic lactobacilli. The suspensions were supplied to bees three times, once a week. Before and during administration of probiotics, samples of honey bees from each hive were taken every week and numbers of lactobacilli, *P. larvae*, enterobacteria and coliform bacteria were determined in their digestive tracts. Four weeks after the first administration of probiotic-pollen solution the numbers of lactobacilli were increased approximately by 0.5 log. Before starting this experiment, *P. larvae* (approx. 10^7 CFU.ml^{-1}) were detected in digestive tracts of honey bees. In the experimental group after 3 weeks and in the control group after 4 weeks, no viable counts of *P. larvae* were found. The numbers of enterobacteria, coliform bacteria and *Bacillus* sp. decreased in both groups. During the experiment the health and condition of the hives were monitored. In addition, also monitored were: the development of the bee colony, the number of dead bees, the amount of hive debris, the aggressiveness of the bees, and the amount of honey spun; later during the autumn treatment also the fall of *Varroa jacobsoni* was noted. In the experimental and control groups, we recorded a decrease in the amount of hive debris. Only in the experimental group was a slightly above-average development of the bee colony, slightly above-average honey yields and a 70 % lower drop of *Varroa mites*. Based on these results, we assumed that the probiotic-pollen solution had a positive influence on the composition of microbiota in bee digestive tracts and it can increase resistance to *P. larvae*. It also had a positive effect on the health and condition of the bee colony. Probiotic-free pollen solution showed similar but weaker effects.

Key words: administration; condition; microbiota; *Paenibacillus larvae*; probiotics

INTRODUCTION

The American foulbrood is one of the most serious diseases of honey bees, resulting in high mortality rates, being spread worldwide and causing major economic losses. It has been classified as a disease subject to the reporting obligation in terms of the OIE list of transmissible diseases. The disease is caused by the Gram-positive rod-shaped bacterium, *Paenibacillus larvae* [2]. Administration of antibiotics is of minor effect as the antibiotics only affect vegetative forms of *P. larvae*, while the spores continue to spread. Moreover, residues of these drugs remain in bee products, primarily in honey, contributing to the increasing antibiotic resistance of pathogens. These are the reasons why the use of antibiotics against American foulbrood has been prohibited under the European Union legislation. Infection by *P. larvae* is dealt with in a radical way – by eradicating the hives and all the combustible material by burning. Therefore, it is necessary to find an effective method of prevention or therapy of this disease. Excellent possibilities are offered by probiotics. Probiotic bacteria are defined as live microorganisms which, when administered to a host in adequate amounts, confer them health benefits [8]. These bacteria are common commensals of the gut and other body surfaces in humans, animals and insects [10]. Insects have different components to their immune system. It consists of 2 parts, social and individual immunity, both parts are connected [4]. The social defence is based on the cooperation of thousands of individuals to combat parasites or other pathogens (cleaning of colonies and immobilization of pathogens). Individual immunity consists of cellular and humoral immunity [7].

This study deals with the testing of administration of probiotic lactobacilli in bee hives to monitor their impact on the composition of intestinal microbiota of honey bees and condition of hives (the development of the bee colony, the number of dead bees, the amount of hive debris, the aggressiveness of the bees, the amount of honey spun and the drop of *Varroa* mites in autumn).

MATERIALS AND METHODS

Probiotic lactobacilli

Lactobacillus brevis B50 BiocenolTM (CCM 8618) and *Lactobacillus plantarum* P12 used for this experiment were isolated from the digestive tracts of adult healthy honey bees. Both strains were identified by rep-PCR and MALDI TOF-MS method. These strains showed in previous studies positive growth and probiotic properties (good growth properties, ability of autoaggregation, production of organic acids, ability to survive storage at –20 °C for long time) and ability to inhibit the growth of *P. larvae* [12]. The night cultures of both strains were cultured in MRS broth (Merck) at 37 °C under aerobic conditions. These cultures were 3 times washed in physiological solution by centrifugation at 600 × g for 30 min. The sediment was re-suspended in autoclaved tap water and concentration of lactobacilli were adjusted to 10^7–10^8 CFU.ml^{-1}.

Probiotic-pollen suspension

For each application and each experimental hive there was prepared half of a litre of probiotic-pollen suspension composed of 50 g of pollen and probiotic lactobacilli culture. For control hives was prepared half of a litre of pollen suspension composed of 50 g of pollen in autoclaved tap water without addition of probiotic lactobacilli.

Design of the experiment

Six bee colonies (3 experimental and 3 control) were used for the experiment. Probiotic-pollen suspensions were administered to the experimental hives and honey bees in control hives received pollen suspensions without probiotic lactobacilli. The solutions were administered to honey bees three times, once a week (Fig. 1).

Analysis of intestinal microbiota of honey bees

Before and during administration of suspensions, approximately 30 bees from each of colony were taken every week. Digestive tracts (intestine, stomach and rectal sack) were used for the evaluation of microbiota composition. Numbers of bacteria were determined by plating on selective media after ten-fold dilution.

Lactobacilli were cultured in MRS agar (Oxoid, Basingstoke, UK) for 48 h at 37 °C under anaerobic conditions (Gas Pak Plus, BBL). *Paenibacillus larvae* were grown in MYPGP agar (yeast extract 15 g, K_2HPO_4 3 g, sodium

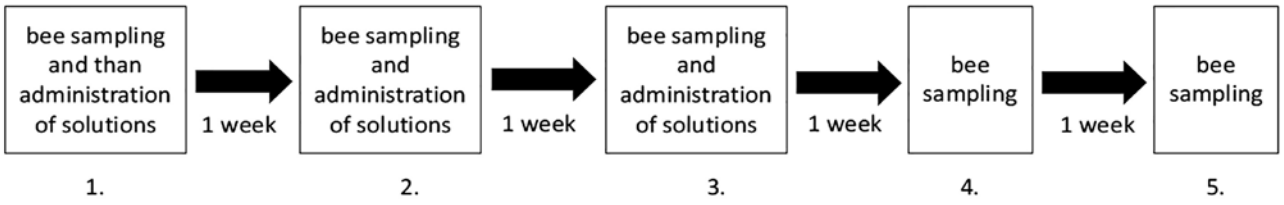

Fig. 1. Experimental design

pyruvate 1 g, D(+) glucose 2 g.l^{-1}, agar 20 g.l^{-1}, pH 7.2) for 48 h at 37 °C. Endoagar (Oxoid, Basingstoke, UK) was used for the cultivation of *Enterobacteriaceae*, Mc Conkey agar (BBL, Becton Dickinson, Cockeysville, USA) for cultivation of coliform bacteria and DCP (dextrose casein peptone) agar (10 g enzymatic casein hydrolysate, 5 g glucose, 0,04 g bromcresol red, 12 g.l^{-1} agar, pH 6.8 ± 0.2) for cultivation of *Bacillus* sp. They were incubated for 24 h at 37 °C.

Clinical examination of honey bee colonies

Throughout the experiment, the health and condition of the bee colonies was monitored, including the development of the bee colony, the number of dead bees, the amount of hive debris, the aggressiveness of the bees, the amount of honey spun, later during the autumn treatment also the drop of *Varroa* mites.

RESULTS

No problems with intake of probiotic-pollen or pollen suspensions were recognized during the study.

Analysis of intestinal microbiota of honey bees

After administration of probiotic-pollen suspension the counts of lactobacilli showed a tendency to increase during the experiment. In two last samplings the counts of lactobacilli were significantly higher in the experimental colonies compared to the control ones (Fig. 2).

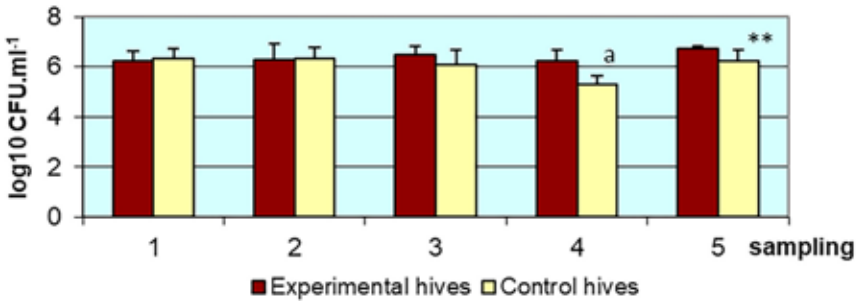

Fig. 2. The counts of lactobacilli expressed as log10 CFU.ml^{-1} ± SD (n = 9) in the digestive tracts of honey bees before and after administration of probiotic-pollen or pollen suspension

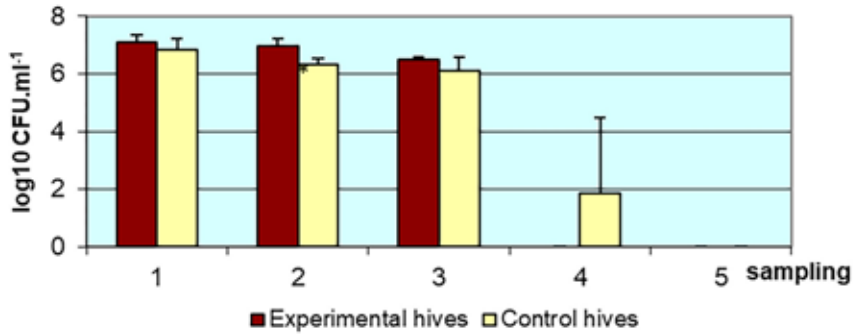

Fig. 3. The counts of *Paenibacillus larvae* expressed as log10 CFU.ml^{-1} ± SD (n = 9) in the digestive tracts of honey bees before and after administration of probiotic-pollen or pollen suspension

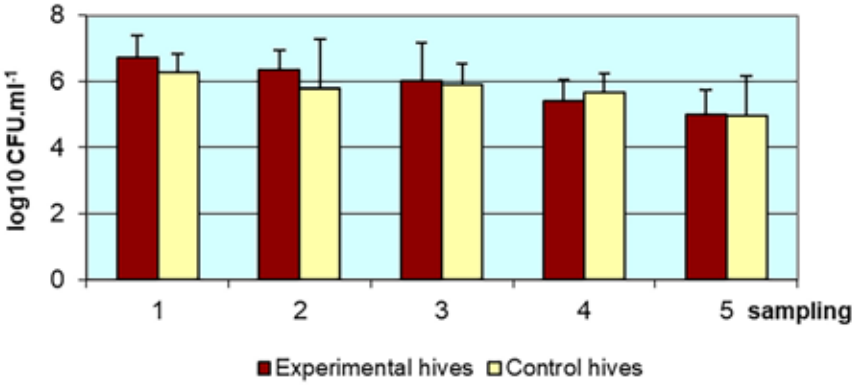

Fig. 4. The counts of *Bacillus* sp. expressed as log10 CFU.ml^{-1} ± SD (n = 9) in the digestive tracts of honey bees before and after administration of probiotic-pollen or pollen suspension

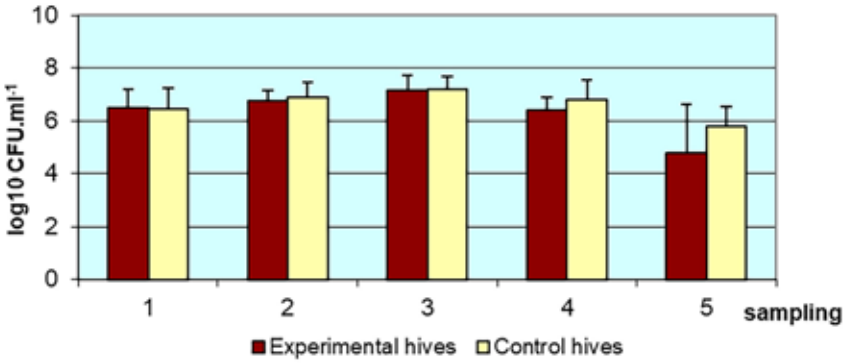

Fig. 5. The counts of coliform bacteria expressed as log10 CFU.ml^{-1} ± SD (n = 9) in the digestive tracts of honey bees before and after administration of probiotic-pollen or pollen suspension

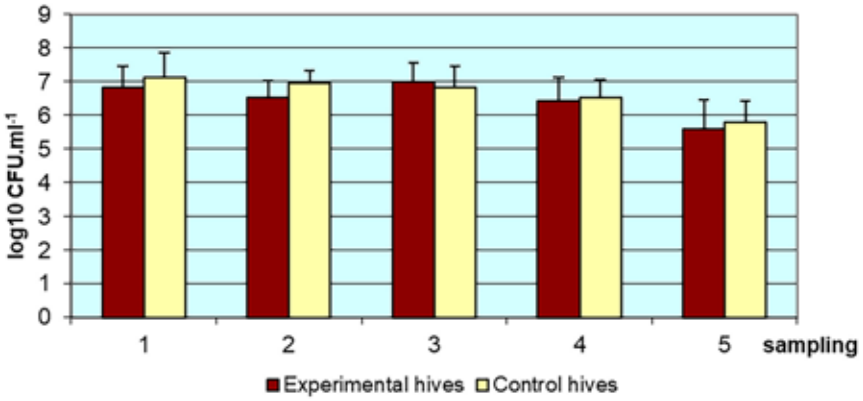

Fig. 6. The counts of Enterobacteriaceae expressed as log10 CFU.ml^{-1} ± SD (n = 9) in the digestive tracts of honey bees before and after administration of probiotic-pollen or pollen suspension

Before starting the experiment, *P. larvae* (in concentration approximately 10^7 CFU.ml^{-1}) were found in the digestive tracts of honey bees. After three administrations of probiotic-pollen suspension, *P. larvae* completely disappeared from the digestive tract. After three administrations of pollen suspension to control colonies, the counts of *P. larvae* were significantly reduced ($P < 0.001$) in comparison with the 3rd sampling and at the last sampling there were detected no viable *P. larvae* (Fig. 3).

Before starting the experiment, the counts of *Bacillus* sp. in the digestive tracts of honey bees were between 10^6–10^7 CFU.ml^{-1}. The counts in experimental groups were higher than in control groups, but not significantly. The counts gradually decreased during the experiment,

in experimental groups the decline was more pronounced compared to control groups, but the difference was insignificant (Fig. 4).

During the experiment, the counts of coliform bacteria and *Enterobacteriaceae* decreased by 1–1.5 log after administration of probiotic-pollen and pollen suspensions by 1–1.5 log, but no significant differences between control and experimental groups were observed (Fig. 5 and 6).

Clinical examination of honey bee colonies

Based on the clinical examinations of the hives during the experiment, we concluded that the administered experimental suspensions did not have a negative effect on the hives. On the contrary, a decrease in the amount of hive debris was recorded in both the experimental and control groups, which indicated an intensification of the cleaning instinct of the bees, which was directly proportional to the condition of the bee colony. Only in the experimental group there was noted a slightly above-average development of the bee colony, slightly above-average honey yields and, surprisingly, by up to 70 % lower drop of *Varroa* mites (Table 1).

Table 1. Clinical examination of honey bee colonies

	Control group	Experimental group
Amount of hive debris	below average	almost none
Development of the bee colony	average	above average
Honey yields	average	above average
Drop in *Varroa* mites	average	up to 70 % lower

DISCUSSION

Probiotics represent one of the promising possibilities for replacement of antibiotics in prevention and therapy of many diseases in animals and people. Our results indicated the ability of *L. brevis* B50 BiocenolTM (CCM8618) and *L. plantarum* P12 to inhibit the growth of *P. larvae*. They stabilize the microbiota of the digestive tract of honey bees. Similar conclusions were presented in the study by C a n g a n e l l a and B a l s a m o [5], who demonstrated the inhibitory effect of lactobacilli against *P. larvae* and *P. alvei* (European foulbrood). F o r s g r e n et al. [9] also observed the ability of lactobacilli and bifidobacteria isolated from the stomach of honey bees to inhibit the growth of *P. larvae*. They administered such lactobacilli to bee larvae through the mother bread and noted significantly lower numbers of infected larvae. A number of authors [1, 3] confirmed the antagonistic action of different bacterial species against *P. larvae*. The gut microbiota of honey bees was shown to have low diversity, and LAB represent an important part of it [11]. The normal bee microbiota is involved in the breakdown and utilization of pollen, degradation of environmental toxic compounds, or activation of bee immune system to prevent colonization of pathogenic microorganisms [6]. Colonisation of the gut by beneficial microbiota can prevent a proliferation of spores *P. larvae* and thus prevent infection. Administration of pure pollen without addition of probiotic lactobacilli also showed a positive effect on the composition of intestinal microbiota of honey bees and contributed to elimination of *P. larvae* from their digestive tract. Pollen can stimulate the immune response of honey bees, because it naturally contains lactobacilli, nutrients which increase the food quality, and some immunostimulating substances (e. g. vitamins, fatty acids. enzymes, etc.). Pollen is given to bee larvae from the third day of life. The greatest content of pollen is in the food given to drones larvae. Therefore, they are more resistant to infection of *P. larvae* than the worker bees larvae. Queen larvae receive the least amount of pollen in their diet and are therefore the most susceptible to disease [13, 14].

CONCLUSIONS

We observed a positive effect of administration of probiotic-pollen or pollen suspension to investigated honey bees reflected in the following:

- honey bees willingly drank the probiotic-pollen and pollen solutions,
- *P. larvae* disappeared from digestive tract of honey bees after administration of the tested solutions,
- the counts of *Enterobacteriaceae*, coliform bacteria and *Bacillus* sp. were reduced by 1–1.5 log,
- a decrease in the amount of hive debris was recorded,
- only in the experimental group there was observed a slightly above-average development of the bee colony, slightly above-average honey yields and 70 % drop in *Varroa jacobsoni*.

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MULTILOCUS SEQUENCE TYPING AS A USEFUL TOOL FOR THE STUDY OF THE GENETIC DIVERSITY AND POPULATION STRUCTURE OF *CRYPTOSPORIDIUM* SPP.

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ABSTRACT

One of the most important aquatic parasites in industrialized countries, *Cryptosporidium* spp., is a major cause of diarrheal disease in humans and animals worldwide. The contingent evolution of cryptosporidia with hosts, host adaptation, and geographic variation contributed to the creation of species subtypes, thereby shaping their population genetic structures. Multilocus typing tools for population genetic characterizations of transmission dynamics and delineation of mechanisms for the emergence of virulent subtypes have played an important role in improving our understanding of the transmission of this parasite. However, to better understand the significance of different subtypes with clinical disease manifestations and transmission risks, a large number of samples and preferably from different geographical areas need to be analyzed. This review provides an analysis of genetic variation through multilocus sequence typing, provides an overview of subtypes, typing gene markers for *Cryptosporidium parvum, Cryptosporidium hominis, Cryptosporidium muris* and *Cryptosporidium andersoni* genotypes and an overview of the hosts of these parasites.

Key words: *Cryptosporidium*; Multilocus Sequence Typing MLST; PCR

INTRODUCTION

Cryptosporidium spp. belongs to protozoan pathogens that infect the gastrointestinal epithelium of a wide range of vertebrates, including humans. Globally, the average prevalence of *Cryptosporidium* is thought to be ~7.6 %, with rates as high as 4.3 % in developed countries and 10.4 % in developing countries. Some areas report a prevalence of this parasite as high as 69.6 % [14]. In immunocompromised individuals, *Cryptosporidium* spp. are responsible for severe diarrhoea, stunted growth, and cognitive impairment in children in developing countries. It has recently been associated with colon cancer [35, 37, 48]. In infectious stages, oocysts of *Cryptosporidium* spp. are transmitted through the faecal-oral route and are highly resistant to disinfection. For this reason, cryptosporidia are considered the causative agents of waterborne and foodborne diseases [15, 65]. The only drug approved for the treatment of cryptosporidiosis in humans is nitazoxanide. However, treatment failure is common in diseases caused by *Cryptosporidium* spp., and no vaccines are available [6].

Clear and unambiguous descriptions of species are essential for understanding their epidemiology, pathogenicity and the development of effective therapeutics [17]. We currently know that most *Cryptosporidium* spp. are not host-specific. However, the oocysts of many *Crypto-*

sporidium spp. are identical or overlap in size and lack other distinguishing features [16]. Molecular tools are needed to understand taxonomy and epidemiology [61]. In recent years, many new species and genotypes of *Cryptosporidium* spp. have been confirmed, and many *Cryptosporidium* spp. were reclassified based on morphological and biological data.

Cryptosporidium is one of more than 300 genera in the phylum Apicomplexa, which contains more than 6000 species [44]. For a long time, *Cryptosporidium* was classified as a coccidia, a parasite of the order Eucoccidiidae, based on the similarity of its life cycle with another enteric coccidia [29]. However, *Cryptosporidium* was later speculated to be closely related to gregarines, that are parasites which form a diverse and primitive group that mostly parasitizes invertebrates. This similarity has been described based on molecular and biological similarities, including the presence of a "feeding" organelle in developmental stages, based on the absence of sporocysts and, last but not least, based on epicellular localization in host cells [8, 20, 28, 46]. The whole genome sequencing (WGS) of the gregarine, *Ascogregarina taiwanensis*, supported a monophyletic relationship between the gregarine and cryptosporidia but demonstrated significant differences in the metabolic capacity of the cryptosporidia [2]. In 2014, based on 18S ribosomal data, Cavalier-Smith revised the taxonomy, transferred *Cryptosporidium* spp. from coccidia to gregarines, and created a new subclass, called the *Cryptogregaria* within the class *Gregarinomorphea* [9]. In 2019, Adl et al. modified the class to the subclass *Cryptogregarinorida* within *Gregarinasina* [3]. Transcriptomic analysis of a broader spectrum of apicomplexan parasites suggests that *Cryptosporidium* represents a distinct lineage from gregarines [24, 34, 47] and further taxonomic revision is needed.

Before 1971, there were only three valid species within the genus *Cryptosporidium*: *Cryptosporidium muris* [53], *Cryptosporidium parvum* [54] and *Cryptosporidium meleagridis* [49]. Greater interest did not emerge until 1993, when a massive waterborne outbreak affected more than 400,000 residents of Milwaukee, Wisconsin [31]. In the mid-1990s, molecular detection and identification methods appeared, which became the basis for taxonomic identification and epidemiological studies. Interest in cryptosporidiosis has resurfaced after the Global Enteric Multicenter Study (GEMS) of more than 22,500 children in Africa and Asia, conducted from December 1, 2007, to March 3, 2011, revealed that *Cryptosporidium* is the second most crucial diarrheal pathogen after rotavirus in children up to 5 years of age [27]. There are currently at least 44 verified species of *Cryptosporidium* (many of which were initially identified as genotypes and later described as species); six avian *Cryptosporidium* spp., four species of *Cryptosporidium* spp. in fish, one in amphibians, four in reptiles, and 29 species of *Cryptosporidium* spp. in mammals. In addition to the 44 species, more than 120 genotypes have been reported, and perhaps many of these will be formally described as species in the future. Valid names for *Cryptosporidium* spp. are essential to elucidate *Cryptosporidium* taxonomy and understand its transmission dynamics. Genotypes often have multiple names in the literature and GenBank, leading to confusion and inaccuracy. All species and genotypes were summarized in the work by Ryan et al. [46].

The greatest diversity of *Cryptosporidium* spp. and genotypes has been described in rodents; namely 14 species and more than 57 genotypes. This is not surprising, as rodents include more than 2,000 species, representing 40% of all mammals, and inhabit all continents [12]. A wide range of other species have also been reported in rodents, including the human infectious species *C. parvum*, *C. meleagridis* and *C. ubiquitum* [21, 26]. Fourty four species and more than 120 genotypes, 19 species and four genotypes have been reported in humans. However, 95% of diseases are caused by two species, *C. hominis* and *C. parvum*, which are very crucial in public health and they are responsible for several sporadic diarrheal outbreaks [7, 41]. *C. parvum* is a zoonotic species and infects a wide range of animal hosts, including humans, whereas *C. hominis* infects only humans [42]. The genomes of *C. parvum* and *C. hominis* (reference strains IOWA and TU502) have been sequenced [2, 62], and their genome sizes are 9,11 and 9,16 Mb. Genome comparison showed that *C. hominis* and *C. parvum* are very similar and show only 3–5% sequence divergence, without large insertions, deletions or rearrangements. Genomic data for genome representatives are available online [11].

Multilocus analysis

Multi-locus typing has become one of the most valuable strategies to address the genetic diversity or variability of microorganisms such as fungi [50]; protists [10]; and

bacteria [5]. The study of alleles of different loci increases the discriminative power of typing schemes, allowing the detection of genetically distinct lineages. Two main strategies were developed in multilocus analysis. One is based on comparative analysis of the size of amplified fragments of multiple loci (Multilocus Fragment Typing-MLFT) and identification of the length/size of polymorphisms; and the second strategy is focused on the comparative analysis of nucleotide sequences (Multilocus Sequence Typing-MLST), which allows the identification of differences in length and variations of nucleotides [5, 10, 22, 23, 40, 43, 50]. Although MLFT can provide good differentiation and it is relatively fast and inexpensive to perform, MLST is considered the gold standard because it is able to unambiguously identify genomic variations such as single nucleotide polymorphisms (SNPs) or point mutations, small insertions and deletions, inversions, and translocations and deletions or duplications [1, 13, 39, 58].

Various molecular tools are available to distinguish between different *Cryptosporidium* spp., pathogenic for humans. Molecular typing studies based on a single locus are limited in their ability to capture population structure, which is better explored using a multilocus approach. To better understand transmission dynamics, genetic diversity and population structure of *Cryptosporidium* spp., it is, therefore, necessary to study markers with high resolution. Multilocus sequence typing (MLST), widely used to study the population biology of many microorganisms [36, 55], has been used for subtyping primarily for *C. hominis* and to a lesser extent for *C. parvum* [32, 51]. Studies from Scotland and the United States have shown that *C. hominis* exhibits a clonal population structure, as it exhibits a lower number of subtypes and a much stronger linkage disequilibrium (LD) compared to *C. parvum* [51]. Significant variation at minisatellite loci is in the form of SNPs that may be missed by typing techniques based on length polymorphism alone. MLST is based on both the length of polymorphisms and SNPs and therefore increases the discriminative power of typing methods. Sequence length polymorphism was studied at loci GP60, CP47, TP 14, MS5, MS9, and MSC6-7, while loci CP56 and Mucin-1 were monomorphic in size. Among all markers studied, GP60 showed the highest gene diversity [41, 43]. Many subtypes in the GP60 family were of the same length, even though the sequences were different. In 2017, Y a d a v et al. [63] discovered that MLST based on the length of polymorphism and SNPs showed that only the CP47 gene is monomorphic, while the study by G a t e i et al. [19] described all other loci as polymorphic except for HSP70. G a t e i et al. [19] identified six subtypes of CP47 and six subtypes of GP60. In a study by Y a d a v et al. [63], up to seven subtypes of GP60 were listed based on the length of polymorphism, and all subtypes were different, except the 847 bp. Although three subtypes were observed in both studies based on the length of polymorphism at the MSC6-7 locus, these were distinct from each other. The Mucin-1 locus had 11 copies of a 63 bp minisatellite repeated in both studies. In both studies, three subtypes were observed based on SNPs. Based on this analysis, the UPGMA tree of Calcutta isolates generated twenty multilocus subtypes, and the Bayesian-derived MLST phylogram showed four major subtype groups. The UPGMA tree generated nine multilocus subtypes, and the MLST phylogram derived by the neighbour-joining method also showed nine groups of subtypes [63]. Although MLST is a better tool for studying transmission dynamics of *Cryptosporidium* subtypes due to its high resolution, to further understand the significance of different subtypes with clinical disease manifestations and transmission risks, it will be necessary to analyze a large number of samples and preferably from different geographical areas. It follows that the 60-kDa glycoprotein (GP 60) is one of the predominant typing gene markers for *C. parvum* genotypes [59]. So far, 15 subtypes of *C. parvum* have been described, including IIa to IIi and IIk to IIp, in which families IIa and IId are defined as zoonotic pathogens [45, 59].

While GP60 showed the highest resolution in MLST, other markers including CP47 and MSC6-7 also showed high gene diversity. The CP47 locus has a classic microsatellite gradual mutation and offers a good alternative for *C. hominis* and *C. parvum* typing. Unlike GP60, CP47 has a high resolution for both length of polymorphism-based and MLST-based typing and discriminates *C. hominis* and *C. parvum* by size without overlap in TAA repeat number. Similarly, MSC6-7 had 28 polymorphic sites and strong LD to both GP60 and CP47; making it a useful minisatellite locus for both *C. hominis* and *C. parvum* typing [19].

Subsequently, multilocus sequence typing (MLST) tools based on four minisatellite and microsatellite loci were developed to analyze genetic variation in *C. muris* and *C. andersoni* [18]. For example, until now the iden-

tification of *C. andersoni* isolates from humans has been based only on the analysis of the SSU rRNA gene or the COWP gene. Epidemiological data from different hosts document the host range or host specificity of *C. andersoni*. Cattle are considered the main animal host of *C. andersoni*, although it is occasionally found in other animals such as dromedary camels, sheep and hamsters [57, 64]. A search for microsatellite and minisatellite sequences in the *C. muris* genome was performed on March 8, 2007. The first 2,500 sequences (tracks 1649410657 to 1649413156) in the *C. muris* whole genome sequencing database were obtained from the Institute for Genomic Research (TIGR, now the J. Craig Venter Institute) [25]. Microsatellite and minisatellite sequences in the sequences were identified using Tandem Repeat Finder software [52].

A search for tandem repeats in 2,500 short sequences obtained from the *C. muris* genome-wide sequencing project identified 101 sequences with microsatellites and minisatellites. Based on the nature (mostly absence of imperfect repeats) and length (>6 copies for minisatellite targets and >10 copies for microsatellite targets) of the repeats and the availability of suitable sequences for primer design (except sequences with short or AT-rich 5'- or 3'- adjacent nucleotide sequences) 13 loci were selected from 101 potential targets, including 6 microsatellite loci and 7 minisatellite loci. Primers for nested PCR were designed for each locus (Tab. 1).

Because DNA sequencing rather than fragment length measurement was used to determine the polymorphism, the final PCR product was larger than that normally used for microsatellite and minisatellite analysis, with expected PCR products ranging from 307 bp to 751 bp. Four loci, CM-MS1, CM-MS2, CM-MS3 and CM-MS16, were selected for sequence polymorphism evaluation using a total of 44 DNA extractions from *C. muris* and *C. andersoni* samples. Of these, 41, 39, 24, and 38 DNA preparations were amplified at the CM-MS1, CM-MS2, CM-MS3, and CM-MS16 loci, respectively. These PCR products were successfully sequenced [18].

Sometimes the difference was only a slight modification of the repeat, such as one of the two minisatellite regions in the *C. muris* variant at the CM-MS1 locus, while sometimes the repeat sequences were completely different, such as those at the CM-MS3 locus. In total, there were 10, 5, 5, and 6 subtypes for *C. muris* and 2, 3, 4, and 1 subtypes for *C. andersoni* at loci CM-MS1, CM-MS2, CM-MS3, and CM-MS16, respectively (Tab. 2). *C. andersoni* also had repetitive sequences distinct from those of *C. muris* at the CM-MS2 and CM-MS3 loci (Tab. 3).

Currently, 18 MLST subtypes have been found in *C. andersoni* isolates from cattle [18, 30, 38, 56, 66]. By providing additional genetic data, it will help in solving the problems of tracing the source of infection/contamination and in elucidating the transmission dynamics of human cryptosporidiosis caused by *C. muris* and *C. andersoni*.

Sequence polymorphism in *C. muris* and *C. andersoni* was predominantly in the form of differences in copy number of microsatellite and minisatellite repeats. In contrast, both *C. hominis* and *C. parvum* have extensive single nucleotide substitutions in the nonrepetitive regions of most microsatellite and minisatellite targets. The coding nature of the targets was probably not responsible for the differences observed between gastric and intestinal *Cryptosporidium* spp., as most microsatellites and minisatellites in *Cryptosporidium* occur in the coding regions of protein genes due to the presence of few introns and short intergenic regions. This difference may reflect intrinsic biological and genetic differences between gastric and intestinal *Cryptosporidium* species, as suggested by previous data on phylogenetic relationships and G/C content in SSU rRNA genes between the two groups of *Cryptosporidium* spp. [60]. Biological differences are known to exist in *C. andersoni*. *C. andersoni* isolates in Japan, the so-called Kawatabi strain, differ from *C. andersoni* isolates in other areas in their ability to infect SCID mice [33]. The genetic difference between the two biological types of *C. andersoni* is not yet clear. In order to better understand the geographic distribution of *C. andersoni* and the genetic determinants of host specificity, it is again necessary to include more genetic loci and a large number of samples from different geographic areas.

CONCLUSIONS

Our understanding of the extent of genetic diversity and zoonotic potential of *Cryptosporidium* species and genotypes has been greatly improved by the development of molecular detection and typing tools, particularly the gp60 typing tool, as it showed the highest genetic diversity of all markers studied. We recorded the lowest number of subtypes of the species monitored by us in *C. hominis*,

Table 1. Primers used in multilocus sequence typing, nature of genetic diversity, annealing temperatures used in PCR, and expected sizes of PCR products for *C. hominis* and *C. parvum*

Gene	The nature of polymorphism	Primer	Sequence (5′ - 3′)	Annealing temperature [°C]	Locations of fragments [bp][a]	Citation
HSP70 in chromosome 2	Minisatellites – 12 bp repeats and SNPs	F1	ACCTATGAAGGTATTGATT	55	1030–1100	[63]
		R1	TTAGTCGACCTCTTCAACAGTTGG			
		F2	CAGTTGCCATCAGTAGAG	45		
		R2	CAACAGTTGGACCATTAGATCC			
Chom3T in chromosome 3	SNP	F1	TTGCAGGAATGGTCGTATTT	50	480–680	This review
		R1	TCTGAGTCTTCCCTCAATTTCT			
		F2	CGGTAAAGTTACCAGTTG	50		
		R2	GGCAAAACTATCACTGAGAA			
Gp60 in chromosome 6	Microsatellites – TCA, TCG, TCT repeats and SNPs	F1	ATAGTCTCCGCTGTATTC	50	800 – 850	[4]
		R1	GGAAGGAACGATGTATCT			
		F2	TCCGCTGTATTCTCAGCC	50		
		R2	GCAGAGGAACCAGCATC			
CP47 in chromosome 6	Microsatellites – TAA, TGA/TAG	F1	GCTTAGATTCTGATATGGATC TAT	43	380 – 500	This review
		R1	AGCTTACTGGTCCTGTATCAGTT			
		F2	ACCCCAGAAGGCGGACCAAGGTT	55		
		R2	GTATCGTGGCGTTCTGAATTATCAA			
CP56 in chromosome 6	SNP	F1	CTCACAGAGTAAAAATCACTT	50	660–750	This review
		R1	GAACGCAAATATTAAGAAAAATTGAG			
		F2	TTGGCAATGTTGTCTTTTTCCA	50		
		R2	ATATGTAATCTGGCGCCAAAG			
Mucin 1 in chromosome 6	Minisatellites – 63 bp repeats and SNPs	F1	ACTGATGTGTCAAGTGGCAATC	58	650 – 900	This review
		R1	TTACAGTTATGAGTTGCTGGT			
		F2	TTGATGATTCAGAATCATCTGACT	55		
		R2	GTGAGTTCTTCTTCATCTGTATAG			
MSC6-7 in chromosome 6	15 bp minisatellites and SNPs	F1	ATTGAACAAACGCCGCAAATGTACA	55	545–564	This review
		R1	CGATTATCTCAATATTGGCTGTTATTGC			
		F2	GCTATTTGCTATCGTCTCACATAACT	55		
		R2	CTACTGAATCTGATCTTGCATCAAGT			

which shows the clonal structure of the population with much stronger linkage disequilibrium. Continuous addition of new data on subtypes of *Cryptosporidium* contributes to the understanding of the role of genetic recombination, the mechanism of host adaptation and interspecies transmission. They will also be useful in understanding the significance of the unique distribution of these subtypes in some geographic areas, such as the dominance of *C. parvum* subtypes in humans, or *C. andersoni* in cattle.

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Table 2. The nature of microsatellite and minisatellite repeats in four genetic loci

Locus	Repetition		
	C. muris	Variant *C. muris*	*C. andersoni*
CM-MS1	$(TAAAGGGAGAGA)_{3}$ $(GAACGAGATAGG)_{14-18}$	$(TAAAGAGAGAGA)_{6}$ $(GAACGAGATAGG)_{14}$	$(TAAAGGGCGAGA)_{3}$ $(GAACGAGATAGG)_{13-14}$
CM-MS2	$(CCATATCCC)_{3-4}$ $(CCATACCTC)_{3}$	$(CCCATTCCT)_{4}$	$(CCATACCTC)_{10-11}$
CM-MS3	$(TGTTGG)_{8-9}$; $(GCTGCA)_{6}$	$(TGTTGG)_{7}$; $(TGC)_{10}$	$(TGTTGGTGTTGCTGT)_{2}$ $(TGCTGCAGCTGC)_{2-3}$
CM-MS16	$(CTTCTTCAT)_{9-2}$	$(CTTCTTCAT)_{8}$	$(CTTCTTCAT)_{14}$

Numbers in subscript indicate number of nucleotide repeats

Table 3. MLST subtypes of *C. andersoni* from different hosts around the world

The host	Subtype number	MS1	MS2	MS3	MS16	Reference
Sheep	1	A2	A4	A2	A1	[56]
	1	A2	A5	A2	A1	[56]
Hamster	2	A3	A4	A2	A2	[56]
Camel	1	A6	A4	A2	A1	[56]
	1	A6	A5	A2	A1	[56]
Cattle	3	A1	A2	A4	A1	[18]
	1	A1	A3	A4	A1	[18]
	34	A1	A4	A4	A1	[56, 66, this review]
	2	A2	A1	A2	A1	[18]
	1	A2	A1	A3	A1	[18]
	1	A2	A3	A1	A1	[18]
	1	A2	A3	A2	A1	[18]
	3	A2	A3	A4	A1	[15]
	6	A2	A4	A2	A1	[56, 66, this review]
	6	A2	A4	A4	A1	[56, 66, this review]
	1	A3	A4	A4	A1	[56]
	3	A4	A4	A2	A1	[66, this review]
	82	A4	A4	A4	A1	[56, 66, this review]
	6	A5	A4	A4	A1	[56, this review]

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MOLECULAR, MORPHOLOGICAL AND CLINICAL CHARACTERISTICS OF SPONTANEOUS CANINE COLORECTAL CANCER – A REVIEW

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ABSTRACT

Cross-species comparison analysis studies are of immense importance in veterinary and human oncological research. Of the various non-rodent species available, dogs have gained most attention as potential animal models for the study of colorectal cancer. Domestic dogs developed evolutionally through a mutually beneficial relationship with humans. Because dogs share the same environment as humans, they are exposed to the same potentially harmful substances which may act as carcinogens in both species. Intestinal adenocarcinomas in dogs are naturally occurring heterogeneous tumours, which have the characteristics of sporadic human malignancies and therefore are more suitable for detailed oncological study than most xenograft or genetically modified rodent models. Furthermore, the canine genome has been comprehensively analysed and sequenced to a 7.6-fold coverage, and a very accurate version of this sequencing is available for study. The purpose of this manuscript is to present a comprehensive review of published data related to colorectal cancer in dogs. In addition, data regarding interspecies comparison of molecular events driving canine and human intestinal carcinogenesis is presented.

Key words: canine adenoma; canine adenocarcinoma; canine animal models; canine colorectal cancer

INTRODUCTION

Colorectal cancer is a heterogeneous epithelial malignancy with many histological subtypes, variable clinical presentation, and a poor prognosis in many patients [33]. At the time when the diagnosis of colorectal cancer is made, approximately 25 % of patients have locally advanced disease and 20 % present with distant metastasis [3]. Because of this, there is an urgent need to conduct detailed research studies, which hopefully will lead to improvement of our ability to prevent, diagnose, and treat this disease. To identify causal genetic alterations driving carcinogenesis, the development of cross-species comparison analysis studies is of immense importance [18]. Of

the various non-rodent species available, dogs have gained most attention as potential animal models for the study of colorectal cancer [40]. The purpose of this manuscript is to present a comprehensive review of published data related to the topic of colonic and rectal adenocarcinomas in dogs. Additionally, we also focus on an interspecies comparison of molecular events driving canine and human intestinal carcinogenesis.

MATERIALS AND METHODS

Dogs as potential animal models for oncological research

Companion animals like the dog potentially are valuable models for the study of human cancer for several reasons: firstly, domestic dogs developed evolutionally through a mutually beneficial relationship with humans, which started at least 15000 years ago and may go back as far as 100,000 years [32, 36]. Because they share the same environment as humans, they are exposed to potentially harmful substances and dietary factors which may act as carcinogens in both species [2, 29, 37]. Indeed, known risk factors for the development of cancer in dogs include obesity as well as other inappropriate quantitative and qualitative dietary factors, exposure to ionizing radiation, environmental air pollutants and other substances which are also known to be involved m in the process of carcinogenesis [23, 40]. Secondly, colorectal carcinomas in dogs are naturally occurring heterogeneous cancers which share the characteristics of sporadic human malignancies and therefore fit better for the purpose of efficient oncological study than most xenografts and genetically modified rodent models [34].

Over the past several years, progress in molecular genetics and other basic sciences has been able to provide valuable scientific data which may be used to improve oncological research [18]. The genome of the dog has been comprehensively analysed and sequenced to a 7.6-fold coverage, and a very accurate version of this sequencing is available for study [23]. This differs from other analysed mammals like, for example, cats in which only a 2.8-fold coverage of the genome is available [27]. Conducting comprehensive oncological research based on bioinformatical analysis is, therefore, more readily carried out in dogs than cats [23]. Furthermore, when compared with humans, the genome of dogs undergoes wide-spread genomic rearrangement [16, 23]. Because of these advantages, the study of sporadic canine colorectal adenocarcinoma is extremely valuable when studying the basic and clinical aspects of the corresponding tumours of humans [18, 29].

Epidemiology, anatomical localization, and morphology of canine colorectal tumours

Compared to humans, canine gastrointestinal tract neoplasms are less often encountered, accounting for 3 % to 10 % of all tumours in this species [8, 14]. However, in dogs, the alimentary tract tumours are more prevalent compared with the sporadic gastrointestinal tumours occurring in cats or other mammals, underscoring the value of dogs as comparative animal model for human oncological studies [18, 25].

The alimentary tract tumours are more prevalent in some canine breeds than in others [11, 31]. For example, a study conducted by Saito and colleagues demonstrated that gastrointestinal tract tumours develop significantly more commonly in Jack Russell terriers and Miniature Dachshunds compared with other dog breeds [31]. Miniature Dachshunds are reported to be predisposed to the development of inflammatory polyps in the large intestine, and these may progress to adenomas and eventually adenocarcinomas [30].

In general, although tumours of the digestive system can develop throughout the gastrointestinal tract of dogs, both benign and malignant neoplasms are found significantly more often in the large intestine [8] with up to 60 % affecting the large intestine [7]. Of these, 50 % to 60 % are malignant [1]. Histogenetically, most malignant tumours are of epithelial origin, with adenocarcinoma being the most common histological diagnosis [25]. Other less commonly encountered large intestine malignancies include: lymphomas, haemangiosarcomas, leiomyosarcomas and plasma cell neoplasms [9, 21].

Regarding more specific localizations of intestinal malignancies within the colon of dogs, adenocarcinomas have a significant predilection for the distal part of the descending colon and the rectal area [35]. Similarly, benign lesions such as adenomatous polyps are also more often encountered in the distal colonic area and rectum [14, 35]. These polyps are the most common benign tumours in the region of the gut [1]. Other less commonly encountered benign tumours are colonic leiomyomas and fibro-

mas [25]. Similarly, as in humans, adenomas localized in the sigmoid and descending colon and the rectum usually morphologically manifest as pedunculated polyps [8]. In the minority of cases which are present in the transverse or ascending colon, adenomatous polyps usually exhibit a sessile and annular appearance and may be the cause of strictures with narrowing of the luminal space [35].

Molecular pathogenesis in canine colorectal adenocarcinoma

One of the reasons why dogs gained attention as potential animal models for the study of colorectal cancer are the interspecies similarities in activated oncological pathways leading to the development of intestinal adenocarcinoma [17, 18].

T a n g et al. [34] analysed copy number abnormalities and other genetic alterations in canine colorectal carcinomas. Studying of 10 spontaneously occurring tumours in dogs showed a high degree of genetic homology between sporadic canine and human tumours. T a n g et al. [34] in their study, also demonstrated amplification of 5 % to 22 % of the canine genome in the analysed adenocarcinoma samples. As is the case in humans, they showed that the total number of altered genetic sequences directly correlated with the stage of progression of the oncological disease, and with the microsatellite instability status. When mapping genetic alterations in dogs, they noted that the analysed mutated canine genes which caused activation of signalling pathways leading to the development of colorectal cancer in dogs, are analogous to oncological pathways implicated in the formation of human intestinal adenocarcinomas. Overall, a significant overlap of copy number alterations was demonstrated when comparing canine and human colorectal cancer, and tumours taken from these two species were allocated according to the histological subtype and not the species [34].

As is the case in human digestive system malignancies, perturbations in the tumour suppressing gene adenomatous polyposis coli (APC) have been found to be present in colonic adenocarcinomas of dogs [40]. Y o u m a n s and colleagues [40] analysed the results of exon-sequencing performed in 23 spontaneously developed canine colorectal tumours, including eight benign adenomatous polyps and 13 invasive intestinal adenocarcinomas. Mutations in the APC were the most frequently identified genetic alterations noted in both adenomas and adenocarcinomas. Large deletions of ≥10 bases as well as single and two base mutations were identified in approximately 70 % of both benign and malignant canine colonic tumours. Many of these genetic alterations were present in the proximity of the mutation cluster region. Besides being a very common genetic alteration, these results suggest that mutations in the APC are an early event in the process of carcinogenesis of colorectal epithelial tumours in dogs [40].

Not all molecular events driving tumorigenesis in dogs have yet been fully elucidated, and further research is required.

The adenoma to carcinoma sequence in canine colorectal tumours

Besides the interspecies homology seen when comparing malignant colorectal tumours in dogs with the corresponding lesions in humans, canine adenomatous polyps also share many homologies to analogous benign lesions in humans [28].

Stem cells of the colonic mucosa are present at the bottom of epithelial crypts, and they proliferate in this localization. Later, the daughter cells migrate into upper parts of the crypt where they lose their ability to divide, and they start to differentiate [10]. This process of proliferation and differentiation is tightly controlled by the wingless-related integration site (WNT) signalling pathway and involves the β-catenin protein [24].

Mutations leading to disturbances in the WNT molecular signalling axis are of major importance in human adenoma formation and colorectal carcinogenesis [41]. Considering the molecular characteristics of colorectal adenomatous polyps in dogs, cytoplasmic and abnormal nuclear accumulation of β-catenin protein was demonstrated immunohistochemically in tumour cells of canine colorectal adenomas [24]. These results suggest that dysregulation in the WNT signalling pathway, as in humans, is an important pathogenetic mechanism involved in the process of canine colorectal adenoma formation and progression [24]. Similar to the well documented adenoma to carcinoma progression in humans, canine colonic adenomas show a tendency to progress to invasive adenocarcinomas through malignant transformation [12]. These precursor lesions progressively acquire genetic alterations, which confer survival advantages and invasive potential of the tumour cells of the adenomatous lesion [35]. However, the molecular events driving carcinogenesis in canine adeno-

matous polyps seem to differ, at least partially, from those in humans because the acquisition of Tp53 mutations is not a common event in the case of colorectal adenomas in dogs [39]. Overall, high-grade dysplastic features can develop in canine adenomatous polyps, which progress into frank malignancy in 17 % to 50 % of this species [35].

Not all invasive adenocarcinomas of the large intestine in dogs develop from benign adenomatous polyps [31]. Beside the described adenoma to carcinoma sequel, another important concept of carcinogenesis in the alimentary tract is the *de novo* carcinoma theory [20]. Based on this cancer formation concept, the primary non-invasive tumour lesions have a macroscopically flat appearance and remain in this phenotypical form even after progression of the lesion and the development of microinvasion into the lamina propria [22, 31]. Since no grossly visible prominent polypoid lesions are identified in these cases, such lesions are easily missed using routine visualization methods [20]. Furthermore, the biological behaviour seems to differ in the case of macroscopically flat-shaped colorectal adenocarcinomas, which are reported to be highly malignant, even though the tumours are relatively small [15, 20].

Histological features of canine colorectal tumours

Several types of malignant tumours in the colonic and rectal area can be distinguished according to the WHO Histological Classification of Tumours of the Alimentary System of Domestic Animals [13]. Using this classification, epithelial intestinal malignancies can be divided into: the acinar (tubular) adenocarcinoma, the mucinous adenocarcinoma, the signet-ring cell adenocarcinoma, the papillary adenocarcinoma, the undifferentiated carcinoma category, and the adenosquamous carcinomas [13]. As is the case in large intestine epithelial malignancies of humans, intratumoural histological heterogeneity is a very common phenomenon in canine colorectal adenocarcinomas. The most common form of mixed adenocarcinoma is the combination of acinar (tubular) and papillary morphology. The presence of various growth patterns in a given adenocarcinoma case represents a challenge for accurate histopathological classification [31]. Considering other epithelial malignancies, pure squamous cell carcinomas in the large intestines of dogs are even rarer than in humans, with very few reported cases [19, 26].

S a i t o et al. [31] analysed the biological behaviour and prognostic implications of the various histological subtypes of tubular (acinar), papillary and mixed tubulopapillary subtypes of intestinal adenocarcinomas. No significant differences in the incidence rate of invasion and metastasis were noted. However, they suggested that dividing colorectal epithelial malignancies into polypoid and non-polypoid categories has more prognostic relevance. In their study, they demonstrated invasion and metastasis in 21 % of the cases of the polypoid type and 100 % of the non-polypoid type of adenocarcinomas. Canine adenocarcinomas with a non-polypoid growth pattern may be therefore considered as a more aggressive malignant subtype than polypoid growing tumours [31].

Clinical signs and diagnosis of canine colorectal tumours

Because of the interspecies similarities in the sense of anatomical localization and gross morphological features, perhaps not surprisingly the clinical signs related to the tumour growth are similar when comparing canine with human colorectal adenocarcinomas [1, 29].

Clinical signs related to the presence of neoplasia occluding the proximal parts of the colon are in the form of anorexia, vomiting, bleeding, weight loss and intussusception. Clinical signs related to the growth of adenocarcinomas in the rectum tend to be different [1, 25]. Classically described in German shepherd dogs, the cardinal clinical signs of rectal adenocarcinoma are constipation, tenesmus, dyschezia, and haematochezia [8]. The faeces in such affected dogs are often described as “ribbon-like” and such an appearance is caused by narrowing of the rectal lumen because of the progressively enlarging tumour mass [1].

Considering the diagnosis of tumorous masses in the rectum of dogs, digital examination is easy to perform and is known to be the most sensitive method used to detect these masses [5]. In early rectal masses, digital examination is known to be more sensitive than ultrasonography and proctoscopy [5, 6]. If large rectal masses and lesions characterized by deep infiltration are noted, then proctoscopy with subsequent biopsy is indicated [1]. In the case of rectal tumours, rectal proctoscopy has been found to be superior to flexible endoscopy. Besides providing better visualization, rigid proctoscopy allows the veterinary physician to use rigid biopsy forceps [38]. Ample tissue samples, which should also contain parts of the submucosa, can be obtained with the proper use of rigid biopsy forceps [1]. Such deep biopsy samples are of major im-

portance to properly differentiate benign adenomas from invasive colorectal adenocarcinomas, because both benign and malignant lesions can manifest macroscopically with the same gross appearance [5].

Compared with rectal tumorous masses, invasive adenocarcinomas localized in the ascending or descending colon are not readily accessible and are therefore more difficult to diagnose [5]. If the tumour is localized in the more distal parts of the descending colon, rigid proctoscopy may be performed [38]. However, this method does not provide access to the transverse and proximal colon in dogs. Colonoscopy may be indicated when there is suspicion of a neoplastic process in these areas [5]. Colonoscopy has the disadvantage that it requires cleaning the colon of faecal material and the use of anaesthesia of the patient [4]. To limit the number of colonoscopies required, abdominal ultrasonography is indicated before the performance of this procedure. Abdominal ultrasonography can detect lymphadenopathy and more obvious signs of widespread metastatic involvement in the abdominal area [1, 5, 38].

CONCLUSIONS

Dogs are an attractive animal model for the oncological study of colorectal cancer because of the interspecies similarities which allow us to recapitulate molecular etiopathogenesis, morphological characteristics and clinical features in humans. Furthermore, intestinal adenocarcinomas in dogs are naturally occurring heterogeneous cancers, which share the characteristics of sporadic human colon cancer and therefore fit better for the purpose of efficient oncological study than most xenografts or genetically modified rodent models. However, not all molecular events driving carcinogenesis in dogs are yet fully elucidated, and further research is required.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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EFFICACY OF DIFFERENT METHODS OF GENERAL ANAESTHESIA IN PIGS AND THEIR INFLUENCE ON HAEMATOLOGICAL PARAMETERS

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ABSTRACT

This study focused on how haematology parameters differ with different types of anaesthesia that is given to piglets. In this study 18 piglets were used divided into three groups. In each of these experimental groups a different combination of anaesthetics was used. The first group received the combination of azaperone, diazepam, and ketamine. In the second group, we used the combination of azaperone, xylazine, and ketamine. In the third group, the anaesthesia was induced by azaperone, diazepam, butorphanol and ketamine. Blood samples were taken from the piglets three times throughout the procedure and the relevant haematological parameters were determined. With respect to haematological values, the combination used in group 1 appeared as the best for the anaesthesia of piglets. The haematological values, such as MCV (mean corpuscular volume), were similar in all groups with the exception of the third group where the MCV and the MCH (mean corpuscular haemoglobin) were significantly lower 24-hour post anaesthesia compared to the other two groups. Lymphocytes in the first two groups showed a steady decrease, while in the third group their levels increased. The opposite trend was observed for the segmented neutrophils that showed a steady increase over the 24-hour period in the 1st and 2nd group while in group 3 their levels decreased. Abolishment of pedal and nasal reflexes to external stimuli applied every 15 min post ketamine administration was observed. The piglets in the 1st group showed no reflexes to external stimuli, while the worst responses were observed in the 2nd group where many of the piglets started to show reflexes very early within the experimental time.

Key words: anaesthesia; haematological parameters; pigs

INTRODUCTION

General anaesthesia is a medically-induced loss of consciousness with concurrent loss of protective reflexes due to anaesthetic agents. During this state, the patient is unarousable to verbal, tactile, and painful stimuli which facilitates the performance of surgical tasks. The purpose of using anaesthesia before surgeries is to minimize the stress and pain with as little side effects as possible to be able to perform various medical procedures. Situations in which the patient is going to experience low or high levels of pain will determine the type of anaesthesia as well as the length of care required after the procedure [10].

Premedication, analgesia and anaesthesia is often used before procedures that require more attention. Popular combined anaesthesia induced by xylazine and ketamine

lasts approximately 20–30 minutes, which is the general length of anaesthesia [5].

When anaesthesia needs to be prolonged, it is possible to administer an additional dose of the same anaesthetic to prolong it. However, the overall length of anaesthesia depends then considerably on the dosage, but giving additional dose will prolong the effect by approximately 10–20 minutes [2].

Premedication is a common practice frequently used before anesthetizing the swine. Premedication assists with putting the swine into a more sedated stage in which it is easier to induce anaesthesia and achieve recovery. One of the most common premedications used in piglets is azaperone. This sedative is fast acting, and when used intramuscularly its effects should appear 5–10 minutes after injection [1].

Anaesthesia puts the swine into an unconscious state; however, many anaesthetics do not have analgesic effects. Therefore, in order to ensure safe surgical procedures, an analgesic is administered together with the anaesthesia. There are groups of drugs that are most commonly used with anaesthetics, such as opioids, non-steroidal anti-inflammatory drugs (NSAIDs), and alpha-2 agonists. All these groups of analgesics are suitable for providing analgesia to swine; however, the opioids have a better analgesic and sedative effect compared to the NSAIDs. After major surgeries, the analgesics such as opioids are administered for several days post-surgery. Butorphanol and buprenorphine that are opioids are commonly used in swine. Butorphanol has an analgesic effect lasting 4–6 hours and has less pronounced side effects, while buprenorphine can be given every 12 hours and the onset of action is within 30–60 minutes [4].

Another group of analgesics known as the non-steroidal anti-inflammatory drugs (NSAIDs) is a group of organic acids involved in reducing the amount of inflammatory mediators. NSAIDs are not generally as effective against high level of pain compared to the opioids. In general, NSAIDs can be combined with opioids to give a more pronounced analgesic effect against the treatment that causes severe pain. Similar to opioids, the NSAIDs can be used post-surgery to ensure analgesia. The common NSAID analgesics include aspirin, meloxicam, and ketorolac [4].

In situations in which visceral analgesia is preferred, alpha-2 agonists may be the analgesics of choice. Alpha-2 agonists include xylazine, detomidine, and medetomidine. The analgesia effects of alpha-2 agonists involve peripheral and central alpha receptors [12]. Alpha-2 receptors are located in the dorsal horn of the spinal cord; stimulation of the alpha-2 receptors is located at the periaqueductal grey regions of the midbrain. This region is important as this is the pain modulation location that releases epinephrine [9].

One of the most popular uses of injectable anaesthesia is ketamine. Ketamine is an injectable anaesthetic with fewer side effects that could cause central nervous system issues. The use of ketamine will allow the eye and laryngeal reflexes during anaesthesia. It should be mentioned that the blocking of NMDA (N-methyl-d-aspartate) receptors are most important for the anaesthetic and analgesic effects. Also the use of ketamine does not suppress the cardiovascular functions but causes increased sympathetic outflow that increases the heart rate and arterial blood pressure. It is important to note that ketamine induces a significant increase in the cerebral blood flow, intracranial pressure and cerebrospinal fluid pressure as a result of cerebral vasodilation. Finally, ketamine will cause increased salivation and secretion of mucus in the respiratory tract that can be reduced by administration of an anticholinergic drug. Piglets can also show signs of tachycardia, hypertension, and poor muscle relaxation. The muscle rigidity can be reduced by administration of acepromazine, diazepam, xylazine, and medetomidine in combination with ketamine. The use of diazepam or xylazine with ketamine induces deep anaesthesia and good muscle relaxation. The combination of butorphanol, medetomidine and ketamine will provide anaesthesia lasting approximately 98 minutes. The combination of medetomidine and ketamine induces longer duration of muscle relaxation than other combinations such as xylazine and ketamine. Medetomidine and ketamine does cause a moderate cardiovascular stimulation with minimal respiratory effect [4].

During the procedure, it is necessary to constantly monitor the piglets and check for any abnormalities every 20–30 minutes. When the piglet is under anaesthesia, there can develop situations when the piglet may have difficulties without realising them. The respiration rate and hearth rhythm should be checked every 20–30 minutes. It is also important to monitor the body temperature. During some procedures, the piglet will rest on warming pads to stabilize its temperature, since anaesthesia also reduces the body temperature [5].

After termination of anaesthesia in swine, it is also im-

portant to consider the postoperative issues that could occur. During recovery from anaesthesia the animals should often be kept in a warmer place. Because of one of the side effects of anaesthesia, the body temperature of the pig can be drastically reduced. The decrease in body temperature as well as the pig′s inability to increase it can also contribute to changes in haematological parameters. The reduced body temperature as well as the potential decrease in cardiovascular functions caused by the use of medications will decrease blood circulation in the body. Reduced circulation will result in an overall decrease in blood volume. For any study involving the measurement of blood levels in an animal it would be ideal to withdraw the blood earlier rather than later in the postoperative management [9].

The goal of this study was to contribute to understanding how anaesthesia can affect the haematological values of swine. Changes in haematological parameters in piglets were observed in relation to combinations of anaesthetics and sedatives and the time immediately after anaesthesia and after 24 hours. Evaluated was also effectiveness of individual combinations of drugs used for induction of general anaesthesia in piglets on the basis of measurements of vital functions and the evaluation of reflexes in the duration of anaesthesia.

MATERIALS AND METHODS

The experiment was carried out on 18 piglets, aged 10–20 days. They were divided into three groups, every group consisted of 6 piglets. Piglets in the 1st group were given azaperone (4 mg.kg^{-1} body weight – b. w.), diazepam (1 mg.kg^{-1} b. w.) and ketamine (30 mg.kg^{-1} b.w.).

In the 2nd group the anaesthesia was induced by azaperone (5 mg.kg^{-1} b. w.), xylazine (2 mg.kg^{-1} b. w.), and ketamine (20 mg.kg^{-1} b. w.) and in the 3rd group by azaperone (4 mg.kg^{-1} b. w.), diazepam (1 mg.kg^{-1} b. w.), butorphanol (0.2 mg.kg^{-1} b. w.), and ketamine (20 mg.kg^{-1} b. w.).

The respiratory rate, heart rate, and temperature of piglets were measured prior to the injection of the first anaesthetic dose of azaperone. Also, prior to the injection of azaperone, blood samples were collected for comparison with the following haematological results. After the injection of azaperone the piglets were left undisturbed in a quiet environment for 15 minutes and after administration of diazepam or xylazine there was a pause of 10 minutes before injection of ketamine. After the administration of ketamine, the piglets were checked every 15 minutes for the respiratory rate, heart rate, temperature, and response to stimuli for the next 75 minutes. Blood samples were collected after 60 min and 24 hours post anaesthesia.

In order to compare the haematological profile of the piglets in individual groups, the results were statistically evaluated using the ANOVA test. To detect significant differences between the groups we used the Tukey's test in GraphPad Prism 3.0. The $P < 0.05$ was considered significant.

ETHICAL CONSIDERATIONS

The project of the study was approved by the Ethics Committee of the University of Veterinary Medicine and Pharmacy in Košice, permit No. EKVP/2022-23.

RESULTS

Comparison of results obtained in groups anaesthetized with different combination of drugs showed some significant differences. At the beginning of the experiment the counts of leukocytes (Leu) were significantly higher in group 1 in comparison with groups 2 and 3. However, in group 2, their counts increased by 24 hours and the difference compared to group 1 was insignificant. After 24 hours post anaesthesia the counts of erythrocytes (Er) were the highest in group 3 compared to the other two groups. Results of haematocrit at 1 hour and 24 hours post anaesthesia were similar in all three groups (Table 1).

MCV (mean corpuscular volume) at the start of the trial also showed levels very similar in groups 1 and 2. In the 3rd group there was detected a gradual decrease from the initial level to the post 24-hour level significantly different from groups 1 and 2 (Table 1).

As far as MCH (mean corpuscular haemoglobin) was concerned, we observed that 1-hour post ketamine administration there was a significant difference between group 1 and group 3 ($P < 0.01$) and 24-post ketamine significant differences, between group 1 and group 3, as well as between group 2 and group 3 ($P < 0.01$).

The haemoglobin levels showed no significant differences among groups, all three groups showed a slow in-

Table 1. Comparison of blood parameters between experimental groups

Parameter	Group 1	Group 2	Group 3	Ref. range
Hk, 0 hour [$L.L^{-1}$]	0.30 ± 0.02^{a}	$0.34 \pm 0^{a, c}$	0.30 ± 0.04^{c}	
Hk, 1 hour [$L.L^{-1}$]	0.28 ± 0.01	0.30 ± 0	0.30 ± 0.01	0.3–0.5
Hk, 24 hours [$L.L^{-1}$]	0.31 ± 0.02	0.31 ± 0.02	0.33 ± 0.02	
MCV, 0 hour [fl]	65 ± 3.01	65 ± 4.3	59.3 ± 8	
MCV, 1 hour [fl]	65 ± 3.3^{b}	63 ± 4	58 ± 7.8^{b}	50–68
MCV, 24 hours [fl]	64 ± 2.7^{b}	63 ± 3.5^{c}	$57 \pm 9.2^{b, c}$	
Er, 0 hour [$T.L^{-1}$]	5.6 ± 2	5.1 ± 0.5	6 ± 0.1	
Er, 1 hour [$T.L^{-1}$]	4.3 ± 0.4	4.9 ± 0.5	7.1 ± 3	5–8
Er, 24 hours [$T.L^{-1}$]	4.8 ± 0.5^{b}	5.2 ± 0.6	7 ± 1.8^{b}	
Leu, 0 hour [$G.L^{-1}$]	$14 \pm 2.5^{a, b}$	$10 \pm 2.6^{a, c}$	$6.8 \pm 2.2^{b, c}$	
Leu, 1 hour [$G.L^{-1}$]	13.2 ± 3.2^{b}	11 ± 3.1	7 ± 3^{b}	11–18
Leu, 24 hours [$G.L^{-1}$]	17 ± 10.1	19 ± 10.2	7 ± 1.8	

Hk – haematocrit; MCV – mean corpuscular volume; Er – erythtrocytes; Leu – leukocytes; $L.L^{-1}$ – litres of cells per litre of blood; fl – femtolitres; $T.L^{-1}$ – $Tera.L^{-1}$ ($10^{12}.L^{-1}$) ; $G.L^{-1}$ – $Giga.L^{-1}$ ($10^{9}.L^{-1}$);
[a] – G1:G2 $P < 0.05$; [b] – G1:G3 $P < 0.05$; [c] – G2:G3 $P < 0.05$

Table 2. Comparison of leukogram between experimental groups (in percentage)

Parameter	Group 1	Group 2	Group 3	Ref. range
Ly, 0 hour [%]	62 ± 5.2	63 ± 4.1	68 ± 0.7	
Ly, 1 hour [%]	62 ± 2.3	60 ± 4.2	63 ± 2.1	39–62
Ly, 24 hours [%]	60 ± 2.8^{b}	61 ± 7.8^{c}	$76 \pm 8.5^{b, c}$	
Neu-S, 0 hour [%]	30.3 ± 7	31.2 ± 4.1	28.2 ± 0.71	
Neu-S, 1 hour [%]	31.2 ± 1.47	33.2 ± 4.3	33.5 ± 0	28–47
Neu-S, 24 hours (%)	33 ± 3.2^{b}	36 ± 10^{c}	$20 \pm 9.2^{b, c}$	
Neu-B, 0 hour [%]	4.67 ± 2^{b}	3.7 ± 0.81	2 ± 0^{b}	
Neu-B, 1 hour [%]	3.8 ± 1	4 ± 1.4	2.7 ± 1	0–4
Neu-B, 24 hours [%]	4 ± 1.7	3.5 ± 1	3.2 ± 1	
Eo, 0 hour [%]	3 ± 1.4^{b}	1.7 ± 1.5	0.2 ± 0^{b}	
Eo, 1 hour [%]	2.7 ± 1.5^{b}	1.5 ± 0.83^{c}	$0,0^{b, c}$	0.5–11
Eo, 24 hours [%]	$2.2 \pm 0.7^{a, b}$	0.7 ± 0.82^{a}	0.2 ± 0.71^{b}	

Ly – lymphocytes; Neu-S – segmented neutrophils; Neu-B – band, unsegmented neutrophils; Eo – eosinophils;
[a] – G1:G2 $P < 0.05$; [b] – G1:G3 $P < 0.05$, [c] – G2:G3 $P < 0.05$

Table 3. Changes in respiration rate (number per minute) in experimental groups post ketamine administration

Groups	0 min	15 min	30 min	45 min	60 min	75 min
Group 1	66.8 ± 2.83	59.3 ± 19.8	72.2 ± 3.5	61.7 ± 14.1	64.3 ± 0	65.3± 28.3
Group 2	66.7 ± 22.6	54.5 ± 9.9	76.3 ± 1.41	97.7 ± 7.07	96.7 ± 5.7	89.8 ± 4.24
Group 3	51.3 ± 5.7	58.7 ± 1.41	48 ± 12.7	56.7 ± 17	51.7 ± 8.5	49.7 ±1.41

0, 15, 30, 45, 60, 75 min – respiration rate at 0, 15, 30, 45, 60 and 75 min post ketamine administration

Table 4. Comparison of pulse (number per minute) among experimental groups

Groups	Pulse 0	Pulse 15	Pulse 30	Pulse 45	Pulse 60	Pulse 75
Group 1	129 ± 9.9	103 ± 24.0	122 ± 10.6	115.7 ± 4.2	116.5 ± 5.7	125.5 ± 7.8
Group 2	132.8 ± 1.4	128 ± 28.3	139.5 ± 23	138 ± 18.4	137 ± 15.6	131 ± 56.6
Group 3	125 ± 7	151.7 ± 7.1	148 ± 1.4	137.3± 26.9	151 ±11.3	126.3 ± 8.5

Pulse 0, 15, 30, 45, 60, 75 – pulse at 0, 15, 30, 45, 60 and 75 min post ketamine administration

Table 5. Comparison of body temperature [°C] among experimental groups

Groups	Temp 0	Temp 15	Temp 30	Temp 45	Temp 60	Temp 75
Group 1	39.4 ± 0.07	37.6 ± 0.92	36.6 ± 1.7	36 ± 2.12	35.6 ± 2.48	35.0 ±2.47
Group 2	39.8 ± 0.14	38.45 ± 0.42	38.9 ± 1.13	38.5 ± 0	37.4 ± 0.85	37.3 ± 0.35
Group 3	38.3 ± 0	39.95 ± 1.56	36.7 ± 1.48	36.45 ± 1.5	36.2 ± 1.41	35.7 ± 0.28

Temp 0, 15, 30, 45, 60, 75 – body temperature at 0, 15, 30, 45, 60 and 75 min post ketamine administration

Table 6. Abolishment of reflexes to stimuli in individual experimental groups post ketamine administration

	P–15 min	N–15 min	P–30 min	N–30 min	P–45 min	N–45 min	P–60 min	N–60 min
Group 1	100 %	100 %	100 %	100 %	66 %	66 %	33 %	33 %
Group 2	83 %	66 %	83 %	16 %	0 %	0 %	0 %	0 %
Group 3	100 %	100 %	83 %	83 %	50 %	50 %	50 %	50 %

E 1 – Experimental group 1; E 2 – Experimental group 2; E 3 – Experimental group 3;
P–15, P–30, P–45, P–60 – pedal reflex at 15, 30, 45, 60 min post ketamine administration;
N–15, N–30, N–45, N–60 – nasal reflex at 15, 30, 45, 60 min post ketamine administration

crease throughout the initial phase up to the last sampling. They ranged from 10.2 g.dl^{-1} to 11 g.dl^{-1} which was still within the normal reference range.

Lymphocyte (Ly) levels in groups 1 and 2 showed a slow decrease, however in group 3 we observed an opposite as there was an increase at 24 hours. Segmented neutrophils (Neu-S) showed an opposite trend, in groups 1 and 2 they steadily increased from the initial levels up to the 24-hour period while in group 3 they drastically decreased within 24 hours far below those in the other two groups. Eosinophil (Eo) counts steadily decreased in groups 1 and 2 while their levels in group 3 were much lower (below the reference range) and differed significantly from those in groups 1 and 2 (Table 2).

Within the experiment it was also important to look at the recordings of respiration, pulse, and temperature among the piglets. For the first 15 minutes the respiration in all 3 groups of piglets gradually decreased. After that the respiration in group 3 continued to decrease while increasing respiration was observed in groups 1 and 2. After 45 minutes of observation, respiration in group 1 was similar until the end of the recording, respiration in group 2 was increased and that in group 3 continued to decrease (Table 3).

The pulse rate of the piglets in group 1 decreased until 60 min post ketamine administration, then returned to the initial level. In group 2 the changes in pulse rate were insignificant while pulse rate in group 3 was increased from 15 min up to 60 min of recording and by 75 min dropped to the initial level (Table 4).

At the beginning of the experiment the body temperature of piglets in all 3 groups were normal. As the time progressed, the temperature of piglets continued to decrease. From the start of the recordings, the decrease was steady and similar in all groups, with the exception of 15 min post ketamine administration in group 3 (Table 5).

The last observation involved pedal and nasal responses to a stimulus. We observed whether the piglets responded to an external stimulus applied every 15 minutes. In the first group none of the piglets showed any signs of reflex for the first 30 minutes, which was assessed as 100 % negative response. At 60 min post ketamine administration, the majority of piglets showed signs of reflex.

In group 2, at first 15 min post ketamine administration, there were some positive responses to the stimuli. As the time continued, piglets showed more positive reflexes and by 45 min post ketamine administration all piglets reacted to stimuli.

In the third group the piglets showed no signs of reflexes within the first 15 min post ketamine administration. After 30 min there were some signs of reflexes and after 45 min until the end of recording (60 min) half of the piglets in this group showed signs of a reflex when tested. (Table 6).

DISCUSSION

Our results of the examination of piglet blood showed that the combinations of drugs used to induce anaesthesia resulted in changes in haematological parameters. The increase in leukocyte count observed in this study was within the reference range so this would not be a cause for concern.

The areas that should be looked at on the basis of our results involved haematocrit and haemoglobin. The reference range of haematocrit for piglets is 0.3–0.5 L.L^{-1} [11]. Results of haematocrit were similar in all three groups and corresponded to the reference range (Table 1). The reference range for haemoglobin in piglets is 9–14.4 g.dl^{-1} [11]. The results obtained in our experiment showed that the haemoglobin in piglets ranged from 10.2 g.dl^{-1} to 11 g.dl^{-1} which was still within the normal reference range for piglets.

The experiments conducted by other authors were similar to ours although they used different anaesthesia combinations to see whether there were haematological differences between the groups. In the study by L e r v i k et al. [8] that was similar to ours, piglets were given combinations propofol-ketamine-dexmedetomidine or propofol-ketamine-fentanyl. The dexmedetomidine group showed a higher haemoglobin level compared to the fentanyl group, but still within the normal range. In the above study both combinations provided stable cardiovascular conditions in normovolaemic, healthy pigs. Based on cardiovascular response and depression of nociceptive withdrawal reflex, dexmedetomidine apparently provided superior analgesia to fentanyl.

Xylazine has the effect of cardiac depression as well as muscle relaxation. However, when given at higher doses, xylazine can cause vomiting and higher cardiac depression [7]. G ó m e z de S e g u r a et al. [3] wrote that xylazine

did not induce adequate sedative or analgesic effects in pigs at any dosage tested. However, cardiovascular effects were considerable. At lower dosages, cardiovascular effects were characterized by bradycardia and biphasic blood pressure response, initial hypertension was followed by hypotension. At higher dosages, severe hypotension with moderate bradycardia was followed by marked bradycardia. The results obtained in our experiment showed that hypothermia was observed in group 2 that was administered combination of ketamine and xylazine and azaperone for sedation. In this group we also observed tachypnoe due to increased stress and short duration of anaesthesia. We can assume that the hypotension induced by xylazine also contributed to a decrease in the body temperature of piglets. In our study the haematocrit and haemoglobin levels in group 2 were similar to those in the other groups that did not receive the combination with xylazine.

One of the important areas is the response to stimulation that the piglets had experienced during the anaesthesia. Our results showed that piglets did not respond to the stimulus applies early after the ketamine administration, however later, after 45 minutes, many of the piglets showed pedal and nasal reflexes to external stimuli. The reaction to stimulation is an important indicator how well the piglets responding to the anaesthesia. This observation is similar to that reported by T a n a k a et al. [13] who observed that the use of anaesthetics such as ketamine combined with other anaesthetic drugs is able to keep the piglets in a deep sedation with adequate muscle relaxation.

From the results of the responses to stimuli it can be concluded that the worst analgesic effect was achieved in the piglets from group 2 anaesthetised by combination with xylazine. In this group some piglets were seen to show a reflex response as early as 15 minutes into the experiment.

In a similar experiment it was revealed that the combination involving xylazine also showed that the animals being tested showed poorer recovery score and quicker time to the end of the anaesthesia. As the piglets anaesthetised with xylazine combination showed the poorest results the authors concluded that xylazine does not give the best analgesia [7].

CONCLUSIONS

The results of this study on piglets showed that the best anaesthetic depth from the point of view of responses to external stimuli was achieved with combination used in group 1. All piglets in this group showed no reflex response up to at least 30 min after administration of ketamine. In groups 1 and 3 where most of the piglets were under anaesthesia for longer time the body temperature decreased after 1 hour of anaesthesia.

Counts of erythrocytes and leukocytes in blood samples from group 3 showed little variations. A decrease in erythrocyte counts was detected in group 1 while leucocytes increased in groups 1 and 2 in comparison with the initial sample. At the last sampling, segmented neutrophils in the third group were significantly lower in comparison with groups 1 and 2 while the lymphocytes in this group were significantly higher compared to the other two groups. With respect to haematological values the combination used in group 1 appeared as the best for anaesthesia of piglets.

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LYME BORRELIOSIS IN DOGS: BACKGROUND, EPIDEMIOLOGY, DIAGNOSTICS, TREATMENT AND PREVENTION

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ABSTRACT

Lyme borreliosis (LB) is a multisystemic tick-borne disease that can affect many organs and have various clinical manifestations in dogs. We attempted to summarise various aspects of Lyme disease: i.e., pathogenesis, epidemiology, benefits and risks of diagnostic approaches, treatment options, and prevention in dogs. Several diagnostic bottlenecks for LB in dogs and humans are compared. Because the occurrence of LB in both humans and dogs is closely related, monitoring its prevalence in dogs as sentinel animals is an excellent aid in assessing the risk of Lyme disease in a given geographic area. Although clinical symptoms in humans help clinicians diagnose LB, they are ineffective in dogs because canines rarely exhibit LB symptoms. Despite significant differences in sensitivity and specificity, serological two-step detection of antibodies against *Borrelia* spp. (ELISA and Western blot) is the most commonly used method in humans and dogs. The limitations of the assay highlight the need for further research to develop new clinical markers and more accurate diagnostic tests. Due to the lack of a specific all-encompassing LB test, a definitive diagnosis of LB remains a difficult and time-consuming process in human and veterinary medicine. Understanding the disease prevalence and diagnostics, as well as preventing its spread with effective and timely treatment, are fundamental principles of good disease management.

Key words: *Borrelia burgdorferi*; borreliosis; diagnostics; dogs; Lyme disease; treatment

INTRODUCTION

Lyme borreliosis (LB) or Lyme disease is a tick-borne zoonotic infection that primarily occurs in the temperate regions of Europe, North America, and Asia. Its geographic distribution is rapidly expanding due to climate change, which affects tick survival, host populations, and human behaviours [42]. Lyme disease is caused by *Borrelia burgdorferi* sensu lato (s.l.) complex genospecies and is generally transmitted by ticks of the *Ixodes* complex, with *I. ricinus* being the major vector in Europe and *I. scapularis* in the United States [84]. The frequency of *B. burgdorferi* infection in humans and dogs is related to the tick population density and biotope location [34]. Dogs are frequently used as seroindicators for risk assessment of Lyme disease in geographic areas [8, 28]. Sensitive de-

tection of tick-borne disease-causing organisms in dogs is diagnostically important for veterinarians, pets, and their owners, and epidemiologically important for public health surveillance [53].

The purpose of this review is to provide a more comprehensive understanding of: the pathogenesis, epidemiology, benefits and risks of diagnostic approaches, treatment options, and prevention of Lyme borreliosis in dogs. We attempted to highlight fundamental issues and differences in diagnostics in dogs versus commonly used human tests.

Etiologic agent and its characteristics

Lyme disease is caused by spirochetes *B. burgdorferi* sensu lato (s.l.) complex which was considered a single species after its discovery in the 1980s [42]. Currently, the complex consists of at least 22 recognized or proposed genospecies that can be naturally transmitted among different vertebrate species and ticks of the genus *Ixodes* [58]. The predominant tick vectors in Europe and Asia are *I. ricinus* and *I. persulcatus*, while in North America it is *I. scapularis* or *I. pacificus* [86],

Borrelia burgdorferi s.l. is a group of gram-negative, spiral-shaped, microaerophilic bacteria with irregular coils, 0.2–0.3 µm in diameter and 10–40 µm in length, included in the family *Borreliaceae*, within the order *Spirochaetales*, the class *Spirochaetia* and phylum *Spirochaetes*. In the recent past, there have been a controversial proposal to modify the taxonomy of the family *Borreliaceae* into two genera, *Borrelia* and *Borreliella*, in order to reflect their genetic and phenotypic divergence [5]. Some authors recommend that the spirochetes responsible for relapsing fever retain the generic name *Borrelia* and those causing Lyme borreliosis, including species of the *Borrelia* burgdorferi s.l. complex, should be given the new name *Borreliella* [6, 35]. However, proposed changes have been contested and remain under debate [60].

At least five genospecies (*B. garinii, B. afzelii, B. burgdorferi* sensu stricto (s.s.), *B. bavariensis* and *B. spielmanii*) can cause Lyme borreliosis in humans in Europe, of which only *B. burgdorferi* s.s. has been known to cause borreliosis in North America [84, 86]. Occasionally, *B. bissettii* and *B. mayonii* are reported in patients in the USA [32, 70]. *B. garinii* is most commonly associated with neuroborreliosis, which affects a higher proportion of human patients in Europe than in the United States. *B. afzelii* infections are mostly manifested with a rare chronic skin condition such as *acrodermatitis chronica atrophicans* while *B. burgdorferi* s.s. seems to be the most arthritogenic with Lyme arthritis as the most frequent form of borreliosis in the USA [83, 84]. Pathogenic significance of other species such as *B. lusitaniae, B. bissettii* and *B. valaisiana* in humans and animals are still questionable [18, 57, 59].

Three human genospecies have been identified as pathogenic to dogs [29]: *B. burgdorferi* s.s., *B. afzelii*, and *B. garinii* (Table 1). So far, no other species has been reported that infect dogs.

Table 1. Genospecies in the *B. burgdorferi* s.l. complex of relevant pathogenicity for humans and dogs

Genospecies	Typical vectors	Main reservoir	Pathogenicity	Epidemiological distribution
***B. burgdorferi* sensu stricto**	*I. scapularis, I. pacificus, I. ricinus, I. persulcatus (?)*	mammals, birds	humans, dogs	North America, Europe
B. garinii	*I. ricinus, I. persulcatus*	birds	humans, dogs	Europe, Asia
B. bavariensis	*I. ricinus, I. persulcatus*	small mammals, birds	humans	Europe, Asia
B. afzelii	*I. ricinus, I. persulcatus*	small mammals	humans, dogs	Europe, Asia
B. spielmanii	*I. ricinus, I. persulcatus*	garden dormouse	humans	Europe

Data reviewed from [55]

Spirochetes have several periplasmic flagella that are anchored to the ends of the bacterium, allowing them to move at a typical undulating and rotational speed of up to 2 mm per minute. *Borrelia* spp. can freely pass through the endothelium of blood vessels and overcome the blood-brain barrier. They are able to enter fibroblasts, dendritic cells and macrophages, in which they survive for a relatively long time [46]..

Various surface lipoproteins (OspA – OspF, DbpA, DbpB, CspA, VlsE, BptA, p13, p66, BesC, BamA, Lmp1, BB0405 and others) are found on the outer membrane of *B. burgdorferi*, which play important roles in virulence, host-pathogen interaction and in maintaining the enzootic cycle of *B. burgdorferi*. Several immunodominant surface lipoproteins have been identified that can recognize and bind host proteins, accelerate bacterial adherence to host cells, and evade the host immune response through antigenic variations as well as activation of the complement system [41]. *B. burgdorferi* s.l. is able to adapt under different environmental conditions and temperatures during the transition from the vector to the host (homothermic, poikilothermic, heterothermic vertebrates) which is reflected in different gene expression of some lipoproteins: OspA expressed mainly during colonization of the tick is replaced in the host by OspC, crucial in an early mammalian infection [72]. OspA and OspC are considered excellent candidates for the development of new vaccines, and understanding of their antigenic structure with its natural diversity essential for the correct interpretation of the immune response induced by vaccination or infection [19, 39]. Another outer surface lipoprotein VlsE (variable major protein-like sequence, expressed) is an antigenically variable protein that evades the host immune response by constantly changing its surface epitopes [66] and replaces the OspC protein on the outer surface of *B. burgdorferi* during persistent infection [92].

Under unfavourable conditions, *Borrelia* spp. are able to form extracellular membrane cystic forms (blebs) with reduced metabolic activity that probably have a defensive function against the penetration of antibodies and antibiotics. Cystic forms can reversibly change into metabolically active spirochetal forms and are responsible for "recurrent" or "dormant" LB. *Borrelia* can thus persist unrecognized in the host's body for several years [78].

Clinical manifestations

Lyme borreliosis is a multisystemic disorder with distinct spectra of clinical manifestations in humans and dogs. In humans, the infection can begin asymptomatically or with influenza-like symptoms (fever, headache, mild stiff neck, arthralgia, and myalgia), but typical LB symptoms can be divided into three stages: early localised (*erythema migrans*), early disseminated (neuroborreliosis, carditis, ocular manifestations), and late disseminated neuroborreliosis and carditis (*acrodermatitis chronica atrophicans*, arthritis) [55, 83]. *Erythema migrans*, a common skin rash of early localised infection that affects approximately 70–80 % of human patients [77], is rarely been detected under the dog fur, so the disease often goes unnoticed until later symptoms appear [4, 52]. Clinical symptoms of LB in humans can be very similar in Europe and in the USA, however, there are some differences due to the greater variety of *B. burgdorferi* s.l. genospecies in Europe. Neuroborreliosis is the most common disseminated form of human LB in Europe, followed by Lyme arthritis, and, on rare occasions, borrelial lymphocytoma, *acrodermatitis chronica atrophicans*, and Lyme carditis [83].

Because the majority of the infected dogs (95 %) show no clinical signs, bacteria can often spread to other tissues and cause more severe manifestations such as: polyarthritis, transient fever, anorexia, lethargy, neurological dysfunctions, and/or lymphadenopathy, particularly in the prescapular or popliteal nodes [4, 50, 52]. Arthritis is the most common syndrome, affecting one to a few joints, particularly the carpal joints, with or without swelling, causing lameness or shifting-leg. In dogs, signs of neurologic (facial paralysis, seizures, aggression) or cardiac manifestations (myocarditis or conduction abnormalities with bradycardia) are uncommon and poorly documented [82]. Furthermore, chronically infected dogs may develop serious Lyme nephritis, associated with rapidly progressive glomerulonephritis, that is less common than Lyme arthritis [51]. Because the pathogenesis of Lyme nephritis has not been experimentally replicated, it is thought to be caused by the deposition of antigen-associated immune complexes in the kidneys [38].

Incidence and prevalence

Canine vector-borne diseases, including LB, have been a matter of concern in Europe for several decades, with changes in prevalence and distribution observed. Further-

more, tourism, traveling with dogs, and importing dogs from endemic areas all contribute significantly to the spread of canine vector-borne diseases to new areas [74, 100].

Monitoring the prevalence of *B. burgdorferi* s.l. in ticks has been considered pivotal in the public health risk assessment of LB. *Ixodes* spp. which is an important vector of LB because its uninfected stages (larvae, nymphs, adults) can feed on infected wildlife reservoirs, become infected, and then transmit the infection to other mammals when taking their next blood meal. As ticks are indiscriminate in their choice of host, *B. burgdorferi* s.l. can be transmitted from wild animals to companion animals and humans [81]. Changes in climate and land use can have a major impact on the population size of many insect vectors, ticks and wildlife reservoirs, such as rodents or migratory birds [74], even though S t r n a d et al. [91] have found that the prevalence of *B. burgdorferi* s.l. in *I. ricinus* ticks remains reasonably constant over recent decades despite tick spreading into higher latitudes and altitudes. After summarising published data since 2010 to 2016 [91], the meta-analysis showed that the overall mean prevalence of *B. burgdorferi* s.l. in ticks in Europe is 12.3 % with the highest incidence in Central Europe (Austria, Czech Republic, Germany, Hungary, Poland, Slovakia, and Switzerland) and Balkan Peninsula (Romania, Serbia, and Bosnia and Herzegovina), with 19.3 and 18.5 %, respectively. Slovakia has a long-standing tradition in researching the ecology of ticks and the epidemiology of tick-borne diseases. *Borrelia* prevalence in questing ticks in Slovakia belongs to the highest in Europe, however, it varies significantly in suburban forests from 4.4 % in northern Slovakia [67] up to 53.2 % in eastern Slovakia [97]. In general, the prevalence of *B. burgdorferi* s.l. in ticks in urban habitats is lower, but they still pose a risk for disease transmission to humans and dogs [85].

Prevalence estimates of LB in dogs are often inaccurate due to a lack of visible clinical signs and no national surveillance system for companion animal diseases. However, screening tests for canine antibodies to *B. burgdorferi* are widely used in diagnostic laboratories and by veterinarians and the results are presented by the Companion Animal Parasite Council (CAPC) for estimating *B. burgdorferi* seroprevalence in the US and Canada [51]. Based on the data collected by the CAPC [13], 3.82 % of canine serum samples were positive for *B. burgdorferi* from over 11 million samples submitted in 2022 with the highest seroprevalence in the New England, mid-Atlantic and upper Midwest regions, ranging from 5.34 % up to 15.66 %. Moreover, CAPC estimates that the number of detected positive canine samples probably represents less than 30 % of total canine seropositivity to *B. burgdorferi* in the US. In general, overall *B. burgdorferi* seroprevalence among dogs in the US has been declining since 2016 (6.43 %) when compared to 2022 (3.82 %). Humans and dogs share many of the same risk factors for encountering *B. burgdorferi*-infected ticks due to their close association; thus, dogs serve as excellent sentinels for human Lyme disease risk. This link is highlighted by comparing maps of human Lyme disease prevalence with maps of *B. burgdorferi* seroprevalence in dogs [73].

Individual countries frequently monitor canine Lyme disease in Europe, but aggregated data may be useful in understanding its distribution on a larger scale. M i r o et al. [63] used point-of-care ELISA testing data to map the distribution and seropositivity of dogs for selected canine vector-borne diseases (*Anaplasma* spp., *Ehrlichia* spp., *Borrelia burgdorferi, Leishmania* spp., and *Dirofilaria immitis*) in Europe since 2016 through 2020. *Borrelia burgdorferi* antibody positivity was concentrated in Northern and Eastern Europe with higher positivity rates (> 5 %) in Austria, the Czech Republic, Estonia, Finland, Germany, Lithuania, the Netherlands, Norway, Poland, Slovenia, Sweden and Switzerland and lowest rates (< 1 %) in Andorra, Croatia, Greece, Hungary, Italy, Malta, Portugal, Romania and Spain. The highest positivity of the test was recorded in Sweden (13.3 %) and the lowest in Greece (< 0.1 %). Annual European test positivity rates decreased from 3.3 % in 2016 to 2.4 % in 2020 for *B. burgdorferi* [63]. G o o s s e n s et al. [33] tested 448 hunting dogs and 75 healthy dogs living in the countryside of the Netherlands for antibodies against *B. burgdorferi* by a whole-cell ELISA. The dogs were of different breeds and age. Antibodies against *B. burgdorferi* were detected in 18 % of hunting dogs and 17 % of pet dogs. In the group of hunting dogs, individuals older than 24 months appeared to have a greater risk of being exposed (22 %) than younger dogs (9 to 11 %), and in addition, the seroprevalence among hunting dogs over 24 months of age remained stable at approximately 22 %. No significant rise in seroprevalence in dogs older than 24 months may indicate that seropositivity after *B. burgdorferi* infection in dogs is rather short, approximately 1 year compared to humans whose seroprevalence

can last considerably longer [33]. Another study examined 846 dog sera for the presence of anti-*Borrelia* antibodies by using ELISA with a mixture of *B. garinii, B. afzelii, B. burgdorferi* s. s. antigens revealed 283 positive samples with mean seroprevalence 33.5 % in dogs [87].

Interestingly, there may have been a breed predisposition of Bernese Mountain Dogs for Lyme borreliosis due to their higher seropositivity against *Borrelia burgdorferi* s. l. in some regions in Europe [30, 69], although it was not sufficiently verified [30]. On the other hand, not having discovered any ticks on a small or medium dog breed can be used as a significant indicator for *Borrelia* seronegative status [9]. However, no correlation between the number of seropositive dogs and their size and gender was reported in some studies [23, 11].

In the United States, ~30,000 to 40,000 cases of human Lyme disease are reported to the Centers for Disease Control and Prevention (CDC) each year [15]. Similarly, the number of cases in Europe has increased steadily since 1990 and more than 360,000 cases having been reported over two decades. Between 1990 and 2010, the highest average incidence rates of human Lyme disease in Europe were found in Belarus, Belgium, Croatia, Norway and Serbia (< 5/100 000), Bulgaria, Finland, Hungary, Poland and Slovakia (< 16/100 000), the Czech Republic, Estonia, and Lithuania (< 36/100 000) and Slovenia (< 130/100 000) [102]. The incidence of human Lyme borreliosis has been slightly increasing in Slovakia since 2010 [85].

Methods of detection of Lyme disease

The diagnosis of canine borreliosis is based on: the epidemiological case history, duration of tick exposure, compatible clinical symptoms, exclusion of other diseases, response to antibiotics and laboratory evidence of infection. In dogs, it is difficult to definitively attribute a particular set of clinical signs to underlying *B. burgdorferi* infection because most dogs infected with *B. burgdorferi* will never develop signs. Since the *erythema migrans* is rarely found in dogs, the diagnosis is based on various laboratory techniques including culturing, histological examination of thin sections, serological tests and polymerase chain reaction (PCR) [56, 62, 89]. We are reviewing some of the routinely used diagnostic techniques for LB.

The direct detection methods

For many years, direct detection methods such as cultivation or microscopy have been the "gold standard" for detecting and classifying bacterial infections. As *Borrelia* spp. cannot be identified by standard optical microscopy or by Gram staining, a dark-field microscopy or phase-contrast microscopy have become a more accurate method in routine diagnostics [20].

In vitro culture is a relatively reliable method for the demonstration of *B. burgdorferi* in clinical samples even though it requires special growth media and long incubation periods. It is rarely sensitive enough because bacterial load in tissue samples or body fluids is generally low with the exception of skin samples from human patients. Accordingly, *B. burgdorferi* is detected most frequently in collagen-rich tissue including skin in experimentally infected dogs [47]. Cultivation is still widely used in the field of LB research during preparation of cultures for experimental work and for the preparation and control of materials in antigen production, and occasionally in clinical investigations [71].

The indirect detection methods

The indirect detection of *B. burgdorferi* by identifying specific antibodies in serum has become an important tool in diagnosing LB. However, the presence of antibodies can only indicate previous exposure to pathogen, but does not prove clinical disease in a given patient [45, 84, 93].

The indirect immunofluorescence assay (IFA) is one of the first established serological methods for the LB testing for Borrelia-specific immunoglobulins M (IgM) detectable within 7 days of infection and immunoglobulins G (IgG) in a few days later. Despite its limitations such as low specificity and cross-reactivity of the antibodies with other antigens, such as heat-shock proteins and flagellar antigens, which often lead to false-positive results, IFA is still used to detect antibodies against *Borrelia* spp. in veterinary practices in Germany and other European countries [2, 11]. In the study by B a r t h et al. [7] IFA-IgG antibodies directed against *B. burgdorferi* s. l. were detected in 51 of 200 serum samples, resulting in a prevalence of 25.5 %. The sensitivity and specificity of IFA-IgG were 76.6 % (95 % confidence interval [CI] 46.87–86.72) and 87.1 % (95 % CI 80.06–91.90), and 26.3 % (95 % CI 11.81–48.79) and 81.0 % (95 % CI 73.64–86.71) for IFA-IgM, respectively. Based on their data, both IFAs had very low sensitivity and specificity and should not be recommended for screening purposes.

In recent years, two-tiered test methods, including infection-specific tests using recombinant antigens, have been developed. The test has two components: a highly sensitive screening enzyme-linked immunosorbent assay ELISA to filter out negative samples with high fidelity and a confirmation assay Western blot (WB) used in a second step to further characterise positive samples or distinguish infected from vaccinated animals [10, 11, 45]. Serological tests are based on the detection of anti-*Borrelia* antibodies produced by patients against antigens mostly on the surface of *B. burgdorferi*. A flagellin B (FlaB or 41 kD flagellin), a major component of the periplasmic flagellar filament crucial for bacterial mobility and OspC, a lipoprotein needed for the establishment of early localised infection, were described as early immunodominant antigens after infection. The C6 antigen, a peptide based on the 6th invariable region (IR6) of a surface lipoprotein VlsE that provides an antigenic disguise of *B. burgdorferi*, is the foundation of the C6 peptide assay [48]. All of the antigens mentioned above are expressed during the early stages of infection and are currently used as target antigens in many serological tests to detect anti-*Borrelia* antibodies produced by patients. Some of their drawbacks are their cross-reactivity and high variability. FlaB immunologically cross-reacts with many other bacterial flagellins, and a high percentage of healthy non-*B. burgdorferi* infected individuals can have antibody reactivity with it. OspC, although less cross-reactive, is a highly variable protein with 24 serotypes. The least cross-reactive antigen expressed after infection is VlsE (IR6). However, IR6 does not bind IgM well, and has more variability than originally thought. The reality is that sensitivity of serological tests is lower in the early stage of the disease and they are incapable to prove an active infection due to a larger number of *B. burgdorferi* antigens recognized. There is a need to develop simpler, more sensitive, and more specific assays [22, 24]. C6 ELISA is a relatively reliable diagnostic tool using a specific synthetic peptide, the 25-mer C6 peptide of VlsE. The region is highly conserved among different *B. burgdorferi* s.l. genospecies and highly immunogenic in the canine host. C6 ELISA has clearly demonstrated that a peptide containing a specific epitope can improve both sensitivity and specificity when compared to whole protein-based assays. However, the C6 assay has limitations that have precluded its adoption as a stand-alone assay. Hence, improved serological assays are needed, and it is likely that a multipeptide assay based on peptides containing specific epitopes from multiple key *B. burgdorferi* antigens could solve many of the issues of current LB serodiagnosis [10, 22, 49].

The kinetic enzyme-linked immunosorbent assay (KELA) is a diagnostic method in ELISA format using whole-cell sonicates for the detection of *Borrelia* antibodies. Limitations of KELA include the possibility of cross-reactions with non-Borrelia-specific antibodies, and the inability to distinguish vaccination from natural infection. However, due to its convenient use and the possibility for automation, KELA has become more popular than other immunoassays despite some of its shortcomings [40, 54].

Many different assays are available as rapid tests in veterinary practice or as more sophisticated laboratory tests. Recently, VetScan FLEX4 Rapid Test (Abaxis, Inc., Union City, CA) and the VetScan canine rapid Lyme test (Abaxis, Union City, CA, USA) have been launched as new assays to detect tick-borne pathogen antibodies and heartworm antigen simultaneously. Both tests are able to detect antibodies reactive to C6 of the VlsE on the surface of *B. burgdorferi* [31, 48, 53]. The SNAP 4Dx Plus Test (IDEXX Laboratories, Inc., Westbrook, ME) can similarly identify antibodies or infection with multiple tick-borne pathogens and canine heartworm antigen in a single assay. Each test kit consists of a coated matrix with 5 blue spots in the result window. These spots contain specific peptides as antigens for the detection of antibodies against *B. burgdorferi* s.l., *Anaplasma phagocytophilum*, and *Ehrlichia canis*, as well as specific capture antibodies for the detection of *Dirofilaria immitis* antigen. Lyme disease detection is based on identification of anti-*Borrelia* antibodies with C6 ELISA technology in clinically and subclinically infected dogs. Out of 200 canine serum samples tested by SNAP 4Dx Plus Test, 10.5 % were determined as positive with a relatively high sensitivity and specificity (84.2 %, 98.5 %, respectively) [7]. G e t t i n g s et al. [31] have evaluated the cross-reactivity of five commercially available and reference laboratory *B. burgdorferi*-based tests (SNAP 4Dx Plus, VetScan canine Lyme rapid test, Lyme Quant C6, the *B. burgdorferi* titre indirect fluorescent antibody test (IFA) and Accuplex4) on six laboratory-raised dogs infected with *B. turicatae* a causative agent of tick-borne relapsing fever. Three of these tests reacted to anti-*B. turicatae* antibodies. Five of six seroconverted dogs to *B. tu-*

ricatae were tested positive on at least one of the tests. The highest magnitude of cross-reactivity was detected for the whole-cell IFA. The three most reactive dogs in the study had measurable antibody levels above 10 U.ml^{-1} with the quantitative C6 ELISA. However, these results are below the positive threshold for the test (30 U.ml^{-1}) and would have been reported as negative. Those three dogs also had colour development on the test line of the VetScan test which can be considered positive according to manufacturer´s instructions. The study has highlighted concerns in evaluation of the results obtained by *B. burgdorferi* diagnostic tests due to significant cross-reactivity to other *Borrelia* spp. that can complicate diagnosis determination and surveillance of Lyme disease in dog.

Polymerase chain reaction (PCR)

PCR has a higher diagnostic sensitivity comparable to that of culture in tissues [47]. Moreover, given the fact that the number of organisms in clinical samples is low and unequally disseminated, the chance of detection is reduced. Clinical manifestation, type of samples investigated and target genes used for PCR also influence sensitivity of PCR [2].

H o v i u s et al. [36] determined simultaneous infection of *B. burgdorferi* s.l. in organ tissues and skin from naturally infected dogs using PCR-coupled DNA-DNA hybridization. Chou et al. [17] used a quantitative PCR assay for the detection of *Borrelia burgdorferi* DNA in formalin fixed, paraffin-embedded tissues of 58 dogs (38 were classified as positive or equivocal for LB on the basis of clinical signs, serologic findings, and pathologic abnormalities) and compared the results with immunohistochemical staining of tissues from seropositive dogs. *Borrelia burgdorferi* DNA was amplified from tissue samples from only 4 dogs (7 %), all of which had been classified as having positive or equivocal results for Lyme borreliosis. They concluded that while it is possible to detect *B. burgdorferi* DNA in formalin-fixed, paraffin-embedded tissues, intact *B. burgdorferi* DNA is rarely found in tissues from naturally infected dogs, including those with presumptive Lyme borreliosis.

The potential of metabolomics in Lyme disease diagnostics

In the field of multi-omics with a focus on genomics, transcriptomics, proteomics and metabolomics, a significant development has been observed in research of various diseases, including infectious diseases, to comprehend relationships between molecular signatures and phenotypic manifestations of a particular disease [21]. Metabolomics involves the quantitative detection of multiple small molecule metabolites in biological fluids, cells and tissues which provides an efficient method for monitoring altered biochemistry that is closely related to the current disease or therapeutic status. Urine and blood serum or plasma are the most commonly used biofluids for metabolomics because both contain hundreds to thousands of detectable metabolites and can be obtained non- or minimally invasively [65, 95]. Despite the many benefits of metabolomics application in nutrition science, toxicology, environmental studies, and biomedicine, particularly for the identification of new disease biomarkers and novel insights into disease pathogenesis, metabolomics has not been fully utilized in veterinary medicine when compared to human medicine. Nonetheless, metabolite profiling in veterinary research can complement our understanding of pathogenesis, diagnostics and treatment of human diseases [94]. There are a few studies demonstrating metabolomics application for disease research in dogs such as: obesity, heart disease, intestinal dysbiosis, bladder cancer, lymphoma, diabetes mellitus, anxiety-related disorders [14], however, none of them investigates Lyme disease or other zoonotic infections. In the study by H o x m e i e r et al. [37] metabolomics approach was used to examine the dynamics of survival and multiplication of spirochetes in tick vectors prior to transmission to the vertebrate host by tick saliva. Using gas chromatography coupled to mass spectrometry (GC-MS), they identified statistically significant differences in metabolic profile between uninfected *Ixodes scapularis* nymphal ticks, *Borrelia burgdorferi*-infected nymphal ticks, and *Borrelia mayonii*-infected nymphal ticks by measuring metabolism every 24 hours over the course of their blood meals up to 96 hours. A study focused on the metabolites such as purines, amino acids, carbohydrates and fatty acids during a blood meal and statistically confirmed differences in their amounts.

Currently, there is a rapid development in the detection and monitoring of diseases using urinary metabolomics which can represent a great potential for the identification of specific biomarkers, reflects the current state of the organism and provides comprehensive information on non-invasive monitoring of disease [96, 104]. Recent-

ly, there have been some studies investigating metabolomics approach in diagnostics of Lyme disease in its early stage searching for potential biomarkers in humans. P e g a l a j a r-J u r a d o et al. [68] used a metabolomics approach to detect urinary metabolites in patients with early stage of Lyme disease, infectious mononucleosis, and healthy controls. Analysis and identification of metabolites revealed dysregulation of several metabolic processes in early stage of Lyme disease compared to healthy controls or mononucleosis, including tryptophan metabolism. Due to the increased catabolism of tryptophan by indoleamine 2,3-dioxygenase (IDO) in infectious diseases, including Lyme disease, tryptophan metabolites in the kynurenine metabolic pathway have been identified and quantified. Their study confirmed significantly elevated kynurenine levels in patients with early stage of Lyme disease compared to healthy controls and significantly reduced tryptophan levels in the patients with disseminated infection compared to patients with localised infection. The results of their study suggest that the metabolic pathway leading to quinoline acid production differs in patients with early-stage Lyme disease and infectious mononucleosis. The study provided further evidence for the use of urinary metabolic profiling to differentiate early stage borreliosis from related diseases. M o l i n s et al. [64] detected metabolites in serum samples from patients with early stage Lyme disease, other diseases and healthy individuals using liquid chromatography-mass spectrometry (LC-MS) method. The result of the study was a metabolic biosignature of 95 molecular features that distinguished patients with early Lyme disease from healthy controls. By statistical adjustment, the biosignature was reduced to 44 molecular features and patients with early Lyme disease and healthy controls were correctly classified with a sensitivity of 88 % (84–95 %) and a specificity of 95 % (90–100 %). In addition, metabolomic biosignature correctly diagnosed 77–95 % of patients with early-stage Lyme disease with a negative serological result.

Treatment

Treatment of LB is based on treating spirochetal infection and managing pain of Lyme arthritis. Since the discovery of Lyme disease causative agent, the antimicrobial therapy is recommended. Recently, many different classes of antibiotics have been described for eradication of the causative agent of LB (Table 2). *Borrelia* organisms are sensitive to tetracyclines, penicillins, macrolides and cephalosporins. They are used during the early and late stages of the disease, and may be given orally or intravenously. Beta-lactams and tetracyclines have also shown to be effective and are widely used in human and veterinary medicine to treat patients with Lyme disease. As the first choice for most sick dogs with suspected LB, doxycycline is recommended due to its easier administration and efficacy against coinfections. Doxycycline can be prescribed for puppies and kittens since the age of 4 weeks in some countries. However, some veterinarians in the field prefer amoxicillin for doxycycline-sensitive or growing dogs.

Table 2. Antibiotics used in the treatment of LB (data reviewed from [51])

Antibiotic		Route; dosage; frequency; duration of use
Cephalosporins	Cefovecin	SC; 8 mg.kg^{-1}; 2 times, 14 days apart; 28 days
	Cefotaxime	IV; 20 mg.kg^{-1}; 3 times daily; 14–30 days
	Ceftriaxone	IV or SC; 25 mg.kg^{-1}; once daily; 14–30 days
Tetracyclines	Doxycycline or minocycline	PO or IV; 10 mg.kg^{-1}; 1–2 times daily; 30 days
Penicillins	Amoxicillin	PO; 20 mg.kg^{-1}; 3 times daily; 30 days
Macrolides	Erythromycin	PO; 25 mg.kg^{-1}; 2–3 times daily; 30 days
	Azithromycin	PO; 22 mg.kg^{-1}, once daily; 10–20 days
	Clarithromycin	PO; 7.5–12.5 mg.kg^{-1}; 2 times daily; 30 days

po – peroral; sc – subcutaneous; iv – intravenous

Other classes of antispirochetal antibiotics are applied in the case of tetracycline intolerance [51].

Veterinarian has a more difficult task in the treatment of LB compared to human disease, since it is often difficult to determine the onset of infection due to the lack of clinical signs reported in humans. LB symptoms in dogs are mostly manifested as an acute monoarticular or polyarticular lameness with joint swelling, fever, lethargy, and mild local lymphadenopathy, usually in young, often large breed dogs with an active/outdoor lifestyle. A critical factor of successful treatment is its initiation, therefore it should start as soon as possible with a focus on suppressing infection and pain. However, in most cases veterinarians begin treatment at the stage where spirochetes have already spread to various tissues [45, 51, 90].

Parenterally and orally administered antibiotics show high efficacy in treating LB that can be seen as a rapid response of the patient to antibiotics typically occurring within 1–2 days. Many experimental studies have shown that antibiotics greatly reduce the number of spirochetes demonstrated by the low incidence of culture-positive tissues and temporary disappearance of *B. burgdorferi*-specific DNA. The most effective antibiotic classes for the supportive therapy of dogs are beta-lactams and tetracyclines with long treatment course (4 weeks) due to the protracted biological behaviour of *Borrelia*. The best drug, dosage and duration of treatment for affected dogs are unknown [45, 51, 90].

In the study by Straubinger [90], antibiotic-treated dogs with ceftriaxone, azithromycin, doxycycline revealed a decrease in antibody titers established by KELA and Western blot. On the contrary, titres in 4 untreated control dogs have shown rapid growth within the first 90 days after tick exposure and continued increasing slightly throughout the experiment.

Recently, Wagner et al. [101] reported a comparative study of cefovecin (2 injections, 14 days apart) efficacy along with 4 weeks treatment of doxycycline or amoxicillin. They compared the outcome of cefovecin (long-acting cephalosporin) treatment of beagles experimentally infected with *B. burgdorferi* to doxycycline and amoxicillin as recommended standard antibiotics. Clinical outcome associated with LB symptoms was low because transient lameness was developed only in 2 out of 32 infected dogs confirmed either serologically (SNAP, Quant C6 or Multiplex) or using PCR amplification of *Borrelia* DNA in skin biopsy. After infection, 12 out of 32 dogs had a detectable *Borrelia* DNA in their skin biopsies. In conclusion, all tested antibiotics were effective against *B. burgdorferi* as the rapid elimination of spirochetes was measured in the skin as well as levels of circulating antibodies to *B. burgdorferi* were reduced. A significant difference was detected in a decrease of joint lesions of cefovecin-treated dogs compared to untreated dogs.

Despite effective treatment in the majority of early cured Lyme disease cases, relapse may occur after antibiotic administration is discontinued. The causative agent *B. burgdorferi* s.l. is capable of establishing a persistent infection in the host. Some studies have shown that PCR positivity in absence of culture positivity may also occur in dogs after antibiotic treatment [4, 89]. It is not necessarily caused by insufficient treatment or improper dosing of the antimicrobial, but it can indicate that the spirochetes may have become non-pathogenic. However, it is not known whether the lack of pathogenicity is irreversible. It remains questionable whether there is enough evidence to predict disease relapse or reinfection or it is induced by more fundamental genotypic or phenotypic alteration of the pathogen [51, 103]. Patients with early infection who recover after antibiotic therapy are susceptible to reinfection [44].

Moreover, problems with treatment may arise due to several immune evasion tactics employed by *B. burgdorferi* [3, 79] and due to the mechanism of persister formation [75]. As a response to the altered conditions, the atypical pleomorphic forms of *Borrelia* can occur. Hostile environment signals activate conversion of spirochetes to their persistent forms. Multiple morphologies of *Borrelia* such as looped or ring shaped, blebs, round bodies (RB) or cysts and colonies aggregates have been described [75]. Persister formation is a reversible process that leads to the rise of a *Borrelia* spp. cell population, particularly biofilms, with different susceptibility to conventional antibiotics. However, successful antimicrobial treatment should eliminate all morphological forms of the microorganism [76]. It was found that most frequently used doxycycline and amoxicillin reduced the spirochetal structures comparably by ~85–90 %. On the other hand, doxycycline increased the number of round body forms about twofold. Metronidazole, tinidazole, and tigecycline significantly decreased both the spirochete and the round body forms of *B. burgdorferi*. Quantitative analysis of biofilm-like colonies showed only 30–40 % reduction of doxycycline or

amoxicillin compared to tinidazole (~50–55 %) [76].

F e n g et al. [27] studied the use of drug combination against *B. burgdorferi* persisters *in vitro* using a SYBR Green/PI viability assay. Currently recommended Lyme antibiotics such as doxycycline or amoxicillin in combination with other antibiotics proved to be more efficient, while the combination of daptomycin, cefoperazone (or cefuroxime) and doxycycline eradicated the most resistant microcolony forms of *B. burgdorferi* persisters. Another study indicated two other triple drug combinations against amoxicillin-induced round body model, artemisinin/cefoperazone/doxycycline and sulfachlorpyridazine/daptomycin/doxycycline [26].

The efficacy of antibiotic treatment used in dogs showing signs of acute arthritis should be rapid (1–3 days) if the clinical signs are a consequence of LB. If it is necessary, then analgesic treatment (e. g., gabapentin for neuropathic pain) is started. Treatment with nonsteroidal anti-inflammatory drugs is less preferred to avoid the necessary "wash-out" period and to reduce the risk of gastrointestinal ulceration if subsequent glucocorticosteroid therapy is indicated for suspected immune-mediated polyarthropathy in unresponsive dogs. If relapse occurs before or after finish of antibiotic treatment, secondary diagnoses should include other infectious diseases, immune-mediated diseases, soft tissue trauma (e. g., ligament or meniscal tears), septic arthritis, or degenerative joint disease [51].

Prevention

As Lyme borreliosis is transmitted via the tick bite, prevention of tick attachment and feeding must be seen as the first obligation of any tick-control agent. The final agent potency should be 100 % at killing the tick before it is able to transmit the pathogen [80]. Many formulations (e. g. collars, spot-ons and orals) and modes of action (contact vs. systemic efficacy) have been used for the transmission blocking of tick-borne pathogens and tick vectors within different transmission times. The capability of Seresto® collars (imidacloprid 10 % + flumethrin 4.5 %) to prevent transmission of *Borrelia burgdorferi* s. l. and *Anaplasma phagocytophilum* by naturally infected ticks was evaluated in two studies with 44 dogs. This collar is highly effective in preventing tick and flea infestations on cats and dogs and has also shown to successfully prevent transmission of a range of pathogens including *Ehrlichia canis* and *Babesia vogeli*. The Seresto® collar was tested for its ability to prevent transmission of *Borrelia burgdorferi* s. l. or *Anaplasma phagocytophilum* from *I. ricinus* at 2 months and from *I. scapularis* ticks at 1 and 7 months after application. Acaricidal efficacy as well as pathogen transmission blocking of *Borrelia burgdorferi* s. l. and *Anaplasma phagocytophilum* was shown to be 100 % for all time points evaluated [43].

The development of an effective vaccine would help preventing the spread of the disease in humans and dogs. However, it is necessary to take into account that immunity to the infection is strain-specific and decreases after one year of infection [25]. The main goal of current research is to detect and characterise a specific antigen that induces persistent protection of the immune system [1].

Borrelia vaccines for dogs are worldwide administered to dogs in endemic areas. Commercially produced vaccines induce strong antibody response to one or more outer surface proteins (Osp) and other antigenic proteins of *B. burgdorferi*, which can be detected by IFA, WB, and whole cell antigen-based assays. Nevertheless, it is not possible to distinguish a vaccinated dog from a naturally exposed one using serological methods.

In 2003 and 2011, vaccination with four vaccines (RECOMBITEK® Lyme, Boehringer-Ingelheim Animal Health; LymeVax®, Zoetis; Galaxy® Lyme, Merck Animal Health; Nobivac® Lyme vaccine, Merck Animal Health) were monitored. Despite characteristic immune response to vaccination in all monitored groups, all samples at all sampling times were negative for *B. burgdorferi* antibodies in the SNAP 4Dx Plus test and the Lyme Quant C6 assay which demonstrates the absence of test reactivity with serum antibodies of vaccinated dogs [88].

Several different types of *B. burgdorferi* s. s. vaccines are currently commercially available in the US, including several bacterins (e.g., LymeVax®, Zoetis; Nobivac® Lyme, Merck Animal Health), recombinant OspA subunit vaccines (e.g., RECOMBITEK® Lyme, Boehringer Ingelheim), and a chimeric recombinant OspA and OspC vaccine (VANGUARD® crLyme, Zoetis) [99]. So far, there are no available experimental field trials examining the efficacy of canine *B. burgdorferi* vaccines [98]. In Europe, lysate vaccines produced with *B. burgdorferi* s. s., *B. garinii* and *B. afzelii* are on the market, however, more pathogenic species may be present in ticks and complete cross-reactive protection of the vaccine-induced antibodies is not documented [16].

CONCLUSIONS

This study was written to summarise knowledge of the epidemiology, clinical manifestations, diagnostic approaches, treatment, and prevention of Lyme borreliosis in dogs, with a focus on the strengths and limitations of various assays used to diagnose borrelial infection. Subclinical infections are common in dogs and on top of that spirochetes are difficult to detect in canines. As a result, a definite diagnosis of LB remains a complicated and time-consuming process in human and veterinary medicine, resulting in many differential diagnoses due to the lack of a specific all-encompassing test for LB. Moreover, the late immune response with delayed antibodies production and late clinical manifestations often complicate diagnosis and efficient treatment. Commonly used serological tests (mostly ELISA and Western blot) show varying sensitivity and specificity due to cross-reactions with other pathogens and a lack of their standardisation. Another developing approach based on biomarker discovery specific for Lyme disease represents a potential for the identification of early disease stage and its differentiation from other diseases. In conclusion, the high prevalence of *Borrelia* spp. in our latitudes, as well as the need for early and targeted veterinarian intervention in the treatment of Lyme disease in dogs, necessitate further research into the remaining challenge of developing precise and rapid diagnostic tests.

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SYNCHRONIZATION OF OVULATION AND TIMED INSEMINATION IN LACTATING DAIRY CATTLE

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ABSTRACT

Successful reproduction of lactating dairy cattle, or in other words its reproductive performance is a limiting factor in the profit ratio of cattle breeding regarding market production. The aim of this research was to evaluate the success rate of synchronization protocols Ovsynch, Presynch/Ovsynch, and Double Ovsynch within the period of 24 months. The success rate of the 1st insemination, 2nd insemination, and after more than 2 inseminations was evaluated. The cattle were of Holstein Friesian breed with a milk yield of 10 200 kg. The animals were sorted into two categories – heifers (protocol Ovsynch) and primiparous, multiparous cows (protocols Presynch/Double Ovsynch). The research proved a 54 % success rate in the category of heifers after the 1st insemination. In the category of primiparous and multiparous cows was the success rate of 41 % after the 1st insemination, 39 % after the 2nd insemination, and 52 % after more than 2 inseminations for the Presynch/Ovsynch protocol. The success rate for Double Ovsynch protocol was 45 % after the 1st insemination, 42 % after the 2nd insemination and 51 % after more than 2 inseminations. The results for the given geographic region of Slovakia exceeded the average.

Key words: dairy cow; Double Ovsynch; heifers; Ovsynch; Presynch; reproduction; synchronization protocols; timed insemination

INTRODUCTION

Nowadays, cattle breeding concerns nearly every country in the world and the issue of reproduction belongs to its inseparable parts. Reproduction is considered as the mainstay of production in this area. If there is no reproduction, there is no production either. Cattle farms are influenced by the constant pressure of economic production and the dairy industry. This is one of the reasons why it is important to present the production results; to ensure continuous reproduction. Since the reproductive performance in lactating dairy cows is not optimal, it led to the development of various strategies in the reproductive management industry aimed at improving the use of artificial insemination (AI) and pregnancy rates in dairy herds [14].

Synchronization programmes have become a standard part of current management of dairy cows in the dairy industry. Many of the synchronization protocols also allow timed insemination (TAI) to avoid the practical difficulties associated with oestrus detection [8].

Other factors, such as weather, season, nutrition, temperature, climate, and the management of the farm itself, along with reproductive indicators, affect the reproduction and functioning of cattle farms. The use of timed insemination after synchronization of ovulation has become one of the most widely used reproductive technologies developed in the last 40 years. Nowadays, different variants of this technology are in use all over the world, especially in milk production [16]. Artificial insemination is also important for the genetic improvement, especially in dairy cattle breeds [18].

According to global statistics, the fertility of Holstein cattle has been gradually decreasing each year by 0.5 % in the last four to five decades with a current increase in productivity due to breeding by 1 % or more [3].

At present many synchronization protocols and their variations, as well as modifications with subsequent artificial insemination are very popular. Many of them are based on the use of gonadotropin hormone and prostaglandin and their functions. The oldest used synchronization protocol is the Ovsynch protocol and its subsequent variations. Ovsynch allows artificial insemination (AI) to be performed at the exact optimal time without checking the ovaries and uterus. The use of these protocols in reproduction allows efficient synchronization of oestrus cycles and subsequent insemination of dairy cows without oestrus detection, which is time-consuming and difficult to perform for farms with many cattle. It is for this reason that Ovsynch became the first tool for the application of AI and an alternative to the detection of oestrus in dairy cattle [10].

The female's natural cycle is physiologically influenced by sex hormones and their secretion is controlled by the central nervous system. The main role in controlling the sex hormones is the hypothalamus – pituitary axis. The hypothalamus sends the GnRH hormone to the pituitary gland to release FSH and LH. These subsequently act on the ovaries, which produce their own hormones, such as oestrogen and progesterone. These hormones have a feedback effect on the hypothalamus. In this way, the regular sexual cycle of females is ensured. The length of oestrous cycle in female cattle usually lasts 17–24 days (on average 21 days). Day 0 in the oestrous cycle is the day when oestrus and other specific behaviour such as vocalization, displeasure, and mounting herd mates begin to manifest itself [15]. Heifers often have a shorter oestrus than dairy cows. Irregular sexual cycles can occur due to: improper postpartum care, the occurrence of endometriosis and pyometra, functional disorder of ovarian activity, such as atrophy, acyclia, ovarian cysts and others.

It can be concluded that the number of cattle in the territory of the Slovak Republic is decreasing every year. In 2020, according to DATAcube there were 442,289 cattle of which 122,049 were dairy cows, compared to 2010, when the number of individuals was 467,125 of which 159,260 were dairy cows. Despite the decreasing trends in cattle breeding, the overall percentage of pregnancy after the first insemination hardly changes over the years. According to Burdych, Kocmánek et al. [1], pregnancy after the 1st insemination in Holstein cattle is around 37.7 % by 2020 compared to 2010, when it was 35.3 %. There was also a significant improvement in milk production in Holstein cattle. According to Burdych, Kocmánek et al. [1] the average presents 10,254 kg of milk in 305 days, while in 2010 it was 8,912 kg of milk in 305 days.

The purpose of this study was to evaluate the success of chosen protocols in a period of 24 months. The Ovsynch, Double Ovsynch and Presynch protocols were used in certain categories of farm animals.

MATERIALS AND METHODS

Farm and animals

In this study Holstein cattle and their crossbreeds were evaluated. The herd is currently undergoing Holsteinization and in 2020 consisted of 482 dairy cows and 173 heifers. In 2021 the number of dairy cows was 479 and the number of heifers represented 162 animals.

A farm chosen for study was located in central Slovakia, housed animals of the Holstein cattle breed and their crossbreeds with the Montbéliarde breed. In 2020, there were 65.5 % crossbreeds on the farm, but their share of the Holstein breed ranged from 93.74 to 50 %. In 2021, the percentage of crossbreeds on the farm, with a share of 93.74 % to 50 % Holstein breed, was 61.6 %. The animals were divided into two groups: heifers after the first insemination and dairy cows after the first or more pregnancies. The results were evaluated by the Breeding Service of the Slovak Republic and collected through the breeding information system PLIS. Artificial insemination was carried

out with insemination doses with a minimum concentration of 10 million viable spermatozoa. These insemination doses were subjects of andrological examinations. Peyetted insemination doses were supplied by Slovak Biological Services and Insemas a. s. In 2020, insemination doses were used from bulls with the following codes, BKM – 7, BKM – 23, DIN – 5, DIN – 2, FDE – 1, HGE – 1, MGL – 11, MGL – 14, MGL – 48, SRE – 6, SRE – 10, SRE – 11. In 2021, the following codes were used: BKM – 28, BKM – 29, MGL – 11, MGL – 48, MGL – 50, MGL – 83, SRE – 10, SRE – 31, SRE – 57. Insemination doses came from black Holstein bulls and only certain doses came from sex-sorted semen.

Synchronization protocols

Heifers

The Ovsynch protocol was performed on a group of heifers. This protocol consisted of administration of GnRH (gonadothropin releasing hormone) on day 0, followed by administration of PGF2α (prostaglandin F2 alpha) 7 days later. GnRH was again administered after 48 hours, and timed artificial insemination took place after 12–18 hours (Fig. 1). Pregnancy control was carried out on the 32nd day after insemination by USG examination.

Primiparous and multiparous cows

For the second category of primiparous and multiparous cows Presynch/Ovsynch (PO) protocol was used. The Presynch/Ovsynch synchronization protocol consisted of synchronization that started on day 0. Preparation based on PGF2α was administered and its application was repeated after 14 days. After 12 days, a preparation based on GnRH was applied and after 7 days of its administration, the preparation with PGF2α was administered again and after 56 hours GnRH was applied (Fig. 2). At the end, a timed artificial insemination took place 16 hours after the last application of the hormonal preparation.

When the farm transitioned to the Double Ovsynch (DO) (October 2021) synchronization protocol, the preparations were applied as follows: on day 0, the GnRH-based preparation was applied to the dairy cows and after 7 days followed the application of the PGF2α preparation. GnRH preparation was applied 3 days later, and the process was repeated in 7 days. After the following 7 days, the PGF2α preparation was administered. The next application of GnRH was in 56 hours and timed artificial insemination took place approximately 16 hours later (Fig. 3)

The PGF2α-based preparations with the trade name Cyclix 250 micrograms/ml inj. (Virbac) were adminis-

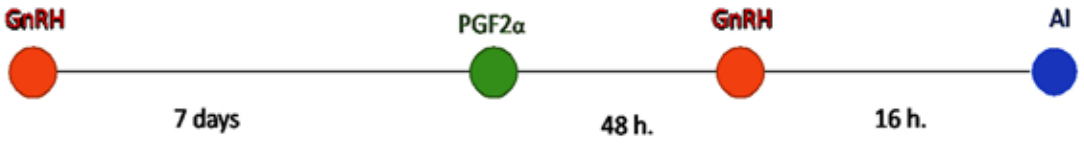

Fig. 1. Timeline for the Ovsynch synchronization protocol
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Fig. 2. Timeline for the Presynch/Ovsynch protocol
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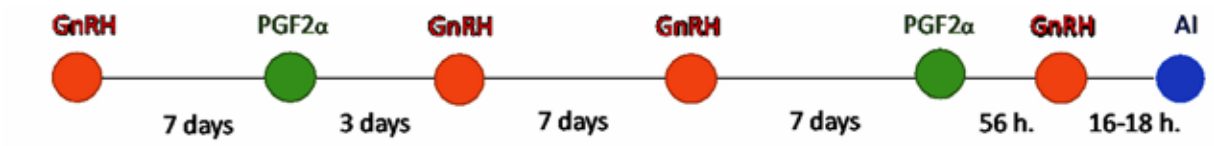

Fig. 3. Timeline for Double Ovsynch protocol
Source: custom imagine

tered to the animals without distinction with the active substance *cloprostenolum natricum* administered in the amount of 2 ml per animal and Enzaprost T (Ceva) with the active substance dinoprostum, administered in the amount of 5 ml per animal. Preparations based on GnRH with the commercial name Ovarelin 50 μg.ml^{-1} (Ceva), with the active substance gonadorelin was administered in the amount of 2 ml per animal, Acegon 50 50 μg.ml^{-1} (Zoetis) with the active substance gonadorelin administered in the amount of 2 ml per animal, injection solution for cattle and Gonavet inj. (Veyx) administered in the amount of 2 ml per animal with the active substance gonadorelinum. These preparations were chosen based on availability. In both categories, after the pregnancy confirmation on the 32nd day, a re-check pregnancy test was held in the period between the 60th and 90th day after insemination.

ETHICAL STATEMENT

The results and protocols evaluated in this study were obtained from the investigated farm therefore ethical approval was not required.

STATISTICAL ANALYSIS

Statistical evaluation was performed in the statistical program GraphPad Prism version 3.00 using Fisher´s exact test. The results were considered significant if the probability value was higher than 95 % ($P < 0.05$).

RESULTS

In the heifers, the success rate of pregnancy after the 1st insemination was on average 54 % in the experimental period. This period lasted from January 2020 to December 2021, but in some months the synchronization was not performed. The evaluation was in the process for 16 months. In the category of dairy cows after one or more births, we recorded the results of pregnancy success after 1st, 2nd and >2 inseminations. The pregnancy rate success, in the period from January 2020 to September 2021, after the 1st insemination was 41 % for the synchronization protocol Presynch/Ovsynch (PO), after the 2nd insemination on average 39 % and 52 % after 2> inseminations. In the period from October 2021 to December 2021, the success rate after the 1st insemination for the Double Ovsynch (DO) synchronization protocol was 45 %, after the 2nd insemination 42 % on average, and after >2 inseminations on average 51 %. No differences were found between PO and DO synchronization protocols. Nevertheless, better but statistically insignificant results were obtained for the DO synchronization protocol.

The service period for the entire period represented an average of 125 days and an intermediate period of 407 days. The insemination interval in cows after one or more births was on average 70 days. The insemination index for the entire observed period was 1.65 for heifers and 2.0 for cows after the 1st and additional births. The results are summarized in Table 1.

Table 1. Summarization of the results obtained after using different synchronization protocols

	Ovsynch	Presynch/Ovsynch	Double Ovsynch
Pregnancy rate after 1st insemination	54 %	41 %	45 %
Pregnancy rate after 2nd insemination	–	39 %	42 %
Pregnancy rate after > 2 inseminations	–	52 %	51 %
Insemination index	1.65	2	2
Insemination interval	–	71	65

DISCUSSION

The synchronization of ovulation is used almost continuously in milk production today and has become an integral part of daily practice, as well as timed artificial insemination. Synchronization of ovulation includes not only the application of hormonal preparations, but also: a proper and regular management of the herd, control over the overall health of the animals, animal welfare, and the success of the protocols. The administration of hormonal preparations was determined according to the relevant protocol. The administration of the first preparation is referred to as day 0 and it continues according to the composition of the given protocol. However, it should be noted that their application does not solve the causes of low fertility [3].

The purpose of the study was to evaluate the success of chosen protocols in a period of 24 months. The Ovsynch, Double Ovsynch and Presynch protocols were used in certain categories of farm animals. The success rate of conception after the first, second and more than 2 inseminations with simultaneous use of artificial insemination (AI) was evaluated.

Synchronization protocols that allow TAI and minimize visual detection of oestrus must be practical to implement within the daily operation of a dairy farm, otherwise the protocol would fail due to a lack of compliance [4]. The success rate of the Ovsynch protocol applied in the group of heifers was 54 %. However, we must point out that the result during the year could have been affected by heat stress, environment, herd management and many other factors. Thermal stress acts as a negative factor on the health and biological functions of dairy cows and manifests itself in the reduced milk production and reduced reproductive capacity [11]. The Ovsynch protocol is the oldest used synchronization protocol. In addition to its original use for synchronizing ovulation, this protocol is also used as a therapeutic method for many ovarian diseases, silent oestrus and heat stress [10]. According to their results, N a v a n u k r a w et al. [9] reported a pregnancy rate of 37.3 % when using the Ovsynch protocol. P u r s l e y and W i l t b a n k [12] reported that for heifers treated with the Ovsynch protocol, they achieved a pregnancy rate of 35.1 % and Y i l m a z et al. [17] obtained a pregnancy rate of 32.5 %. The use of the Ovsynch protocol seems to be an effective and advantageous method. However, this method has been shown to be more effective for dairy cows than for heifers [10]. However, the success of the Ovsynch synchronization protocol is closely related to the synchronization of the ovulatory follicle and corpus luteum (CL) [12]. In the herd for the category of heifers, synchronization of ovulation with the following TAI was omitted in some months due to the absence of suitable animals on the farm that could undergo this method. Overall, the pregnancy rate percentage for this category covers a period of 16 months. The Presynch/Ovsynch synchronization protocol was performed in the category of cows after one or more births. The time period for this synchronization protocol was from January 2020 to September 2021. Subsequently, there was a change in the synchronization protocol used by the farm itself. The success rate of the Presynch/Ovsynch protocol in the given period, for a certain category of animals, was 41 % after the 1st insemination. The success rate after the 2nd insemination and the > 2 insemination was 39 % and 52 %, respectively.

The presynchronization protocol has become one of the most widely used protocols to synchronize the oestrous cycle and ovulation for the first AI postpartum in lactating dairy cows. Synchronization may begin in the period between the 30th and 35th day after parturition [3, 6]. A study by K a r a k a y a - B i l e n et al. [7] pointed out the success of the pregnancy rate on the 31st day of the Presynch synchronization protocol at 59.3 %, when using the conventional type of insemination dose, and the pregnancy rate on the 31st day of 35.5 % when using the sex-sorted type of insemination dose. G i o r d a n o et. al. [6] recorded a pregnancy rate after AI using PO at a 12-day interval in primiparous dairy cows on day 39 of 44.7 % and in multiparous dairy cows 32.4 %, for a 14-day interval in primiparous dairy cows 38.7 % and multiparous 34.7 % for TAI. C o l a z o et al. [2] recorded a pregnancy rate after AI on day 60 in primiparous animals of 55.8 % and 32.9 % in multiparous animals [2].

Our results of the period October 2021 to December 2021 for the synchronization protocol Double Ovsynch were as follows: 45 % after 1st insemination, 42 % after 2nd insemination and 50 % after > 2 inseminations. However, the evaluation of this period is short and therefore the results may be distorted and incomplete. A study by S o u z a et al. [13] reported a pregnancy rate of 49.7 % using the Double Ovsynch protocol with a pregnancy rate of 65.2 % in primiparous animals and 37.5 % in multiparous animals. Double Ovsynch appears to be a better choice for

the treatment of anovulatory conditions [13]. A study by G i o r d a n o et al. [5] reported the effectiveness of DO on the 29th day as 38.7 % and on the 39th day as 35.3 % pregnancy rate.

However, for an objective evaluation of Double Ovsynch, we would need a longer research period to obtain more accurate results.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

CONCLUSIONS

We monitored the use of synchronization protocols in high-yielding dairy cows of the Holstein Friesian breed and their crossbreeds. The results partially approach the global parameters and the results reported after synchronization when synchronization protocols were used. The use of these synchronization protocols requires precision and discipline of the breeder, as this is the only way to achieve the desired effect of synchronizing the sexual cycle, ensuring the desired pregnancy and reproductive process, since the staff and the management of the company is not able to cover the methods and reproductive procedures of classical reproduction in cattle breeding. The tendency to increase the production of dairy cows suggests, that this method is currently highly effective and recommended for practice.

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