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CONTENTS

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BEHAVIOURAL RESPONSE AND WEIGHT OF BROILER CHICKENS REARED UNDER DIFFERENT ARTIFICIAL LIGHT SOURCES

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ABSTRACT

This study evaluated the behavioural response and weight of broiler birds at the 4th and 6th week of age when raised under incandescent (ICD) and compact fluorescent light (CFL). At 2 weeks of age, a total of 216 Arbor Acres birds were randomly allotted into 3 treatments: Control (no artificial light), ICD (60 watts, 800 lm) and CFL (40 watts, 2000 lm). Artificial lighting was provided from 8 p. m. to 4 a. m. daily. Data collected were subjected to One-way ANOVA using Minitab 17 through a general linear model (GLM) procedure and means were separated using Tukey's test software. At 4 weeks of age, the presence of light significantly increased ($P < 0.05$) the weight, as well as the occurrence of feeding, drinking, sitting, standing, walking, running and jumping behaviour. ICD and CFL had similar effects on feeding and jumping behaviour. At 6 weeks of age, the presence of light had no significant effect ($P > 0.05$) on the frequency of drinking and jumping behaviour. However, the weight, the frequency of feeding, standing, walking and running behaviour increased ($P < 0.05$) under the two light sources compared to the control. Also, birds raised under CFL displayed a higher frequency ($P < 0.05$) of inactive/resting and locomotor behaviour compared to birds reared under ICD which had a higher frequency of drinking behaviour. From this study, the use of CFL throughout the production stages is encouraged in poultry facilities due to the resultant increase in the activity levels of broilers and the reduced energy cost.

Key words: artificial light behaviour; broiler; compact fluorescent light; incandescent light; weight

INTRODUCTION

It has been demonstrated that a relationship exists between animal welfare, animal behaviour and the environment [9]. Light is the most important of all environmental factors to broilers [18], and management tool in modifying broiler behaviour because it influences the brain organization that influences the behavioural reaction [12] and ca-

pacity to adapt to stress. Light is characterized by source, intensity, wavelength range and length of the photoperiod [16]. Broiler behaviour is categorized into consummatory behaviour, comfort behaviour, locomotor behaviour, social behaviour, inactive behaviour, and aggressive behaviour, exploratory/foraging and reproductive behaviour [10]. The consummatory behaviour includes feeding and drinking. Feeding behaviour in broiler birds seems to be regulated only by satiety mechanism in contrast to hunger mechanisms in red jungle fowl, this may be attributed to intense selection for fast growth through different breeding programs [4].

In the past, incandescent lights (ICD) of various sorts and intensities have been utilized in poultry houses because of their low price [18], yet are at present, being eliminated because of their moderately high power utilization. Poultry farmers have been changing to more energy-efficient light [24] like fluorescent light, particularly the compact fluorescent light (CFL). CFL offers a lower dimension of energy utilization for a comparable light output [8], is less expensive [18], lasts longer, and does not transmit ultraviolet light. Studies have been carried out to identify the light sources that enhance consummatory, locomotor and inactive behaviour in laying birds [6, 18, 22, 23], but researches are scarce on the effect of these light sources on broiler chicken behaviour. Some of the behavioural studies carried out on broiler chickens tend to focus more on light intensities, light spectrum, housing conditions etc., rather than on the light source [2, 3, 5, 7, 20, 21]. Since compact fluorescent light is the available alternative to incandescent bulbs used in Nigeria poultry farming, it is imperative to evaluate the behavioural responses of broiler birds reared under these two light sources, and making more scientific information available in this regard as it has already been reported by A d e l e y e et al. [1] that the presence of artificial lighting in the evening (8 p. m. to 4 a. m.) in poultry houses enhances the feeding and drinking of broiler birds which in turn improves the conversion of feed to meat within a short period.

MATERIALS AND METHODS

Experimental site

The experiment was carried out at the Teaching and Research Farm, Federal University of Agriculture Abeokuta, Ogun State, Nigeria, with latitude 7°13'27"N and longitude 3°25'29"E and an altitude of 76 m above sea level. The experimental site was located in the rain forest vegetation zone of south-western Nigeria. The area was characterized by a humid climate, a mean annual rainfall of about 1037 mm, as well as a mean temperature and humidity of 34.7 °C and 83 %, respectively (Google Earth, 2020).

Experimental animals

Two hundred and sixteen (216) day-old broiler chicks of Arbor Acres strain were used for this study. The chicks were brooded for two weeks using rechargeable lamps as a light source and the birds were fed *ad libitum* throughout the experiment with commercial feed. Broiler starter feed with crude protein (CP) of 23 % and metabolizable energy of 2800 kcal.kg^{-1} was fed between 0—4 weeks of age, while broiler finisher with crude protein of 18 % CP and metabolizable energy of 3000 kcal.kg^{-1} was fed between the weeks of 5—6. All vaccinations and medications were administered as and when due. After 2 weeks of brooding, the birds were randomly distributed into three experimental treatments.

Experimental treatments

A completely randomized design (CRD) was used to assign birds to the different light sources; control (without artificial light), incandescent (ICD, 60 watts, 800 lm), compact fluorescent light (CFL, 45 watts, 2000 lm) from 2 weeks of age (post-brooding). The bulbs (Philips Gls ICD 60 W bulb and HL011906-2, China Full spiral 80 W CFL Energy Saver) were purchased based on what was available to farmers in the south-west, Nigerian local market. The birds were randomly distributed into 3 treatments. Each treatment was assigned 72 birds which were balanced for weight. The treatments were further divided into six replicates with each replicate containing twelve (12) birds. The housing system used was a deep litter system with each replicate having a dimension of 150 cm by 90 cm. Lights were on at night from 8 p. m. to 4 a. m. daily, while birds were allowed access to natural lighting during the day. Each treatment was demarcated to prevent light penetration from one into another.

Weight measurement

Weekly weight of the birds was determined during the study to monitor the weight changes.

Behavioural recordings

With the aid of an eight (8) units Closed-Circuit Television (CCTV) with infrared cameras embedded, behavioural recordings were done. The infrared was necessary to capture the control group which had no light source. At the 4th and 6th weeks of age (starter and finisher phase), the frequency of bird's feeding, drinking, sitting, standing, walking, running, jumping behaviour were recorded throughout the artificial lighting period (3 consecutive days recording per week). Behavioural recordings were watched on an hourly basis by adopting a scan sampling method of 10 min interval using an ethogram (Table 1).

Statistical analysis

One-way analysis of variance (ANOVA) was used to analyse the effects of treatment on behavioural expressions as well as the birds' weight at the 4th and 6th week of age. This was done using Minitab 17 [17] through a General linear model (GLM) procedure, and separation of means was done using Tukey's test; $P < 0.05$ was considered statistically significant.

Ethical considerations

The study was approved by the Departmental Ethical Committee (FUNAAB-APH17-02).

RESULTS

Effect of light sources on the mean frequency of behavioural pattern of broiler birds

The frequency of occurrence of all of the behaviour (feeding, drinking, sitting, standing, walking, running and jumping) in the birds were influenced by the presence of light at week 4 (Table 2). Birds subjected to ICD and CFL had significantly ($P < 0.05$) higher drinking and feeding

Table 1. An ethogram describing the behaviour of birds observed during the study

Consummatory	Feeding	Head extended towards available feed resource while beak appears to be ingesting feed
	Drinking	Beak in contact with drinking water
Inactive/Resting	Sitting	Abdomen touching the litter
	Standing	The bird is motionless with no apparent movement of legs
Locomotory	Walking	Moving forward taking one or more steps
	Running	Walking very fast
	Jumping	Lifting both feet away from the litter. No part of the body is in contact with the litter

Adapted from: Villagrá et al. [22]

Table 2. Effect of CFL and ICD light sources on the mean frequency of behaviour of broiler birds (week 4)

Behavioural categories	Parameters	Control	ICD	CFL	SEM	P value
Consummatory	Feeding	3.35^{b}	5.09^{a}	5.63^{a}	0.69	0.00
	Drinking	2.15^{b}	2.87^{a}	$2.50^{a,b}$	0.21	0.02
Inactive	Sitting	7.88^{a}	5.51^{c}	6.82^{b}	0.69	0.00
	Standing	0.42^{c}	0.90^{b}	1.70^{a}	0.36	0.00
Locomotor	Walking	3.99^{c}	6.09^{b}	7.11^{a}	0.92	0.00
	Running	0.01^{c}	0.46^{b}	1.07^{a}	0.31	0.00
	Jumping	0.03^{b}	$0.04a^{b}$	0.07^{a}	0.01	0.04

Mean values that do not share the same superscripts across the rows differ significantly
ICD—Incandescent; CFL—Compact Fluorescent Light; SEM—Standard Error of Mean

Table 3. Effect of CFL and ICD light sources on the mean frequency of behaviour of broiler birds (week 6)

Behavioural categories	Parameters	Control	ICD	CFL	SEM	P value
Consummatory	Feeding	2.31[b]	3.35[a]	3.62[a]	0.40	0.00
	Drinking	1.94	2.11	1.70	0.12	0.11
Inactive	Sitting	6.74[a]	4.97[b]	5.61[b]	0.52	0.00
	Standing	0.16[b]	0.30[a]	0.35[a]	0.06	0.00
Locomotor	Walking	2.49[b]	3.17[a]	3.56[a]	0.32	0.00
	Running	0.00[c]	0.11[b]	0.20[a]	0.06	0.00
	Jumping	0.02	0.03	0.02	0.01	0.09

Mean values that do not share the same superscripts across the rows differ significantly
ICD—Incandescent; CFL—Compact Fluorescent Light; SEM—Standard Error of Mean

frequency relative to those in the control group. The frequency of standing, walking, running and jumping exhibited by birds subjected to CFL was significantly higher ($P < 0.05$) than the control.

At week 6, drinking and jumping behaviour exhibited by birds were not affected by the presence of light (Table 3). The frequency of occurrence of feeding, sitting, standing, and walking behaviour of birds reared under ICD and CFL were similar. However, birds subjected to CFL exhibited a higher frequency of running behaviour.

Effects of light sources on hourly occurrence of consummatory behaviour

The average feeding frequency of broiler birds based on hours under different light sources at week 4 is shown in Figure 1. Under both light sources, the peak of feeding frequency was observed at 9 p. m. and the least at 1 a. m. The average feeding frequency of broiler birds on an hourly basis at six weeks of age is shown in Figure 3. Across all treatments, the least of the average feeding frequency was observed at 8 p. m. and the peak at midnight. The presence of light influenced feeding behaviour compared to the control, but the frequency of feeding behaviour exhibited by birds under the light sources was very similar.

Average drinking frequency on an hourly basis at week 4 is shown in Figure 2. Birds under ICD light showed a higher drinking frequency over others, except at 1 a. m., 3 a. m. and 4 a. m. while the control has the lowest drinking frequency from 10 p. m. till 4 a. m.

At six weeks of age, there was a steady increase in the drinking frequency of birds raised under ICD as shown in Figure 4. The expression of the behaviour reached its peak at midnight followed by a sharp decline.

Effects of light sources on the weight of broiler birds

Presented in Figures 5 and 6 is the total weight of the birds at the 4th and 6th week of the experiment, respectively. Birds reared under both artificial light sources had significantly ($P < 0.05$) higher weight than those in the control treatment. Birds subjected to both light sources had similar weights.

DISCUSSION

In this experiment, the presence of light influenced the frequency of feeding and drinking behaviour in birds. Birds raised in the control group under no artificial light exhibited less feeding and drinking behaviour as compared to those raised during the night under the two light sources (ICD and CFL), with a higher rate of feeding and drinking. The results of this study agree with the findings of Mohammed et al. [18], who noted that the presence of artificial light increased the frequency of feeding behaviour during the night. Birds may have been motivated to perform consummatory behaviour due to the presence of light which might have enhanced visualization of the feeders and drinkers. The frequency of the occurrence

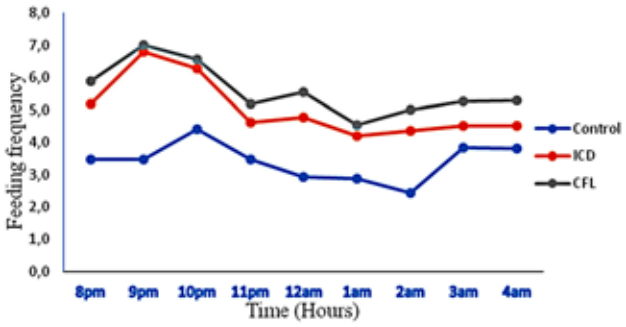

Fig. 1. Hourly average frequency of feeding behaviour of birds under control, ICD and CFL at 4 weeks
ICD—Incandescent; CFL—Compact Fluorescent Light

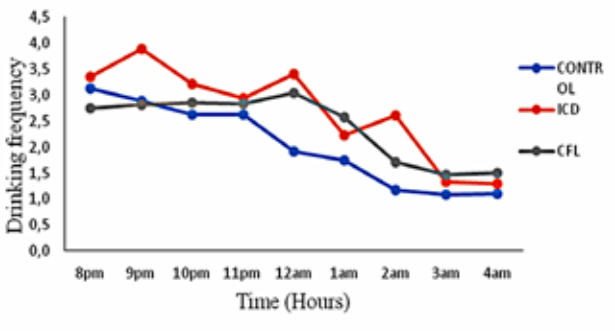

Fig. 2. Hourly average frequency of drinking behaviour of birds under control, ICD and CFL at 4 weeks
ICD—Incandescent; CFL—Compact Fluorescent Light

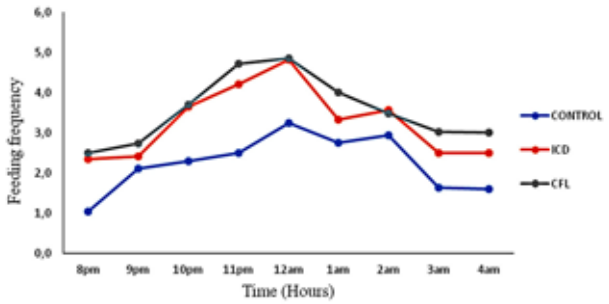

Fig. 3. Hourly average frequency of feeding behaviour of birds under control, ICD and CFL at 6 weeks
ICD—Incandescent; CFL—Compact Fluorescent Light

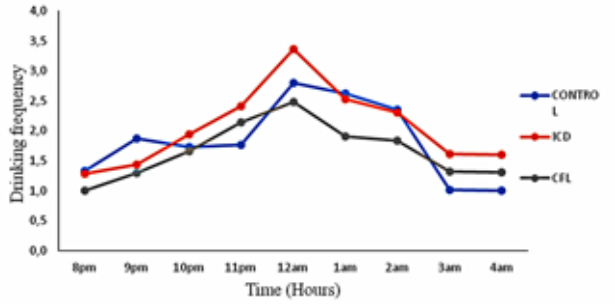

Fig. 4. Hourly average frequency of drinking behaviour of birds under control, ICD and CFL at 6 weeks
ICD—Incandescent; CFL—Compact Fluorescent Light

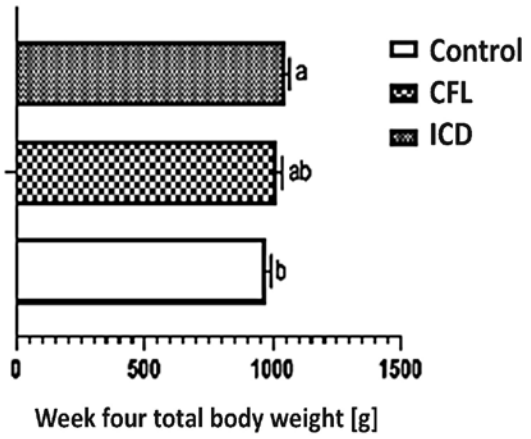

Fig. 5. Total weight of birds under control, ICD and CFL at 4 week of age
ICD—Incandescent; CFL—Compact Fluorescent Light

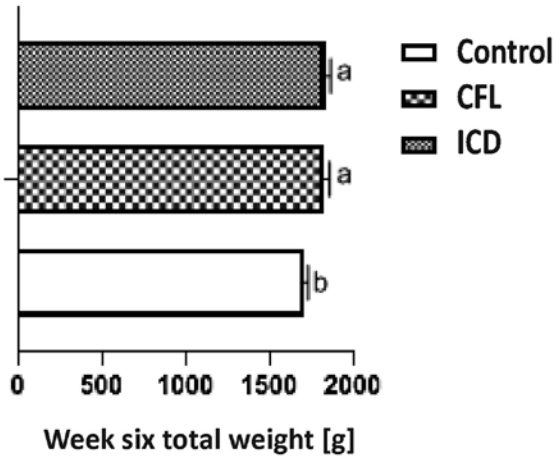

Fig. 6. Total weight of birds under control, ICD and CFL at 6 week of age
ICD—Incandescent; CFL—Compact Fluorescent Light

of feeding behaviour in broiler birds reared under ICD and CFL were similar. This result aligned with the research of Vanderberg and Widowski [23], who found that although the presence of light influenced the feeding behaviour, the differences observed in the frequency of the feeding behaviour of birds under the light sources were negligible. This also further supported researchers who also noted that minor differences were observed in the feeding behaviour of laying birds under different light sources [18, 22]. Therefore, none of the light sources highly influenced the feeding behaviour better than the other. The average hourly frequency of feeding behaviour exhibited by birds showed the response of birds in each treatment from 8 p.m. to 4 a.m. For birds at 4 weeks of age, the feeding frequency based on hourly average. Figure 1 revealed that birds reared under both light sources exhibited a higher frequency of feeding behaviour when compared to the controls. Under both light sources, the peak of occurrence of the feeding behaviour was observed in the second hour (9 p.m.). This observation could be attributed to an increase in bird's activity at the onset of lighting. The feeding pattern in the control group almost appeared to be consistent with the other groups. The reason(s) for this remains unclear, but since the animals were supplied with feed simultaneously, one would expect the animals to access the feed almost immediately. At the 6th week of age, the frequency of feeding behaviour was similar under both ICD and CFL and the feeding frequency (Figure 3) peaked across all treatments at midnight.

At 4 weeks of age, the frequency of drinking behaviour exhibited by birds reared under ICD was higher compared to the controls. A possible explanation for this might be as a result of the heat dissipated by ICD against CFL bulb type. For a given quality of light, ICD produces more heat, thus necessitating a need for thermoregulation. Surprisingly in the 6th week, the presence of light did not influence the frequency of drinking behaviour in birds, that is, no difference was found in the drinking behaviour of the broiler birds raised in the control group (without any artificial light) and those raised under the two different light sources. A possible explanation for this might be that since the locomotor behaviour is generally reduced at 6 weeks (due to age and weight), the frequency at which birds visited drinkers reduced generally across treatments. The bird's frequency of drinking based on an hourly average (Figure 4) showed the response of birds in each treatment from 8 p.m. to 4 a.m. At 4 weeks of age across all treatments, birds exhibited a high frequency of drinking behaviour at the onset of lighting and a low frequency at the end of the lighting period. The reason behind this observation remains unclear. However, it appears as though, the bird drinks more frequently at a young age than when they are old as the mean frequency at 6th week dropped numerically at this age. This is because the reduced mean frequency of drinking did not attain statistical significance. The fluctuation in the behavioural expression of birds reared under ICD could be a result of heat dissipated by the bulb. Generally, at week 6, the frequency of drinking behaviour increased from the onset of artificial light, and the peak was observed at the fifth hour (midnight) before a decline. The midnight peak was due to the refilling of the birds' drinkers that took place every midnight during the study.

The impact of the consummatory behaviour was reflected in the weight of birds measured at the 4th and 6th week of life. Higher weights were recorded in birds raised under both artificial light sources when compared to the control group. Birds reared under both light sources had similar weight. This is in accordance with the findings of Ghuffar et al. [13] who observed no significant difference in the mean body weight of broilers housed under ICD and fluorescent light, and Ingram and Hatten [14] whose report on light restriction in broilers showed the lowest mean weight gain in birds with restricted lighting regimen in broilers.

The occurrence of inactive behaviour (sitting and standing) was greatly influenced by the presence of light. The frequency of sitting behaviour exhibited by birds supplied with artificial light was reduced when compared to control, thus the absence of light enhanced sitting behaviour. This can be explained by the fact that the presence of light motivated birds to perform physical activities, thus the frequency of sitting behaviour was reduced under the light sources. In contrast, the frequency of standing behaviour exhibited by birds in the treatments supplied with artificial light increased. A possible explanation for this is that the absence of light inhibited the expression of standing behaviour. Birds that were reared without artificial light performed more of sitting and less of standing behaviour, while those reared under artificial light performed more of standing and less of sitting. At 4 weeks, a higher frequency of sitting and standing behaviour in birds reared under CFL compared to those under ICD, further support the

idea of Mohammed et al. [18], who reported that sitting behaviour was lowest for birds raised under ICD light albeit with different intensity. Since birds raised under CFL performed more inactive/resting behaviour than those under ICD, this may be because there was a need for a counterbalance for the energy expended on physical activity, by resting. At 6 weeks, the frequency of sitting and standing behaviour exhibited by birds under ICD and CFL were similar. This supported the study of Kristensen et al. [15] who stated that sitting behaviour was not influenced by light sources. Mohammed et al. [18] also reported that light sources do not affect standing activity in birds. A possible explanation for this is that, regardless of the light source, the time spent resting (sitting) increased as broilers grew older, which is thought to be another consequence of increased body size and decreased mobility [4, 26].

The frequency of occurrence of locomotor behaviour was improved by the presence of light. Broiler birds raised with artificial light exhibited a higher frequency of walking, running, and jumping behaviour compared to those in the control group. This might be due to the presence of light facilitates birds' vision and act as a motivation to perform locomotor behaviour. It is worthy of note, that at 6 weeks, jumping behaviour was not influenced by the presence of light, and this could be due to an increase in age and body weight. Another important finding was that at the 4th week, higher frequency of walking and running behaviour was exhibited by birds reared under CFL when compared to those raised under ICD. The frequency of jumping behaviour exhibited by birds under CFL was higher when compared to the control group. These present findings are consistent with other researches, which revealed that physical activities are higher under CFL than ICD [6, 18]. A possible explanation for this result might be the high luminous efficacy (2000 lm) of 40 watt CFL compared to 60 watt ICD (800 lm) used during these experiments. Luminous efficacy ($lm.w^{-1}$) is the brightness of the light produced by a bulb. Activity levels of birds are known to be positively correlated to light intensity [6, 11]. Low light intensity reduces activity [20], and brighter light fosters increased activity level in broiler birds [19]. Although, at 6 weeks of age, the frequency of occurrence of walking behaviour exhibited by birds raised under ICD and CFL was very similar. This may be because regardless of the light source, as the body size of broiler birds increases, mobility decreases [25].

CONCLUSIONS

This study allowed us to conclude that the use of CFL increased the activity levels of broiler chickens during the 4th and 6th weeks of age as revealed by the mean frequency of the locomotor behaviour, while the drinking behaviour was exhibited more by birds under the ICD light source. Furthermore, the weight of broiler chickens during the 4th and 6th weeks of age was significantly improved by the use of artificial light in the rearing facility.

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CONFLICT OF INTEREST

All the authors declare no known conflict of interest.

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IN VITRO INHIBITORY ACTIVITY OF CELL-FREE SUPERNATANTS OF *LACTOBACILLUS* SPP. AND *BACILLUS* SPP. AGAINST *FUSARIUM GRAMINEARUM*

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ABSTRACT

In this study, the antifungal activity of cell-free supernatant (CFS) of *Lactobacillus* spp. (*Lactobacillus plantarum* CCM 1904; L81, *Lactobacillus fermentum*; 2I3, *Lactobacilus reuteri*; 2/6, L26;) and *Bacillus* spp. (*Bacillus subtilis* CCM 2794, *Bacillus licheniformis* CCM 2206) against two strains of *Fusarium graminearum* CCM F-683 and *Fusarium graminearum* CCM 8244 were investigated *in vitro*. All tested CFS of *Lactobacillus* spp. were able to inhibit the growth of both strains of *Fusarium graminearum*. The highest inhibitory effect (IE) (56.5 %) against *F. graminearum* CCM F-683 was observed for CFS *Lactobacillus fermentum* (2I3) at the minimum inhibitory concentration (MIC) (2.25 ± 0.56 mg.ml^{-1}). CFS of *Lactobacillus reuteri* (2/6) showed the best IE (40.0 %) against *F. graminearum* CCM 8244 (2/6) at the MIC 1.25 mg.ml^{-1}. However, no inhibitory effect of *Bacillus subtilis* and *Bacillus licheniformis* CFS against both strains of *F. graminearum* were observed, even at the highest tested concentration of 5.0 mg.ml^{-1}.

Key words: *Bacillus* spp.; biological protection; inhibitory effect; *Lactobacillus* spp.; microscopic filamentous fungi

INTRODUCTION

Fusarium graminearum is one of the most important fungal pathogens of cereal grains (Fig. 1). It is the causal agent of *Fusarium* head blight (FHB) in wheat and barley [15]. FHB or scab is a plant disease of economically significant importance worldwide [9]. *F. graminearum* causes direct damage, poor quality of grains and losses of crop and contamination of the raw grains and processed wheat products with mycotoxins [25].

The most important mycotoxin produced by *Fusarium graminearum* is deoxynivalenol (DON, vomitoxin) and its acetylated derivatives. The presence of deoxynivalenol, in a host-pathogen interaction, can be a virulence factor in *Fusarium graminearum* wheat infection [21]. In addition, deoxynivalenol and other *Fusarium secondary* metabolites

Fig. 1. Sporulation of *Fusarium graminearum*, which is manifested by orange colouration (own figure)

(zearalenone, fumonisins and other) are very toxic compounds for animals and humans and can cause the so-called mycotoxicosis [17]. Mycotoxicosis are manifested as a variety of clinical signs that depend on factors such as: type and concentrations of mycotoxins; duration of exposure; species; genus; age and health status of the individual [11].

Nowadays, there are various options to eliminate the growth of *Fusarium graminearum*. In addition to chemical antifungals, adsorbents, antioxidants and biologically active substances are used [22]. According to K o k k o n e n [12], lactic acid bacteria are involved in the biological adsorption of mycotoxins and prevent their absorption from the digestive tract into the blood of humans and animals. The ability of the genus *Lactobacillus* to suppress the growth of *Fusarium* microscopic filamentous fungi has been reported [5]. Bacterial endophytes, *Bacillus subtilis* and *Bacillus licheniformis* are biological tools to protect plants from phytopathogens. They can enter and colonize plants without causing any signs of plant disease. Endophytes penetrate plants from the soil and roots and subsequently spread into leaves, flowers and fruits [2]. *Bacillus* spp. can protect plant hosts by producing many antimicrobials molecules and induced systemic resistance [23]. The strains of the genus *Lactobacillus* and *Bacillus* can be used for the formulation of cell-free supernatants (CFS). CFS are mixtures derived from broth cultures. The separation and removal of cells can be performed using several physical and mechanical processes. CFS can be obtained through two main operations, centrifugation and filtration, or in combination with other technologies according to the desired final product [3, 19]. Many studies of CFS deal with their utilization in medical and food sectors. The studies on the biostimulant and biocontrol properties of CFS in plants are limited to *in vitro* tests [19].

The aim of this study was to determine the inhibitory effect of CFS of various species of the genera *Lactobacillus* and *Bacillus subtilis* and *Bacillus licheniformis*.

MATERIALS AND METHODS

Tested strains

In the experiment, 9 strains of microorganisms (Table 1) were used. The strains of *F. graminearum* CCM F-683, *F. graminearum* CCM 8244, *Lactobacillus plantarum* CCM 1904, *Bacillus subtilis* CCM 2794 and *Bacillus licheniformis* CCM 2206 were obtained from the Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic). Other strains of the genus *Lactobacillus* were provided by the Department of Microbiology and Immunology of the University of Veterinary Medicine and Pharmacy in Košice.

Table 1. The tested strains

Species	Number
Fusarium graminearum	CCM F-683
Fusarium graminearum	CCM 8244
Bacillus subtilis	CCM 2794
Bacillus licheniformis	CCM 2206
Lactobacillus plantarum	CCM 1904
Lactobacillus fermentum	2I3
Lactobacillus plantarum	L81
Lactobacillus reuteri	2/6
Lactobacillus reuteri	L26

Preparation of fungal inoculum

The inoculum suspension was prepared from 10-day old culture of *F. graminearum* (CCM F-683 and CCM 8244) grown on potato dextrose agar (PDA, HiMedia Laboratories Pvt. Ltd., Mumbai, India). Twenty ml of physiological saline containing 0.1 % Tween 80 was applied on

the culture surface and a microbiological stick was used to release conidia from the mycelium. The conidia were then collected with a Pasteur pipette. The density of the fungal inoculum was adjusted to McFarland 2 (10^6 CFU.ml^{-1}) by adding saline and using a densitometer (Pliva-LaChema a. s., Brno, Czech Republic). The prepared inoculum was diluted with potato dextrose broth (PDB) to a concentration 10^6 CFU.ml^{-1}, which was used for the microdilution testing.

Preparation of cell-free supernatants (CFS) from *Lactobacillus* spp. and *Bacillus* spp.

To prepare CFS from *Bacillus subtilis* (Bs) and *Bacillus licheniformis* (B1) strains a 24-hour old culture grown on B10 agar at 35 °C was used. Using a sterile inoculation loop (mesh volume 10 µl), bacteria colonies were picked up, and 50 ml of B10 broth, in an Erlenmeyer flask was inoculated. The cell-free supernatant from individual *Lactobacillus* strains was prepared by inoculating 50 ml of MRS broth with colonies of cells grown under anaerobic conditions on MRS agar at 35 °C for 48 h, using a Gas pack system. Multiplication of *Lactobacillus* spp. and *Bacillus* spp. was performed in a thermostat under aerobic conditions, at 35 °C, by shaking the Erlenmeyer flasks on a horizontal shaker (Orbital Shaker—Biosan) at 170 rpm. To obtain CFS, the suspensions formed by *Bacillus subtilis* (Bs) and *Bacillus licheniformis* (B1) were centrifuged twice for 20 minutes at 6000 rpm, after 24 hours of incubation. Pure, filtered (Q-Max syringe filter, pore size 22 µm) CFS was used to test *F. graminearum* growth inhibition. *Lactobacilli* supernatants: *Lb. fermentum* 2I3 (L1), *Lb. reuteri* L26 (L3), *Lb. plantarum* L81 (L4), *Lb. reuteri* 2/6 (L5) and *Lb. plantarum* CCM 1904 (L6) were prepared in the same manner, after 48 hour of incubation.

Test procedure

The microdilution method was used to determine the inhibitory effect of cell-free supernatants. The experiment was performed in 96-well sterile microplates (U shaped), and PDB was used as the medium. Undiluted (100 %) sterile CFS (200 µl) was added into the well No. 1 and, subsequently, a series of two-fold dilutions was applied to wells No. 2—10. After application of the fungal inoculum (100 µl) to each dilution of CFS, the final concentrations ranged from 5 to 0.01 mg.ml^{-1}. Each *Fusarium* strain was tested in five rows (A—E). Row H, which served as a negative control, contained only individual concentrations of CFS (100 µl) diluted in PDB (100 µl). Column 11 was used as a blank containing 200 µl of PDB. Column 12, a positive control, contained 100 µl of PDB and 100 µl of inoculum. The microtitre plates were incubated for 72 h at 25 ± 2 °C, in the dark [6]. After 48-hours of incubation, 15 µl of 0.1 % resazurin solution was added into rows A—E and H [13]. The inhibitory effect was determined by measuring the absorbance of the content of wells at 635 nm using an ELISA reader (Dynex Technologies, Inc., Virginia, USA) and calculated according to the formula:

$$IE\ (\%) = \frac{(PC - NC) - (S - NC)}{(PC - NC)} \times 100$$

IE—the inhibitory effect,
PC—the absorbance of the positive control,
NC—the absorbance of the negative control,
S—the absorbance of the sample.

The lowest concentration of the tested CFS that completely inhibited the growth of *Fusarium graminearum* mycelium was considered the minimum inhibitory concentration (MIC).

Statistical analysis

The statistical functions of the MS Excel software were used to evaluate the MIC results and the data obtained in this study were reported as means ± standard deviations (SD).

RESULTS

Evaluation of the inhibitory effect of cell-free supernatants

Table 2 shows the results of the evaluation of MICs and the inhibitory effect of CFS strains of *Lactobacillus* spp.: *L. fermentum* 2I3 (L1), *L. reuteri* L26 (L3), *L. plantarum* L81 (L4), *L. reuteri* 2/6 (L5), *L. plantarum* CCM 1904 (L6) and *Bacillus* spp.: *Bacillus subtilis* CCM 2794 (Bs) and *Bacillus licheniformis* CCM 2206 (B1).

The best inhibitory effect against both *Fusarium* strains was observed at a concentration of 1.25 mg.ml^{-1}, in CFS L3 (10.8 % and 7.9 %, respectively), L4 (5.4 % and 12.3 %), L5 (6.1 % and 40.0 %) and L6 (8.4 % and 12.5 %). CFS L1 was effective on *F. graminearum* CCM F-683 at MIC

Table 2. MICs (mg.ml^{-1}) and the inhibitory effect (%) of cell-free supernatants of *Lactobacillus* spp. and *Bacillus* spp. *against F. graminearum* strains

Tested strains	*F. graminearum* CCM F-683		*F. graminearum* CCM 8244	
	x ± SD [mg.ml^{-1}]	IE	x ± SD [mg.ml^{-1}]	IE
L1	2.25 ± 0.56	56.5	2.5 ± 0	39.1
L3	1.25 ± 0	10.8	1.25 ± 0	7.9
L4	1.25 ± 0	5.4	1.25 ± 0	12.3
L5	1.25 ± 0	6.1	1.25 ± 0	40.0
L6	1.25 ± 0	8.4	1.25 ± 0	12.5
Bs	≥ 5.0 ± 0	0	≥ 5.0 ± 0	0
Bl	≥ 5.0 ± 0	0	≥ 5.0 ±0	0

x—mean values of MIC; SD—standard deviation; IE (%)—inhibitory effect; L1—*L. fermentum* 2I3; L3—*L. reuteri* L26; L4—*L. plantarum* L81; L5—*L. reuteri* 2/6; L6—*L. plantarum* CCM 1904; Bs—*Bacillus subtilis* CCM 2794; Bl—*Bacillus licheniformis* CCM 2206

2.25 ± 0.56 mg.ml^{-1} and on *F. graminearum* CCM 8244, at MIC 2.5 mg.ml^{-1}, with inhibitory effect 56.5 % and 39.1 %, respectively. CFS from *Bacillus subtilis* and *Bacillus licheniformis* did not show a sufficient inhibitory effect even at the highest tested concentration of 5.0 mg.ml^{-1}.

DISCUSSION

The use of beneficial microorganisms in agricultural, is an increasingly and successful strategy aimed at the elimination of plant pathogens. Biological control by microorganisms is related to the restriction of the use of chemical in agriculture [1, 4]. Many species of *Lactobacillus* have antifungal activity against microscopic filamentous fungi. *Lactobacilli* species, *L. acidophilus, L. brevis, L. casei, L. fermentum, L. plantarum, L. reuteri, L. rhamnosus and L. sakei* are often described as producers of antifungal compounds [14]. The antifungal activity of individual strains of the genus *Lactobacillus* is associated with the synthesis of organic acids, fatty acids, esters of fatty acids, hydrogen peroxide, bacteriocins and other secondary metabolites [20]. Antifungal activity of CFS probably depends of the overproduction of substances with antifungal effects (low molecular weight and thermally stable substances, bacteriocins) [7]. However, their antifungal activity is usually poor or moderate [14]. In our experiments, CFS from *Lactobacillus fermentum* at MIC 2.25 ± 0.56 mg.ml^{-1} was the most effective against *F. graminearum* CCM F-683 (56.5 %) and CFS from *Lactobacillus reuteri* at MIC 1.25 mg.ml^{-1} against *F. graminearum* CCM 8244 (40.0 %). Several literature sources point to antifungal efficacy of *Lactobacillus* and *Bacillus bacteria*, which produce substances capable of inhibiting the growth of microscopic filamentous fungi *Fusarium* [8, 10]. *Bacillus subtilis* and *Bacillus licheniformis* can enhance growth of the plant and biomass production through synthesis of various phytohormones, fixation of nitrogen, solubilisation of phosphate and production of ammonium ion. Large number of metabolites of these endophytes activate plant and protect them against harmful bacteria and fungi [24]. The antifungal properties of the *Bacillus subtilis* are mainly attributed to the lipopeptides (fengycin, surfactin and mycosubtilin), which are produced by various strains of this species [16]. In our study, we did not observe an inhibitory effect of CFS from *Bacillus subtilis* even at the highest tested concentration of 5 mg.ml^{-1} against *F. graminearum*. According to Mihalache et al., [16] the combination of lipopeptides from *B. subtilis* showed potent inhibitory activity at concentrations as low as 5 µg.ml^{-1} against the phytopathogenic fungi *F. oxysporum*. This antifungal activity is related to the inhibition of spore germination and the irreversible damage of the hyphae cell wall. However, the results of antagonism test showed that the strain *Bacillus licheniformis* could reduce and inhibit the mycelium growth of various plant pathogens *in vitro* and *in vivo* [18].

CONCLUSIONS

Cell-free supernatants of non-pathogenic microorganisms, such as strains of *Lactobacillus* spp. and soil microorganisms *Bacillus subtilis* and *Bacillus licheniformis* deserve a special attention in the future as fungicides. The obtained partial results point to the possibility of their practical application in agricultural practice, as well as to the further direction of scientific research. The use of these bacterial strains creates a space for the study of the synergistic effect of their mutual combinations, possible with other products of natural origin.

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GROSS MORPHOLOGY OF THE BRAIN AND SPINAL CORD OF THE AFRICAN PYGMY HEDGEHOG (*ATELERIX ALBIVENTRIS*)

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ABSTRACT

The African pygmy hedgehog (*Atelerix albiventris*) is an insectivorous animal, native to Africa. The central nervous system (CNS) consists of the brain and the spinal cord, protected by the cranium and vertebral column respectively. Assessment of the gross appearance and morphometries of the African pygmy hedgehog CNS were carried out using six adults (3 males and 3 females). The gross examination showed the brains to be lissencephalic, with relatively large olfactory bulbs, similar to that observed in some rodents. The rootlets of the first cervical spinal nerves were observed to emerge before the *foramen magnum*. Linear measurements were obtained from both the brain and spinal cord. The mean weight of the animals was 199.00 ± 16.09 g, with the males having an average body weight of 183.50 ± 12.02 g and the females 206.80 ± 11.95 g. Although not statistically significant, the males had a higher encephalisation quotient (0.40 ± 0.08) relative to the females 0.36 ± 0.04). The values for the brain weight, length of spinal cord and heights of the telencephalon and diencephalon at different points were higher in the males, while the spinal cord weight, length of brain and cerebellar height were higher in the females. The spinal cord showed slight enlargements at the cervical, thoracic, lumbar and sacral segments. This study aimed to provide baseline data for the study of the gross appearance and neuromorphometrics of the hedgehog, with possible application in regional anaesthesiology and comparative wildlife neuroanatomy.

Key words: *Atelerix albiventris*; brain; morphometrics; spinal cord

INTRODUCTION

The central nervous system (CNS) consists of the brain and spinal cord. The brain is encased in the skull, while the spinal cord is continuous caudally with the brain and is protected by the vertebral column [21].

Atelerix albiventris (also known as the four-toed hedgehog) is the smallest of the African hedgehogs, belonging

to the family *Erinaceidae*, subfamily *Erinaceinae*. It is one of the four species of the genus *Atelerix*. These hedgehogs are phenotypically small, short tailed and spiny, possessing a pointed muzzle. They have a well-developed sense of smell, sight and hearing [19]. They are terrestrial and placental mammals, nocturnally active and classified as "least concern" on the International Union for Conservation of Nature (IUCN). Their occurrence is widespread in Africa, they can be found in human habitation and are sexually dimorphic [6, 10]. The hedgehogs are an important group of insectivores, they have relatively small brains with little neocortex, and for some decades have been employed to better understand the initial stages of the brain evolution of mammals [4]. In more recent years, African hedgehogs are becoming increasing popular as pets, with a lifespan of 1—4 years in the wild [11]. In spite of the increasing importance of the hedgehog (as pets and experimental models), there are few studies detailing its anatomy in the absence of disease. These animals are kept as pets in many climes, resulting in them being in close contact with humans. This, among other reasons makes it imperative to study and understand the anatomy of this unique spine-bearing animal.

This study, the first in a series on the anatomy of the hedgehog CNS, aimed to assess the gross morphology and morphometrics of the brain and spinal cord of the African pygmy hedgehog. It is hoped that this study will lead to a better understanding of the hedgehog CNS, its similarity to other mammalian insectivores, provide the basis for future research as well as contribute to the knowledge of African pygmy hedgehog neuroanatomy.

MATERIALS AND METHODS

A total of six clinically healthy African pygmy hedgehogs (3 females and 3 males) were obtained from the forests around Ibadan, Oyo State, Nigeria. The animals were examined and determined to be free of any abnormality or deformity that could interfere with this study.

Each animal was weighed with a digital kitchen weighing scale (Electronic Kitchen Scale, Camry® EK5350, error of 1 g, China) and sedated using an overdose of ketamine HCl injection, (150 $mg.kg^{-1}$) and xylazine (6 $mg.kg^{-1}$) intramuscularly, adapted from Catania et al. [4]. Intracardiac perfusion commenced after cessation of respiration and lack of response to vigorous tactile stimuli. Intracardiac prefusion was performed using 0.9 % normal saline followed by 4 % paraformaldehyde (PFA), each solution about 300 ml. The cauda equinae could not be dissected out because it was very tiny, too fragile and delicate and was easily damaged.

Linear measurements and total weight were obtained on the brains and spinal cords before severing the brains immediately caudal to the obex. Individual weights and relative weights of the brain and spinal cord (relative to body mass) were also determined per animal.

A total of 16 gross linear parameters were measured on the brain and spinal cord. All linear measurements were determined with the aid of either a digital vernier calliper (Neiko®, sensitivity of 0.01 mm) or a centimetre rule, and were recorded in millimetres. Weights of the brain and spinal cord were obtained using an electronic digital pocket scale (Camry® Model EHA901, error of 0.01 g) recorded in grams.

The parameters measured and their landmarks are defined below in Figures 1 and 2:

- **Length of brain (LOB):** Distance from the most rostral part of the olfactory bulb to the most caudal aspect of the cerebellum.
- **Length of brain and spinal cord (LOBSC):** Distance from the most rostral part of the brain to the most caudal part of the spinal cord measured *in situ*.
- **Length of cerebrum (LOC):** Distance from the most rostral point of the olfactory bulb to the most caudal aspect of the cerebrum, at the transverse fissure where it articulates with the cerebellum.
- **Length of cerebellum (LOCB):** Distance from the most rostral end of the cerebellum where the cerebellum makes contact with the cerebrum, to the most caudal point or far extremity rostral to the *medulla oblongata*.
- **Width of cerebrum (WIOC):** Maximum distance between the lateral aspects of the two cerebral hemispheres.
- **Width of cerebellum (WIOCB):** Maximum distance between the most lateral aspects of the cerebellum.
- **Height of cerebrum (HOC):** Distance from the ventral aspect of the brainstem to the most dorsal aspect of the cerebrum.
- **Height of cerebellum (HOCB):** Measured from the highest point of the median vermis to the roof of the fourth ventricle.
- **Height of olfactory bulb (HOOB):** This was measured

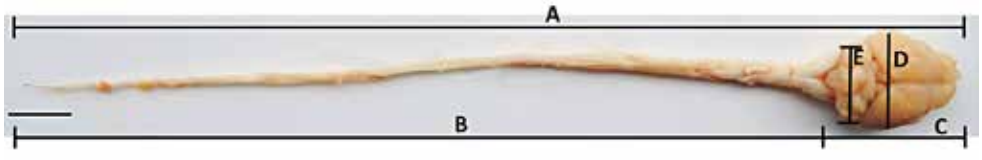

Fig. 1. Dorsal view of the brain and spinal cord of the African pygmy hedgehog, showing the length of the brain and spinal cord (A), the length of the spinal cord (B), the length of the brain (C), width of the cerebrum (D) and width of the cerebellum (E). Scale bar—1 cm

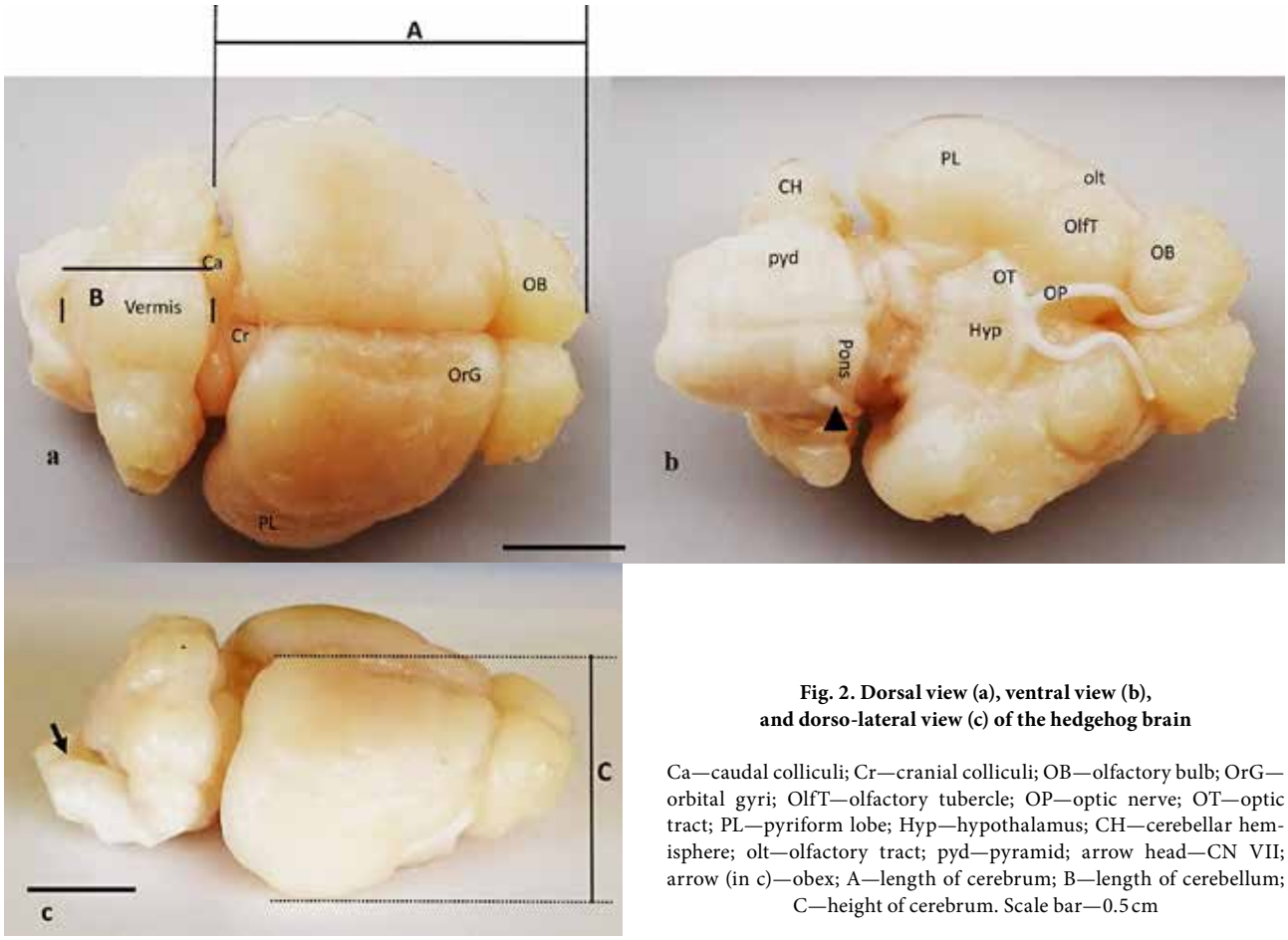

Fig. 2. Dorsal view (a), ventral view (b), and dorso-lateral view (c) of the hedgehog brain

Ca—caudal colliculi; Cr—cranial colliculi; OB—olfactory bulb; OrG—orbital gyri; OlfT—olfactory tubercle; OP—optic nerve; OT—optic tract; PL—pyriform lobe; Hyp—hypothalamus; CH—cerebellar hemisphere; olt—olfactory tract; pyd—pyramid; arrow head—CN VII; arrow (in c)—obex; A—length of cerebrum; B—length of cerebellum; C—height of cerebrum. Scale bar—0.5 cm

from the dorsal aspect of the olfactory bulb to the ventral aspect.

- **Height of brain at the level of pons (HOBP):** Distance from the dorsal aspect of the brain to the ventral aspect of the pons, at the root of the trigeminal nerve (cranial nerve V).
- **Length of spinal cord (LOSC):** Measurement of the spinal cord from its rostral limit, at the emergence of the first spinal nerve, to the tip of the *conus medullaris*.
- **Diameter of the cervical enlargement of the spinal cord (CESC):** Measurement of the thickness/width of the cranial expanded portion of the spinal cord.
- **Diameter of the thoracic enlargement of the spinal cord (TESC):** Thickness/width of the spinal cord, at the thoracic enlargement.
- **Diameter of the lumbar enlargement of the spinal cord (LESC):** Thickness/width of the spinal cord, at the lumbar region.
- **Diameter of the sacral enlargement of the spinal cord (SESC):** Thickness/width of the spinal cord, at the sacral region.
- **Number of vertebrae (NOV):** The total number of cervical, thoracic, lumbar, sacral and coccygeal vertebrae that form the vertebral column.

The external features of the brain were photographed and visually examined from the dorsal, ventral and dorso-lateral views (Figs. 1 and 2). Naming of structures was adapted from texts and previous reports [12, 20, 21]. Encephalization quotient (EQ) was calculated based on the formula described by C a i r ó [3].

Statistical analysis

All data obtained were analysed with ANOVA, with Bonferroni post-test (Graphpad prism version 5, La Jolla, USA), and the level of significance was calculated at $P < 0.05$.

Ethical statement

All experimental protocols conformed to the ethics and guidelines for the care and use of experimental animals by the University of Ibadan, Ibadan, Oyo state, Nigeria and the National Institute of Health (NIH), USA. Ethical approval was obtained from the Animal Care Use and Research Ethics Committee (ACUREC) of the University of Ibadan, Nigeria, code UI-ACUREC/17/0023. Every effort was made to reduce suffering and bring any pain from handling to the barest minimum.

RESULTS

General appearance of the CNS

The most rostral structures of the telencephalon were the two distinct olfactory bulbs, which appeared relatively large compared to other mammalian species. Immediately caudal to the bulbs were the two separate cerebral hemispheres. The cerebrum was mostly lissencephalic (lacking gyri) in appearance, but the demarcation of the orbital gyri and the pyriform lobes were distinct. The *corpora quadrigemina* was observable from the dorsal aspect, with the caudal colliculi being bigger and more laterally extended. The ventral surface showed a clearly demarcated olfactory bulb, well-defined olfactory tubercle and tract, with the olfactory tract being more laterally placed. Caudal to the cerebrum was the cerebellum (comprising the median vermis and the two lateral hemispheres). Beneath these structures was the brain stem, which continued caudally as the spinal cord. The spinal cord possessed a cervical enlargement, and subsequently enlargements at the different segments (thoracic, lumbar and sacral). The mean EQ was 0.37 ± 0.05, with the males being 0.40 ± 0.08, while the females had a non-significant lower value of 0.36 ± 0.04. Of note was the fact that the first cervical spinal nerves were noted to emerge midway around the region of the occipital condyles.

Morphometrics

The results for linear measurements are presented in Table 1 as means ± Standard Deviation (SD).

The mean weight of the African pygmy hedgehog used was 199.00 ± 16.09 g, males 183.50 ± 12.02 g and females 206.80 ± 11.95 g. No statistically significant difference was observed between male and female body weights ($P > 0.05$).

Of all the parameters measured, the females were observed to record higher values in only nine parameters (weight of spinal cord, lengths of brain and of cerebellum, widths of cerebrum and cerebellum, height of cerebellum, diameters of the thoracic, lumbar and sacral enlargements of the spinal cord). The values obtained for the length of the cerebrum was similar in both genders (male 11.53 ± 0.23; female 11.57 ± 1.50). All other parameters showed higher values in the male, although no statistically significant difference ($P > 0.05$) was observed in all parameters measured (Table 1).

The number of vertebrae was consistent in all animals assessed. The total number of vertebrae was 33, with the vertebral formula C7T13L6S4Cd3.

The spinal segment length yielded varying results along the different regions of the spinal cord. The thoracic spinal segment had the most contribution to the spinal cord length as well as the number of spinal nerves while the sacral spinal segments contributed the least.

DISCUSSION

The knowledge of the gross anatomy of the nervous system is an essential tool in treating neurologic diseases, conditions of the CNS and in some medical manoeuvres like inducing local anaesthesia. Morphometric evaluations of the CNS and its surrounding tissues have proven useful in medical imaging, as in histopathological studies [5].

Although males are usually heavier than females of the same age and species, it was not so in this study probably due to the fact that the specific age of the animals examined were not known, since the hedgehogs were

Table 1. Morphometric values of the brain and spinal cord of the African pygmy hedgehog, data presented as means ± SD

	PARAMETERS (unit)	TOTAL (n = 6)	MALE (n = 3)	FEMALE (n = 3)
1	**Weight of animal** [g]	199.00 ± 16.09	183.50 ± 12.02	206.80 ± 11.95
2	**Weight of brain and spinal cord** [g]	2.00 ± 0.17	1.99 ± 0.21	2.01 ± 0.16
3	**Weight of brain** [g]	1.50 ± 0.16	1.52 ± 0.19	1.49 ± 0.16
4	**Weight of spinal cord** [g]	0.49 ± 0.08	0.44 ± 0.10	0.53 ± 0.02*
5	**Encephalization quotient**	0.37 ± 0.05	0.40 ± 0.08	0.36 ± 0.04
6	**Length of brain** [mm]	21.24 ± 0.96	20.74 ± 0.57	21.61 ± 1.08*
7	**Length of brain and spinal cord** [cm]	13.98 ± 0.85	14.34 ± 0.68	13.71 ± 0.95
8	**Length of cerebrum** [mm]	11.55 ± 1.07	11.53 ± 0.23	11.57 ± 1.50
9	**Length of cerebellum** [mm]	6.25 ± 0.59	6.22 ± 0.60	6.28 ± 0.67*
10	**Length of spinal cord** [mm]	11.84 ± 0.85	12.23 ± 0.61	11.55 ± 0.0.96
11	**Width of cerebrum** [mm]	13.84 ± 1.95	12.04 ± 1.28	15.19 ± 0.93*
12	**Width of cerebellum** [mm]	11.97 ± 1.12	11.06 ± 0.58	12.66 ± 0.92*
13	**Height of cerebrum** [mm]	8.63 ± 0.46	8.79 ± 0.72	8.50 ± 0.19
14	**Height of cerebellum** [mm]	6.36 ± 0.69	6.36 ± 0.79	6.37 ± 0.73*
15	**Height of olfactory bulb** [mm]	4.58 ± 0.30	4.45 ± 0.24	4.68 ± 0.33
16	**Height of brain at the pons** [mm]	7.89 ± 0.41	8.19 ± 0.47	7.67 ± 0.21
17	**Cervical enlargement diameter** [mm]	3.12 ± 0.13	3.10 ± 0.22	3.14 ± 0.04
18	**Thoracic enlargement diameter** [mm]	2.21 ± 0.22	2.05 ± 0.26	2.32 ± 0.07*
19	**Lumbar enlargement diameter** [mm]	1.41 ± 0.12	1.35 ± 0.16	1.46 ± 0.06*
20	**Sacral enlargement diameter** [mm]	0.74 ± 0.09	0.68 ± 0.11	0.79 ± 0.05*
21	**Number of vertebrae**	33.00 ± 0.00	33.00 ± 0.00	33.00 ± 0.00

*—Indicates values which were higher in females relative to males
No statistically significant difference was observed in all parameters measured ($P > 0.05$)

captured from the wild, only the age range was known. This is however similar to other reports in goats [15]. The brain of the hedgehogs in this study accounted for about 0.85 ± 0.24 % of the total body weight which is considerably less than the 2 % reported in humans [2, 18], while that of the spinal cord accounted for about 0.26 % of the total body weight. The relative spinal cord weight of 0.26 % is the same as the value reported for the African giant rat (0.26 %) and about half of what was reported in the rabbit [7].

Although slight and not statistically significant, the height, width and length of the cerebellum were higher in the females. This may probably indicate that the females likely possess finer motor dexterity and better cognitive ability, as cerebellar volume has been shown to have a positive correlation to general intelligence and fine motor dexterity; while also contributing to several aspects of cognition [17].

In spite of the fact that the males recorded higher values for the brain weight and height, the females recorded slightly higher values for the length of the brain. This difference may be attributed to the longer length of cerebellum in the females. The heavier brain weight recorded in the males is similar to what was obtained in pigs [14], although the male pigs recorded higher values for brain length while the females recorded higher values for brain height.

The encephalization quotient (EQ) is regarded as "a more complex and approximate alternative to measures of cognition. It takes into account the allometric effects of widely divergent body sizes" [3]. Values obtained are similar to those observed in the domestic pig, 0.39 [13], considerably smaller than that observed in the shrew, 2.54, and the dolphin, 5 [3]. In this study, the males showed a higher EQ value, although not statistically significantly different. Since more recent studies are taking EQ to calculate intelligence [1, 3, 8, 13], it will be interesting to assess which of the genders in this species will be shown to be more intelligent, noting that the values for the linear measurements of the cerebellum were higher in the females.

The African pygmy hedgehog spinal cord was observed to begin at the same level with the middle of the occipital condyles, where the most cranial rootlets of the first cervical spinal nerve roots emerge. However, previous reports state the cranial limit of the spinal cord to be at the *foramen magnum* in all domestic animals [9, 20].

Apart from the smaller number of the caudal bones, the vertebral formula was similar to that observed in the African giant rat [16].

CONCLUSIONS

This study provides basic topographic and macro-morphometric reference data on the African pygmy hedgehog brain and spinal segments that may be useful in applied research for regional anaesthesiology, diagnostic medical imaging in brain and spinal cord diseases, veterinary neuroanatomy and also comparative and regional anatomy. Data documented from this may provide a basis for future research as well as contribute to the knowledge of African pygmy hedgehog anatomy.

CONFLICT OF INTEREST

The authors declare there are no conflicts of interest.

ACKNOWLEDGEMENT

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ICHTHYOSIS IN DOGS—CONGENITAL DERMATOLOGIC DISORDER

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ABSTRACT

The skin provides protective functions, such as thermoregulation, resorption, provision of immune responses, storage and sensory functions, which all play an important role in the internal stability of the organism. The skin has 3 major layers: the epidermis, the dermis and subcutis. The outermost protective layer of the epidermis, the stratum corneum, consists of 20 to 30 overlapping layers of anucleate cells, the corneocytes. Ichthyosis is an autosomal recessive congenital skin disease, in which the corneocytes form defects that appear like individual steps of the stratum corneum. Ichthyosis is characterized by excessive scaling over the entire body surface and is not curable; the symptoms can only be alleviated. Several genetic variants have been identified in specific dog breeds: *PNPLA1* in the Golden Retrievers, *SLC27A4* in the Great Danes, *NIPAL4* in the American Bulldogs, *TGM1* in the Jack Russel Terriers, *ASPRV1* in the German Shepherds, which cause different forms of nonepidermolytic ichthyosis and *KRT10* in the Norfolk Terriers, which causes epidermolytic ichthyosis. When classifying breeds of dogs predisposed to ichthyosis, it is necessary to determine the presence of defective genes in the genome of the individual animals involved in mating.

Key words: dog; gene; hereditary disease; keratin; skin

INTRODUCTION

Ichthyosis represents a group of rare congenital skin disorders characterized by excessive scaling caused by defective formation of the stratum corneum, which is the main skin barrier. It makes life very unpleasant for the suffering patients. The disease is caused by changes in the genes that encode a wide range of molecules, including enzymes, structural proteins and lipids, involved in the formation of the stratum corneum. Ichthyosiform dermatoses affect humans, dogs, cattle, rats, cats and pigs [4, 7, 9, 18, 26, 27].

The skin is the largest organ of the organism and forms an important outer barrier between the factors of the ex-

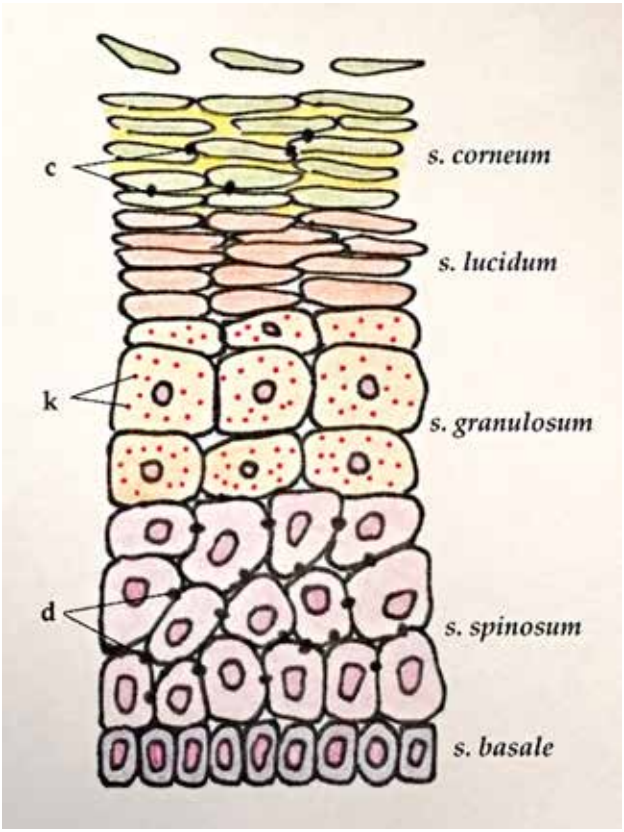

Fig. 1. The epidermis
c—corneodesmosomes; d—desmosomes; k—keratohyalin granules

ternal environment and the internal environment of the organism. In addition to providing a protective barrier against the different effects (physical, chemical, ultraviolet radiation, microbial, etc.) of the environment, the basic functions of the skin include thermoregulation, resorption, provision of immune responses, storage and sensory functions. If the skin is not in order, the whole internal stability of the organism may be disturbed [15, 34].

The skin

The skin has 3 major layers: the epidermis (outermost layer), the dermis (middle layer) and subcutis (innermost layer). The thickness of the skin is not the same over the entire body surface. Other important parts of the skin include: skin appendages (such as hair and claws) and subcutaneous muscles and fat [28, 35].

The outer layer of the skin, the epidermis (Fig. 1), is composed of several cell types, including keratinocytes, melanocytes, Langerhans cells and Merkel cells. In the process of the keratinization (cornification) new epidermal cells are created near the base of the epidermis and migrate upwards and differentiate from basal keratinocytes to the highly specialized corneocytes at the same time. The outermost protective layer of epidermis, stratum corneum, consists of 20 to 30 overlapping layers of polyhedral anucleate cells, the corneocytes. Corneodesmosomes play an important role, they contribute to the cohesiveness of the stratum corneum by connecting corneocytes to each other [35]. On the healthy skin, there is always a layer of dead skin cells, which are continuously removed and replaced by cells from the lower layers. The melanocytes are located mostly at the base of the epidermis and produce the skin and hair pigment called melanin. The Langerhans cells are representative of immune processes in the skin and provide phagocytosis and antigen presentation. Merkel cells are specialized cells associated with the sensory organs in the skin. Their role in animals is to provide the sensory information from whiskers and the deep skin areas called tylotrich pads [19, 29, 35].

There are no blood vessels in the epidermis and its nutrition is ensured through tissue fluid. Under the stratum corneum are other epidermal layers: stratum lucidum, stratum granulosum with keratohyalin granules, stratum spinosum and stratum basale. In the connection zone between the epidermis and the dermis is located the basement membrane, which can be damaged by skin diseases, including a number of autoimmune conditions [35].

The dermis is made up of connective tissue, and contains sebaceous and sweat glands, claws, blood vessels, nerves and hair. The blood vessels that supply the epidermis with nutrients are located in the dermis. Their role is also to regulate skin and body temperature. Sensory and motor nerves are present in the dermis and ensure the skin's response to the sensations of touch, pain, itch, heat and cold. A large amount of water is bound in the dermis, and in addition there are hyaluronic acid, elastic, collagen and reticular fibres.

The hair follicles in dogs are compound. The hair follicle has a central hair that is surrounded by 3 to 15 smaller secondary hairs, all exiting from one pore. The hair coat protects the skin from physical and ultraviolet light damage, and helps regulate the body temperature. The sebaceous glands secrete an oily substance (a mixture of fatty acids called sebum) into the hair follicles and onto the skin. These oil glands are present in large numbers near the paws, back of the neck, rump, chin, and tail area. Sebum is important for maintaining the skin soft, moist, and pliable.

The sweat glands in dogs are present only on the feet [28, 33, 35].

The innermost layer of skin is the subcutis (hypodermis) which is formed with the subcutaneous fat and muscles. The main functions of the subcutaneous fat are insulation, a reservoir for fluids, electrolytes and energy and a shock absorber [28].

Ichthyosis

Congenital malformation is a deviation that occurs during the intrauterine development of the fetus. It arises either on a hereditary basis or without a hereditary burden. A missing or altered chromosome or a missing and altered gene can cause the development of a birth defect. Congenital skin diseases in dogs are caused by the presence of a faulty gene [8, 24].

Ichthyoses belong to a heterogeneous group of genetically determined dermatoses characterized primarily by excessive skin scaling over the whole body surface. The name ichthyosis comes from the Greek word ichtys (fish), because the peeling skin resembles fish scales [8, 9]. The disease has been known for a long time, it occurs in several breeds of dogs, but it was not until 2012 that the genetic reason was confirmed [9]. In individual affected breeds, these are mostly mutations in different genes, which are transferred to the gene pool of the offspring. The mutations present in the genes cause defects in the formation of the stratum corneum in the epidermis. However, spontaneous mutations may occur in dogs of each breed or crossbreeds [24].

Although it does not seem so, the stratum corneum performs very important functions. It is the key layer that restricts water movement into and out of the skin. Even its slight damage results in increased transepidermal water loss [21, 32]. This was also confirmed by the increased water losses in dogs with atopic lesions [31]. The continuous desquamation largely ensures the barrier function of the skin and at the same time a certain resistance to microbial pathogens. In addition to the physical exclusion of pathogens, the stratum corneum also contains natural antimicrobial peptides such as defensins and cathelicidins. All abnormalities in the corneal layer may predispose the dog to bacterial and yeast infections [8]. The process of the stratum corneum formation is complex, with every single step being controlled by genes. If an erroneous entry of genetic information is present and one step in the process of forming this layer is disrupted, the formation of the original stratum corneum is impossible. The process of stratum corneum formation consists of several important steps: bundling of the keratin to establish the corneocyte core, replacement of the cell membrane with a thick cornified envelope, and formation of the lipid lamellar bilayers and desquamation. The final form of the stratum corneum is a tough hydrophobic but biochemically active layer of the corneocytes between lipid layers. When any step is changed, the stratum corneum barrier function is disrupted and an attempt is made to repair it. A state of lipid upregulation occurs to replenish lipids into the stratum corneum, causing the epidermis to become hyperplastic [8, 24].

In veterinary medicine, two forms of ichthyosis are described: non-epidermolytic and epidermolytic. The non-epidermolytic ichthyosis is characterized by hyperkeratosis and epidermal hyperplasia with hypergranulosis and the presence of vacuoles and lysis of the keratinocytes within the spinous and granular cell layers. defects arise in the formation step. It has been found in several purebred dog breeds, such as the Golden Retriever, Great Dane, American Bulldog, Jack Russell Terrier, German Shepherd and other breeds [1, 9, 13, 20, 22—25]. The epidermolytic ichthyosis was described in the Norfolk terrier and sporadically occurs in other dogs, e. g., Rhodesian Ridgeback or Labrador cross. The defects arise in the formation step of keratin formation (i.e. formation of the corneocyte core) [5, 11, 24].

Ichthyosis in Golden Retrievers

Ichthyosis as genodermatosis is best known in Golden Retrievers (Fig. 2), sometimes referred as lamellar ichthyosis (LI). In Golden Retrievers, ichthyosis is manifested by adherent scales of various size (from small to large) with a wide range of colorization; at the beginning with whitish scales, later from grey to brown and at the end with blackish scales are presented. The range of ichthyosis forms in this breed varies, ranging from mild, moderate to severe [12]. A mild form of ichthyosis is more common, it is often pronounced especially on the ventral trunk. The range of forms of ichthyosis in this breed varies, ranging from mild, moderate to severe associated hyperpigmentation. The glabrous skin is rough and looks like emery. The clinical signs of a fully manifested disease include lesions of exfoliation and hyperpigmentation that are generally not itchy. The lesions are located mainly in the axilla, thorax, flanks and

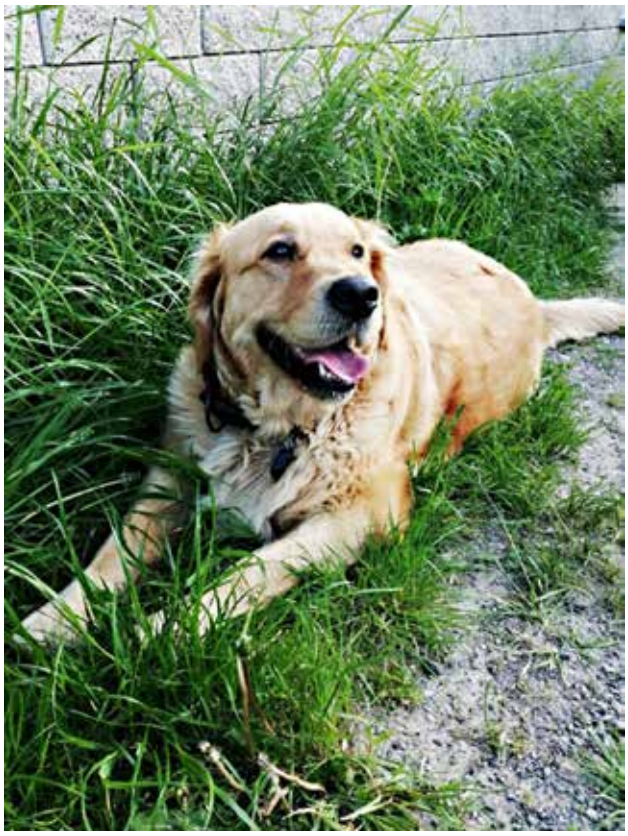

Fig. 2. Ichthyosis is typical for Golden Retriever breed

inguinal region with symmetrical mounting. The area of the paws, nose and ear canals are not usually affected. In dogs, it appears before the 1st year with moderate to severe degree, but it may not appear until adulthood. The formation of skin scales lasts for the whole life of the individual, but the disease may be manifested with a recurring exacerbation and/or remission. Secondary bacterial, mycotic or parasitic infections can be created and it may lead to pruritus [3, 10, 12, 22, 24].

On the histological examination, laminated or compact orthokeratotic hyperkeratosis and numerous persistent corneodesmosomes are usually recorded in the stratum corneum. The stratum granulosum is without significant involvement [12].

Golden Retrievers ichthyosis belongs to autosomal recessive inherited diseases. The disease has been known for a long time, but the exact cause was published in the year 2012; namely it is an insertion-deletion mutation in exon 8 of the gene *PNPLA1* (patatin-like phospholipase domain-containing protein 1), that leads to the formation of a premature stop-codon, with loss of 74 amino acids and is assumed to arise to a non-functional protein causing alterations in the keratinization process A mutant *PNPLA1* supposes to harbour potential causative mutations for human ichthyosis in European and North African populations and its discovery in Golden Retrievers has helped elucidate ichthyosis in humans [9]. A recessive simple Mendelian mode of inheritance applies to ichthyosis [25]. The disease occurs in individuals who inherit the faulty mutated gene from both parents, and in diagnostics are marked as positive/positive (homozygous). Heterozygous, individuals (positive/negative), who have one faulty gene present are clinically healthy but pass the gene on other offspring. When covering two heterozygotes (2 × positive/negative), there is a possibility that puppies with ichthyosis (positive/positive) of 25 % will be born [9]. Homozygous dogs will transmit the *PNPLA1* variant to 100 % of their lineage. The reliability of the *PNPLA1* mutation in predicting clinical signs of ichthyosis in dogs is unclear. It is not yet known whether *PNPLA1* is the only gene involved or whether other genes or environmental factors may play a role in the development of ichthyosis in Golden Retrievers [10]. In veterinary diagnostics, a genetic test by PCR using buccal swabs or whole blood is available to detect wild-type, heterozygous and homozygous dogs and helps to plan the right healthy breeding. Homozygous individuals must not be allowed to breed and each breeding dog should be examined for the *PNPLA1* presence. The homozygous puppies can exhibit clinical signs suggestive of ichthyosis prior to 1 year of age but in adulthood become subclinical or have moderate form of ichthyosis. The breeders often do not consider mild or moderate scales and hyperpigmentation as symptoms of hereditary disorder and they can unknowingly use these dogs in breeding and spread ichthyosis further [30]. Several studies have been performed to determine *PNPLA1* mutation in Golden Retriever individuals. In the year 2018, G r a z i a n o et al. [10] found that the prevalence of the *PNPLA1* gene variant in Italian breeding programs was high and that only 21 % of the sampled dogs represented the wild-type. A study in Germany reported the result in puppies: 19 % of wild type, 53 % of heterozygous and 28 % of homozygous and in adult dogs it was similarly: 18 % of wild type, 47 % of heterozygous and 35 % of homozygous [30].

In addition to the genetic test, the diagnosis is made by classical examination methods used in the veterinary practice. However, determining the correct diagnosis often

depends on the veterinarian's subjective view and experience and diagnostic possibilities. In a differential diagnosis, sebaceous adenitis, atopy, demodicosis and other parasitic skin disease, hormonal and metabolic disease, must be excluded. A skin biopsy is sometimes required to make a definitive diagnosis [24].

Ichthyosis cannot be cured. At the present, there is no effective treatment for ichthyosis; they can only supress the symptoms of the disease and maintain them to a certain acceptable extent. The proper treatment of an ichthyotic dog includes regular combing, bathing in special shampoos, applying emollient ointments to help maintain a skin barrier and prevent excessive water loss. A quality diet is essential; supplements of omega fatty acids are ideal [24].

Ichthyosis in Great Danes

Ichthyosis in Great Danes occurs with characteristic strong wrinkles in the head and legs area immediately since birth. The puppy's face looks like Shar Pei's. The skin on the head and legs, mostly around the nose and eyes has an oily appearance, and a yellow, greasy material gradually covers the skin in other areas of the body as well. Puppies may have trouble opening their eyes. Fine white to yellow dry flaking scales gradually begin to appear all over the body in the coat. The skin is dry, inelastic and lichenified. Glabrous skin in the axilla and inguinal area are very dry and has a leathery appearance. Ichthyotic changes with advancing age, may lead to secondary infections and inflammatory skin lesions, which occur mainly in-between wrinkles due to the increased exudative character of the skin. The prognosis is bad [14, 25].

The skin histopathological examination shows typical symptoms of lamellar ichthyosis: diffuse, orthokeratotic hyperkeratosis, follicular keratosis, focal keratin plugging, and acanthosis with vacuolization of keratinocytes without epidermolysis. The accumulation of an amorphous material within hair canals and sebocytes found with different staining patterns, which were observed in samples of ichthyotic Great Dane until electron microscopy, have not been reported in other breed suffering from ichthyosis [25].

In the genetic analysis of a Great Dane genome, a wide significant peak was detected on chromosome 9 at 57—58 Mb in the region of *SLC27A4*. The mutant transcript of *SLC27A4* showed an in-frame loss of 54 base pairs in exon 8, which resulted in the loss of 18 amino acids [14]. Defective *SLC27A4* was not found in genotyping 413 controls from 35 different breeds of dogs and seven wolves; it occurs only in Great Danes. In the analysis of skin biopsies in ichthyosis patients was found that mutant *SLC27A4* protein to induce an uneven distribution of lipids and disturb the formation of the skin barrier [17] and this was confirmed in the study of 15 cases of Great Dane puppies affected with ichthyosis [14].

Ichthyosis in American Bulldogs

The disease appears shortly after the birth of the puppies. The first symptom is a dishevelled pelage with generalized soft and white scales, which are recognizable in 1 to 2-week-old animals. Other symptoms are light brown and mildly erythematous skin of the abdomen and diffusely adherent light brown scales. These clinical signs persist into adulthood in a milder or more severe form. A diffuse and severe scaling with large white scales loosely adherent to the skin or in the hair on the dorsum and lateral thighs may be present. Adherent grey scales are present on the muzzle, around the eyes and on the pinna. Pruritus is present in the case of secondary infection, most often in Malassezia overgrowth [2, 4, 23].

A light microscopic examination records diffuse laminated to compact orthokeratotic hyperkeratosis with hypergranulosis and mild acanthosis. The epidermis has a prominent granular layer, and multifocal granular layer keratinocytes display, a perinuclear clear space and the stratum corneum contains randomly arranged layers of variably sized, often enlarged corneocytes. The keratin bars in the stratum corneum are closely apposed [23].

Ichthyosis in the American Bulldog is caused by a deleterious molecular defect in the *NIPAL4* gene encoding ichthyin. The ichthyin, a transmembrane protein is encoding by 6 exons that resides on canine chromosome 4, composed of 404 amino acids and expressed in the granular layer of the epidermis. It is thought to play a role in lipid metabolism during the epidermal development and works as a Mg^{2+} transporter. The inability to create the normal stratum corneum is caused by non-functional truncated ichthyin protein and leads to the specific symptoms of ichthyosis. 35.6 % of American Bulldogs are heterozygote (carriers) for the disease and 5.4 % are affected with ichthyosis [2, 4, 23].

Ichthyosis in Jack Russel Terriers

Nonepidermolytic ichthyosis in the Jack Russel Terrier is associated with insertion of a long-interspersed nucleotide element (LINE-1) in the transglutaminase 1 (*TGM1*) gene. This ichthyosis form is homologous with *TGM1*-deficient LI in humans *TGM1*. The symptoms are typical for ichthyosis and appeared as early as the puppy's age. The affected dogs have a generalized severe form of hyperkeratosis with large adherent scales. In histological examination, the laminated to compact hyperkeratosis without epidermolysis was observed and on transmission electron microscopy, the stratum corneum was thickened and intercorneocyte spaces were extremely narrow. The first three corneocyte layers contained tonofilaments that were irregular, coarse and wavy [6, 20].

Ichthyosis in German Shepherds and in other breeds

In German Shepherds a novel form of ichthyosis was described, which is caused by a de novo missense variant in the canine *ASPRV1* gene. *ASPRV1* encodes "aspartic peptidase, retroviral-like 1" also known as skin aspartic protease (SASPase), which is involved in profilaggrin-to-filaggrin processing [1, 16]. The novel variant had arisen by a de novo mutation event that must have occurred in either one of the parental germlines or during early embryonic development of this patient. The symptoms in the affected female include: severe scaling of the skin with mild pruritus, generalized hypotrichosis, allopathic lesions with severe exfoliation of greyish scales and mild erythema, comedones on the abdomen and in the perivulvar area. The symptoms developed immediately after birth [1].

In English Springer Spaniels, Labrador Retrievers and West Highland White Terriers the cases of ichthyosis were confirmed, but molecular identification has not been documented [24].

Epidermolytic ichthyosis in Norfolk Terrier Dogs

An autosomal recessive form of epidermolytic ichthyosis has been observed in Norfolk Terrier Dogs. This was caused due to a splice-site mutation in the gene encoding keratin 10 (*KRT10*). The mutation is associated with activation of at least three cryptic or alternative splice sites, which resulted in transcripts containing premature termination codons. The affected dogs displayed generalized and pigmented hyperkeratosis with epidermal fragility. In histologic examination, epidermolysis with hyperkeratosis, a decrease in tonofilaments and abnormal filament aggregations were found in the upper spinous and granular layer of keratinocytes [5].

CONCLUSIONS

Ichthyosis is an autosomal recessive inherited skin disease, which affects specific breeds of dogs e.g. Golden Retriever, Great Dane, American Bulldog, Jack Russel Terrier, German Shepherd and Norfolk Terrier. Ichthyosis is caused by the presence of defective genes, that affect the creation of corneocytes and individual steps in the process of stratum corneum formation. The main clinical sign of a fully manifested disease is abnormal skin scaling of the entire body surface. The disease is not treatable and makes life unpleasant for sick dogs and their owners. In veterinary practice, genetic tests are important in the diagnosis of ichthyosis. Tests are increasingly used not only in clinical practice, but also in dog breeding. The breeder creates new generations of dogs and should approach the health of their offspring responsibly. An important role here is played by a veterinarian, who should explain to the owners the importance of genetic tests and the possibilities of using their results. The veterinarian should also emphasize that the sick individual must not be used in breeding and that the carrier is a healthy dog, which can be used in breeding and have healthy offspring if the partner is carefully selected.

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MILK UREA CONCENTRATION TO THE EVALUATION OF NITROGEN EFFICIENCY TRANSFORMATION ON DAIRY FARMS

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ABSTRACT

The aim of this study was to evaluate the effects of nutrition on the milk urea nitrogen (MUN) concentration; on the transformation of N in the farm's conditions; and there-by allow the milk urea nitrogen concentration to serve as a tool to maximize the protein nutrition and the metabolism of the cows. The relations evaluated by linear or multiple regression confirmed that the highest nutritional effects of the crude protein (CP) on the MUN concentration, which represented a 69.3 % variation in the MUN content. According to the CP content in the total mix ration (TMR) and MUN content (3150 milk samples) under farm conditions, a regression relationship was determined for the estimated of MUN ($mg.dl^{-1}$) = $-13.2 + 0.16 \times CP$ ($g.kg^{-1}$ dry matter). For multiple regression, the rate of variation expressed by this relationship increased to 72 for nutrient content and 78.3 % for nutrient intake in the TMR. The efficiency of nitrogen utilization (ENU) determined by calculations based on the MUN content according to the regression equations represented a negative correlation ($P < 0.0001$; $R2 = 0.854$) with respect to the CP content in the TMR and that the increased CP content by 1 % in the range of 14 to 18 % in the TMR decreased the ENU by 1.48 %. Validation of the models for prediction of nitrogen transformation (ENU) for practical application on the farms determined the best equation, which used the available data from the routine analysis of Breeding services of Slovakia. After taking into consideration of our breeding conditions, it was confirmed that the equation of ENU had taken into account the MUN, in addition to the amount of the milk produced.

Key words: milk urea nitrogen MUN; N utilization efficiency; protein nutrition

INTRODUCTION

Protein metabolism in ruminants is a complex process influenced by the fermentation of carbohydrates and the degradation and synthetic capacity of rumen microorganisms. The optimal content of nitrogen in the formulation

of daily ration presents such an amount of crude protein (CP) in the total mix ration (TMR) which on the one hand prevents production losses at the lower intake of CP, and on the other hand does not lead to metabolic load and losses of nitrogen in the urine when the content of CP in TMR increases. In dairy cows, the proteins supplied from the feed undergo five major metabolic transformations, which include:

1. degradation of feed proteins and microbial protein synthesis in the rumen,
2. enzymatic digestion and absorption of amino acids in the small intestine,
3. metabolic transformation in tissues, synthesis and secretion of milk proteins,
4. catabolism and excretion of unused proteins,
5. their metabolites being excreted in the faeces and urine [14].

The level of protein nutrition in farm conditions can be evaluated:

a) by analysing the nutritional composition of TMR and assessing daily intake of nutrients. This requires an accurate assessment of the daily CP intake of the production group, which is in practice limited mainly due to inaccuracy in the prediction of dry matter intake [17];
b) by monitoring the production parameters by evaluation of milk production and milk protein [8];
c) by monitoring the metabolites (the concentration of urea in milk and/or blood) for evaluation of protein transformation [16].

A significant tool for the evaluation of protein nutrition is the determination of the efficiency of nitrogen utilization (ENU), defined as the ratio between the content of nitrogen excreted in the milk and the amount of nitrogen intake utilized as CP from the TMR. The ENU is influenced mainly by the protein concentration in the TMR and the content of the urea nitrogen in the milk [16]. Urea presents the final metabolite of nitrogen transformation of ammoniacal nitrogen absorbed from the rumen after degradation of feed proteins [22], as well as deamination products of the absorbed amino acids (aAA) not used in proteosynthesis, due to aAA imbalances or lack of energy [25]. Thus, transformed nitrogen compounds form a proportion of urea in the blood that has three metabolic pathways in dairy cows:

(I) recycling of urea through saliva and through the rumen wall back into the rumen, which is an important source of nitrogen for the synthesis of microbial proteins;
(II) excretion of urea into milk;
(III) urea excretion which via the kidneys is the major route of excretion of excess nitrogen from metabolism into the urine [13].

Milk urea is mainly derived from blood urea, and a direct relation between milk urea and blood urea has been confirmed [2, 4]. The results of the measurements of the milk urea (MU) or milk urea nitrogen (MUN) provides valuable information on the nutritional status, the level of excretion of N, and is an ecological burden of the environment [16] and enables the evaluation of the efficiency of nitrogen utilization (ENU) from feed into milk protein [24]. Nitrogen, which is not transformed into milk protein, is metabolized in the tissues, where it presents metabolic load and also an ecological burden for the environment after excretion from the body.

The aim of our study was to analyse the relation of nutrition (nutrient concentration and daily nutrient intake in TMR) to MUN concentration under farm conditions and to evaluate the efficiency of metabolic transformation of nitrogen to milk protein and validation of models for ENU prediction.

MATERIALS AND METHODS

The evaluations were carried out in 30 herds with an average annual production of milk of between 8,500 and 9,500 kg per cow. For evaluations, we selected dairy cows in the first lactation phase according to days in milk (DIM; 21—130 DIM), or according to daily milk production. Feed rations of dairy cows were predominantly based on corn and alfalfa silage, supplemented with different carbohydrate's feeds (cereal grains and cereal grain by-products) and protein supplements (soybean meal and rapeseed meal) provided as TMR *ad libitum*. At the farms studied, the daily feed intake and nutritional composition of TMR were intensively monitored throughout the year and dairy cows of all of the farms were kept in a free-stall housing system.

Analysis of TMR

The samples of the prepared TMR on the farms were taken from the feed manger on the control day and were analysed for dry matter (DM), which consistent of: crude protein (CP), acid and neutral detergent fibre (ADF, NDF),

and starch and ether extract (EE) contents according to conventional methods [6]. DM was determined by weight upon drying the sample at 105 °C under the prescribed conditions. The CP content was determined by Kjeldahl method using a 2300 Kjeltec Analyser Unit (Foss Tecator AB, Hoganas, Sweden). Fat (as ether extract) was determined by the device Det-Gras (JP SELECTA, Spain). And ADF and NDF were determined using Dosi-Fiber Analyser (JP SELECTA, Spain) and the content of starch was determined by polarimetry. The net energy of lactation (NEL) was calculated using regression equations according to National Research Council (NRC) [17].

Analysis of production parameters

All farms were evaluated by the Breeding Services of the Slovak Republic on the control day of milk collection, in the framework of checking the performance of milk yield. Milk samples were analysed for the total protein content, fat, lactose, and urea concentration by a near-infrared spectrophotometric assay using MilkoScan FT^{+} and BENTLEY FTS at the Central Analytical Laboratory of Milk with accreditation under the registration number 096/5878/2015/2. The analysed urea in milk (MU) was converted to urea nitrogen in milk (MUN) using the MUN equation (mg.dl^{-1}) = MU (mg.dl^{-1}) × 0.4667 [19].

Statistical analysis

The average values, descriptive statistics, and variability of the examined markers, as well as the influence of factors on these properties, were studied using software XLSTAT2018. The evaluation of the regression equations for the determination of the ENU have consisted in the validation of models to the correctness and accuracy based on the mean bias [12] and the root mean square prediction error (RMSPE) [3] or residual error [12].

RESULTS

Nutrient content of rations, feed intake and milk production in groups of dairy cows

The average concentration of nutrients in TMR, daily intake of nutrients and the production parameters of dairy cows on the farms are presented in Table 1.

The average milk production was 35.4 ± 6.4 kg.d^{-1} (range 23.2 to 47.9), the content of milk protein was 3.15 ± 0.2 % and milk fat was 3.62 ± 0.4 %. The content of MU was 27.4 ± 3.6 mg.dl^{-1} and MUN 12.79 ± 1.67 mg.dl^{-1} (with ranges 19.74 to 35.7 and 9.21 to 16.64 respectively). Fluctuations of production parameters reflect the different content of nutrients and the composition of ration, genetic production potential, and the order of lactation. The parameters of milk production and composition of TMR showed significant differences in the chemical composition of nutrients against actual production and composition of the milk.

The relation of milk urea concentration to management of nutrition

A total of 30 TMRs were analysed and evaluated to confirm the dependence between the variable concentrations of nutrients in daily rations for the production groups and the MUN concentrations in 3150 individual milk samples of dairy cows. The analysed nutritional and production parameters (concentration or daily nutrient intake in TMR, milk production, and composition) were statistically processed to test in their relation as independent variables to MUN concentration as a dependent variable.

The relationships were evaluated by simple linear regression (Table 2) based on the determination coefficient (R2) and the level of statistical significance (P). The independent variables that were statistically significant ($P < 0.05$) in models of simple linear regression were transferred to a model of multiple linear regression (Table 3) to identify the causes of variation of MUN concentration.

In single linear regression, the evaluation of the relation of the nutrient content in the TMR and the MUN confirmed the most statistically significant positive relationship ($P < 0.001$) between the average concentration of the MUN and the CP content ($R^2 = 0.693$). In contrast, the negative correlation was confirmed between the MUN concentration and the content of starch, NFC, and NEL ($P < 0.001$). The content of crude protein in the TMR was the best single marker for the MUN estimation on average for the production group and represented 69.3 % of the total variance determined by the regression relationship MUN (mg.dl^{-1}) = −13.2 + 0.16 × content CP (g.kg^{-1} DM; Fig. 1). The nutrient intake confirmed the positive correlation between the CP intake and the concentration of MUN ($R^2 = 0.307$, $P < 0.001$) and negative correlation with the starch intake ($R^2 = 0.224$, $P < 0.01$) and NFC intake ($R^2 = 0.185$, $P < 0.05$).

Table 1. Nutritional composition of TMR, production and composition of the milk

	Average	SD	Minimum	Maximum
Nutrients in TMR [% DM]				
Crude protein	16.20	0.8	14.42	17.85
NEL	6.76	0.2	6.38	7.13
NDF	34.44	2.6	28.04	38.75
ADF	20.69	1.6	16.07	24.68
Starch	25.01	3.3	16.93	29.78
NFC	37.99	3.4	31.65	48.95
NEL/CP	0.42	0.03	0.37	0.48
Starch/CP	1.55	0.3	1.02	2.07
NFC/CP	2.35	0.3	1.89	3.17
Daily intake of nutrients [kg.day^{-1}]				
Dry matter	22.35	1.3	20.76	24.90
CP intake	3.62	0.2	3.20	4.02
NEL intake	150.89	10.6	134.09	171.83
NDF intake	7.68	0.7	6.07	8.69
ADF intake	4.62	0.5	3.42	5.40
Starch intake	5.60	0.9	3.66	7.03
Production and composition of milk				
Milk yield [kg.d^{-1}]	35.40	6.5	23.20	47.90
Milk protein [%]	3.15	0.2	2.77	3.46
Milk fat [%]	3.62	0.4	2.95	4.55
Yield of milk protein [kg.d^{-1}]	1.11	0.2	0.77	1.46
Yield of milk fat [kg.d^{-1}]	1.26	0.20	1.00	1.67
Milk urea [mg.dl^{-1}]	27.40	3.58	19.74	35.66
Milk urea nitrogen [mg.dl^{-1}]	12.79	1.67	9.21	16.64

TMR—total mix ration; CP—crude protein; ADF—acid detergent fibre; NDF—neutral detergent fibre; NEL—net energy of lactation; NFC—non-fibre carbohydrate; SD—standard deviation

The independent variables from the single linear regression were added in the statistical evaluation into the multiple regression by the "stepwise method" at the significance level of $P < 0.05$ and eliminated variables at the significance level of $P > 0.1$. The accuracy of the model was evaluated by the adjusted R2, P-value, Akaike information criterion, and the root mean square error. The relation of the average of concentrations of the MUN of dairy cows in the production groups in multiple regression (Table 3) to the nutrient concentration in the TMR confirmed a high correlation ($P < 0.0001$) to the CP and NDF with a 72.0 % level of variability on the concentration of the MUN.

The multiple regression confirmed the impact of the nutrient intake on the level of the average concentrations of the MUN, with a significant relation of the daily intake of the CP ($P < 0.0001$) on the MUN concentration and with a minor impact of the daily starch intake and dry matter intake of the TMR. Selected nutritional factors affected the

Table 2. Linear regression between MUN in milk and nutrients content of TMR, daily intake of nutrients, milk yield and composition

Items	Slope	SD	r	R^2	P
Concentration of nutrients in TMR [% DM]					
CP	1.600	0.201	0.832	0.693	< 0.0001
NEL	–3.223	1.329	0.417	0.174	0.022
NDF	0.181	0.105	0.310	0.096	0.096
Starch	–0.247	0.072	0.542	0.294	0.002
NFC	–0.233	0.072	0.649	0.273	0.003
Starch/CP	–4.208	0.816	0.698	0.487	< 0.0001
NFC/CP	–3.895	0.698	0.726	0.527	< 0.0001
NEL/CP	–44.05	6.119	0.806	0.649	< 0.0001
Daily intake of nutrients in TMR [kg.d^{-1}]					
Dry matter	–0.118	0.214	0.105	0.011	0.587
CP intake	3.791	1.076	0.554	0.307	0.001
NEL intake	–0.040	0.026	0.281	0.079	0.133
NDF intake	0.388	0.421	0.170	0.029	0.365
Starch intake	–0.786	0.276	0.473	0.224	0.008
NFC intake	–0.623	0.247	0.430	0.185	0.018

TMR—total mix ration; CP—crude protein; ADF—acid detergent fibre; NDF—neutral detergent fibre; NEL—net energy of lactation; NFC—non-fibre carbohydrate;

Table 3. Multiple regression of nutritional effect on MUN concentration

Concentration of nutrients in TMR [% DM]			
Items	**Coefficient**	**SD**	**P**
Intercept	–16.799	3.473	< 0.0001
CP	1.552	0.190	< 0.0001
NDF	0.216	0.099	0.038
Precision of the model			
Adjusted R^2	–	–	0.720
RMSE	–	–	0.803
AIC	–	–	–10.302
P	–	–	< 0.0001
Daily intake of nutrients [kg.day^{-1}]			
Intercept	5.171	2.425	0.043
CP intake	6.359	0.968	< 0.0001
Starch intake	–0.576	0.192	0.006
Dry matter intake	–0.548	0.205	0.013
Precision of the model			
Adjusted R^2	–	–	0.783
RMSE	–	–	0.707
AIC	–	–	–17.074
P	–	–	< 0.0001

TMR—total mix ration; CP—crude protein; NDF—neutral detergent fibre; AIC—Akaike information criterion; RMSE—root mean square error

concentration of the MUN at the level of 78.3 % in dairy cows. The statistical evaluation of the multiple regression confirmed more significant effects of the daily intake of nutrient on the variability of the MUN concentration compared to the content of nutrients in the TMR.

Evaluation of the efficiency of nitrogen utilization from feed to milk

The efficiency of nitrogen utilization (ENU) determined as a percentage of the nitrogen ingested that is transformed into milk protein (empiric model), that is calculated from the production of milk protein (yield of milk protein) and CP intake in the ration according to equation ENU (%) = [100 × N milk/N intake] [7, 17]. In the farms, the estimated efficiency of utilization (Table 4) was determined on average at 30.1 ± 5.0 % with significant fluctuations in farm values with the range from 2″ 1.1 % to 39.5 %.

The efficiency of the nitrogen utilization according the MUN content for the production group of dairy cows was estimated by using the regression equations from meta-analytical assessments of the balance experiments [15, 24] or in combination with the amount of milk produced [11]. Thus, the calculated ENU according to individual authors (Table 4) gives a lower range of value fluctuation of the mean and in relation to the CP content in the TMR the higher negative correlation was confirmed.

The dynamics of the ENU changes, calculated from the analysed MUN content, expressed as the average value of regression equations of selected authors in farms (Fig. 1), shows a negative correlation ($P < 0.0001$; $R^2 = 0.729$) in relation to the CP content in the TMR, where a 1 % increase in the CP in the range of 14 to 18 % in the TMR, reduces the efficiency of the use of nitrogen from feed to milk by 1.48 %.

The ENU determined by the transformation of N to milk protein (empiric model) in relation to the CP content of the TMR is expressed by the equation ENU % = 90.5 – 3.73 CP % confirmed a negative correlation ($R^2 = 0.350$; $P = 0.001$), where a 1 % increase in the CP in the range of 14 to 18 % in the TMR, reduces the efficiency of the use of nitrogen from feed to milk by 3.73 %.

Table 4. Predicted efficiency of N utilization (ENU) in relation to CP content in TMR

Author	ENU [%]	Range	R^2	P
N Milk/N Intake	30.1 ± 5.0	21.1—39.5	0.350	0.001
Nousianen et al., 2004 [16]	28.7 ± 1.1	26.0—31.2	0.693	< 0.0001
Huhtanen et al., 2015 [11]	30.6 ± 1.8	26.7—33.2	0.363	0.0004
Wattiaux, Ranathunga, 2016 [24]	28.2 ± 1.9	23.6—32.5	0.666	< 0.0001
Average (1—2—3)	29.2 ± 1.4	26.0—32.1	0.729	< 0.0001

TMR—total mix ration; ENU—efficiency of nitrogen utilization

Regression calculated from the evaluated data:

ENU % = 90.5 – 3.73 × CP %; $R^2 = 0.350$; $P = 0.001$

ENU % = 53.217 – 1.4823 × CP %; $R^2 = 0.729$; $P < 0.0001$

Regression equations by authors:

Nousianen et. al. [16]:

ENU % = –0.73 × MUN + 38

Huhtanen et al. [11]:

ENU % = 23.8 + 0.7 × MY – 0.0064 × MY2 – 0.27 × MUN – 0.01 × BW

Wattiaux, Ranathunga [24]:

ENU % = 44 – 1.24 × MUN

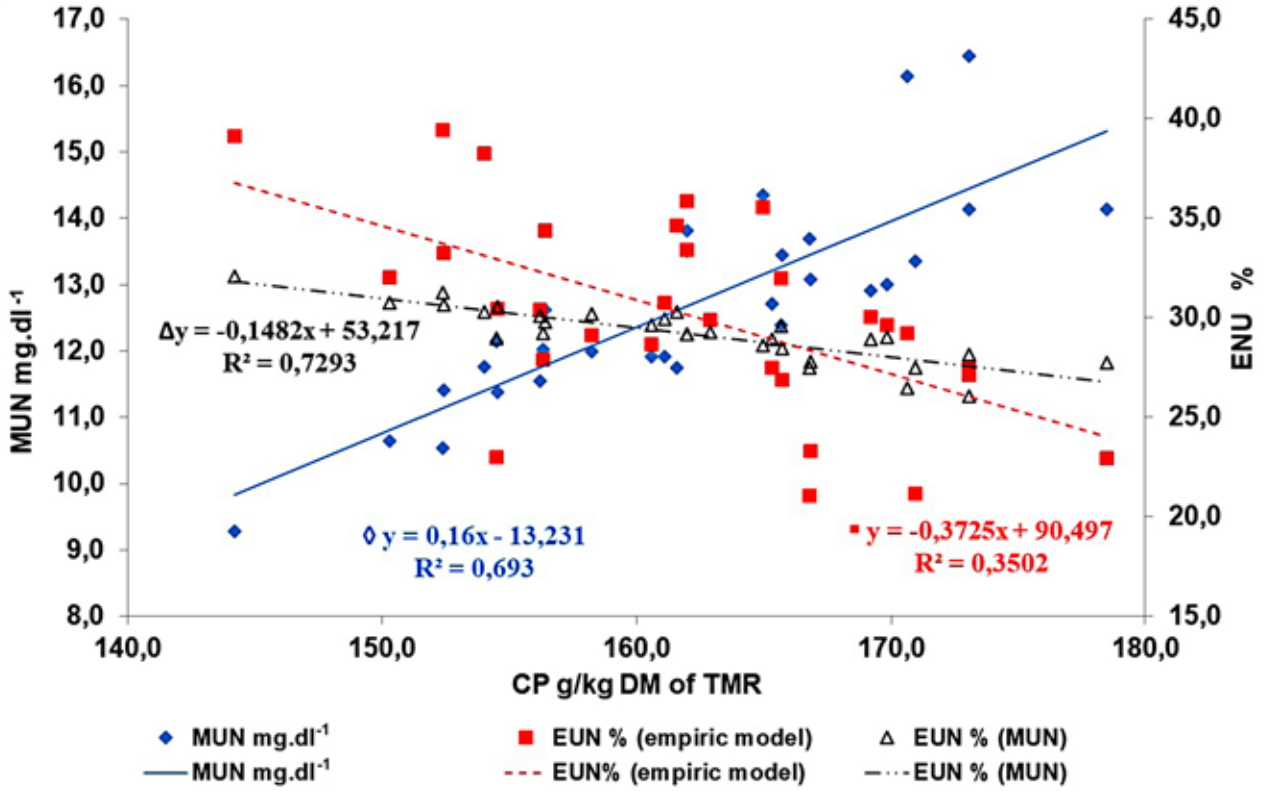

Fig. 1. Effect of CP content on MUN concentration and efficiency of N utilization

Table 5. Validation models for predicting the ENU according to the MUN content compared to the calculation by the empirical model

	Model A Huhtanen et al. [16]	Model B Nousiainen et al. [11]	Model C Wattiuax, Ranathunga [24]
ENU % by Empiric model	30.15 ± 5.0	30.15 ± 5.0	30.15 ± 5.0
ENU % by Equation by MUN	30.63 ± 1.8	28.74 ± 1.1	28.23 ± 1.9
Mean bias	0.48	–1.41	–1.92
Residual error	3.39	4.52	4.47
RMSPE	3.43	4.73	4.87
Regression of bias against empirical model for the ENU evaluation			
R^2	0.976	0.976	0.923
Slope (probability)[a]	–0.678 (P < 0.0001)	–0.9029 (P < 0.0001)	–0.848 (P < 0.0001)

A—coefficient of determination and statistical probability of existence of slope coefficient (F test); RMSPE—root mean square predictive error

Validation of models for determining efficiency of nitrogen utilization

Selected models of the regression equations to determine the ENU based on the MUN content were validated for accuracy and precision compared to the empirical model of the ENU according to the metabolic transformation of nitrogen (N milk/N intake). The results are summarized in Table 5. The precision of the model was evaluated by residual error (RE) and root mean square predictive error (RMSPE). By evaluation of the regression of the individual values of bias in the models, was confirmed with the negative linear relationships with coefficients of the slope at the statistical significance level of $P < 0.0001$ (Table 5).

The determination of accuracy in our studies were assessed by the mean bias [12] as the mean value of bias between values of the ENU estimated from the models of the equation according to the MUN and the values of the ENU empirical model according to metabolic transformation N (N milk/N intake). Using the appropriate parameters to determine accuracy and precision, model A was the most accurate to predict the ENU in breeding conditions compared to models B and C. The observed differences were based on the fact that model A, in the calculation of the ENU values took into account, in addition to the urea nitrogen in the milk, also, the amount of produced milk whereas B and C only took the content of the MUN. According to these parameters of precision and accuracy of model A, using a regression relationship [11] was best suited to predict the ENU in breeding conditions and was a good way to evaluate programs of nutrition to maximize the efficiency of nitrogen utilization from feed to milk.

DISCUSSION

Urea nitrogen in milk (MUN), is commonly used to monitor feeding programs and control the nitrogen load on the environment [1]. At the feeding trials, where the TMR contained corn and alfalfa silage, average the milk production of 35.4 ± 6.6 kg per day in high producing dairy cows in the first lactation phase we analysed the concentrations MUN as an average of 12.8 ± 1.7 mg. dl^{-1} on a large number of farms (30/3150) with the CP content (16.2 ± 0.8 %), starch content (25.1 ± 3.3 %) and NEL (6.76 ± 0.2 MJ). At comparable nutritional and production parameters the published values from the meta-analysis [23] confirmed the MUN content of 13.1 ± 3.6 mg.dl^{-1} for North America.

In north-western Europe, the MUN content achieved an average value of 12.5 ± 5.1 mg.dl^{-1}, at lower production (25.5 ± 4.5 kg) and at half the starch content (13.2 ± 8.4 %). The MUN level ranging from 8 to 12 mg.dl^{-1}, indicate optimal intake and utilization of nitrogen from the feed ration [16, 23]. Levels of MUN above 12 mg.dl^{-1} indicate an increased loss of ingested nitrogen, excreted in the urine in the form of urea urinary nitrogen. For high producing dairy cows with an annual milk yield of 12,000 kg, the recommended MUN is an average value of above 14.5 mg.dl^{-1} [13] or more precisely from 10 to 16 mg.dl^{-1} [24].

The observations confirm that the content of the CP in the TMR is the most important nutritional factor affecting the concentration of MUN, which is closely associated with the excretion of nitrogen in urine and can be applied in commercial herds as a biomarker of protein nutrition in dairy cows [16]. The higher level of regression dependence between the concentration of MUN and CP content was confirmed in experimental conditions with the coefficient of determination in the range of $R^2 = 0.78$ [16] and 0.84 [4] than our analysis ($R^2 = 0.69$ [3]. Evaluation of the analysed nutritional relations in farming conditions by the multiple regression method, together with the content of the CP, confirmed the influence of the energy concentration (NEL) and the NDF ($R^2 = 0.720$) with minority proportion on the increase of the coefficient of determination and variations in the concentration of the MUN. The same tendencies with comparable results in experimental conditions in the evaluation of nutritional influences were confirmed by N o s i a i n e n [15], where the expressed CP/ME ratio on fluctuations of the MUN was most affected by different CP contents ($R^2 = 0.876$) in the feed ration.

The efficiency of N conversion from feed into milk protein ranges from 25 to 35 % in dairy cows and is mainly influenced by the content of the CP in feed and level of milk production [10]. The observed ENU in the farms showed proximity according to the evaluation method at the level of values of the mean, but a different range of fluctuations of individual values for some of the farms. Studies by some authors [9, 10, 21] found the ENU under commercial conditions at the level of 28 %, with a fluctuation between 18 % and 35 %. Also, a study by P a c h e c o [20] reported the average value of ENU at the level of 29 % with a range of fluctuations from 19 to 40 % between farms, which are

values comparable to our results obtained from calculation according to the metabolic conversion.

By calculation of ENU, according to analysed content of the MUN, there was observed a decrease in ENU by 1.48 % when the content of the CP in the TMR increased by about 1 %, which was a lower decrease as compared to the value of 1.86 % determined by Olmos Colmenero, Broderick [18], or 1.76 % determined by Broderick [5]. On the other hand, the ENU determined according to a nitrogen metabolic transformation evaluation (N milk/N intake) confirmed a negative correlation (R^2 = 0.350, P = 0.001), with a decrease efficiency of N utilization of 3.72 % at the increase of the CP content in the TMR by 1 %. The regression relationship, expressed by equation y = –0.372x + 90.5, at the values of the CP below 15 % in DM TMR, overestimates the ENU and at the values over 17 % of the CP underestimates the EUN, compared to the biologically accepted efficiency of nitrogen transformation from feed to milk [10]. The performed evaluations of efficiency according to metabolic transformation (empiric model) obtained from production records of the farms are loaded by the degree of inaccuracy of the evaluation of dry matter intake.

CONCLUSIONS

The highest nutritional effect on the urea nitrogen content in milk (MUN) has the CP concentrations in the TMR (with a coefficient of determination R^2 = 0.693), where an increase in the CP content by 1% resulted in an increase in the MUN by 1.6 $mg.dl^{-1}$. The efficiency of nitrogen utilization (ENU) from feed to milk, determined by the balance method, expressed as a percentage of nitrogen intake, varied from 21.1 to 39.5 with a decrease in N utilization efficiency of 3.72 % while increasing the content of the CP in the TMR by 1 %. The efficiency of nitrogen utilization calculated according to the MUN content using regression equations gives on average ENU 28.2—30.6 % with a decrease in the ENU by 1.48 % with an increase in the content of the CP in the TMR by 1 %. The validation of ENU models from the MUN concentration under farm conditions confirmed the ENU as the most accurate regression equation for determining the ENU (%) = 23.8 + 0.7 × MY – 0.0064 × MY^2 – 0.27 × MUN – 0.01 × BW. The evaluation of milk urea nitrogen at the farm level provides the possibility to adjust the content and proportion of proteins and carbohydrates in the feed ration to optimize the protein needs of lactating dairy cows, increasing the efficiency of nitrogen transformation from feed to milk and reduces the excretion of nitrogen in urine as a tool to reduce environmental degradation.

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OZONIZATION OF WATER, RETENTION OF OZONE AND DEVITALIZATION OF *ESCHERICHIA COLI* IN WATER BY OZONE

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ABSTRACT

The aim of this study was to observe the efficiency of ozone transferred by an airstone bubble diffuser, using two ozone generators with different output of ozone (5 g.h^{-1} – G1; 15 g.h^{-1} – G2). The retention of ozone in ozonised distilled and potable water and the devitalisation effects on *E. coli* in the water were also noted. Ozone was introduced to two types of potable water of different composition intended for mass consumption, (MC)^{a} and (MC)^{b}, distilled water, and well water intended for individual consumption. The devitalisation effect of ozone on *E. coli* in well water (WW) and added to potable and distilled water was observed. The results of our study showed that under the conditions used, the level of ozone during ozonisation with G1 increased more rapidly in distilled water and after termination of ozonisation, the retention of ozone in distilled water was a little lower in comparison with the potable water. The devitalisation of *E. coli* either naturally present in the water or added to it required the level of ozone close to or equal to 0.25 mg.l^{-1}.

Key words: *E. coli*; ozone; ozone retention; ozonised water

INTRODUCTION

Ozone, an allotropic form of oxygen, is an inorganic gas (CAS n. 10028-15-6) constituted by three oxygen atoms (O_3) arranged in a bent structure, where the distance among oxygen atoms is 1.26 Å. It easily decomposes into oxygen (O_2) and one single, very reactive oxygen atom. Ozone is present in nature and its concentration in the atmosphere is approximately 0.04 parts per million (ppm: 1 ppm ~ 2 mg.m^{-3}). The solubility of ozone in water is 49.0 ml.100^{-1} ml (at 0 °C), tenfold than oxygen, thus causing an immediate reaction with any biomolecule in biological fluids. Its density (2.14 kg.m^{-3}) is higher than that of air, letting it concentrate close to the ground in indoor environments. Being a powerful oxidant, it is able to react with organic molecules containing double or triple bonds. This is the origin of its bactericidal, virucidal, and fungi-

cidal actions, which are used in water treatment, odour control and medicinal applications [10].

The applications of ozone are wide, varied and still expanding. They include disinfection of drinking water, groundwater, waste water, air purification, disinfection of surfaces, medical applications (dentistry, disinfection of external wound, possible treatment of diseases), grain and feed remediation [32], disinfection of stored foods, disinfection in dairy industry (milking equipment, udder, rear legs) and others. [15].

Ozone therapy has been utilized and extensively studied for many decades. Its effects are proven, consistent and with minimal side effects. Used to treat infections, wounds and multiple diseases, O_3's effectiveness has been well-documented. Ozone has been known to be used to treat as many as 114 different diseases [4].

The inhibitory and lethal effects of ozone on pathogenic microorganisms have been observed since the 19th century, and the most cited explanation of these effects is based on the disruption of their envelopes through peroxidation of phospholipids and the interaction with proteins [31]. As mentioned by Hudson et al. [8], the gas state allows ozone to get to areas that are difficult to reach and to disinfect much more than just surfaces.

Ozone has a broad antimicrobial spectrum and each microorganism species has inherent sensitivity to the gas [3]. Present at low concentration levels, dissolved O_3 is effective at destroying a wide variety of pathogenic bacteria including *E. coli*, salmonella, *Legionella pneumophila*, *Staphylococcus* spp., *Streptococcus faecalis* and *Mycobacterium tuberculosis*. However, contact time varies from one bacteria to another; *Mycobacterium tuberculosis* requires a contact time six times as long as *E. coli* in order to achieve the same level of microorganisms inactivation. Gram positive bacteria and spore forming bacteria have been found to be more resistant but are still sensitive to the disinfectant effect of O_3 [25].

Inactivation of viruses by ozone is the result of reactions between this oxidant and the biomolecules constituting the essential structures, external and internal, of the target organism. Other oxidant species generated through ozone reactions, such as hydroxyl radical and singlet oxygen may also contribute to some extent to the inactivation of viruses during this process [19]. Ozone can react with the nucleic acids of the viral genome, following molecular diffusion through the external structures towards the nucleic material. The external structures are the capsid proteins and, only for some species, the proteins and lipids of the viral envelope, such as in the case of SARS-CoV-2.

Although the existing scientific literature supports the effectiveness of ozone in the inactivation of viruses, there are very few studies about it on the SARS virus and still not a single study about its efficiency of inactivation of SARS-CoV-2. Therefore, in the absence of scientific literature, it is possible to assume that ozone is equally effective in inactivating SARS-CoV-2, however specific studies must be conducted to know also the ozone dose and effective exposure times [2].

Ozone is also effective against protozoan cysts present in water, unlike a number of other competing disinfectants. O_3 appears to more easily penetrate through protozoal cyst walls compared to other disinfectants such as chlorine. Some protozoal cysts are more resistant than others; for example, *Cryptosporidium* oocysts have been found to be ten times more resistant than *Giardia* cysts to ozonisation [16, 17, 25].

O_3 is highly effective at destroying a wide range of pathogenic organisms but the concentration of O_3 required to deactivate microorganisms will differ depending on the particular microorganism in question. In all case, a critical concentration threshold value of O_3 must be reached [25].

Ozone generators are currently being promoted as effective tools for removing pollution and odours from indoor air, but it must be remembered that ozone is associated not only with adverse health effects but also with safety issues. Ozone generators are high voltage electric machines, with all the safety implications involved. Moreover, the corona effect, whichever the frequency of the applied current, produce a broad spectrum of radio frequencies depending on many different parameters [13]. The European Chemical Agency (ECHA) safety guidance forbids the entire room where the ozone generator is deployed to pacemaker bearers. The actual extension of the area where the electromagnetic field exceeds the reference levels set for the protection of general public and workers should be addressed in the risk assessment.

Human activities in industry and agriculture have been scaling up in order to meet the needs of a growing population. Such activities are having drastic consequences on the environment, such as water pollution. The main sources of water pollution include domestic and industrial sewage and animal waste produced in agriculture. An important

strategy that has been put in place throughout the EU in order to protect water sources against contamination is the use of Zones of Hygiene Protection (ZHPs) [20].

However, protecting water sources alone does not ensure that these sources are free from contamination. The quality of drinking water must be monitored regularly. Establishing the microbiological content of drinking water is essential in determining the presence of waterborne bacteria, viruses, protozoa and helminths that may have the potential to cause disease, in particular Gastro-Intestinal Track (GIT) diseases [30].

However, the detection of all of the potential waterborne microorganisms is expensive and time consuming. Therefore, routine microbiological tests should involve the detection of the indicator bacteria, total coliform bacteria and *E. coli*, being the most common to assess the quality of drinking water [29].

All drinking water intended for mass domestic water supply undergoes treatment and disinfection. The most commonly used large scale strategies for drinking water disinfection include use of chlorine, UV radiation, and ozone. Each of them has some advantages and disadvantages.

Chlorine is highly effective at inactivating some of the most significant microorganisms present in water sources. However, chlorinated water contains higher concentrations of by-products such as chloroform and trihalomethanes (THMs) which have carcinogenic properties [25]. The advantage of UV radiation is that there are no remaining chemical residues and no production of toxic by-products, however, there is no residual effect and some previously inactivated microorganisms may recover due to photo-reactivation.

Ozone is more effective than chlorine in destroying viruses and bacteria. This method has many advantages: the ozonisation process utilizes a short contact time (approximately 10 to 30 minutes); there are no harmful residuals because ozone decomposes rapidly. Ozone is generated onsite, and thus, there are fewer safety problems associated with shipping and handling. However, low dosage may not effectively inactivate some viruses, spores and cysts; ozonisation is a more complex technology; and it has short-lasting residual effects and suspended solids significantly reduce viral inactivation by ozone [26].

Generation of O_3 for water or air treatment takes place in a number of steps. First of all a source of O_2 is required. This O_2 must then be fed into an O_3 generator to be converted into O_3 and O_3 must be transferred to the intended gas or aqueous environment. The final step may involve the destruction of any unreacted O_3 via an off-gas destruction system or alternatively recycling of O_3 back into the system [15]. O_3 gas transfer into water poses more of a challenge than transfer into air. The most widely used and efficient method of O_3, the bubble diffusion, involves the injection of O_3 gas into the water through porous diffusers which create small rising bubbles of O_3 within the water. O_3 that comes into contact with the water dissolves and reacts with organic and inorganic compounds within the water, as well as with microorganisms. The efficiency of ozonisation is related to the size of the diffuser pores, as well as the height of water vessel used (effect on contact time).

Once ozone enters into water, it becomes highly unstable and rapidly decomposes through a complex series of reactions [7, 24].

Contact time (CT) is crucial to the effectiveness of ozonisation as predicted by Chick's Law. For example, the standard CT value used in the bottled water industry is 1.6 $mg.l^{-1}$ min. CT value should increase with increasing pH [25].

Ozonised water is defined as the water obtained after the ozonisation (a process of infusing water with ozone) of water. During the ozonisation of water there are no chemical reactions between ozone and pure water. The only chemical reactions take place between ozone and organic, inorganic, or biological material present in the water. The solubility of ozone in water depends on different variables: pH, temperature, salts contents, and others. The half-life of ozone in water also depends on these variables [12].

The aim of this study was to observe the efficiency of ozone transfer by airstone bubble diffuser, retention of ozone in distilled and potable water and disinfectant effect of ozone in water naturally contaminated or spiked with *E. coli*.

MATERIALS AND METHODS

Materials

Ozone generators used in this study are depicted in Figures 1 and 2.

According to the manufacturer's recommendations, this generator is suitable for the disinfection of the inte-

Fig. 1. Ozone generator 1: Q-005 with output of 5 g.h^{-1}

Ozone unit: air cooling ceramic tube, 20 000 hours lifespan, power: 230 V AC, 80 W. Ozone source: ambient air. Manufacturer: FANDS Trade s. r. o., Valaliky, Slovakia. Source: http://www.technik.fands.eu/Generator-ozonu-Q-003B-3g-hod-d51.htm

Fig. 2. Ozone generator 2: FST-I-15 with output 15 g.h^{-1}

Ozone unit: air cooling ceramic tube, 10 000 hours lifespan, power: 230 V AC, 4000 W. Ozone source: ambient air. Manufacturer: FANDS Trade s. r. o., Valaliky, Slovakia. Source: http://www.technik.fands.eu/fotky35619/fotos/_vyr_6115g-vent.jpg, 2017

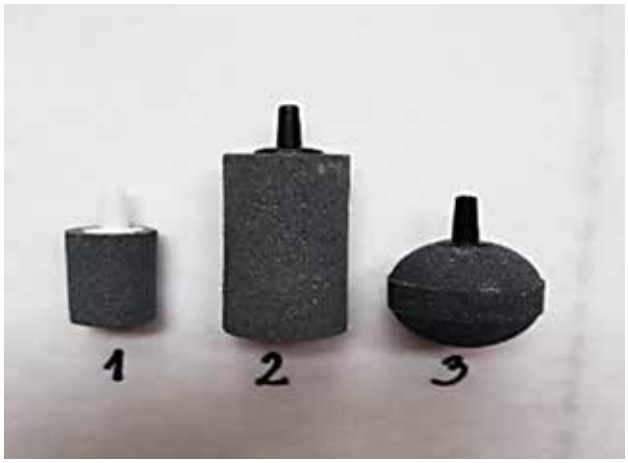

Fig. 3 Airstone bubble diffusers

riors of ambulances, pharmacies, use in households, small facilities etc. It is effective in rooms of size 60—300 m^3 at concentration 2—30 mg.m^{-3}. It can also be used for disinfection of water.

Manufacturer's recommendations: Suitable for premises up to 1200 m^3. This ozone generator can be used for disinfection of public facilities including restaurants, sports centres, playrooms, commercial canteens. Can also be used in pig and poultry housing and in food processing and food storage premises.

Ozone concentration in water was measured with the apparatus F-DOZ30 which allows one to read the levels of dissolved O_3 directly from the monitor. HACH Lange 2-canal multimeter (Germany) equipped with pH (PHC301 and conductivity (CDC401) electrodes was used for determination of water pH and conductivity.

Other materials required for our experiments included large glass cylinders (1000 ml), thermometers, plastic pipes with airstone bubble diffusers (Fig. 3, diffuser 1) relaying ozone from the generator to the water.

Endo agar (EA) was the culture medium used for cultivation of *E. coli*. On this agar, *E. coli* can be visualised as dark red colonies with a metallic shine.

Methods

Water pH and conductivity of water was determined using Hach Lange 2-canal multimeter and the relevant electrodes according to [21].

Counts of *Escherichia coli* were determined by membrane filtration method and cultivation on Endo agar (EA) at 43 °C for 24 hours [22].

Ozonisation trials:

1. **Efficiency of ozone transfer by airstone bubble diffuser and retention of ozone in distilled and potable water**

   We investigated the efficiency of ozone transfer through an airstone bubble diffuser and the time duration required to reach 0.25 mg.l^{-1} dissolved ozone in water

while using ozone generators 1 (G1) and 2 (G2). Ozonisation was performed in duplicate (results are presented as an average) in a laboratory (water and air temperature 20 °C, 49—53 % relative humidity) using distilled water (D_1, D_2) and potable water intended for mass consumption $(MC)^a$, $(MC)^b$ [6].

Potable or distilled water was poured up to mark to glass cylinders (1 litre volume, height of water column 36 cm) and ozone was introduced to the bottom of each cylinder through a bubble diffuser attached to the end of a plastic pipe, which extended from the respective ozone generator. The top of the cylinder was covered during ozonisation in order to minimize the escape of ozone. Small rising bubbles of ozone gas within the water could be observed. Generator G1: after the G1 was switched on, we measured the concentration of ozone in distilled (D_1) and potable water $(MC)^a$ in the intervals of 30, 60, 90, 120 and 150 minutes. After 150 min of ozonisation, the generator was switched off, the covers were removed and concentration of ozone was measured in 30-minute intervals up to 270 min from the beginning of the experiment.

Generator G2: Concentration of the dissolved ozone in potable water $(MC)^b$ was measured in the intervals of 30, 40 and 50 min from the beginning of ozonisation. In addition, retention of ozone in this water was observed after the termination of ozonisation upon reaching the effective level of ozone (0.25 $mg.l^{-1}$) in intervals of 10, 30, 60 and 120 min.

2. **Devitalisation effect of ozone in two types of potable water $(MC)^a$ and $(MC)^b$, in distilled water (D_2) spiked with *E. coli*, and in well water naturally contaminated with *E. coli***

Experiments were carried out in glass cylinders (1 litre volume, 36 cm water column). Cultivation of samples were done on Endo agar [22].

Generator G1: Potable water $(MC)^a$ previously ozonised to the level of 0.25 $mg.l^{-1}$ of O_3 was spiked with 1 ml of *E. coli* culture and samples for cultivation of *E. coli* were collected in intervals of 10, 20, 30 and 40 minutes. Counts of *E. coli* were determined in well water (IC) naturally contaminated with *E. coli* (2100 $CFU.l^{-1}$) in intervals of 1, 2, 5 and 10 min.

Generator G2: in trial one. counts of *E. coli* were determined in distilled water (D_2) previously ozonised to the level of 0.25 $mg.l^{-1}$ of O_3 and spiked with *E. coli* in intervals of 10, 30, 50 min and 2 and 24 h. In trial two, after spiking the ozonised distilled water with *E. coli* we continued with ozonisation and examination in intervals of 10, 30, 50 and 120 min.

RESULTS

Results of pH and conductivity of two types of potable water, distilled water and well water used in the study are presented in Table 1.

Efficiency of ozone transfer and retention of ozone

Ozone generator G1

The concentration of the dissolved ozone introduced continuously to potable water intended for mass consumption $(MC)^a$ and to distilled water (D_1) by G1 is presented in Fig. 4 and the level of residual ozone measured after the ozonisation was switched off is shown in Fig. 5.

Supported by previous studies, a concentration of 0.25 $mg.l^{-1}$ of dissolved ozone in water has a disinfecting effect of 99.99 % particularly on *E. coli* [14]. This level was reached after 120 min of ozonisation in distilled water, but not in potable water that required more than 120 min of ozonisation with G1, and was slightly exceeded after 150 min (0.26 $mg.l^{-1}$). The efficiency of ozonisation of distilled water was slightly higher throughout the process (Fig. 3).

Table 1. Results of pH and conductivity of water used in the experiments

Parameter	Range/Limit for potable water	$(MC)^a$	$(MC)^b$	WW	D_1	D_2
pH	6.5—9.5	7.59	6.8	7.43	7.06	7.26
Conductivity [$mS.m^{-1}$]	125	43.9	32.2	131.7	2.4	8.72

$(MC)^a$, $(MC)^b$—water suitable for mass consumption [6]; WW—well water; D_1—distilled water used in ozonisation trial 1; D_2—distilled water used in ozonisation trial 2

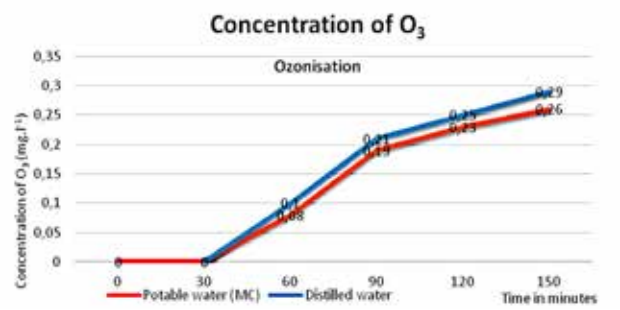

Fig. 4. Concentration of ozone in potable (MC)[a] and distilled water (D_1) during ozonisation with G1

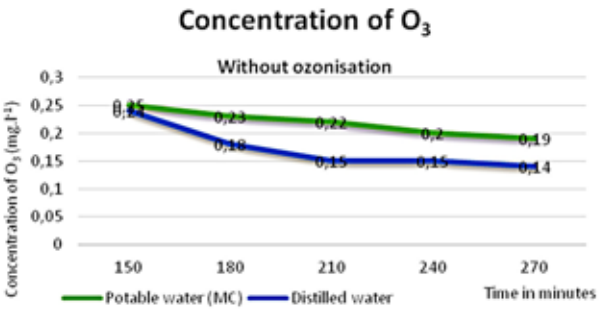

Fig. 5. Retention of ozone in potable (MC)[a] and distilled water (D_1) after termination of ozonisation

Table 2. Concentration of ozone in potable water (MC)[b] during ozonisation with G2

Duration of ozonisation [min]	Concentration of O_3 [mg.l^{-1}]	pH	Conductivity [mS.m^{-1}]
0	0	6.8	32.2
30	0.14	6.58	34.6
40	0.25	6.15	37.5
50	0.31	6.0	40.7

After termination of ozonisation (150 min), the level of ozone decreased more rapidly in distilled water but the decrease was surprisingly slow and after 270 min from the beginning of ozonisation both potable and distilled water still contained 0.19 and 0.14 mg.l^{-1} of dissolved ozone, respectively (Fig. 5).

Ozone generator G2

This generator was used for ozonisation of potable water (MC)[b] with lower initial pH and conductivity compared to water ozonised with G1 (MC)[a]. Results of the relevant parameters during ozonisation of this water and after termination of ozonisation are presented in Tables 2 and 3.

According to expectation, when using ozone generator with higher output the target concentration of 0.25 mg.l^{-1} dissolved ozone for the disinfection of 99.99 % *E. coli* was reached after 40 minutes of ozone exposure. This appears to be the optimal time needed to achieve the target concentration of ozone; however, the composition of ozonised water may play some role in this respect. The pH of water decreased and conductivity increased during ozonisation.

The decrease in concentration of dissolved ozone was more rapid in comparison with the decrease observed after ozonisation of potable water (MC)[a] with G1.

Table 3. Retention of ozone in potable water (MC)[b] after termination of ozonisation

Time intervals* [min]	Concentration of O_3 in potable water [mg.l^{-1}]
0	0.25
10	0.20
30	0.11
60	0.04
120	0

*Time intervals after reaching the effective level of ozone, starting at the termination of ozonisation

Devitalisation effect of ozone on *E. coli*

Results of ozonised potable water (MC)[a] spiked with 1 ml of *E. coli* culture in comparison with the control are presented in Table 4.

The effect of ozone was evident as no *E. coli* were detected in ozonised water after 10 minutes of exposure to ozone.

Ozone was also introduced into well water (WW) intended for individual consumption (Table 5). However, this particular water did not comply with the requirements on potable water [6].

Table 4. Results of ozonised potable water (MC)[a] containing 0.25 mg.l^{-1} of dissolved ozone, spiked with *E. coli*

Time [min]	Concentration of ozone [mg.l^{-1}]	Ozonised potable water + *E. coli* [CFU.l^{-1}]	Non-ozonised potable water + *E. coli* [CFU.l^{-1}]
0	0.25	3.2×10^5	3.2×10^5
10	0.17	0	9.8×10^3
20	0.20	0	6.0×10^3
30	0.22	0	5.6×10^3
40	0.16	0	4.6×10^3

Table 5. Counts of *E. coli* in well water (WW) during ozonisation with G1

Time [min]	Concentration of ozone [mg.l^{-1}]	Well water [CFU.l^{-1}]
0	0.00	2100
1	0.07	1600
2	0.17	25
5	0.26	0
10	0.36	0

Table. 6. Counts of *E. coli* in distilled water (D_2) initially containing 0.25 mg.l^{-1} of dissolved ozone, spiked with *E. coli*

Sample collection intervals	Dissolved O_3 [mg.l^{-1}]	*E. coli* [CFU.l^{-1}]
0 min	0.25	1.5×10^8
10 min	0.21	0
30 min	0.11	0
50 min	0.05	0
2 hours	0	0
24 hours	0	0

Table 7. Counts of *E. coli* in distilled water (D_2) initially containing 0.25 mg.l^{-1} of dissolved ozone and undergoing continual ozonisation after spiked with *E. coli*

Sample collection intervals [CFU.l^{-1}]	Dissolved O_3 [mg.l^{-1}]	*E. coli* [CFU.l^{-1}]
0 min	0.25	1.5×10^8
10 min	0.31	0
30 min	0.35	0
50 min	0.41	0
120 min	1.0	0

E. coli present in the well water were devitalised within 5 min of ozonisation with the less efficient generator G1. By this time dissolved ozone reached the level considered effective against *E. coli*.

In trial one, 1 ml of *E. coli* culture was added to previously ozonised distilled water (D_2) containing an initial concentration of 0.25 mg.l^{-1} of dissolved ozone. Samples of distilled water were taken for cultivation at 10, 30 and 50 minutes and 120 min after ozonisation. The results can be seen in Table 6.

The concentration of dissolved ozone decreased with time up to the 50 min of observation when it dropped to the lowest detected level. *E. coli* added to this water did not survive the exposure to the effective level of ozone.

In trial two, 1 ml of *E. coli* culture was added to previously ozonised distilled water (D_2) containing 0.25 mg.l^{-1} dissolved ozone and undergoing continual ozonisation via an airstone bubble diffuser. Samples of water spiked with *E. coli* were taken immediately after addition of *E. coli* (0 min) and at 10, 30, 50 and 120 min of continuous ozonisation. The results can be seen in Table 7.

Our results showed that within 10 minutes of ozonisation, the level of dissolved ozone in the distilled water had increased from 0.25 mg.l^{-1} to 0.31 mg.l^{-1} and continued to increase up to 1.0 mg.l^{-1} at 120 min. It was observed that *E. coli* could not be detected when concentration of dissolved ozone was 0.25 mg.l^{-1} or higher.

DISCUSSION

The use of ozonised water is gaining on importance in many segments of human activities, such as treatment of water, food and agricultural industries and medicine. As a result of this there is available a wide range of different types of ozone generators that differ in their characteristics and thus also in their output. Thus, the customers should be able to select the optimum configuration for their specific use. However, the extensive offer produces some difficulties, as the scientific studies present results obtained under different conditions and affected by various factors which makes it sometimes very difficult to arrive to definite conclusion regarding the suitability of a particular generator.

The aim of this study was to contribute to the available information about ozonisation of water and the effect of this treatment on *E. coli* in water. Two types of ozone generators with different output of ozone (G1: 5 g O_3 per h; G2: 15 g O_3 per h) were used.

Results of ozonisation of potable and distilled water at 20 °C showed that the increase in O_3 level during ozonisation with G1 was more rapid in distilled water where the concentration of 0.25 mg.l^{-1}, that should be effective against *E. coli* [14], was reached after 120 min while in potable water after 150 min. Initial pH of this potable water (MC)[a] was 7.59 and of distilled water 7.06 and initial conductivity of potable water was 43.9 mS.m^{-1} and of distilled water 2.4 mS.m^{-1}.

When different potable water (MC)[b] with pH = 6.8 and conductivity equal to 32.2 mS.m^{-1} was ozonised with the higher output generator (G2), the effective concentration of ozone in the distilled water was reached after 40 min and ozone was retained for 60 min (0.04 mg.l^{-1}).

After termination of ozonisation with G1 (150 min), the level of ozone decreased more rapidly in distilled water. The decrease was surprisingly slow and after 270 min from the beginning of ozonisation both potable and distilled water still contained 0.19 and 0.14 mg.l^{-1} of dissolved ozone (Fig. 5). This is in contrast with the study by Andoyo et al. [1] who explored the stability of ozone in distilled, double distilled and tap water at different temperature (5 °C, 25 °C, 40 °C), pH, water type and several variations of mineral content. According to their results, the retention time of ozone in acid pH was longer than that in the alkaline pH, and high level of minerals has a shorter retention time compared to low minerals samples. However, in this study there was no mention about the ozone output of the ozone generator used. The authors reported that after 5 and 10 min of ozonisation the level of ozone reached 4.3 and 5.8 mg.l^{-1}, respectively, which is a much higher level than that investigated in our study. The authors also noted that ozone is a reactive compound that readily binds to other compounds when ozone concentrations are high; nevertheless, at high concentrations ozone tends to be unstable. Mineral content affects the diffusion of the ozone bubbles into the water, the higher the mineral content, and the easier the ozone to be decomposed and thus causes the retention time of ozone to be shorter. [13]. According to Von Gunten [18], ozone firstly decomposed inorganic compound then organic compounds will be decomposed afterwards. In the condition of water that contains many minerals such as tap water, ozone more easily decompose into O_2 (oxygen) and O* (free oxygen ions), while in condition of water with less minerals such as distilled water, ozone was decomposed longer. The mineral content of the types of water in the study by Andoyo et al. [1] was characterised by the content of Ca while in our study we reported conductivity of water. Conductivity is a measure of the ability of water to pass an electrical current. It is affected by the presence of inorganic dissolved solids such as chloride, nitrate, sulfate, and phosphate anions (ions that carry a negative charge) or sodium, magnesium, calcium, iron, and aluminium cations (ions that carry a positive charge). Organic compounds like oil, phenol, alcohol, and sugar do not conduct electrical current very well [27].

In our study we used two generators with different output of ozone. In water ozonized at lower rate the ozone was retained for longer time despite its higher pH (7.59 versus 6.8) and higher conductivity (43.9 versus 32.2). Thus, the retention of ozone may be affected by the rate of its relaying into water and, potentially by the use and type of bubble diffuser.

Changes in pH and conductivity during ozonisation of potable water (MC)[a] with G1 were minimal but observed a decrease in pH from 6.8 to 6.0 and rise in conductivity from 32.2 to 40.7 mS.m^{-1} in potable water (MC)[b] during ozonisation with generator G2 (Table 2). This differed from the results of Subedi et al. [23] who reported no remarkable effect of ozonisation on pH and conductivity but observed a significant effect on chemical properties (nitrate, hardness, iron) when using dielectric barrier dis-

charge unit to produce highly oxidising ozone molecules. These authors also reported an effective removal of faecal coliforms from water from 3 different sources.

During ozonisation, oxidation of organics with ozone gives rise to intermediate compounds. Carbon dioxide as a product of oxidation plus water increase conductivity of water. Also, impurities present in air from which ozone water was generated can increase the conductivity of water [28].

Ersoy et al. [5] comprehensively evaluated the inactivation mechanism of ozone on *E. coli* and *E. faecalis*. The results supported the idea that ozone targeted mainly cell walls of these bacteria. According to the TTC dehydrogenase relative activity and flow cytometry results, ozone was more effective for Gram-positive cells inactivation. *E. faecalis* cells were inactivated totally in a very short time, whereas much more time was required for the total inactivation of *E. coli* cells. In their study, conductivity increased during first 5 min followed by decrease after treatment with 1 $mg.l^{-1}$ ozone and contact times of 30 s, 1, 5, 10 and 20 min. Treatment for 30 s resulted in the release of intracellular components (i. e. DNA and protein) for both types of cells. Cell wall integrity was disturbed as indicated by leakage of K^+ ions and cellular organic molecules.

According to Manousaridis et al. [11], ozone is very unstable both in the gaseous phase and in solution, decomposing into hydroxy (·OH), hydroperoxy ($\cdot HO_2$) and superoxide ($\cdot O_2^-$) radicals. The reactivity of ozone is attributed to the great oxidizing power of these free radicals, making ozone a potent disinfectant in water treatment and the food industry. The above authors evaluated the effect of ozone gas on *Escherichia coli* O157:H7 inoculated on an organic substrate, and the efficacy of ozonised water in controlling the pathogen. Water was ozonised at 45 $mg.l^{-1}$ for 15 min and *E. coli* O157:H7 was exposed for 5 min to the ozonised water immediately after ozonisation, and after storage for 0.5, 1.0, 1.5, 3.0, and 24 h at 8 °C. Ozonised water was effective in inactivating of *E. coli* O157:H7 in all treatments. Furthermore, refrigerated ozonised water stored for up to 24 h was effective in the control of *E. coli* O157:H7.

The effect of ozonation on the rate of disinfection of *Escherichia coli* was investigated as a function of ozone concentration, ozonation duration and flow rates. Ozone was generated *in situ* using corona discharge method using compressed oxygen stream and depending on the oxygen flux the ozone concentrations ranged from 0.91–4.72 $mg.l^{-1}$. The inactivation was faster at lower pH than at basic pH. Molecular ozone was more effective in disinfection than hydroxyl radicals [33].

Among the factors that affect the action of ozone on microorganisms, highlighted are pH, temperature, and composition of the medium. In addition, organic compounds can compete with microorganisms for ozone [9]. This justifies the greater efficiency of microorganism inactivation by ozone in substrates with less organic matter.

One of the goals of our study was to examine devitalization effect of ozone in potable and distilled water spiked with *E. coli* and in well water containing *E. coli* bacteria. Exposure of *E. coli* added to potable (MC)[a] and distilled water previously ozonised to the level of 0.25 $mg.l^{-1}$ of ozone caused complete devitalization of these bacteria within 10 minutes. Generator G1 was used to ozonise well water (WW) containing 2100 $CFU.l^{-1}$ of *E. coli*. Within 2 min of action of very low concentrations of ozone (0.07—0.17 $mg.l^{-1}$ *E. coli* counts decreased to 25 $CFU.l^{-1}$ and within 5 min when the level of 0.26 $mg.l^{-1}$ ozone was reached, these bacteria were devitalised completely. These results confirmed the high effectiveness of ozonised water against *E. coli*, an important indicator of contamination of water with animal and human feces.

CONCLUSIONS

Results of our study conducted under laboratory conditions showed that the level of ozone during ozonisation with G1 increased more rapidly in distilled water compared to the concentration of ozone in potable water and after termination of ozonisation, retention of ozone in distilled water was a little lower in comparison with potable water with higher pH and conductivity. At higher rate of ozonisation, pH of distilled water decreased and conductivity increased during ozonisation. Complete devitalisation of *E. coli* that was either present in water or added to it required the level of ozone close to or equal to 0.25 $mg.l^{-1}$ considered as effective against *E. coli*.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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IMPACT OF DIETARY HUMIC SUBSTANCES SUPPLEMENTATION ON SELECTED MINERALS IN MUSCLES OF BROILER CHICKENS

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ABSTRACT

In this study, we assessed the effect of humic substances on the changes of some mineral (Ca, Mg, Cu and Zn) content in the thigh and breast muscle of broilers. Group 1 (G1) was supplemented with 0.7 % Humac Natur Mycosorb (HNMy), and G2 with 0.3 % HNMy. The control group (GC) received a basal diet without any supplements. In the breast muscle of broilers from the group G1 we found a statistically significantly higher ($P < 0.05$) Ca content and significantly reduced ($P < 0.05$) in the thigh muscle Ca content from the group G2 compared to the control group (GC). A statistically significant increase in Mg content ($P < 0.05$) was analyzed in breast muscles in the group G1 and also significant reduction ($P \leq 0.01$) in Cu in the breast and thigh muscles was found in the group G1 and in the breast muscle from the group G2 compared to the GC. Significantly lower content of Zn ($P \leq 0.05$) was found in the breast and thigh muscles of broilers in the group G2 and strong positive correlation ($r = 0.9093$) were observed between Ca in breast muscle from the group G2 and Ca in thigh muscle from the control group. A negative correlation ($r = –0.7656$) was shown for Mg between thigh muscle from the group G2 and Ca in thigh muscle from the GC. A strong negative correlation was confirmed ($r = –0.9221$) for Zn content in breast muscles between groups G2 and G1. The high positive correlation for Zn was occurred between breast muscle from the control group and thigh muscle from the group G1 ($r = 0.9786$).

Key words: AAS; broilers; calcium; copper; humic substances; magnesium; zinc

INTRODUCTION

The nutritional value and the outstanding taste of chicken meat have given rise to an increase in its consumption in a number of countries. Chicken meat is a popular ingredient in our diet, people consume it mainly for its organoleptic qualities (colour, taste), but also for nutritional reasons—full of proteins, vitamins and minerals. This means that the search for the new ways to improve

the production yield of this animal species is of considerable interest. In recent years, there is growing interest in the use of humic compounds in animal feeding. The supplemental humic substances (HS) as a g warowth-promoting agent has multiple health effects and nutritional benefits for domestic animals [15]. The effect of humic compounds used in animal nutrition on rearing performance improved the results obtained for growth rate, feed utilization, meat quality, egg yield, and egg shell thickness and strength [1, 3, 10, 16, 26]. Some studies investigated the effect of using humic acids (HA) as growth promoter in poultry and obtained positive results [9,17]. Humic substances have shown strong affinity for binding various substances, such as heavy metals [11, 13], minerals [5] and aflatoxins [19, 25]. It has been indicated that HA had differentiated effects upon trace elements in rats. Plasma iron levels were hardly affected, while copper and zinc levels were initially suppressed with a tendency for recovery after 60 days. Feeding humic substances increased levels of some essential minerals (such as Ca, Mg and Fe) in serum, liver and poultry muscles [22]. There are interactions between the individual chemical elements [2], the mutual manifestation manifests itself synergistically or antagonistically, which takes place in feed, in the digestive tract, as well as in the process of tissue and cellular metabolism.

The aim of this study was to find out the influence of humic substances on the changes of mineral content (Ca, Mg, Cu and Zn) in the thigh and breast muscle of broiler chickens.

MATERIALS AND METHODS

One-day-old chickens of hybrid ROSS 308 were randomly divided into 3 groups (n = 30). The average body weight of the chickens was 37.30 g. **Dietary treatments were as follows:** the broilers were fed commercial feed mixture BR1, diet for fattening broilers within 10 days of age, BR2 diet for growing to 30 days of age and BR3 final feed mixture (AGROCASS plus, Ltd. Čaňa, Slovakia) for the duration of the experiment (42 days).

Ethical statement

The experiment was approved by the Ethics Committee of the University of Veterinary Medicine and Pharmacy in Košice, the Slovak Republic. All procedures in this study were performed in accordance with the principles of the European Directive on the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Parliament and Council, 2010).

Composition of BR1 diet for fattening

Maize 35.00 %; wheat 35.00 %; soybean meal 21.30 %; dried blood 1.25 %; limestone 1.00 %; monocalcium phosphate 1.00 %; salt 0.10 %; lysine 1.20 %; methionine 0.60 %; premix 0.50 %. Chemical composition: metabolic energy 12.01 IU; nitrogenous substances 22 %; ash 6 %; fat 2.5—5.0 %; crude fibre max. 4.00 %; nonphytate phosporus min. 0.42 %; Ca min 0.9 %; Na min 0.15 %; retinol 12 500 IU.kg^{-1}; Cholecalciferol 3000 IU.kg^{-1}; alfa-tocoferol 50 IU.kg^{-1}. Antioxidants: propylgallat 100 mg.kg^{-1}. Coccidiostats: narazin 70 mg.kg^{-1}.

Composition of BR2 diet for growing

Maize 40 %; wheat 35 %; soybean meal 18.70 %; limestone 1.05 %; monocalcium phosphate 0.70 %; salt 0.15 %; lysine 1.15 %; methionine 0.46 %; premix 0.50 %. Chemical composition: metabolic energy 12.03 IU; nitrogenous substances 19.5 %; ash 4—6 %; fat 6—8 %; crude fibre max. 4.50 %; nonphytate phosphorus min. 0.40 %; Ca min. 0.85 %; Na min. 0.14 %; retinol 12 500 IU.kg^{-1}; cholecalciferol 3000 IU.kg^{-1}; alfa-tocoferol 40 mg.kg^{-1}. Antioxidants: propylgallat 100 mg.kg^{-1}. Coccidiostats: salinomycinat sodium 70 mg.kg^{-1}.

Composition of BR3 final diet

Maize 37 %; wheat 36.80 %; soybean meal 20 %; limestone 1.12 %; monocalcium phosphate 1 %; salt 0.20 %; lysine 0.98 %; methionine 0.40 %; premix 0.50 %. Chemical composition: metabolic energy 12.37 IU; nitrogenous substances 19 %; ash 4—6 %; fat 6—10 %; crude fibre max. 4.00 %; nonphytate phosphorus min. 0.40 %; Ca min 0.85 %; Na min 0.14 %; retinol 10 000 IU.kg^{-1}; chol ecalciferol 2000 IU.kg^{-1}; alfa-tocoferol 30 mg.kg^{-1}. Antioxidants: propylgallat 100 mg.kg^{-1}.

The control group (GC) was fed with basal diet without any supplement. The experimental group G1 was supplemented in feed with 0.7 % Humac Natur Mycosorb (HNMy). Group G2 was supplemented in feed with 0.3 % Humac Natur Mycosorb (HNMy). The Humac Natur Mycosorb was obtained from HUMAC Ltd. Košice, Slovakia.

Composition of Humac Natur Mycosorb

Powder (particle size up to 100 μm), humic substances 60 %, fulvic acid 5 %, Ca 42.278 $g.kg^{-1}$, Mg 5.100 $g.kg^{-1}$, Fe 19.046 $g.kg^{-1}$,Cu15 $mg.kg^{-1}$,Zn37 $mg.kg^{-1}$,Mn442 $mg.kg^{-1}$, Co 1.24 $mg.kg^{-1}$, Se 1.67 $mg.kg^{-1}$, V 42.1 $mg.kg^{-1}$, Mo 2.7 $mg.kg^{-1}$.

During fattening chickens had access to water and feed *ad libitum*. They were reared on deep litter and microclimatic conditions complied with the requirements for fattening of broilers. The temperature was gradually decreased from 33 °C on day 1 to 21 °C on day 42 and kept constant afterword. The relative humidity was maintained between 50—70 %. After fattening, the animals were stunned and killed by cervical dislocation. Subsequently, breast and thigh muscle samples were taken for further laboratory examination. The muscle samples were immediately frozen and stored at –20 °C until analysed. The analysis consisted of digestion (5 ml HNO_3 and 1 ml HCl per 1g of sample) in a Milestone mineralization system (MLS 1200 Mega) with microwave decomposition technology. Analysis of samples for the presence of calcium, magnesium, copper and zinc was performed on an AAS (Unicam Solar 939, UK) by the flame method (Table 1). The methodology presented in the List of Official Methods and Laboratory Diagnostics of Food and Feed (Bulletin of the Ministry of Agriculture SR, 2004) was used for the determination.

Statistical analysis

The differences between means were determined, according to the unpaired t-test using GraphPad Prism 6 software. Correlations between pairs of elements in each tissue were determined by Pearson correlation analyses. Some of these correlations were highly influenced by the samples that had undetectable mineral concentrations and only samples with detectable mineral levels were included in the analysis. Only significant correlations with an r value > 0.3 are reported.

RESULTS AND DISCUSSION

The values presented in the Tables 2 and 3 are the average values calcium (Ca), magnesium (Mg), copper (Cu) and zinc (Zn) from 6 samples of breast and thigh muscle from each experimental group. The addition of humic substances in feed ensures good animal health and has a positive effect on production parameters and does not require withdrawal periods.

In the breast muscle of broilers from the group G1 we found a statistically significantly higher ($P < 0.05$) Ca content (0.51 $g.kg^{-1}$) compared to the control group (0.43 $g.kg^{-1}$). On the other hand, the addition of 0.3 % HNMy (G2) to feed significantly reduced ($P < 0.05$) Ca content in the thigh muscle (0.32 $g.kg^{-1}$) compared to the control group (0.48 $g.kg^{-1}$), but Ca was slightly increased in the breast muscle (0.47 $g.kg^{-1}$). Probably, the low concentration of Ca in the thigh muscle occurred due to chelating effects of humic substances that are influenced by their large number of carboxylic acid side chains. Mariam et al. [14] found an increased Ca content in poultry meat (1.72 $g.kg^{-1}$) compared to the values found by us. Similarly, Straková et al. [23] when comparing the nutritional content of broiler and pheasant muscle in both sexes, found that higher levels of Ca and Mg in breast muscle (males 2.15; 1.54 $g.kg^{-1}$ and females 2.03; 1.47 $g.kg^{-1}$) and in thigh muscle (males 1.80; 1.12 $g.kg^{-1}$ and females 1.67; 1.13 $g.kg^{-1}$). After feeding humate, increased levels of some essential minerals (such as Ca, Mg, Al and Fe) in serum, liver and muscles were recorded by Stepchenko et al. [22]. The relatively low Ca levels in chicken muscle (13.83 ppm) were

Table 1. Working conditions of atomic absorption spectrometer for analysis of minerals

Element	Fuel	Support	Flame stoichiometer	Wavelength [nm]	Spectral band pass [nm]
Calcium	Acetylene	Air	Oxidizing	422.7	0.5
Magnesium	Acetylene	Air	Oxidizing	285.2	0.5
Copper	Acetylene	Air	Oxidizing	324.8	0.5
Zinc	Acetylene	Air	Oxidizing	213.9	1.0

Table 2. The content of calcium and magnesium in breast and thigh muscle of broilers

Group	Muscle	Ca [g.kg^{-1}]	Mg [g.kg^{-1}]
GC	Breast	0.43 ± 0.07	0.69 ± 0.08
	Thigh	0.48 ± 0.06	0.67 ± 0.12
G1	Breast	0.51 ± 0.09*	0.80 ± 0.06*
	Thigh	0.51 ± 0.12	0.59 ± 0.04
G2	Breast	0.47 ± 0.06	0.73 ± 0.07
	Thigh	0.32 ± 0.06*	0.67 ± 0.12

The data are means of 6 samples of breast and thigh muscles from each group; GC—diet without the addition of humic substances; G1—diet with the addition of 0.7 % Humac Natur Mycosorb; G2—diet with the addition of 0.3 % Humac Natur Mycosorb; *—P ≤ 0.05

Table 3. The content of copper and zinc in breast and thigh muscle of broilers

Group	Muscle	Cu [mg.kg^{-1}]	Zn [mg.kg^{-1}]
GC	Breast	11.72 ± 1.12	26.62 ± 1.59
	Thigh	11.62 ± 1.18	28.35 ± 4.05
G1	Breast	8.83 ± 1.08**	25.18 ± 2.59
	Thigh	8.50 ± 0.77**	25.75 ± 3.05
G2	Breast	9.35 ± 0.62**	22.45 ± 2.82*
	Thigh	10.07 ± 0.69	24.38 ± 2.85*

The data presented are means of 6 samples of breast and thigh muscles from each group; GC—diet without the addition of humic substances; G1—diet with the addition of 0.7 % Humac Natur Mycosorb; G2—diet with the addition of 0.3 % Humac Natur Mycosorb; *—P ≤ 0.05 ; **—P ≤ 0.01

reported by Ebeledike et al. [4]. A statistically significant increase in Mg content ($P < 0.05$) was analyzed in breast muscle (0.80 g.kg^{-1}) in broilers from the group G1 compared to Mg content in the muscles (0.69 g.kg^{-1}) of broilers from the control group (Table 2).

Differentiated effects have been shown by humic acids to trace elements, especially copper and zinc [8]. Humates can act as a potent metal chelator, it is possible that HA chelates the extracellular ions of some elements and transfers them to the cells, which can be explained as the reason for the increase in concentration.

Our study reports that concentrations of copper and zinc in muscles after the addition of humates were lower compared to values in muscles in control chickens (Table 3). Regarding the essential elements, the average concentrations of the monitored elements in the muscles of chickens in this study did not exceed the maximum permissible limits.

In the control group of broilers, higher levels of Cu were found in the breast and thigh muscles (11.72; 11.62 mg.kg^{-1}) than in the muscles of broilers from the groups G1 (8.83; 8.50 mg.kg^{-1}) and G2 (9.35; 10.07 mg.kg^{-1}). A significant reduction in Cu in the breast and thigh muscles was found in the group G1 ($P \leq 0.01$) compared to the control group. A similar significant reduction ($P \leq 0.01$) was observed only in breast muscle in the group G2. Copper is an element that presents itself as essential but also potentially toxic. The highest concentrations of copper are found in the liver, lower content in the kidneys and the lowest in muscle. Several authors report lower or nearly equal levels

Table 4. Correlation coefficients between minerals (Ca, Mg, Cu, Zn) in the breast muscle (BM) and the thigh muscle (TM) in the control group

Control	Ca BM	Mg BM	Cu BM	Zn BM	Ca TM	Mg TM	Cu TM	Zn TM
Ca BM	1.0	–0.1875	**0.8333**	**0.8159**	0.4450	–0.4808	0.2157	0.3002
Mg BM	–	1.0	–0.1481	–0.4029	0.0745	0.5084	0.2878	**0.7248**
Cu BM	–	–	1.0	0.3919	0.0915	**–0.6771**	–0.3028	0.2873
Zn BM	–	–	–	1.0	0.6061	–0.2322	0.5566	0.0162
Ca TM	–	–	–	–	1.0	–0.1211	0.6754	–0.0255
Mg TM	–	–	–	–	–	1.0	0.5623	0.4637
Cu TM	–	–	–	–	–	–	1.0	0.3339
Zn TM	–	–	–	–	–	–	–	1.0

of the Cu in chicken muscle in Brazil 0.3—3.5 mg.kg^{-1} [6]; in Turkey 0.5 to 12.3 mg.kg^{-1}1 [24] ; 0.27—0.82 mg.kg^{-1} in China [7]. S k a l i c k a et al. [18] recorded in the experiment lower levels of Cu (6.18 0—7.88 mg.kg^{-1}) in breast and thigh muscle (6.13—6.98 mg.kg^{-1}), where the feed mixture was added with the addition of 0.7 % Humac Natur Monogastric with the addition of formates. After iron and zinc, copper is the third most abundant trace element in the body. In Pakistan, M a r i a m et al.[14] monitored copper and zinc concentrations in some animals and found that all sample values in the study were below than the permissible limits. Poultry muscle recorded the lowest concentrations of copper and zinc (12.86 mg.kg^{-1}, 28.52 mg.kg^{-1}, resp.).

In our study we found higher levels of Zn in the breast and thigh muscles (26.62; 28.35 mg.kg^{-1}) than in the muscles of broilers from the groups G1 and G2. The significantly lower content of Zn ($P \leq 0.05$) was found in the breast and thigh muscle of broilers from the group G2 (22.45; 24.38 mg.kg^{-1}) compared to the control group. A decrease in Zn levels in the breast and thigh muscles was also observed in the group G1. K h a n et al. [12] found much higher values in the thigh muscle (107.4 ± 7.60; 106.6 ± 7.37 and 106.78 ± 7.48 mg.kg^{-1}) and breast muscle (107.82 ± 7.66; 107, 4 ± 7.49 and 107.95 ± 7.73 mg.kg^{-1}) taken from three different districts. H u et al. [7] found Zn values of 3.27—17.90 mg.kg^{-1} in the poultry muscle from the food markets region in southern China.

Correlation analysis revealed some relationships between the content of elements in breast and thigh muscles (Table 4).

In the control group, correlations were observed in the breast muscle between Ca and Zn (r = 0.8159) and between Ca and Cu (r = 0.8333). Similarly, positive correlation was observed between Zn in the thigh muscle and Mg in the breast muscle (r = 0.7248). A negative correlation was found for Mg in the thigh muscle and Cu in the breast muscle (r = –0.6771).

The effect of different addition levels of Humac substances on the correlation coefficients of mineral elements in the breast and thigh muscles are shown in Tables 5 and 6.

The calcium content in the breast muscle confirmed a strong positive correlation between the control group and the group G2 (r = 0.7195). The strong positive correlation (r = 0.9093) for Ca was found in the thigh muscle from the control group and in the breast muscle from the group G2. On the other hand, in the group G2, the medium negative correlation was shown (r = –0.6724) for Mg between thigh muscle and breast muscle. Similarly, significantly negative correlation (r = –0.7656) was found between in the thigh muscles, between Mg from the group G2 and Ca from the control group. A high positive correlation (r = 0.7643) were observed in the thigh muscles between Mg content from group G1 and Ca content from the control group.

Other studies examined the relationship between mineral concentrations, and the relationship of mineral concentration physicochemical characteristics in muscles of Japanese Black steers. Magnesium (Mg), potassium (K) and zinc (Zn) concentrations had negative correlations with fat content, but sodium (Na), manganese (Mn), copper (Cu) and molybdenum (Mo) concentrations were not correlated with fat content. The results of the present experiment sug-

Table 5. Correlation coefficients between Ca and Mg in experimental groups in the breast muscle (BM) and the thigh muscle (TM)

		GC			G1				G2			
		Ca TM	Mg BM	Mg TM	Ca BM	Ca TM	Mg BM	Mg TM	Ca BM	Ca TM	Mg BM	Mg TM
GC	Ca BM	0.4450	−0.1875	−0.4808	−0.2282	0.0878	0.5815	0.1301	0.7195	0.6928	−0.0468	−0.3848
	Ca TM	1.0	0.0745	0.1211	−0.4361	−0.5810	0.5292	0.7643	0.9093	−0.0296	0.5504	−0.7656
	Mg BM	–	1.0	0.5084	−0.4079	−0.2611	−0.1884	−0.2331	0.3581	0.1949	0.6002	−0.4305
	Mg TM	–	–	1.0	−0.6571	0.3087	−0.2331	−0.1622	0.0193	−0.3916	0.7616	−0.4236
G1	Ca BM	–	–	–	1.0	−0.2879	−0.5541	0.2185	0.1579	−0.1822	−0.1406	−0.2687
	Ca TM	–	–	–	–	1.0	0.1663	0.0030	−0.5112	−0.0556	−0.4483	0.4957
	Mg BM	–	–	–	–	–	1.0	0.5775	−0.4199	−0.1343	0.3848	−0.2887
	Mg TM	–	–	–	–	–	–	1.0	−0.5853	0.0318	−0.0911	−0.4236
G2	Ca BM	–	–	–	–	–	–	–	1.0	0.1869	0.6189	−0.4726
	Ca TM	–	–	–	–	–	–	–	–	1.0	−0.2383	−0.2407
	Mg BM	–	–	–	–	–	–	–	–	–	1.0	−0.6724

GC—control group; G1—diet with the addition of 0.7 % Humac Natur Mycosorb; G2—diet with the addition of 0.3 % Humac Natur Mycosorb

Table 6. The correlation coefficients between Cu and Zn in experimental groups in the breast muscle (BM) and the thigh muscle (TM)

		GC			G1				G2			
		Cu TM	Zn BM	Zn TM	Cu BM	Cu TM	Zn BM	Zn TM	Cu BM	Cu TM	Zn BM	Zn TM
GC	Cu BM	−0.3028	0.3919	0.2873	−0.3693	−0.6987	0.7110	0.2207	0.4947	0.5946	−0.7570	−0.1164
	Cu TM	1.0	0.5566	0.3339	−0.1777	−0.0438	−0.7415	0.7003	−0.1334	−0.6652	0.5717	−0.1182
	Zn BM	–	1.0	0.0162	−0.3813	−0.1836	−0.1108	0.9786	0.0519	0.1456	0.0300	−0.3661
	Zn TM	–	–	1.0	0.1577	−0.8666	−0.2526	0.0235	−0.0108	−0.5038	0.0691	−0.1198
G1	Cu BM	–	–	–	1.0	−0.0719	−0.4372	−0.3850	−0.5413	−0.1427	0.3780	0.0054
	Cu TM	–	–	–	–	1.0	0.1395	−0.2734	−0.2188	0.0149	0.3357	0.0972
	Zn BM	–	–	–	–	–	1.0	−0.0907	0.6415	0.8109	−0.9221	0.2245
	Zn TM	–	–	–	–	–	–	1.0	−0.0207	−0.0302	0.1907	−0.3661
G2	Cu BM	–	–	–	–	–	–	–	1.0	0.4836	−0.8403	0.7301
	Cu TM	–	–	–	–	–	–	–	–	1.0	−0.7852	0.2258
	Zn BM	–	–	–	–	–	–	–	–	–	1.0	−0.4701

GC—control group; G1—diet with the addition of 0.7 % Humac Natur Mycosorb; G2—diet with the addition of 0.3 % Humac Natur Mycosorb

Table 7. Correlation coefficients between minerals (Ca, Mg, Cu and Zn) in experimental group G1 in the breast muscle (BM) and the thigh muscle (TM)

	Ca BM	Mg BM	Cu BM	Zn BM	Ca TM	Mg TM	Cu TM	Zn TM
Ca BM	1.000	–0.5541	–0.4731	0.5100	–0.2879	0.2185	0.7377	–0.1839
Mg BM	–	1.000	–0.0731	–0.1465	0.1663	0.5775	–0.4301	0.6523
Cu BM	–	–	1.000	–0.4372	0.7905	–0.5029	–0.0719	–0.385
Zn BM	–	–	–	1.000	–0.0443	0.1395	–0.1530	-–0.0907
Ca TM	–	–	–	–	1.000	0.0030	–0.0420	–0.4738
Mg TM	–	–	–	–	–	1.000	0.2620	0.3385
Cu TM	–	–	–	–	–	–	1.000	–0.2734
Zn TM	–	–	–	–	–	–	–	1.000

G1—diet with the addition of 0.7 % Humac Natur Mycosorb

Table 8. Correlation coefficients between minerals (Ca, Mg, Cu and Zn) in experimental group G2 in the breast muscle (BM) and the thigh muscle (TM)

	Ca BM	Mg BM	Cu BM	Zn BM	Ca TM	Mg TM	Cu TM	Zn TM
Ca BM	1.000	0.6189	–0.8349	0.6496	0.1869	–0.4726	–0.2770	–0.9277
Mg BM	–	1.000	–0.3096	0.2136	–0.2383	–0.6724	0.0977	–0.6988
Cu BM	–	–	1.000	–0.8403	–0.4381	0.1597	0.4836	0.7301
Zn BM	–	–	–	1.000	0.8228	–0.3821	–0.7852	–0.4701
Ca TM	–	–	–	–	1.000	–0.2407	–0.8698	0.0345
Mg TM	–	–	–	–	–	1.000	0.0636	0.2970
Cu TM	–	–	–	–	–	–	1.000	0.2258
Zn TM	–	–	–	–	–	–	–	1.000

G2— diet with the addition of 0.3 % Humac Natur Mycosorb

gest that mineral concentrations reflect some traits such as fat content but also the composition of myofiber type and the intracellular fluid volume in the muscle [11].

As regards the trace metals, a strong negative correlation was confirmed (r = –0.9221) for Zn content in breast muscle between the groups G2 and G1. A strong positive correlation was observed also between Zn in the breast muscle from the group G1 and Cu in the thigh muscle from the group G2 (r = 0.8109). Similarly, positive correlation was demonstrated between Zn content in thigh muscle from the groups G1 and Zn content in breast muscle from the control group (r = 0.9786). In the group G2 was found positive correlation (r = 0.7301) between Cu content in the breast muscle and Zn content in the thigh muscle and high negative correlation (r = –0.8403) in the breast muscle between Zn content and Cu content in the breast muscle.

S k a l i c k á et al. [20] showed significant correlation between minerals in the liver and thigh muscle. The negative correlation was confirmed between Cd in muscle and Cr in muscle (r = –0.947) and Cu in liver and Cd in muscle (r = –0.885). The results of this study demonstrate antagonism among selected elements.

In addition, significant correlations were observed between copper and most essential elements in breast and thigh muscles of broiler chickens. Tables 7 and 8 summarise the correlation analysis between minerals in breast and

thigh muscles in experimental groups G1 and G2.

After addition of 0.7 % HNMy to diet we observed a strong positive correlation between Cu content in the breast muscle and Ca content in the thigh muscle (r = 0.7905). Also between Cu content in the thigh muscle and Ca in the breast muscle (r = 0.7377). S k a l i c k á et al. [21] in the study with addition 0.7 % Humac Natur in the diet for broiler chickens were found negative correlation between the elements: Ca and Cu (r = –0.6582) in the breast muscle.

On the contrary, after addition of 0.3 % HNMy to the diet we observed negative correlations in the thigh muscle between copper and calcium (r = –0.8698). A strong negative correlation was observed between Zn content in the thigh muscle and Ca content in the breast muscle (r = –0.9277). On the other hand, in the study with 0.5 % Humac Natur in the diet there was a positive correlation between Cu in the thigh muscle and Ca in the breast muscle (r = 0.8881) [21].

CONCLUSIONS

Increased attention as an alternative to feeding antibiotics in poultry production has been paid to ecological additives. Organic additives are more acceptable by consumers. According to the results of this experiment, the use of the 0.7 % and 0.3 % Humac Natur Mycosorb as feed supplement contributed to the increase in the Ca and Mg content in the breast and thigh muscles of broilers. Changes in concentrations of elements observed in the muscle of chickens after the addition of humates included in this study were caused by mutual interactions. The mechanism involves formation of chelate bonds with the elements. To sum up, the Humac Natur can be considered a good feed supplement which positively affects the nutritional value of chicken meat.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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MOLECULAR AND BIOLOGICAL CHARACTERIZATION OF *HAEMAPHYSALIS LEACHI* (ACARI: IXODIDAE) IN NIGERIA WEST AFRICA

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ABSTRACT

Ticks constitute a serious threat to the wellbeing of humans and other animals. The accurate identification of ticks is paramount in epidemiological investigations. Genetic markers have been identified and used to overcome the limitations of phenotypic identification of ticks. In this study, the cytochrome c oxidase 1 (*Cox1*) gene was amplified and sequenced for the identification of *Haemaphysalis leachi*, the putative vector of *Babesia rossi* in Nigeria. Amplification was successful in 34 out of 39 (87 %) ticks collected from dogs in three Nigerian states with sequence homology of 99 % to *H. leachi* in Genbank (GenBank: MN663156.1). Maximum Likelihood phylogenetic analysis showed significant grouping of *H. leachi* sequences in independent monophyletic subclade with a bootstrap value of 100 %. Genetic distance analysis of *H. leachi* identified in this study indicated a very low level of intraspecific diversity (0.016 %) compared to 0.150—0.190 % interspecific distance to other *Haemaphysalis* species. The number of eggs laid by engorged female ticks maintained in the laboratory ranged from 885 to 2190 and was proportional to the ticks' initial weight. The mean value of other biological parameters; female engorgement weight, pre-oviposition period, oviposition period, total egg mass, egg size, efficiency rates of female ticks in converting their food reservoir to eggs and incubation period are, 147.5 mg, 7.8 days, 13.2 days, 59.5 mg, 485.5 × 348.7 µm, 41.2 % and 26.2 days, respectively. This study reports the first molecular identification of *H. leachi* in Nigeria.

Key words: *Cox1*; identification; Ixodidae; Nigeria; PCR; sequencing

INTRODUCTION

There are nearly 170 described species of ticks in the genus *Haemaphysalis* Koch, 1844 (Acari: Ixodidae) worldwide [12]. The subgenus *Rhipistoma* Koch, 1844 has 33 species occurring in a wide range of climatic regions ranging from rain forest to desert areas mostly in sub Saharan Africa, as well as in Europe, and Asia [12, 29]. Members of this subgenus primarily parasitize carnivores, rodents, hyraxes and hedgehogs [12, 15]. Renewed interest in the study of *H. (R.) spinulosa* subgroup of the *H. (R.) leachi* has led to the redescription of new taxa in addition to the four existing valid taxa in the group [2, 3, 28, 29]. In the tropical regions, ticks are ranked second to mosquitoes in terms of veterinary and public health importance. Specifically, *H. leachi* has been incriminated in the transmission of the infectious agent *Babesia rossi* to dogs and *Rickettsia conorii* to humans among others [18, 23].

Phenotypically, *H. leachi* possesses characteristic conspicuous lateral extensions to palp articles 2, forming mouthparts with a distinctive conical shape. Other distinctive features are the presence of medium length spurs on the coxae 4 of males compared to very conspicuous spurs on coxae 4 of *H. punctata* and *H. sulcata*. The presence of spurs on the ventral surface of palp articles 2 of female *H. spinulosa* differentiates it from *H. leachi*. However, *H. paraleachi*, *H. punctaleachi* and *H. moreli* are difficult to distinguish from *H. leachi* [29]. Therefore, ticks in the *H. (R.) leachi* group have been considered one of the most difficult from a taxonomic point of view [3]. This then calls for caution before making definite statements about the identity of specimens within this group. In this context, the conventional methods based on morphology and tick's ecology for species determination is inadequate, especially when dealing with morphologically similar taxa, damaged specimens, and where immature stages are not described or are engorged [22, 30]. This can be overcome by using molecular markers which are highly conserved and easy to amplify suitable regions of the genome using the polymerase chain reaction (PCR), sequence analyses and alignment of the data with reference sequences [4, 20].

The mtDNA encoded cytochrome oxidase subunit I (*Cox1*) gene has been identified as a species-level marker that produces a high standard barcode for phylogenetic and taxonomic studies of arthropods including ticks [9, 19, 20]. To date, there is no such study on identification of *Haemaphysalis* spp. using a well-defined molecular approach in Nigeria. Therefore, the aim of this study is to utilize the *Cox1* gene to verify the morphological status of *H. leachi* and to determine its biological characteristics in this African country.

MATERIALS AND METHODS

Ethical statement

Approval for this study was granted by the Animal Use and Care Committee (AUCC), National Veterinary Research Institute (NVRI) Vom, Nigeria, number AEC/03/21/15. Oral consent for permission to collect the ticks was obtained from dog owners.

Collection of ticks

Ticks were collected from naturally infested owned dogs in households or in veterinary clinics in 11 states and the Federal Capital Territory (FCT) Abuja, Nigeria between August 2018 to July 2019 (Fig. 1). Ticks were also removed from dog carcasses submitted for post-mortem at the veterinary pathology laboratory, College of Veterinary Medicine, FUNAAB. In all, ticks were removed from 472 dogs using forceps and kept in labelled ventilated tubes according to each host and transported to the Entomology Laboratory, National Veterinary Research Institute (NVRI), Vom, Nigeria.

Morphological identification of ticks

All tick samples were preliminary identified to ge-

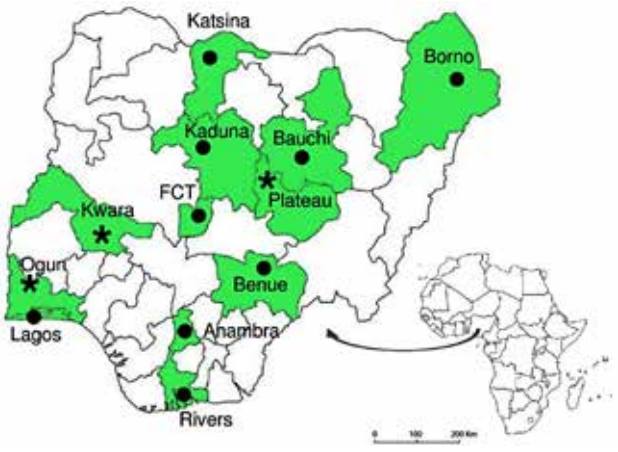

Fig. 1. Map of Nigeria, West Africa showing tick sampling locations (shaded) and states where *H. leachi* were identified (asterisk)

nus-level based on external morphological characteristics under a stereo microscope (Zeiss, Stemi DV4) using specific illustrated morphological taxonomic keys [28, 29]. *Haemaphysalis* ticks were identified based on characteristic conspicuous lateral extensions to palp articles 2, forming mouthparts with a distinctive conical shape, rectangular basis capitulum, absence of eyes, presence of festoons and absence of ventral plates in males [29]. Only ticks morphologically identified to belong to the genus *Haemaphysalis* were included in this study. Partially or fully engorged female ticks were placed individually in labelled ventilated tubes for fecundity studies.

Biological studies

Six engorged female ticks were weighed individually and placed in coded sterile ventilated tubes. The tubes were placed in an incubator at 27 °C, relative humidity of 85 % with 12-hour photoperiod. The ticks were monitored daily for the commencement of egg laying. Ticks were weighed and eggs laid counted daily. Ten eggs were randomly picked from each clutch daily and measured (length and width), under a calibrated microscope (Nikon, Eclipse E100). This was continued until egg laying ceased as evidenced by constant weight of the ticks over five consecutive days. Biological parameters; female engorgement weight (FW), egg mass weight (EMW), pre-oviposition period (POP); incubation period (IP); efficiency rates of female ticks in converting their food reservoir to eggs (ERCE), were calculated according to D i p e o l u et al. [7] and S z a b ó et al. [25].

Molecular identification of ticks

DNA extraction from ticks

Genomic DNA was extracted from 39 adult ticks morphologically identified as *H. leachi*. Ticks were removed from 70 % ethanol and washed in three changes of phosphate buffered saline (PBS), before DNA extraction using the QIAmp DNA Mini Tissue extraction kit (QIAGEN Hilden, Germany) according to manufacturer's instructions with slight modifications. Cell lysis was achieved at 56 °C overnight. Other procedures are as stated in the manufacturer's instructions. Eluted DNA were preserved at –20 °C until PCR analysis.

Amplification of cytochrome c oxidase 1 (*Cox1*) by conventional PCR

The identity of the ticks was molecularly confirmed by PCR targeting the 710 bp of the cytochrome c oxidase (*Cox1*) gene using the primers HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') and LCO1490 (5'-GGT CAA ATC ATA AAG ATA TTG G-3') under a modified cycling conditions reported by F o l m e r et al. [11]. The reaction was carried out in a volume of 50 µl, which contained 1.25 U Taq DNA polymerase (Fermentas), 5 µl 10× Taq reaction buffer (including 15 mM $MgCl_2$), 5 µl PCR nucleotide Mix (0.2 mM each), 1.5 µl (1 µM final concentration) of each primer, 5 µl template DNA. PCR grade water (BioConcept, Switzerland) was used to make up the volume. The cycling conditions were as follows; an initial denaturation step at 95 °C for 2 min was followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 48 °C for 1 min and extension at 72 °C for 1 min. The final extension was performed at 72 °C for 7 min. The PCR was carried out using the Applied Biosystem thermal cycler (GeneAmp 9700) in the Molecular Biology Unit, Parasitology Division, NVRI Vom.

The resulting PCR products were electrophoresed on a 1.5 % agarose gel stained with SafeView stain from Applied Biological materials Inc. (Canada) to check the size of the amplified fragments by comparison to 100 bp DNA Ladder from New England Biolabs (Ipswich, MA, USA) under a blue light transilluminator (Cleaver Scientific UK). The amplified products of the expected size were sequenced at a commercial facility (Macrogen Europe B.V Amsterdam, the Netherlands). The sequences obtained were manually corrected by visual analysis of the electrophoregram using Bioedit v7.0.5.3 [13] and were compared with sequences in the GenBank database by using the BLAST program (http:www.ncbi.nlm.nih.gov/BLAST).

Phylogenetic and pairwise distance analyses

The sequences that allowed the species-level identification were aligned with the corresponding sequences of *Haemaphysalis* tick species available in GenBank using multiple sequence alignment with Clustal Omega (https://www.ebi.ac.uk/Toos/msa/clustalo/). Phylogenetic relationships based on the alignment of *Cox1* sequences were performed to analyze the phylogenetic status of the *H. leachi* in this study. Data were analysed using the Maximum likelihood (ML) approach while *Ixodes ricinus* (GenBank:

AY945440) was used as outgroup. The phylogenetic tree was performed using Kimura 2-parameter as substitution model in MEGA 5 [27]. Branch supports were estimated by the bootstrap analysis of 1000 replicates. Pairwise analysis was conducted on the nucleotide sequences obtained in this study and six nucleotide sequences in GenBank. The distance divergence was computed based on Kimura 2 parameter model.

Statistical analysis

Data generated in this study were entered into an Excel spreadsheet (Microsoft Corporation, Redmond, WA) and analyzed accordingly. Data were summarized using descriptive statistics to obtain the mean, range standard deviation and 95 % confidence interval for each parameter assessed in the study.

RESULTS

A total of 5,670 ticks (nymph, adult and larva) were collected from 472 owned dogs in 11 states of Nigeria and the Federal Capital Territory (FCT), Abuja between August 2018 to July 2019 (Fig. 1). The ticks were identified to genus level under a stereomicroscope based on the diagnostic morphological characters. Among all the 5,670 ticks collected, 166 (2.9 %) of them were morphologically identified to the genus *Haemaphysalis*, Kwara state (n = 4), Ogun state (n = 7) and Plateau state (n = 155) (Fig. 1). Based on the number of *Haemaphysalis* ticks from each study location a subset of 39 were selected for molecular identification. Amplification of the 710 bp of the *Cox1* was successful in 34 specimens; 2/2 (100 %) from Kwara state, 4/5 (80 %) from Ogun state and 28/34 (82 %) ticks from Plateau state. Sequencing results confirmed the ticks as *H. leachi* with 99 % identity to *H. leachi* in Genbank (GenBank: MN663156.1). Sequences generated in the study were deposited in the GenBank under the accession numbers MW558147-MW558149.

Phylogenetic analyses showed significant grouping of *H. leachi* sequences in this study with sequence in GenBank in independent monophyletic subclade with a bootstrap value of 100 %. The phylodendograms showed that the *H. leachi* sequences identified in this study were clustered together and with *H. leachi* from USA (GenBank: MN663156.1) with 100 % bootstrap value. Other *Haemaphysalis* species such as; *H. erinacei*, *H. longicornis*, *H. qinghaiensis* and *H. flava* as well as *Dermacentor* and *Rhipicephalus* species formed distinct clusters (Fig. 2).

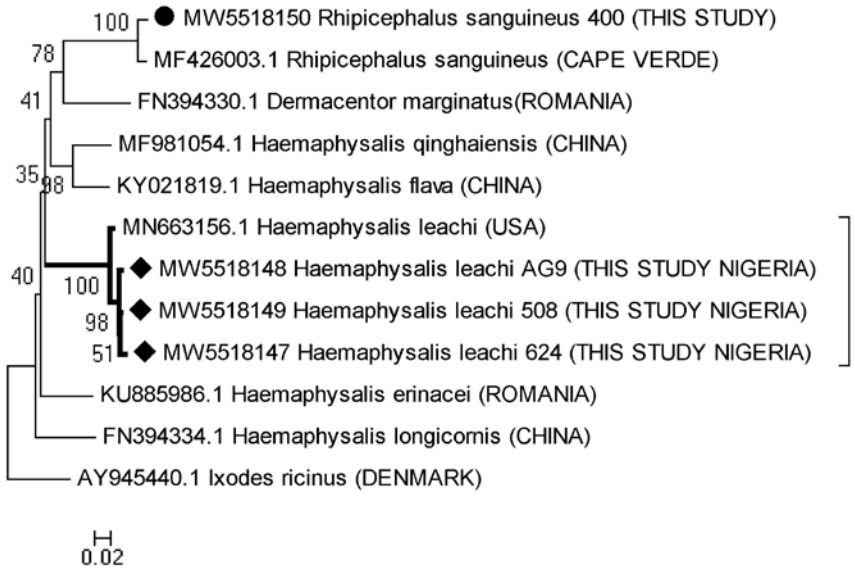

Fig. 2. Maximum Likelihood tree of 12 sequences (including one outgroup) from GenBank and sequences of *H. leachi* (black rhombus) and one sequence of *R. sanguineus* (black circle) identified in the present study. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches

Table 1. Pairwise distance of *Cox1* of 3 *H. leachi* from this study with 5 *Haemaphysalis* and 1 *Ixodes* ticks

S/No.	Species (Accession number)	1	2	3	4	5	6	7	8	9
1	***H. leachi* (MW5518149)**	–	0.003	0.004	0.005	0.017	0.016	0.018	0.017	0.020
2	***H. leachi* (MW5518147)**	0.007	–	0.004	0.006	0.019	0.019	0.020	0.020	0.022
3	***H. leachi* (MW5518148)**	0.008	0.009	–	0.005	0.017	0.017	0.019	0.017	0.020
4	***H. leachi* (MN663156)**	0.019	0.022	0.016	–	0.017	0.015	0.018	0.016	0.019
5	***H. erinacei* (KU885986)**	0.150	0.166	0.154	0.145	–	0.016	0.017	0.016	0.019
6	***H. flava* (KY021819)**	0.174	0.190	0.178	0.159	0.138	–	0.015	0.011	0.017
7	***H. longicornis* (MF666902)**	0.182	0.184	0.182	0.171	0.144	0.151	–	0.014	0.018
8	***H. qinghaiensis* (MF981054)**	0.168	0.178	0.164	0.158	0.155	0.098	0.144	–	0.017
9	***I. ricinus* (AY945440)**	0.232	0.232	0.230	0.222	0.176	0.200	0.182	0.215	-

Table 2. Biological parameters of *H. leachi* maintained under standard conditions

Biological parameters		Tick sample code						Range	Mean ± SD
		159	163	597	610	616	588		
FW [mg]		128	124	144	127	150	212	124—212	147.5 ± 33.3
POP [days]		6	9	8	7	9	8	6—9	7.8 ± 1.2
OP [days]		12	11	12	13	15	16	11—16	13.2 ± 1.9
NEL		1690	1407	885	1095	1419	2190	885—2190	1447.7 ± 458.9
EMW [mg]		76	58	36	49	62	76	36—76	59.5 ± 15.6
ES (μm)	**Length**	465	455	474	427	465	465	427—474	485.5 ± 16.6
	Breath	344	316	353	362	352	365	316—365	348.7 ± 17.7
TFW		29	26	27	24	26	48	24—48	30 ± 3.66
WL (%)		99 (77.3)	98 (79.0)	117 (81.3)	103 (81.1)	124 (82.7)	164 (77.4)	98—164 (77.3—82.7)	117.5 ± 25 (79.8 ± 2.2)
ERCE [%]		59.4	46.8	25.0	38.6	41.3	35.8	25—59.4	41.2 ± 11.5
IP [days]		23	28	24	25	29	28	23—29	26.2 ± 2.5

Engorged female weight (FW); egg mass weight (EMW); pre-oviposition period (POP); oviposition period (OP); egg size (ES); incubation period (IP); efficiency rates of female ticks in converting their food reservoir to eggs (ERCE), weight loss (WL); number of eggs laid (NEL); tick final weight (TFW)

The pairwise distance analysis of *H. leachi* showed that the local species is genetically different from *H. leachi* from USA (GenBank: MN663156.1) with low genetic distance value of 0.02 % (Table 1). Genetic distance analysis of *H. leachi* collected from all the three localities also indicated a very low level of intraspecific diversity/variability of 0.016 %. However, interspecific distance analyzed by the pairwise comparison revealed that *H. leachi* sequences from this study genetically differ from other *Haemaphysalis* spp.; *H. erinacei*, *H. longicornis*, *H. qinghaiensis* and *H. flava* with slightly higher genetic variation values from 0.15—0.190 % (Table 1).

The average engorgement weight of female *H. leachi* in this study was 147.5 mg, ranging from 124—212 mg. On the average, a female tick lays 1448 eggs within 11—16 days oviposition period which was preceded by 6—9 days pre-oviposition period. The egg mass was correlated to the initial weight of the ticks (Table 2). The number of eggs laid increased daily reaching a peak at 3—5 days into oviposition before it gradually declined. Similarly, there was a gradual increase in the size of the eggs before it then reduced toward the end of the oviposition period. The maximum size, length × breadth (L × B) of the eggs recorded in this study was 474 μm × 365 μm [(mean ± SD) 485.5 ± 6.6 × 348.7 ± 17.7)]. The efficiency rates of female ticks in converting their food reservoir to eggs was 25—59.4 % resulting in the loss of 77.3—82.7 % of the initial weight (Table 2). The eggs were spherical to oval in shape and dark brown to mahogany in colour with glistening surfaces.

DISCUSSION

To a large extent, tick identification in Nigeria and other less scientifically developed countries relies on morphological criteria, which is fraught with challenges especially when dealing with ticks of similar morphological features, those with damaged mouth parts or immature stages [19, 30]. However, accurate identification of ticks is of great significance for disease investigation and formulation of effective tick control measures. *Haemaphysalis leachi*, also known as the yellow dog tick, is a three-host tick adapted to feeding on domestic dogs in tropical and sub-tropical areas, especially in sub-Saharan Africa [29]. The difficulty of the determination of ticks belonging to the *H. (R.) leachi* group from a taxonomic point of view has been reported [2]. Therefore, the main objective of this study was to employ PCR and sequencing approaches in order to overcome the limitations associated with the phenotypic method of tick identification and to obtain data on some biological characteristics of *H. (R.) leachi* group in Nigeria.

Ticks collected from different ecological zones of Nigeria and morphological identified as belonging to the genus *Haemaphysalis* were subjected to PCR targeting the *Cox1* gene. The results from this study showed that the *Cox1* gene was successfully amplified from ticks sampled from three states in Nigeria with high sequence identity to *H. leachi* in GenBank confirming for the first time by molecular approach the presence of this tick species in Nigeria. Indeed, the phylogenetic tree inferred by the ML method placed the sequences of *H. leachi* from this study in the same cluster with *H. leachi* sequences in the GenBank. So far, this tick species has been morphologically identified on dogs in three states located in the Guinea and the Savanna regions of Nigeria [17]. The results in this study supports the earlier morphological identification of *H. leachi* from some parts of Nigeria. It can be surmised from both the morphological and molecular studies that *H. leachi* is present in the Guinea and Savanna, but not the Sahel region of Nigeria. The low rainfall and high ambient temperature characteristic of the Sahel ecological zone of Nigeria may not favour the survival of this tick.

Pairwise and phylogenetic analyses revealed that genetic diversity at both inter- and intra-species levels between the tick samples in Nigeria and sequences deposited in GenBank was low; 0.007—0.82. This was depicted in the high bootstrap value in the phylogenetic tree, attesting to the utility of the mitochondrial *Cox1* gene is for determination of genetic variation either by interspecies or intraspecies of ticks. Similar findings have been reported among *H. punctata*, *H. hystricis* and *H. bispinosa* in Romania, Malaysia and India, respectively [4, 5, 9, 10]. Of interest is the fact that *H. leachi* has been reported as the putative vector of *B. rossi* [29], which is considered the most pathogenic of the large babesias infecting dogs [21]. Indeed, pallor of the mucosa was observed in carcasses of dogs examined at the Department of Veterinary Pathology, FUNAAB, where some of the ticks were collected. This could be due to anaemia as a result of blood sucking by the ticks or the effects of haemoparasite transmitted by the ticks. The confirmation of its presence in some states of Nigeria may explain

the frequent detection of *B. rossi* in Nigerian dogs [1, 16, 24]. Some doubts remain, however, as to whether *H. leachi* or *H. elliptica* or both are involved in the transmission of *B. rossi* to dogs, following the reclassification of *H. leachi* to *H. elliptica* in South Africa by Apanaskevich et al. [3]. It is intriguing that some yellowish coloured ticks sampled from dogs in this study did not yield readable sequences in the *Cox1* reaction. Indeed, the ticks phenotypically identified and confirmed as *H. leachi* by PCR and sequencing in this study were dark brown in colour, possibly due to blood engorgement. Therefore, more studies are needed in order to update, reconcile and resolve earlier phenotypic and genotypic identities of these species. None of the samples tested in this study had similar identity with *H. elliptica* sequences in GenBank on BLASTn. In fact, *H. elliptica* did not appear on the BLASTn search list. This may be due to lack of the sequences of *H. elliptica* in the GenBank, underscoring to the need for more studies on the members of *Haemaphysalis* genus.

Biological characteristic determined in this study showed that the mean female engorgement weight of *H. leachi* was 1147.5 mg. Several factors such as climate, hosts and tick species have been reported to influence the engorgement weight of female ticks [6, 26]. The daily egg laying in *H. leachi* follows a Type 1 pattern where there was an initial low egg production followed by a gradual increase reaching peak production before it starts to decline as previously observed in *A. ariegatum* [7]. The total number of eggs laid by individual *H. leachi* varied proportionately with initial engorgement weight in accord with earlier observation for other tick species [8, 14, 26]. This could be explained by the fact that the blood meal that constitute the bulk of the tick mass is required for the egg production process as well as other biological activities of the tick during the period of oviposition [6, 26]. This could be surmised from the fact that approximately 80 % of the initial weight of the ticks was lost during the period of oviposition. However, it has also been reported that there is a maximum effective engorgement weight beyond which the number of eggs laid is not influenced by the engorgement weight [6]. This phenomenon was not observed in the *H. leachi* ticks collected in this study. Other biological parameters such as, the POP, EMW, IP and the ERCE values obtained for *H. leachi* in this study differed from those reported for *R. sanguineus* collected from dogs [14, 26]. Furthermore, the egg morphology of *H. leachi* in this study differs from those of *R. sanguineus, A. maculatum* and *D. variabilis* [6, 7, 8]. This could be due to tick species and /or host differences.

CONCLUSIONS

In conclusion, the identity of *H. leachi* has been confirmed in Nigeria by the amplification and sequencing of the *Cox1* gene, also, preliminary data on its biological characteristic has been provided. As the putative vector of *B. rossi*, veterinarians should be cautious and accord priority to the proper diagnosis and treatment of this protozoan parasite as well as the control of this tick species. Additional studies are required to ascertain the various species of the *Haemaphysalis* genus in the various ecological zones of Nigeria and to determine their veterinary and public health significance.

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CULTIVABLE ORAL MICROBIOTA IN PUPPIES

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ABSTRACT

The oral microbiota has been shown to be different in children born by caesarean section and delivered vaginally. The aim of this study was to investigate the oral microbial diversity in healthy puppies and to determine whether the birth mode affects the composition of the oral microbiota. A total of 19 puppies from 4 dams were included in the study. The puppies were divided into two groups depending on the birth mode, vaginal delivery (vaginal born VB) or caesarean delivery (caesarean section CS). On the seventh day after birth, swabs of the oral cavity were taken. All samples were analysed by bacteriological cultivation under aerobic and anaerobic conditions. Bacterial colonies were identified by Sanger sequencing of 16S rRNA. A total of 64 bacterial strains belonging to 10 genera were obtained from the oral swabs. The genera *Staphylococcus* (30.23 % VB and 47.62 % CS) and *Enterococcus* (25.58 % VB and 33.33 % CS) were the most abundant in both groups. The genera *Escherichia* (18.60 %) and *Enterobacter* (16.28 %) were largely present in puppies delivered vaginally, they were not found in puppies born by caesarean section. The other detected genera were present at lower proportions (< 5 %) and varied between the groups. The oral microbiota of the puppies in the litter was similar, but differed between litters and between groups. Based on these results, we can assume that the birth mode affects the oral microbiota of puppies.

Key words: microbial cultivation; oral bacteria; puppies

INTRODUCTION

It has long been believed that under normal conditions, intrauterine foetal development occurs in an aseptic environment and the colonization begins at birth. However, recent studies have shown that amniotic fluid is colonized by oral microorganisms in up to 70 % of pregnant women [15]. In addition, a similar composition of the microbial community was detected in samples of the maternal oral cavity, amniotic fluid, placenta, and neonatal oral cavity

[20]. To date, it has not been investigated whether canine amniotic fluid contains bacteria. However, genera *Staphylococcus, Streptococcus, Actinomyces, Arthrobacter, Cutibacterium, Bacillus, Kocuria, Fingoldia, Pasteurella, Moraxella, Escherichia* and *Pantoea* have been isolated from the placenta of puppies [22].

The mode of birth has an impact on the oral bacterial community of infants. Vaginally delivered infants exhibit a greater bacterial diversity compared to infants delivered by caesarean section [4, 11]. After birth, the newborn is exposed to a wide range of microbes from a variety of sources, including the environment in which it lives and maternal bacteria. The maternal microbiome is considered a key factor in the initial colonization of the neonatal microbiome [2]. Early colonization of the mucosa of mammalian host plays a pivotal role in the maturation of the host's immune system [23]. The composition of the oral microbiota in the first days of life appears to be a very important factor in achieving and maintaining good health in the following years [20].

There have been several studies which focused on the oral microbiology of babies [2, 20], kittens [17] or foals [6]. However, there has been no study focused on the oral microbiology of puppies that we know of which have used conventional culture or molecular methods.

The aim of this study was to investigate the microbial diversity of the oral cavity in healthy puppies and examine whether there are variations in the oral microbiota between puppies delivered vaginally and by caesarean section.

MATERIAL AND METHODS

Study population and swab collection

Nineteen puppies from 4 litters were enrolled in this study: Rottweiler (n = 7), Border Terrier (n = 5), Maltese (n = 4) and Czechoslovak Wolfdog (n = 2). The Rottweilers and the Malteses were born vaginally. The Border Terrier and the Czechoslovak Wolfdog were born by caesarean section. Oral swab samples were taken on the 7th day after birth at the Clinic of Small Animals, University of Veterinary Medicine and Pharmacy in Košice. Samples were collected for 15—20 seconds while rotating the swab (Amies Agar Gel Transport Swabs) throughout.

Ethical statement

All procedures involving animals followed the guidelines stated in the Guide for the Care and Use of Animals (Protocol number 3323/16-221/3) which was approved by the State Veterinary and Food Administration of the Slovak Republic and by the Ethics Commission of the University of Veterinary Medicine and Pharmacy (Košice, Slovakia). The animals were handled in a humane manner in accordance with the guidelines established by the relevant commission. All applicable international, national and institutional guidelines for the care and use of animals were followed.

Microbiological analysis of samples

The oral samples were inoculated onto Brain Heart Infusion agar (BHI; HiMedia, Mumbai, India), deMan-Rogosa-Sharpe agar (MRS; Carl Roth GmbH, Karlsruhe, Germany), M-Enterococcus agar (ME; Becton, Dickinson and Co., Le Pont de Claix, France), Mitis Salivarius agar (MSA; Sigma Aldrich, Steinheim, Germany); supplemented with 1 % potassium tellurite solution (Sigma Aldrich, Steinheim, Germany), and blood agar. The blood agar (BA) was prepared as Trypticase soy agar (Carl Roth GmbH, Karlsruhe, Germany), and supplemented with 5 % of sterile defibrinated ram's blood. BHI plates were cultured under aerobic conditions, ME, MSA and BA plates were cultured under aerobic and anaerobic conditions (BBL GasPak™ Plus, Becton, Dickinson and Co., Maryland, USA), and MRS plates were cultured under anaerobic conditions. The plates were incubated at 37 °C for 48 hours at aerobic and 72 hours at anaerobic cultivation. Colonies with different morphological characteristics such as growth form, shape, size and colour were selected and subsequently sub-cultured to obtain pure bacterial cultures. The purity of the culture was determined by staining according to Gram.

16S rRNA sequencing of isolates

Bacterial DNA was extracted from the individual isolates using a DNAzol® Direct (Molecular Research Centre Inc., Cincinnati, USA) according to the manufacturer's recommendations. The PCR was carried out in a thermal cycler (TProfesional Basic, Biometra GmbH, Göttingen, Germany) using the universal primers, 27F and 1492R, and OneTaq 2X Master Mix with Standard Buffer (New England Biolabs, Foster City, USA). The PCR mixtures were subjected to an initial denaturation phase of 5 min

at 94 °C, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 3 min and a final extension step at 72 °C for 10 min. The amplified PCR products were separated on 2 % agarose gel and sent for Sanger sequencing using primer 1492R (Microsynth, Wien, Austria). All sequencing results were analyzed using Geneious 8.0.5 program (Biomatters, Auckland, New Zealand) and compared to the NCBI GenBank database using the BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST/) to identify the isolates.

RESULTS

Across all samples, a total of 64 bacterial strains were obtained from the oral swab samples. The bacteria belonged to 3 phyla, namely: *Actinobacteria, Firmicutes* and *Proteobacteria*. In the first group of puppies born vaginally (VB), *Firmicutes* accounted for 55.81 % and *Proteobacteria* for 44.19 %. In the second group of puppies born by caesarean section (CS), *Firmicutes* accounted for 90.48 %, and both *Actinobacteria* and *Proteobacteria* for 4.76 %. In general, bacterial taxa belonged to 10 genera. Seven genera were detected in the VB group and six genera in the CS group. Genera *Staphylococcus* (30.23 % VB and 47.62 % CS) and *Enterococcus* (25.58 % VB and 33.33 % CS) were the most abundant in both groups. The representation of individual genera in both groups is shown in Fig. 1.

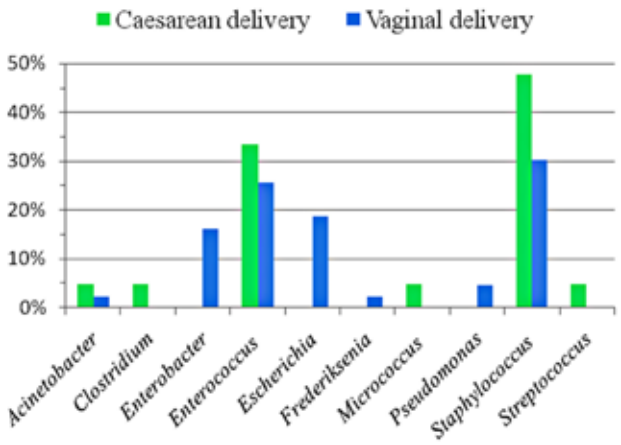

Fig. 1. The representation of bacterial genera in the oral cavity of puppies

Table 1. Prevalence of oral bacterial species in puppies in relation to delivery mode

Species (Phyllum)	Caesarean delivery (n = 7)	Vaginal delivery (n = 10)
Acinetobacter pittii **(Proteobacteria)**	1	1
Clostridium perfringens **(Firmicutes)**	1	0
Enterobacter ludwigii **(Proteobacteria)**	0	7
Enterococcus avium **(Firmicutes)**	0	2
Enterococcus canintestini **(Firmicutes)**	2	1
Enterococcus faecalis **(Firmicutes)**	5	8
Escherichia coli **(Proteobacteria)**	0	8
Frederiksenia canicola **(Proteobacteria)**	0	1
Micrococcus luteus **(Actinobacteria)**	1	0
Pseudomonas aeruginosa **(Proteobacteria)**	0	2
Staphylococcus epidermidis **(Firmicutes)**	1	3
Staphylococcus hominis **(Firmicutes)**	1	0
Staphylococcus haemolyticus **(Firmicutes)**	5	0
Staphylococcus pseudintermedius **(Firmicutes)**	3	10
Streptococcus fryi **(Firmicutes)**	1	0

The most common bacterial species in the CS group were *Enterococcus faecalis* and *Staphylococcus haemolyticus*, while in the VB group *Enterococcus faecalis, Escherichia coli* and *Staphylococcus pseudintermedius* were the most common. *Acinetobacter pittii, Enterococcus canintestini, Enterococcus faecalis, Staphylococcus epidermidis* and *Staphylococcus pseudintermedius* were present in both groups. All detected oral bacterial species in relation to the mode of delivery are shown in Table 1. The number of species isolated from individual puppies varied. On average, three species were isolated from puppies in the CS group. The number of isolated species was higher in puppies in the VB group, on average 4.3 species.

DISCUSSION

To our knowledge, this is the first study to focus on bacteria in the oral cavity of puppies. Previous studies of the canine oral microbiota have concentrated on the sampling of adult dogs. In this study, a total of 3 bacterial phyla, Actinobacteria, Firmicutes and Proteobacteria, which are commonly detected in the oral cavity of adult dogs [3, 13], were detected by culture-dependent methods. The phylum Actinobacteria was represented only by a species Micrococcus luteus isolated from one CS-born puppy, while the Firmicutes had a high proportion in all samples. Bacterial species belonging to the phylum Firmicutes accounted for more than 90 % of the microorganisms in the CS group.

The most frequently isolated bacteria were members of the genus *Staphylococcus*, specifically species: *S. epidermidis, S. hominis, S. haemolyticus* and *S. pseudintermedius*. In the study by Suepaul et al. [18], *S. epidermidis* and *S. haemolyticus* were isolated from canine and human mouths, while *S. hominis* were isolated only from humans and *S. pseudintermedius* was not detected. On the other hand, in the study by Sahin-Tóth et al. [14], *S. pseudintermedius* was commonly isolated from dogs from the mouth, nose and skin. *S. pseudintermedius* has been shown to be transmitted from the dam to puppies at birth and may persist in the offspring for a long time. However, intrauterine transmission is also plausible because this bacterium has also been isolated from samples of placenta and meconium of puppies born by caesarean section [22].

The second most numerous genus in this study was the genus *Enterococcus*. Enterococci are natural inhabitants of the mammalian gastrointestinal tract. Generally, enterococci are considered low pathogenic bacteria with the potential to improve health as a probiotic. *Enterococcus canintestini* and *Enterococcus faecalis* were isolated from faecal samples and vaginal mucosa of dogs [9, 10, 22]. Enterococci are considered in humans to be a transient component of the oral microbiota [8]. In the study by Isaiah et al. [5], enterococci were significantly present in the oral samples from patrol and narcotics detection dogs.

Large proportions of cultivable microbiota from the oral cavity of puppies in the VB group were represented by *Enterobacter* species and *Escherichia coli*. On the other hand, *Enterobacter* species and *Escherichia coli* were not detected in the CS group. *Enterobacter* species are part of the microbiota of the gastrointestinal tract of mammals, while other *Enterobacter* species may be present on skin, in water, certain foods, soil, and wastewater. Several *Enterobacter* species are responsible for causing many nosocomial infections [12]. *Enterobacter ludwigii*, isolated in this study, is a fermentative Gram-negative environmental species and an accidental human pathogen that belongs to the *Enterobacter cloacae* complex [16]. *Escherichia coli* along with *Lactobacillus, Pseudomonas, Staphylococcus* and *Streptococcus* are highly prevalent in the first several months of an infant's life, before tooth eruption [21]. Unclassified *Escherichia-Shigella* sp. was the most abundant saliva taxon of adult dogs in the study by Rupparell et al. [13].

Compared to adult dogs, genera such as *Actinomyces, Corynebacterium, Neisseria* or *Pasteurella*, commonly isolated from the oral cavity of adult dogs [3], were not isolated from the oral cavity of puppies. However, bacteria commonly isolated from the skin [19] or vaginal mucosa [22] of adult dogs were isolated. Potentially pathological species like *Clostridium perfringens* and *S. haemolyticus* were detected in the oral cavity of puppies in the CS group. *Acinetobacter pittii*, isolated from both groups, is an important nosocomial pathogen in humans and animals [1, 7].

CONCLUSIONS

The findings of this study provide new information about the oral microbiota in healthy puppies. The oral microbiota of puppies differs significantly from the oral microbiota of adult dogs. Oral microbiota also differs between puppies born vaginally and born by caesarean sec-

tion. The mode of birth seems to affect the bacterial colonization of the oral cavity of puppies in the first days of life. Further studies focusing on the oral microbiota of puppies, its development and the factors influencing it are necessary to identify physiological and pathological bacterial composition of oral microbiota in dogs.

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FELINE EOSINOPHILIC KERATITIS—A REVIEW

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ABSTRACT

Eosinophilic keratitis is a disease occurring in cats, horses and rabbits. Its clinical signs include blepharospasm, discharge, chemosis, conjunctival hyperaemia and the presence of corneal ulceration. The typical signs of the disease are white to pink plaques on the cornea accompanied with keratitis. The diagnosis of the disease has to be confirmed with cytology examination and the presence of eosinophils and mast cells in the specimen. Local application of corticosteroids and cyclosporine has good therapeutic effect and has been recommended in all affected species. In samples collected from cats, the presence of feline herpes virus DNA has been reported. Eosinophilic keratitis might be caused by an aberrant immune response or reaction to unknown allergic stimuli. The primary cause of the disease is currently unknown.

Key words: corneal plaque; cyclosporine; feline eosinophilic keratitis

INTRODUCTION

Feline eosinophilic keratitis (FEK), also known as proliferative keratitis or eosinophilic keratoconjunctivitis, or firstly described as corneal eosinophilic granuloma, is a chronic corneal disease [2, 10]. D e a n, M e u n i e r [4] suggested an alternative name for the disease as eosinophilic ocular surface disease as it affects not only the cornea but also the conjunctiva and sometimes the third eyelid. The clinical signs of the disease appear as an immune mediated response to unknown antigenic stimuli [22]. The disease usually occurs in cats [3, 6, 9, 12, 13, 14], horses [18] and rabbits [8]. The clinical signs can appear bilaterally but in most cases (66 % of cases) [14] and (80 % of cases) [22] unilateral appearance is observed. In some cases at the beginning, unilateral appearance can be observed while with progressing the eyes can be affected [22]. FEK is most often observed in middle aged, young, castrated male cats [12, 14] The most common affected breed is Domestic Short-haired cat (DSH). The analysis of 35 affected cats revealed that 30 of them were DSH, three were Domestic Long-haired, and one Siamese and one Maine Coon [22].

The clinical signs of FEK are usually typical changes of the cornea—inflammation with the white plaques, granulation and sometimes ulceration. The disease has a progressive character, in the initial stages the lateral or nasal corneal quadrants are affected. With the development of the disease, the entire cornea can be affected leading to vision impairment [11, 22]. The diagnosis is confirmed with cytological examination of the cornea. The basic recommended therapy in the case of FEK, is the topical application of dexamethasone, prednisolone and cyclosporine [3, 22].

There are few reports regarding the eosinophilic keratoconjunctivitis in rabbits. The clinical signs in this species included bilateral purulent discharge, dacryocystitis, conjunctivitis, and ulcerative keratitis, conjunctiva with hyperaemia, chemosis and typical white plaques on the bulbar and palpebral conjunctiva, as well as the eyelid margins. Cytology confirms the presence of macrophages, lymphocytes, plasma cells and polymorphonuclear cells containing intracytoplasmic eosinophilic granules. As reported in the literature; the treatment included ofloxacin eye drops and topical neomycin-polymyxin B-dexamethasone eye solution four times daily and artificial viscous tears. The improvement of the status was observed within one week. Dexamethasone eye drops twice daily were applied as continuation of the therapy while relapse has been observed in one case after 20 days of application with 0.2 % cyclosporine ointment. Despite these observations, cyclosporine is recommended in rabbits affected with eosinophilic keratoconjunctivitis. Similarly in cats, infectious diseases and environmental factors have been considered as a possible aetiology of eosinophilic keratoconjunctivitis in rabbits [8, 24, 25].

Similarly to cats and rabbits, in horses eosinophilic keratoconjunctivitis manifests as a raised band of 1 to 2 mm subepithelial plaques on the cornea. The additional clinical signs include blepharospasm, epiphora, yellow-to-white mucoid discharge, chemosis, conjunctival hyperemia, and corneal ulceration. The presence of eosinophils and segmented neutrophils, mast cells, plasma cells, and lymphocytes during cytology examination can be revealed. Histological examination of samples collected from the equine cornea showed the presence of degenerated collagen fibres infiltrated by eosinophils, neutrophils, lymphocytes, plasma cells, and macrophages. The therapy in horses is similar to that recommended for cats and rabbits. It has been reported that a 3 week treatment with corticosteroids caused complete resolution of the corneal changes [18, 26].

AETIOLOGY

The aetiology of the disease is uncertain [22]. It is presumed to be an immune-mediated disease or the reaction to unknown allergic stimuli. Analysis of this aberrant immune response suggest type I or IV hypersensitivity reaction in the case of FEK [16, 17]. It has been detected that Feline herpes virus type 1 (FHV-1) is present in corneal scraping in 76 % of the cats affected with FEK [13, 22]. The diagnostic tests performed in a study conducted on 33 affected cats included cytological examination of the cornea and conjunctiva and real-time quantitative polymerase chain reaction (PCR) for the detection of feline herpesvirus type 1 (FHV-1). It has been revealed that viral DNA was present in 54.4 % of the cats [4]. To compare, only 5.9 % positive for FHV-1 DNA samples has been detected in the samples collected from healthy feline corneas. Additionally, roles for mycoplasma and chlamydia in cases of feline conjunctivitis and keratitis have been described but none of the reports have pointed to it in eosinophilic/ proliferative keratitis [5].

DIAGNOSTICS

The typical clinical signs of FEK are single or multiple focal plaques on the corneal surface. The changes resemble granulation tissue and can be white, yellow or pink [11]. The inflammatory process of the disease involves blepharospasm with a mucoid sometimes purulent discharge, superficial vascularisation and oedema of the cornea. Additionally, conjunctivitis with severe chemosis with rarely cobblestone appearance can be observed [12, 14]. In some cases, the third eyelid can also be affected and eyelid margins depigmented [11]. Corneal ulceration is observed on an eye affected with the disease in 25 % of the affected cats. According to the reports, corneal defects are usually superficial and heal within 5 days of antibiotic therapy [15].

The diagnosis of the disease is based on clinical signs. It should always be confirmed with a cytological examination. In cytology, the samples collected from the cornea, the presence of eosinophils, mast cells and neutrophils are confirmed. Additionally, hyperplastic or/and dysplastic epithelial cells can be observed [11]. In a study conducted on 35 cats affected with FEK, 97.1 % had eosinophils present and 71.4 % mast cells in the corneal cytologic specimens;

Fig. 1. A—White corneal plaques in a cat with eosinophilic keratitis; B—The same eye as in A after 7 days of therapy with topical dexamethasone 3 times daily; C—The same eye as in A, B, after 21 days of therapy with topical dexamethasone 3 times daily

the cells typically absent in unaffected feline cornea. Therefore, the characteristic clinical signs—changes on the cornea and cytology results are pathognomonic in the case of feline eosinophilic (proliferative) keratitis. Lymphocytes, and plasma cells are less often present in specimens but they can also occur in the case of FEK [22]. FEK is reported to affect both corneal epithelium and stroma [16, 17].

Samples from superficial layers of the cornea contain squamous epithelial cells, cellular debris, including eosinophilic granules, and mast cells with lower numbers of intact eosinophils. According to Prase, Winston eosinophils and lymphoid cells are predominantly visible in deeper scrapings [17], (more eosinophils than mast cells). It has been also reported that mast cells are most frequent in scrapings that avoid the white surface exudate [19].

The differential diagnosis of FEK include: FHV-1, keratitis, keratomycosis, corneal neoplasia, acid fast granuloma and foreign body granuloma [12, 14].

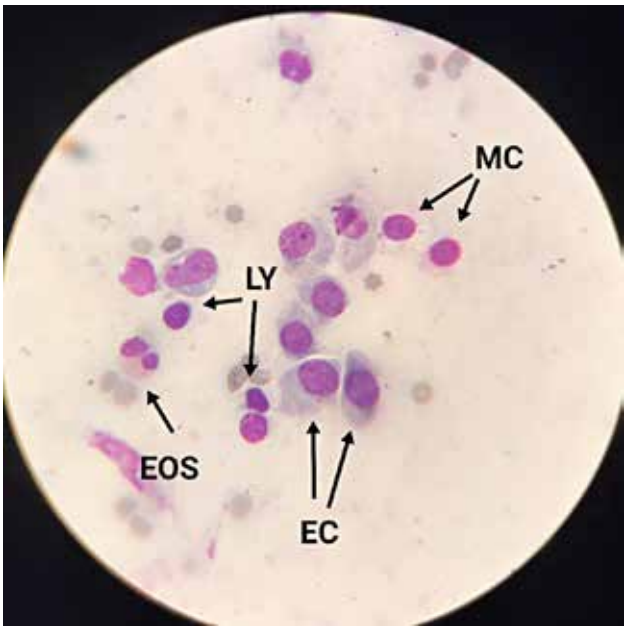

Fig. 2. Cytology sample from corneal lesion of affected cat

We can see different populations of cells, with the highest number of epithelial cells (EC), some with dark brown pigment granules. Mast cells (MC) with purple staining cytoplasmatic granules, eosinophil (EOS) with segmented nucleus and eosinophilic cytoplasmatic granules and small lymphocytes (LY) are also present

TREATMENT

Corticoids are the drugs of choice in the case of FEK. Traditionally, they are applied topically or systemically. Due to knowledge regarding the presence of FHV-1 DNA in cases of FEK, treatment using corticosteroids requires caution. Immunomodulatory drugs may develop reactivation of a virus from latency and a worsening of the status [11]. Good results were also observed in cases treated with ophthalmic antiviral agents or famciclovir, although in many cases additional immunomodulatory therapy was required. Additionally, in cases with higher risk of ulceration, it is recommended to use famciclovir with systemic treatment of corticosteroids while corticosteroids may lead to recrudescence FHV-1 that lead to dendritic ulceration [11, 14]. Oral administration of megestrol acetate was also recommended, although its side effects including adrenocortical suppression, *diabetes mellitus,* mammary hyperplasia, neoplasia and also behavioural changes, and hair loss makes it rarely a drug of choice in the case of FEK and patients should be always controlled during therapy [11].

Cyclosporine is an alternative treatment. It is recommended in cases with ulceration while cyclosporine does not affect re-epithelialization of the cornea and does not have cytotoxic effects [21]. However, the development of marginal blepharitis during the treatment of FEK with cyclosporine was reported in 1.5 % of cats [22]. Additionally, intolerance including irritation, chemosis and conjunctival hyperaemia were also reported in some of the cats treated with cyclosporine [1, 22]. Cyclosporine applied in commercially available ocular ointment in 0.2 % concentration had good therapeutic effects only in 2 from 5 cats, as the 3 developed ocular irritation [1]. The other study revealed improvement after treatment of the same ointment in one cat as well in 4 cats treated with 1 % cyclosporine in corn oil [20]. The analysis of 35 cats treated with 1.5 % cyclosporine in corn oil twice and three times daily for 5 months revealed good results in the vast majority of cases affected with FEK [22]. Other studies suggest also good tolerance in felines for 1—4 % cyclosporine in olive oil [7].

Analysis of safety and therapeutic effects of allogeneic feline adipose-derived mesenchymal stromal cells revealed also good therapeutic effects. The cells were implanted subconjunctivally around the ocular surface affected with lesions. The study was performed in five cats and in all cases the reduction of clinical signs were observed. During the therapy none of the animals had systemic or local complications. In this study improvement of the status was observed in the first 4 weeks, while corneal cytology was negative for eosinophils and mast cells after 2 months of treatment. Complete remission of the disease was observed in cats after 6 months [23].

In severe cases of FEK superficial keratectomy was recommended, although in most cases a topical treatment (prednisone, dexamethasone) will be satisfactory [15, 16]. Most cases have a good response to a treatment, although disease has to be controlled while recurrence has been observed in 64 % of the cats [12]. Feline eosinophilic keratitis is a disease that cannot be cured, but only controlled. It is recommended for long-term and in some cases, life-long treatment with a required low effective dose.

CONCLUSIONS

The feline eosinophilic keratoconjunctivitis is a common disease in ophthalmic practices. The typical signs of the disease need to be confirmed with cytology. The exact pathogenesis of this proliferative keratoconjunctivitis is still unknown but it is presumed to be the result of hypersensitivity reaction to an unknown stimuli. This hypothesis is supported by the response to immunomodulation therapy, however the disease requires further research.

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