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ANTIBIOTIC RESISTANCE OF *ESCHERICHIA COLI* ISOLATED FROM BROILER CHICKENS

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ABSTRACT

The purpose of this study was to detect the antibiotic resistance of forty-one *Escherichia coli* isolates from the intestinal contents of slaughtered broiler chickens using the disk diffusion method according to Kirby-Bauer. Mueller-Hinton agar plates were inoculated with 0.1 ml overnight broth cultures of individual *E. coli* isolates and the disks with the following concentrations of antibiotics were applied onto them: ampicillin (10 µg), cefotaxime (30 µg), gentamicin (10 µg), streptomycin (10 µg), azithromycin (15 µg), tetracycline (30 µg), ciprofloxacin (30 µg) and levofloxacin (3 µg). After the incubation at 37 °C for 16—18 hours, the inhibition zones were measured and interpreted in accordance with the Clinical and Laboratory Standard Institute (CLSI) zone diameter breakpoints. Almost all *E. coli* isolates showed resistance to tetracycline (92.68 %), most of them were resistant to gentamicin (75.61 %) and levofloxacine (70.73 %). Phenotypic resistance to tetracycline was further confirmed with the help of the Polymerase Chain Reaction (PCR) procedure focused on the presence of specific tet(A) and tet(B) genes. These genes were detected in all 41 *E. coli* isolates. On the contrary, *E. coli* isolates were highly susceptible to both azithromycin and streptomycin. In conclusion, the study highlighted the role of commensal *E. coli* bacteria isolated from the intestines of broiler chickens as an important reservoir of tetracycline resistance genes.

Key words: antibiotics; broilers; disk diffusion method; *Escherichia coli*; PCR

INTRODUCTION

Escherichia coli comprises a significant part of the normal microflora of all warm-blooded animals. This bacterium constitutes a major concern to the public health and food safety issues as it is more than just a harmless intestinal inhabitant; it can also be a highly versatile, and frequently also deadly pathogen.

The gastrointestinal tract of broiler chickens is inhabited by more than 900 bacterial species and this microbial

community consists of both commensal and pathogenic bacteria [1, 22]. Several different *E. coli* strains cause diverse intestinal and extra-intestinal diseases by means of virulence factors that affect a wide range of cellular processes [20]. However, in immunocompromised humans and other animals, even the normal non-pathogenic *E. coli* strains are capable of causing infection [6].

Antibiotics are used worldwide in food-producing animals for many reasons, including prevention of diseases, treatment of infections, growth promotion and increased production. In past decades, the inappropriate use of antibiotics in human and veterinary medicine has led to an increasing rate of antimicrobial resistance [13] and a rapid spread of drug-resistance among both pathogenic and commensal bacteria [14]. Therefore, the European authorities laid down general and specific principles of official controls on the products of animal origin intended for human consumption in order to ensure the compliance with feed and food laws, including animal health and animal welfare rules. At all stages of the food production chain, the food business operators must ensure that food products meet the requirements of food law and that those requirements are being adhered to in an effective way [17, 18].

Tetracyclines which are commonly used in poultry farming have been reported as one of the drugs against which bacteria are most resistant. Tetracycline resistance has been reported in poultry even without any previous administration of this antibiotic [2]. Due to frequent occurrence, *E. coli* strains resistant to tetracycline can be used as an indicator of antibiotic-resistant bacteria in poultry farming [8].

Therefore, our study was focused on the detection of tetracycline resistance genes in *E. coli* bacteria inhabiting the intestines of commercial broiler chickens.

MATERIALS AND METHODS

E. coli isolates were obtained from the intestinal contents of 18 broiler chickens (cross COBB 500) slaughtered at the age of 42 days in a private poultry slaughterhouse Hydina Slovensko s. r. o. in Košice (Slovakia). Immediately after evisceration, the intestines were transported to the Department of Food Hygiene and Technology of the University of Veterinary Medicine and Pharmacy in Košice, while being kept permanently at a refrigeration temperature. Microbiological testing had commenced as soon as the samples arrived at the laboratory. The appropriate decimal dilutions of the intestinal content in a sterile 0.85 % saline were spread in a volume of 0.1 ml on the surface of Endo Agar plates (HiMedia, India) and incubated at 37 °C for 24 hours. Colonies with typical appearance were further identified and confirmed using the biochemical test kit ENT 16 fp (Diagnostics Inc., Slovakia) where the reference strain *Escherichia coli* CCM 4225 (Czech Collection of Microorganisms, Czech Republic) was used as a positive control.

Table 1. Antibiotic susceptibility limits for Enterobacteriaceae [4]

Test/Report group	Antimicrobial agent	Disk content	Interpretive Categories and Zone Diameter Breakpoints (nearest whole mm)		
			S	I	R
Penicillin	Ampicillin	10 µg	≥ 17	14—16	≤ 13
Cephems	Cefotaxime	30 µg	≥ 26	23—25	≤ 22
Aminoglycosides	Gentamicin	10 µg	≥ 15	13—14	≤ 12
	Streptomycin	10 µg	≥ 15	12—14	≤ 11
Macrolides	Azithromycin	15 µg	≥ 13	–	≤ 12
Tetracycline	Tetracycline	30 µg	≥ 15	12—14	≤ 11
Fluoroquinolones	Ciprofloxacin	5 µg	≥ 21	16—20	≤ 15
	Levofloxacin	5 µg	≥ 17	14—16	≤ 13

S—susceptible; I—intermediately resistant; R—resistant

The susceptibility of *E. coli* isolates to eight antibiotics was tested by the Disk diffusion method according to Kirby-Bauer [4]. Bacterial suspensions adjusted to a 0.5 McFarland standard were spread in a volume of 0.1 ml on the surface of Mueller-Hinton agar plates (HiMedia, India) and the disks with the following concentrations of particular antibiotics (Oxoid, United Kingdom) were applied: ampicillin (10 µg/disk), cefotaxime (30 µg/disk), gentamicin (10 µg/disk), streptomycin (10 µg/disk), azithromycin (15 µg/disk), tetracycline (30 µg/disk), ciprofloxacin (30 µg/disk) and levofloxacin (3 µg/disk). The diameters of inhibition zones were measured after incubation at 3 °C for 16—18 hours. In accordance with CLSI zone diameter breakpoints (Table 1) the individual *E. coli* isolates were reported as susceptible (S), intermediate resistant (I), or resistant (R) to a particular antimicrobial agent [5].

The presence of tet(A) and tet(B) genes (577 bp and 634 bp) associated with resistance to tetracycline was determined by the Polymerase chain reaction and the set of primers shown in Table 2. The PCR mixture in a volume of 20 µl contained 2.5 µl of template DNA, 0.3 µl of each primer (ProScience Tech s. r. o., Slovakia), and 4.0 µl of HOT Firepol® Blend Master Mix (Solis BioDyne, Estonia). Amplification in the DNA thermo-cycler (Techne, United Kingdom) has started with an initial denaturation at 95 °C for 12 minutes and was followed by 25 cycles of denaturation at 95 °C for 20 seconds, annealing at 56 °C for one minute and elongation at 72 °C for 2 minutes. The process was completed with a final extension at 72 °C for 10 min. After electrophoresis in 1.5 % agarose gel stained with the GelRed™Nucleic Acid gel stain (Biotium Inc., USA), the amplicons were visualised by the UV transilluminator Mini Bis Pro® (DNR Bio-Imaging Systems Ltd., Israel). The 100 bp DNA ladder (Solis BioDyne, Estonia) was used as a size standard.

Table 2. Primers used for the detection of tetracycline resistance [16]

Resistance genes	Sequences	Size [bp]
***tet*(A)**	(F) GGT TCA CTC GAA CGA CGT CA (R) CTG TCC GAC AAG TTG CAT GA	577
***tet*(B)**	(F) CCT CAG CTT CTC AAC GCG TG (R) GCA CCT TGC TGA CTC TT	634

RESULTS

Forty-one *E. coli* isolates were obtained by inoculation of the chicken intestinal contents on the surface of Endo Agar selective-differential medium. These formed typical pink/red colonies with a metallic sheen. The identity of suspect isolates was further confirmed biochemically using the commercially distributed biochemical test kit ENT 16 fp.

Antimicrobial susceptibility testing

The results of the disk diffusion method in forty-one *E. coli* isolates are shown in Table 3. In this study, a high degree of resistance to almost all antibacterial agents tested was detected among *E. coli* isolates (Fig. 1). Resistance to tetracycline was observed most frequently (38 isolates), followed by that to gentamicin (31 isolates), levofloxacin (29 isolates) and ciprofloxacin (28 isolates). In opposite, resistances to azithromycin (3 isolates) and streptomycin (5 isolates) were the least frequent.

All of the *E. coli* isolates showed resistance to at least one of the eight antibiotics tested. Only one isolate was resistant to a single antibacterial substance (ampicillin). Resistance to two antibiotics was confirmed in 5 *E. coli* isolates. Among the remaining 38 isolates of *E. coli*, 9 were simultaneously resistant to three antibiotics, 12 isolates

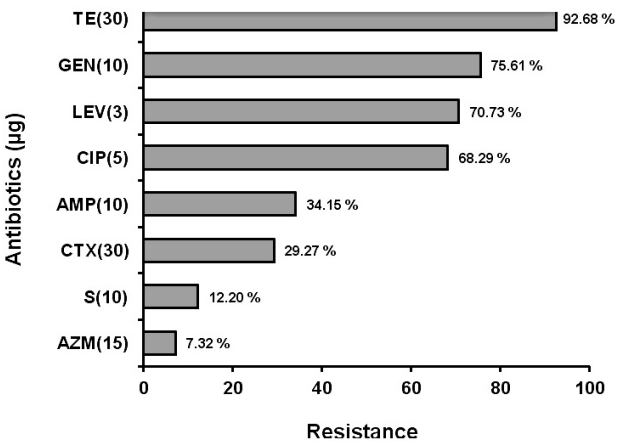

Fig. 1. Resistance of *E. coli* isolates to selected antibiotics

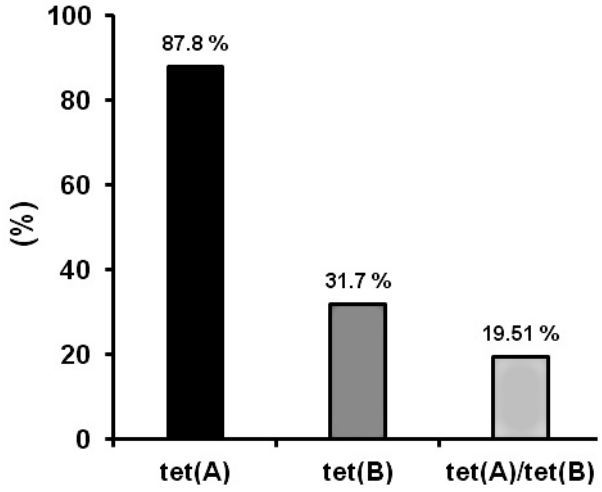

Fig. 2. Occurrence of tetracycline resistance genes in *E. coli* isolates

showed resistance to four antibiotics, 11 isolates were resistant to five antimicrobial agents and another 3 isolates even to 6 out of 8 antibiotics tested.

PCR detection of tetracycline resistance genes

As seen in Table 3, the resistance to tetracycline was phenotypically manifested in almost 93 % of the *E. coli* isolates. However, the PCR method has detected the presence of tetracycline resistance genes in all 41 isolates tested. Resistance to tetracycline was encoded by the tet(A) gene in 36 *E. coli* isolates, with the presence of tet(B) gene confirmed in 13 *E. coli* isolates. Eight isolates possessed both tetracycline resistances genes tested (Figs. 2, 3).

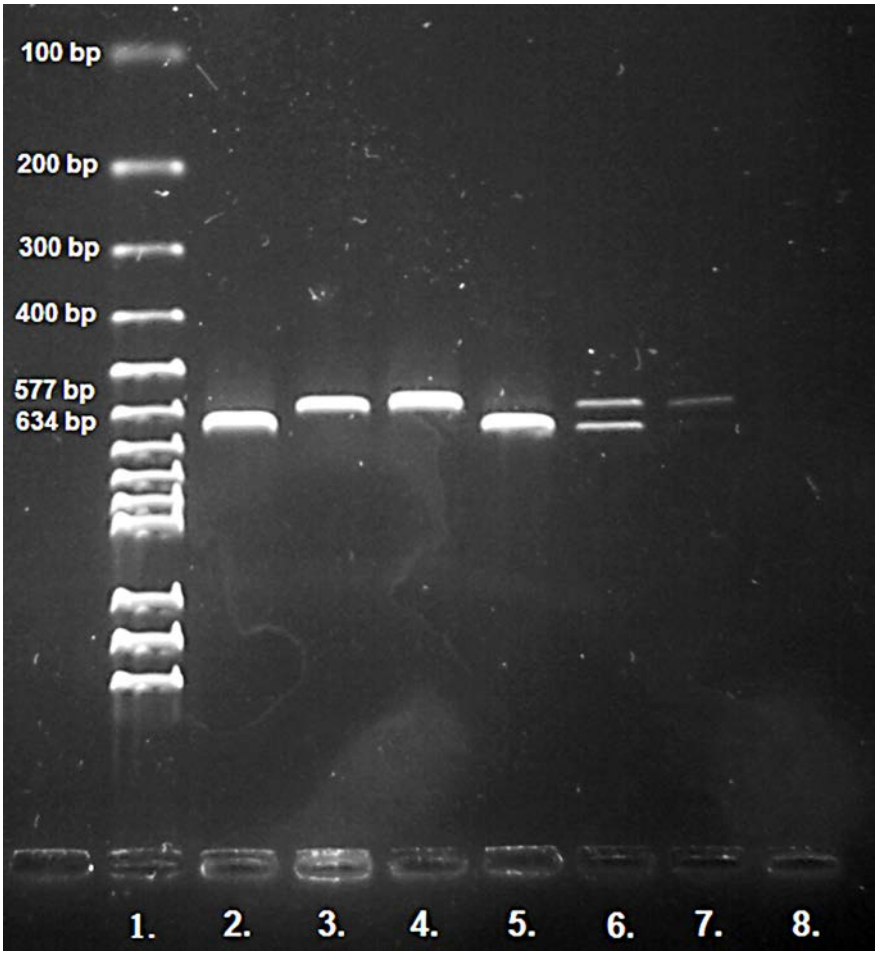

Fig. 3. Detection of tet(A) and tet(B) genes in *E. coli* isolates

Line 1: ladder (100 bp); Lines 2—7: *E. coli* isolates T9, T10, T11, T14, T15, T26;
Line 8: negative control; 577 bp: tet(A); 634 bp: tet(B)

Table 3. Evaluation of inhibition zone diameters (mm) for selected antibiotics in *E. coli* isolates

Isolate	AMP_{10}	CTX_{30}	AZM_{15}	TE_{30}	LEV_{3}	CIP_{5}	GEN_{10}	S_{10}
T1	6 (R)	29 (S)	6 (R)	8 (R)	9 (R)	10 (R)	20 (S)	6 (R)
T2	6 (R)	30 (S)	28 (S)	6 (R)	9 (R)	8 (R)	20 (S)	18 (S)
T3	6 (R)	30 (S)	25 (S)	9 (R)	13 (R)	12 (R)	19 (S)	15 (S)
T4	6 (R)	28 (S)	13 (S)	20 (S)	25 (S)	25 (S)	19 (S)	17 (S)
T7	6 (R)	28 (S)	20 (S)	12 (I)	12 (R)	12 (R)	20 (S)	14 (I)
T8	6 (R)	31 (S)	22 (S)	19 (S)	6 (R)	11 (R)	25 (S)	20 (S)
T9	6 (R)	27 (S)	29 (S)	9 (R)	6 (R)	6 (R)	20 (S)	7 (R)
T10	6 (R)	27 (S)	20 (S)	7 (R)	22 (S)	23 (S)	20 (S)	17 (S)
T11	6 (R)	29 (S)	28 (S)	8 (R)	9 (R)	11 (R)	22 (S)	6 (R)
T12	6 (R)	26 (S)	27 (S)	8 (R)	20 (S)	17 (I)	16 (S)	15 (S)
T13	15 (S)	13 (I)	22 (S)	10 (R)	11 (R)	15 (R)	6 (R)	30 (S)
T14	14 (R)	6 (R)	17 (S)	6 (R)	9 (R)	6 (R)	7 (R)	28 (S)
T15	15 (R)	13 (I)	24 (S)	9 (R)	10 (R)	7 (R)	7 (R)	25 (I)
T16	19 (S)	16 (S)	9 (R)	6 (R)	12 (R)	22 (S)	7 (R)	33 (S)
T17	9 (R)	6 (R)	25 (S)	7 (R)	8 (R)	7 (R)	9 (R)	30 (S)
T18	19 (S)	6 (R)	27 (S)	7 (R)	12 (R)	5 (R)	6 (R)	27 (S)
T26	20 (S)	10 (R)	25 (S)	10 (R)	10 (R)	7 (R)	6 (R)	30 (S)
T27	20 (S)	11 (R)	23 (S)	9 (R)	20 (S)	25 (S)	6 (R)	30 (S)
T28	20 (S)	11 (S)	23 (S)	10 (R)	12 (R)	10 (R)	9 (R)	30 (S)
T29	15 (S)	9 (R)	27 (S)	7 (R)	9 (R)	6 (R)	7 (R)	25 (I)
T30	12 (R)	8 (R)	25 (S)	7 (R)	14 (I)	14 (R)	6 (R)	27 (S)
T31	15 (S)	14 (I)	26 (S)	9 (R)	8 (R)	7 (R)	7 (R)	26 (S)
T32	16 (S)	15 (S)	24 (S)	9 (R)	12 (R)	14 (R)	8 (R)	28 (S)
T33	20 (S)	15 (S)	24 (S)	11 (R)	14 (R)	11 (R)	7 (R)	25 (I)
T34	19 (S)	13 (I)	7 (R)	9 (R)	13 (R)	13 (R)	7 (R)	26 (S)
T35	14 (I)	6 (R)	23 (S)	7 (R)	10 (R)	9 (R)	7 (R)	26 (S)
T36	16 (S)	15 (S)	21 (S)	8 (R)	13 (R)	13 (R)	7 (R)	26 (S)
T49	15 (S)	7 (R)	24 (S)	8 (R)	18 (S)	20 (I)	6 (R)	28 (S)
T52	16 (S)	15 (S)	22 (S)	7 (R)	20 (S)	22 (S)	6 (R)	26 (S)
T53	17 (S)	19 (S)	27 (S)	7 (R)	12 (R)	17 (I)	6 (R)	28 (S)
T61	17 (S)	6 (R)	29 (S)	8 (R)	10 (R)	6 (R)	6 (R)	27 (S)
T62	15 (S)	14 (I)	25 (S)	10 (R)	15 (I)	13 (R)	6 (R)	27 (S)
T63	15 (S)	11 (R)	25 (S)	6 (R)	20 (S)	22 (S)	6 (R)	25 (I)
T64	16 (S)	11 (R)	23 (S)	7 (R)	10 (R)	7 (R)	7 (R)	27 (S)
T65	17 (S)	15 (S)	23 (S)	6 (R)	24 (S)	25 (S)	6 (R)	27 (S)
T66	15 (S)	15 (S)	18 (S)	11 (R)	12 (R)	14 (I)	7 (R)	30 (S)
T67	14 (S)	14 (I)	27 (S)	7 (R)	14 (I)	14 (I)	7 (R)	19 (R)
T68	15 (S)	15 (S)	23 (S)	7 (R)	6 (R)	10 (R)	6 (R)	25 (I)
T69	14 (I)	13 (I)	24 (S)	7 (R)	8 (R)	7 (R)	10 (R)	26 (S)
T70	17 (S)	17 (S)	24 (S)	6 (R)	21 (S)	19 (I)	7 (R)	25 (I)
T72	17 (S)	15 (S)	20 (S)	9 (R)	12 (R)	7 (R)	6 (R)	10 (R)

S—susceptible; I—intermediately resistant; R—resistant

DISCUSSION

Bacteria *E. coli* frequently contaminate food-producing animals as well as foods of animal origin. Some strains supple a part of the common human and other animal intestinal microflora, while others may cause diseases. Avian *E. coli* strains are opportunistic pathogens that are capable of extra-intestinal infection after the exposure of birds to various stressors [21].

Nowadays, antimicrobial resistance is a problem of global public health and food security. This phenomenon can spread globally among microbial species and genera and the mechanisms are not fully understood. Currently, the food chain is seen as one of the most important pathways of spreading antimicrobial resistance [15]. The major factor selecting for antimicrobial resistance in bacteria is antibiotic use, followed by crowding and poor sanitation [7]. These three factors are typical of intensive poultry farming and explain the high prevalence and degree of resistance in faecal *E. coli* of poultry in this particular study and others.

In this study, most bacteria isolated from the intestinal contents of broiler chickens showed a multi-resistant phenotype. The prevalence of tetracycline, gentamicin and ciproflaxin resistance was noticeably high, indicating that intestinal *E. coli* could serve as a reservoir of antimicrobial resistance genes. Almost 93 % of *E. coli* isolates showed a phenotype of tetracycline resistance. However, the presence of tet(A) and/or tet (B) genes were detected in all 41 isolates. Tetracycline resistance genes tet(A) and tet(B) detected in this study were also found in resistant *E. coli* isolates of human and other animal origin in other studies. Soufi et al. [20] reported high rates of antimicrobial resistance among *E. coli* isolates, and the values were similar to the ones in our study. These findings might be linked to the excessive use of sulphonamides, tetracyclines and penicillins in food-producing animals that can result in the selection and transmission of antimicrobial resistance [6]. A high prevalence of tetracycline resistant *E. coli* isolated from broiler chickens was also reported by Sengelov et al. [19]. The authors detected tet(A) in 41.2 % and tet(B) in 52.9 % of the isolates from healthy broilers. In isolates originated from diseased broilers, tet(A) was present in 72.2 % and tet(B) in 27.8 % of the strains. The results of Momtaz [12] confirmed that among 57 *E. coli* isolates from chicken meat samples, resistance to tetracycline had occurred most frequently (91.2 %) and was followed by the resistance to sulfamethoxazol (45.6 %), chloramphenicol and trimethoprim (29.8 %). The antimicrobial resistance profile of *E. coli* strains from broilers of West Azerbaijan province confirmed the presence of tetracycline resistance genes in 54.5 % of the isolates. Among them, 47.7 % were positive for tet(A), 9 % for tet(B) and 2.3 % for both of the above mentioned tetracycline resistance genes [11]. On the contrary, Zibandeh et al. [24] reported the tet(A) to be the only tetracycline resistance gene detected in 72.5 % of *E. coli* isolates taken from the chickens on the day before slaughter.

Tetracyclines are broad spectrum antibiotics used in all food-producing animals (including poultry) because they are cheap and easily available. Therefore, the widespread use of tetracyclines often leads to resistance [3] not only among pathogenic, but also commensal intestinal bacteria, such as *E. coli* [8], resulting in the transmission of resistant bacterial strains from poultry to humans via the food chain [9]. In *E. coli*, the genes tet(A), tet(B), tet(C), tet(D), and tet(E) are associated with an efflux mechanism and make an important part of the tetracycline resistance [3].

It is a well-known fact that integrons play an important role in the dissemination of antimicrobial resistance among Gram-negative bacteria. These genetic structures are able to capture, excise and express genes, frequently included in mobile elements such as plasmids [6]. Therefore, molecular methods, and especially polymerase chain reaction, have been widely used to study antimicrobial resistance genes. As reported by Soufi et al. [20], most of the integrons were detected in food isolates. Marchant et al. [10] noted the correlation between the presence of integrons and resistance to tetracycline in *E. coli* isolates from healthy broiler chickens. The authors found integrons in 49 % of the chicken isolates belonging to the oldest (1999) and the latest (2006) available Spanish surveillance programs, while resistance to tetracycline was determined in 94 % of integron-positive isolates.

However, it should be taken into account that the co-selection of multi-resistant bacteria by the use of different antimicrobial agents, for which resistance genes are associated in the same microorganism, could also occur [23]. Further studies should be done to recognize the main reasons of how commensal non-pathogenic *E. coli* ends up in the wrong place and start acting as pathogenic, and causing harm to the organism.

This study highlighted the role of commensal *E. coli* bacteria isolated from broiler chickens as an important reservoir of antimicrobial resistance genes. During the processing, these bacteria can easily be ingested, enter the human intestines, proliferate and render the person a carrier of these resistant microorganisms. Ultimately, the occurrence of resistant bacteria results in poor human medicine practice, causing a big impact on human health.

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BROAD SPECTRUM DETECTION OF ANTIBIOTIC RESIDUES IN POULTRY MEAT BY A MULTI-PLATE ASSAY

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ABSTRACT

The objective of this study was to use the "Screening test for antibiotic residues" (STAR) as a broad-spectrum detection method for antibiotic residues in poultry meat. The STAR method is a microbiological inhibition assay (a five plate test) where the development of inhibition zones (IZs) indicates the presence of antibiotic residues in meat samples. By using the STAR method, in a total of 13 poultry products providing 18 meat samples (14 muscle and 4 skin) and 18 corresponding juice samples, 11 out of the 18 samples were positive for containing antibiotic residues. Based on muscle alone (which is the matrix validated for use in the STAR method), 6 of the 14 muscle samples were positive for antibiotic residues. The STAR method as a screening technique proves advantageous as it is relatively easy to perform and of a low cost. Furthermore, the STAR method not only indicates the presence or absence of antibacterial substances, but simultaneously, a positive sample gives an indication of the antibiotic family present due to the use of five different bacterial test organisms. Families of antibiotics pre-identified due to positive samples in the results of this study include aminoglycosides (one out of 18), beta-lactams and sulphonamides (6 out of 18), and macrolides (5 out of 18). Such pre-identification of the antimicrobial families allows for a targeted confirmatory analysis. However, one could argue that the STAR method is laborious and time consuming. Overall, given the potential for false positive/negative results, further confirmatory method analysis of the samples must be performed to ensure that the results and conclusions drawn here are true.

Key words: antibiotics; meat; residues; screening

INTRODUCTION

Throughout the world, there is an emerging public health crisis and a growing concern regarding the potential for the development of antimicrobial resistance (AMR) that can occur as a result of the selective pressure and dissemination of resistance caused by unscrupulous misuse and inappropriate antimicrobial usage [1, 9, 13, 19]. Over time,

the restriction of antibiotic usage has been encouraged, if not enacted through legislation, in an attempt to prevent such AMR development due to non-rational veterinary antimicrobial use [10]. However, despite this, by 2030, the global use of antimicrobials in food animal production is expected to increase by 67 % compared to the 2010 usage levels [18, 19].

Antimicrobial usage within the veterinary industry can result in the presence of antimicrobial residues in food of animal origin [15]. Such presence of antimicrobial residues can result in direct toxicity or indirectly in AMR. Antimicrobial residue levels in the edible tissues of livestock, including poultry, should be monitored via detection methods (screening and subsequent confirmation) and assessed regularly to ensure that the food of animal origin is safe and wholesome for consumers and does not contain antimicrobial residues at levels which exceed the established Maximum Residue Limits (MRLs) as defined by legislation [4, 13].

The purpose of screening methods is to rapidly identify a desired analyte (antibiotic residues in this instance) where present, using ideally a simple (meaning requiring minimal resources and able to be performed by an unskilled personnel) and high throughput method [2]. Where a screening method results in a positive result for a sample, a confirmation is performed to determine if the sample is indeed positive (for antibiotic residues), identify the substance(s) present; and, in relation to antibiotic residues present in the meat, determine if these residues are at levels above the MRL which would result in condemnation of the meat product [8]. Plate tests are microbiological inhibition assays used as the main screening method for antibiotics in slaughter animals in Europe [15]. Plate tests rely on the principle that when antimicrobial (antibiotic) residue-containing samples are applied, the growth of the bacteria will be impeded resulting in the formation of inhibition zones (IZs) as a result of diffusion of the antibiotic from the sample [2, 15].

This study concerned the use of the STAR method as a broad spectrum multi-plate assay method for the detection of antibiotic residues in poultry meat. By its completion we aimed to determine whether antibiotic residues were present in the samples and also to evaluate the STAR method as a screening method for a broad spectrum detection of antibiotic residues. We also wanted to compare and draw conclusions from the results given by the use of different matrices, despite the STAR method being validated only for use in muscle (and milk).

MATERIALS AND METHODS

Sample material

A total of 13 poultry products from the UK and Ireland were used for residues analysis. The 18 samples with sample numbers 1 and 6—9 being thigh; sample numbers 3—5, 10—12 and 15 being breast; sample number 2 being a chicken portion; sample number 13 being a wing; and sample numbers 14, 16—18 being skin which was used as a different matrix for 4 products in addition to muscle (namely sample numbers 15, 8, 7 and 6, respectively). For one product, the muscle from 2 different locations (breast—sample number 15; wing—sample number 13) were used. For all samples, in addition to the skin or muscle matrix used, also the juices were collected for testing.

Sample analysis

The study used the STAR method in the manner prescribed by the STAR protocol of Gaudin and Fuselier [5]. The test organisms used were: *Bacillus stearothermophilus* ATCC 10149, grown at pH 7.4 (with the addition of Trimethoprim at a 1 % concentration equalling 0.005 μg. ml^{-1} agar medium), *Bacillus cereus* ATCC 11778, grown at pH 6, *Bacillus subtilis* B. G. A., grown at pH 7.2, *Escherichia coli* ATCC 11303, grown at pH 8, and *Kocuria rhizophila* ATCC 9341 (formerly *Micrococcus luteus*), grown at pH 8. Prior to use, the meat samples were stored at –18 °C (deep-freeze freezer temperature) and the removal of samples for analysis from the freezer occurred to allow slight defrosting prior to sample preparation. For each muscle sample, a sterile cork borer of 8 mm diameter was used to remove a cylindrical plug measuring approximately 2 cm in length (and 8 mm in diameter). Then the muscle sample was gently pushed out from the borer (using forceps) and cut into slices of approximately 2 mm thick, using a sterile scalpel. Where the skin was attainable, it was removed from the corresponding muscle via manual manipulation and scissors were used to cut a small section from the skin similar in size and shape to the muscle slices taken. The prepared tissue (muscle or skin) slices were transferred to the test plates using sterile forceps. The juice was obtained from the skin and muscle samples via thawing the tissue samples

(placed in 15 ml capped plastic graduated cylinder centrifuge tubes with the cap loosened in the test tube racks) using a microwave oven set on the defrost setting. For juices, antibiotic test paper discs (diameter of 9 mm from Albert Lab Science, Germany) were used to soak up approximately 30 µl from the juice obtained for each sample and then placed onto the test plates using sterile forceps. Onto each plate, 3 tissue (muscle or skin) slices were placed as well as the juice-soaked test paper discs corresponding to the same product and type of sample from which these slices were obtained; resulting in 6 samples per plate. Testing occurred in duplicate. Placement was aimed to be approximately 1 cm away from the rim of the plate and other samples. The prepared plates inoculated with test bacteria and with samples were incubated as follows: *Bacillus stearothermophilus* incubated at 55 °C for 15 hours, *Bacillus cereus* incubated at 30 °C for 18 hours, *Bacillus subtilis* incubated at 30 °C for 18 hours, *Escherichia coli* incubated at 37 °C for 24 hours, and *Kocuria varians* incubated at 37 °C for 24 hours.

Evaluation of results

For each plate, individual tissue samples (skin or meat) and juice-soaked test paper discs were visually analysed for the presence or absence of IZs. The presence of an IZ indicated the diffusion of antimicrobial substances present within the sample/juice for which the test organism inoculated on the test plate was sensitive; resulting in the inhibition of test organism growth creating the IZ seen. Where the clear IZs were present, the zones were measured using a digital caliper (with a precision of 0.01 mm) at 3 points from the boundary of the tissue sample or the test paper disc to the outer limits of the IZ. The measurements were recorded and a mathematical average was taken to determine one value (from 3) for each IZ where present. Samples were deemed positive where an IZ equal to or more than a width of 2 mm was found on the test plates inoculated with four of the test organisms: *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli* and *Kocuria rhizophila*. For the test plate inoculated with *Bacillus stearothermophilus*, samples were deemed positive where an IZ equal to or more than a width of 4 mm was found. According to the protocol of Gaudin and Fuselier [5], if at least one plate out of the 5 plates containing different test bacteria was positive, the sample was considered overall positive for containing antibacterial residues. Positive (quality) control plates inoculated with test organisms were validated for systematic respect of operating conditions using standard solutions containing reference antibiotics.

RESULTS AND DISCUSSION

Based on all matrices used, 11 out of the 18 samples were positive for containing antibiotic residues. Based on the muscle as the matrix alone (which is the matrix validated for use in the STAR method), 6 of the 14 muscle samples were positive for containing antibiotic residues. Based on additional matrices not validated for use within the STAR method, 3 of the 4 skin samples and 3 of the 18 juice samples (juice as obtained from meat samples/muscle and skin) were positive for containing antibiotic residues. Note that no IZs were produced when *Bacillus cereus* was the organism, nor when meat was the matrix used when *Bacillus subtilis* was the organism, and nor where juice was the matrix used when *Escherichia coli* was the organism. Table 1 shows a summary of the overall results for the samples using the STAR method. Table 2 gives the results of the STAR method based on the mean inhibition zone measured (in mm) with standard deviation (SD) for all organisms and all plates. Figures 1—5 present the plates for sample numbers 1—6 for each individual organism (*Bacillus stearothermophilus*, *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli* and *Kocuria rhizophila*, respectively).

According to the plates for *Bacillus stearothermophilus*, based on meat (muscle and skin) as the matrix, IZs were produced for all samples. Furthermore, based on juice as the matrix for the *B. stearothermophilus* plates, IZs were produced for 5 of the 18 samples (sample numbers 3, 4, 6, 8 and 9); with positive results given for 3 of the 14 muscle samples (sample numbers 1, 6, and 13) and 3 of the 4 skin samples (sample numbers 14, 16 and 17). For the *B. stearothermophilus* plates, based on SD values and given the spread of data, one could argue that sample numbers 6 and 14, where muscle and skin was used as the matrix respectively, could be false positives; whilst sample number 18, where skin was used as the matrix, could be a false negative. According to the plates for *Bacillus cereus*, no IZs were produced for any of the samples on any of the plates. According to the plates for *Bacillus subtilis*, an IZ of a size greater than 2 mm indicating a positive result was produced for sample number 3 with juice as the matrix. No other IZs were produced for the *B. subtilis* plates. According to

Table 1. Summary table giving the overall results for the samples using the STAR method

Organism →		*Bacillus stearothermophilus*		*Bacillus cereus*		*Bacillus subtilis*		*Escherichia coli*		*Kocuria rhizophila*	
Matrix →	Overall Result	Juice	Meat	Juice	Meat	Juice	Meat	Juice	Meat	Juice	Meat
Sample no. ↓											
1 Thigh	Positive	–	+	–	–	–	–	–	–	–	–
2 Portion	Positive	–	–	–	–	–	–	–	–	+	–
3 Breast	Positive	–	–	–	–	+	–	–	–	–	–
4 Breast	Positive	–	–	–	–	–	–	–	–	+	–
5 Breast	Negative	–	–	–	–	–	–	–	–	–	–
6 Thigh	Positive	–	+	–	–	–	–	–	–	–	–
7 Thigh	Negative	–	–	–	–	–	–	–	–	–	–
8 Thigh	Negative	–	–	–	–	–	–	–	–	–	–
9 Thigh	Negative	–	–	–	–	–	–	–	–	–	–
10 Breast	Negative	–	–	–	–	–	–	–	–	–	–
11 Breast	Positive	–	–	–	–	–	–	–	–	–	+
12 Breast	Negative	–	–	–	–	–	–	–	–	–	–
13 Wing	Positive	–	+	–	–	–	–	–	–	–	+
14 Skin	Positive	–	+	–	–	–	–	–	–	–	–
15 Breast	Positive	–	–	–	–	–	–	–	–	–	+
16 Skin	Positive	–	+	–	–	–	–	–	–	–	–
17 Skin	Positive	–	+	–	–	–	–	–	–	–	–
18 Skin	Negative	–	–	–	–	–	–	–	–	–	–

the plates for *Escherichia coli*, based only on muscle as the matrix, IZs were produced for 4 of the 14 samples (sample numbers 2, 4, 5 and 6). None of these IZs produced were of a size great enough to result in a positive result; and no other IZs were produced for the *E. coli* plates. According to the *Kocuria rhizophila* plates, based on muscle as the matrix, IZs were produced for 10 of the 14 samples (sample numbers 2—6, 10—13 and 15). Furthermore, based on juice as the matrix for the *K. rhizophila* plates, IZs were produced for 8 of the 18 samples (sample numbers 1—6, 8 and 15). Based on skin as the matrix for the *K. rhizophila* plates, an IZ was produced for one of the 4 samples (sample number 14). For the *K. rhizophila* plates, positive results were given for 3 muscle samples (sample numbers 11, 13 and 15) and 2 juice samples (sample number 2 and 4). For the *K. rhizophila* plates, based on SD values and given the spread of data, one could argue that sample numbers 2 (juice), 11 (muscle) and 13 (muscle) could be false positives; whilst sample numbers 1 and 15, where juice was used as the matrix, could be false negatives.

The STAR method detected the presence of inhibitory substances and so gave positive results for some samples; and in doing so, it detected antibiotics from the families including aminoglycosides (indicated by the *B. subtilis* plates), β-lactams and sulphonamides (indicated by the *B. stearothermophilus* plates), and macrolides (indicated by the *K. rhizophila* plates). The STAR method is validated for use only in the case that muscle samples and milk are the matrix in accordance with the European Decision 2002/657/EC [3] and with an internal guideline of validation as defined by the authors of the validation paper [6, 7]. As such, the positive results given by juice samples

Table 2. Results of screening for the presence of antibiotic residues in samples using the STAR method for all organisms and all plates

Organism	*Bacillus stearothermophilus*		*Bacillus cereus*		*Bacillus subtilis*		*Escherichia coli*		*Kocuria varians*	
Matrix →	Juice	Meat	Juice	Meat	Juice	Meat	Juice	Meat	Juice	Meat
Sample no. ↓	Mean IZ ± SD	Mean IZ ± SD	Mean IZ ± SD	Mean IZ ± SD	Mean IZ ± SD	Mean IZ ± SD	Mean IZ ± SD	Mean IZ ± SD	Mean IZ ± SD	Mean IZ ± SD
1 Thigh	–	**4.01** ± 0.51	–	–	–	–	–	–	1.87 ± 0.95	–
2 Portion	–	1.74 ± 0.99	–	–	–	–	–	0.69 ± 0.19	**2.33** ± 0.34	1.04 ± 0.11
3 Breast	0.84 ± 0.35	0.99 ± 0.44	–	–	**4.38** ± 0.21	–	–	–	0.88 ± 0.58	1.03 ± 0.25
4 Breast	1.35 ± 0.29	3.65 ± 0.34	–	–	–	–	–	0.53 ± 0.02	**2.49** ± 0.35	1.43 ± 0.30
5 Breast	–	1.29 ± 0.12	–	–	–	–	–	0.85 ± 0.20	1.42 ± 0.10	1.44 ± 0.44
6 Thigh	1.95 ± 0.26	**4.42** ± 0.72	–	–	–	–	–	0.60 ± 0.03	1.08 ± 0.50	1.04 ± 0.20
7 Thigh	–	2.15 ± 0.16	–	–	–	–	–	–	–	–
8 Thigh	1.16 ± 0.82	3.28 ± 0.03	–	–	–	–	–	–	0.60 ± 0.28	–
9 Thigh	0.55 ± 0.16	3.46 ± 0.12	–	–	–	–	–	–	–	–
10 Breast	–	2.23 ± 0.40	–	–	–	–	–	–	–	1.43 ± 0.47
11 Breast	–	2.41 ± 0.22	–	–	–	–	–	–	–	**2.20** ± 0.44
12 Breast	–	1.26 ± 0.12	–	–	–	–	–	–	–	0.64 ± 0.15
13 Wing	–	**5.34** ± 0.68	–	–	–	–	–	–	–	**2.39** ± 0.90
14 Skin	–	**4.31** ± 1.19	–	–	–	–	–	–	–	1.47 ± 0.24
15 Breast	–	2.91 ± 0.52	–	–	–	–	–	–	1.67 ± 0.44	**3.61** ± 0.27
16 Skin	–	**6.81** ± 1.18	–	–	–	–	–	–	–	–
17 Skin	–	**4.40** ± 0.11	–	–	–	–	–	–	–	–
18 Skin	–	3.86 ± 1.14	–	–	–	–	–	–	–	–

Mean IZ—Average Inhibition Zone (mm); SD—standard deviation; Bold numerals indicate a positive result

and skin samples are questionable; especially whereas it is known that the matrix used can impact upon the results given [14, 17]. The results show evident of a discrepancy between whether an IZ is produced and whether an IZ produced yields of a positive result based upon the type of matrix used when comparing muscle vs. juice vs. skin. Also, given the SD and the spread of data, false negative and false positive results are possible; with a false negative being of a more significant concern given where a negative result is produced, means no further testing is performed.

Given the use of calipers for the measurement of IZs associated with antibiotic residue presence, the STAR method could be defined as a semi-quantitative method used as a qualitative screening assessment; where the diameter of the IZ produced theoretically correlates and so is directly proportional to the level/concentration of antibiotic residues found within a sample [11, 12, 16]. Note that there is some subjectivity in relation to determining at which points to measure on an IZ and also the respective distance of the IZ. This subjectivity is limited given that 3 points are measured and then the average is taken to give the IZ size; however, this could prove significant in relation to the SD in cases where the results could be deemed as false positives or false negatives.

The STAR method proved of value as a screening method due to its relative ease to perform, low cost, robustness and ability to detect a wide range of antibiotics at a level of satisfactory sensitivity. However, the STAR method proved laborious and time consuming. Furthermore, the STAR

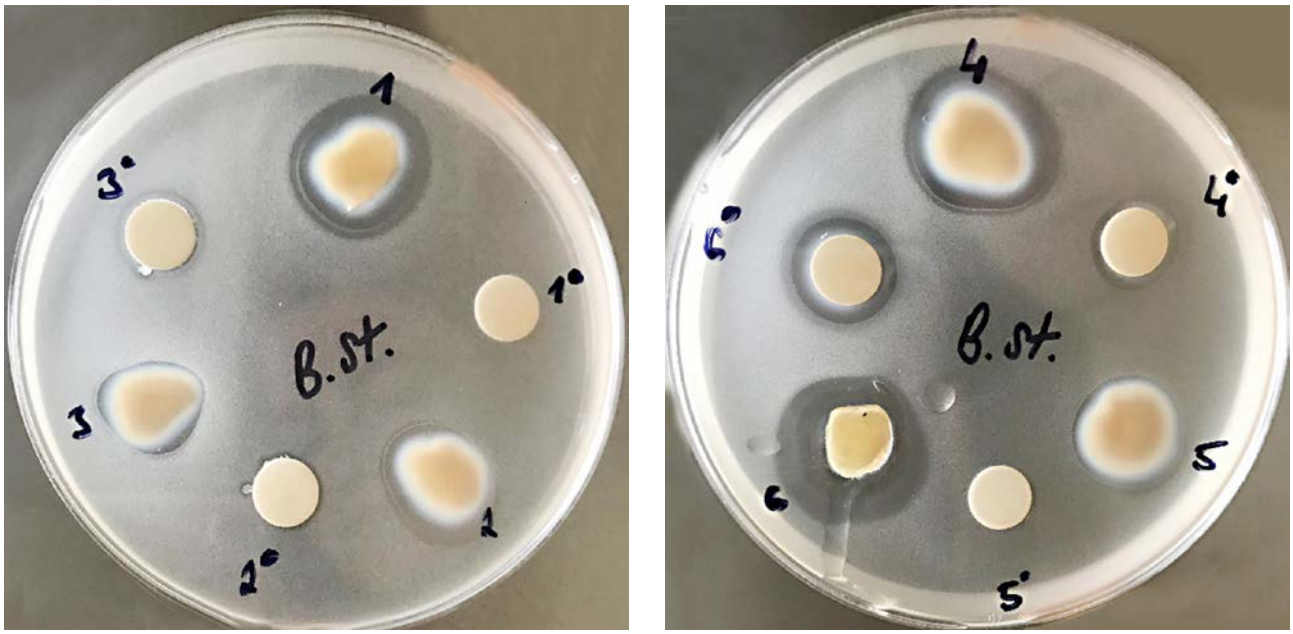

Fig. 1. Images showing the *Bacillus stearothermophilus* plates post-incubation for sample numbers 1—6 where IZs are present for all meat samples and for samples 3•, 4• and 6• where juice was the matrix

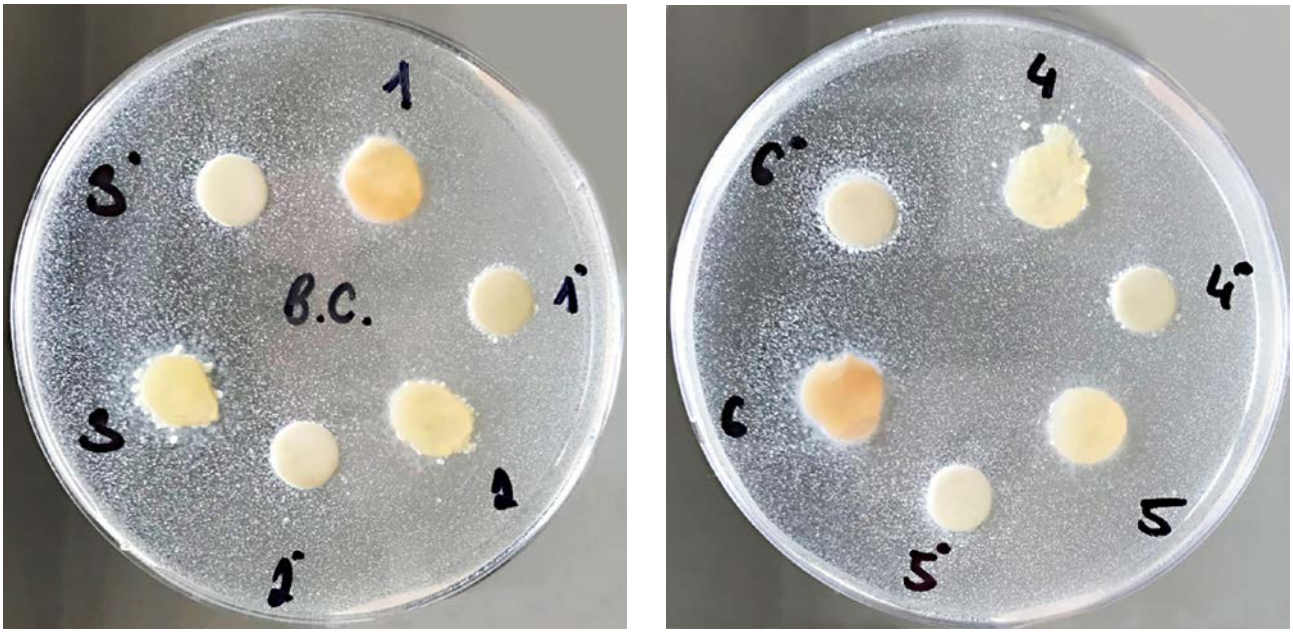

Fig. 2. Images showing *Bacillus cereus* plates post-incubation for sample numbers 1—6 (no IZs present)

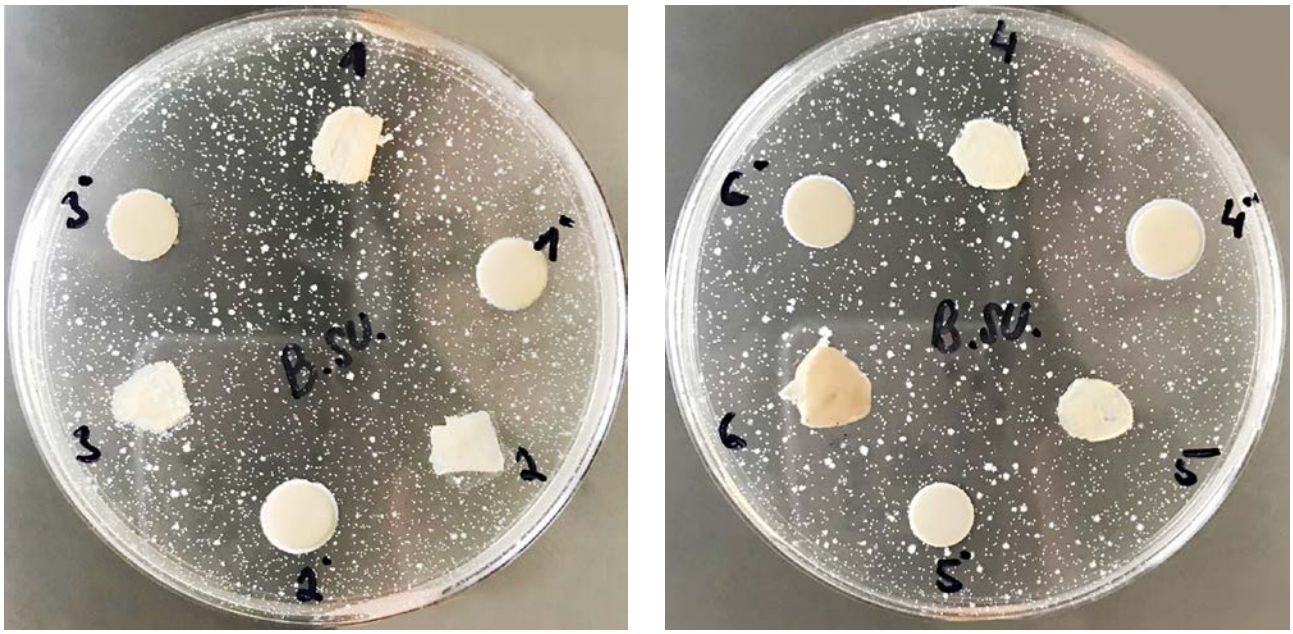

Fig. 3. Images showing the *Bacillus subtilis* plates post-incubation for sample numbers 1—6 where no IZs are present except for sample number 3• where juice was used as the matrix

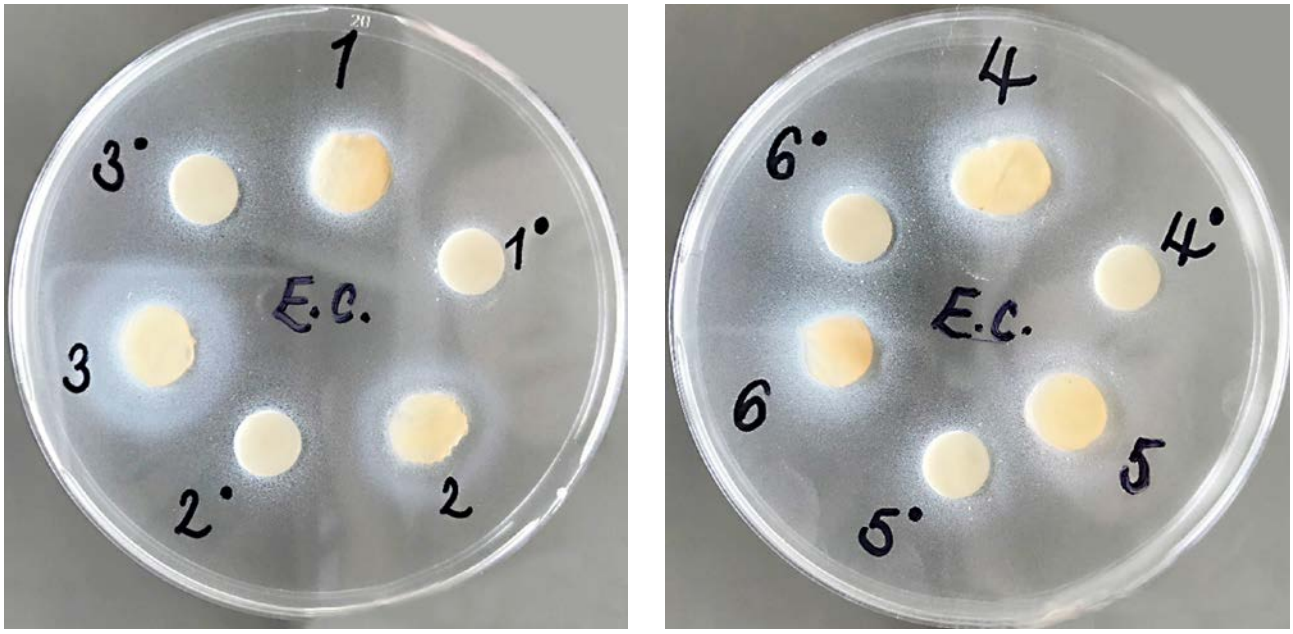

Fig. 4. Image showing the *Escherichia coli* plates post-incubation for sample numbers 1—6 where IZs are present for sample numbers 2, 4, 5 and 6 where muscle was the matrix used

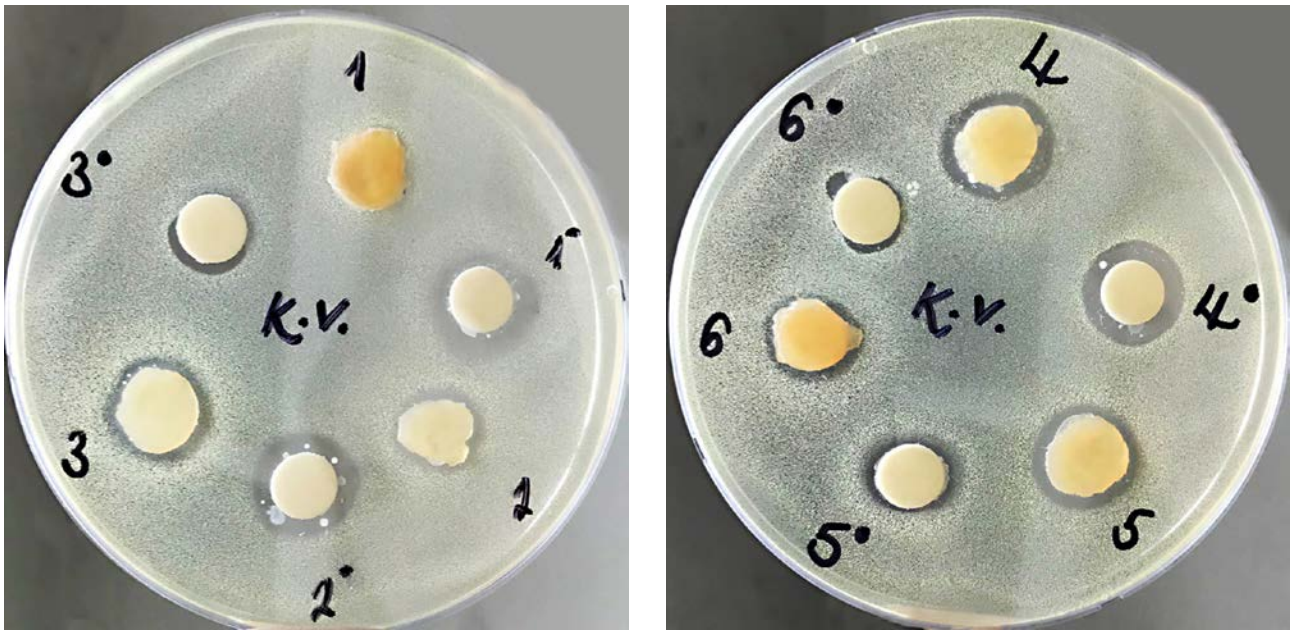

Fig. 5. Images showing the *Kocuria rhizophila* plates post-incubation for sample numbers 1—6 showing IZs present for meat samples 2—6 and for juice samples 1•—6•

method may not detect certain antibiotics or prove less sensitive in relation to other methods (as seen by the validation studies performed in 2010 by G a u d i n et al. [7].

To promote this work, one could perform further experimentations of the same poultry products and samples with other screening methods for comparison. Additionally, the experiment using the STAR method could be repeated to determine if the same results are attained. Moreover, the STAR method could be evaluated in relation to a larger sample size. In relation to the effect of the matrix on the STAR method's performance, no experimentations or other studies have been performed and published which looked at using matrices other than muscle, like meat, juice and skin, using the STAR method. Given the discrepancies discussed, further experimentations are needed to ascertain whether other matrices can be validated for use in the STAR method so that reasonable conclusions can be drawn from the results.

CONCLUSIONS

This study concerned the broad-spectrum detection of antibiotic residues in poultry meat by a multi-plate assay via use of the STAR method. Families of antibiotics pre-identified due to positive samples in the results of this work include aminoglycosides, beta-lactams, sulphonamides and macrolides. As a targeted confirmatory analysis was not performed, we are unable to determine where false positive and false negative results arose as given by the use of the STAR screening method. Therefore, to be able to draw any definitive conclusions from this study, further confirmatory analysis must be performed.

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POTENTIAL HEALTH RISK TO HUMANS RELATED TO ACCUMULATION OF BRODIFACOUM AND BROMADIOLONE IN THE WHEAT GROWN ON RODENTICIDE CONTAMINATED SOIL

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ABSTRACT

The aim of this study was to determine in a model experiment the potential residues of bromadiolone and brodifacoum in the wheat grown on soil treated with these rodenticides and to compare them with the respective acceptable daily intake (ADI) in order to obtain information lacking in the scientific literature. The study focused on the level of residues of chronic rodenticides Broder G, with the active ingredient brodifacoum, and DERATION G, with the active ingredient bromadiolone, in wheat (*Triticum* spp.). The preparations were used in the form of granular bait. In the wheat grown on the soil treated with 100 g.m^{-2} of the preparation BRODER G, the brodifacoum residues ranged from 0.012 to 0.0218 mg.kg^{-1}, while the treatment of soil with 500 g.m^{-2} resulted in residues ranging between 0.0344 and 0.0436 mg.kg^{-1}. When using the preparation DERATION G, bromadiolone residues ranged between 0.012 and 0.018 mg.kg^{-1} after the treatment of soil with 100 g.m^{-2} and between 0.030 and 0.0428 mg.kg^{-1} after the treatment with 500 g.m^{-2}. We observed that the acceptable daily intake was exceeded significantly in all of the cases and the residual levels depended on the rodenticide dose. In the case of brodifacoum, the ADI was exceeded more than 700-fold at a dose of 100 g.m^{-2} and more than 1400-fold at a dose of 500 g.m^{-2} of soil. With bromadiolone, the ADI was exceeded 150-fold at a dose of 100 g.m^{-2} and more than 350-fold at a dose of 500 g.m^{-2}. This indicates the risk to consumers from such crops.

Key words: brodifacoum; bromadiolon; cereal crop; food safety; residues

INTRODUCTION

Targeted, unauthorised or unintentional application of chemicals and their spreading and persistence in the environment presents risks to humans and other animals, and has negative effects on biodiversity in general.

Managing populations of commensal rodents is essential. By sharing our environment, they can transmit to humans, directly or indirectly, more than 40 zoonotic patho-

gens, such as Yersinia pestis, hantavirus and Leptospirosis species. They can cause the destruction or degradation of cereals, accounting for nearly 10 % of the grain crops in the world, with considerable variation depending upon the country [2].

Due to the neophobic behaviour of rodents, the only really effective molecules that can be used to manage their populations are anticoagulant rodenticides (ARs), sometimes also referred to as chronic rodenticides. Their delayed action, with mortality occurring several days after consumption, makes them effective in controlling neophobic species. ARs are usually classified into two generations. The activity of ARs of the first generation (warfarin) is now largely limited by the genetic resistance phenomenon. The second generation of ARs (bromadiolone, difenacoum, brodifacoum, flocoumafen and difethialone), sometimes referred to as superwarfarins, are chemically modified forms of warfarin where substituted phenyl rings are attached to the 4-hydroxycoumarin moiety [19]. Second generation ARs have been used since the 1980s for pest management. They are currently the most widely used rodenticide compounds and are authorized through various legislative provisions.

Some recent research makes an effort to implement a third generation of ARs which is based on the stereochemistry concept (difenacoum enriched in trans-isomers), which would be efficient against resistant strains of rodents and be less persistent and thus less involved in secondary poisonings [9, 10].

These anticoagulant compounds used as rodenticides inhibit vitamin K 2,3-epoxide reductase, affecting vitamin K-dependent coagulation factors in the liver and causing spontaneous internal bleeding and, at lethal doses, death [39].

In the past, the availability of an effective antidote, vitamin K1, was considered an advantage of anticoagulant rodenticides. Early administration of this antidote, i. e. before haemorrhage affects the vital centres, will prevent death. However, while the anticoagulant actions of superwarfarins are ultimately responsible for their lethality, additional targets exist that could lead to pathological consequences or contribute to their toxicity. In addition to proteins in the coagulation cascade, vitamin K is a cofactor for several other proteins throughout the body, and modeling studies suggest there may be over 100 targets for GGC (γ-glutamyl carboxylase). Lower vitamin K levels will therefore reduce the GGC-dependent carboxylation of these proteins, including osteocalcin, a hormone involved in energy metabolism, fertility, brain development, and bone remodeling [27] and the matrix Gla protein, important for vascular decalcification [34]. In addition to their roles in clotting, vitamin K–dependent proteins are vital to several other processes, including ones that help maintain the normal brain function and homeostasis [20]. Thus, unlike warfarin overdose for which 1—2 days of treatment with vitamin K is effective, treatment of superwarfarin poisoning with vitamin K is limited by its extremely high cost and can require daily treatment for a year or longer.

Due to their increased tissue persistency and bioaccumulation in the environment, non-target poisoning by second generation ARs is commonly described in wildlife. Different species are affected by this exposure, such as red foxes, European minks, bobcats and various raptors such as red kite, buzzards, kestrels, peregrine falcons, and eagle owls [26, 30]. Besides potential adverse sublethal effects on wildlife, there is also a concern for human exposure through consumption of meat from wild game animals that carry brodifacoum residues [4].

With regard to the high toxicity of chronic rodenticides to animals, monitoring of their influence on other non-target species has been initiated. B o o t h et al. [3] described acute toxicity of brodifacoum to invertebrates, such as snails and terrestrial species of crabs. E a s o n et al. [13] examined poisoned wild pigs and goats for the presence of brodifacoum residues and B o o t h et al. [3] investigated toxic effects of brodifacoum on earthworms.

Because of the well-known use-related risks, ARs were identified by the European Union as candidates for future comparative risk assessment and substitution.

Regulation (EU) No. 528/2012 [32] which concerns the making available on the market and use of biocidal products state conditions for approval and authorization of biocidal products, including products used for the control of mice, rats or other rodents, by means other than repulsion or attraction. Since brodifacoum and bromadiolone fulfil the criteria set in Article 5(1) of this Regulation, the approval of these rodenticides in product type 14 should normally not be renewed. However, this raised an issue of sufficiently effective alternatives that would present no practical or economical disadvantages. On the basis of an input from stakeholder organisations, companies, non-governmental bodies, independent experts and na-

tional bodies, the Biocidal Products Committee (BPC) presented in 2016 an opinion on the application for renewal of the approval of the active substance: Brodifacoum, Product type: 14, ECHA/BPC/113/2016, and Bromadiolone, Product Type 14, ECHA/BPC/111/2016, both adopted 16th June 2016 [14, 15]. This opinion was based on the conclusion that currently no significant and effective alternative to anticoagulant rodenticides is readily available. Non-chemical controls or prevention methods for rodent control, such as mechanical, electrical or glue traps, may not be sufficiently efficient and may raise further questions as to whether they are humane or whether they cause unnecessary suffering to rodents. Alternative active substances approved for use as rodenticides may not be suitable for all user categories or efficient for all rodent species. Rodent control currently relies largely on the use of anticoagulant rodenticides, the non-approval of which could lead to insufficient rodent control. This may not only cause significant negative impacts on human or animal health or the environment, but also affect the public's perception of its safety with regard to exposure to rodents or the security of a number of economic activities that could be vulnerable to rodents, resulting in economic and social consequences. On the other hand, the risks to human health, animal health or the environment arising from the use of products containing brodifacoum and bromadiolone can be mitigated if they are used according to certain specifications and conditions.

Specific conditions for the use of rodenticide preparations based on brodifacoum and bromadiolone were set by the Commission Implementing Regulations (EU) 2017/1380 and 2017/1381 [7, 8].

Other rodenticides that currently are registered to control rodents are bromethalin, cholecalciferol and zinc phosphide. These compounds are not anticoagulants. Each is toxic in other ways [17].

Brodifacoum is a bromylate derivative of hydroxycoumarin that was used for the first time in 1975 to control rodents resistant to coumarin [25]. The solubility of brodifacoum in water is 0.004 $mg.l^{-1}$ [23]. Brodifacoum has been supported and evaluated as a rodenticide in the following use situations: in and around building and in sewers. The target species are brown rat, black rat and the house mouse. This rodenticide is not readily biodegradable. It is hydrolytically stable to hydrolysis (t½ > 1 year). The degradation time DT50 value of 157 days indicate that brodifacoum would be persistent and immobile in soil. The exposure to the groundwater is unlikely. Under basic conditions (high pH), brodifacoum is not likely to be adsorbed onto soils or sewage sludge due to the ionisation of the molecule; whereas under acidic conditions (low pH), brodifacoum is likely to be adsorbed onto soils or sewage sludge as the molecule is in its neutral or non-ionised form [11].

Bromadiolone was used for the first time in 1976 also to control rodents resistant to warfarin. It is more effective and at the same time less toxic than other chronic anticoagulant rodenticides of the 1st and 2nd generation [28]. Bromadiolone has been evaluated as a rodenticide against rats and mice for the following use patterns: in and around buildings (professional and non-professional use), sewers (professional use only), open areas (professional use only) and waste dump (landfill) perimeters (professional use only). The solubility of bromadiolone in water is 0.002 $mg.l^{-1}$ [23]. Bromadiolone is not readily biodegradable under environmentally relevant conditions or during sewage treatment processes. It is also not inherently biodegradable. No hydrolysis was found at pH 7, or 9, so hydrolysis of bromadiolone is not expected to be a significant process in the environment. Bromadiolone is quickly degraded in soil under aerobic conditions with an estimated DT50 value between 4 and 53 days (at 12 °C, extrapolated from 20 and 25 °C, LiphaTech), however degradation led to the formation of unidentified soil metabolites which persisted in significant quantities for > 1570 days. Laboratory soil column leaching and aged leaching studies performed by LiphaTech indicated that bromadiolone and any potential degradation products, even if released indirectly to soil in small quantities, are not likely to move through the soil profile and are unlikely to reach groundwater in significant quantities [12].

Commission Directives 2009/92/EC and 2010/10/EU [5, 6] dealt with the relevant issues by drawing attention to high persistence, bioaccumulation and toxicity of ARs. The estimated daily exposure that will not cause harmful effects throughout the human life is referred to as Acceptable Daily Intake (ADI) in $mg.kg^{-1}$ body weight. ADI for brodifacoum has been estimated as 0.0000005 $mg.kg^{-1}.day^{-1}$ which corresponds to NOEL (No Observable Effect Level) equal to 0.001 $mg.kg^{-1}.day^{-1}$. ADI for bromadiolone has been estimated to 0.000002 $mg.kg^{-1}.day^{-1}$, which corresponds to NOEL for bromadiolone equal to 0.004 $mg.kg^{-1}.day^{-1}$ [35].

EFSA [16] has reviewed the maximum residue levels (MRLs) currently established at the European level accord-

ing to Article 12 of Regulation (EC) No. 396/2005 [31] for the pesticide active substance bromadiolone. Considering that this active substance was not authorised for use on edible crops within the EU, no MRLs were established by the Codex Alimentarius Commission (codex maximum residue limits) and that no import tolerances were notified to EFSA, residues of bromadiolone were not expected to occur in any plant or animal commodity. Even though information on the limit of quantification (LOQ) of bromadiolone was provided by the European Union Reference Laboratories for Pesticide Residues, the available data were not sufficient to derive a residue definition for enforcement against potential illegal uses.

Most studies related to the risk of ARs focused on the analysis of rodenticides in biota. Probably, because of their low solubility in water, very few studies investigated the presence of rodenticides in water. Recently some studies related to the authorised use of ARs indicated that anticoagulants can reach sewage waters that are treated in wastewater treatment plants (WWTP) and pass to treated water and sewage sludge [22, 23, 33]. Thus they may be discharged into rivers or applied to soil manured with sludge.

With regard to the risk of the presence of ARs in agricultural soil and their translocation to crops, even with the professional use of anticoagulant rodenticides, one must also consider the potential changes in the integrity of pellets after being subjected to precipitations. Also the rules of correct agricultural practice may occasionally be violated. Thus grain crops, for example wheat, which are used directly for human consumption (breadstuffs), or as feed for farm animals (concentrate), may be exposed to ARs. For the 2019 crops, FAO's forecast for world wheat production is 757 million tonnes, which would place this year's output 4 percent above the 2018 level [18].

The aim of our study was to investigate the potential risk to humans related to the consumption of wheat grown on soil treated with second generation ARs (brodifacoum, bromadiolone).

MATERIALS AND METHODS

The rodent control preparations BRODER G and DERATION G (granular baits) used in this study are based on anticoagulant rodenticides of the 2nd generation, brodifacoum and bromadiolone, respectively. The study was carried out in five subsequent years 2013—2017. Wheat (*Triticum* spp., order *Cyperales*, family *Poaceae*) was sown to open boxes of dimensions 1× 1 × 0.5 m, that contained loam soil suitable for growing wheat.

Two open boxes were used for each of two AR doses and additional two were used as a control. The boxes were placed outside and were exposed to precipitations.

In the second half of March of each year, rodenticides were placed onto the surface of the soil in the respective boxes at doses of 100 $g.m^{-2}$ and 500 $g.m^{-2}$ of each preparation and worked into the top 5 cm layer. Then 7.5 g of wheat grains (*Triticum* spp.) were sown to each box to the depth of about 25—50 mm. No other agrotechnical measures were taken throughout the vegetation period. In early August, at harvest time, the wheat was scythed manually and respective samples (whole spikes) were collected and homogenized. Control samples were obtained from wheat grown on untreated soil. This procedure was repeated for an additional 4 years using fresh soil free of rodenticide residues.

The samples collected were thoroughly homogenized and 10 g aliquots were extracted for 15 min with 25 ml of a mixture of chloroform + acetone (1 : 1). The solvent was then decanted through a filter paper and the extraction was repeated once more with a fresh solvent. The extracts were combined and the solvent was evaporated at approximately 40 °C in a rotating vacuum evaporator. The residuum was dissolved in l ml of methanol and 1 ml of the mobile phase was added to remove co-extracted proteins. The solution was then transferred into a centrifugation tube containing n-hexane and centrifuged for 10 min at 3500 rpm. The cleared lower layer was filtered and used for HPLC chromatographic analysis (Agilent, 1200 Series HPLC System, Agilent Technologies, USA).

The chromatographic analysis was carried out on a column LiChrospherR 100 RP-18 (5 µm) with mobile phase acetonitrile (A) + acetate buffer, pH 4.6 (B), ratio 60 + 40, at a flow rate of l $ml.min^{-1}$, injected aliquot 10 µl and UV detection at 265 and 310 nm.

The statistical evaluation was carried out using GraphPad Prism Anova. The P threshold value for statistical significance was set at $P < 0.05$.

RESULTS

Residues of brodifacoum and bromadiolone determined between 2013 and 2017 are presented in Table 1. The treatment of soil with 100 g.m^{-2} dose of preparation BRODER G resulted in wheat residual levels ranging between 0.012 and 0.0218 mg.kg^{-1} while the dose of 500 g.m^{-2} produced residues ranging between 0.0344 and 0.0436 mg.kg^{-1}. Treatment of soil with 100 g.m^{-2} of DERATION G resulted in residues in wheat between 0.012 and 0.018 mg.kg^{-1} while the dose of 500 g.m^{-2} produced residual levels between 0.030 and 0.0428 mg.kg^{-1}.

A comparison with ADI for brodifacoum in humans (0.0000005 mg.kg^{-1}.day) for a 60 kg individual (0.00003 mg.kg^{-1}.day) showed that the rodenticide dose of 700 g.m^{-2} soil caused a significant increase in brodifacoum in wheat that exceeded the respective ADI 400-fold in 2014 and more than 700-fold in 2015 (Table 1).

A dose of 500 g.m^{-2} soil of BRODER G resulted in more than 1100-fold higher level in 2014 and more than 1400-fold higher level in 2015 and 2017 in comparison with ADI for a 60 kg man.

The precipitations in the area of the study varied considerably between individual years and months. In the years 2014, 2015 and 2017, they were distributed relatively evenly, in 2013 they were more intensive in the second half of the study period and in 2016 most of them occurred in April and May. No correlation was found between the residues in wheat and the mean monthly precipitations during the growing season (April—August).

A comparison with bromadiolone ADI for humans (0.000002 mg.kg^{-1}.day^{-1}) considering a 60 kg individual (0.00012 mg.kg^{-1}.day^{-1}) showed that the rodenticide dose of 100 g.m^{-2} soil resulted in 100-fold higher level in 2015 and 150-fold higher level in 2016 than the level acceptable for 60 kg individual (Table 1).

Table 1. Residues of brodifacoum and bromadiolone in control wheat and wheat grown on soil treated with these rodenticides at doses 100 g.m^{-2} and 500 g.m^{-2}

Years	2013	2014	2015	2016	2017
Mean monthly rainfall (mm) during the study period (April—August)	73.6	120.8	52.6	89	89.4
Control ADI 60 kg man, brodifacoum mg.day^{-1}			**0.00003**		
Residues of brodifacoum in wheat – BRODER G dose 100 g.m^{-2} soil					
Residues of brodifacoum mg.kg^{-1} in control wheat	0	0	0	0	0
Residues of brodifacoum mg.kg^{-1} in treated wheat	0.0196*	0.012*	0.0218*	0.02*	0.0178*
Residue—ADI multiple	653.3	400	726.6	666.6	593.3
Residues of brodifacoum in wheat—BRODER G dose 500 g.m^{-2} soil					
Residues of brodifacoum mg.kg^{-1} in control wheat	0	0	0	0	0
Residues of brodifacoum mg.kg^{-1} in treated wheat	0.040*	0.0344*	0.043*	0.038*	0.0436*
Residue—ADI multiple	1333.3	1146.6	1433.3	1266.6	1453.3
Control ADI 60 kg man, bromadiolone mg.day^{-1}			**0.00012**		
Residues of bromadiolone in wheat—DERATION G dose 100 g.m^{-2} soil					
Residues of bromadiolone mg.kg^{-1} in control wheat	0	0	0	0	0
Residues of bromadiolone mg.kg^{-1} in treated wheat	0.017*	0.014*	0.012*	0.018*	0.014*
Residue—ADI multiple	141.6	116.6	100	150	116.6
Residues of bromadiolone in wheat—DERATION G dose 500 g.m^{-2} soil					
Residues of bromadiolone mg.kg^{-1} in control wheat	0	0	0	0	0
Residues of bromadiolone mg.kg^{-1} in treated wheat	0.040*	0.03*	0.036*	0.0428*	0.035*
Residue—ADI multiple	333.3	250	300	356.6	291.6

*—significant at $P < 0.001$

The dose of 500 g.m^{-2} soil of DERATION G resulted in 250-fold higher level in 2014 and more than 350-fold higher level in 2016 in comparison with the ADI for 60 kg man.

DISCUSSION

Anticoagulant poisons have an ongoing history of worldwide use for the control of pest vertebrates, particularly rodents. Control of rodents is important not only to reduce the very high economic losses caused by rodents in both animal and plant production but also the losses associated with disease transfer which are frequently much higher. Balancing benefits of rodent control and the potential costs of applying anticoagulant rodenticides to the environment remains a challenge.

Superwarfarins like BDF and DiF represent an emerging threat to human life. The increased use of these agents has raised the risk of accidental bait ingestion or incidental exposure to residual material present in the environment following widespread dispersals [19].

There is also a significant risk of unintentional exposure to superwarfarins as a result of their use to eradicate rodents. In 2001, close to 20 tons of rodent bait containing 0.002 % BDF, for a total of 360 g, was released into the environment owing to a transport accident on the east coast of South Island in New Zealand [29]. Measurements were performed immediately after the spill and up to 21 months later. While the levels in water and sediment declined to below detectable limits, within 9 days brodifacoum was detectable in the shellfish for up to 21 months after the spill and calculated to require up to 31 months before the levels were acceptable for human consumption. In 2010, 700 kg of similar bait was accidentally dropped into Lake Kirirua in New Zealand [21]. It was estimated that 10 bags sank intact, and water and sediment measurements did not detect significant brodifacoum up to 1 month later. The absence of brodifacoum in the few animals tested may be due to its poor solubility in water. Although residual brodifacoum levels are considered safe with respect to their effects on anticoagulation, they may have actions that occur at significantly lower levels.

Generally, the currently used chronic anticoagulant rodenticides are toxic to both humans and other animals. Their slow onset of effect and death of rodents after several days results in their increased effectiveness. The concentration of the active ingredients in the bait in rodenticides of the 2nd generation, e. g. bromadiolone or brodifacoum, is 0.005 %. Such baits act sufficiently after acceptance of only one dose, as 3 g of the bait have already lethal effects. Preparations of the 3rd generation, e. g. difenacoum, contain 0.0025 % of the active ingredient, which is below the threshold of taste sensitivity of rats which equals to 0.005 % [37].

Our investigations confirmed the presence of residues of ARs dependent on the dose of bait incorporated into soil.

Incorporation of BRODER G at a dose of 100 g.m–^{2} of soil resulted in the residues of brodifacoum in wheat that ranged between 0.012 and 0.0218 mg.kg^{-1} and the dose of 500 g.m^{-2} of the soil produced residues between 0.0344 and 0.0436 mg.kg^{-1}.

After incorporation of DERATION G at a dose of 100 g.m^{-2} of soil, the residues of bromadiolone in wheat ranged between 0.012 and 0.018 mg.kg^{-1} and at a dose of 500 g.m^{-2} of soil between 0.030 and 0.043 mg.kg^{-1}.

From among all descriptions dealing with the use of anticoagulant rodenticides those that investigated their elution from soil and potential residues in plants date back mostly to the eighties and nineties of the past century [1, 24, 36, 38].

A study was carried out with ^{14}C-bromadiolone in four types of soil. With a soil rich in clay and organic compounds, bromadiolone stayed in the superficial layer and scarcely moved. However, in soil poor in clay and organic compounds, 67 % of the added bromadiolone was eluted [36]. Since anticoagulant rodenticides are not intended for direct application to growing crops, no residues in plant food stuffs are expected. Unlike conventional crop protection products, which must be applied over relatively large crop areas, rodenticides are applied to discrete sites in the form of low concentration baits. Even if the bait is spilled, it will not be taken up by plants. Hall and Priestley [24] monitored the metabolism of ^{14}C-brodifacoum in soil under aerobic conditions after applying it at a nominal rate of 0.4 mg.kg^{-1} and incubating for up to 52 weeks. A mean total of 35.8 % of the applied radioactivity was recovered as $^{14}CO_2$ within the test period. ^{14}C-brodifacoum was the major radiolabeled component in the soil extracts throughout the 52 weeks. Under the conditions of the study, the half-life of brodifacoum was calculated to be 157 days. A study was carried out with ^{14}C-bromadiolone in four types of soil. The rodenticide was degraded significantly with half-lives ranging from 1.8 to 7.4 days [38].

The study published by Askham [1] was inspired by the crop use of currently registered rodenticides during the spring and late summer when rodents have been found to re-emerge and growers did not have any practical means of controlling their expanding population. Several aspects were investigated including the degree at which a commercially prepared rodenticide bait might withstand precipitations regimes and the subsequent potential crop contamination. In addition to other aspects, the translocation potential of bromadiolone from a growing medium to wheat was investigated. One hundred ml of a test solution containing 2199.39 μg ^{14}C-bromadiolone was applied to the soil surface of 12 growing wheat plants. An additional 100 ml of hydroponic solution (1389.03 μg ^{14}C-bromadiolone) was used for 12 wheat plants. The application of bromadiolone onto soil resulted in 353 ppb of bromadiolone in leaves and stems of wheat after 16 days while the level of bromadiolone in shoots grown in hydroponic solution was in the 2 to 3 ppm range, probably due to the lack of soil binding sites.

For public health reasons, ARs are applied in public infrastructures and sewage systems, especially in large urban areas where rodents can obtain food and shelter. They are released to sewage waters and reach wastewater treatment plants (WWTPs) through solubilisation of AR baits when applied to eliminate or control rodents in sewage systems, water channels, or due to agricultural runoff [23]. Gómez-Canela et al. [22] tested the hypothesis that WWTP effluents can be a source of anticoagulants to receiving waters and that these can affect aquatic organisms and other non-target species. In their study, they determined the occurrence of 11 anticoagulants in WWTPs receiving urban and agricultural wastewaters. Warfarin was the most ubiquitous compound detected in influent waters and was partially eliminated during the activated sludge treatment, and low nanograms per liter concentration were found in the effluents. Other detected compounds were: coumatetralyl, ferulenol, acenocoumarol, flocoumafen, brodifacoum, bromadiolone, and difenacoum at concentrations of 0.86—87.0 ng.l^{-1}. Considering the water volumes of each WWTP, daily emissions were estimated to be 0.02 to 21.8 g.day^{-1}, which means that WWTPs contribute to the loads of anticoagulants to the receiving waters.

However, there is another important risk related to the discharge of anticoagulants to wastewaters. The products of aerobic biological treatment of urban wastewater (sewage) are treated water and sewage sludge. Because sewage sludge contains macronutrients (nitrogen and phosphorus), micronutrients (iron, zinc, copper, …) and a high percentage of organic matter, it has valuable agronomic properties and is applied as fertilizer in many countries. The use of sewage sludge must take into account the nutrient needs of plants but should not compromise the quality of soil or surface water and groundwater [23]. Gómez-Canela and Lacorte [23] developed a multiresidue method based on the soil-liquid extraction in combination with an optimized cleanup and liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the determination of ARs in sludge and used this method to evaluate the presence of ARs in 27 centrifuged WWTP sludge intended to be used as agricultural fertilizer. Bromadiolone was detected in sludge of six out of 27 WWTPs at concentrations between 4.77 and 7.73 ng.g^{-1}. This rodenticide has a low solubility which explains its accumulation in activated sludge with respect to the other ARs. Brodifacoum, the most toxic of the 2nd generation ARs was only detected in two of the 27 WWTP at concentrations of 15.2 and 17.4 ng.g^{-1}. The authors calculated the mass loads of ARs on a monthly basis considering the ΣAR concentration and the sludge generated in each WWTP. The minimum and maximum ΣAR loads were 0.04 and 65.8 g per month, respectively. Taking into account the mass loads generated by the WWTP every year, about 1.9 kg of anticoagulants are estimated to be eventually discharged to agricultural soils in north-east Spain. Their presence may trigger the accumulation of ARs in animals that prowl landfills and farmlands.

CONCLUSIONS

The results obtained in our study, investigating wheat grown on bait-contaminated soil, proved the presence of residues of both rodenticides, brodifacoum and bromadiolone in wheat exceeding considerably the ADI for these ARs. This indicates that despite the low solubility of both tested anticoagulant rodenticides, the field crops grown on soil exposed to the relevant baits may present considerable loads on the food chain. The acceptable daily intake was significantly exceeded in all the cases in dependence on the soil-incorporated bait dose. With brodifacoum and the 100 g.m^{-2} dose, the ADI was exceeded more than 700-fold and with the 500 g.m^{-2} dose even more than 1400-fold.

With bromadiolone and the 100 g.m^{-2} dose the ADI was exceeded 150-fold and after incorporation of 500 g.m^{-2} of the soil, the ADI in wheat was exceeded more than 350-fold. With regard to the recent studies that found the presence of rodenticide residues in water treated by WWTP and in sewage sludge that may be used in agriculture for irrigation or manuring, our results indicate the need for additional investigation of potential translocation of anticoagulant rodenticides to crops and the risks to their consumers.

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GOAT COLOSTRUM—SOURCE OF TOXIGENIC *BACILLUS CEREUS*

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ABSTRACT

The aim of this study was to evaluate the toxigenic potential of *Bacillus cereus* strains isolated from frozen goat colostrum. Of the 50 phenotypically suspected *B. cereus* isolates, 39 (78.0 %) were confirmed as *B. cereus* by the polymerase chain reaction (PCR) method based on the gyrB gene detection. In these isolates, genes encoding the production of haemolysin BL (Hbl), a complex of non-haemolytic enterotoxins (Nhe) and emetic toxin were detected by the PCR method. In 36 (92.3 %) confirmed *B. cereus* isolates, genes encoding at least one type of toxins of interest were detected. In all toxigenic isolates, we found the presence of genes for Nhe production, and in 16 (41.0 %) of the isolates, genes encoding both Nhe and haemolysin BL were shown. Eight (20.5 %) of the emetic strains of *B. cereus* were identified. The emetic toxin production gene was always detected simultaneously with genes encoding non-haemolytic enterotoxin production. The ability to produce BL haemolysin and non-haemolytic enterotoxins were confirmed by the immunochromatographic method. In summary, goat colostrum can be a significant source of toxigenic strains of *B. cereus*.

Key words: cereulide; haemolysin BL; non-haemolytic enterotoxin; PCR

INTRODUCTION

Bacillus cereus is a Gram-positive, facultative anaerobic, and spore-forming rod. As a soil associated ubiquitous organism, *B. cereus* is commonly found in food products where they can cause spoilage [6]. *B. cereus* is the causative agent of two different types of food-borne illnesses: the emetic syndrome, caused by ingestion of a preformed emetic toxin (cereulide) in the food, and the diarrheal syndrome, caused by different toxins that can be formed in the food but also in the small intestine [5, 8]. The occurrence of these diseases are mainly associated with the consumption of foods made from cereals and potatoes, as well as vegetable dishes, minced meat dishes, milk and rice [8].

The diarrheal syndrome has been associated with three toxins: a single protein named cytotoxin K, and two heat-labile complexes—haemolysin BL and non-haemolytic enterotoxin (both composed of three protein components). The emetic syndrome is caused by the heat-resistant toxin cereulide [6].

As evidenced by a number of studies, raw milk, dairy products and dairy farm environments are a common source of toxigenic *B. cereus* [4, 5, 6, 12, 13, 15]. Simultaneously, psychrotrophic *B. cereus* strains capable of growing at temperatures below 8 °C can also be isolated [16]. The incidence of *B. cereus* in raw milk can be dramatically affected by the housing conditions [4]. Milk and dairy products are more commonly associated with the occurrence of a diarrheal form of the disease [10]. Nevertheless, in comparison with other food matrices the risk of toxin-induced diarrheal illness through consumption of contaminated milk may be limited [5]. According to a study of M a et al. [13], *B. cereus* was the dominant bacterial species found in goat milk powder (42.22 % from 249 identified species). The high occurrence of *B. cereus* in raw goat milk has also been shown in a study by Z h a n g et al. [23].

The objective of our study was to evaluate the toxigenic potential of *B. cereus* strains isolated from frozen goat colostrum samples.

MATERIALS AND METHODS

Bacterial strains

A total of 186 samples of frozen goat colostrum obtained from a local producer of food supplements were examined for the presence of *Bacillus cereus*. The individual samples of goat colostrum were collected from goats from different herds (accurate data were not provided by the producer). The samples (25 g) were incubated at 37 °C for 24 h in 225 ml of Buffered Peptone Water (Oxoid Ltd, Basingstoke, Hampshire, UK). The enriched samples were inoculated onto Mannitol Yolk Polymyxin B agar (MYP; HiMedia Laboratories Pvt. Ltd., Mumbai, India) and incubated at 30 °C for 24 h. A total of 50 (26,9 %) phenotypically suspected *B. cereus* strains (appeared as colonies with typical morphology on MYP agar and complete haemolysis on blood agar) were obtained and included in the study.

DNA isolation

For DNA isolation, colonies grown on blood agar plates were used (after incubation at 30 °C for 24 h). The DNA isolation was carried out by means of heating the bacterial cells in 200 µl sterile saline at 100 °C for 20 min with consecutive centrifugations at 10 660 × g for 6 min. The supernatant was used as a template.

Polymerase chain reaction

The polymerase chain reaction (PCR) was used for species confirmation and detection of genes encoding emetic toxin (cereulide), non-haemolytic enterotoxin (Nhe) and haemolysin BL (Hbl). A total of 4 multiplex polymerase chain reactions (mPCR) were carried out with specific primers to detect gyrB gene and toxicity determined by various *B. cereus* genes. The 16S rRNA gene was targeted as an internal control gene in the presence of bacterial DNA. The sequences of the primers used are given in the Table 1, the composition of the reaction mixture was adapted to PPP Master Mix use (Top-Bio, Ltd., Prague, Czech Republic). A total reaction volume of 25 µl was made up of 23 µl of the master mix and 2 µl of the template DNA. Amplification was performed on a PTC-200 thermocycler (MJ Research Watertown, Massachusetts, USA).

PCR 01: This was used for the detection of the gyrase B gene (*B. cereus* species confirmation), gene encoding the non-ribosomal peptide synthetase (which plays a role in the production of emetic toxin) and the highly conserved regions of the bacterial 16S rRNA (internal control). The PCR amplification involved: the initial denaturation step of 10 min at 95 °C, followed by 30 cycles, each including 1 min of denaturation at 94 °C, 1 min of annealing at 54 °C, 1 min of elongation at 72 °C, and the completion with a final elongation at 72 °C for 5 min.

PCR 02: This was used for the detection of the hblA and hblC genes encoding the production of haemolysin BL. The PCR amplification involved: the initial denaturation step of 4 min at 94 °C, followed by 36 cycles, each including 30 s of denaturation at 94 °C, 1 min of annealing at 62.5 °C, 1 min of elongation at 72 °C, and the completion with a final elongation at 72 °C for 7 min.

PCR 03: This was used for the detection of the nheA and nheC genes encoding production of the non-haemolytic enterotoxin. The PCR amplification involved: the initial denaturation step of 2 min at 94 °C, followed by

Table 1. PCR primers used in the study

Target gene	Primer	Primer sequence (5´– 3´)	Amplicon size [bp]	Reference	Annealing	PCR
gyrB	BC1	ATT GGT GAC ACC GAT CAA ACA	365	Yamada et al. (1999) [22]	54 °C	BC 01
	BC2	TCA TAC GTA TGG ATG TTA TTC				
NRPS*	CER1	ATC ATA AAG GTG CGA ACA AGA	188	Horwood et al. (2004) [11]	54 °C	BC 01
	CER2	AAG ATC AAC CGA ATG CAA CTG				
16Sr RNA	InKo1	GGA GGA AGG TGG GGA TGA CG	241	Martineau et al. (1996) [14]	54 °C	BC 01
	InKo2	ATG GTG TGA CGG GCG GTG TG				
hblA	HBLA1	GCT AAT GTA GTT TCA CCT GTA GCA AC	873	Rowan et al. (2003) [17]	62.5 °C	BC 02
	HBLA2	AAT CAT GCC ACT GCG TGG ACA TAT AA				
hblC	HBLC-N	AAT AGG TAC AGA TGG AAC AGG	399	Rowan et al. (2003) [17]	62.5 °C	BC 02
	HBLC-C	GGC TTT CAT CAG GTC ATA CTC				
hblD	HBLD-N	AAT CAA GAG CTG TCA CGA AT	439	Rowan et al. (2003) [17]	54 °C	BC 04
	HBLD-C	CAC CAA TTG ACC ATG CTA AT				
nheA	nheA344S	TAC GCT AAG GAG GGG CA	499	Ghelardi et al. (2002) [7]	54 °C	BC 03
	nheA843A	GTT TTT ATT GCT TCA TCG GCT				
nheB	NBF	TTT AGT AGT GGA TCT GTA CGC	743	Guinebretière et al. (2002) [9]	54 °C	BC 04
	NBR	TTA ATG TTC GTT AAT CCT GC				
nheC	NCF	TGG ATT CCA AGA TGT AAC G	683	Guinebretière et al. (2002) [9]	54 °C	BC 03
	NCR	ATT ACG ACT TCT GCT TGT GC				

* NRPS—non-ribosomal peptide synthetase

35 cycles, each including 1 min of denaturation at 94 °C, 1 min of annealing at 54 °C, 2 min of elongation at 72 °C, and the completion with a final elongation at 72 °C for 5 min.

PCR 04: This was used for the detection of the nheB gene (non-haemolytic enterotoxin) and hblD gene (haemolysin BL). The PCR amplification involved: the initial denaturation step of 2 min at 94 °C, followed by 35 cycles, each including 1 min of denaturation at 94 °C, 1 min of annealing at 54 °C, 2 min of elongation at 72 °C, and the completion with a final elongation at 72 °C for 5 min.

The amplified products were separated by electrophoresis on a 2 % agarose gel in 0.5× TBE buffer, followed by consecutive staining with ethidium bromide and visualization on a UV transilluminator.

Duopath® Cereus Enterotoxins GLISA

The ability of the strains to produce the diarrheal enterotoxins of Nhe and Hbl were verified by the use of the commercially available Duopath® Cereus Enterotoxins GLISA immunoassay kit (Merck KGaA, Darmstadt, Germany). The tests were performed according to the manufacturer's instructions. A strain was considered positive if the red lines appeared on both of the test and control zones.

RESULTS

Out of the 50 phenotypically suspected *B. cereus* isolates included in the study, 39 (78 %) were confirmed by the PCR method as *B. cereus*. In 36 (92.3 %) of the iso-

lates, the genes encoding for the production of at least one type of the toxins of interest were detected. The detection of genes encoding Nhe, Hbl and emetic toxin (cereulide) production were performed only in the confirmed *B. cereus* isolates. The summary of the results is given in Fig. 1 and Table 2.

At the same time, genes encoding the production of all three toxins (cereulide, Hbl, Nhe) were not detected in any of the isolates. As shown in Fig. 1, the detection rate of non-haemolytic enterotoxin genes among all *B. cereus* positive isolates was 30.8 % (12 isolates). In 16 (41.0 %) of the *B. cereus* isolates, genes encoding both non-haemolytic enterotoxin (Nhe) and haemolysin BL (Hbl) production were shown.

In our study, all 3 genes (hblA, hblC, and hblD) encoding the individual components of this toxin were always detected simultaneously. However, there was observed a different variability of the nheA, nheB and nheC genes in the goat colostrum *B. cereus* isolates (Table 2). Genes encoding all three components of both haemolysin BL (hblACD) and non-haemolytic enterotoxin (nheABC) were detected simultaneously only in six *B. cereus* isolates (15.4 %). In all potentially enterotoxigenic strains of *B. cereus*, the immunochromatographic method confirmed the ability to produce the observed enterotoxins.

A total of 8 (20.5 %) emetic strains of *B. cereus* were detected (Table 2). The emetic toxin production gene was always detected simultaneously with genes encoding for the production of a complex of non-haemolytic enterotoxins (Figure 1).

DISCUSSION

The genes nhe encoding non-haemolytic enterotoxin are thought to be present in all *B. cereus* strains [20]. Also, other studies have confirmed the high proportion of nhe genes in toxigenic *B. cereus* isolated from food [4, 9, 19]. The results of our work confirmed these assumptions.

The Hbl enterotoxin complex consists of B, L1 and L2, and its enterotoxigenic activity appears when all 3 components of the Hbl complex are present [2, 18]. In our study, the simultaneous presence of all hblA, hblC, and hblD genes encoding the production of haemolysin BL was demonstrated in 41 % of the *B. cereus* isolates, always in combination with genes for non-haemolytic enterotoxin.

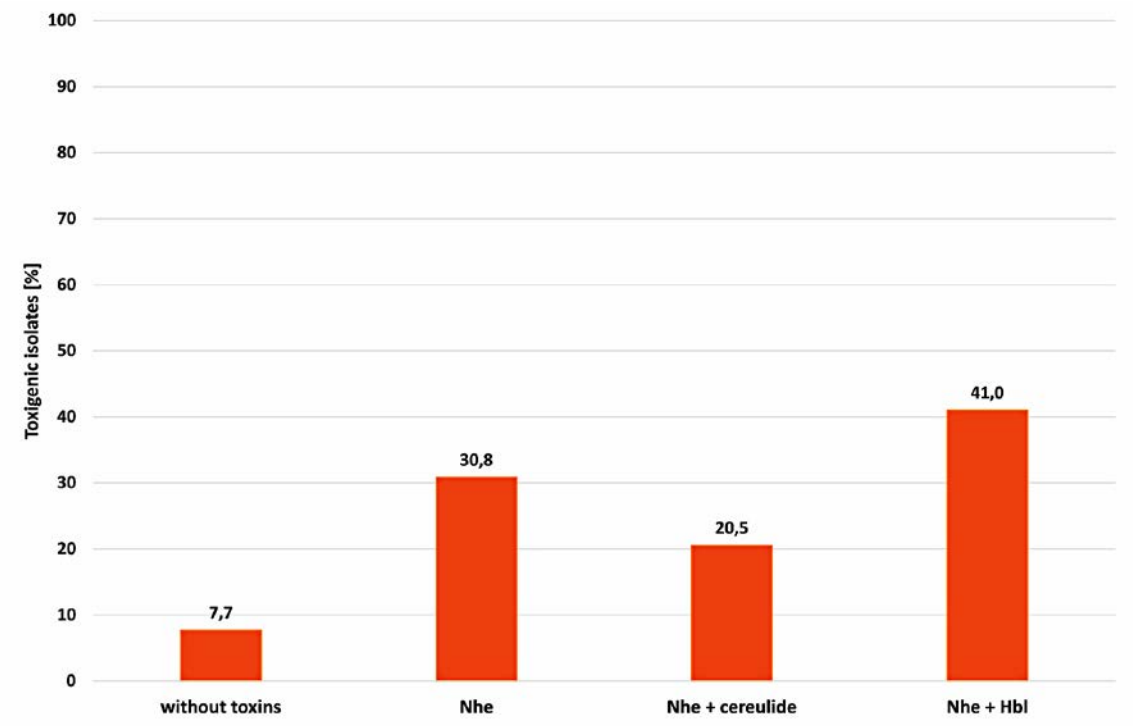

Fig. 1. Toxigenic potential of *B. cereus* isolated from goat colostrum (n = 39)
Nhe—non-haemolytic enterotoxin; Hbl—haemolysin BL; cereulide—emetic toxin

Table 2. Detection of toxin-encoding genes in B. cereus isolated from goat colostrum (n = 39)

Isolate	Cereulide	Haemolysin BL			Non-haaemolytic enterotoxin		
	cer	hblA	hblC	hblD	nheA	nheB	nheC
BC 505	–	+	+	+	+	+	+
BC 506	+	–	–	–	+	+	+
BC 507	–	+	+	+	+	+	+
BC 508	+	–	–	–	+	–	+
BC 511	–	–	–	–	–	+	+
BC 512	+	–	–	–	+	+	+
BC 513	–	–	–	–	–	–	–
BC 515	–	–	–	–	–	–	–
BC 516	–	–	–	–	+	–	+
BC 518	+	–	–	–	+	+	+
BC 519	–	+	+	+	+	+	–
BC 520	–	+	+	+	+	+	–
BC 521	+	–	–	–	+	–	+
BC 522	–	+	+	+	+	–	+
BC 523	–	+	+	+	+	+	–
BC 524	–	+	+	+	+	–	+
BC 525	–	–	–	–	–	+	+
BC 526	–	+	+	+	+	–	+
BC 527	–	+	+	+	+	–	+
BC 531	+	–	–	–	+	+	+
BC 533	–	+	+	+	+	+	+
BC 534	+	–	–	–	+	+	+
BC 536	–	–	–	–	–	+	+
BC 537	–	+	+	+	+	+	+
BC 539	–	+	+	+	+	+	–
BC 540	–	–	–	–	+	+	+
BC 541	–	–	–	–	+	+	+
BC 542	+	–	–	–	+	+	+
BC 543	–	–	–	–	–	–	–
BC 544	–	+	+	+	+	+	+
BC 546	–	–	–	–	+	–	+
BC 549	–	+	+	+	+	–	+
BC 550	–	+	+	+	+	+	+
BC 552	–	–	–	–	+	+	+
BC 553	–	–	–	–	+	+	+
BC 554	–	–	–	–	–	+	–
BC 556	–	–	–	–	–	+	–
BC 557	–	–	–	–	–	+	+
BC 558	–	+	+	+	+	–	+
Total (%)	8 (20.5)	16 (41.0)	16 (41.0)	16 (41.0)	30 (76.9)	26 (66.7)	30 (76.9)

Similar results were obtained by Cui et al. [4], who detected 55 % Hbl-positive *B. cereus* strains isolated from raw milk samples. Interestingly, the toxigenic strains of *B. cereus* have not yet been shown to produce Hbl as a separate enterotoxin [3].

The occurrence of *B. cereus* emetic syndrome is mainly associated with the consumption of rice and rice dishes, but also with foods of higher starch content, such as pasta or noodles [20]. Cressey et al. [3] reported that most of the *B. cereus* strains are capable of producing either diarrheal or emetic toxins. In contrast, Beattie and Williams [1] detected 36 % of *B. cereus* strains capable of producing both toxins simultaneously. In our study, we detected 20.5 % of emetic strains that also produced non-haemolytic enterotoxin. From this point of view, goat colostrum can be considered as a significant source of emetic *B. cereus*. Conversely, a relatively low proportion of emetic strains have been shown by the results of a study conducted in Sweden to monitor the occurrence of emetic toxin in *B. cereus* isolates from raw cow's milk samples from different farms. Of the 722 isolates, only 11 (1.5 %) emetic strains were detected [21]. The low incidence of emetic *B. cereus* strains was also shown in a study by Seong et al. [19], who detected 5 (7 %) emetic strains among 71 food isolates.

CONCLUSIONS

Recently, the popularity of goat's milk and its products has increased. Goat colostrum is increasingly used as a part of dietary supplements. *Bacillus cereus* spores very well withstand adverse environmental conditions as well as many technological operations used in food production. The results of our study confirm that goat colostrum may be a significant reservoir of toxigenic *B. cereus* strains, which may be able to produce not only different types of enterotoxins but also emetic toxins under appropriate conditions. From this point of view, the occurrence of toxigenic *Bacillus cereus* in goat colostrum could be a risk for consumers.

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APPRAISAL OF THE ANTIOXIDATIVE POTENTIAL OF *ALOE BARBADENSIS* M. ON ALCOHOL-INDUCED OXIDATIVE STRESS

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ABSTRACT

This investigation estimated the anti-oxidative potential of *Aloe barbadensis* gel extracts in rats against alcohol-induced oxidative stress. Thirty male albino rats (5 each per group) were included in the experiments. Group A (positive control) and B (negative control) were administered 4 mg.kg^{-1} body weight distilled water and 50 % alcohol respectively for 21 days. Groups C and D were administered 50 % alcohol for the first 14 days followed by co-administration of 125 mg and 250 mg.kg^{-1} body weight extract with alcohol respectively for the last 7 days. Groups E and F were administered distilled water for the first 14 days followed by co-administration of 125 and 250 mg.kg^{-1} body weight *Aloe barbadensis* gel extracts with distilled water respectively for the last 7 days. The administration of alcohol resulted in a significant ($P < 0.05$) decrease in the specific activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and reduced glutathione (GSH) levels, while cholesterol (CHO), triglycerides (TAG), nitric oxide (NO) and malondialdehyde (MDA) concentrations were significantly increased when compared to the controls. Co-mobilization with *Aloe barbadensis* gel extracts for 7 days significantly reversed the deleterious effects of alcohol in the treated groups when compared to the alcohol group. This study indicated that *Aloe barbadensis* probably possesses anti-oxidative effects against alcohol–induced oxidative stress in rats.

Key words: alcohol; *Aloe barbadensis*; antioxidant; free radicals; lipid; oxidative stress

INTRODUCTION

Alcohol, which may be recognized as the most widely used psychoactive substances after caffeine is primarily metabolized in the liver to mainly acetaldehyde and generate toxic effects through the production of free radicals [16, 25, 31]. The acetaldehyde is further metabolized to acetate by acetaldehyde dehydrogenase (ALDH) and xanthine oxidase with the generation of some free radicals, namely superoxide radicals [27, 40, 46].

Free radicals are atoms with unpaired electrons in excited states which lead to further generation of more reactive oxygen species (ROS) and reactive nitrogen species (RNS) by chain reaction resulting in oxidative stress which has been implicated in the pathophysiology of many chronic diseases [39, 40, 46]. Although the production of free radicals is a normal physiological process, imbalances between its production and antioxidants system could lead to oxidative stress with the perturbation of various biological membrane functions and structural changes [23, 52]. Thus, there is a need for external antioxidant supplementation that could curb oxidative stress through the use of medicinal plants due to their therapeutic potentials.

Medicinal plants have gained tremendous interest of various researchers as an alternative, cheap and easily accessible regimen for the treatment of oxidative stress-related diseases [40, 42]. Despite this increased interest in the pharmacotherapeutic potentials offered by medicinal plants, only a few have received proper scientific scrutiny. Thus, *Aloe barbadensis* can be rightly considered as a plant of interest [1, 7, 11, 26].

Aloe vera (*Aloe barbadensis* Miller) is a perennial, succulent plant belonging to the Liliaceae family, which has been used as a source of food and medicine around the world for thousands of years [11, 40]. *A. barbadensis* is often referred to as a "healing" plant with the gel commonly used as an antioxidant gel in some industries [11, 26, 40]. The plant consists of two parts, the outer green rind including the vascular bundles and the parenchyma containing the gel, which consists of different types of polysaccharides that are responsible for its therapeutic properties [1, 11, 26]. Various studies have revealed that *A. barbadensis* gel possesses many pharmaceutical activities, including: antimicrobial, anticancer, anti-diabetic, antiulcer, hepato-protective, free radical scavenging and immune-modulatory activities [1, 3, 7, 38]. To the best of our knowledge, the physiological functions, as well as the mechanism through which lyophilized *A. barbadensis* gel extract exerts its antioxidative effect on alcohol-induced oxidative stress in male albino rats, are yet unclear and there has not been any established validated therapeutic antioxidant drug against alcoholic liver diseases, except therapeutic management regime [11, 40]. This study was, therefore designed to estimate the anti-oxidative potential of *A. barbadensis* on alcohol-induced oxidative stress in male albino rats by comparing it with only positive/normal control group using specific activities of; superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), as well as the concentrations of cholesterol (CHO), triglycerides (TAG), nitric oxide (NO), malondialdehyde (MDA) and reduced glutathione (GSH) in various tissues of animal models.

MATERIALS AND METHODS

Plant collection and authentication

Fresh aloe vera (*Aloe barbadensis* M.) plants were obtained from Pure and Applied botany garden and authenticated by a Botanist (Professor D. A. Agboola) in the Department of Pure and Applied Botany, Federal University of Agriculture, Abeokuta, Nigeria. The plant was identified and authenticated with herbarium number FUNAABH0028.

Extraction of aloe vera gel

The aloe vera plants were washed thoroughly with clean water, and then the inner parts (gel) of the leaves were removed into a clean container and blended using an electric blender. The blended gel was stored in a frozen state and lyophilized at Covenant University, Ota, Ogun State, Nigeria.

Experimental animals

The approval of the departmental animal ethical committee (FUNAAB-BCH) was obtained prior to the experiments. The rats were handled with care according to the guide for the care and use of laboratory animal's manuals [37]. Fifty-four male albino rats weighing between 180 and 250 g were purchased from the Department of Veterinary Medicine, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria for this study. The animals were acclimatized for two weeks before the commencement of the experiments. They were housed in plastic cages with good ventilation and were supplied with standard pellets and clean water ad libitum.

A. barbadensis lethal dose (LD50) test

Aloe vera gel extracts LD50 was determined according to the method described by Chinedu et al. [9]. Three rats each were used for the 3 stages of the experiment. Lyophilized aloe vera gel (1500 $mg.kg^{-1}$ body weight) was administered in the first stage and when no sign of toxicity

or death was seen, the dose (3000 mg.kg^{-1} body weight) was given to another 3 rats and then was increased to 5000 mg.kg^{-1} body weight when no death or sign of toxicity was observed in the second stage. Again when no sign of toxicity or death was seen at the third stage (final stage) of testing, it was concluded that the LD50 of the extract was more than 5000 mg.kg^{-1} body weight and hence had a high degree of safety [9].

Experimental design (Table 1)

Forty-five male albino rats weighing 180—250 g were first divided into two groups of 15 animals and 30 animals. The 15 animals were orally given 4 ml.kg^{-1} body weight of distilled water for the first two weeks (14 days) of the experiment, while the other 30 animals were orally administered 4 ml.kg^{-1} body weight of 50 % ethanol for the first two weeks (14 days) of the experiment. After the first two weeks, the 15 animals were further divided into three groups (A, E and F) of 5 animals each; group A served as the positive control and were further administered 4 ml.kg^{-1} body weight of distilled water for another one week (7 days); while group E and F were co-administered 125 mg and 250 mg.kg^{-1} body weight aloe vera gel extract with distilled water respectively for another one week (7 days) of the experiments. Twenty-four animals were selected from the 30 alcohol-treated group and 10 of the animals were further administered the same quantity and concentration of alcohol for another one week which served as the negative control group (B). The remaining 14 alcohol-treated animals were also divided into 2 groups (C and D) of 7 animals each and co-treated with 125 mg and 250 mg.kg^{-1} body weight aloe vera gel extract respectively after 30 minutes of alcohol administration for another one week (7 days) of the experiment (see groups description in Table below). On the 22nd day, 30 animals (5 each per group) were sacrificed after an overnight fasting under a light anesthesia. Blood samples were then collected into clean plain tubes, allowed to stand and clot for 15 minutes and centrifuged at 3000 rpm for 10 minutes to obtain the serum using the refrigerated centrifuge (BK-THRI16, Biobase Industry Shandong). The rats were then dissected and some tissues (kidney, liver and stomach, brain, testes and heart) were excised for biochemical examination, using 10 % homogenate.

Table 1. The groups of rats and their treatment (A—F)

Groups /Treatment	Groups
Distilled water for 21 days	A
50 % Alcohol for 21 days	B
50 % Alcohol (14 days) followed by alcohol + aloe vera (7 days)	C (125 mg.kg^{-1}) and D (250 mg.kg^{-1})
Distilled water (14 days) followed by alcohol + aloe vera (7 days)	E (125 mg.kg^{-1}) and F (250 mg.kg^{-1})

Biochemical assays

The malondialdehyde (MDA) concentration was determined according to the method of Fraga et al. [17]. The nitric oxide (NO) level was assayed by using Griess reagent as described by Sild and Horak [47]. The reduced glutathione (GSH) concentration was determined according to the method described by Moron et al [36]. The specific activities of glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), and catalase (CAT) were determined according to the methods described by Rotruck et al. [43], Calberg and Mannervick [5], Marklund and Marklund [34] and Hadwan and Abed [22] respectively. The isolation of HDL (high-density lipoproteins) and VLDL (very low-density lipoproteins) + LDL (low-density lipoproteins) from the serum was performed according the methods described by Gidez et al. [19]. Triacylglycerol (TAG) and cholesterol concentrations were determined in the serum and the lipoproteins (HDL and VLDL + LDL) isolated, using Randox standard Laboratory kits.

Statistical analysis

The results are presented as the mean ± standard error of the mean. The level of homogeneity among the groups were assessed using a one-way analysis of variance (ANOVA). Where homogeneity occurred, the Duncan test was used to differentiate between the groups. All analyses were done using SPSS (Statistical Package for Social Science version 20.0) with $P < 0.05$ considered statistically significant ($n = 5$).

RESULTS

***Aloe barbadensis* acute toxicity test (LD50)**

No sign of toxicity or death was observed at the three stages of the acute toxicity test of *A. barbadensis* (the rats were administered 1500 mg, 3000 mg and 5000 mg.kg^{-1}

body weight gel extracts respectively). Since no sign of toxicity or mortality was perceived at the maximum dose administered given, the LD50 of *A. barbadensis* is considered greater than 5000 mg.kg^{-1} body weight and hence has a high degree of safety [9].

Effects of *A. barbadensis* on the lipid peroxide levels

The extent of oxidative damage by alcohol consumption was assessed by determining the malondialdehyde (MDA) concentration as an index of lipid peroxidation level in all tissues examined. Significant ($P < 0.05$) increases were observed in the serum, brain, liver, heart, kidney, testes and stomach when compared to the controls (Fig. 1). Co-treatment with *A. barbadensis* resulted in marked ($P < 0.05$) reductions in the MDA levels respectively as compared to the alcohol-treated groups, with 125 mg.kg^{-1} body weight of extract causing 41.7 % (serum), 45.6 % (brain) and 43 % (testis) reduction in the respective tissues. The administrations of the aloe vera extract only showed little or no difference when compared with control groups.

Effects of *A. barbadensis* on nitric oxide (NO) levels

Fig. 2 shows the effects of the *A. barbadensis* on NO levels in the alcohol-intoxicated rats. There was a significant ($P < 0.05$) increase (4.5 %, 3.2 %, 3.6 %, 4.1 %, 5 %, 4.5 % and 4.5 %) in the serum, brain, liver, heart, kidney, testes and stomach respectively of the alcohol treated group (group B) when compared to the control (group A). However, *A. barbadensis* co-treatment significantly lowered the NO levels back to comparable level of the control (group A), while administration of aloe vera extract only showed little or no difference when compared with control (group A).

Effects of *A. barbadensis* on reduced glutathione (GSH) levels

The endogenous non-enzymatic antioxidant status of the cell was assessed using the reduced glutathione (GSH) concentration as an index following alcohol administration in rats as depicted in figure 3. Significant ($P < 0.05$) percentage decreases were observed in the concentrations of GSH in serum (35.9 %), brain (54.5 %), liver (27.9 %), heart (34 %), kidney (33.7 %), testes (64 %) and stomach (35 %) of the alcohol treated animals (group B) when compared to the control (group A). However, 125 mg.kg^{-1} body weight of *A. barbadensis* co-treatment caused 14.5 %, 55.7 %, 32.6 %, 67 % and 84.6 % increase in the serum, liver, heart, testis and stomach, respectively, while 250 mg.kg^{-1} body weight caused 86.8 % and 59 % increase in the brain and kidney respectively when compared to the alcohol treated group (group B).

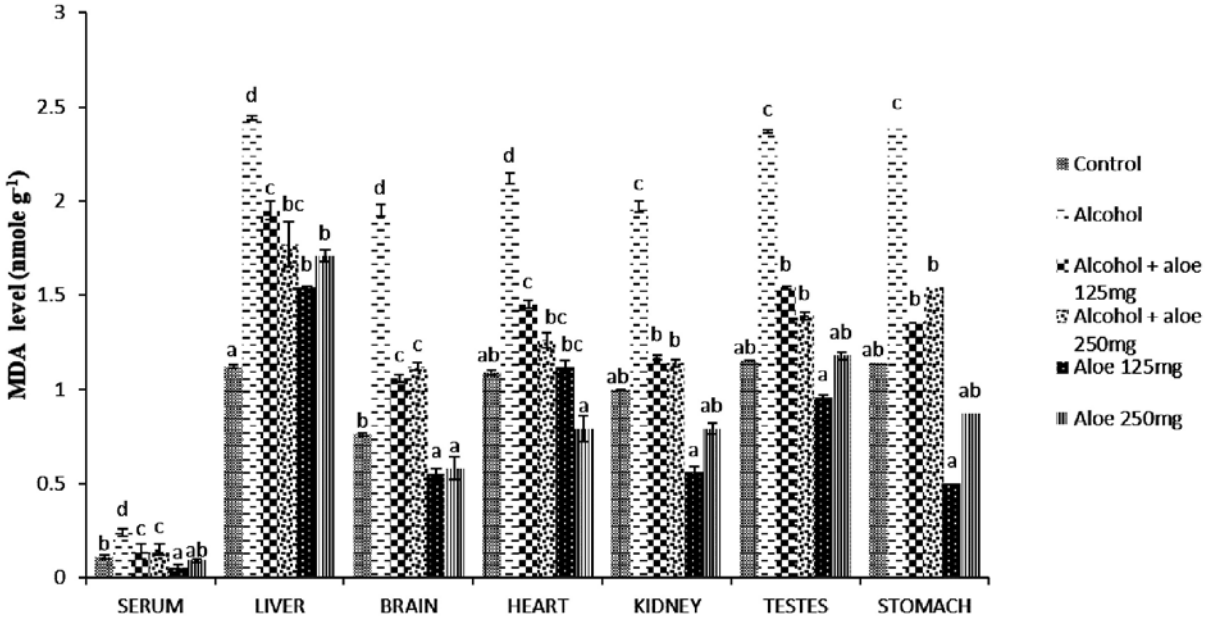

Fig. 1. Effects of *A. barbadensis* on MDA levels in alcohol intoxicated male albino rats
These data were expressed, as the mean ± SEM. The columns with different small alphabets at the top are significantly different at $P < 0.05$. Alcohol + aloe 125 mg—Alcohol + 125 mg.kg^{-1} body weight *A. barbadensis*; Alcohol + aloe 250 mg—Alcohol + 250 mg.kg^{-1} body weight *A. barbadensis* (n = 5)

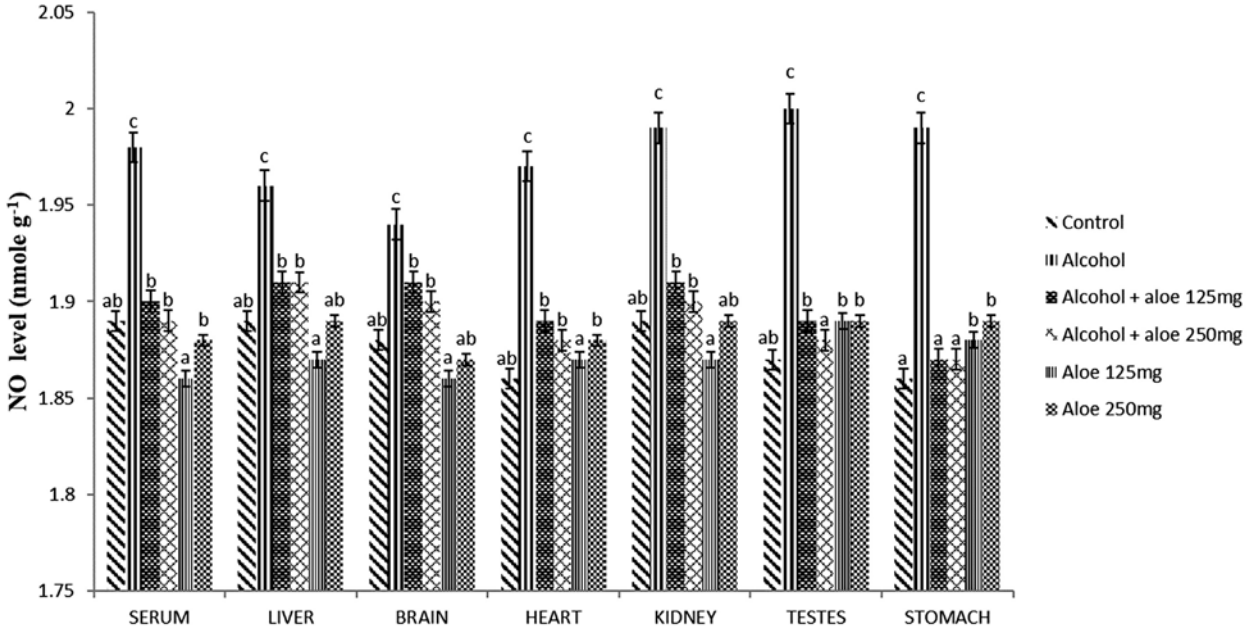

Fig. 2. Effects of *A. barbadensis* on NO levels in alcohol intoxicated male albino rats
These data were expressed, as the mean ± SEM. The columns with different small alphabets at the top are significantly different at $P < 0.05$. Alcohol + aloe 125 mg—Alcohol + 125 mg.kg^{-1} body weight *A. barbadensis*; Alcohol + aloe 250 mg—Alcohol + 250 mg.kg^{-1} body weight *A. barbadensis* (n = 5)

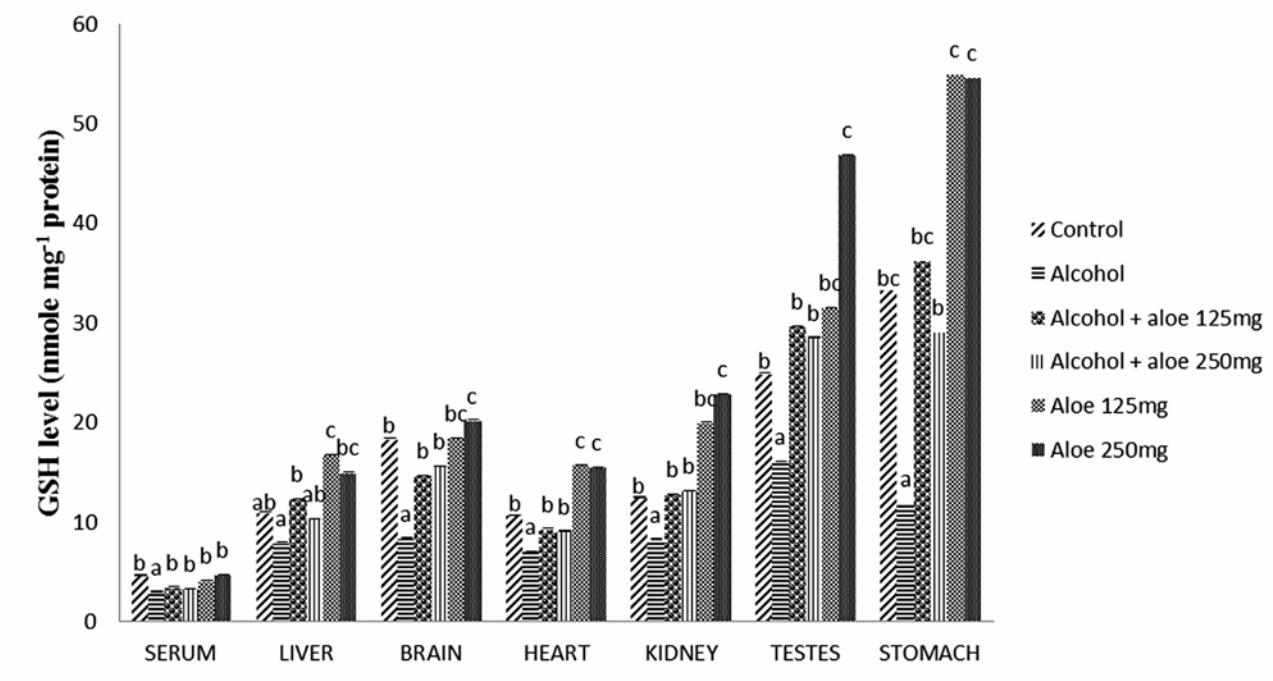

Fig. 3. Effects of *A. barbadensis* on GSH levels in alcohol intoxicated male albino rats
These data were expressed, as the mean ± SEM. The columns with different small alphabets at the top are significantly different at $P < 0.05$. Alcohol + aloe 125 mg—Alcohol + 125 mg.kg^{-1} body weight *A. barbadensis*; Alcohol + aloe 250 mg—Alcohol + 250 mg.kg^{-1} body weight *A. barbadensis* (n = 5)

Effects of *A. barbadensis* gel extract on serum, hepatic and renal antioxidant enzymes

The cellular enzymatic antioxidant status of the animals was estimated using the specific activities of SOD, CAT, GPx and GR in the serum, liver and kidney (Table 2). Note that GR was not done in the serum in Table 2. Alcohol ingestion resulted in a significant ($P < 0.05$) percentage decrease in serum SOD (40.7 %), CAT (31 %) and GPx (14.6 %) specific activities as compared to the control (group A) respectively. An estimated 34 %, 29 %, 34.8 % and 56.6 % decrease in the hepatic SOD, CAT, GPx and GR specific activities respectively as well as 15.9 %, 33 %, 79.9 % and 74.7 % decrease in renal SOD, CAT, GPx and GR respectively were observed. The observed decrease in the specific activities of these enzymes was significantly elevated in *A. barbadensis* co-administered groups in the investigated tissues. The extract (125 $mg.kg^{-1}$ body weight) elevated serum SOD, CAT and GPx specific activities by 71 %, 82.3 % and 10.8 %; while hepatic SOD, CAT and GPx were increased by 65 %, 95 % and 67 %; and renal SOD, CAT and GPx by 71.4 %, 64 % and 79.3 %, respectively, when compared to the alcohol-treated group (group B). The effect of the extract at 250 $mg.kg^{-1}$ body weight was not significantly different from that of the 125 $mg.kg^{-1}$ body weight dose.

Effects of *A. barbadensis* gel extracts on the brain and cardiac antioxidant enzymes.

Table 3 shows the effects of *A. barbadensis* on the brain and heart SOD, CAT, GPx and GR specific activities following alcohol and/or *A. barbadensis* treatments. The alcohol-treated group showed 20 %, 51.6 % and 38 % significant ($P < 0.05$) reduction in the brain SOD, CAT and GPx specific activities respectively. Cardiac specific activities were reduced by 30 %, 42 % and 59 % respectively when compared to the control. However, co-treatment with 125 $mg.kg^{-1}$ body weight of *A. barbadensis* caused an estimated 46 %, 99 % and 22.9 % increase in the brain SOD and CAT specific activities respectively; while the cardiac enzymes (SOD, CAT and GPx) specific activities elevated by 61 %, 42 % and 74.8 % respectively when compared to the alcohol-treated group. The effect of the 250 $mg.kg^{-1}$ body weight dose was not significantly different from the 125 $mg.kg^{-1}$ body weight dose, with exception only in the heart GR specific activities.

Effects of *A. barbadensis* gel extracts on testis and stomach antioxidant enzymes

Following alcohol administration, a significant ($P < 0.05$) decrease (39.9 %—SOD, 26.8 % CAT, 35 %—GPx and 37 %—GR) was observed in the testis while an estimated decrease

Table 2. Effects of *A. barbadensis* gel extract on antioxidant enzymes specific activities in serum, liver and kidney of alcohol-induced oxidative stress rats

Parameter		Control	Alcohol	Alcohol + aloe 125 mg	Alcohol + aloe 250 mg	Aloe 125 mg	Aloe 250 mg
Serum ($U.mg^{-1}$ protein)	SOD	0.59±0.03^{b}	0.35±0.02^{a}	0.60±0.03^{b}	0.58±0.02^{b}	0.69±0.02^{c}	0.74±0.02^{c}
	CAT	4.23±0.09^{b}	2.88±0.12^{a}	5.25±0.15^{b}	5.56±0.29^{b}	6.73±0.30^{c}	5.96±0.25^{c}
	GPx	3.89±0.20^{b}	3.32±0.12^{a}	3.68±0.21^{b}	3.42±0.17^{b}	3.57±0.08^{b}	4.06±0.25^{c}
Liver ($U.mg^{-1}$ protein)	SOD	11.35±0.37^{b}	7.49±0.39^{a}	12.37±0.74^{b}	11.21±0.32^{b}	16.45±0.43c	15.43±0.26^{c}
	CAT	83.78±2.40^{b}	59.37±2.88^{a}	116.0±6.64^{b}	112.0±3.39^{b}	93.37±2.69^{b}	126.0±2.99^{c}
	GPx	6.32±0.24^{b}	4.12±0.17^{a}	6.89±0.32^{b}	5.88±0.39^{b}	7.69±0.37^{c}	8.05±0.09^{c}
	GR	0.83±0.05^{b}	0.36±0.02^{a}	1.21±0.07^{b}	1.08±0.06^{b}	1.25±0.04^{b}	1.50±0.05^{c}
Kidney ($U.mg^{-1}$ protein)	SOD	11.75±0.45^{ab}	9.88±0.38^{a}	14.41±0.26^{b}	15.53±0.12^{b}	17.60±0.13^{b}	20.46±0.97^{c}
	CAT	173.0±1.59^{b}	115.7±5.69^{a}	190.3±2.52^{b}	184.8±8.75^{b}	213.5±7.59^{c}	217.4±7.51^{c}
	GPx	21.14±1.09^{b}	11.75±0.39^{a}	21.07± 0.56^{b}	21.87±0.59^{b}	30.41±2.07^{c}	29.23± 1.66^{c}
	GR	2.97±0.17^{b}	0.75± 0.03^{a}	2.81±0.13^{b}	3.31± 0.12^{b}	4.81±0.20^{c}	5.04±0.20^{c}

These data were expressed as the mean s ± SEM (n = 5). Values with different alphabets across the row for each parameter are significantly different at $P < 0.05$. Alcohol + aloe 125 mg—Alcohol + 125 $mg.kg^{-1}$ body weight aloe vera; Alcohol + aloe 250 mg—Alcohol + 250 $mg.kg^{-1}$ body weight aloe vera; Aloe 125 mg—125 $mg.kg^{-1}$ body weight aloe vera; Aloe 250 mg—250 $mg.kg^{-1}$ body weight aloe vera

Table 3. Effects of *A. barbadensis* extract on antioxidant enzymes specific activities in the brain and heart of alcohol-intoxicated male albino rats

Parameter		Control	Alcohol	Alcohol + aloe 125 mg	Alcohol + aloe 250 mg	Aloe 125 mg	Aloe 250 mg
Brain [U.mg^{-1} protein]	SOD	5.66±0.21^{ab}	4.51±0.14^{a}	6.61±0.38^{b}	7.15±0.41^{b}	8.17±0.31^{c}	9.07±0.56 c
	CAT	89.74±4.4^{b}	43.40±0.97^{a}	87.22±3.41^{b}	91.9±4.34^{b}	102.2±2.53^{b}	106.89±3.96c
	GR	1.86± 0.08^{ab}	0.73± 0.02^{a}	3.07± 0.06^{b}	2.75± 0.16^{b}	4.32±0.15^{c}	5.08±0.08c
	GPx	4.24±0.21c	2.62±0.16^{a}	3.22±0.20^{b}	3.02±0.13^{b}	4.35±0.21^{c}	4.65±0.22 c
Heart [	SOD	8.38± 0.28^{b}	5.83±0.29^{a}	9.43± 0.27^{b}	9.51±0.26^{b}	12.66±0.5^{c}	11.73±0.71 c
	CAT	115.0±7.20^{b}	66.18±2.25^{a}	117.8± 2.96^{b}	123.5±7.31^{b}	121.2±5.68^{c}	136.3±1.29 c
	GR	7.43±0.14^{b}	4.66±0.15^{a}	8.15± 0.27^{b}	6.90± 0.23^{b}	10.42± 0.34^{c}	10.06± 0.27 c
	GPx	0.58±0.04^{b}	0.16±0.01^{a}	0.91±0.02^{c}	0.83±0.03^{b}	0.89±0.04^{c}	0.92±0.02c

These data were expressed as the means ± SEM (n = 5). Values with different number of alphabets across the row for each parameter are significantly different at p < 0.05. Alcohol + aloe 125 mg—Alcohol + 125 mg.kg^{-1} body weight *A. barbadensis*; Alcohol + aloe 250mg—Alcohol + 250 mg.kg^{-1} body weight *A. barbadensis*; Aloe 125 mg—125 mg.kg^{-1} body weight; Aloe 250 mg—250 mg.kg^{-1} body weight aloe vera

Table 4. Effects of *A. barbadensis* extracts on antioxidant enzymes specific activities in the testis and stomach of alcohol-intoxicated male albino rats

Parameter		Control	Alcohol	Alcohol + aloe 125 mg	Alcohol + aloe 250 mg	Aloe 125 mg	Aloe 250 mg
Testis [U.mg^{-1} protein]	SOD	36.21±2.01^{b}	21.75±0.97^{a}	47.78±1.23^{b}	44.20±1.21^{b}	58.64±2.19^{c}	65.10±2.71^{c}
	CAT	352.9±9.78a^{b}	258.1±16.0^{a}	432.9±9.64^{b}	433.7±6.37^{b}	437.2±5.37^{b}	452.5±11.01^{c}
	GPx	34.96±1.24^{b}	26.59±1.46^{a}	42.04±0.24^{bc}	33.77±1.81^{b}	50.60±1.24^{c}	52.51±0.77^{c}
	GR	21.57±1.31^{b}	13.58±0.42^{a}	29.35±0.94^{bc}	28.01±0.79^{bc}	39.67±1.88^{c}	37.01±1.66^{c}
Stomach [U.mg^{-1} protein]	SOD	36.21±2.01^{ab}	21.75±0.97^{a}	47.78±1.23^{b}	44.20±1.21^{b}	58.64±2.19^{c}	65.10±2.71^{c}
	CAT	364.2±9.38^{b}	235.1±5.92^{a}	446.1±11.63^{bc}	360.2±13.37^{b}	474.1±10.03^{c}	524.8±5.11^{c}
	GPx	38.40±1.21^{ab}	29.17±1.73^{a}	55.55±1.46^{b}	53.57±1.09^{b}	76.17±0.95^{c}	74.95±1.55^{c}
	GR	14.41±0.36^{b}	6.16±0.14^{a}	15.07±0.33^{b}	15.72±0.36^{b}	15.54±0.69^{bc}	18.04±0.49^{c}

These data were expressed as the means ± SEM (n = 5). Values with different alphabets across the row for each parameter are significantly different at P < 0.05. Alcohol + aloe 125 mg—Alcohol + 125 mg.kg^{-1} body weight *A. barbadensis*; Alcohol + aloe 250 mg—Alcohol + 250 mg.kg^{-1} body weight *A. barbadensis*; Aloe 125 mg—125 mg.kg^{-1} body weight; Aloe 250 mg—250 mg.kg^{-1} body weight aloe vera

of 37.9 %, 35 %, 31.6 % and 57 % manifested in the stomach SOD, CAT, GPx and GR of the rats respectively when compared to the control. Co-treatment with *A. barbadensis* (both doses) however caused a significant (P < 0.05) increment in the testis and stomach SOD, CAT, GPx, and GR specific activities respectively as compared to the alcohol group (Table 4).

Effects of *A. barbadensis* on serum and lipoproteins TAG concentration

Figure 4 depicts the effects of *A. barbadensis* extracts on the serum triacylglycerol (TAG), HDL-TAG and VLDL+LDL-TAG in alcohol-intoxicated rats. Alcohol ingestion caused a significant increase in the serum TAG (hypertriglyceridemia) while there were no significant differ-

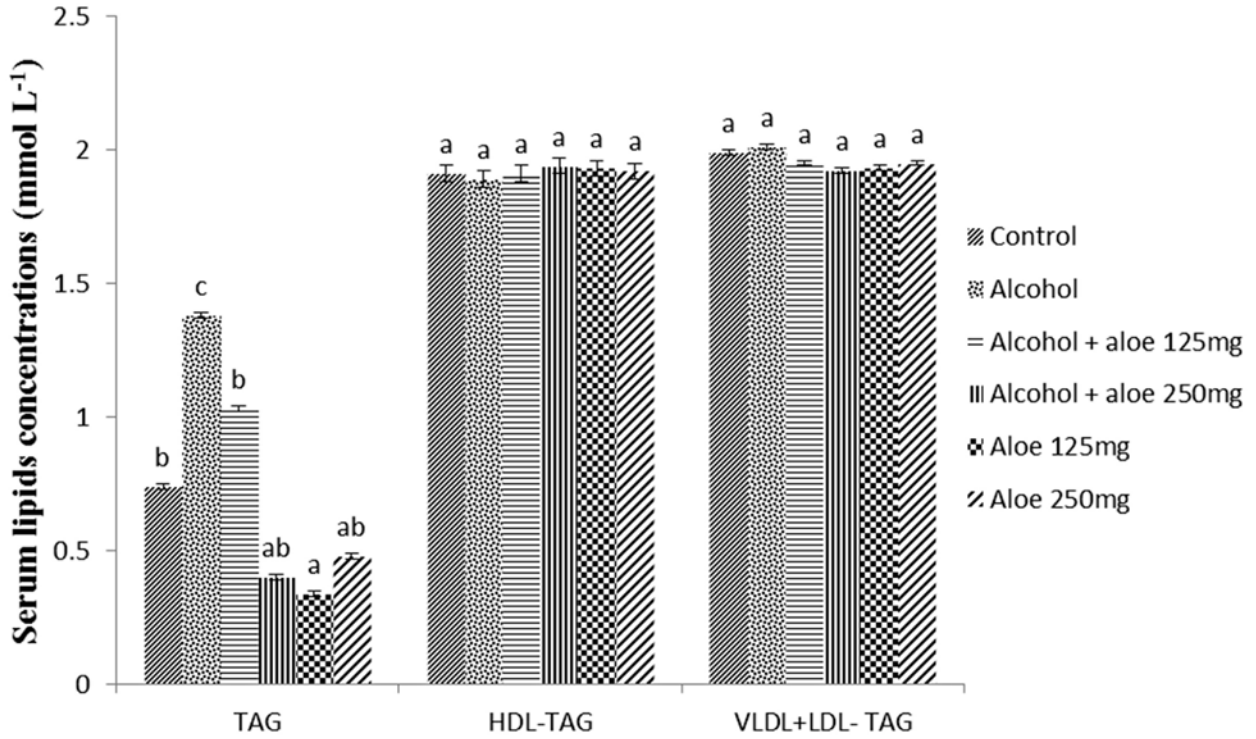

Fig. 4. Effects of A. barbadensis on serum TAG, HDL-TAG and VLDL+ LDL-TAG levels in alcohol-intoxicated male albino rats
These data were expressed as the means ± SEM. Column with different small alphabets at the top are significantly different at $p < 0.05$. Alcohol + aloe 125 mg—Alcohol + 125 mg.kg^{-1} body weight *A. barbadensis*; Alcohol + aloe 250 mg—Alcohol + 250 mg.kg^{-1} body weight *A. barbadensis* (n = 5)

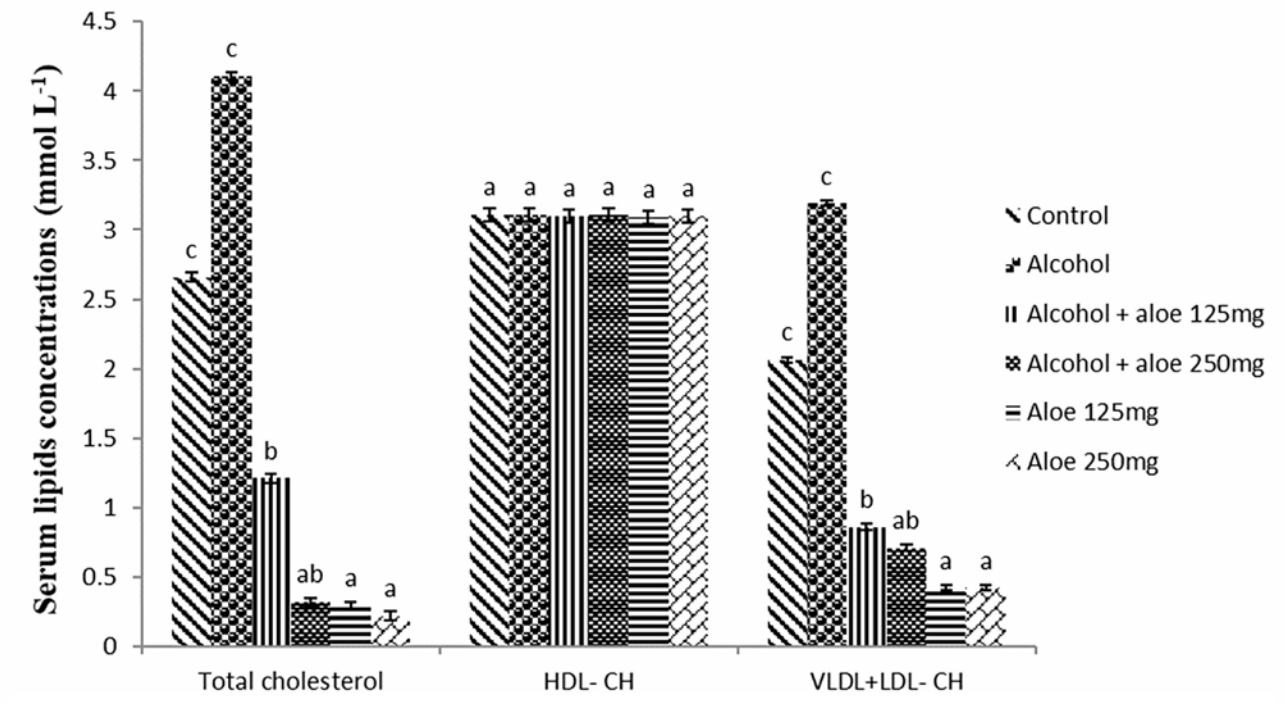

Fig. 5. Effects of *A. barbadensis* on serum cholesterol, HDL-cholesterol and VLDL + LDL-cholesterol levels in alcohol-intoxicated male albino rats
These data were expressed, as the means ± SEM. The columns with different small alphabets at the top are significantly different at $P < 0.05$. Alcohol + aloe 125 mg—Alcohol + 125 mg.kg^{-1} body weight *A. barbadensis*; Alcohol + aloe 250 mg—Alcohol + 250 mg.kg^{-1} body weight *A. barbadensis* (n = 5)

ence in the HDL-TAG and VLDL + LDL-TAG level when compared to the control. The *A. barbadensis* co-mobilized groups (C and D) showed a significant ($p < 0.05$) decrease in the serum TAG levels respectively when compared to the alcohol-treated group. However, the 250 mg.kg^{-1} body weight dose was more effective.

Effects of *A. barbadensis* on serum and lipoprotein cholesterol concentrations

The effects of *A. barbadensis* extracts on the serum cholesterol, HDL-cholesterol and VLDL + LDL-cholesterol in alcohol-intoxicated rats are shown in Figure 5. Alcohol ingestion caused a significant increase in the serum and VLDL + LDL-cholesterol (hypercholesterolemia) while there was no significant difference in the HDL-cholesterol level when compared to the control. The *A. barbadensis* co-mobilized groups showed a significant ($P < 0.05$) decrease in the serum cholesterol and VLDL + LDL-cholesterol levels when compared to the alcohol-treated group respectively, with the 250 mg.kg^{-1} dose proving to be more effective.

DISCUSSION

Cytochrome P450 2E1 (CYP 2E1)—an inducible xenobiotic metabolizing enzyme which is responsible for the metabolism of alcohol in many tissues produces acetaldehyde and at the same time reduces dioxygen to a variety of reactive oxygen species (ROS) such as superoxide anions, hydroxyl and hydroxyethyl radicals [15, 30, 33]. These radicals and lipid peroxides could lead to oxidative stress and inflammation especially when their production overwhelms the antioxidant systems' ability to eliminate them from the system [8, 30, 46]. Malondialdehyde (MDA) and nitric oxide (NO) levels are some of the indices used to measure the degree of peroxidative damage caused by ROS and reactive nitrogen species (RNS) which is capable of altering the membrane structure and function [15, 42]. Reduced glutathione (GSH), an antioxidant and a powerful nucleophile is critical for cellular protection in detoxification of ROS, conjugation and excretion of toxic molecules and control of inflammatory cytokine cascade [4, 42]. The present study sought to evaluate the toxic effects impacted on various tissues of the body by alcohol and possible ameliorative prowess of *A. barbadensis* Miller on such damages.

Our study further confirmed that administration of alcohol resulted in significant ($P < 0.05$) increments in the serum, hepatic and renal MDA and NO levels as well as a marked decrease in GSH concentrations when compared to the control and similar results have been reported by many authors [11, 15, 30, 41, 42]. These increased NO levels observed in this study might be due to inflammation which is seen in alcoholics as a result of inducible nitric oxide synthase (iNOS) that mediates the synthesis of pro-inflammatory cytokines such as IL-1, IL-2, IL-6, and TNF-α and in turn stimulate the synthesis of NO [35, 41]. Similarly, high levels of MDA as seen in the brain especially and other tissues might be due to the presence of easily peroxidizable fatty acids in their membranes [6]. The GSH depletion noticed in various examined organs of alcohol-intoxicated rats might be due to increased utilization of GSH for conjugation processes or the inability of the liver to synthesize it from its precursors [13, 46, 51]. Co-treatment with aloe vera gel extract (125 mg and 250 mg.kg^{-1} body weight) significantly enhanced the antioxidant system by lowering elevated levels of MDA and NO and causing a concomitant increment in the level of GSH. This attenuation of oxidative stress markers might be due to the ability of the gel extracts to alleviate lipid peroxidation and scavenge free radicals produced following alcohol metabolism which could be attributed to the acetyl groups and reductive nature of the monosaccharide in the gel extracts as reported by Chun–hui et al. [10] and Cui et al. [11].

Superoxide dismutase (SOD) catalyzes the dismutation of superoxide anions into hydrogen peroxide (H_2O_2) and oxygen (O_2); the H_2O_2 is then converted by catalase (CAT) or by glutathione peroxidase (GPx) to water (H_2O) and oxygen. The GPx uses GSH in producing an oxidized glutathione (GSSG) which is then converted back to GSH by glutathione reductase (GR) using NADPH—a cyclic antioxidant processes occurring in the cell [42, 46]. Significant reductions in the specific activities of SOD, CAT, GPx and GR in all examined tissues following alcohol administration indicates oxidative stress which might be as a result of free radicals generation [46]. These free radicals are capable of inducing lipid peroxidation by either the inactivation of enzymes or depletion of the non-enzymic antioxidants, such as GSH and NADPH [42]. The improved antioxidant status of the *A. barbadensis* co-treated groups (125 mg and 250 mg.kg^{-1} body weight) might be due to the active polysaccharides in the gel as reported by Cui et al. [11]. This polysaccharide inhibits the activation of NF-kB following

stimulation by alcohol via up-regulation of the IkB-alpha leading to lowered stimulation of hepatic inflammation and oxidative stress [11, 24, 48]. Therefore, *A. barbadensis* gel extracts might be considered a potential regimen against alcohol-induced oxidative stress and inflammation.

Lipids and lipoproteins abnormalities have been shown to play a major role in the pathogenesis and progression of various disease conditions [20, 21]. High cholesterol is a leading risk factor for human cardiovascular disease such as coronary heart disease and stroke, which has led to US recommendations to reduce cholesterol intake [32]. The triacylglycerol (TAG) are the main constituent of fats in both plants and animals; their presence in the blood enable the bidirectional transference of adipose fats and blood glucose from the liver [18, 29]. A lipoprotein is a compound containing both lipids (fat) and protein [49]. They include LDL (low density lipoprotein) often referred to as bad cholesterol that conveys cholesterol from the liver to the extra-hepatic tissues, while HDL (high density lipoprotein) is often referred to as good or happy cholesterol, which is responsible for the reverse cholesterol transport—a process of absorbing cholesterol from the extra-hepatic tissues back to the liver where it under goes catabolic processes thus preventing cholesterol accumulations in those tissues [49, 50].

Alcohol ingestion resulted in elevated levels of TAG (only in the serum) and cholesterol in the serum and VLDL + LDL when compared to the control. Chronic alcohol consumption increases the hepatic NADH/NAD+ ratio, which in turn suppresses mitochondrial β-oxidation of fatty acids and stimulates de novo lipogenesis, thus causing lipid accumulation in hepatocytes which in turn is transported into the blood stream and may be responsible for the observed elevation in TAG and cholesterol in serum and VLDL + LDL [11, 14]. Co-treatment with *A. barbadensis* gel extract reverted the elevated lipids back to normal as compared to alcohol group (group B). This reduction in the lipid level by *A. barbadensis* extracts might be as a result of up-regulation of AMP dependent protein kinase (AMPK-α2) gene expression and down-regulation of sterol regulatory binding protein -1c (SREBP-1c) as reported by C u i et al. [11]. AMPK-α2, a key regulator of lipid metabolism in the liver, is responsible for the inactivation of acetyl-CoA carboxylase (ACC), which results in increased fatty acid oxidation in liver, while SREBP-1c particularly stimulates fatty acid synthesis through up-regulation in the transcription of lipogenic enzymes [2]. This suggests that *A. barbadensis* might possess anti-hyperlipidemic effects which may be attributed to the presence of aloe vera gel polysaccharides (AVGP) as reported by C u i et al. [11] that supplementation of AVGP significantly restored the decreased AMPK-α2 gene expression to a near-normal level.

Many bioactive compounds with different types of molecules and diverse structures from aloe vera gel have been isolated and reported to be responsible for the beneficial effects of the plant by previous researchers [10, 11, 26, 28, 44, 45]; to mention a few are oleic acid, 11,14-eicosadienoic acid—methyl ester, n-hexadecanoic acid, 1,2-benzenedicarboxylic acid—butyloctyl ester, hexadecanoic acid—methyl ester, tetradecanoic acid, 1,2-benzenedicarboxylic acid — diisooctyl ester, squalene, anthraquinones and polysaccharides (primarily acemannan). D e b n a t h et al. [12] also reported that high levels of vanillic acid detected in aloe vera might be responsible for their strong antioxidant activities against DPPH (1,1-diphenyl-2-picryl-hydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), hydroxyl, and superoxide radicals. This study further confirmed the *in vivo* beneficial and pharmacological effects of lyophilized *A. barbadensis* with its less toxicity level at the tested dosages. However, the mechanisms by which this extract confined their beneficial and pharmacological effects are still not established at present and will be subjected to further investigations.

CONCLUSIONS

The results obtained in this study indicate that *A. barbadensis* gel extracts attenuated the alcohol–induced oxidative stress in various organs by enhancing their antioxidative status and also possess the ability to reverse dyslipidemia induced by alcohol. It is of note that the two doses investigated have different way they modulate the parameters and the effects were not much different from each other; although 125 mg.kg^{-1} body weight of *A. barbadensis* appeared to be the therapeutic dose as far as this study was concerned. Therefore, *Aloe barbadensis* probably possesses the ability to alleviate alcohol-induced oxidative stress and dyslipidemia in experimental rats.

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THE EFFECTS OF CLINOPTILOLITE ADMINISTRATION ON THE APPETITE, THE CONSISTENCY OF FAECES AND THE HISTOLOGY OF THE SMALL INTESTINE IN GROWING PIGS

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ABSTRACT

Clinoptilolite (Cp) is the most common and suitable natural zeolite type for many commercial and industrial applications. Recent studies have also shown a high potential of clinoptilolite in various medical applications. The aim of our study was to evaluate the effect of long-term peroral administration of clinoptilolite on appetites, the consistency of faeces, and the histopathology of the intestines of growing pigs. Fourteen Landrace × Large White crossbred pigs of both genders, a few days after weaning (12.95 kg b. w.), were divided into two equal groups. The control group was fed with a basal feed mixture, and the experimental group with a feed mixture supplemented with 2 % of natural zeolite (the commercial preparation "ZeoFeed"). The appetite, clinical state and consistency of the faeces were assessed every day. The blood samples were collected on days 0, 21, and 42 of the experiment. Histological examinations of the intestines from the control and experimental animals were carried out at the end of the experiments. The supplementation of 2 % Cp did not affect neither the appetite nor the clinical state of the pigs. The faecal consistency score in the experimental animals was 18.82 % lower than that of the control piglets. The histopathological evaluations showed protective evidence of the Cp on the intestinal tract wall in the duodenum and jejunum.

Key words: appetite; faeces; growing pigs; zeolite clinoptilolite

INTRODUCTION

Clinoptilolite is a microporous natural form of zeolite which has a three dimensional dense network of aluminosilicates, characterized by plentiful internal spaces in the form of channels and cavities with an internal negative charge, which provides its specific absorptive and ion-exchanging properties. The cavities may entrap molecules of different solids, liquids or gaseous substances, for example: methane, carbon dioxide, ammonia, steam, etc. [22]. Due to its structure and properties, this natural inert and non-toxic material can be used as a slow-release carrier of fer-

tilizers, as well as other agrochemical, pharmaceutical and biochemical active compounds including disinfectants. Natural zeolite can also be used to improve the physical properties of soils and to treat contaminated soils [17].

The specific physicochemical properties of clinoptilolite are the prerequisite for its use also in animals [22]. In veterinary medicine, the supplementation of Cp can be used to protect against ammonia toxicity by binding ammonia *in vivo* [10, 20]. Clinoptilolite also has the ability to bind some polar mycotoxins, especially aflatoxins [18, 19, 24]. Moreover, it provides some protection against zearalenone [16]. Cp is also capable to act as: an antioxidant, haemostatic, anti-diarrheic, growth-promoting and immunostimulating agent [3, 4, 25, 30]. Zeolites improve the ability to resist some diseases, promote some positive effects as an antioxidant and may have antiviral attributes [12].

Despite extensive use of clinoptilolite, the mechanisms underlying the action of Cp in its natural or modified forms are still unclear [7]. For instance, further research is needed to elucidate the direct effects of zeolite on the morphology and physiology of the small intestine [29].

The aim of this study was to evaluate the effects of infeed inclusion of a natural zeolite (clinoptilolite) on the appetite, consistency of faeces, and the histological structure of the intestines in growing pigs.

MATERIALS AND METHODS

Animals

Fourteen clinically healthy pigs (crossbreed Large White × Landrace) of the same age (36 days), with an average body weight of 12.95 kg were randomly divided into two equal groups: experimental and control.

Experimental design

In the experiments lasting 6 weeks, the control pigs were fed with a commercially prepared dry mixture recommended for this category of animals (OŠ-02, Poľnonákup Šariš a. s., Prešov, Slovakia). The experimental pigs were fed the same dry feed mixture supplemented with 2 % of a commercially preparation "ZeoFeed" (natural zeolite—type clinoptilolite) prepared by the company Zeocem a. s. (Bystré, Slovakia). Its mineralogical and chemical composition is shown in Table 1. Feed and water were available *ad libitum*. The animals were housed under standard housing indoor conditions on a solid floor at the Clinic for swine of the UVMP in Košice. The appetite, clinical state and consistency of faeces were evaluated each day after morning feeding. Venous blood was sampled from the ophthalmic sinus on days 0 21, and 42 of the experiments.

Diagnostic testing

The concentrations of leukocytes (Lc) were measured using an animal blood counter (abc TM Vet.).

Assessment of the consistency of faeces

A five-point scale was implemented: 1—Solid; 2—Paste; 3—Thin; 4—Watery; 5—Admixtures (blood, mucus).

Histological examinations

After termination of the experiments the animals were euthanized. The intestinal tissue samples were flushed with a physiological saline, fixed in 10 % formalin, dehydrated in ethanol solutions with increasing concentration, and embedded in paraffin. The 5μm thick sections were prepared, stained with haematoxylin and eosin and examined under a light microscope Zeiss Axio Lab A1, and documented with a camera Axio Cam ERc 5.

Statistical processing of results

The statistical processing of the results was performed by assessment of means (x) and standard deviations (SD) in both groups of pigs. The significance (P) of differences in the means of corresponding variables were evaluated by the Student's t-tests.

RESULTS

The appetites of the animals during the entire period of the experiments were excellent without any apparent changes. It was determined that all of the pigs had their body temperature, pulse and respiratory rate in the normal physiological ranges (39.3 ± 0.3 °C; respiration 25—40; and pulse 80—100). A comparison of the concentration of white blood cells (Table 2) with the reference range (11.0—18.0 $G.l^{-1}$) [28] indicated a leucocytosis in the control group at the 1st and 2nd collection, while in the experimental group a leucocytosis was seen only at the 2nd collection.

The most liquid faeces were observed up to five days after the beginning of the experiments in the control group.

On the other hand, the faecal consistency score was relative stabile throughout the experiment in the Cp treated group.

The histopathological changes were detected in the duodenum and jejunum. The mucosa and submucosa of these segments of the small intestine of the both control and experimental animals revealed apparent inflammatory infiltrations. The infiltrates occurred in the lamina propria mucosae, tela submucosa and in the epithelial lining. They consisted mainly of lymphocytes, but also some neutrophils, macrophages, and plasma cells at different locations were seen. The mucosa of the duodenum and jejunum in the control animals revealed diffuse degenerative histopathological changes. The intestinal villi were reduced in size, mostly disintegrated and completely devoid of the epithelial covering. The general morphology of the Lieberkűhn crypts as well as other deeper layers of the intestinal wall were not affected. On the contrary, in the experimental group, the intestinal villi were comparatively larger, mostly regular in shape, and covered by the continuous epithelium (Fig. 2).

Table 1. Mineral and chemical composition of ZeoFeed clinoptilolite

Parameter	Content
Clinoptilolite of sediment origin	Not less than 80 %
Clay minerals	Not more than 20 %
Particle size	0.01—0.2 mm
Loss on drying	≤ 6 %
SiO_2	62—73 %
Al_2O_3	11—14 %
Si: Al ratio	4.8—5.40
CaO	2—5.50 %
Na_2O	0.2—1.5 %
Fe_2O_3	0.7—2.3 %
K_2O	2.2—3.4 %
MgO	0.5—1.2 %
TiO_2	0.1—0.3 %

DISCUSSION

The supplementation of 2 % Cp during forty-two days of experiment did not influence the appetite or the clinical state of the pigs. It is in accordance with the studies by Prvulović et al. [15] and Papaioannou et al., [12] where Cp ingestion was well tolerated by pigs. Several

Table 2. Concentration of white blood cells in $G.l^{-1}$ (x ± SD)

Sampling Day	0	1st	2nd
Experimental group	16.83 ± 3.02	16.95 ± 0.82	20.65 ± 2.13
Control group	16.28 ± 4.33	22.45 ± 4.01	23.13 ± 4.81

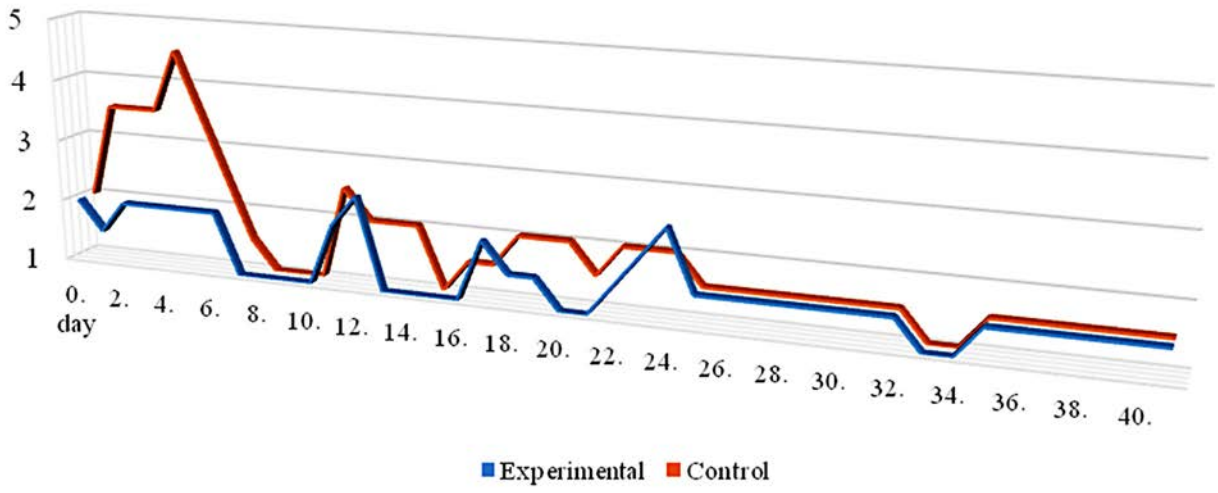

Fig. 1. Faecal consistency score

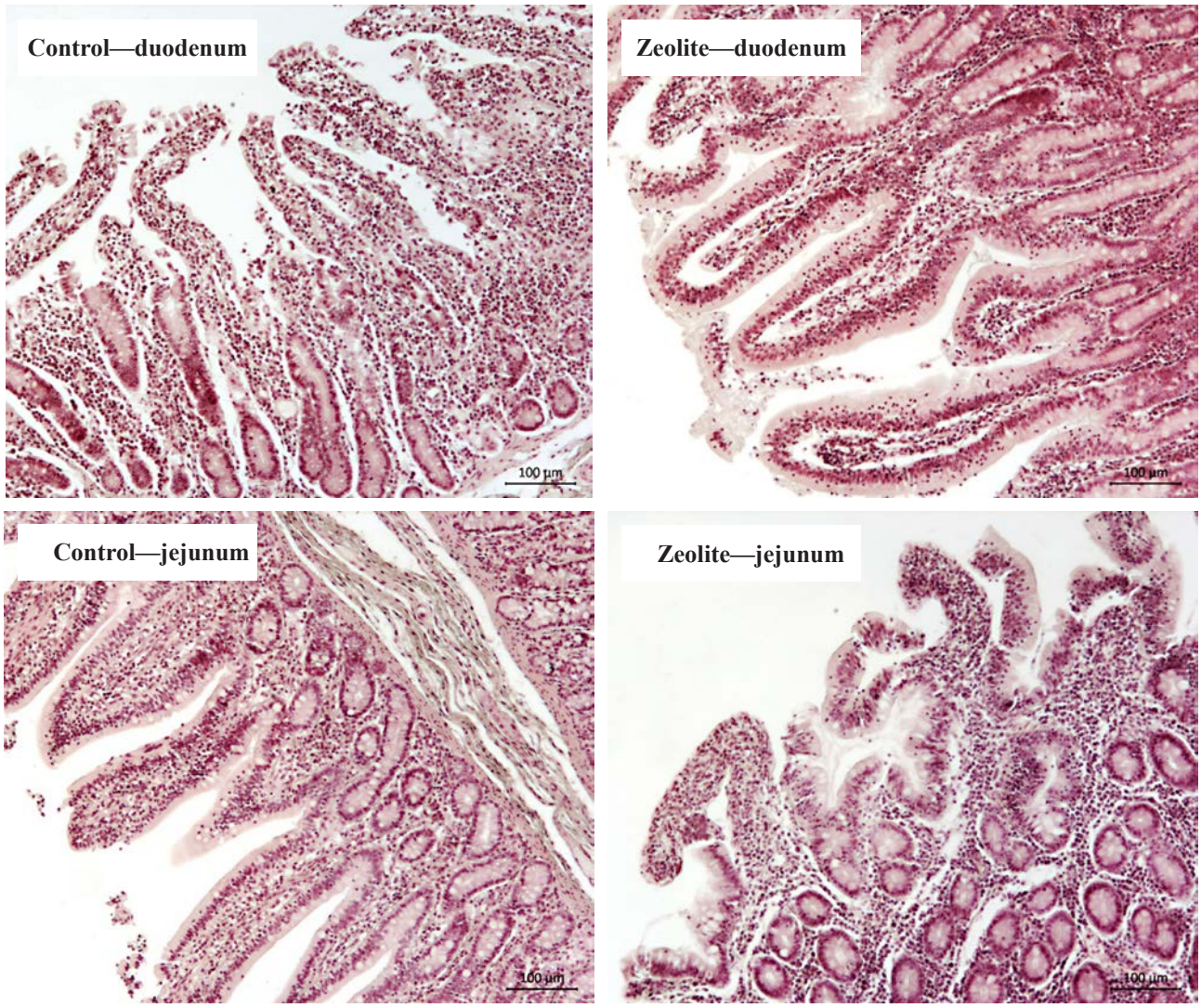

Fig. 2. Representative microphotographs showing histopathological changes and inflammatory infiltrations of duodenum and jejunum, haematoxylin—eosin. Magn. ×100

toxicological studies as well as haematological, biochemical and histopathological analyses dealing with natural Cp proved that this compound is non-toxic and safe for use in human and veterinary medicine [1, 15, 21, 26].

The supplementation of Cp to the basal diets in our experiment decreased the faecal consistency score. It was by 18.82 % lower (more solid faeces) than in the non-treated control piglets. In a similar five-week study [25] of Cp supplementation, there was recorded about 12.96 % decrease in diarrhoea severity score compared to non-treated piglets. The action of natural zeolite in the digestive system is interpreted as prolongation of the retention time of digesta in the gut of pigs [11]. Similarly, P r v u l o v i ć et al. [15] described a longer retention time of the digesta in the gut of pigs, slower passage of ingesta through the gastrointestinal tract and increased microbial activity in the small intestine leading to better utilization of nutrients. Also, the water adsorption properties of the natural zeolites resulted in drier and more compact faeces [13].

Pigs in the early phases of growth, up to around 25 kg live weight, tend to be less tolerant to feed ingredients of low digestibility and thus in this age group, the feed specification is more critical. Profound physiological changes occur as a result of abrupt weaning, notably a change of high pH to low pH in the stomach; a change in the profile of digestive enzymes, particularly with respect to dealing with starch. At the same time, stunting of the intestinal villi reduces the absorptive capacity in the ileum. These changes render the gut more susceptible to pathogens [6]. P a p a i o a n n o u et al., [13] found that Cp zeolite can adsorb dietary substances linked to intestinal hypersensitivity and restore digestive enzyme activity.

The incidences of diarrhoea have been reported as 32 % [2] and 39 % [5] for the period from weaning up to 14 days post-weaning. Nabuurs [8] reported an incidence of diarrhoea of 40 % during the first, 69 % during the second and 50 % during the 3rd post-weaning week of piglets reared under commercial conditions. It would appear that the piglets in this study showed extensive diarrhoea, but it should be noted that the faeces score in this study was not equivalent to overt diarrhoea [27]. In our experiments, the most liquid faeces—thin to watery consistency—was observed up to five days after the beginning of the experiment (day 7 post-weaning) in the control group. On the contrary, denser, pasty consistency in the experimental group was observed in parallel. The consistency of faeces did not stabilise up to day 25 of the experiment (day 27 post-weaning).

The published data provides evidence that piglets with more liquid faeces have shorter intestinal villi, which may reflect that shorter villi result in decreased faeces consistency [27]. Nabuurs et al. [9] showed on a herd level that the mean villus height in diarrhoeic herds was relatively lower than in the specific pathogen-free (SPF) herds. The villus height and crypt depth may influence the pathogenesis of post-weaning diarrhoea, as suggested by Nabuurs et al. [9], through the absorptive and secretive properties of small intestine enterocytes [14]. Our histological examination showed more severe changes in the duodenum and jejunum of the control group. These changes could be associated with leucocytosis recorded on days 21 and 42 of the experiment. On the other hand, the experimental group (leucocytosis on day 42 of the experiment) had comparatively larger intestinal villi that were regular in shape and were covered by continuous epithelium. These morphological results in the small intestine after supplementation of Cp are in accordance with the studies of Subramaniam and Kim [23], and also Wawrzyniak [29] where dietary zeolite caused morphological changes in the intestinal villi and improved intestinal function in pigs and in female broilers. An increase in the villus height enhances the surface area for nutrient absorption thus it increases nutrient digestibility [23].

The most important factors for clinoptilolite performance are the concentration, purity and type of supplemented clinoptilolite as well as the growth phase of animals [15].

Our experiment allowed us to conclude that the dietary addition of 2 % of natural zeolite clinoptilolite affected positively the faecal consistency score and histological findings in duodenum and jejunum of growing pigs.

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OVARIAN ACTIVITY AND THE SIZE OF OOCYTES IN OVARIAN FOLLICLES IN SEXUALLY MATURE GILTS IN INDIVIDUAL SEASONS

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ABSTRACT

The aim of this study was to evaluate ovarian activity and the size of oocytes in ovarian follicles in sexually mature Landrace-Yorkshire gilts in relation to the individual seasons of the year. The study was carried out on 240 gilts slaughtered at an abattoir during the four yearly seasons. The size and weight of the ovaries, the number of follicles and corpora lutea (CL) according to individual size categories were evaluated. The oocytes were aspirated from follicles and their sizes were measured. Our evaluation of the size of the ovaries showed that they were the largest in autumn, when their mean length reached 25.8 ± 3.4 mm, while in winter their mean length was 24.2 ± 2.9 mm. The smallest weight of the ovaries was determined in autumn (mean 5.7 ± 1.4 g) and the highest in spring (mean 6.2 ± 2.2 g). The largest number of follicles in the ovaries of the gilts was recorded during the autumn months, with a predominance of follicles up to 3 mm (mean number 17.9 ± 7.5). The largest number of corpora lutea was observed in spring (mean number 12.1 ± 2.6) and the smallest in winter (mean number 6.1 ± 1.1). The oocytes from follicles of up to 3 mm size, were the smallest in spring (mean size $16.99 \times 10^3 \pm 3.42 \times 10^3$ μm²) and the largest in winter (mean size $18.90 \times 10^3 \pm 2.99 \times 10^3$ μm²). In total, the largest oocytes were aspirated from 4—6 mm follicles in autumn (mean size $19.60 \times 10^3 \pm 5.37 \times 10^3 \pm$ μm²). The values recorded indicated that the seasons affected the ovarian activity and the growth of oocytes in gilts.

Key words: follicles; oocytes; season, sexually mature gilts

INTRODUCTION

The successful reproductive performance of gilts and sows are based upon the active functioning of their ovaries, characterised by intensive folliculogenesis. Numerous growing and ripening ovarian follicles ensure conditions for favourable conception abilities of gilts and sows and are an optimum source of oocytes for the use in embryo transfers.

Folliculogenesis is the process of the development of follicles that are the basic structures of mammalian ovaries. It is one of the most important phases of development of the female reproductive organs that commences already during their foetal life [12]. Extensive studies have shown that after reaching sexual maturity, folliculogenesis involves ripening and ovulation of minimally 14—20 follicles during each subsequent oestrous cycle. However, every population of these follicles is morphologically and biochemically heterogenic. The differences in the number of granulosa cells, activation of lutropin receptors (LH) receptors or concentration of steroids in the follicular fluid are recorded even in follicles of the same size.

It is evident that the quality of follicles affects the quality of the oocytes [11]. The pre-ovulation development of follicles and oocytes can thus affect positively or negatively the formation, development and survival of embryos.

The aim of this study was to analyse ovarian activity in sexually mature gilts and to evaluate the size of oocytes according to individual size categories of follicles in the individual seasons of the year.

MATERIALS AND METHODS

Ovarian activity was evaluated on ovaries from 240 Landrace-Yorkshire gilts, 9—10 months old with weights of 150—160 kg. The ovaries were obtained after slaughtering the animals at the abattoir. The ovaries were evaluated for their size and weight and the number of follicles and corpora lutea (CL), according to individual size categories. In follicles the size categories were up to 3 mm, 4—6 mm, 7—9 mm, 10—11 mm and 12 mm and more, in corpora lutea up to 5 mm, 6—9 mm, 10—11 mm and 12 mm and more. Using an insulin syringe BD Micro-Fine Plus 0.5 ml (BD Medical, Franklin Lakes, USA), the follicular fluid with oocytes were aspirated and applied to Petri dishes (Gama group, a. s., České Budejovice, Czech Republic) and filled up to one third with sodium chloride 0.9 % infusion solution (KO, Košice, Slovak Republic). The Petri dishes were marked with the size category of follicles from which the oocytes were aspirated. The identification of the oocytes in the dishes were carried out under a stereoscopic microscope STM 723 (Kapaoptic, Kvant, Bratislava, Slovak Republic) at 20—40-fold magnification (Fig. 1). Using an adjusted Monoject tuberculin syringe 1 cc (Covidien, Mansfield, England) with a piece of IUI pipette, we transferred the oocytes to the Petri dishes containing clear sodium chloride 0.9 % infusion solution and after calibration of the microscope we photographed them using a camera 1,3 MP Moticam 1SP (Motic China Group Co., Ltd., HongKong, China). The size of oocytes (Fig. 2) were measured by a "Software Motic Images Plus 2.0" (Motic China Group Co., Ltd., HongKong, China).

The statistical evaluations of the results were carried out by the software "Microsoft Office Excel 2013" (Microsoft Corporation, Redmond, USA) and the significance of differences of two sets of data were determined by the Student t-test.

RESULTS AND DISCUSSION

The functionality of the reproductive apparatus of the sows is affected by a number of factors. One of them is the season of the year. It has been demonstrated that the sea-

Fig. 1. Search for oocytes

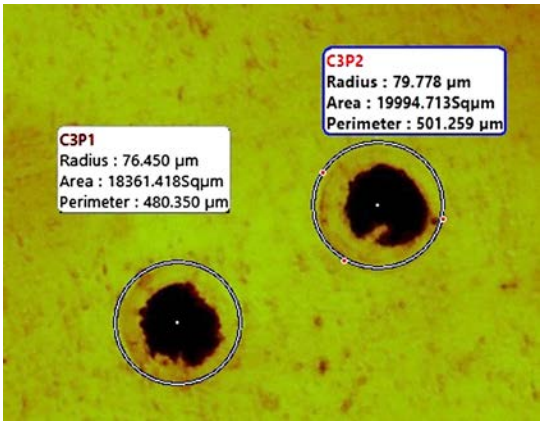

Fig. 2. Oocyte measurement

sonal conception abilities of gilts and sows change and that may cause disturbances of the oestrous cycle and embryonal mortalities may occur. The reasons for these disorders are alterations of synthesis and release of gonadotropins from the hypophysis [10], particularly insufficient production of lutropin, which affects the activity of the luteal cells of the corpora lutea and causes a decreased production of progesterone. The low production of progesterone by the ovaries of gilts results in the reduced developmental competence of the oocytes [2]. Yoshizawa et al. [13] stated that an optimum concentration of progesterone during folliculogenesis in gilts is necessary for the oocyte to reach its full developmental potential. Gupta, Uhm, Lee [6] and Bertoldo et al. [3] reported that oocytes obtained from the corpora lutea containing ovarian follicles of gilts and sows exhibited higher developmental potential than oocytes obtained from follicles that did not possess CL. It was recorded that the concentration of progesterone varies with the seasons and its marked decrease is associated with a reduced conception rate or increased embryonal and foetal mortality rates in gilts and sows. Our evaluation of ovarian size (Fig. 3) revealed that the smallest ovaries were observed in winter (mean size 24.2 ± 2.9 mm) and the largest in autumn (mean size 25.8 ± 3.4 mm). The ovaries of gilts reached the highest weight (Fig. 4), in spring (mean weight 6.2 ± 2.2 g) and the lowest one in autumn (mean weight 5.7 ± 1.4 g).

The above findings correspond with the largest number of ovarian follicles in gilts (mean number 24.8 ± 7.7) in autumn (Fig. 5) that resulted in enlarged volume of ovaries, and the highest number of *corpora lutea* (mean number 12.1 ± 2.6) in spring (Fig. 6) that affected the weight of the gilts' ovaries. In autumn, the predominant size of the follicles was up to 3 mm (mean number from 13.7 ± 6.3 to 17.9 ± 7.5). The ovarian follicles of size 4—9 mm were found most frequently in autumn and winter (mean number 6.9 ± 2.0 and 6.9 ± 1.8, resp.).

It has been assumed that the quality of oocytes and their fertilization ability as well as maturation competence are affected by the size of the follicles from which they originated [1]. Oocytes are present in ovarian follicles in all stages of folliculogenesis and can be obtained already from follicles 2—5 mm in size [8]. However, there is an assumption that with the increasing size of the follicle, the environment that affects the quality of the oocyte and its fertilization ability improves as its maturation competence improves. With the growth of follicles, the number of layers and counts of columnar cells surrounding the oocytes increase [7]. Oocytes obtained from larger follicles (3—8 mm) exhibit increased intensity of meiotic maturation and higher cytoplasmic activity than oocytes obtained from small follicles (up to 3 mm). It was reported that higher proportion of oocytes collected from larger follicles developed to metaphase II compared to oocytes from small follicles [7, 9]. During

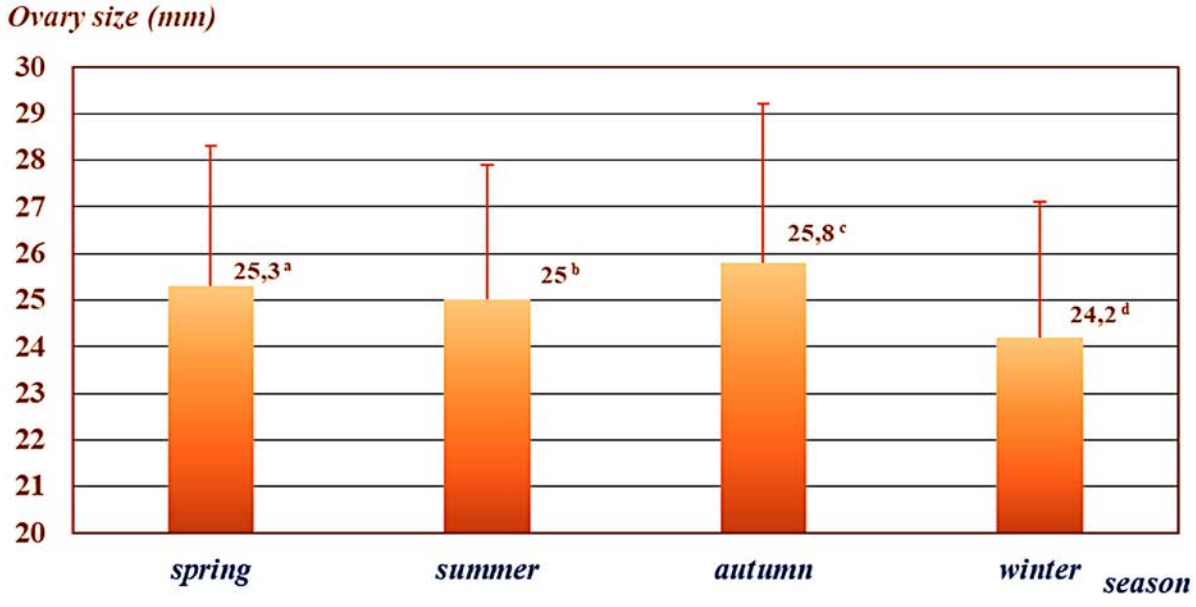

Fig. 3. The mean size of the ovaries of gilts during the seasons
t-test: b : c, d = P < 0.05; a : d = P < 0.01

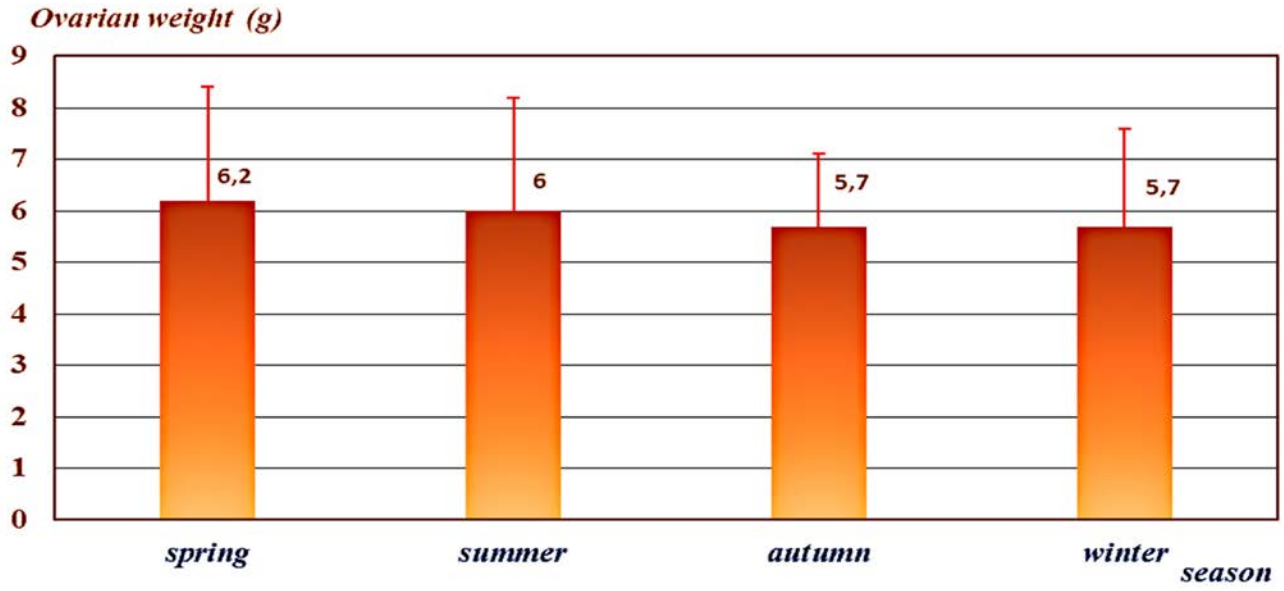

Fig. 4. The mean weight of the ovaries of gilts during the seasons
t-test: P > 0.05

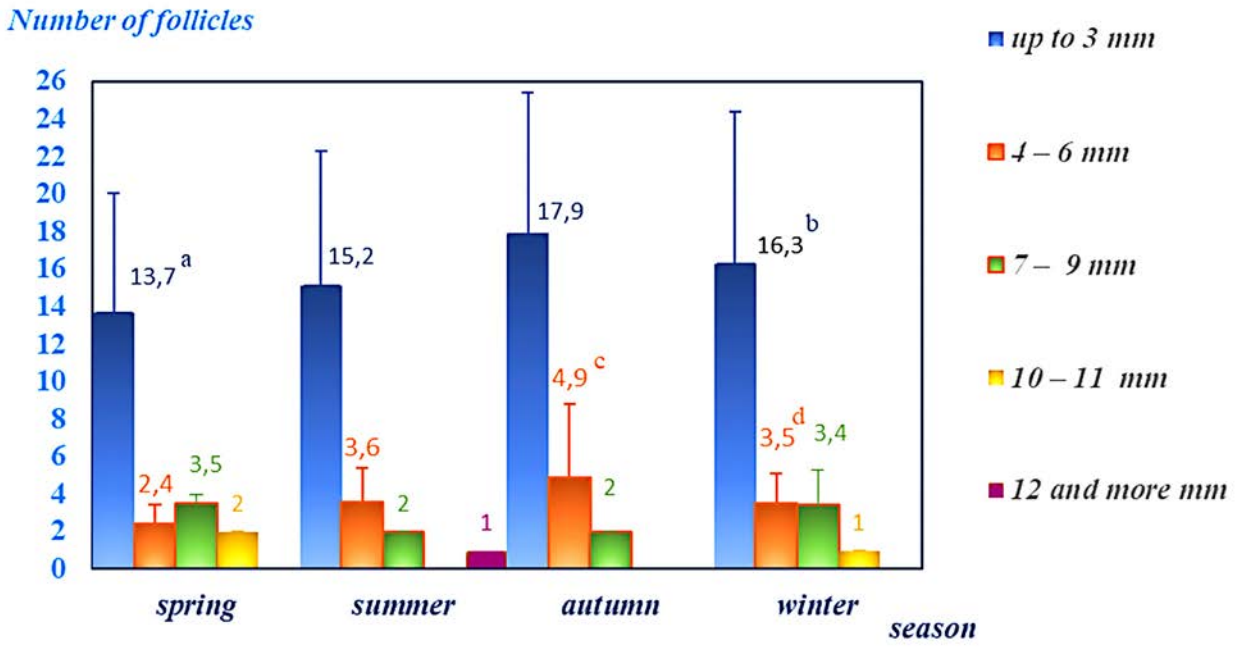

Fig. 5. The mean number of follicles per ovary in gilts during the seasons
t-test: c : d = P < 0.05; a : b = P < 0.01

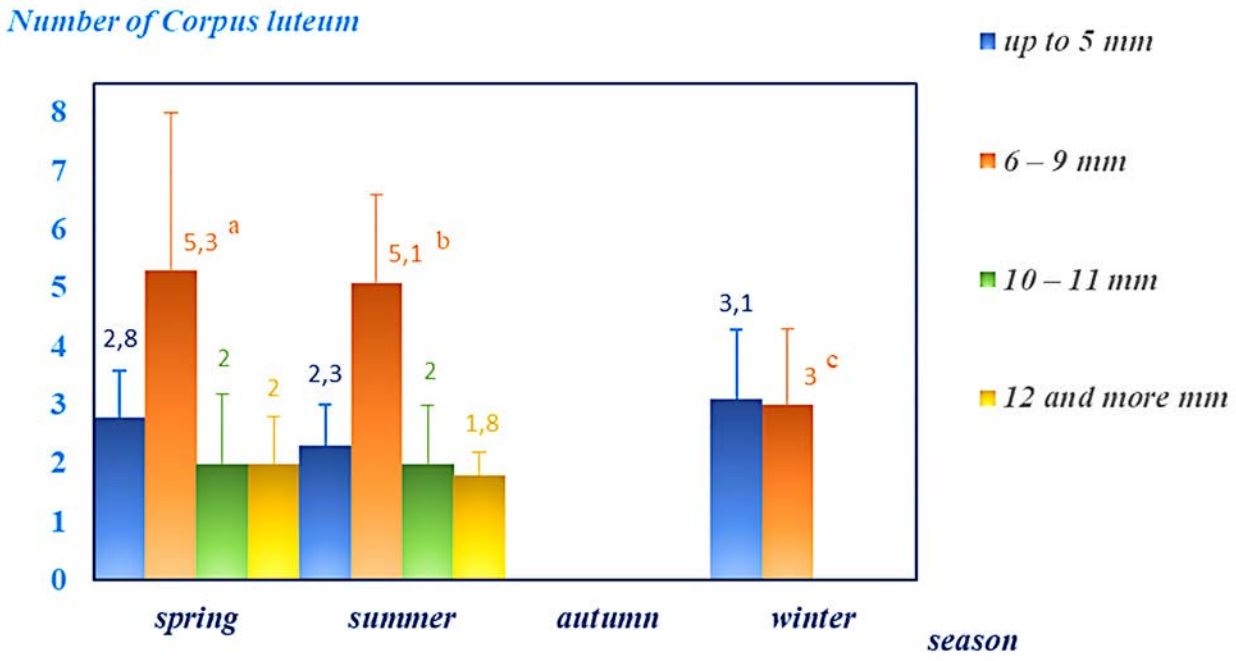

Fig. 6. The mean number of Corpus luteum per ovary in gilts during the seasons
t-test: c : a, b = $P < 0.05$

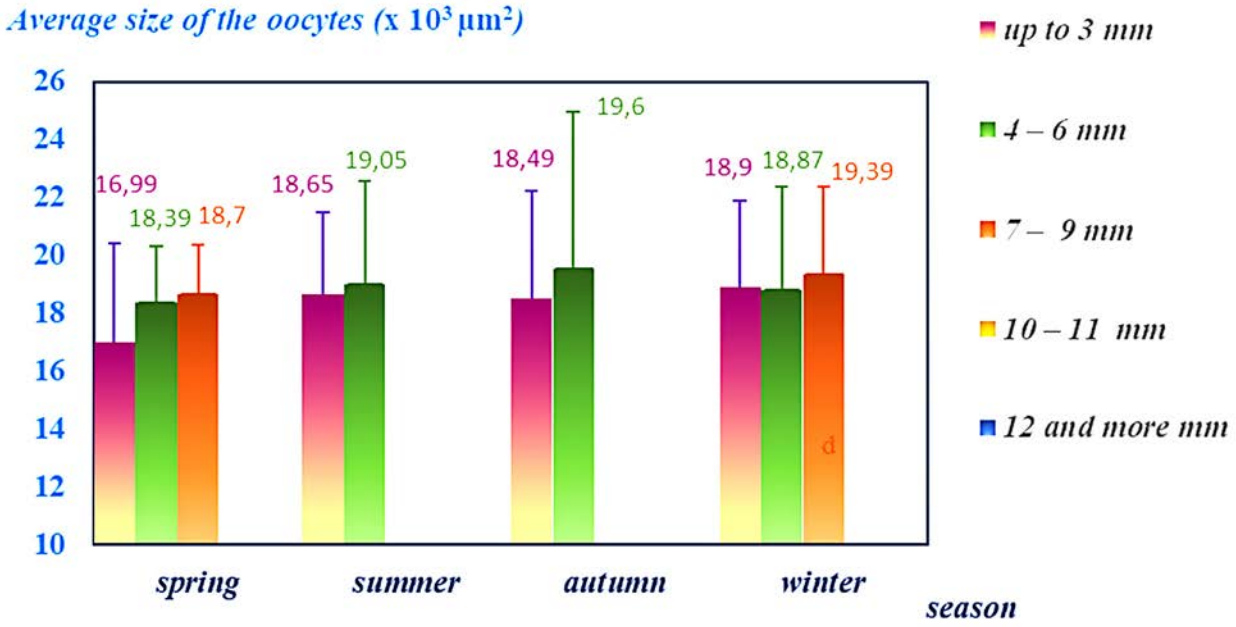

Fig. 7. The average size of the oocytes of gilts during the seasons
t-test: $P > 0.05$

in vitro cultivation, such oocytes exhibit a higher ability to develop to the blastocyst stage than oocytes collected from smaller follicles. The concentrations of steroid hormones, particularly progesterone (P4) and 17-beta estradiol (E2) increases in the follicular fluid of growing follicles which is positively reflected in terms of an increased developmental competence of oocytes [7]. Differences in the concentrations of these steroid hormones were recorded also between ovarian follicles of pre-pubertal and sexually mature gilts [5]. The concentration of these hormones was substantially lower in follicles of pre-pubertal gilts compared to follicles of the same size collected from sexually mature gilts. Similarly, oocytes obtained from follicles of pre-pubertal gilts at *in vitro* fertilization showed markedly a higher degree of polyspermy than oocytes from follicles of sexually mature gilts. This indicates insufficient cytoplasmic maturation in pre-pubertal gilts and the fact that the age of gilts also affects the developmental competence of oocytes. Griffin et al. [4] observed that the developmental competence of oocytes during *in vitro* fertilization correlated with the size of oocytes; the larger the oocyte, the higher the probability of its good developmental competence. The size of oocytes is again in correlation with the size of the follicles. Only sufficiently large follicles contain the optimum number of cells necessary for the adequate developmental competence of oocytes, numerous receptors for gonadotropic hormones and optimum concentrations of steroids in the follicular fluid. When evaluating the size of oocytes collected from follicles of gilts of size up to 3 mm, we detected different sizes of oocytes during individual seasons (Fig. 7). The smallest were oocytes collected from follicles up to 3 mm size in spring (mean size $16.99 \times 10^3 \pm 3.42 \times 10^3$ μm^2) and the largest were those collected in winter (mean size $18.90 \times 10^3 \pm 2.99 \times 10^3$ μm^2). In total, the largest were oocytes collected from 4—6 mm follicles in autumn (mean size $19.60 \times 10^3 \pm 5.37 \times 10^3$ μm^2). The results obtained in this study confirmed the influence of seasons on ovarian activity and oocyte growth in gilts and sows.

CONCLUSIONS

The fertilization abilities and developmental competences of oocytes are affected by a number of factors including the age of gilts, their nutritional status during mating, the seasons and the growth of the follicles. Without acceptation and optimisation of the influence of these factors on folliculogenesis and thus also on the development and maturation of oocytes, the breeders will be unable to ensure the required reproductive parameters in pig farming and the experimental workers will not reach the expected results of *in vitro* fertilization in embryo transfer programmes developed for pigs.

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THE DISTRIBUTION OF COLLAGEN AND ELASTIC FIBRES IN THE LACTATING BOVINE MAMMARY GLAND

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ABSTRACT

Collagen and elastic fibres are generally present in organs whose normal function requires great resistance and elasticity. The aim of this study was to localize the collagen and elastic fibres in the stroma of the bovine lactating mammary gland and to determine their role in the process of milk ejection. For this purpose, the histochemical staining for collagen and the immunohistochemical method for the detection of elastin were used. The accumulation of scattered collagen fibres was observed between and inside the lobules where they formed distinct septa. Between secretory alveoli, the collagen fibres were found to be concentrated into two incomplete layers surrounding the blood capillaries. Bundles of elastic fibres in high density were located in the interlobular spaces. A dense network of elastic fibres was located between adjacent alveoli. Elastic membranes were located beneath the secretory epithelium. The high concentration of the collagen and elastic fibres indicated, that both types of fibres play a significant role in the resistance during the secretory stage and in the recoil of the mammary gland after milk ejection.

Keywords: collagen; elastic fibres; mammary gland; bovine

INTRODUCTION

Fibrillar collagen in the stroma has been described to be the most abundant extracellular matrix constituent which maintains the structure of most interstitial tissues and organs, including skin, gut, and exocrine glands. The stroma surrounding secretory units and interlobular septa of the mammary gland is composed of supporting and free cells and fibrous elements embedded in the extracellular matrix. The scanning electron microscopy image of mammary acini showed individual mammary epithelial cells surrounded by oriented collagen fibres [1].The ultrastructural localization of V-type collagen revealed that it is mainly located on the basal surface of epithelial, but not beneath the myoepithelial cells. In the 100-day-old virgin and lactating rat mammary glands V-type collagen was mainly present in the interstitial connective tissue and in association with blood vessels. It was not present in the basement

membrane region surrounding the ducts in mature virgin glands, but it was present in this region in neonatal and lactating glands [20].

It has been well demonstrated, that the extracellular fibrillar structures composed of various types of collagens, such as collagen fibrils and basement membranes, have a supportive function in various tissues and organs [4]. Fibrillar collagen deposition has been quantified in the rat mammary gland across the pregnancy, lactation, and involution cycle, and the ratio of collagen to epithelial cells differed by an order of magnitude depending on the developmental state [14].The density and spatial alignment of the three-dimensional collagen architecture define mechanical tissue properties, i.e. stiffness and porosity, which guide or oppose cell migration and positioning in different contexts, such as morphogenesis, regeneration, immune response, and cancer progression [21]. Several other proteins such as fibronectin and proteoglycans bind to collagens and affect the organization of collagen fibres, and thereby have effects on the mechanical milieu [17].

The elastin is a highly elastic protein in connective tissue and allows many tissues in the body to resume their shape after stretching or contracting. Elastin is also an important load-bearing tissue in the bodies of vertebrates and is used in places where mechanical energy is required to be stored. It is present in large amounts in the organs the elastic properties of which are essential for their function, such as arteries and lungs. The elastic fibres, unlike collagen fibres, were generally not described in the mammary gland. The aim of this study was to localize collagen and elastic fibres in the lactating bovine mammary gland.

MATERIALS AND METHODS

Mammary gland tissue samples were obtained from five lactating cows (Holstein) at a slaughter house. The samples were placed in 0.1 mol phosphate buffered 10% formalin for 24 hours at room temperature, dehydrated and embedded in paraffin. The 5 mm sections were deparaffinised and rehydrated. For collagen fibres the Azan staining method was used. For the detection of elastic fibres, the sections were pretreated with 3 % H_2O_2 in methanol for 30 min and preincubated with 2 % goat serum. Afterwards, the sections were incubated at 4 °C overnight with monoclonal anti-elastin antibody (Sigma), dilution 1 : 5000. The sections were washed twice in a phosphate-balanced salt solution (PBS) and incubated with a biotinylated polyvalent secondary antibody for 45 min and incubated with avidin-biotin-peroxidase complex (Vectastain ABC kit; Vector, Burlingame Calif., USA). The peroxidase activity was visualized with 0.05 % 3´.3´- diaminobenzidine (DAB) and 0.03 % v/v H_2O_2. Some sections were counter-stained with Mayer's haematoxylin. The negative controls were performed by omitting the primary antibody.

RESULTS

The light microscopic study of a bovine lactating mammary gland revealed, that connective tissue septa consisted of bundles of fibres with fibroblast and different amounts of free cells. An accumulation of scattered collagen fibres was observed between the lobules where they formed the dominant structure, being denser in the interlobular and less dense in the intralobular connective tissue septa. The blood capillaries located between alveoli and arterioles and venules between lobules were closely surrounded by fibres.

Collagen

In the stained histologic sections on the bovine mammary gland, the collagen fibres in the interlobular space were seen to form a dominant component of the supporting tissue. A high accumulation of the collagen fibres was observed between the lobules where they formed a layer of surrounding blood vessels and ducts (Fig. 1). As the duct diameter increased, collagen fibres size and number also increased. From the gross interlobular tissue, collagen fibres enter the lobes that divided them into smaller compartments. Among the secretory alveoli, collagen fibres were concentrated into two incomplete layers surrounding the blood capillaries.

Elastin

An immunohoistochemical study revealed strong positive reaction for elastin confined to elastic fibres in the connective tissue stroma. The bundles of elastic fibres forming bands of different thickness were located in the broad interlobular space (Fig. 2). Regularly, a distinct layer of the elastic fibres was also seen in the intralobular septa. Between more separated alveoli, the elastic fibres formed

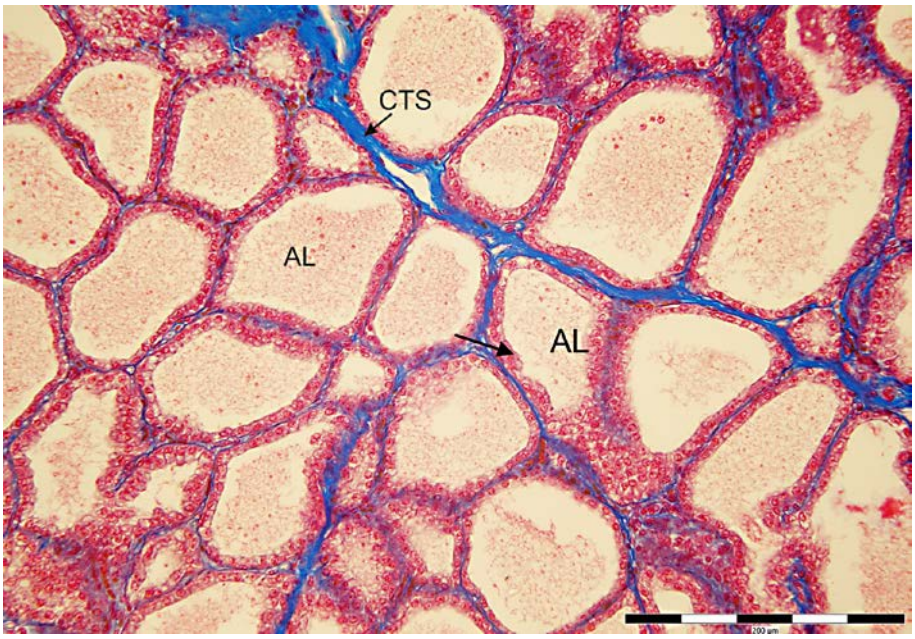

Fig. 1. Section of lactating mammary gland. Azan staining. Thick connective tissue septa (CTS) rich in collagen fibres are in the interlobular and in intralobular space. Fine layer of collagenous fibres is located between the secretory alveoli (AL)

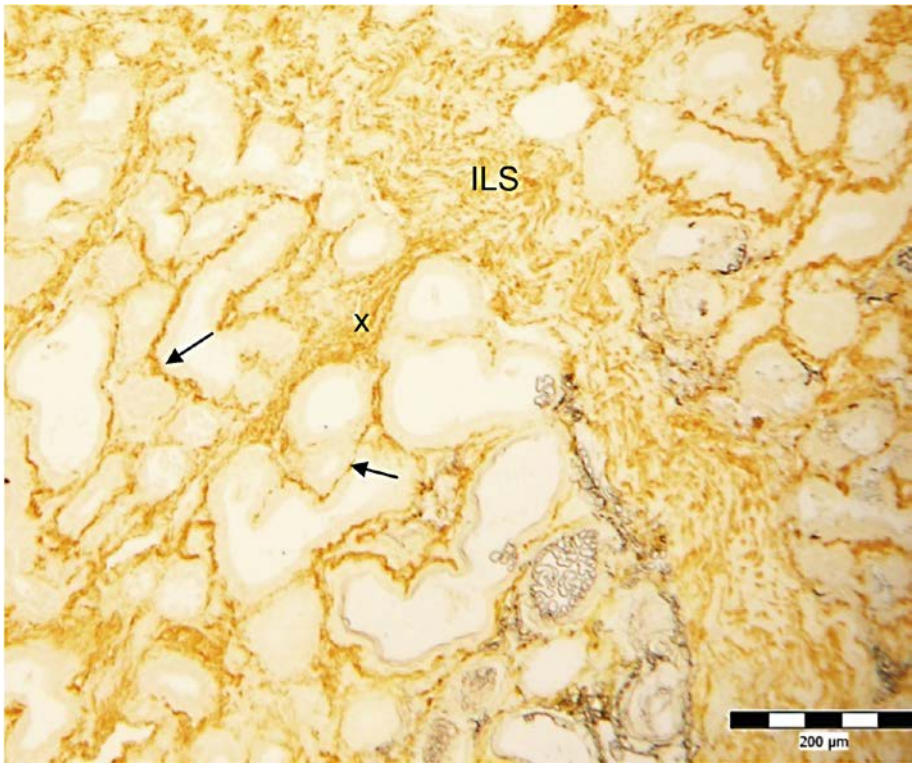

Fig. 2. Immunostaining for elastin. Elastic fibres are accumulated in the interlobular septa (ILS). A dense subepithelial plexus of elastic fibres was among most alveoli (arrows). Inside the parenchyma elastic fibres form a dense plexus (x)

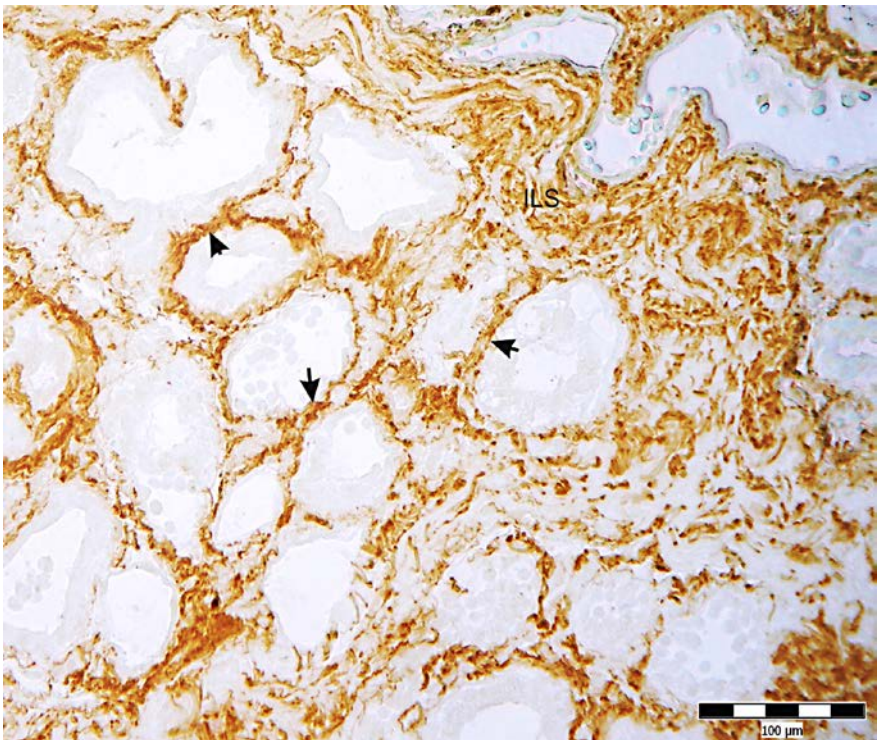

Fig. 3. Immunostaining for elastin. Positive reaction was confined to elastic fibres accumulated in the interlobular interstitial septa (ILS). A dense subepithelial plexus of elastic fibres was also observed among alveoli (arrows)

loose network. High concentration of elastic fibres were found between the alveoli where elastic fibres were often concentrated into membranes located beneath the secretory epithelium (Fig. 3). In this area the elastic fibres were closely associated with the basal lamina that separated the epithelium from the less underlying elastic-rich stromal compartment.

DISCUSSION

Collagen is a component of trabecules in exocrine glands. In the lactating mammary gland collagen fibrils were dominantly present in the interlobulary space, whereas between individual acini a small amount of collagen fibres was found. Periductal connective tissue and distinctive populations of collagen fibrils were associated with ductal epithelia of the mammary gland and other exocrine glands [3]. Collagen was not present in the basement membrane region surrounding the ducts in mature virgin glands but was present in this region in neonatal and lactating glands [20]. Fibrillar collagen deposition has been quantified in the rat mammary gland across the pregnancy, lactation, and involution cycle, and the ratio of collagen to epithelial cells differed by an order of magnitude depending on the developmental state [14]. It seems that the differences coincide with different secretory activity of exocrine glands. Following weaning, high levels of fibrillar collagen are deposited between involuting acini. Ultrastructural localization of V-type collagen reveals that it is mainly located on the basal surface of epithelial, but not beneath myoepithelial cells. In addition, V-type collagen is located on some interstitial collagen fibres that are in close proximity to the basal surface of both epithelial and myoepithelial cells [20].

The fibroblast is the most common cell that creates collagen. Despite the fibroblasts, other types of cells like macrophages were found in the connective tissue stroma of the mammary gland. Studies by Ingman et al. [6] revealed a role of macrophages in collagen fibrillogenesis and in the organization of the structure of terminal end buds. According to the authors, macrophages promote collagen fibrillogenesis around terminal end buds of the developing mammary gland [6]. It is probable that these cells play the same role in the adult body in the process of milk production. Collagen fibres increased deposition in regressing mammary lobules in comparison to lactating lobules. Collagen-

associated proteins known to influence cross linking are similarly regulated including elastin, fibrillin 1, decorin, lumican, and biglycan [18].

Despite their mechanical function, collagen fibres are dynamic structures in the mammary gland. The fibrillar c ollagens and stromal extra cellular matrix are the major structural proteins that affect the mechanical environment of mammary epithelial cells. In addition to providing a biochemical ligand for several receptors, collagen provides structural support for the gland, which is appreciated when one sees the relationship of collagen fibres to the epithelial cells [17]. Collagen fibres among the alveoli are in close contact with secretory cells and the mammary epithelial cells respond to the stiffness of a collagen matrix [13, 17]. Cross-linking of collagen fibres and collagen arrangement will affect the mechanical proper ties of the extracellular matrix [15].

The interstitial connective tissue of several exocrine glands revealed that the intra- and extraglandular ducts were surrounded by a specialized connective tissue for the mechanical support of the ducts and glands. The increase of connective tissue has been observed in mice, rats and humans, suggesting that associated tensional changes are required for the massive remodeling that occurs with involution [2, 5, 7].

The elastic fibres in the mammary gland were seen to be much more dense than those observed in other exocrine glands [9, 10, 11] as was previously supposed. Elastic fibres form bridges between cells and their matrix [12]. In the rat submandibular salivary gland, elastic fibres in the ductal system were found to separate from several layers of longitudinal elastic fibres by a capillary-rich zone sparse in elastic fibres except for fine angular ones [9]. In the bovine mammary gland, elastic fibres occurred as individual, branching and anastomosing fibres localized near the basement membrane of the alveoli where they may form an elastic membrane. In the intralobular and in the larger extralobular ducts such membranes were not observed and elastic fibres were present in dense network. The high number of elastic fibres and the high microfibril elastin ratio indicated that most of the elastic fibres were secreted, probably by fibroblasts and myofibroblasts which have been seen in the interalveolar and in interlobular connective tissue. Light microscopic studies indicate that elastin is made up of repeating self-similar structures at many length-scales [19]. The main components of elastic fibres elastin and fibrillin-containing microfibrils play a structural and mechanical role in the arteries and their essential function is to provid elasticity and resilience to the tissues [8].

It is supposed that in mammary glands, the myoepithelial cells contractions are the principal factor concerned with the "let-down" and the ejection of milk [16]. According to F o s c h i n i et al. [1] other factors such as interlobular smooth muscle contraction, vascular changes, and elastic recoil of the stroma appear to play minor roles, if any, in this phenomenon. Our results indicated, that smooth muscle cells are altered in the lactating mammary gland with collagen and elastic fibres. The presence of elastic fibres in the mammary gland reflects their utility in the process of secretion, and mainly after ejection of milk. We assume that the high density of elastic fibres may play an important role—that is to allow proliferation and expansion of the alveoli and consequently the whole gland during lactation and after milk let-down to allow the mammary gland to return to its original state.

In conclusion, the fibrous elements of the lactating mammary gland consist of collagenous and elastic fibres located mainly in intralobular and interlobular septa. Along the adjacent alveoli, elastic fibres form elastic membranes. Elastic and collagen fibres, along with myoepithelial cells, may participate in the recoil of the gland after milk ejection.

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HIGHLY PATHOGENIC AVIAN INFLUENZA H5N1 (HPAI/H5N1) VIRUS SEARCH FROM WILD BIRDS IN GHANA

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ABSTRACT

Highly pathogenic avian influenza (AI) disease has occurred in many countries globally adversely affecting domestic poultry production. Ghana recorded her first outbreak of a highly pathogenic avian influenza (HPAI) in 2007 on a small scale commercial farm in Tema. Since then, there have been numerous outbreaks. The source of these outbreaks is not conclusive. The role of wild birds in the epidemiology of avian influenza outbreaks in Ghana is not known. This study sought to investigate the role of wild birds in the outbreaks of Highly Pathogenic Avian Influenza (HPAI H5N1) in Ghana, particularly in Southern Ghana. Wild birds were trapped and sampled through mist netting. The faecal and tracheal samples were analysed using a One-Step Real Time Reverse Transcription Polymerase Chain reaction (RT-PCR) with primer sets targeting the matrix protein gene of the Avian influenza virus. Sera samples were subjected to multispecies competitive Enzyme Linked Immunosorbent Assay (ELISA) for anti-AI virus antibodies. Three hundred and twenty two (322) wild birds were trapped and sampled. Birds sampled included 87.3 % (281/322) resident birds and 12.7 % (41/322) migratory birds. The migratory birds included intra-African migrants 12.2 % (5/41) and Palearctic migrants 87.8 % (36/41). Avian influenza virus and antibody were neither detected in these swabs nor sera samples, respectively. The study documented the absence of AI in resident and migrant wild birds in the study area and suggest that wild birds may not be responsible for the outbreaks of AI in the poultry. However, sustained surveillance is recommended to ascertain a nationwide successful prevention and control strategy to stay the tide of any future intruding AI outbreaks.

Key words: Highly Pathogenic Avian Influenza Virus (HPAI H5N1); migratory birds; resident birds; southern Ghana; wild birds

INTRODUCTION

Globally, wild birds play an important role in the ecosystem such as, forest regeneration through plant pollination

and seed dispersal and provide ecotourism [15]. Wild birds are natural reservoirs for avian influenza (AI) viruses and play a major role in the evolution, maintenance, and spread of AI viruses [2, 14]. They host a wide diversity of subtypes, and provide a dynamic population for viral evolution and transmission to domestic flocks and mammals (12). Wild birds such as migratory birds have been implicated in the transmission of highly pathogenic avian influenza (bird flu), West Nile virus and so many others [16]. Studies have shown that low pathogenic avian influenza virus has been detected in waterfowl and shorebirds species [7] and highly pathogenic avian influenza virus has been detected in wild birds, especially ducks [9]. Varied strains of avian influenza virus have been detected in many countries, H5N8 in wild ducks in Netherlands [9], H5N1 was detected in wild bird in Bavaria [10], H5N8 has been confirmed in Nigeria [18].

Surveillance for diseases including AI in wild birds is sporadic, geographically biased, and often limited to the last outbreak virus. Ghana reported several outbreaks of highly pathogenic avian influenza (HPAI H5N1) in the southern and middle belts of the country. The first outbreak of AI (H5N1) was in 2007 on a small scale commercial poultry. Several outbreaks have occurred in Ghana after the first outbreak. There have been outbreaks of HPAI H5N1 in 2015 [5] and H9N2 subtype reported in poultry in Ghana in 2019 [1]. The outbreaks affected the poultry industry as about 40,000 birds were culled. Other economic loss to Ghana include the cost in compensation to the affected farmers, disease control, capacity building in diagnosis and surveillance [5]. The source of these outbreaks is not conclusive. Many projections to the source of outbreaks have been made. Wild birds have been implicated, however, the role of wild birds in the epidemiology of avian influenza outbreaks is not known in Ghana. More so, the source of the first outbreak is still not clear [16].

The aim of this study was to investigate the role of wild birds in the outbreaks of Highly Pathogenic Avian Influenza (HPAI H5N1) in Ghana, particularly in Southern Ghana.

MATERIALS AND METHODS

Study Area

The study locations were selected based on previous AI outbreaks in Ghana in Southern Ghana (Fig. 1) which included the Greater Accra Region (Ayi Mensah, Achimota and Densu Delta Ramsar Site) and Eastern Region (Assin-Foso, Akim-Oda and Brema Asikuma). Samples were collected from free ranging healthy wild birds and quarantined wild birds.

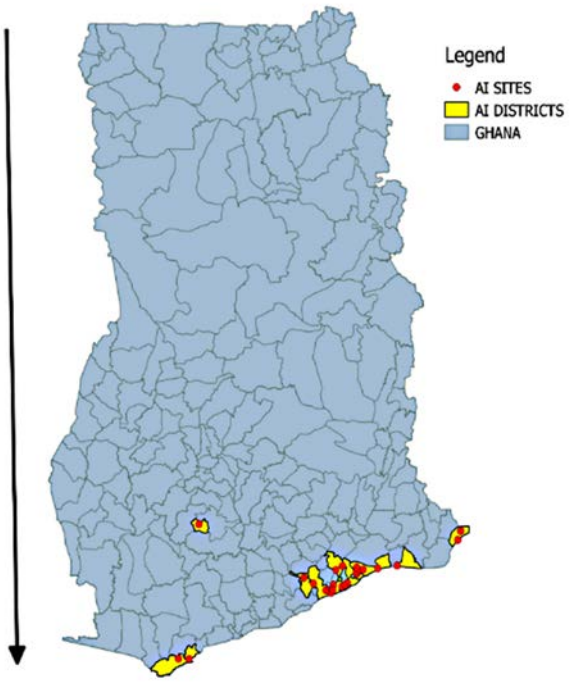

Fig. 1. Map of Ghana showing the Avian Influenza Outbreak Sites and Districts

Wild bird trapping and sample collection in the field

Mist net trapping was done in the early hours of the nights and mornings targeting the peak period of the wild bird activities. These ready opened trap nets were visited and inspected regularly at intervals of 30 minutes, to check for captures. Captured birds were immediately removed to avoid struggles and injuries. Birds were kept in cotton bird bags in a shady field camp until sampled. They were restrained by trained assistants using the ringer hold's method [3]. Whole blood (0.5 ml) from the jugular vein or wing vein [6], as well as cloacal and tracheal swabs were collected from each bird. After sampling, the birds were safely released back into the wild. Tracheal and faecal swabs were placed in tubes containing viral transport medium (Thermo Scientific™ Sterilin™ UTM (Universal Transport Medium). Whole blood was obtained from the jugular vein or wing vein [8] using 27 G ½ needle and a 1 ml syringe and placed in non-heparinized capillary tubes. A total of

322 wild birds were sampled by the end of the study. The wild birds sampled from the various sampling sites included in the surveillance are in Fig. 2.

Swabs from the field were immediately placed in a thermos flask containing ice packs and transported to the Accra Veterinary Laboratory. The swabs and sera samples were stored at –18 °C [17] at the Accra Veterinary Laboratory until use. The sera were harvested into a clean plain tube, covered and labelled appropriately.

Sample analyses

Real time RT-PCR and multispecies competitive Enzyme Linked Immunosorbent Assay (ELISA) were employed in this study.

Molecular detection

RNA Extraction: Up to five cloacal and throat samples were pooled based on species and sampling location. RNA was extracted using purification of viral RNA spin protocol with QIAamp viral RNA Mini kit (Q i a g e n, 2010).

Real time RT-PCR: The pooled samples were tested for the highly conserved gene of Influenza type A gene. The determinations of positive pools were done individually and subsequently tested for H5. Primers and probes specific to influenza A matrix gene were used in conjunction with the PikoReal Time PCR system (Life Technologies Corp, Carlsbad, CA) (Table 1) with thermocycler conditions (as in Table 2) and described by L o r e n z et al. [11].

Serology Test

The presence of antibodies against the nucleoproteins and matrix proteins of avian influenza virus were determined by ELISA Test using the Flocktype AIV Ab ELISA kit (Lot No: 254111033) produced by Qiagen (Germany).

Ethical Clearance

The work was undertaken as part of government official veterinary surveillance during the AI outbreak. The first author was the Government Wildlife Veterinarian of Wildlife Division of the Forestry Commission of Ghana and has

Table 1. PCR primers, probe, and sequence

Specific test	Primer/ probe	Sequence 5′ -3′
Influenza type A M-gene	M25-F	AGATGAGTCTTCTAACCGAGGTCG
	M124-R	TGCAAAAACATCTTCAAGTCTCTG
	M64	FAM-TCAGGCCCCCTCAAAGCCGA-TAMRA

Table 2. Thermocycling conditions for M-gene

RT	Initial Denaturation	Denaturation	Annealing
50 °C	95 °C	94 °C	60 °C
20 min	15 min	45 sec	45sec
		40 Cycles	

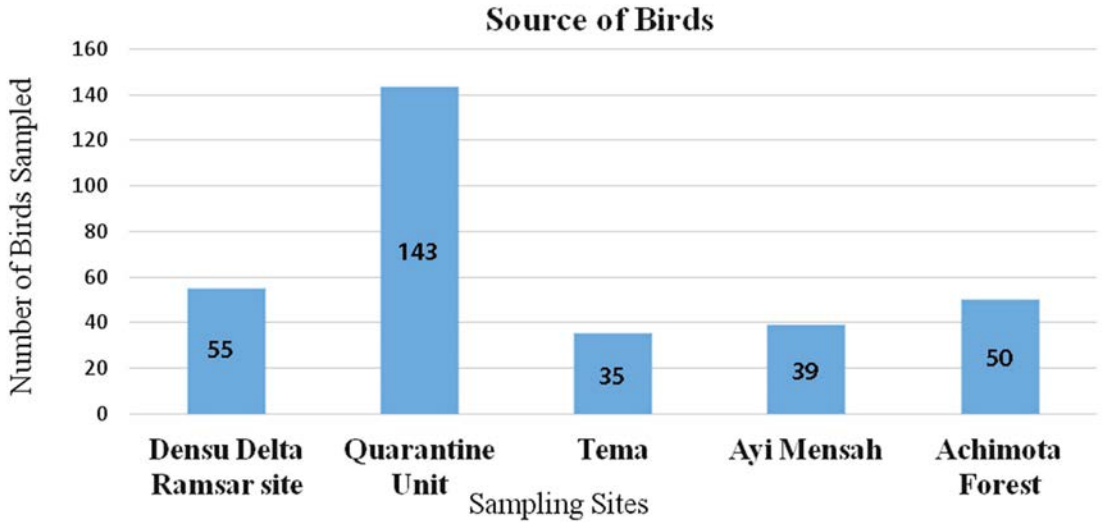

Fig. 2. Locational distribution of wild birds in previous Avian Influenza outbreak sites in Ghana

possessory right to the data. No written permission was needed and hence not issued.

RESULTS

Descriptive characteristics of wild birds

The classification of birds by location, migratory status and the bird species sampled are presented in Figures 2, 3 and 4, respectively. The majority (45 % (145/322)) of the wild birds in this study were quarantined birds from the eastern region of Ghana (Fig. 2). These birds were legally collected by Wildlife export facilities for export (Wildlife business). The total numbers of wild birds for the study were 322. Those categorized as Ghana resident birds were 87.3 % (281/322) while the remaining 12.7 % (41/322) were migratory birds. About 12.2 % (5/41) of the 41 migratory birds, were intra-African migrants and 87.8 % (36/41) were Palearctic migrants (Fig. 3). We trapped and sampled 8 migratory species overwintering in Ghana (Fig. 4).

Reverse Transcription Polymerase Chain Reaction

The M-gene was amplified from the positive control tested by PikoRT-PCR. Detectable levels of M-gene were recorded at 28.31 cycles. The maximum amplification was recorded at 2400 relative fluorescence units (RFU).

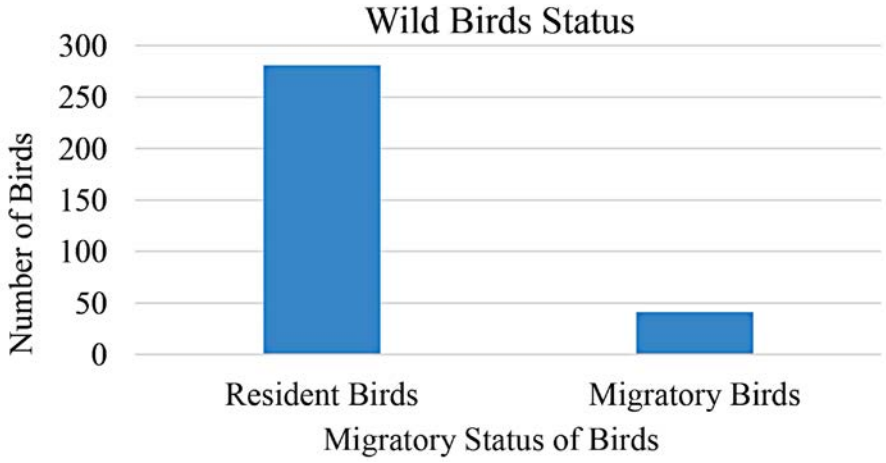

Fig. 3. Distribution of resident and migratory wild birds from previous Avian Influenza outbreak sites in Ghana

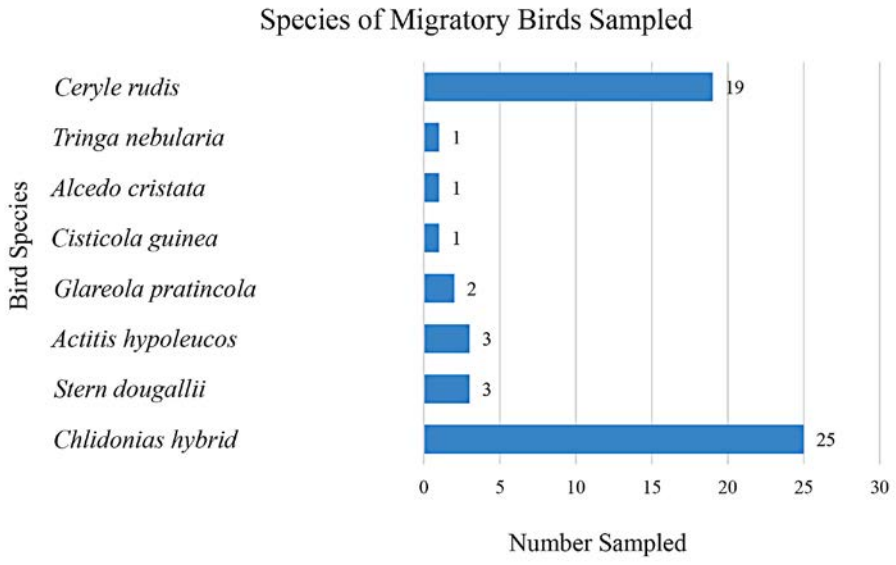

Fig. 4. Species variation of wild birds sampled from previous Avian Influenza outbreak sites in Ghana

Influenza Type A M-gene was not amplified from the negative control tested by PikoRT-PCR. Internal amplification control recorded a maximum amplification of about 55 RFU. No influenza Type A M-gene was amplified from the 322 swab samples tested by PikoRT-PCR. Internal amplification control of all the samples were less than that recorded in the positive control sample.

Three hundred and eighteen (318) sera were collected for testing. No antibody was detected in all sera (Table 3).

DISCUSSION

Studies reported in other African countries and Europe support the hypothesis that wild birds are carriers of avian influenza viruses and play a role in the epidemiology of avian influenza viruses [2, 14].

This is not evident in this study given the time frame and wild bird species that were sampled. Also, given the fact that 12 % of the wild birds in this study are palearctic migrants, there was no evidence of avian influenza virus as well as antibodies to signify exposure.

The failure to detect AI virus and the absence of the AI antibodies may be a true picture of AI in wild birds in Ghana in the absence of other factors such as diagnostic failure probably because of lack of validation of the method for wildlife samples to eliminate false negative results or poor collection and/or handling of samples on the field or during storage prior to sample analysis.

There were no detectable antibodies in the sera of the birds (Table 3). The serological prevalence values of the samples were below 0.3. Thus, these wild bird species may not have been exposed to avian influenza virus and neither the HPAI H5N1 virus.

Influenza Type A M-gene was amplified from the positive control and none was amplified from the negative control. This indicated that the primers and probes for detecting the Influenza type A M-gene and the control samples were valid. Cloacal and throat swab samples from all the wild bird species that were sampled tested negative for influenza Type A virus M-gene. Hence, no influenza virus, both HPAI and LPAI was found in the wild bird species sampled, nor in samples from AI outbreaks areas in Ghana, as similarly documented by F e n t e n g et al. [4]. The outcome of this work is also not surprising because it is also consistent with the report of the absence of H5N1 in wild birds in Europe [13].

Table 3. Enzyme linked immunosorbent assay for HPAI H5N1 antibody in resident and migrant wild birds in Ghana

Samples	Number	Positives	Prevalence %
Throat samples	322	0	0
Cloacal samples	322	0	0
Sera	318	0	0

CONCLUSIONS

There was no evidence of Avian Influenza virus or any antibodies in all of the wild birds sampled from areas where AI previously occurred in Ghana. The continued AI surveillance in all seasons nationwide is needed in order to be able to establish the status and/or role of wild birds in relation to AI transmission so as to aid the prevention and control of AI programs nationwide and by extension, globally.

ACKNOWLEDGEMENTS

The Directorate of Veterinary Services and Staff especially of the Accra Veterinary Laboratory were very supportive in laboratory analysis of the samples. Dr. Sherry Mawuko Johnson read the manuscript and provided useful comments. The authors wish to thank the Wildlife Division of the Forestry Commission of Ghana for waiving the cost of providing a permit for the study and providing Field Staff to assist with the field study.

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THE IMPACT OF MASTITIS ON REPRODUCTIVE PARAMETERS OF DAIRY COWS

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ABSTRACT

The aim of this study was to evaluate the occurrence of mastitis and its impact on the reproductive parameters in a herd of 180 dairy cows. Based on the herd records 127 cows of Slovak spotted cattle and their crosses with red Holstein were selected for study between 1—2 months after calving. The examination of the health status of the mammary glands consisted of: the clinical examination of the udder, the California mastitis test (CMT) supplemented by the collection of mixed milk samples, and the laboratory examination of bacterial pathogens causing the mastitis. In addition to the mammary investigation, reproduction indicators such as the length of the insemination interval, the service period, the intercalving period and the insemination index were also analyzed. The results of this study indicated: a high incidence of mastitis (41.6 %), especially latent (21.2 %), subclinical (15.7 %) and clinical (4.7 %) forms were most common in the herd. The most frequently isolated bacteria from the infected milk samples were: coagulase negative staphylococci (54.1 %), *S. aureus* (16.9 %), *Streptococcus* spp. (15.0 %), *A. viridans* (7.5 %) and *Ent. faecalis* (6.4 %). According to the available literature, the optimum values of the intercalving period were 365—400 days, the insemination interval 55—80 days, the insemination index 1.2—2 and the service period 60—110 days. In comparison, our results showed increased, unsatisfactory reproductive values in the group of dairy cows with clinical mastitis. While in healthy cows as well as in groups of cows with latent and subclinical mastitis, all of the reproductive indicators were within the optimal levels.

Key words: dairy cows; intercalving period; mastitis; pathogens; service period

INTRODUCTION

Cattle rearing is a very important part of livestock production. Its role in agriculture involves mainly the production of milk and meat which are essential components of human nutrition. From the point of view of the economy, cattle breeds with high milk yield [20] are of greatest interest in the Slovak Republic, but due to their high milk production, the reproductive indicators essential for repeated pregnancy are often worsened [3].

Securing the reproductive cycle requires a lot of attention and skill from the breeder. It is important that the farmer be able to control the reproductive cycle of cows (Fig. 1), manage the detection of oestrus, maintain an identification system with records of cows mated and calved. It is also necessary to use high quality semen and the correct time of insemination and pay attention to the formation of groups of dairy cows according to the appropriate age category. In addition, close observation of animal hygiene requirements on the farm is required in order to ensure the good health of these productive animals [17].

The economic value of dairy cows is determined mainly by their milk yield and longevity, because milk is the main source of income on Slovak dairy farms [20]. The economic losses are mainly due to the prolongation of the service period and intercalving period the nonstandard length of which reduces milk yield during the subsequent lactation. Losses that arise due to poor fertility are affected by more factors such as the milk production, the purchase price of milk, the price of calves, the course of the lactation curve, individual characteristics of the cows and more [15].

In addition to reproduction management, one of the important roles of dairy farmers is to control the incidence of diseases, especially mastitis. Often due to the lack of symptoms, the identification of subclinical mastitis is a frustrating problem for dairy farmers and veterinarians to overcome in order to ensure not only the animal health but also the hygienic quality of the produced milk [6, 19].

Based on the intensity and severity of the clinical signs, mastitis is usually divided into subclinical and clinical disease. In clinical mastitis (CM), the signs range from mild to severe and can be systemic, local, or milk related, whereas in subclinical mastitis (SM) no signs are observed. The most prominent signs of CM are swelling, heat, hardness, redness or pain of the udder. The milk of a cow with CM has a watery appearance, and flakes, clots or pus is often present. During SM the udder and milk appear normal, but the infection is still present [16]. Subclinical mastitis is more common than CM. It is estimated that in the herd of dairy cows there are approximately 15–40 undetected cases of SM for each case of CM. The increase in somatic cell counts (SCC) are associated with reduced milk production to the tune of 60 to 140 litres per cow per year in animals with SM [1].

Postpartum diseases, especially mastitis, can have a major impact on the reproductive performance of dairy cows. Due to the signs, it is insufficient or difficult to detect the oestrus cycle in mastitis cows resulting in a prolongation of the insemination interval and service period [9]. Therefore, the aim of this work was to evaluate the prevalence of

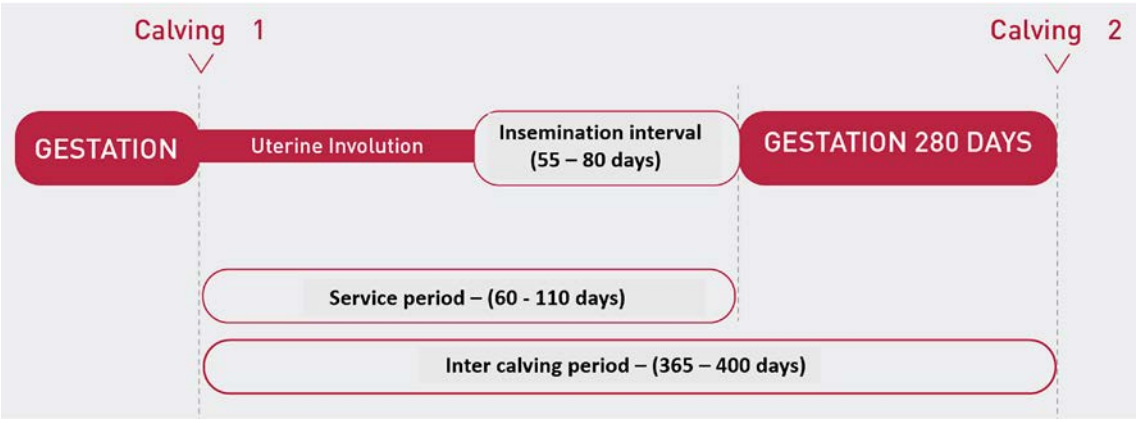

Fig. 1. Reproductive cycle of dairy cows [4]

mastitis and its impact on the reproductive parameters in dairy cows.

MATERIALS AND METHODS

Dairy cows and milking

This study was conducted on a herd of 180 cows of Slovak pied cattle breed and their crosses with Red Holstein with the average milk yield of 7,228 kg per lactation. The farm was located in the Trencin region (west Slovakia) with two stables for dairy cows and heifers. The cows were milked twice a day in a herringbone milking parlor DeLaval 2x10 (Tumba, Sweden) (Fig. 2), with the first milking starting at 3:30 a.m. and the second at 3 : 30 p. m. First, the wet udder hygiene was performed with water to remove dirt from the udder and teats. Subsequently, the udder was thoroughly wiped with disposable wipes soaked in a disinfectant solution (Valiant – ABS CZ s. r. o.).

The first milk from each quarter was hand milked into a dark-bottomed pot, and the milk was subjected to sensory analysis. The milking and pulsation vacuum was set at 42 kPa. The pulsation ratio was 60 : 40 at a rate of 52 cycles per min and termination was automatically signalled when the milk flow dropped to 0.2 $l.min^{-1}$. After the milking process, the teats were disinfected by teat-dipping with IODERM 5000 (Hypered Czech s. r. o.). The milk was stored in refrigerated milk tanks at +5 °C and removed daily around 11 : 30 a. m.

Animal selection

A total of 127 cows of the 180 dairy cows were selected for study based on the data provided from the milk recording control for the purpose of their investigation at intervals of 1—2 months after calving. The herd monitoring was carried out during one lactation period of each cow at the turn of 2017/2018. During this period, a complex examination of each cow was performed for mastitis with the sta-

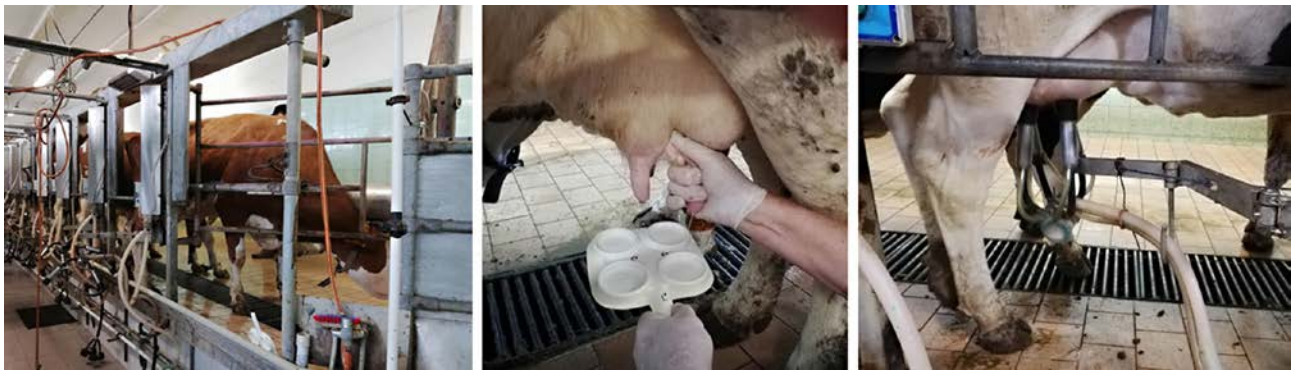

Fig. 2. Herringbone milking parlour with CMT evaluation and milking

Fig. 3. Bacterial pathogens isolated from clinical mastitis cases
From the left: *S. aureus*, *S. warneri* and *Str. dysgalactiae*

tistical analysis of the reproductive indicators, such as the insemination interval, the insemination index, the service period and the intercalving period.

Udder exanimation and laboratory analyses

A thorough evaluation of the udder health in the 127 lactating cows included a veterinary history, clinical examination, sensory analysis of milk from forestripping of each udder quarter followed by a CMT (Indirect Diagnostic Test, Krause, Denmark) (Fig. 2). The milk from every quarter was mixed with the reagent and the result was read as trace, score 1—4 or negative depending on the gel formation in the milk sample according to Jackson and Cockcroft [7].

Afterward (10 ml) each of the mixed milk samples from each cow was collected aseptically for bacteriological cultivation in accordance with the guidelines of the National Mastitis Council [13]. The cooled samples were immediately transported to the laboratory of the University of Veterinary Medicine and Pharmacy in Kosice.

The bacteriological examinations and identifications were performed according to generally accepted principles [12]. The milk samples (10 µl) were inoculated on a Columbia Blood Agar Base (Oxoid, UK) with 5 % of defibrinated ram blood in a Petri dish and incubated for 48 h at 37 °C (Fig. 3). The dishes were examined after 24 and 48 h of incubation. The suspected colonies were inoculated and cultured on selective media and growth-confirmed colonies of *Staphylococcus* spp., *Streptococcus* spp. and *Enterobacteriacae* spp. were further identified biochemically using the STAPHYtest 24, STREPTOtest 24, respectfully. ENTEROtest 24 (Erba-Lachema, CZ) and the software TNW Pro 7.0 (Erba-Lachema, CZ).

Mastitis forms

Based on the diagnostic methods of mastitis, the individual forms were classified as follows: Latent mastitis is characteristic only with the presence of bacterial pathogens in samples of milk without changing its consistency and somatic cell count (SCC). Subclinical mastitis is characterized by a positive CMT score, bacteriological cultivation, increased SCC, and a reduced milk yield without clinical signs. Clinical mastitis is characterized by a positive CMT score, bacteriological cultivation, higher level of SCC, a change in the consistency of the milk, and a reduced or loss of milk production with clinical signs.

Statistical analysis

The average values of the reproductive parameters of dairy cows were analysed by the one-way ANOVA. A Dunnett's Multiple Comparison was used to compare mastitis groups of dairy cows with a healthy (control) group. The level of significance was set to $P < 0.05$.

RESULTS AND DISCUSSION

Worldwide, mastitis is known as a multifactorial disease, and it is closely related to the production system and the environment. The incidence of mastitis increases when the immunological and antioxidant defense mechanisms of the mammary gland are impaired. Dairy cows are exposed to numerous genetic, physiological, and environmental factors associated with both the host and pathogens that can compromise host immunity and increase the incidence of mastitis [2, 21].

Table 1 shows the prevalence of mastitis and bacterial agents of intramammary infections (IMI) in dairy cows. The results demonstrated a high incidence of mastitis (41.6 %), especially latent (21.2 %), subclinical (15.7 %) and clinical (4.7 %) forms were most common in the study herd.

According to Ozenc et al. [14] more than 140 different microorganisms are considered to cause mastitis. Bacteria are the most common causative factor, recognized in more than 95 % of mastitis cases. Globally, the most common mastitis-causing bacteria in dairy cows and small ruminants are *S. aureus* and coagulase-negative staphylococci (CoNS), as well as streptococci and E. coli, which may have a similar or higher prevalence than that of staphylococci.

From our results, the most common bacteria pathogens were CoNS (*S. chromogens*, *S. warneri* and *S. xylosus*), which were found mainly in the latent and subclinical forms. The bacteria *S. aureus*, *S. chromogenes*, *Str. dysgalactiae* and *E. coli* were the most frequently isolated from the clinical forms of mastitis (Table. 1).

A similar incidence of IMI was reported by Kivaria and Noordhuizen [8] who isolated *Staphylococcus* spp. followed by *Streptococcus* spp., *E. coli* and *Klebsiella* spp. The CoNS have been increasingly isolated from dairy cows and are reported to be the leading cause of environmental mastitis. In addition to latent and subclinical forms, CoNS are often isolated from clinical and persistent mastitis [10, 18, 21].

Table 1. Bacterial agents of mastitis

Isolated bacteria	n	Latent		Subclinical		Clinical	
		n	%	n	%	n	%
Staphylococcus spp.							
Staphylococcus aureus	9	3	2.3	4	3.1	2	1.6
Staphylococcus chromogenes	14	11	8.6	2	1.6	1	0.8
Staphylococcus warneri	4			3	2.3	1	0.8
Staphylococcus xylosus	2	1	0.8	1	0.8	–	–
Streptococcus spp.							
Streptococcus bovis	4	2	1.6	2	1.6	–	–
Streptococcus dysgalactiae	4	3	2.3	–	–	1	0.8
Other bacteria							
Aerococcus viridans	7	4	3.1	3	2.3	–	–
Escherichia coli	4	1	0.8	2	1.6	1	0.8
Enterococcus faecalis	5	2	1.6	3	2.3	–	–
Total infected samples	**53**	**27**	**21.2**	**20**	**15.7**	**6**	**4.7**

n—number of examined mixed milk samples

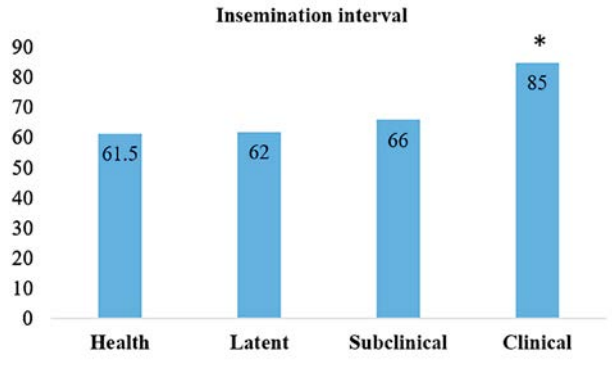

Fig. 4. Insemination interval of selected dairy cows
* P < 0.05—significant differences between selected groups

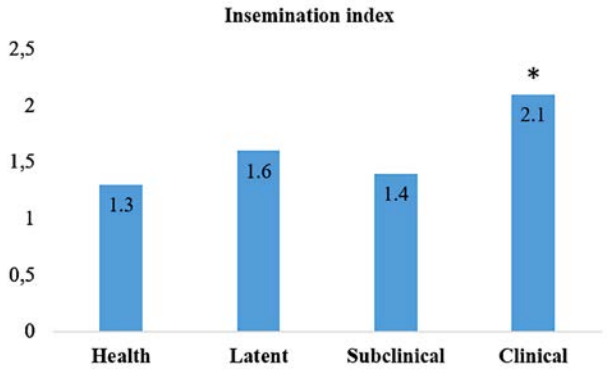

Fig. 5. Insemination index of selected dairy cows (in days)
* P < 0.05—significant differences between selected groups

In the studies by Blowey and Edmondson [2] and Lange et al. [10] more than 20 CoNS were isolated from mastitis milk samples; most of them were *S. chromogenes*, *S. haemolyticus, S. epidermidis, S. simulans* and *S. xylosus*.

Good fertility of dairy cows depends on the following indicators as: length of insemination interval, calving after the first insemination, insemination index, the length of service period and the intercalving period [9].

According to Fahey et al. [5] a satisfactory insemination interval is considered to be between 60—80 days and over 80 days is considered unsatisfactory. In our study, the insemination interval was 61.5 to 85 days, with the longest interval in dairy cows with clinical mastitis and the shortest in healthy cows (Fig. 4). The insemination index indicates how many inseminations were necessary to induce pregnancy. The average insemination index ranges from 1.2 to 2.0. If it is higher than 2.0, it indicates fertility disorders, as confirmed in our study in cows with clinical mastitis (Fig. 5).

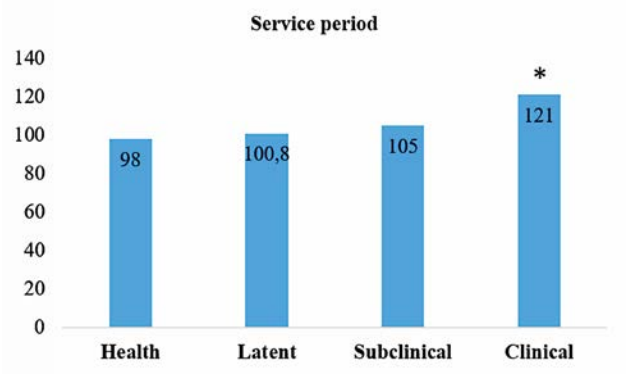

Fig. 6. Length of service period of selected dairy cows (in days)
* P<0.05—significant differences between selected groups

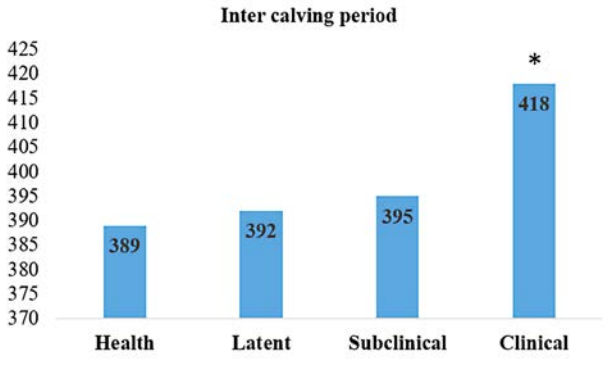

Fig. 7. Length of intercalving period of selected dairy cows (in days)
* P < 0.05—significant differences between selected groups

The service period is economically one of the most important indicators. It is the period between the date of calving and the date of successful conception. The ideal service period is 85 days but may be longer in high-performance animals. The causes of service period prolongation can be found in the inadequate monitoring of oestrus, especially for non-fertilized cows, but also for physiological and health reasons. For high-yield dairy cows, a service period from 110 to 125 days can be tolerated, but only when the interval calving does not exceed 400 days [11]. The results of our study revealed that in dairy cows with clinical mastitis, the length of service period was at the cut-off value (121 days) but with a significantly increased intercalving period (418 days) which is considered unsatisfactory in both cases (Fig. 6). Conversely, the best milk yield (7,392 litres) was found in healthy dairy cows with a service period of up to 98 days. In dairy cows with clinical mastitis in addition to increased service period, we observed excessively long intercalving period which resulted in reduced milk yield (6,421 litres).

The intercalving period is the period between two calving in cows and in general, the length of this intercalving period should be between 365 and 405 days. According to a study by Říha and Hanuš [15], when the intercalving period in dairy cows with milk yield above 7,000 kg of milk increases from 365 days to 405 days, 20 % of the milk production may be lost while in dairy cows with lower milk yields, the loss amounts only to about 5 %.

In our studied groups of dairy cows, the length of the intercalving period ranged from 389 to 418 days. In healthy dairy cows, the average intercalving period was 389 days and the longest was observed in dairy cows with clinical mastitis (Fig. 7) in which it also resulted in longer insemination intervals. This indicates that the prolongation of the intercalving period also affects the length of the insemination intervals. In dairy cows with long intercalving periods, there may be less pronounced oestrus, problems with its detection, reproductive cycle disturbances, or the prolongation may be caused by other external factors.

CONCLUSIONS

All reproductive parameters were recorded as unsatisfactory only in the group of dairy cows with clinical mastitis. The clinical IMI significantly affected the health of dairy cows, not to mention the reduced milk production and increased reproductive parameters compared to the healthy cows. It should be pointed out that the effectiveness of generally established anti-mastitis methods to reduce environmental bacteria in combination with major mastitis pathogens are usually limited. Therefore, dairy farmers should still look for the most effective methods of cattle breeding using rational management of the reproductive cycle.

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