Veterinarija ir Zootechnika

Volume 80(1)
2022

Veterinarija ir Zootechnika

LITHUANIAN UNIVERSITY OF HEALTH SCIENCES

Volume 80(1)
2022

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Issues per Year – 4. From 2016 - 2.

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Publication Information:

„VETERINARIJA ir ZOOTECHNIKA"
A scientific journal and the Official Organ of the Veterinary Academy,
Lithuanian University of Health Sciences (LSMU VA).

ISSN 1392-2130 (Print)
ISSN 2669-2511 (Online)

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Layout Rūta Atie
Printed by LSMU Academic Publishing Department, A. Mickevičiaus 9, LT-44307 Kaunas, Lithuania. Edition of 10 copies.

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Volume 80(1)
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Volume 80(1)
Pages 1–76
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Comparing Health and Welfare of Pigs Farmed in Conventional and in Organic Systems Housed Indoors

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Keywords: *organic farming, parasitic diseases, free range husbandry, behaviour, African swine fever, tail biting.*

Abstract. *Since 2014, it has been forbidden to keep pigs outside in Estonia, because of African swine fever. This study compared the welfare and health of pigs raised in indoor conventional and organic systems in Estonia. Selected parameters for comparison were included: stocking densities, behaviour, a human-animal approach test, lameness, dirtiness, tail biting, skin wounds and faecal samples for parasitic egg counts. Organic farms had poorer human-animal interactions compared with conventional farms, but were better in regards to the social and exploratory behaviour among pigs. They were also slightly better regarding lameness and dirtiness compared with conventional farms. Organic farms had less tail biting and skin wounds than conventional farms. Of 16 faecal samples from organic farms, 12 had parasite eggs, while none of the samples from conventional farms were positive. Welfare problems remain to be solved on organic pig farms irrespective of whether they are kept indoors or outdoors, but there are benefits to pig welfare under organic regulations even if they have no access to an outdoor area.*

Introduction

It has been argued that pigs reared in intensive indoor conventional farms have lower levels of animal welfare and health compared with pigs reared in less intensive organic farms (Alban et al., 2015), and the arguments have been reviewed by Spoolder (2007). However, reliable and practical worldwide welfare assessment criteria are yet to be developed. It might be impossible to have a common standard, because different countries rear their animals in different environments. Also, there is a diverse understanding of what good welfare is. Nevertheless, there are some protocols (e.g., Welfare Quality® protocol 2009) to assess factors that are considered to be reliable indicators, such as diseases, fertility, morbidity, mortality, animal productivity, stress hormone levels and behaviour (reviewed by Botreau et al., 2007). The Estonian government and the European Union Council have set minimum requirements for organic pig production systems (e.g., Regulation of Estonian Ministry of Agriculture RT I, 11.12.2012, 2 & RT I, 12.10.2018, 8; European Union Council Directive 2008/120/EC; European Union Council Regulation 834/2007/EC & 889/2008/EC). These state that organic farms must have lower stocking densities, have access to outdoors, bedding material should be provided, later weaning, no use of farrowing crates and feeds have to be organic; and there are health treatment restrictions, particularly prophylaxis, compared with conventional farms.

African swine fever was detected in Estonia in September 2014 among the wild boar population, but the first domestic pig farm was infected in July 2015 (Nurmoja et al., 2018). The Estonian Veterinary and Food Board reacted to limit the spread of the disease and passed a decree on 25.07.2014 that forbade swine to have access to outside and the storage of their feed outside. Since then all swine in Estonia have been kept inside all year around (Veterinaar ja Toiduameti käskkiri 25.07.2014 no. 117). This might be expected to have an impact on pig welfare in organic systems. This study considered some welfare and health parameters to evaluate the differences of production systems in regards to the new regulation. In addition, organic and conventional pig production *per se* can be compared in the current conditions without the confounding factor of access to outdoors. Observation of behaviour is crucial to ensure that pigs are coping with their environment. The expression of normal behaviours and abnormal behaviours is key to understanding this. Pigs living in stressful environments can show abnormal behaviours, such as tail and ear biting and floor licking (Arney et al., 2018; Zimmermann et al., 2012).

The aim of this study was to evaluate and compare the health and welfare of pigs kept on farms in conventional systems and on organic farms where the pigs are kept indoors. The selected animal-based indicators were lameness, dirtiness, skin wounds, tail and ear biting, shoulder ulcer, human-animal interactions, social and exploratory behaviours,

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parasite infection and organ changes found on *post-mortem* inspection. The collapse of the organic pig sector in Estonia following the restrictions described above (the number of adult pigs declined from 1,455 in 2014 to 534 in 2018 (Põllumajandusamet, 2020)) limited the population size available for their inclusion in this study, and this paper has been presented as a case study rather than a statistically robust comparative analysis.

Materials and Methods

This research was based on data from three organic pig farms and three conventional pig farms in Estonia, which were collected from July to October 2019. All of the organic farms were certified at the latest in 2005 according to the Estonian Organic Agriculture register. Collected data from the farms included assessment of an easily evaluated welfare protocol and health parameters *via* observation, collection of faecal samples for quantitative faecal flotation analysis for parasitic eggs, and description of management and husbandry procedures from an interview with the farm representative. Assessments were made on finishing pigs, weaners and dry sows. Piglets and lactating sows were not included. The organic pigs were housed with straw bedding. As there were small animal numbers, all animals were housed in one space, though grouped and separated by fences. Conventional farms were more intensive, larger, with slatted floors and resting area on concrete. Not all animals on the farms were housed in a single space, and not all farms provided environmental enrichments (such as toys).

A standardised on-farm assessment was carried out, with guidance from the literature (Dippel et al., 2014; Welfare Quality® Consortium, 2009). All assessments were made by one observer during a single-day visit to the farms, and sampled pigs were selected randomly. Health and welfare parameters were chosen to evaluate locomotion and skin quality. Also, social and exploratory behaviour and human-animal reaction interactions were evaluated. In total, 402 organic pigs and 612 conventional pigs were assessed. Each health and welfare parameter had their own scoring systems, which are described in Tables 1 and 2. Lameness was assessed from every randomly selected individual pig while moving. If the pig was reluctant to move, the pig was encouraged to move through vocal encouragement by the observer. Dirtiness was evaluated from one side of the pig at a 0.5–1 m distance and scored into three categories. Skin wounds, identified by physical injury where the integrity of the skin was compromised, were also observed from one side of the pig at a 0.5–1 m distance. A shortened tail (identified as such if a part of the tail had been removed), tail biting, ear biting and shoulder ulcer were assessed by observation. Signs of tail and ear bites were recorded as existent when fresh blood was visible on the tail/ear and/or there was evidence of swelling and infection and/or part of the tail/ear was missing.

Human-animal interaction, assessed by the distance permitted by an animal between itself and a human, should become shorter between the stockperson and the animal that is in his care. This

Table 1. Scoring scales of health and welfare parameters (adapted from Dippel et al., 2014; Welfare Quality® Consortium, 2009)

Parameters	Scoring scale
Lameness	0 – No detectable lameness, pig moves easily 1 – Pig moves relatively easily, but there are visible signs of lameness in at least one leg, reluctant to put on weight on the affected leg, but still weight bearing 2 – Lameness is apparent in one or more legs, pig shows compensatory behaviours such as arching the back and dipping the head 3 – Reluctance to walk and bear weight on one or more legs, pig does not want to move when encouraged
Dirtiness	0 – Up to 10% of the body surface is soiled 1 – 10–30% of the body surface is soiled 2 – More than 30% of the body surface is soiled
Skin wounds	0 – No visible skin wounds 1 – Less than 5 skin wounds 2 – 5-10 skin wounds 3 – More than 10 skin wounds
Shortened tail	0 – Tail has natural length 1 – Tail shorter than normal
Tail biting	0 – No visible signs of tail biting 1 – Visible signs of tail biting
Ear biting	0 – No visible signs of ear biting 1 – Visible signs of ear biting
Shoulder ulcers	0 – No visible signs of shoulder ulcers 1 – Visible signs of shoulder ulcers

Table 2. Scoring scales of welfare parameters (adapted from Dippel et al., 2014; Welfare Quality® Consortium, 2009)

Parameter	Scoring system
Human-animal reaction	0 – Observer can touch the pig and it does not flee or flees after touching, but then returns to the observer within 10 seconds 1 – Pig allows the observer to come as close as 0.5 m, but does not allow touching or allows the observer to come as near as 0.5 m, then flees, but returns within 10 seconds, or after allowing touching does not return to the observer within 10 seconds 2 – The pig does not allow the observer to come near it
Social and exploratory behaviour	Negative social behaviour (N) – aggressive behaviour; Positive social behaviour (P) – sniffing, licking, nosing and moving gently away from another pig; Pen investigation (S) – sniffing, licking or nosing floor, wall or pen fittings, except toy and straw; Exploratory behaviour (E) – playing with a toy or straw; Resting (R) – lying down; Other (O) – other behaviours

was assessed in stalls one pig at a time. All assessed pigs were standing during the assessment. The evaluation comprised three stages. First, the observer stood about 0.5 meters away from the selected pig and stayed in a relaxed position for 10 seconds. If the pig did not react, the observer proceeded to the next stage. The observer started slowly moving towards the pig, keeping the hands and arms close to their body. Once the observer reached near to the pig, the observer crouched down in front of the pig motionless for 10 seconds. If the pig did not react, the observer proceeded to the next stage. At this last stage, the observer reached out a hand and slowly attempted to touch the pig between the ears for 10 seconds. If the pig came close to the human in the first stage, the pig was slowly touched, missing out the second stage.

Social and exploratory behaviours were assessed outside of the feeding period in the passageway for 5 minutes. Observations started 2 minutes after the observer approached the group in the passageway to standardise the response of pigs to the observer. During the observation, the observer did not move. For the assessment, all animals were standing. Lying animals were encouraged to stand up through vocal encouragement by the observer. Over a period of 5 minutes, the observer recorded how many animals showed negative or positive social behaviours, explored the pen or the enrichment material, or lay down. If an observed pig showed behaviour distinct from these, such as standing idle, this was classified as "other" behaviour.

Faecal samples (around 10 g) were taken from assessed pigs either from the rectum or from fresh faeces. Sixteen samples from the organic farms and 20 samples from the conventional farms were taken. All samples were stored in a cooling box. If faecal analysis was not done on the same day as collection, then the samples were stored in a refrigerator at +4°C for a maximum period of 16 h. Depending on the size of the farm, 4–8 samples per farm were taken. Faecal analysis was performed according to the Concentration McMasters quantitative faecal flotation method. Around 4.0 g of a faecal sample was taken and 56 mL of tap water was added. Faeces and water were stirred and left to rest in a container for 30 min. Then, 10 mL of the faecal suspension was poured through a single layer of gauze into a test tube. The test tube was centrifuged for 7 min at 1,200 rpm. Shortly before counting, flotation fluid (saturated NaCl with 500 g glucose per litre) was added to a volume of the 4 mL mark and the solution was suspended using a Pasteur pipette. A McMaster counting chamber was then filled with the faecal suspension. Microscopic examinations of parasitic eggs were made at 100x magnification. Results were presented as number of eggs per gram (Roepstorff et al., 1998).

An interview was made with a representative of the farm who had knowledge of the farm's management, husbandry procedures and *post-mortem* inspection data regarding changes in abnormalities. During the interview, questions regarding disinfection methods, manure removal and management, anthelminthic treatment, grouping, animal quarantine, disease history, castration, teeth cutting and tail docking procedures were asked.

The number of farms of each system was small, because the national number of organic farms is only three, and thus analytical statistics were not used as they might be considered to claim significance where this was not justifiable. Statistics of raw data presented here are descriptive and should be considered in the light of an observational case study.

Results

The organic pigs had around twice as much area as the conventionally reared pigs. The mean measured stocking densities were higher than required by law, although this was not the case for individual farms. Some farmers knew how densely they can stock their pigs, because they had already measured the areas and read the law. Other farmers did not know the area size and stocked their pigs according to their experience, particularly in regard to their estimates to minimise tail biting. On the conventional farms, the minimum measured density of sows was 1.94 m^2 and the maximum was 5.75 m^2, and among finishers, the minimum density was 0.82 m^2 and the maximum was 2.06 m^2. On the organic farms, the minimum stocking density of sows was 2.07 m^2 and the maximum was

37.5 m^2, while among finishers, the minimum density was 0.87 m^2 and the maximum was 5.42 m^2. In both systems, there were sows at a higher stocking density than they should be according to the regulations. The farms that had too high densities were aware that they were higher than should be, but they had lack of space to accommodate all the pigs, and thus decided to put more pigs in groups than there should have been.

From the human-animal interaction evaluations, among conventional pigs, 93.6% were scored 0 and 6.4% as 1. There were no conventionally raised pigs that were given score 2. Among organic pigs, 86.3% were scored 0; 9.7% were scored 1 and 4.0% were scored 2. In the conventional systems, higher prevalences of aggressive behaviour (fighting for food and space) among pigs were recorded compared with organic farms (Table 3). And an opposite result was seen regarding positive behaviours; the organic farms had higher prevalences of positive behaviours (sniffing and gently nosing of another pig) than on the conventional farms (Table 3). There were no differences in resting behaviour between the two management systems (Table 3).

On the conventional farms, 97.9% of pigs were not lame, and 2.1% of pigs were lame. Of the latter, 1.8% were scored with a lameness score 1, 0.2% with a score 2 and 0.2% with a score 3. On the organic farms, 99.3% of pigs were not lame, while 0.7% were lame. Lameness score 1 was detected in 0.2% of pigs and 2 in 0.5%. There were no pigs that had a lameness score of 3. Therefore, organic farms had slightly fewer lameness cases. On the conventional farms, 86.8% of pigs were scored clean (score 1), 13.0% were scored slightly dirty (score 2) and 0.2% were dirty (score 3). On the organic farms, 92.8% pigs scored clean, 5.5% scored slightly dirty and 1.7% scored dirty. Pigs on the organic farms were cleaner compared with those on conventional farms, although there were more pigs with score 3 on the organic farms than conventional.

Tail bites were observed on 5.0% (31 pigs) of pigs on the conventional farms and 0.0% on the organic farms. A small proportion of pigs raised on conventional farms (1.1%, 7 pigs) and on organic farms (2.0%, 8 pigs) had their ears bitten. There was no difference for incidences of ear biting between pigs on the organic and conventional farms. There were few observations of shoulder ulcers. On the conventional farms, 0.2% (2 pigs) were affected by shoulder ulcers and 0 pigs were detected with this problem on the organic farms. On the conventional farms, there was a higher prevalence of skin wounds than on the organic farms. On the conventional farms, 9.9% of pigs were observed with skin wounds and there were 2.5% of such pigs on the organic farms. Most skin wounds were located in the middle and hind sections of the body (Table 4). In both production systems, the commonest number of wounds was 5–10 per animal, then fewer than 5 wounds, and least frequently more than 10 wounds were observed (Table 5). In both systems, the material for pens was wood and metal, so the wounds inflicted because of rubbing against the walls should not have influenced the overall scores.

All faecal samples taken from conventional farms were negative for parasites, while only 4 negative samples were from the organic farms (all of which were taken from one farm). Median egg counts per gram on the organic farms were 150, with a mean of 260 (Table 6). Of positive samples, the following species were identified: *Eimeria spp*, *Ascaris suum* and *Strongylida spp*. Of these samples, 7 contained *Eimeria spp*, 6 *Ascaris suum* and 11 *Strongylida spp*. Most frequently seen were *Strongylida spp* eggs with a

Table 3. Social and exploratory behaviours among conventional and organic pigs

	Conventional pigs (%)	Organic pigs (%)
Negative social behaviour	1.6	0.69
Positive social behaviour	14.8	19.7
Pen investigation	33.2	25.9
Exploratory behaviour	2.0	15.5
Resting	11.9	11.7
Other	35.8	26.6

Table 4. Location of skin wounds and percentages in conventional and organic pigs

Skin wound location	% of pigs with skin wounds among conventional pigs (number)	% of pigs with skin wounds among organic pigs (number)
Head-neck	0.7% (4)	0.5% (2)
Middle body	6.5% (40)	0.5% (2)
Hind body	2.1% (13)	0.7% (3)
Legs	0.7% (4)	0.7% (3)

Table 5. Distribution of numbers of wounds in pigs at different body locations

	Conventional farms (no. wounds)			Organic farms (no. wounds)		
	< 5	5–10	> 10	< 5	5–10	> 10
Head-neck	3	1	0	0	2	0
Middle body	8	21	11	1	1	0
Hind body	2	9	2	1	2	0
Legs	3	1	0	1	2	0
Total	16	32	13	3	7	0

Table 6. Total egg counts on conventional and organic farms (epg – eggs per gram)

	Conventional farms (epg)	Organic farms (epg)
Positive farms (% of farms)	0 (0%)	2 (67%)
Mean (min/max)	0 (0/0)	260 (0/1,235)
Median	0	150
Samples taken	20	16

Table 7. Total egg count of *Eimeria spp*, *Ascaris suum* and *Strongylida spp* on organic farms (epg – eggs per gram)

Organic farms			
	Eimeria spp (epg)	*Ascaris suum* (epg)	*Strongylida spp* (epg)
Average (min/max)	87 (0/1,146)	35 (0/288)	137 (0/638)
Median	0	0	36

median of 36 epg, while the highest number of eggs of the groups was *Eimeria spp* with 1,146 epg (Table 7). The intensity between positive samples was variable, ranging from 10 epg to 1,235 epg.

Unfortunately, only two of the conventional and one of the organic production systems knew their herd *post-mortem* inspection data. One conventional farm and one organic farm did not know that it was possible to ask for feedback from the slaughterhouse. One of the organic farms has not slaughtered pigs for the past three years. It was noted that most of the farms did not use *post-mortem* inspection data to improve the pigs' living conditions. The conventional farms that had analysed their *post-mortem* data reported a higher prevalence of respiratory problems compared with other pathologies. Also, there were mild cases of liver spots. One conventional farm reported that they had had problems with urinary tract infections recently according to the *post-mortem data*: around 14% of slaughtered animals. On organic farms, the farmers reported a high prevalence of liver spots, not recalling any other changes that might be of interest. Among the conventional farms, the most often reported pathological problem reported involved the respiratory tract. The prevalence of respiratory disease varied greatly among the conventional farms. The prevalence of respiratory diseases ranged from 25–35% on one conventional farm to 5–7% on another. None of the organic farms reported a high prevalence of respiratory disease, although one farm said that they had had a brief period of a respiratory disease in the herd that had been treated with antibiotics.

On conventional farms, a small number of cases of liver spots during *post-mortem* inspection was found. One farm had a prevalence of 0.0–0.8% while another had around 2%. Regarding two organic farms, the farmers reported that there had been high numbers of parasites found in their animals. One farmer said that they had had several incidences of high numbers of liver spots and they had changed anthelminthic schemes. This indicated that the conventional farms had a very mild infestation of endoparasites in the herd, while organic farms had higher infestation rates of internal parasites. It was noted that the organic farms had no proper disinfection schemes, because they were not able to remove all pigs from the pens for whole-pen disinfection, so they often just cleaned one group at a time after sending specific groups of pigs to slaughter. Also, all of them used deep straw bedding, where new was added on top of old and removed depending on the farm (ranging from once a week to once a year). This might have had a negative effect on the eradication of parasitic eggs in the environment and higher probability of reinfection.

Discussion

Good human-animal interaction scores were observed among 93.6% of conventional pigs and among 86.3% of organic pigs. On the organic farms, the handling frequency of animals was lower

compared with conventional farms, which might have made the animals more cautious of humans. Several studies have shown that the human–animal interaction depends on the workforce and management of the farm (Hemsworth, 1989, 1999; Pearce et al., 1989; Waiblinger et al., 2006), which confirmed the finding that a lower frequency of positive handling causes pigs more stress, thus increasing fearfulness to humans. The human–animal interaction was possibly influenced by the farmers' handling frequency. On one organic farm, the farmer did not see their pigs regularly and they rarely saw other people, and on this farm the pigs would flee at the sight of humans. The slightly poorer scores for organic farms in this regard may thus be explained by this unfamiliarity with humans and not be indicative of poorer stockmanship on organic farms. The low prevalence of exploratory behaviour on the conventional farms was possibly linked to the low availability of enrichment material. On all organic farms, there was straw available for every pig, which would be expected to encourage play behaviour in the pigs. Positive social behaviour and exploratory behaviour are expressed more when pigs have a natural environment, where they can express their normal behaviours (Roy et al., 2019; Studnitz et al., 2007; Van de Weerd et al., 2003). The deep straw bedding provided on organic farms allows pigs to forage, and this might indicate that on organic farms pigs can show exploratory and positive behaviours more often than on conventional farms. Where there was no free access to enrichment material, on the conventional farms, the pigs were forced to express other behaviours more often, such as investigating their surroundings and just standing. This may explain the higher prevalence of pen investigation and other behaviours in the conventionally reared pigs. Negative social behaviours, resting and other behaviours were more prevalent on the conventional farms than on the organic farms. Positive social behaviours and exploratory behaviours were less frequently observed on the conventional farms than on the organic farms.

Lameness was recorded in 2.1% of pigs on the conventional farms and in 0.7% on the organic farms. Several studies have shown that lameness is more prevalent on conventional farms than on organic farms (Leeb et al., 2019; Pluym et al., 2013; Knage-Rasmussen et al., 2014). The use of fully slatted floors is only allowed on conventional farms and it is not required to provide bedding material on conventional farms as it is for organic farms (Council Regulation 889/2008/EC; Council Directive 2008/120/EC), which increases the risk of lameness in conventional systems (Heinonen et al., 2006; Maes et al., 2016). The pigs detected with severe lameness were all on one farm that had had an accident regarding management. They had mistakenly left open the faucet tap with quicklime, thus pigs sat on the caustic alkaline ground and burnt their hind legs. Due to this, they were in too much pain to move properly. Pigs scored with a lameness score 1 or 2 had different reasons for limping (trauma or hoof diseases).

While 86.8% of conventional pigs were scored clean, this compared with 92.8% of organic pigs, therefore, this does not seem to have been different between the two systems. There have been no studies comparing the cleanliness of pigs in different production systems, although several studies state that cleanness indicates good hygiene (Sanchez-Vazquez et al., 2010; Van Breda et al., 2017; Wagner et al., 2018). Straw bedding, which was used only on the organic farms, absorbs moisture, thus leaving pigs cleaner. On one organic farm, the farmer said that the worker constantly forgets to add new straw, and thus the pigs on the farm were dirtier than they ought to have been. This may have influenced dirtiness scores among organic farms' pigs. On conventional farms, there were mostly slatted floors, and thus, the faeces dropped into the passage underneath leaving the floor dry.

Both tail biting and ear biting are linked with similar risk factors, which are high stocking density and absence of enrichment material (Beattie et al., 2005; Brunberg et al., 2011). According to minimum standards, organic farms should have twice as much area as conventional farms and are required to provide bedding material, which conventional farms are not (Council Regulation 889/2008/EC; Council Directive 2008/120/EC). Thus, conventional farms would be expected to have more tail and ear biting than in organic systems. This study did not observe that either tail or ear biting were higher on conventional farms, although there was a slightly higher incidence of tail biting, possibly because of the higher stocking densities and absence of enrichment material. As reported earlier, the stocking densities in conventional farms were twice those on the organic farms. In total, two conventional farms and all three organic farms had enrichments – chains, car tyres, balls or straw, which showed that conventional farms had less enrichment for pigs, thus having higher likelihood of tail biting.

There were few observations of shoulder ulcers among conventionally reared pigs, and no organic pigs were detected with shoulder ulcers. The prevalence of shoulder ulcers varies greatly within the production system, which indicates that it is influenced by the management within farms (Cleveland-Nielsen et al., 2004; Rosendal and Nielsen, 2005). Shoulder ulcers are affected by low body condition scores (Rosendal and Nielsen, 2005). All pigs in the study had good body condition scores, which might have been the reason for the similar and low numbers of shoulder ulcers observed. Skin wounds have been linked with aggression, which can be decreased with less frequent regrouping, smaller group sizes, lower stocking densities and provision of enrichment materials

(Roy et al., 2019; Thomansen et al., 2016; Van de Weerd et al., 2003). As stated by the minimum requirements (Council Regulation 889/2008/EC; Council Directive 2008/120/EC), organic pigs should have twice as much space as conventional systems, and are to be provided with bedding, so the prevalence of skin wounds would be expected to be higher on conventional farms. This was confirmed in this study as more pigs had skin wounds in conventional systems than in organic. The average number of lesions discovered in this study was 5–10 wounds per pig in both systems, although in the conventional systems there were more pigs with wounds than in the organic systems. This was evaluated when pigs had already established a hierarchical order, which might have influenced the results. In newly mixed groups, the number of wounds could be even higher, as Thomansen et al. (2016) and Turner et al. (2006) showed.

None of the 20 faecal samples taken from conventional farms contained parasite eggs, while 12 of 16 faecal samples from organic farms had parasite eggs. Järvis et al. (2012) also discovered that all organic farms in their sample had parasites, compared with 41.9% on large conventional farms. However, at that time in 2012, organic pigs were kept outdoors. The similarity in these rates suggests that it is not only the outdoor keeping of pigs that contributes to the higher parasite infection rates on organic farms; the risk of an endoparasitic infection on organic farms was not higher solely because of them being outdoors, but because of some other factors. One of these factors may have been that the organic farms had poorer disinfection schemes, and it was not considered practicable to do full room disinfection, which would have impaired the eradication of the parasite eggs from the environment. Also, the practice of laying new straw bedding on top of old can be considered important. On one of the organic farms, no parasite eggs were found in any of the faecal samples. It might also be that the likelihood of infection is smaller in low density populations. Regarding the intensity of the parasitic infection it should be considered that parasitic eggs are not distributed equally in a faecal sample. Also, it should be noted that the host immune response is different between individuals, and different species of endoparasites have variable fertility (Järvis et al., 2012). Unfortunately, only two of the conventional and one of the organic production systems knew their herd *post-mortem* inspection data, so it is impossible to make overall conclusions. Nevertheless, the available data showed that organic farms had more liver spots compared with conventional farms, but had fewer signs of respiratory disease. Higher occurrence of liver spots on organic farms was also found by Kongsted and Sørensen (2017) in a three year *post-mortem* data analysis in Denmark. Bonde et al. (2010) reported similar results regarding *post-mortem* reports of respiratory disease as the analysis of the available data in this study.

This study did not contain any analytical statistics because of the small number of farms in the sample; thus, only descriptive statistics are presented. Unfortunately, in Estonia, there are only four organic pig farms (one excluded organic farm had only three sows); thus it was impossible to have a greater sample size in Estonia for this investigation.

Conclusion

Positive findings for the welfare of pigs on indoor organic farms included the following. On organic pig units, the minimum stocking density was twice as much as in conventional farming and the stocking densities were lower than regulations permit. Organic pigs had more positive social behaviours and less negative behaviours compared with those on the conventional farms. In addition, there were higher incidences of exploratory behaviour by these pigs. Tail biting occurred more often on the conventional farms than on the organic farms. Pigs on the conventional farms had more skin wounds than those on the organic farms. Organic farms pigs were scored cleaner compared with conventional farms, but this did not mean that they had better sanitary conditions. Organic farms had worse human-animal interaction scores than on the conventional farms, possibly due to less frequent handling of pigs in those farms. Parasite eggs in faecal samples were found on two of the three organic farms, while all samples taken in the conventional systems were negative. The higher prevalence of parasitic infections on the organic farms compared with the conventional systems in this study suggests that access to outdoors alone is not the only influence on endoparasitic infection prevalence on organic pig farms. Numbers of shoulder ulcers were infrequent. Despite being restricted to indoors, organic pigs had better welfare scores than conventional pigs and poorer parasite scores, as would be expected from organic pigs that are allowed access to an outdoor facility.

Acknowledgements

The study was supported financially with a UFAW (Universities Federation for Animal Welfare) student scholarship for JP. The authors record their thanks to all farm representatives who allowed collection of the data and answered questions with honesty. We also thank Jaanika Pajuste of the Institute of Veterinary Medicine and Animal Sciences of the Estonian University of Life Sciences for help analysing faecal samples.

Ethical Approval

The animals in this study were only observed, and they were not subjected to any interference in their management regime and nor were animals injured or harmed. No blood samples were taken. Body tissue was sampled *post mortem*.

Funding

The study was funded in part with a student scholarship awarded by the Universities Federation for Animal Welfare (UFAW).

Competing Interest

There were no conflicts of interest excepting the student scholarship from UFAW mentioned above.

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Received 25 February 2022
Accepted 7 April 2022

Virulence Genes and Antibiotic Resistance of *Aeromonas hydrophila* Isolated from Marketed Milk

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Keywords: *Egypt, market buffalo milk, virulence genes, antibiotic resistance, A. hydrophila*

Abstract. *This research aims to assess the existence rate, most dangerous virulence genes (aerolysin (aerA) and hemolysis (ahh1)), antibiotics sensitivity, and resistance pattern of Aeromonas hydrophila strains that were isolated from 100 raw marketed buffalo milk samples, which were gathered from Dakahlia governorate, Egypt. The culturally obtained Aeromonas spp. were evidenced in about 87% of the examined raw market milk samples while the biochemical investigation revealed that 72% of the inspected samples were polluted with Aeromonas species. On the other hand, A. hydrophila was detected in about 30% of the examined market milk samples. In addition, molecular detection of aerA and ahh1 virulence factors of 19 A. hydrophila isolates using multiplex PCR was carried out. Then, it was detected that 8 (42.1%) isolates had aerA gene, while 5 (26.3%) isolates possessed ahhl gene and 4 (21.1%) isolates had both aerA and ahh1 genes. Additionally, 2 (10.5%) isolates were negative for the two inspected genes. All A. hydrophila isolates (19) showed resistance against streptomycin antibiotic; the average multiple antibiotic resistance between A. hydrophila isolates was 0.431, and it reached 1 in one strain (positive for aerA gene) as this strain was resistant to all used antibiotics. In conclusion, this study reveals a high incidence of multiple antibiotic resistance (MAR) of A. hydrophila strains that were isolated from marketed milk samples in Dakahlia governorate. Moreover, it indicates the presence of virulence genes.*

Introduction

It is well known that milk as well as milk products are considered as the principal component of nourishment for all ages. Meanwhile, various human digestive diseases and outbreaks have been reported due to ingestion of raw milk and milk products (Verraes et al., 2015).

It is identified that *Aeromonas* species consist of non-spore pointing gram negative rods which are universal in all aqueous environments. It has been identified that all individuals can be infected with *Aeromonas* spp. which may lead to bacteremia. Diarrhea and wound infections are also considered as consequences of this microbe. Unfortunately, young aged children besides immunocompromised persons, are the most affected persons that show signs of diarrhea (Figueras and Beaz-Hidalgo, 2015). It has been assessed that in advanced states, the portion of people that might be vulnerable to suffer from foodborne illness would be close to 20% (Lund, 2015).

Consequently, foodborne problems are growing and causing excessive threats to milk buyers in Africa. According to WHO (2015), it was identified that diet played an important role in the occurrence of diarrheal sicknesses worldwide, as about 600 million cases of illness and 420 000 deaths were informed in 2010.

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The *A. hydrophila* has been known to produce variable potential virulence toxins. Meanwhile, aerolysins *(aerA)* besides hemolysins *(hlyA)* have been considered as the greatest significant hemolytic poisons resulting in the pathogenesis of the disease following infection (Martin-Carnahan and Joseph, 2015).

There are about 30 identified types of *Aeromonas* spp. (Martínez-Murcia et al., 2016), and in humans there are four subgroups of this *Aeromonas* spp. that are involved in human diseases: *A. hydrophila, A. veronii, A. biovar, A. sobria, A. caviae,* and *A. dhakensis*. Moreover, the severity of chronic digestive illness is linked to *Aeromonas* spp. that is found in diverse water besides food (Teunis and Figueras, 2016). Also, *Aeromonas* spp. reveal a main role in the transmission of antibiotic confrontation, constructing these bacteria as a problematic. They have been employed as intermediaries in the transmission of antibiotic confrontation indicators among hospitals besides ecological strains (Varela et al., 2016).

A. hydrophila is insulated from an extensive range of diets such as fish, eggs, milk, dairy products, seafoods, vegetables, meat and its' products (Kamalpreet, 2017). Commanding *A. hydrophila* contagion is so supreme because this bacterium can threat food safety (Talagrand-Reboul et al., 2017) as it has appeared as a significant food-related microbe universally (Pal, 2018). In addition to food, *Aeromonas* spp. are isolated from medical and ecological samples as

they can grow at low temperatures besides yielding toxins. As a result of this, they raise the danger of foodborne contagion dramatically (Bello-López et al., 2019). *Aeromonas* spp. secrete extracellular proteins, as enzymes (proteases and lipases) and cytotoxins which are correlated to their microbial pathogenicity in addition to showing diverse parts in the infectious procedure (Pessoa et al., 2019 & Fernández-Bravo and Figueras, 2020)

This research aims to assess the existence rate, most dangerous virulence genes (aerolysin *(aerA)* and hemolysis *(ahh1)*), antibiotics sensitivity, and resistance pattern of *A. hydrophila* strains that were isolated from 100 raw market buffalo milk samples gathered from Dakahlia governorate, Egypt.

Materials and Methods

Ethics statement

The gathering of specimens that were used in this research monitored the rules of Mansoura University. The procedures of this research were approved by the Research Ethics Committee, Faculty of Veterinary Medicine, Mansoura University (code R/54).

The methods that were useful for sample gathering were directed by the American Public Health Association (A.P.H.A, 1992). One hundred raw market buffalo milk samples (each 250 mL) were haphazardly gathered from different shops and marketplaces in different regions of Mansoura city, Dakahlia governorate, Egypt. The samples were saved in a secure ice box (4 ± 1°C) to be transferred to the laboratory of Food Hygiene and Control Department, Faculty of Veterinary Medicine, Mansoura University for a microbiological check-up. The biosecurity measures during collection of 100 market raw buffalo milk samples from dairy markets were agreed to by OIE (2008). Biosafety measures during sample handing and application of microbiological examination in the laboratory were followed according to the guidelines of the WHO (2004), while the procedures of infection control for research work in laboratories were applied (Burt et al., 2014).

Isolation and identification of *A. hydrophila*

Milk specimens were thoroughly stirred, then we added 25 mL of each specimen to 225 mL of tryptone soya broth, blended well at the time while they were incubated at 37°C for 24 h, and subcultured to the *Aeromonas* selective medium base (Oxoid, CM0833) with supplementary ampicillin for selective quarantine of *Aeromonas* spp. Ampicillin concentration was chosen according to recommendations. The petri plates were incubated at 25°C for 24 h. Five typical opaque green colonies with dark centers were chosen, then purified on nutrient agar slants and kept warm at 37°C for 24 h for more investigation (Palumbo et al., 2001).

Pure cultures of the isolates were morphologically stained by Gram stain (Koneman et al., 1994). Motility and biochemical tests such as: Esculin hydrolysis test, Oxidase test, Arginine hydrolysis, Indole test, Methyl Red Test, Voges Proskauer test, Citrate utilization test, Urease test, Hydrogen sulfide production test, Nitrate reduction test, Gelatin hydrolysis test, Ornithine decarboxylase (ODC), L-lysine decarboxylase (LDC), Arginine decarboxylase (ADH), β- galactosidase (ONPG), and fermentation of sugars (Garrity, 2001). Then 19 isolates from *A. hydrophila* were taken and examined for proteolytic activity (Yucel et al., 2005), lipolytic activity (Collins et al., 1989), and hemolytic activity (Singh and Sanyal, 1997).

Molecular identification of *A. hydrophila*

Genomic DNA extraction of *A. hydrophila*

The DNA was extracted from *A. hydrophila* isolates by genomic DNA extraction kit (Thermo scientific, UK) according to the manufacturer guidelines. Briefly, up to three or five bacterial colonies were taken, homogenized in 200 µL of deionized water, heated at 100°C for 15 min, and centrifuged at 10 000 g for 3 min. The supernatant was then transported to a sterile Eppendorf tube and taken as a DNA template.

Detection of suspected virulence genes in *A. hydrophila* using multiplex PCR

Multiplex PCR protocol was conducted in order to identify *A. hydrophila* species by using specific pairs of primers (Invitrogen, Carlsbad, CA) for detection of aerolysin (*aerA*) and hemolysin (*ahh1*) virulence genetic factors following the methods described by Shah et al., (2009), Meanwhile, the sequence of primers (Pharmacia Biotech) and the PCR product size are illustrated in Table 1. The amplification of virulence genetic factors was achieved following the procedures of Wang et al. (2003) on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany).

PCR amplification was achieved in a 96-well 2720 thermal cycler (Applied Biosystems, Norwalk,

Table 1. Target genes, primers sequences, and amplicon sizes

Target genes	Primers	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	Reference
aerA	*AH-aerA (F)*	*5′ CAAGAACAAGTTCAAGTGGCCA '3*	309	Stratev et al. (2016)
	AH-aerA (R)	*5′ ACGAAGGTGTGGTTCCAGT '3*		
ahh1	*AHH1 (F)*	*5′ GCCGAGCGCCCAGAAGGTGAGTT '3*	130	
	AHH1 (R)	*5′ GAGCGGCTGGATGCGGTTGT '3*		

California, USA). PCR mixture was amplified in a total volume of 25 µL containing 12.5 µL of 2X master mix (Thermo scientific), 1 µL of *ahh1* and *aerA* primers, 5 µL of DNA template and the total volume was completed to 25 µL by DNase/RNase-free H_2O. Then, the amplification cyclic conditions of PCR comprised of: initial denaturation at 95°C for 5 min, 50 cycles at 95°C for 30 sec, 59°C for 30 sec, 72°C for 30 sec, and final elongation at 72°C for 7 min. The quality of PCR products was tested by electrophoresis on 1.5% agarose gels electrophoresis and imagined via UV transilluminator using a 100-bp DNA ladder (Invitrogen, San Jose, California, USA).

Antimicrobial susceptibility testing and multiple antibiotic resistance (MAR) index value

All the identified *A. hydrophila* isolates were examined via the disc diffusion method; sensitivity discs by adjustable concentrations were practiced to define the susceptibility of the quarantined bacterial isolates (Oxoid Limited, Basingstoke, Hampshire, UK). Pure cultures of the identified *A. hydrophila* were cultured in tryptic soy broth (Oxoid CM0129), incubated at 28°C for 8 h, and then streaked via sterile cotton swabs on nutrient agar petri plates. Then, the antimicrobial discs were put in petri plates, incubated at a suitable temperature (37°C) for 24 h, and finally tested for the development of the microbe near the antimicrobial discs. Concerning the diameters of inhibition zones, the examined isolates were categorized as susceptible, intermediate, or resistant (Clinical & Laboratory Standards Institute, 2016). Thus, the antibacterial discs and their condensation in addition to the widths of the areas of suppression for the examined isolates were established in Table 2.

Multiple antibiotic resistance (MAR) index for each isolate was calculated according to the formula specified by Singh et al. (2010) as below:

MAR index = Number of the resistance (isolates categorized as intermediate were measured sensible for MAR index) (a) / Total No. of examined antibiotics (b). MAR index= a/b.

Statistical Analysis

The data were analyzed as numbers and percentages. Multiple antibiotic resistance (MAR) index for each isolate and the total average were calculated by SPSS (Statistical Package for Social Science) software version 16.

Results

Isolation and identification of *A. hydrophila* in examined raw milk

Culturally *Aeromonas* spp. were detected in 87% of examined raw market milk samples while biochemically 72% of the examined samples were contaminated with *Aeromonas* spp. Identification of confirmed species showed that *A. hydrophila, A. trota, A. janda, A. caviae, A. veronii* and *A. fluvialis* were the main isolates by proportions of 30%, 26%, 15%, 13%, 3%, and 2%, respectively. (There were 17 samples from the positive samples (72) that contained two different species of *Aeromonas* spp.; therefore, they were counted two times (89 – 17 = 72%).

From about 94 confirmed cultures isolated from raw market milk samples, 35 (37.2%) isolates could be identified as *A. hydrophila* followed by 26 (27.7%)

Table 2. Antimicrobial discs, concentration, and interpretation of their action on A. hydrophila isolates

Antimicrobial agent	Sensitivity disc content (ug)	Resistant (mm)	Intermediate (mm)	Susceptible (mm)
Cephalothin (CN)	30	14 or less	15–17	18 or more
Ampicillin (AM)	10	13 or less	14–17	18 or more
Nalidixic acid (NA)	30	13 or less	14–18	19 or more
Oxytetracycline (T)	30	14 or less	15–18	19 or more
Meropenem (M)	10	9 or less	10–12	13 or more
Cefepime (FEP)	30	18 or less	19–24	24 or more
Cefazolin (CZ)	30	10 or less	11–14	15 or more
Gentamicin (G)	10	12 or less	13–14	15 or more
Doxycycline (DO)	30	14 or less	15–18	19 or more
Amikacin (AK)	30	12 or less	13–15	16 or more
Ciprofloxacin (CP)	5	15 or less	15–19	20 or more
Cefotaxim (CF)	30	17 or less	18–22	23 or more
Erythromycin (E)	15	13 or less	14–22	23 or more
Streptomycin (S)	10	11 or less	12–14	15 or more
Neomycin (N)	30	12 or less	13–16	17 or more
Sulphamethoxazol (SXT)	25	10 or less	11–15	16 or more

as *A. trota,* 15 (16.0%) as *A. janda*, 13 (13.8%) as *A. caviae,* 3 (3.2%) as *A. veronii* and 2 (2.1%) as *A. fluvialis* (Table 3).

Proteolytic, Lipolytic, and Hemolytic Activities of *A. hydrophila*

A. hydrophila showed proteolytic, lipolytic and hemolytic activities at the ratio of 57.9%, 42.1%, and 21.1%, respectively, while some isolates (5.3%) revealed proteolytic and lipolytic activities at the same time and others (21.1%) had proteolytic and hemolytic activities together also (Table 4).

Molecular identification of *A. hydrophila* using multiplex PCR

Genetic detection of aerolysin (*aerA*) and hemolysin (*ahh1*) genes from 19 *A. hydrophila* isolates were carried out using multiplex PCR technique. There were about 8 (42.1%) isolates that possessed *aerA* gene, while 5 (26.3%) isolates revealed *ahhl* gene and 4 (21.1%) isolates showed *aerA* and *ahh1*genes. However, 2 (10.5%) isolates were negative for the two examined genes (Table 5 and Figure 1, 2).

Antibiotic susceptibility profile of *A. hydrophila* isolates

The antimicrobial drug vulnerability outlines for the 19 *A. hydrophila* isolates that were isolated from raw market milk samples are exposed in Table 6. Higher susceptibility of *A. hydrophila* was reported to be against amikacin (AK) (89.5%) and ciprofloxacin (CP) (78.9%). Also, there was high multiple antibiotic resistance between *A. hydrophila* isolates as shown in Table 7, because the average multiple antibiotic resistance index (MAR) was 0.431, and it reached 1 in one isolate (this isolate is numbered 11 in Figure 2 using multiplex PCR that has a positive result for *aerA* gene), as this isolate was resistant to all the used antibiotics followed by 0.937 (the isolate numbered 1 in multiplex PCR and has positive bands for *aerA* and *ahhl* genes) in the second isolate.

In brief, *A. hydrophila* isolates that are numbered from 1 to 19 in the antimicrobial resistance profile (Table 7) have numbers 11, 1, 2, 3, 12, 13, 14, 4, 15, 5, 16, 17, 6, 7, 8, 9, 18, 19 and 10 when running on agarose gel electrophoresis of multiplex PCR to determine *aerA* (309 bp) and *ahhl* (130 bp) genes (Figures 1 and 2).

Table 3. Frequency distribution and prevalence of *Aeromonas* spp. obtained from examined milk samples

Isolates	Samples		Isolates	
	n	%	n	%
A. hydrophila	30	30	35	37.2
Trota	26	26	26	27.7
Janda	15	15	15	16.0
Caviae	13	13	13	13.8
Veronii	3	3	3	3.2
Fluvialis	2	2	2	2.1

Where total number of examined raw market buffalo milk samples is 100, and total number of *Aeromonas* isolates is 94 isolates.

Table 4. Distribution of proteolytic, lipolytic, and hemolytic activities of 19 *A. hydrophila* isolates isolated from examined milk samples

A. hydrophila	No. of isolates	Proteolytic Activity		Lipolytic activity		Hemolytic activity		Proteolytic +Lipolytic activity		Proteolytic +Hemolytic activity	
	19	n	%	n	%	n	%	n	%	n	%
		11	57.9	8	42.1	4	21.1	1	5.3	4	21.1

Table 5. Incidence of *ahhl,* and *aerA* genes via multiplex PCR of *A. hydrophila* isolates (19) from examined raw milk samples

A. hydrophila	No. of Strains	+ve for *aerA* gene		+ve for *ahhl* gene		+ve for *aerA* and *ahhl* genes		-ve for *aerA* and *ahhl* genes	
	19	n	%	n	%	n	%	n	%
		8	42.1	5	26.3	4	21.1	2	10.5

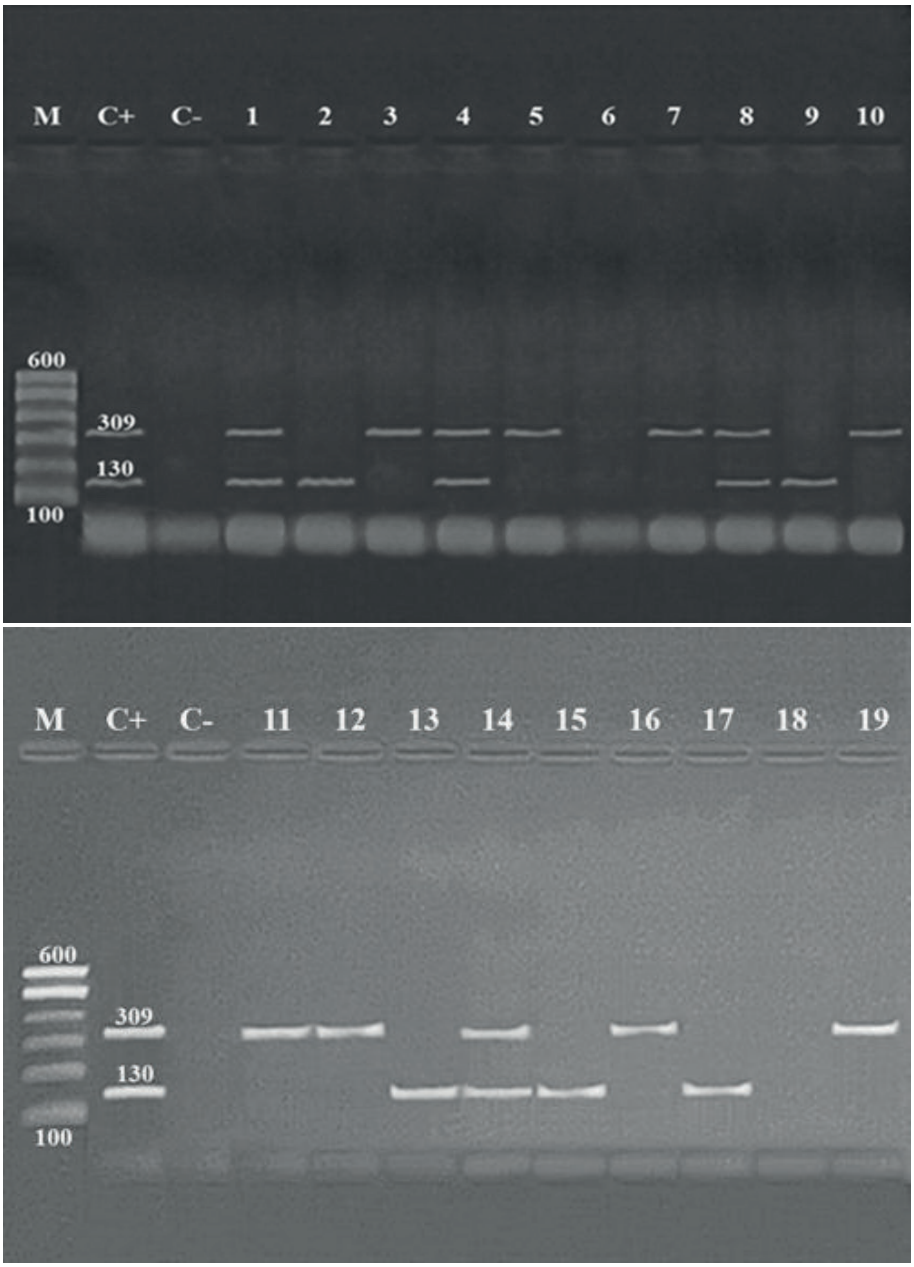
Table 6. Antimicrobial sensitivity of *A. hydrophila* isolates (n = 19)

Drug agent	S		I		R	
	n	%	n	%	n	%
Streptomycin (S)	-	-	-	-	19	100
Erythromycin (E)	-	-	1	5.3	18	94.7
Ampicillin (AM)	1	5.3	2	10.5	16	84.2
Cefazolin (CZ)	3	15.8	1	5.3	15	78.9
Cephalothin (CN)	8	42.1	1	5.3	10	52.6
Doxycycline (DO)	8	42.1	2	10.5	9	47.4
Cefotaxim (CF)	9	47.4	3	15.8	7	36.8
Sulphamethoxazol (SXT)	10	52.6	2	10.5	7	36.8
Neomycin (N)	11	57.9	2	10.5	6	31.6
Gentamicin (G)	12	63.2	1	5.3	6	31.6
Oxytetracycline (T)	12	63.2	3	15.8	4	21.1
Cefepime (FEP)	13	68.4	2	10.5	4	21.1
Nalidixic acid (NA)	14	63.7	1	5.3	4	21.1
Meropenem (M)	14	63.7	2	10.5	3	15.8
Ciprofloxacin (CP)	15	78.9	2	10.5	2	10.5
Amikacin (AK)	17	89.5	1	5.3	1	5.3

Table 7. Antimicrobial resistance profile of *A. hydrophila* strains (n = 19)

No	Antimicrobial resistance profile	MAR index
1	S, E, AM, CZ, CN, DO, CF, SXT, N, G, T, FEP, NA, M, CP, AK	1
2	S, E, AM, CZ, CN, DO, CF, SXT, N, G, T, FEP, NA, M, CP	0.937
3	S, E, AM, CZ, CN, DO, CF, SXT, N, G, T, FEP, NA, M	0.875
4	S, E, AM, CZ, CN, DO, CF, SXT, N, G, T, FEP, NA	0.812
5	S, E, AM, CZ, CN, DO, CF, SXT, N, G	0.625
6	S, E, AM, CZ, CN, DO, CF, SXT, N, G	0.625
7	S, E, AM, CZ, CN, DO, CF, SXT	0.500
8	S, E, AM, CZ, CN, DO	0.375
9	S, E, AM, CZ, CN, DO	0.375
10	S, E, AM, CZ, CN	0.313
11	S, E, AM, CZ	0.250
12	S, E, AM, CZ	0.250
13	S, E, AM, CZ	0.250
14	S, E, AM, CZ	0.250
15	S, E, AM, CZ	0.250
16	S, E, AM	0.187
17	S, E	0.125
18	S, E	0.125
19	S	0.062
	Average 0.431	

S: Streptomycin; E: Erythromycin; AM: Ampicillin; CZ: Cefazolin; CN: Cephalothin; DO: Doxycycline; CF: Cefotaxim; SXT: Sulphamethoxazol; N: Neomycin; G: Gentamicin; T: Oxytetracycline; FEP: Cefepime; NA: Nalidixic acid; M: Meropenem; CP: Ciprofloxacin; AK: Amikacin



Figures (1, 2). Agarose gel electrophoresis of multiplex PCR of *aerA* (309 bp) and *ahhl* (130 bp) genes for a description of *A. hydrophila* (n = 19).

Lane M: 100 bp ladder as a molecular size DNA marker.
Lane C+: Control positive *A. hydrophila* for *aerA* and *ahhl* genes.
Lane C–: Control negative.
Lanes 1, 4, 8 & 14: Positive *A. hydrophila* isolates for *aerA* and *ahhl* genes.
Lanes 2, 9, 13, 15 & 17: Positive *A. hydrophila* strains for *ahhl* gene.
Lanes 3, 5, 7, 10, 11, 12, 16 & 19: Positive *A. hydrophila* strains for *aerA* gene.
Lane 6 & 18: Negative *A. hydrophila* strain for *aerA* and *ahhl* genes.

Discussion

Aeromonas spp. are linked to food poisoning and certain human illnesses such as gastrointestinal disorders in addition to extra-intestinal contagions like skin infections, shocking wound contagions, as well as lower breathing tract/urinary tract contagions (Batra et al., 2016).

In our study, 72 (72%) raw market milk samples contained *Aeromonas* spp. bacteria. But lower results were recorded by Ahmed et al. (2014) (32%), ElBalat et al. (2014) (32%), Sadek et al. (2017) (36%), and Hammad et al. (2018) (25%).

The examination process was focused on the prevalence of *Aeromonas* spp. and the infection with these bacteria occurs more due to ingestion of contaminated diets. In addition, *A. hydrophila* is the most identified *Aeromonas* spp. in raw milk and milk by products (ElBalat et al., 2014).

A higher existence rate of motile *Aeromonads* was identified in raw milk as the bacterium can pollute the udder through the teat, then proliferate in the mammary tissue, and then can be released in milk (EL-Shemawy and Marth, 1990). Thus, higher existence of *Aeromonas* spp. in raw milk samples reflects inappropriate hygienic procedures of milking and allocation.

The prevalence of *A. hydrophila*, *A. trota*, *A. janda*, *A. caviae*, *A. veronii*, and *A. fluvialis* is shown in Table 3. Opposing results with a lower prevalence ratio were reported by ElBalat et al. (2014) (8% of milk samples were contaminated with *A. hydrophila* spp. while 12% of samples revealed *A. trota* and *A. janda* spp. (Zeinhom and Abdel-Latef, 2014), *A. hydrophila* was found in 24% (Alrazakkazal and Abdullah, 2016), *A. hydrophila* was detected in 7% of the examined milk samples as revealed by Tahoun et al. (2016), But *A. hydrophila* was detected in 8% of the examined raw milk samples (Sadek et al., 2017). Also, *A. hydrophila* was found in 16% of the examined milk samples and four isolates of *A. hydrophila* (3.3%) were isolated from raw milk samples as reported by Abdulaal (2019). While the high prevalence ratio of *A. hydrophila* spp. was 40% in the examined food samples as informed by Enany et al. (2013).

Concerning *A. caviae,* similar results were recorded by ElBalat et al. (2014) (10%) and Sadek et al. (2017) (12%), while higher results were reported by Enany et al. (2013) (31.7%). In addition, about 94 strains of *Aeromonas* spp. were taken from raw milk specimens as shown in Table 3. The other prevalence ratios were reported by ElBalat et al. (2014) (25%), while higher outcomes such as 54.3% were documented by Eid et al. (2013). Regarding *A. trota* and *A. janda*, ElBalat et al. (2014) declared other prevalence ratios such as 40% and 25% respectively which were different from our findings. Also, *A. veronii* was detected in 3% of the examined milk samples (Table 3). It had an extensive variety of hosts and may cause diarrhea and sepsis in individuals (Fernandez-Bravo et al., 2020).

Ahmed et al. (2014) informed that *A. hydrophila* showed proteolytic and lipolytic activities at the ratios of 41.7% and 16.7%, respectively. Meanwhile, Al-Oqaili et al, (2016), Simon et al. (2016), and Sadek et al. (2017) proved that all the examined isolates showed 100% proteolytic and lipolytic activities. Also, all the examined *A. hydrophila, A. caviae* and *A. sobria* had 100% β-hemolytic actions.

On the other hand, our results revealed higher proteolytic, lipolytic and hemolytic activities of *A. hydrophila* isolates (Table 4). Proteinases and lipases enzymes from psychotropic bacteria are documented to be the chief spoilage enzymes of milk products (Sorhaug and Stepaniak, 1991).

According to Citterio and Biavasco, (2015), *A. hydrophila* is the most virulent type of *Aeromonas* spp. In addition, these species produce many virulent toxins, contain structural components that are linked to adhesion, cell virulence, and escape from the phagocytosis process. Another factor that helps them in the induction of the poisoning process is the specific extracellular toxins such as aerolysin which cause lysis as well as toxicity of the cells.

Agreeing to our results that are presented in Table 5 and Figures 1 and 2, similar results were reported by Seker et al. (2015) as they demonstrated that about 9 (40.9%) strains of *A. hydrophila* contained *aerA* gene. Also, Tawab et al. (2017) recorded that *A. hydrophila* and *A. caviae* isolates were positive for numerous virulence genetic factors such as *hly, act, ast*, and *aer* , while Seker et al. (2015) revealed contrasting results to ours, as they reported that about 15 (68.2%) isolates of *A. hydrophila* were positive for *hlyA* gene, while 7 (31.8%) isolates of *A. hydrophila* were noticed to have *hlyA* and *aerA* genes together and nothing of these genetic factors remained achieved from 5 (22.7%) isolates. Also, higher occurrences of these genes were documented by Simon et al. (2016). In addition, Sadek et al. (2017) reported the occurrences of *aer*A and *ahh*1 genetic factors in the examined *A. hydrophila* spp. with the percentages of 66.7% and 77.8%, respectively.

Also, Hammad et al. (2018) reported that the prevalence of *aerA* and *ahh1* genes was 34.9% and 20.6%, respectively, and 13 isolates had no hemolysin gene; besides, another 8 hemolytic isolates showed no virulence genetic factor.

So, the foodborne illness created by *Aeromonas* spp. could be resulted from colonization, and intoxication as the bacteria discharge endotoxins as a consequence to their development in foods (Edberg and Browne, 2007).

As presented in Figure 1, it is clear that the isolate of *A. hydrophila* in lane 6 had neither *aer*A nor *ahh*1 genes although it revealed a hemolytic action on sheep blood agar (Table 4). Similar results were reported by Sadek et al. (2017) who illustrated that the hemolytic action of *A. hydrophila* may be reasoned for genes other than *aer*A and *ahh*1 genes.

Biofilm development is the main virulence aspect in pathogenic microbes. It consists mostly of proteins, DNA, and polysaccharides (Singh et al., 2017). This construction provides the antimicrobial confrontation between *Aeromonas* strains (Dias et al., 2018). *Aeromonas* spp. are sensitive to monobactams, aminoglycosides, carbapenems, cephalosporins, and fluoroquinolones (Codjoe and Donkor, 2017).

Antimicrobial drug resistance of 19 *A. hydrophila* strains isolated from raw market milk samples is shown in Tables 6 and 7. The isolated *A. hydrophila* showed variable resistance to different antimicrobial agents. The spreading of antimicrobial confrontation between food borne microbes may be due to the prolonged use of drugs in the animals used for human consumption (Deng et al., 2016). Previous studies on the confrontation of these strains that were obtained from milk and its products are scarce.

Seker et al. (2015) found that *A. hydrophila* revealed a resistance to ampicillin, cefazoline, gentamycin, amikacin, ciprofloxacin, and cefotaxime of 100%, 81.8%, 4.5%, 5.3%, 4.5%, and 4.5%, respectively. Also, Sadek et al. (2017) reported that *A. hydrophila* isolates showed 100% resistance to ampicillin, erythromycin and amoxicillin antibiotics, while they showed sensitivity against kanamycin, ceftriaxone, ciprofloxacin, and trimethoprim-sulfamethoxazole in the ratios of 22.2%, 55.6%, 100%, and 0.0%, respectively. Moreover, Strateva and Odeyemibc (2016) reported that *A. hydrophila* was resistant to commercial antibiotics.

On the other hand, Odeyemi and Ahmad (2017) stated that *Aeromonas* spp. were completely resistant (100%) to ampicillin, trimethoprim, novobiocin, and sulphamethoxazole. However, isolates were susceptible to tetracycline (100%), oxytetracycline (24.5%), kanamycin (5.7%), and gentamicin (5.7%).

The findings of Salem et al. (2020) are similar to ours. They demonstrated that the isolated *A. hydrophila* from Nile tilapia were highly susceptible to amikacin and ciprofloxacin antibiotics. And these results are dissimilar to those of Eid et al. (2013) who stated that 100% of *A. hydrophila* were susceptible to amikacin.

Also, 100% of the isolated *A. hydrophila* were resistant to ampicillin and this result is controversial to our study as only 84.2% were resistant to ampicillin, and Salem et al. (2020) reported that the most resistant *A. hydrophila* spp. which were isolated from Nile tilapia in various localities in Egypt against ampicillin and erythromycin (83.3%). The lowest sensitivity of *A. hydrophila* was reported with erythromycin (5.3%). Our study agreed with the results obtained by Eid et al. (2013) who presented that *A. hydrophila* spp. varied in their susceptibility and resistance to different antibiotics. These results illustrated the uncontrolled use of antibiotics in animals. Also, the environmental differences may play a role in antibiotic resistance. Another theory for antibiotic resistance is the presence of resistant plasmids against the antimicrobial drugs (Seker et al., 2015).

The isolated *A. hydrophila* spp. revealed a multiple antibiotic resistance (MAR) that extended from 0.062 to 1 in one isolate and to 0.937 in the second strain with an average of 0.431 (Table 7). The MAR index is an effective, usable, and low cost method that is used in basis checking of antibiotic resistant bacteria (Sandhu et al., 2016). The MAR index that is greater than 0.2 means the higher contamination risk and higher use of antibiotics in the field (Rotchell and Paul, 2016) as the MAR is of vital importance to public health as the recently developing multi-drug resistance strains do not respond to treatment with the traditional antibiotics leading to severe health problems, as long clinic stay, cure failure, and death.

Conclusion

The present context established the presence of pathogenic multi-drug resistance *A. hydrophila* in some milk samples collected from markets in Dakahlia governorate, Egypt. This pathogen may be considered as a potentially hazardous one for human health conditions as the isolated *A. hydrophila* showed virulence belongings on the foundations of proteolytic, lipolytic, and hemolytic activities in addition to the existence of *aer*A and *ahh*1 genes in most *A. hydrophila* isolates. Also, its resistance to different antibiotics was detected while amikacin was the greatest effective antibiotic against *A. hydrophila*. Pollution is caused through management and processing of milk and its products should be evaded particularly by possession of clean procedures and pasteurization of milk.

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Received 9 November 2021
Accepted 18 May 2022

State of Disaster Preparedness of Pet Owners for Ensuring the Safety of their Families and Companion Animals

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Keywords: *action plan, companion animals, disaster resilience, pet ownership.*

Abstract. *Growing urbanization and the related demand for resources together with the climate change appear to be among the factors responsible for the intensified frequency and severity of disasters worldwide. At the same time, urbanization is related not only with the increasing flow of inhabitants to the big cities but also with an increase in the number of pet ownership in seeking social, psychological and health benefits. But when a disaster strikes, companion animals are affected as much as humans. Their survival depends on the preliminary preparedness of their guardians for disaster response and recovery. For this purpose, the present study investigated the level of disaster preparedness among 335 pet owners in Bulgaria through an anonymous written questionnaire. The results showed that 64.86% of the participants in the survey were women, 52.24% of all respondents were 19–24 years old and 75.45% were keeping pets at the moment of filling in the survey.*

The study found that 87.16% of the respondents were well-informed about the likelihood of disaster hazards in their residential area. Pet owners were prepared to approach the relevant public health authorities (89.55% of them), respectively the animal health services (82.88%) in case of emergency. Only 36.72% of all pet keepers had a prepared disaster family plan, with another 28.96% of the respondents having developed a disaster pet action plan for their animal companions. If emergency evacuation is needed, more than 66% of the respondents would take their pets with them during relocation. This intention was statistically significant in women and those pet owners who were familiar with the potential disaster hazards.

Introduction

As per the general classification of the International Disaster Database (EM-DAT) maintained by the Centre for Research on the Epidemiology of Disasters – CRED, disasters are distributed in two main groups – natural and technological (EM-DAT, 2020). Data records (CRED, 2020) show that worldwide during the last decade the frequency of natural disasters has increased, with the rise of hydrometeorological (floods, storms, heat waves) and climatological disasters (droughts, wildfires) rather than geophysical ones (earthquakes, volcanic eruptions) (Anonymous, 2015). This trend together with multiple factors like soil erosion and deforestation (UNEP, 2014; Olsson et al., 2019), informal and poorly planned urban settings (Di Martire et al., 2012; Brown et al., 2014) and coastal or low-lying cities (UNEP, 2010) lead to increased vulnerability of people and their livelihoods to environmental hazards.

Under the terms of the Sendai Framework for Disaster Risk Reduction 2015–2030 (United Nations Office for Disaster Risk Reduction – UNDRR, 2015), people's vulnerability to disasters could be overcome through a comprehensive approach which includes the following four priorities: understanding disaster risk; strengthening disaster risk governance to manage disaster risk; investing in disaster risk reduction for resilience; enhancing disaster preparedness for effective response and to "Build Back Better" in recovery, rehabilitation and reconstruction (UNDRR, 2015).

Disaster resilience of the community is highly dependent on the awareness, preparedness, communication and education of people from all societal groups. Low levels of disaster preparedness were found to lead to failure in household evacuation (Heath et al., 2001a), thus threatening the health and lives of all family members, including animals, emergency responders and the general public as well (Bernard et al., 2010; Smith et al., 2015; Trigg et al., 2015b; Baker et al., 2018). Human casualties were even reported in fatal attempts for saving 'stock, property or pets' in disasters (Coates, 1999; Thompson, 2013).

At the same time, inclusion of non-human animal companions in pre-disaster planning activities was argued to impact positively the latter animal owners' response to safety measures as evacuation, shelter boarding, well-being, etc. (Farmer et al., 2016; Thompson et al., 2017; Farmer & De Young, 2019) due to the established strong human-non-human animal bond (Nusbaum et al., 2007; Travers et al., 2017).

At the international level, guidelines on disaster

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management and risk reduction in relation to animal health, animal welfare and veterinary public health were issued by the World Organisation for Animal Health (OIE, 2016). Within the guidelines, it was assumed that, due to recent disaster events, a particular need has aroused to bring all components of disaster management together in cohesive response plans at both national and international levels using a multidisciplinary (thus, multi-agency) approach to achieve optimal efficiency and effectiveness (OIE, 2016). Still, local communities are further apart from achieving such goals, sometimes due to limited access to the resources required, like enough housing options for people and their animals, lack of pre-disaster planning of animal owners, poor coordination between the emergency services for humans and animals, unwillingness for evacuation (Taylor et al., 2015; Yamazaki, 2015; Squance, 2018). However, when a disaster strikes it usually causes huge losses in livelihoods and the communities have no choice but to respond and cope with the event (Wood et al., 2013).

Although there is still a gap in logistics, coordination and even legislation in some countries, research has confirmed the widely recognized need for a joint approach by householders, emergency responders and animal rescue teams (Mcclure & Kerr, 2011; Taylor et al., 2014; Glassey, 2018) in order to relocate the whole family, including pets. This necessity provoked our scientific interest to investigate the present level of disaster preparedness among the pet owners in Bulgaria with a focus on their familiarity with the most probable disasters in their residential area, knowledge in the local emergency services, preparation of emergency family and pet action plans and supplies, and evacuation decisions.

Materials and Methods

Design of Survey

Fifth-year veterinary students at Trakia University – Stara Zagora and their families and friends participated voluntarily in the survey in the period April 2019 – June 2020. Each student willing to participate was given a paper multiple-choice questionnaire to fill it in anonymity with additional four more questionnaires for distribution to family members and relatives. All filled questionnaires were returned to the author (n = 335), representing heterogenous respondents throughout Bulgaria. This study did not need ethics approval.

The questions were distributed in several sections. Briefly, the first section (questions 1–4) contained questions on the participant's demographic data, such as age, gender, residence (capital city, city-administrative centre, small town, village), previous or current experience with pet animals. The second section (questions 5–8) focused on the respondents' awareness and knowledge about the main hazards and potential disasters, as well as the relevant structures and institutions responsible for the public health and animal health in their residential area. For the purpose of the study, within the Public Health Services were included the main institutions and organizations responsible for protection of human health like emergency call centre 112, fire departments, police, town hall, medical centres and hospitals, Emergency Department, Red Cross Committee, etc. Similarly, under the Animal Health Services were gathered state and private institutions and enterprises in the field of animal health and welfare protection like veterinary authorities, veterinary clinics and dispensaries, animal shelters, animal hotels, etc.

The third section (questions 9–11) contained statements about the pet owners' preparedness for disastrous events, i.e. a developed action plan for their families and for their companion animals with provision of the most needed supplies for survival in the first hours after the event. The last question studied the respondents' intention to evacuate with or without their pets in case the rescue team could not evacuate the animals at the same time with the people.

The filled questionnaires were returned to the author filled and coded with numerical values; thus, each text answer was converted into a number for easier data analysis.

Statistical Analysis

Data received were statistically processed (IBM SPSS-Inc., 2019, SPSS Reference Guide 26 SPSS, Chicago, USA). The study parameters were analyzed through descriptive statistics (frequency distribution tables), correlation analysis (Pearson correlation coefficient) and Student t test. A two-sided $p < 0.05$ was considered significant. The results afterwards were presented on diagrams (Excel, Windows 10).

Results

Respondents' demographics varied in age and residence (Table 1). Most of the participants in the survey were women (64.86%), aged 19–24 (52.24%), graduated from a high school (66.77%) and studying for their university degree (58.21%). The majority of the respondents were with urban background, living in the capital city and administrative cities throughout the country (85.63% in total), while only 14.37% of them came from rural settings. Regarding the participants' relationship with companion animals, the study found that the majority of them were taking care for pets at that moment (75.45%), while 24.25% of them had owned pets previously.

Depending on the environmental and infra-structural characteristics of their residential area, the respondents had to answer whether they were familiar with the most common hazards and potential disasters (flood, fires, storms, etc.) (Figure 1). The majority of them, 87.16%, stated they were aware of the disasters which could occur in the area. Another

Table 1. Demographic profile* of pet owners in the survey

Respondents' Demographics	Count	Percentage
Age (years)		
1) ⩽ 18	7	2.09
2) 19–24	175	52.24
3) 25–29	64	19.10
4) 30–60	82	24.48
5) 61–64	5	1.49
6) 65+	2	0.60
Gender		
1) Male	117	35.14
2) Female	216	64.86
Residence		
1) Capital city	14	4.19
2) City–Regional administrative centre	189	56.59
3) City–Municipal administrative centre	78	23.35
4) Town	5	1.50
5) Village	48	14.37
Pet keeping experience		
1) Previous experience	81	24.25
2) Current experience	252	75.45

*Values may not total 100% for each variable because of non-responders and rounding of values

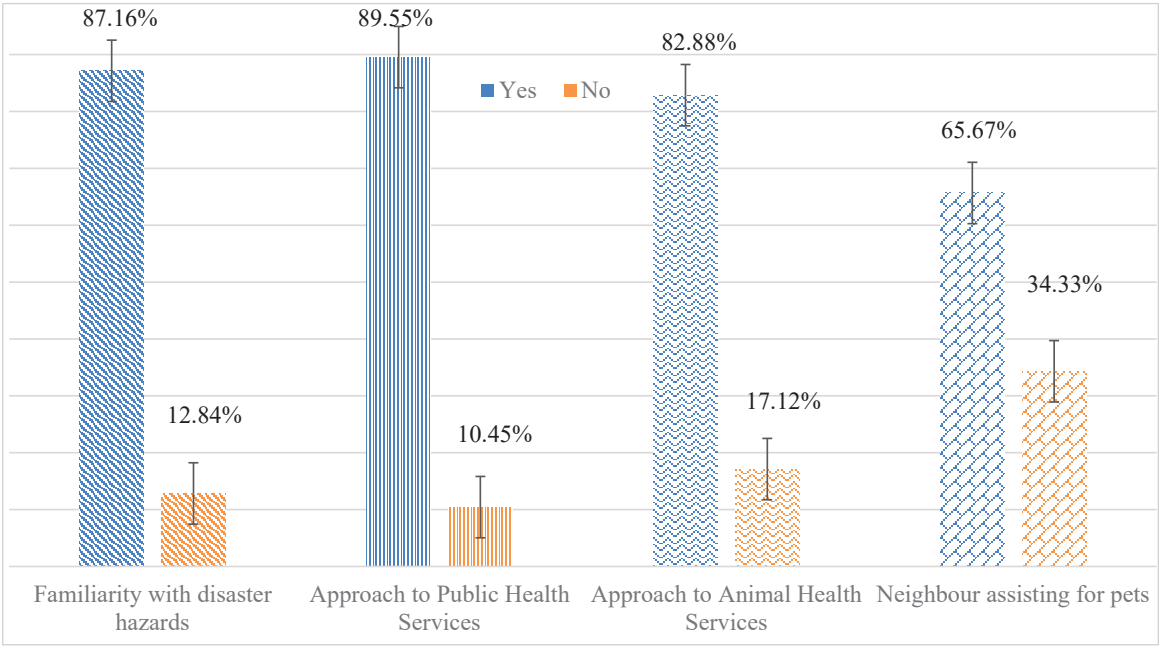

Figure 1. Respondents' distribution regarding their knowledge on disaster hazards and preparedness to contact health authorities or neighbours for help; with Std. Error presented on the error bars

proportion of 89.55% of the respondents declared they had knowledge where and how to approach local Public Health Services (emergency call centre 112, fire departments, police, town hall, hospitals, Emergency Department, Red Cross Committee, etc.) for assistance in case of disastrous event. A similar share of 82.88% of pet owners stated they could approach the Animal Health Services for the safety of their pets if needed (veterinary authorities, veterinary clinics and dispensaries, animal shelters, animal hotels, etc.). Asked to provide additional information on the preparation for their companion animals evacuation in disasters, another 65.67% of the participants in the survey said they could rely on the help of a neighbour (the neighbour had a key for the house, knew the location of the evacuation exits from the building, knew the animals and they were familiar with him/her, etc.).

Even though weak, a positive correlation was established between the age of the participants in the survey and their knowledge on the relevant institutions for public ($r = 0.1857$; $p = 0.001$) and animal health in the region ($r = 0.1294$; $p = 0.023$) supposing that elder respondents were better prepared to contact the authorities. A negative correlation was found between the pet owners' residence and the possible neighbour's help in a disaster situation, i.e., residents from big cities were less likely to rely on such assistance for their pets ($r = -0.1208$; $p = 0.033$).

The study found significant differences between the group of respondents who were familiar and prepared to contact the Public Health Services, respectively Animal Health Services, and gender, in favour of women being more prepared ($t[333] = 7.9506$, $p < 0.001$ for public health institutions; $t[333] = 5.3989$, $p < 0.001$ for animal health structures). The same statistically significant differences were found for urban residents (from cities-administrative centres) who appeared to be better prepared to approach health authorities to ensure their own and family safety, respectively the wellbeing of their pets ($t[334] = 24.8488$, $p < 0.001$ for public health institutions; $t[334] = 23.2836$, $p < 0.001$ for animal health structures). Those respondents who were taking care of pets at the moment of filling in the survey were found significantly to rely on a neighbor's assistance for evacuation of their animals ($t[334] = 11.8462$, $p < 0.001$).

The state of disaster preparedness of companion animal owners was investigated with regard to the availability of a developed disaster action plan (Figure 2). Approximately one-third or 36.72% of the respondents stated that they had prepared a disaster family plan and a backpack with the most needed supplies for survival in the first hours after the event, together with a list of contacts for emergency cases. A smaller share of 28.96% of the participants had developed a disaster plan for evacuation and survival of their pets. However, the Student *t* test found significant differences between the absence of disaster plans for family members and the current status of pet-keeping by the respondents ($t[334] = 3.4890$, $p = 0.0005$), which indicated that present pet owners seemed not to arrange disaster family plans. A weak positive correlation was found between the prepared action plans for family members and the respondents' awareness on disaster hazards in the area ($r = 0.2098$; $p < 0.001$). Again, a positive correlation was established between the available disaster plans for pets and the respondents' familiarity with the local animal health structures ($r = 0.1839$; $p = 0.001$).

Adequate disaster preparedness among the population affected their decision-making for evacuation in case of disaster outbreak. The majority of the respondents, 66.06%, stated that if a safety evacuation was ordered they would take their pets with them. On the other hand, 33.94% of the participants in the survey would leave without their companions if the rescue team could not evacuate the animals at the same time as people (Figure 3).

A negative correlation was found between the decision for evacuation and the female respondents ($r = -0.1621$; $p = 0.004$) indicating that women were more likely to take the animals with them.

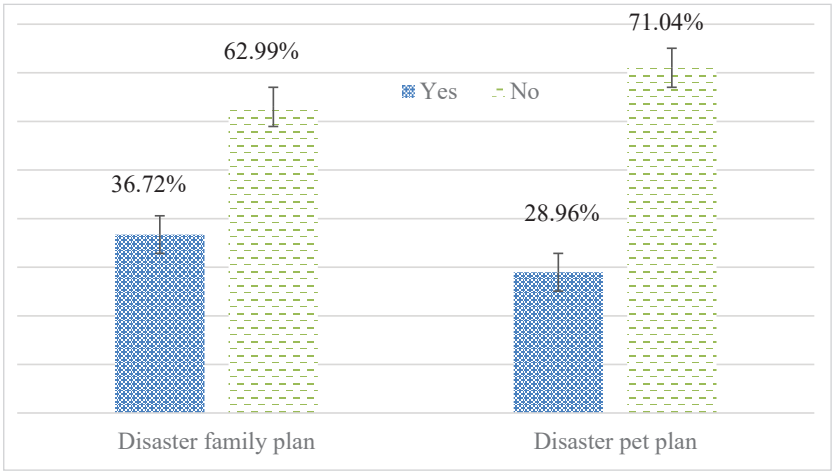

Figure 2. Availability of disaster action plans for the respondents' families and pets

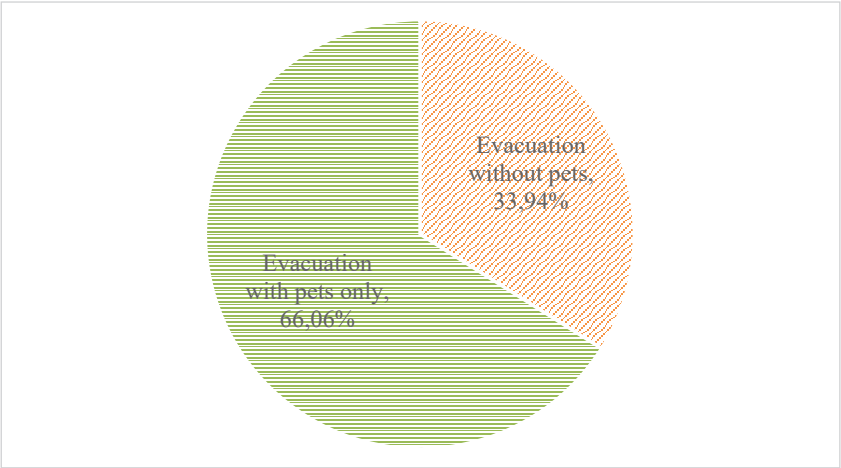

Figure 3. Respondents' decision for evacuation in case of disaster outbreak

This gender difference was confirmed by the *t* test as women showed a statistically significant intention not to abandon their pets during evacuation ($t[333] = -8.3612$, $p < 0.001$). Also, respondents who indicated their familiarity and awareness on the potential disaster hazards in the region were found to choose evacuation with their animals ($t[335] = -16.7364$, $p < 0.001$).

Discussion

Pet-keeping is reported to have increased in the last decades, as ten years ago approximately 60% of all US households owned at least one pet (Case, 2011) compared with 67% at present, according to the 2019–2020 National Pet Owners Survey (APPA, 2020). For Europe, an increase in the number of companion animals is also confirmed since 2010 (FVE Report, 2018). In our study, the share of the respondents who take care of pets at home at the present moment appeared to be 75.45%.

Many animal guardians consider their pets to be a part of their families (Case, 2011; Farmer et al., 2016) and form a strong emotional and psychological bond with them (Trigg et al., 2015a) with a significant positive impact on the human comfort and wellbeing (Hunt et al., 2012; Nusbaum et al., 2007; Travers et al., 2017).

At the same time, there is not a clear understanding among the public about the primary responsibility for safeguarding non-human animal companions during disasters and emergencies (Travers et al., 2016; Travers et al., 2017). As main caregivers, animal owners have to provide all necessary resources for the wellbeing of their pets but this engagement is found to be highly dependent on the extent of pet attachment (Shore et al., 2005). Meanwhile, in emergency events, animal guardians could be incapable to adhere to evacuation orders, due to various reasons as lack of housing options (Chadwin, 2017), lack of knowledge / a chaotic approach of the relevant emergency services (Garde et al., 2013), or emotional issues (Taylor et al., 2015). However, the results from our study showed that pet owners' preparedness to approach the public and animal health authorities was in a positive correlation with their age, indicating elder respondents to be better informed whom to contact ($p < 0.05$).

Research on the evacuation behaviours of pet owners who experienced a range of natural disasters found that 30% of them turned to neighbours and friends for assistance and another 8% called the emergency services (Taylor et al., 2015). In comparison, our study found that pet owners' disaster preparedness in hypothetic disaster situations was connected to a great extent to a call to the relevant local public and animal health authorities, as declared by the majority of the respondents (89.55% for public health and 82.88% for animal health services), or to seeking an assistance by a neighbour for 65.67% of the pet owners. Neighbour support and debrief for animals have also been cited among the measures during the recovery phase after the disaster event (Thompson et al., 2014). At the same time, Decker et al. (2010) identified a lack of preparedness in the local communities coupled with underutilization of the emergency agencies and non-human animal shelters as a resource. Indirectly, the ineffective use of disaster preparedness resources within communities is confirmed by Heath et al. (2001b) who reported that 90% of owners made housing arrangements for their pets for evacuation purposes without assistance. However, our study found a weak positive correlation between the respondents' familiarity with the local animal health structures and their disaster planning for pets ($p < 0.05$).

A step forward in improving public response to disasters is development of legislation which would require animals to be included in the community disaster plans. Some arguments in favour of this idea are given by Irvine (2007) and Farmer et al. (2016) who have provided information on law development regarding evacuation of pets and made suggestions for inclusion of animals in the state/local disaster mitigation plans. Furthermore,

householders are also encouraged to develop a written action plan that includes pets and animals and to relocate themselves and their animals before the disastrous event (Thompson et al., 2017). In line with these recommendations, our study found that approximately one-third of the respondents (36.72%) had a prepared disaster family plan but a smaller share of them (28.96%) included a part for evacuation of pets. These values appeared to be lower than those reported by Yamazaki (2015) who found that less than 50% of animal owners were engaged in different types of pet-related disaster preparations at the time of the Fukushima earthquake.

Pre-disaster planning with a priority on pet evacuation is found to be among the risk factors which influence the successful household evacuation and disaster response. Research indicates that animal owners may delay evacuation due to concern for their companions (Baker et al., 2018; Travers et al., 2017; Graham & Rock, 2018) which could result in a failure in household relocation (Heath et al., 2001c; Hunt et al., 2012). Heath et al. (2001a, 2001b) reported that during a hazardous event or disaster approximately 50% of the households evacuated without their pets. For comparison, Yamazaki (2015) reported that 41.2% of animal owners were able to evacuate with their pets during a disastrous earthquake, thus indicating a failure in companion animal evacuation for the rest 58.8% of the householders. However, results differ when preliminary intentions of the animal owners for relocation are investigated. Our study found that 66.06% of the respondents would take their pets with them if emergency evacuation was ordered, while 33.94% of the respondents stated that they would leave without their pets if it was not possible to evacuate together. In anticipated disasters, other studies have found even higher percentage – around 70% (Taylor et al., 2015); and overall 74.5% (Hesterberg et al., 2012) of the animal owners declared that they had planned to keep all their pets with them if evacuated. Furthermore, we found that this statement was gender dependent as our respondents-women showed a statistically significant intention not to abandon their pets during evacuation ($p < 0.05$).

Although pet ownership was not found to be a statistical risk factor for evacuation failure (Hunt et al., 2012), animal guardians have been reported to behave in a way that may compromise their own safety in disaster situations (White, 2012; O'Dwyer & Thompson, 2018). Taking into consideration the established strong human-animal bond and its impact on the decision-making for evacuation by the pet owners, some authors recommend the inclusion of animal ownership as a factor in the disaster mitigation and preparedness plans (Squance et al., 2018; O'Dwyer & Thompson, 2018). As a result, communities could cope better during disasters when companion animals receive protection alongside with their human families (Mcclure & Kerr, 2011; Travers et al., 2016).

Conclusion

The demographic profile of pet owners in the present study was represented mainly by young women in their university undergraduate degrees, as well as by residents with an urban background. The majority of the respondents were currently caring for pets. A very high proportion of the pet keepers were aware of the most likely disasters in their residential area, indicating a high level of preparedness to contact the relevant authorities in emergency events, which correlated positively with their age.

Regarding the pre-disaster planning activities, only one-third of the respondents had a prepared disaster family plan in advance, fewer of them included their pets in such an action plan and made provisions for the animal evacuation and survival. However, the majority of pet owners stated their intention to relocate with their animals in case evacuation orders were issued. This intention was gender dependent as women were found statistically significantly determined not to abandon their non-human companions during evacuation. The same decision was statistically confirmed among the respondents who were better informed about the likelihood of the disaster hazards in the area.

The established level of disaster preparedness among the pet owners in Bulgaria indicated the necessity of further development of the emergency management at national and regional levels with recommendations to general public for preparations of a disaster family plan with inclusion of provisions for pet animals and defined responsibilities among authorities for pet evacuation and relocation.

Acknowledgment

The author expresses her gratitude to all the participants who filled in the questionnaire.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Received 6 January 2022
Accepted 8 February 2022

Effect of Quantitative Feed Restriction during the Growing Period on Growth Performance and Economical Efficiency in Broiler Chickens

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Keywords: *broiler, feed restriction, growth, consumption, economical efficiency.*

Abstract. *Five hundred broilers chickens were used to study the effect of quantitative feed restriction during the growth period on the zootechnical performance and economical efficiency. Chickens were raised collectively during the starter period and a part of the growing period. At 24days of age, chickens were divided into three groups: control group, fed ad libitum, and two experimental groups (R1 and R2) restricted, respectively, to 10% and 20% of the daily feed intake of the control between 32 and 42 days of age. At 43 days, chickens of R1 and R2 were re-fed ad libitum until the end of the raising (49 days) where 13 randomly selected chickens from each group were slaughtered to record the weight of the heat carcass. Results showed that the average final live weight and the average heat carcass weight were similar in the three groups. Restricted animals recovered, through compensatory growth, the weight to reach the level of the control group. Quantitative feed restriction allowed to save 641.1g and 1282.1g feed per chicken, respectively, in R1 and R2 groups. The quantitative feed restriction provides a significant economy in feed costs, a reduction in carcass cost price and, consequently, a significant improvement in farmers income.*

Introduction

The poultry sector in Algeria has seen significant development in recent decades. Population growth and changes in feeding habits that have accompanied the country's urbanization are the main determinants of this development (Kaci, 2015). The strong development of the poultry sector contributes to job creation and reduction of animal protein deficits (Kaci, 2009). The national poultry industry is undergoing changes that create new constraints. Indeed, the basic factors necessary for its operation (maize and soybean, biological materials, veterinary products, etc.) are exclusively imported (Mouhous et al., 2015). The foreign currency resources allocated annually to this sector are very important and constantly increasing (Belaid-Gater et al., 2021b).

Under these conditions, the formulation of the feed is made almost exclusively with corn and soybean meal. The value of imports of these two raw materials amounts annually to about 1 billion US dollars (Mouhous et al., 2021). As feed is the most important component of expenses in broiler farming (more than 60%), the cost price per kilogram of chicken is highly impacted and dependent on the fluctuation of corn and soybean meal prices on the world market (Belaid-Gater et al., 2019).

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The high growth rate of chickens increases the deposition of fat in the carcasses, which leads to human health problems, metabolic and skeletal disorders in poultry, feed wastage through ad libitum feeding and high mortality (Baghbanzadeh & Decuypere, 2008). Also, the syndrome of sudden death (Khurshid et al., 2019) and ascitis (Kalmar et al., 2013) are common and well-known health and safety complaints in fast-growing broilers..

Broiler farmers are looking for high productivity with reduced production costs. This objective is closely related to the quantitative feed restriction practiced in broilers to induce compensatory growth, improve feed utilization efficiency and reduce maintenance requirements in the growth and finishing phases (Teimouri et al., 2005) but also to reduce the incidence of metabolic diseases (Sahraei, 2014). This restriction decreases the fat content in the carcass and decreases the incidence of disease associated with a high growth rate, such as ascites (Afsharmanesh et al., 2016). This will ultimately result in lower feed and production costs, resulting in higher quality, leaner and cheaper meat with lower mortality rates (Zubair & Leeson,1996a; 1996b). In addition, recent studies on the topic, such as those of Trocino et al. (2020), report that feed restriction stimulates activity during and after the restriction phase without any relevant effect on the stress state of the chickens.

In this sense, the aim of this study was to investigate, under the real conditions of local production, the

effect on the growth, slaughter and economical performance of broilers, of a phase of quantitative feed restriction during the growth period.

Materials and Methods

Presentation of the experimental site

This experiment was carried out in a poultry house of the Institute of Technologies and Specialized Agricultural Means (ITMAS) located in the province of Tizi-Ouzou (Algeria). The study started on February 3, 2019, (installation of the birds) and finished on March 23, 2019, i.e., a 49-day period. The house is an open type building with a total surface of 129 m^2 and the occupied surface is 90 m^2. It is provided with a natural ventilation system assisted by three small ventilators and three small extractors. The heating is provided by a pancake brooder of 1450 kcal. The temperature inside the building has changed inversely with the age of the chickens and has decreased from 33°C to 21°C. The humidity level recorded varied between 50% and 70%. The lighting was combined (natural and artificial). Feed distribution was manual, with feeders adapted to the age of the chickens. The chickens were watered ad libitum and manually at the starter period and automatically during the growing and finishing periods.

Feeds and animals

Five hundred day-old chicks (mixed sexes) of Cobb 500 strain were delivered from a private hatchery located in the same area. The rearing period was divided into three periods. The start period was from the 1st to the 13th day of age, the growth period from the 14th to the 42nd day of age and the finishing period from the 43rd to the 49th day of age. Three different diets were used in this experiment: a starter diet (crumbled) a growth diet (pellet) and a finish diet (pellet). These three diets are available on the local market, in high demand and used by many breeders, and manufactured and marketed by a private feed factory in the region. During the starter period (13 days) and the first 11 days of the growing period (24 days), all birds were raised collectively in the same and conventional broiler rearing conditions. At 24 days of age, the chickens were divided into three equal groups taking into account the number of chickens (163 chickens each) and the total weight of the group. The control one (C) was fed ad libitum and the two experimental groups R1 and R2 were subjected to quantitative dietary restriction for 11 consecutive days (from 32 to 42 days of age) with two levels of restriction, respectively, 10% (R1) and 20% (R2) of the daily intake of the control group. Ad libitum re-feeding with the finishing feed began at 43 days of age of the chickens and ended on the last day of the experiment, i.e., at 49 days of age. The field conditions were reproduced, i.e., how broilers are raised in local production conditions. In addition, during the animals' distribution, the groups were as homogenous as possible, including the proportion of males and females.

Measures performed or calculated

Mortality monitoring wascarried out daily. During the seven weeks of the experiment, weekly monitoring of individual body weight was carried out. A daily control of the quantities of consumed feed (quantities distributed – quantities refused) was carried out per group and per period: before restriction (adaptation period from 24th to 32nd day), during restriction (from 32nd to 42nd day) and after restriction (from 43rd to 49th day).

The economic efficiency (AD = Algerian Dinar) was calculated according to the model of Harouz-Cherifi et al. (2018).

Economic efficiency (%) = [(Weight gain income in AD/kg – Total feed cost in AD/kg) / Total feed cost in AD/kg] × 100.

Weight gain income, AD /kg = total weight gain (kg) * price per kg live weight (in AD).

Total feed cost (feed loads) = the total amount of feed consumed (kg/chicken) * price per kg of feed.

Gross margin (AD) = Weight gain income (AD) - Total feed cost (AD).

Cost/benefit ratio = Total cost of feed consumed/ gross margin.

The price of the feed, which was bought on the local market, was 52 AD /kg.

Slaughter

At the end of the experiment, 13 randomly selected chickens from each group were slaughtered without fasting. The total live weight per slaughter cage and the weight of the hot carcass were recorded.

Chemical analysis

The raw materials of which the feed is composed are corn grain, soybean meal, wheat bran, soybean oil, synthetic amino acids (DL-Methionine, L-Lysine), salt, phosphate, calcium, mineralovitamin complex andan anticoccidian (Coccidiostat).

The chemical analyses of diets (Table 1) covered dry matter, crude ash, crude protein (NA652-1992), fat (NA654-1992), calcium (AFNOR) and phosphorus (NA657-1992).

Statistical analysis

The different results were expressed by means ± standard error of means. The recorded and/ or calculated data were subjected to an analysis of variance using software R 3.6.1(www.r-project.org), with the restriction level as the only variation factor. Analysis of variance was used to assess the effects of feed restriction on growth performance and slaughter parameters. Significant differences between the means of the various variables were determined by using the Duncan test. The results were considered different when the value of p was lower than 0.05.

Table 1. Chemical composition (% DM) of the three types of diets used in the test

Parameter	Starter diet	Growth diet	Finishing diet
Dry matter	83.83	88	89.5
Crude ash	5,4	5.6	5.6
Organic matter	94.6	94.4	94.4
Crude protein (N×6.25)	21,49	21.74	20.66
Crude fat	4.4	4.87	3.7
Calcium	1.1	0.93	0.7
Phosphor	0.68	0.74	0.6
Metabolizable energy(kcal/kg)[a]	3811.42	3832.62	3807.43

DM: Dry matter
N: nitrogen
[a]: Estimate from the equation of Zarghi et al. (2010):
ME (MJ /kg DM) = 16.063-0.115EE-0.027CP.

Results

The crude protein, calcium and phosphorus contents of the starter diet are higher than those recommended by the Cobb 500 strain guide (Table 1). The growth diet contains a crude protein content (Table 1) that is 1.75% higher than the standards recommended in the strain guide. The protein concentration of the finishing diet also exceeds the nutritional recommendations of the strain by 2.66%. Calcium and phosphorus levels in the growing diet exceed the nutritional recommendations of the strain guide (Vantress, 2018). For the finishing diet, the phosphorus content is higher than the recommended intake according to the strain guide (Vantress, 2018), in contrast to the calcium content.

During the restriction period (11 days), as expected and contrary to the pre-restriction period, daily consumption per chicken was significantly lower in restricted groups R1 and R2 compared with the control group(p = 0.002;Table 2). After the return to ad libitum feeding (post-restriction period), there was an increase in feed intake in the chickens that were restricted, resulting in a similar average feed daily intake in the three groups during this period.

The average live weights (Table 3), obtained at the end of the trial (49 days of age), are similar between group C and groups R1 and R2, respectively, 3416.34 g, 3456.38 g and 3432.10 g (p > 0.05). However, the highest level of compensatory growth was noted for the most severe level of restriction with an average daily gain (ADG) of 95.43 g/day during the post-restriction period, P = 0.022(Table 4).

In terms of absolute values, the feed conversion seems to be at the same level in the three groups or even more interesting in the restricted groups with an overall feed conversion ratio (at 49 days) of 1.77 for group R1, 1.78 for group R2 and 1.90 for the control group (Table 4).

No mortality was recorded in the different groups during the trial. Throughout the restriction phase, the chickens' health status was good, and only two chickens died in group R1 on the last day, probably due to the stress of handling (the final weighing).

The average hot carcass weight (Table 5) was similar in the three groups (C, R1 and R2).

The parameters in Table 6 were calculated according to the model of Harouz-Cherifi et al. (2018) as précised in Material and Methods, without a statistical study.

The feed restriction, at the end of the growth period, resulted in an improvement of 20% and 42% in economic efficiency, respectively, for groups R1 and R2, compared with that of the control one (Table 6). Similarly, restriction reduced the total feed cost per chicken by 9.52% and 19.97% for groups R1and R2 respectively, compared with the control one. This reduction is explained by the amount of feed saved due to the restriction of 10% and 20% of the control's consumption, which are 641.1 g and 1282.1 g per chicken, respectively.

Table 2. Average daily feed consumption per chicken (g/d) according to the level of restriction before, during and after restriction

Period \ Groups	C	R1	R2	SEM	P
Before restriction (1–31days)	151.04	150.22	144.65	8.23	0.949
During restriction (32–42days)	210.89b	177.85a	169.89a	5.77	**0.002**
After restriction (after 43days)	186.29	183.01	200.63	5.53	0.404

C: Control. R1:Group with 10% of restriction. R2: Group with 20% restriction.
Values followed by different letters on the same line are significantly different.

Table 3. Comparison of average live weight (g) according to restriction level and period

Period \ Groups	C	R1	R2	SEM	P
Before restriction (1–31 days)	1673.46	1665.80	1652.80	13.99	0.152
During restriction(32–42 days)	2305.91a	2247.62b	2258.60b	19.73	0.038
After restriction (after 43 days)	3416.34	3456.38	3432.10	18.94	0.682

C: Control. R1: Group with 10% of restriction. R2: Group with 20% restriction.
Values followed by different letters on the same column are significantly different.

Table 4. Average daily gain (ADG) and the feed conversion ratio (FCR) according to the level of feed restriction

Average Daily Gain (ADG)(g/d)				
Groups \ periods	Before restriction	During restriction	After restriction	Global ADG
C	85.16	91.91	66.98a	70.13
R1	84.07	89.72	85.44b	70.97
R2	72.50	92.13	95.43b	70.46
SEM	11.67	09.37	17.63	2.13
P	0.113	0.336	0.022	0.174
Feed Conversion Ratio (FCR)				
Groups \ periods	Before restriction	During restriction	After restriction	global FCR
C	1.62	1.91	2.24	1.90
R1	1.52	1.97	2.04	1.77
R2	1.81	1.61	2.76	1.78
SEM	1.64	1.13	1.96	1.56
P	0.167	0.052	0.057	0.178

C: Control. R1: Group with 10% of restriction. R2: Group with 20% restriction.
Global ADG and global FCRwere calculated for all the experimental period (before, during and after restriction).

Table 5. Effect of feed restriction on hot carcass weight (g) of chickens

Group	C	R1	R2	SEM	P
hot carcass weight (g)	2549.16	2732.72	2670.90	53.10	0.367

C: Control. R1: Group with 10% of restriction. R2: Group with 20% restriction.
Values followed by different letters on the same line are significantly different.

Table 6. Production economy per 49-day-old chicken in the three groups (C, R1 and R2)

Parameters	C Group	R1 Group	R2 Group
Average live weight of a day-old chick (g)	49.7	49.7	49.7
Average live weight at 49 days (g/chicken)	3416.4	3456.4	3432.1
Total weight gain (kg)	3.37	3.41	3.38
Selling price AD-Euro/kg live weight	250–2.11	250–2.11	250–2.11
Income as live weight gain AD-Euro/kg	842.5–7.1	852.5–7.18	845–7.12
Total feed consumed/chicken (kg)	6.41	5.8	5.13
Price per 1kg of feed (AD-Euro)	52–0.44	52–0.44	52–0.44
Total feed/chicken cost (AD-Euro/kg)	333.3–2.81	301.6–2.54	266.8-2.25
Economical efficiency (%)	153	183	217
Gross margin (AD-Euro)	509.2–4.29	550.9–4.64	578.2–4.87
Cost/benefit ratio	0.65	0.54	0.46

C: Control. R1: Group with 10% of restriction. R2: Group with 20% of restriction. AD: Algerian Dinar, (01 euro=118.7 DA during the essay period).

The restricted groups also enabled an improvement in the income per kg of meat produced, with a reduction of 31.72 AD for R1 and 66.56 AD for R2 for each kg of meat produced. In addition, restricted groups R1 and R2 had better cost/income ratios than the control one.

Discussion and Conclusion

Chemical composition of the feeds

According to Larbier and Leclercq (1992), fillet yields are better when the requirements for a minimum feed conversion are optimized during the first two rearing phases. According to thesame authors, when growing chickens are provided with the required energy, the excess of protein moderately reduces the appetite without affecting growth and raises the protein level by 1 point (10 g/kg diet) resulting in a 3% increase in water consumption.

According to Drogoul et al. (2004), for a given energy concentration, the crude protein and amino acid content of the diet decreases as the age of chickens increases. A combined and balanced intakeof calcium and phosphorus in the feedis necessary for bone growth; calcium plays a determining role in phosphorus availability (Narcy et al., 2009).

In addition, nutritionists use wide safety margins to ensure adequate phosphorus intake, which is crucial for skeletal integrity and growth performance in broilers (Flaten et al., 2003).

According to Waldenstedt (2006), the calcium content must be less than 0.70% after 28 days of the age of chickens. Globally, the nutrient intakes of the three types of diets, especially nitrogen, exceed the recommendations, which have negative repercussions on the costs of these diets, generate significant nitrogen rejections and indicate the lack of adequate knowledge regarding the formulation aspect at the level of the feed mills.

Feed consumption and growth performance

The resultsof daily consumption per chicken are in agreement with those reported in the literature (Novele et al., 2008; Malpotra et al., 2017). Those of increased feed intake in chickens that were restricted are in agreement with those recorded by Zomrawi et al. (2019). However, the opposite was recorded by Bouallegue & Aschi (2015) who explained their results by the severity of the level of restriction applied, with the restricted chickens receiving the same amount of feed for 8 successive days while those of the control group gradually increased their consumption. Our results were caused by the low level of restriction applied in this experiment. Chickens from groups R1 and R2 were restricted for 11 successive days. The amounts consumed by these chickens were calculated on the basis of the daily consumption of those in the control group. The control chickens gradually increased their consumption. The difference in average daily consumption recorded per restricted chickens during the 11 days of restriction ranged from 350 g to 450 g less compared with those which were fed ad libitum.

The results of the mean live weights, obtained at the end of the trial (49 days of age), are in agreement with those reported by Khetani et al. (2009) who reported that restricted chickens expressed compensatory growth after re-feeding ad libitum. When animals are restricted, a negative effect on body weight growth occurs; restricted animals grow less than animals fed ad libitum. However, this effect will disappear during the ad libitum feeding period due to compensatory growth. Compensatory growth has been reported by the literature (Lee & Leeson, 2001; Jang et al., 2009). Thus, the level of feed restriction or/and duration of restriction showed a negative correlation with the growth of restricted chickens (Urdaneta-Rincon & Leeson, 2002).

The average daily gain (95.43 g/day), during the post-restriction period, is slightly higher than recommended, at the same time, by the Cobb 500 strain guide, and confirms the results recorded by Leeson and Zubair (1997).

The average final live weight at 49 days (3434.94 g) recorded in this study was better than those (2760 g) reported by Mouhous et al. (2012) recorded at 57 days of age in private farms in the same region (Tizi-Ouzou) or those reported by Mouhous et al. (2014) for the Béjaia region, which borders Tizi-Ouzou. However, it is slightly lower than the average weight of the Cobb 500 strain and is the weight recommended by the strain guide at 49 days, i.e., 3506 g.

The results of health status are in agreement with those reported in the literature regarding the reduced mortality rate and sudden restriction death syndrome (Bhat & Banday, 2000; Urdaneta-Rincon & Leeson, 2002; Boostani et al., 2010). In addition, this restriction did not affect the chickens' welfare according to Belaid-Gater et al. (2021a). Jang et al. (2009) reported a significant increase in cytokine levels of blood in the feed-restricted groups, which could positively impact the health and welfare of the chickens. This was noted even when the quantitative feed restriction was strict (less than 75% of the ad libitum amount).

The results of the feed conversion were similar to those reported by several authors, notably Sahraei (2012) and Bouallegue and Aschi (2015). The value of the overall feed conversion ratio recorded for the two restricted groups (1.77) was slightly better than the performance of the Cobb 500 strain (1.82) recommended, at the same age as the chickens, by the strain guide.

The average hot carcass weight is very interesting and indicates that the experimental chickens managed to regain growth during their re-feeding ad libitum period after feed restriction and to reach the weight of the control group with reduced feed intake. Jayasiri et al. (2019) also reported this type of result.

For indication, the average carcass yield in the

restricted groups was 78.44% compared with 74.61% in the control one. The recommended yield for chickens of this strain at this age by the Cobb 500 Guide is 75.42%. Lee & Leeson (2001) suggest that there was no loss in meat yield if chickens achieve compensatory growth during their re-feeding period after feed restriction.

Economical efficiency

The results of the economic study of this experiment showed the advantage of the feed restriction and confirmed the results of the bibliography, notably those of Sahraei (2012), Zomrawi et al. (2019), Trocino et al. (2020). They were in agreement with those of Proudfoot & Hulan (1982) and Jayasiri et al. (2019), who reported that chickens restricted and then fed *ad libitum* had a higher benefit than those fed ad libitum without restriction (control).

In conclusion, the results of this experiment confirm the advantages of quantitative feed restriction in broiler farming. Applied at two levels (10% and 20%) during the late growing period (32 to 42 days of age), it did not affect the health status or zootechnical performance of the chickens. It induced sufficient compensatory growth to reach a final live weight and hot carcass weight similar to the control group with an appreciable economy of feed consumed.

The quantitative feed restriction mainly results in a significant economy of feed costs in broiler production, which is very profitable for the breeders but also, indirectly, for the consumers.

Conflict of Interests

The authors declared that there is no conflict of interests.

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Received 7 January 2022
Accepted 12 April 2022

Evaluation of the Effects of *Urtica Dioica* L. Supplementation on Egg Quality and Blood Parameters in Laying Hens

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Keywords: *Urtica dioica, layers, egg quality, biochemical parameters*

Abstract. *The purpose of the current research was to study the influence of dietary supplementation of nettle (Urtica dioica) on laying performance, egg quality and blood serum biochemical parameters of layers. A total of 60 laying hens (42 weeks old) from Lohman Klassik Brown breed were randomly allocated into three groups: a control and two experimental groups (n = 20 hens per group). All layers received compound feed with the following nutritional value: 2710 Kcal/kg metabolizable energy; 16.44% crude protein; 3.32% crude fats; 4.58% crude fibres; 3.73% Ca; 0.49% P. The hens from the experimental groups received 0.3% (experimental group 1) and 0.5% (experimental group 2) of dried nettle with the diet. Both experimental groups had significantly higher egg yolk pigmentation ($p < 0.001$) compared with the control group. A significantly lower egg yolk cholesterol content was found in hens from experimental group 1 ($p < 0.05$). Nettle addition reduced significantly blood serum glucose ($p < 0.01$ and $p < 0.05$ in experimental groups 1 and 2, respectively) as well as the total serum cholesterol content ($p < 0.001$).*

Introduction

The nettle *(Urtica dioica* L., family *Urticaceae***)** is an ordinary plant with extraordinary properties (Bisht et al., 2012; Kregiel et al., 2018), widely grown in different parts of the world and used to promote health. Nettles have a high ratio of nutritious substances (vitamin C, carotenoids, minerals), active compounds such as tannins, formic acid, salicylic acid, thymol and carvacrol and make a readily digestible food (Viegi et al., 2003; Gülçin et al., 2004). Nettles possesse antioxidant, antimicrobial, antifungal and antiviral properties (Rutto et al., 2013, Upton, 2013). Recently, there has been a trend in the animal nutrition to improve the digestibility, gut health, immune response, and quality of the animal products (eggs, meat, milk) by using herbs and their extracts.

Szewczyk et al. (2006) have reported a reduction of monounsaturated fatty acids (MSFAs) and an increase of polyunsaturated fatty acids (PUFAs) in pig's muscle fat by nettle addition to their diet. Khanal et al. (2017) have established a beneficial effect of stinging nettle supplementation on quantity and quality of milk yield as well as on the body condition score in dairy cattle. Influence of diet nettle supplementation has been investigated by Stojčić et al. (2016) with broilers in two alternative housing systems. There were no effects on carcass weight, dressing percentage, abdominal fat and percent of parts of broiler chicken carcass. The authors found out that dietary supplementation of fresh nettle can improve the quality of chicken breast meat better than pasture intake.

The inclusion of 6% nettle in a quail's diet has led to reduction of egg yolk cholesterol, serum total cholesterol and serum triglyceride levels and has not negatively influenced quail performance (Moula et al., 2019). Loetcher et al. (2013a) have used nettles as natural yellow colorant for egg yolk. There was no substantial influence of nettle supplementation on laying performance and general egg quality. Nettle supplementation of layer diets is therefore considered as an effective means to naturally achieve the desired yolk yellowness, without risking unfavourable side-effects.

However, in available literature, there is no sufficient information about the effects of dried and milled nettle in a laying hen's diet.

The objective of our study was to investigate the influence of dried nettle addition on egg production, egg quality, yolk lipid oxidation, and some blood parameters in Lohman Klassik Brown layers.

Materials and methods

The experiment complied with Directive 2010/63/EU on the protection of animals used for scientific purposes, and the experimental procedures were approved by the Ethical Commission of National Research and Development Institute for Biology and Animal Nutrition.

Experimental design

The experiment was conducted in the Poultry Experimental Base of the Institute of Animal Science-Kostinbrod, Bulgaria, with a total of 60

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laying hens at the initial age of 42 weeks from Lohman Klassic Brown breed. The laying hens were randomly divided in three groups: control (n = 20) and two experimental (n = 20 per group), kept in separate pens. The poultry was raised on a deep litter pen on a 16-hour lighting schedule, 70–85% relative air humidity, and 21–24°C air temperature. Water was supplied via nipple drinkers. The trial lasted 50 days: the preparatory period was 10 days, and the experimental period lasted 40 days. During the preparatory period, all the groups received compound feed for layers in the amount of 130 g/day/hen in order to eliminate the influence of the previous diet. During the experimental period, the hens received 130 g/day/hen of this compound feed, whereas the diet of experimental hens was supplemented with 0.3% (experimental group 1) and 0.5% (experimental group 2) dried and milled nettle. In our research, a dry mass of the above ground part of *Urtica dioica* was used as a diet supplement. The chemical composition and antioxidant properties of dried nettle are presented in Table 1.

The ingredients and chemical composition of the diets are presented in Table 2.

The feed nutritive value was determined by the conventional Weende analysis: crude protein, crude fat, and crude fibres (by Weende analysis); the contents of both Ca (BSS 11 374-86, 1990) and P (BSS 4336-73, 1990); the pH value, determined, using a pH meter Stirrer, type OP-951. The metabolizable energy was calculated according to WPSA (1989).

At the beginning and at the end of the trial, the live body weight of the hens from the control and experimental groups was measured.

Table 1. Chemical composition, total antioxidant capacity and pH of nettle

Nettle	
Moisture, %	9.75
Crude proteins, %	24.52
Crude fat, %	2.86
Crude fibre	11.39
Ca, %	3.95
P, %	0.556
Total phenolic content, mg GAE/100 g	357.00
Total antioxidant capacity, mmolTE/100 g	1230.20
pH	8.33

Table 2. Composition and nutritive value of the feed for laying hens

Ingredients, %	Control	Experimental group 1	Experimental group 2
Wheat	63.34	63.04	62.84
Soybean meal	9.0	9.0	9.0
Sunflower meal	14.0	14.0	14.0
Sunflower oil	2.5	2.5	2.5
Nettle	0.0	0.3	0.5
Limestone	9.0	9.0	9.0
Mono calcium phosphate	0.4	0.4	0.4
Complex premix 6015*	1.25	1.25	1.25
Nutritive value			
Metabolizable energy, kcal kg^{-1}	2710	2710	2710
Crude proteins, %	16.44	16.44	16.44
Crude fat, %	3.32	3.32	3.32
Crude fibre, %	4.58	4.58	4.58
Ca, %	3.73	3.73	3.73
P, %	0.49	0.49	0.49

* Complex premix contains: Mn (MnO): 120 mg/kg; Zn (ZnO): 110 mg/kg; Fe ($FeSO_4$): 140 mg/kg; Cu ($CuSO_4$): 18 mg/kg; I ($Ca(IO_3)_2$: 1.80 mg/kg; Se (Na_2SeO_3): 0.35 mg/kg; vitamin A (retinyl acetate): 9900 UI; vitamin D_3 (cholecalciferol): 3000 UI; vitamin E (DL-alpha-tocopherol): 30 mg/kg. It does not contain nutritive antibiotics, synthetic dyes and carotenoids or other stimulants.

The egg production (in percent) for each group was controlled every day. There was no mortality noticed in the experimental groups during the trial.

Thirty eggs from each group, laid within two consecutive days, were taken at the beginning and at the end of the experiment and the following measurements were made:

– The weight of the egg, egg shell with a shell membrane, egg yolk, and albumen were measured with an electronic scale BOECO within 0.001 g;

– The shape index was measured by an index meter;

– The height of albumen and egg yolk as well as the egg yolk width were measured with a calliper (in mm);

– The haugh unit was calculated by the formula:

$$HU = 100 \log (h + 7.17 - 1.7\ W0.37)$$

Where: H is height of the thick albumen (in mm) and W is egg weight;

– The shell thickness (mm) without a shell membrane was measured by a micrometer Amer 25EE with the precision of 0.0001 mm;

– The egg yolk colour was determined visually by the 15 Roche colour fan having a 15 degrees scale.

At the end of the treatment, 10 hens from each group were chosen randomly and blood samples were taken from *Vena cutanea ulnaris*. The serum levels of total cholesterol, glucose, and triglycerides were measured by commercial kits using biochemical analyser BioSystems (S.A. Costa Brava, Spain).

At the end of the experimental period, some lipid fractions of egg yolks of 10 eggs from each group were determined. The total lipids were determined by the method of Bligh and Dyer (1959). The total cholesterol content in the yolk was determined by the method of Schoenheimer-Sperry modified by Sperry and Webb (1950).

At the end of the trial, the lipid oxidation of egg yolk of 6 eggs from each group was evaluated as TBARS according to the method of Castellini et al. (2006). Oxidation products were quantified as malondialdehyde equivalents (mg MDA 100 g^{-1}).

The results were expressed as means with their standard errors. Statistical examination of the data obtained was determined by SPSS, single factor, ANOVA program. A t-test was used to compare the results between control and experimental groups. Statistical significance was set at $p < 0.05$.

Results and discussion

Effect of nettle powder on laying performance

The values of live body weight egg intensity and mortality of the hens from the control and experimental groups are shown in Table 3. The live body weight of laying hens at the beginning of the experimental period varied within narrow range: 1846 g; 1782 g, 1868 g for control, and experimental groups 1 and 2, respectively ($p > 0.05$). This parameter increased by 90 g, 35 g, 78 g for control, and experimental groups 1 and 2, respectively, at the end of the trial ($p > 0.05$). No significant differences ($p > 0.05$) between the groups about these parameters were found.

The hens' laying intensity at the beginning of the experimental period was as follows: control – 86.67%, experimental group 1 – 85%; experimental group 2 – 86.00%. At the end of the treatment, this indicator was 92%, 89% and 89.5% for control and experimental groups 1 and 2, respectively. At the end of the trial, within the groups, there was an increase in laying intensity by 5.33% in the control and by 4% and 3.5% for experimental groups 1 and 2. The differences between the groups were insignificant ($p > 0.05$).

Effect of nettle supplementation on egg quality

The effects of nettle powder supplementation on egg morphological parameters of laying hens are presented in Table 4. According to Song et al. (2000), Toussant and Latshaw (1999), and Wolanski et al. (2007), the egg morphological parameters can be classified into 2 main groups: external (egg weight, shell thickness, shape index) and internal traits (albumin index, yolk index, Haugh unit, egg yolk color). As can be seen from Table 4, the addition of 0.3% and 0.5% dried and milled nettle to the layers diet had no significant effect on egg-, albumen-, yolk- and shell weights; shell thickness; Haugh units; and shape index, albumen index and yolk index. The present finding is in agreement with nettle supplementation of layer diets that showed no considerable effect on internal and external traits (Loetscher et al., 2013).

Yolk color is one of the important factors for egg marketing. In general, consumers *prefer eggs with an orange yolk colour*. To achieve desirable yolk

Table 3. Live body weight, feed intake, egg intensity and mortality of laying hens (X ± SE)

Dietary treatments	Control	Dietary nettle powder (%)	
		0.3	0.5
Initial body weight (g)	1846 ± 27.65	1782 ± 22.63	1868 ± 30.29
Final body weight (g)	1936 ± 28.34	1817 ± 48.92	1946 ± 32.98
Egg intensity (%) Start	86.67 ± 1.44	85.00 ± 2.67	86.00 ± 1.25
Egg intensity (%), End of experiment	92.00 ± 0.82	89.00 ± 1.80	89.50 ± 1.17

Table 4. Effect of dietary nettle powder on egg morphological parameters (X ± SE)

Groups / Indices	Control	Dietary nettle powder supplementation (%)		Control	Dietary nettle powder supplementation (%)	
		0.3	0.5		0.3	0.5
	Start of the experiment			End of the experiment		
Egg weight, g	63.84 ± 0.64	61.36 ± 0.78	62.30 ± 0.76	64.10 ± 0.72	62.06 ± 0.89	62.13 ± 0.88
Albumen, g	41.50 ± 0.45	40.00 ± 0.61	40.17 ± 0.64	41.44 ± 0.49	40.08 ± 0.69	40.05 ± 0.72
Yolk, g	15.56 ± 0.21	14.74 ± 0.21	15.53 ± 0.20	15.96 ± 0.24	15.64 ± 0.22	15.50 ± 0.18
Shell, g	6.93 ± 0.12	6.61 ± 0.09	6.77 ± 0.08	6.54 ± 0.11	6.34 ± 0.15	6.27 ± 0.11
Shell thickness, mm	0.39 ± 0.005	0.38 ± 0.003	0.40 ± 0.003	0.39 ± 0.004	0.39 ± 0.005	0.39 ± 0.005
Haugh units	81.50 ± 1.30	76.20 ± 1.41	77.45 ± 1.33	73.43 ± 1.51	71.38 ± 1.30	71.13 ± 1.42
Shape index %	78.55 ± 0.36	79.50 ± 0.47	80.23 ± 0.40	78.60 ± 0.37	79.40 ± 0.36	79.25 ± 0.44
Albumen index %	9.74 ± 0.29	8.10 ± 0.34	8.69 ± 0.38	7.73 ± 0.30	6.49 ± 0.32	6.74 ± 0.32
Yolk index %	38.30 ± 0.64	37.76 ± 0.70	41.56 ± 0.85	40.52 ± 0.61	38.46 ± 0.51	38.82 ± 0.57
Yolk color	2.89 ± 0.18	2.81 ± 0.19	2.78 ± 0.18	2.93 ± 0.16	4.73 ± 0.27 A***	5.20 ± 0.24 B***

Significance by: * – $p \leqslant 0.05$; ** – $p \leqslant 0.01$; *** – $p \leqslant 0.001$
A – control group / experimental group 1
B – control group / experimental group 2

colour, intensity hens' feed is often supplemented with synthetic carotenoids because they are cheaper. The increased demand of safety animal products during the recent years requires further studies on the possibilities to use various natural sources of carotenoids as layers' diet ingredients (Grigorova and Petkova, 2014). At the beginning of our study, yolk colour intensity in the groups varied within close range – from 2.78 to 2.89 points on the Roche Colour Fan. At the end of the trial, this parameter increased significantly ($p < 0.001$) in both experimental groups compared with the control group (Table 4). Based on the fact supported by scientific investigations of Kang et al. (2003) and Karadas (2006), the carotenoids from feed compound passed unchanged in egg yolk, so the nettle powder used in different amount (0.3% and 0.5%) in our study was a suitable applicant for egg yolk pigmentation.

Egg is one of the major sources of dietary cholesterol, which may lead to lower consumption of eggs of the consumers instead of the scientific thesis that the cholesterol improves lipid profile (Fernandez-Robredo et al., 2008). There are a lot of investigations for reducing the cholesterol in egg yolk and enhancing the nutritional value of egg with supplementation of the hen's diet with plants and herbs (Grigorova et al., 2021; Chowdhury et al., 2002; Chen et al., 2005). In the current research, a significant decrease of total yolk cholesterol in hens receiving 0.3% dried nettle with the diet was established (Table 5).

These results are similar with the findings of Mansoub (2011) in which 2% nettle powder supplementation to the diet of laying hens reduced the total cholesterol and triglycerides in eggs, but are slightly different from the limited effects reported by Keshavarz et al. (2014). The present study is consistent with the findings of Moula et al. (2019) who have reported that nettle at the level of 6% reduced egg yolk cholesterol in quails.

In general, herbs have antioxidant properties, so as a feed ingredient they deserve a special attention for decreasing the oxidation level that may cause the changes in organoleptic properties of eggs. Nettle leaves are rich in polyphenols, mainly flavonoids (i.e., kaempferol, isorhamnetin, quercetin, and their rutinoside or glycoside derivatives) and phenolic acids (i.e., caffeic acid and its ester derivatives, like chlorogenic acid and caffeoylmalic acid) (Upton, 2013). The phenolic compounds of nettle stabilize the lipid peroxidation (Akbay et al., 2003). In recent years, some researchers have presented results according to which supplementation of feed with some plant products indicates reduction of lipid peroxidation in eggs. The antioxidant capacity of nettle (*Urtica dioica*) is achieved mainly by in vitro approaches (Gulcin et al., 2004).

Grigorova et al. (2020) confirms a significant reduction of the lipid peroxidation in egg yolk by feeding laying hens with rosehip rich with lycopene. In the current experiment, there is no significant influence on content of malondialdehyde (MDA), which is the main indicator of lipid peroxidation because of the lack of lycopene in nettle (Table 5). The literature data from investigations about the influence of the nettle powder or extract used in the diet on lipid peroxidation in the egg yolk are very scarce. Nettle was usually used as supplement for poultry meat colour (yellow colour of meat) and

Table 5. Effect of dietary nettle powder on yolk lipids, yolk cholesterol, and lipid oxidation (X ± SE)

Indices \ Groups	Control	Dietary nettle powder supplementation (%)	
		0.3	0.5
Total lipids g / 100 g yolk	36.00 ± 0.59	35.36 ± 0.46	35.34 ± 0.40
Total cholesterol, mg / 100 g yolk	1536.78 ± 39.90	1382.10 ± 31.12 A**	1501.73 ± 44.72
Malondialdehyde (MDA), μg g^{-1}			
At the end of the experiment	0.31	0.38	0.31
Storage 30 days in fridge	1.14	0.91	0.85

Significance by: ** – $p \leqslant 0.01$
A – control group / experimental group 1

improvement of the colour of the yolk with nature colourants. The investigation established with broilers by Keshavarz et al. (2014) revealed that thiobarbituric acid reactive substances (TBARS), as an indicator for meat lipid oxidation after storage, were not influenced by adding nettle powder and nettle extract in broiler diets. Loetscher et al. (2013b) have reported that nettle supplementation in a diet did not improve oxidative stability (TBARS), but strongly intensified skin yellowness compared with the control treatment. In our experiment, the nettle supplementation did not significantly influence reduction of the lipid peroxidation in egg yolk, but there was a slight reduction of the lipid oxidation value compared with the control group.

Serum biochemical parameters

The influence of nettle powder supplementation on some serum biochemical parameters of laying hens is shown in Table 6. The addition of 0.3% dried nettle to the diet significantly decreased serum concentrations of total cholesterol ($p < 0.001$) and triglycerides ($p < 0.01$). The supplementation of nettle powder at 0.5% in the diet decreased ($p < 0.001$) total cholesterol, without affecting triglycerides. The level of the glucose was reduced significantly in the serum of laying hens fed with 0.3% supplemented diet ($p < 0.01$) and 0.5% ($p < 0.05$)

Mansoub (2011) have reported that the serum total cholesterol, triglycerides and LDL concentration were significantly reduced in the group supplemented with 2% nettle powder compared with the control group ($p < 0.05$), but the concentration of serum HDL and glucose were not significantly reduced in the groups compared with the control group. Moula et al. (2019) who studied the nettle effect in quails have reported reduced quail serum total cholesterol and serum triglyceride levels at the level of 6%.

The results of a significant reduction of the level of cholesterol in serum lead to the cholesterol reduction in egg yolk, which is reported in this study.

Conlussions

Our results demonstrated a positive effect of 0.3% and 0.5% nettle addition to the layers diet on yolk colour pigmentation. A significantly lower egg yolk cholesterol content was found in hens from experimental group 1 ($p < 0.05$). Nettle supplementation also reduced significantly blood serum glucose ($p < 0.01$ and $p < 0.05$ in experimental groups 1 and 2, respectively) as well as the total serum cholesterol content ($p < 0.001$). However, laying hens' performance did not alter by dietary nettle supplementation. The results also suggest that nettle could be added to the diets of laying hens to improve egg quality and the serum biochemical profile. Nettle proved unsuitable as a dietary antioxidant in the applied form and concentration, yet the colouring effect on the egg yolk could be interesting for egg production in certain cultures.

Table 6. Effect of dietary nettle powder on some biochemi cal parameters in blood serum of layers (X ± SE)

Indices \ Groups	Control	Dietary nettle powder supplementation (%)	
		0.3	0.5
Glucose, mmol/L	9.22 ± 0.44	7.07 ± 0.64 A**	7.66 ± 0.58 B*
Triglycerides, mmol/L	11.95 ± 1.54	5.59 ± 1.11A**	8.46 ± 1.11
Total cholesterol, mmol/L	2.75 ± 0.17	1.86 ± 0.13 A ***	1.87 ± 0.10 B ***

Significance: * – $p \leqslant 0.05$; ** – $p \leqslant 0.01$; *** – $p \leqslant 0 = 001$
A – control group / experimental group 1
B – control group / experimental group 2

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Received 4 May 2022
Accepted 15 June 2022

Milkability of Improved Valachian, Tsigai and Their Crosses With Lacaune and East Friesian

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Keywords: *dairy sheep, machine milking, milkability, systematic effects.*

Abstract. *Milkability is defined as the ability of an animal to give a regular, complete, and rapid milk secretion by the mammary gland in response to a proper milking technique. Indicators of milk production and milkability of ewes were determined in 359–370 ewes of 9 genotypes. For each ewe, the milk flow was recorded during the individual control measurements. The amount of milked milk was measured in individual time intervals after the attachment of milking cups to teats on udder of the ewe (10 indicators). We processed the obtained data using the REML methodology, with the MIXED procedure of the SAS statistical package. All indicators characterizing milk production and milkability of ewes were statistically significantly influenced by the genotype and the control year factors ($p < 0.001$). The order and stage of lactation were also significant factors in some cases. The machine milk yield of the monitored population of ewes was 318.26 mL on average. The total milk yield was 436.58 mL and the machine stripping ratio was 27.73% on average, ranging from 0 to 95%. The highest machine stripping ratio was determined in the Lacaune breed (37.69%), which had the highest total milk yield (524.69 mL) and one of the highest machine milk yield (332.70 mL). Compared with purebred Tsigai ewes and ewes of the Improved Valachian breed, crossbreeds with specialized dairy breeds (Lacaune and East Friesian) had better milk production and, in some indicators, also better milkability.*

Introduction

The machine milking of Improved Valachian and Tsigai in Slovakia already started during 1960s. Introduction of milking machines required information related to the milkability of ewes and search for the best milking parameters. Therefore, many experiments were carried out concerning milkability of the mentioned breeds in Slovakia between 1960s and 1980s (Mikuš, 1973), in cooperation with France (Labussiere, 1988). However, machine technology did not spread to farms in a larger scale since that time in Slovakia.

Genetic improvement of the milkability is the main tool to improve cheese production, and consequently the income of the producers. Many factors, such as breed, feeding or parity, have an influence on the quantity and the composition of sheep milk (Libis-Márta, 2021).

Milk, a product that is consumed by newborns to develop and grow, is one of the most important products of livestock. It is the main source of nutrition in feeding a human and animal offspring. Increasing demand for cheese made from processed ewe milk indicates that dairy sheep are becoming an interesting economic alternative for farmers. Farms with high-producing dairy sheep usually milk large flocks automatically (by machine milking) and conduct milking twice-daily throughout the lactation period. As a result, more than half of the total daily labor on dairy sheep farms is spent on milking (Marnet and McKusick, 2001), and therefore milking is one of the main reasons that deters people from dairy sheep production.

Milking characteristics and udder morphology are important factors determining milkability in dairy ewes. Machine milking benefits are maximal milk yield with better hygienic properties than hand-milked milk and easier stripping.

Milk flow kinetics is related to milk production (Mioč et al., 2009; Kremer et al., 2015; Kremer and Roses, 2016; Turkyilmaz et al., 2018; Salamon et al., 2019; Panayotov et al., 2018; Dhaoui et al., 2019; Pourlis, 2020; Prpic et al., 2020; Sevov et al., 2018; Vrdoljak et al., 2020; Devi et al., 2022), especially in non-well genetically selected breeds (Mačuhová et al., 2020; Costa et al., 2022). It can indicate the occurrence of a milk ejection reflex, which is crucial for complete milk extraction and, thus, for milk production. Milk within the udder of dairy ruminants can be divided into two fractions: the cisternal fraction, which has already been transferred from the alveoli to the cistern during the intermilking interval (immediately obtainable without prior milk ejection),

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and the alveolar fraction, which can be induced from the udder only if milk ejection occurs during machine milking (Tancin and Bruckmaier, 2001). A ide range of differences between dairy species exists with respect to the proportion of total milk that can be stored within a cistern. For example, following a normal milking interval of 12 to 14 h, the dairy ewe and goat can store up to 75% of the total milk volume within a cistern (Marnet and McKusick, 2001).

Improving milkability is a major issue to consider in breeding dairy species. Milking is the most time-consuming task in dairy farming. The constant increase in the average flock size and its productivity has been contributing to the extension of the milking time. In Slovakia, Lacaune ewes are selected for milk production traits, because of somatic cell count and udder morphology. On the one hand, selection of udder morphology aims to improve milking ability indirectly. Direct assessment of the milk flow can be assessed with automatic milking jars (Marie-Etancelin et al., 2006).

Milk production and milk flow profiles are important parameters to be recorded and evaluated, as being informative about milking management.

Monitoring the milkability of animals allows improving efficiency of milking procedures and reducing farm production costs. It is noted that udder traits like depth, udder attachment or teat placement are correlated with milk production, machine milking extraction or mastitis incidence.

The aim of the presented work was to determine the milk production and milkability of ewes in the breed Improved Valachian (IV), Tsigai (T), Lacaune (LC) and their crossbreeds with a 25%, 50% and 75% genetic share of specialized dairy breeds (SDB) Lacaune and East Frisian. At the same time, we analyzed genetic and non-genetic factors which influence selected indicators qualifying the milkability of ewes during the milking period under machine milking conditions.

Materials and methods

The breed Improved Valachian originated from a coarse wool Valachian breed in Slovakia, where intensive cross-breeding programme started in 1950. To improve wool, meat and milk production, the crossing with a wide range of breeds (Leicester, Lincoln, Texel, Cheviot, Kent and East Friesian sheep) was attempted. The Improved Valachian was recognized as an independent dual-purpose breed (wool-meat and meat-milk, respectively) in 1982. Tsigai and Improved Valachian are very similar in production potential. At present, Tsigai and Improved Valachian are crossed with the purpose to improve their milk production, milkability and prolificacy with specialized dairy breeds as Lacaune and East Friesian. We included biological material from the Center of Animal Production Research Nitra of Institute of Sheep and Goat Breeding Trencianska Tepla in our experiments. In this farming, during the milking period, under machine milking conditions, we determined the milk production and milkability of ewes of various breeding groups over a period of 7 years. The kinetics of milk ejection was monitored during the milking period. The animals were bred and managed within one dairy flock.

The ewes were milked twice a day during the lactation period, in each of the monitored years. Machine milking was performed in a row milking parlor 1 x 24 stalls, with a sliding fixing device (vacuum 38 kPa; number of pulses 140-160 / min pulsation ratio 1:1). The included ewes represented purebred individuals of the Valachian, Tsigai and Lacaune breeds. In addition to the purebred ewes of the breeds Improved Valachian, Tsigai and Lacaune, the experiment also included ewes crossbred with different genetic proportions of improved breeds Lacaune and East Frisian. The crosses created on the basis of the Improved Valachian breed and on the basis of the Tsigai breed were divided into six genotypic groups, with a 25%, 50% and 75% genetic share of Lacaune and East Friesian dairy breeds (IV x SDB 25%, IV x SDB 50%, IV x SDB 75%, T x SDB 25%, T x SDB 50%, T x SDB 75%). We compared the functional and morphological properties of the udder of selected ewes of 9 genotypes (3 purebred breeds, 6 genotype groups of hybrids). Most crosses created on the basis of a breed of Tsigai or the Improved Valachian formed two-breed crossbreeds with a 25%, 50% and 75% genetic share of the Lacaune breed. Three-breed crossbreeds with a 25%, 50% and 75% genetic share of both Lacaune and East Frisian dairy breeds represented a significantly smaller part of the evaluated population (17 ewes, i.e. about 5% of the evaluated population). In the experimental ewes of all 9 genotypes, ewes were presented in the first, second, third and subsequent lactations in each of the monitored years. Most measurements were taken in May and July. Experimental measurements were always performed in the evening, and then in the morning milking. During the milking period, at least 2, and in some years up to 4, milk control measurements were performed. Some ewes were included in the experiment within two or even more years, which shows that we performed up to 8 control measurements of milk on some ewes. The specific number of observations of the selected indicators, depending on genotype, order and stage of lactation, are given in the relevant Tables 2 to 5.

During the individual control measurements, the milk flow was recorded for each ewe, at individual time intervals after the attachment of the teat cups to the udder of the ewe. Certified milk meters standardly used by Breeding Services, š. p. Bratislava for the control of sheep milk yield, were applied, with the measurement accuracy ± 10 mL. In this case, we recorded the amount of milked milk at 10-second intervals until the milk flow stopped and the amount

Table 1. Basic variational-statistical characteristics of selected indicators characterizing milk production and milkability of ewes

Indicators	n*2	$\bar{x}$	s	v	min.	max.
Milk yield in 10 s (MY10s) (mL)	1029	90.13	76.45	84.82	0	400
Milk yield in 30 s (MY30s) (mL)	1218	220.40	100.78	45.73	0	650
Milk yield in 60 s (MY60s) (mL)	1159	307.15	154.09	50.17	0	1200
Machine milk yield (MMY) (mL)	1218	318.26	166.90	52.44	10	1200
Milking time (s)	1218	62.67	16.10	25.69	15	160
Total milk yield (mL)	1218	436.58	197.11	45.15	30	1339
Machine stripping (mL)	1218	118.69	91.85	77.39	0	775
Machine stripping ratio MS/TMY (%)	1218	27.73	15.53	56.00	0	95
Milk yield ratio in 30s MY30s/MMY (%)	1218	53.83	18.35	34.09	0	100
Milk yield ratio in 60s MY60s/MMY (%)	1159	69.35	17.01	24.52	0	100

*[2]n – number of observations; $\bar{x}$ – mean value; s – standard deviation; v – variation coefficient

Table 2. Analysis of covariance of indicators of milk production and milkability of ewes

Source of variance	**(df)**	**Trait**									
		MY10s		MY30S		MY60S		MMY		MT	
		f value	$p > f$	*f* value	$p > f$	*f* value	$p > f$	*f* value	$p > f$	*f*value	$p > f$
Year	5	97.04	< 0.0001	36.68	< 0.0001	24.82	< 0.0001	22.58	< 0.0001	32.69	< 0.0001
Lactation stage	3	0.60	0.6147	2.25	0.0808	8.88	< 0.0001	10.11	< 0.0001	0.37	0.7749
Genotype	8	4.01	0.0001	3.61	0.0004	9.45	< 0.0001	12.92	< 0.0001	7.11	< 0.0001
Parity	2	1.95	0.1434	3.15	0.0043	5.33	0.0050	4.86	0.0080	0.52	0.5947
Days in milk	1	0.41	0.5231	9.76	0.0018	21.87	< 0.0001	38.46	< 0.0001	9.10	0.0026
Source of variance	**(df)**	**Trait**									
		TMY		MS		MS/TMY		MY30s/MMY		MY60s/MMY	
		f value	$p > f$	*f* value	$p > f$	*f* value	$p > f$	*f* value	$p > f$	*f* value	$p > f$
Year	6	32.10	< 0.0001	8.71	< 0.0001	6.64	< 0.0001	8.65	< 0.0001	4.97	< 0.0001
Lactation stage	3	14.52	< 0.0001	1.93	0.1235	0.80	0.4960	3.88	0.0090	1.68	0.1688
Genotype	8	29.30	< 0.0001	21.58	< 0.0001	8.54	< 0.0001	15.69	< 0.0001	9.59	< 0.0001
Parity	2	0.60	0.5469	8.10	0.0003	9.37	< 0.0001	2.74	0.0652	10.15	< 0.0001
Days in milk	1	48.69	< 0.0001	1.52	0.2175	0.25	0.6163	0.30	0.5848	0.00	0.9582

+++ $p < 0.001$; ++$p < 0.01$; +$p < 0.05$

Table 3. Influence of genotype on individual indicators characterizing milk production and milkability of ewes – I

Source of variance				Trait									
				MY10s *1		MY30S*2		MY60S*3		MMY*2		MT*2	
				LSM ± SE		LSM ± SE		LSM ± SE		LSM ± SE		LSM ± SE	
Genotype													
Improved Valachian (100)	**186*1**	**218*2**	**200*3**	**88.79**	**5.584**	**200.93**	**9.116**	**277.46**	**12.666**	**274.35**	**13.001**	**57.65**	**1.268**
IVxSDB (25%) (125)	49	68	67	88.15	10.994	249.87	15.448	341.35	21.052	354.97	22.115	63.47	2.161
IVxSDB (50%) (150)	69	93	91	84.53	8.791	221.87	12.955	324.24	17.666	343.16	18.529	63.05	1.813
IVxSDB (75%) (175)	79	82	82	72.65	8.560	220.56	13.720	337.87	18.663	366.49	19.617	67.31	1.926
Tsigai (200)	**244**	**268**	**244**	**80.52**	**5.027**	**176.42**	**8.411**	**209.21**	**11.766**	**207.60**	**12.004**	**55.12**	**1.171**
TxSDB (25%) (225)	10	18	15	109.87	22.724	226.64	31.429	331.59	43.245	314.55	44.573	56.79	4.281
TxSDB (50%) (250)	135	169	164	89.11	6.422	226.42	9.989	307.06	13.571	327.16	14.226	62.48	1.379
TxSDB (75%) (275)	25	47	47	85.27	13.014	212.24	20.324	305.18	27.383	337.05	28.778	66.43	2.746
Lacaune (300)	**222**	**255**	**249**	**53.61**	**5.294**	**216.59**	**8.642**	**315.18**	**11.773**	**332.70**	**12.312**	**62.88**	**1.194**
Significant differences				100:300+++; 125:300+; 150:300++; 175:300+; 200:300+++; 225:300+; 250:300+++; 275:300+;		100:125++; 100:200+; 125:200+++; 175:200++; 200:250,300+++;		100:125,175++; 100:150,300+; 100:200+++; 125:200+++; 150:200+++; 175:200+++; 200:250,300+++; 200:225,275++;		100:125,150,250++; 100:175,200,300+++; 100:275+; 125:200+++; 150:200+++; 175:200+++; 200:225+; 200:250,275,300++;		100:125,150,250+; 100:175+++; 100:275,300++; 125:200+++; 150:200+++; 175:200+++; 175:225,250,300+; 200:250,275,300+++; 225:275+;	

+++ $p < 0.001$; ++$p < 0.01$; +$p < 0.05$; ns – non-significant; *1, *2, *3 – number of measurements depending on the indicator

Table 4. Influence of genotype on individual indicators characterizing milk production and milkability of ewes – II

Source of variance			Trait									
			TMY*1		MS*1		MS/TMY*1		MY30s/MMY*1		MY60s/MMY*2	
			LSM ± SE		LSM ± SE		LSM ± SE		LSM ± SE		LSM ± SE	
Genotype												
IV (100)	**218*1**	**200*2**	**349.61**	**14.314**	**76.81**	**8.298**	**24.06**	**1.435**	**58.92**	**1.669**	**75.21**	**1.607**
IVxSDB (25%) (125)	68	67	446.98	24.258	91.68	14.022	23.09	2.442	55.06	2.833	72.80	2.671
IVxSDB (50%) (150)	93	91	460.40	20.343	119.88	11.772	27.79	2.046	50.27	2.375	68.89	2.241
IVxSDB (75%) (175)	82	82	495.08	21.544	130.00	12.478	27.96	2.166	46.03	2.514	65.31	2.367
T (200)	**268**	**244**	**278.53**	**13.208**	**73.39**	**7.656**	**27.41**	**1.324**	**64.03**	**1.540**	**71.87**	**1.492**
TxSDB (25%) (225)	18	15	429.11	49.353	115.94	28.721	27.97	4.906	52.93	5.741	73.01	5.480
TxSDB (50%) (250)	169	164	435.95	15.685	110.82	9.104	26.71	1.568	54.45	1.828	69.79	1.720
TxSDB (75%) (275)	47	47	496.30	31.915	159.43	18.507	32.48	3.165	45.59	3.710	63.25	3.464
LC (300)	**255**	**249**	**524.69**	**13.571**	**194.51**	**7.875**	**37.69**	**1.357**	**42.93**	**1.582**	**58.93**	**1.492**
Significant differences			100:125,150,175, 200,250,275,300+ ++; 125:200+++; 125:300++; 150:200+++; 150:300++; 175:200+++; 175:250+; 200:225++; 200:250,275,300+++; 250:300+++;		100:150,250++; 100:175,275,300+++; 125:175+; 125:275++; 125:300+++; 150:200,300+++; 175:200,300+++; 200:250++; 200:275,300+++; 225:300++; 250:275+; 250:300+++;		100:275+; 100:300+++; 125:275+; 125:300+++; 150:300+++; 175:300+++; 200:300+++; 225:300+; 250:300+++;		100:150,275++; 100:175,300+++; 100:200+; 125:175,275+; 100:200++; 100:300+++; 150:200+++; 150:300+; 175:200+++; 175:250++; 200:250,275,300+++; 250:275+; 250:300+++;		100:150,250+; 100:175,300+++; 100:275++; 125:175,275+; 125:300+++; 150:300+++; 175:200,300+; 200:275+; 200:300+++; 225:300+; 250:300+++;	

+++ $p < 0.001$; ++ $p < 0.01$; + $p < 0.05$; ns – non-significant; *1, *2, – number of measurements depending on the indicator

Table 5. The influence of the order and stage of lactation on the indicators characterizing milk production and milkability of ewes

Source of variation					Trait									
					MY10S[*1]		MY30S[*2]		MY60S[*3]		MMY[*2]		MT[*2]	
					LSM ± SE		LSM ± SE		LSM ± SE		LSM ± SE		LSM ± SE	
Parity														
1.	(1)	370[*1]	434[*2]	425[*3]	87.87	4.302	226.25	6.167	323.04	8.546	335.41	8.893	61.76	0.892
2.	(2)	317	348	321	80.76	4.444	214.71	6.862	300.69	9.608	310.88	10.046	61.12	1.039
3+	(3)	342	436	413	79.86	4.804	209.55	7.122	292.64	9.914	306.39	10.335	62.18	1.046
Significant differences					ns		1:3+;		1:2+; 1:3++;		1:2,3++;		ns	
Lactation stage														
40.– 99. day	(1)	184	261	251	91.21	9.183	225.46	12.489	330.04	18.088	334.05	19.063	61.94	2.151
100.–129. day	(2)	324	366	357	86.16	5.191	223.27	7.204	302.46	10.110	305.74	10.660	61.29	1.136
130.–159. day	(3)	289	335	316	81.05	5.106	205.36	7.755	277.06	11.250	293.79	11.545	61.28	1.244
160.–210. day	(4)	232	256	235	72.92	8.347	213.26	12.071	312.28	17.846	336.64	18.417	62.25	2.078
Significant differences					ns		ns		1:3+; 3:4++;		1:2+; 3:4+++;		ns	
Source of variation					**Trait**									
					TMY[*1]		MS[*1]		MS/TMY[*1]		MY30s/MMY[*1]		MY60s/MMY[*2]	
					LSM ± SE		LSM ± SE		LSM ± SE		LSM ± SE		LSM ± SE	
Parity														
1.	(1)	434[*1]		425[*2]	440.33	9.684	226.25	6.167	323.04	8.546	335.41	8.893	61.76	0.892
2.	(2)	348		321	429.85	10.774	214.71	6.862	300.69	9.608	310.88	10.046	61.12	1.039
3+	(3)	436		413	435.36	11.182	209.55	7.122	292.64	9.914	306.39	10.335	62.18	1.046
Significant differences					ns		1:2+;1:3+++;2:3+;		1:2+;1:3+++;2:3+;		1:3+;		1:2++; 1:3+++; 2:3+;	
Lactation stage														
40.– 99. day	(1)	261		251	462.81	19.609	132.18	10.874	26.65	2.157	48.54	2.352	70.21	2.333
100.–129. day	(2)	366		357	420.67	11.311	117.44	6.412	27.03	1.193	53.18	1.340	70.71	1.294
130.–159. day	(3)	335		316	405.87	12.176	112.46	6.872	29.25	1.295	53.90	1.450	67.34	1.443
160.–210. day	(4)	256		235	451.38	18.952	114.57	10.514	30.48	2.084	53.37	2.273	67.07	2.302
Significant differences					1:2++; 1:3+;3:4+++;		ns		ns		1:2++;		ns	

+++ $p < 0.001$; ++$p < 0.01$; +$p < 0.05$; ns – non-significant; *1, *2, *3 – number of measurements depending on the indicator

of milk remained at the same level for at least 20 seconds. If the milk flow was not detectable for 20 seconds using our specific meters, the timekeeper instructed the milker to start the machine stripping. All ewes were machine stripped for another 60 seconds. If the milk flow was noticeable for more than 60 seconds, then machine milking continued and a new machine stripping was done only from the moment when no milk flow was recorded in the previous 20 seconds. The sheep were machine stripped again at the instruction of the timekeeper until the milk flow stopped. We also recorded the amount of milk drawn at 10-second intervals during each machine stripping. Based on the individual recording of the milk release of each ewe in 10 seconds, or at second intervals, we evaluated:

– Milk yield in 10 s (MY10s)
– Milk yield in 30 s (MY30s)
– Milk yield in 60 s (MY60s)
– MY30s/MMY (%)
– MY60s/MMY (%)
– Machine milk yield (MMY) (mL)
– Machine stripping (MS) (mL)
– Total milk yield (mL)
– MS/TMY (%)
– Milking time (s)

The machine milk yield represented the amount of milk drawn after the milking set was put on (without prior udder stimulation) until the milk flow was completed within 20 seconds time interval. Machine stripping represents the amount of milk drawn from the beginning of machine stripping to the withdrawal of the milking set.

Data were processed by REML methodology using a MIXED procedure from the SAS statistical package. The following statistical model with fixed and random effects was applied:

$$yijklm = \mu + Y_i + LSj + GEN_k + P_l + an_m + + a*DIM_{ijklm} + e_{ijklm}$$

where:

y_{ijklm} is an observed trait (see above for details); Y_i – year (a fixed effect with 4 to 7 levels); LSj – lactation stage, a fixed effect with 4 levels (from 40th to 99th lactation day, from 100th to 129th lactation day, from 130th to 159th lactation day and from 160th to 210th lactation day); GEN_k – genotype (breed group; a fixed effect with 9 levels; see above for detail characterization); P_l – parity (a fixed effect with 3 levels; first, second, third and further parity); an_m – animal (random effect); DIM_{ijklm} – days in milk (covariate; 40 to 210 days in milk); e_{ijklm} – the random error.

The differences were statistically significant at $p < 0.05$, or less.

Results

As can be seen from Table 1, we observed a large variability in the evaluated population for all indicators characterizing milk production and milk yield of ewes. The average total milk yield at the level of 436.58 mL is not high, if we consider the fact that in the monitored population there were also high-producing purebred ewes of the Lacaune breed. In the case of selection of sheep for milk production obtained by machine milking, the machine stripping ratio should be reduced, which significantly affects labour productivity and the udder health of machine-milked ewes. For the whole monitored population of ewes, the machine stripping reached on average 318.26 mL, while the range was relatively large (10 to 1200 mL). The machine stripping ratio in the monitored population of ewes was relatively high (27.73; Table 1). When evaluating the milk flow rate, we found that in some ewes, the amount of milk yield in 10 s, 30 s or 60 s was at the level of 400 mL, 650 mL or 1200 mL, and vice versa, some ewes did not run milk at all during this time. In the best ewes, the ratio of milk yield in 30 s or 60 s of the total milk yield was up to 100%.

Our results (Table 2) show that the genotype factor has a statistically significant effect on all monitored production factors. The influence of the factors like lactation sequence, lactation stage and the day of lactation were not so highly statistically significant. On the contrary, the accompanying variable "year" had a statistically highly significant effect on all indicators we surveyed ($p < 0.001$).

Table 3 shows that the most milk yield in 10 s was found in T x SDB crosses (25% SDB) (109.87 ± 22.724 mL) and, conversely, the least milk yield in the first 10 s was found in purebred Lacaune ewes (53.61 ± 5.294 mL). Regarding the amount of milk yield in 30 seconds as an indicator, we found the most milk yield for this indicator in crosses IV x SDB (25% SDB) at the level of 249.87 ± 15.448 mL, and vice versa, the lowest average value for this indicator was found in purebred Tsigai ewes (176.42 ± 8.411 mL). For the indicator of the amount of milk yield in 60 s, we found the highest average value in crosses IV x SDB (25% SDB) (341.35 ± 21.052 mL), and vice versa, in purebred ewes of the breed Improved Valachian, we found the lowest average value for this indicator (277.46 ± 12.666 mL). The indicators evaluated by us: the amount of milk yield in 10 s, 30 s and 60 s well characterize the milk release rate of milked ewes. In practice, the more milk is yield in 60 s, the more advantageous it is for the breeder (more sheep will be milked per unit of time). As expected, we found the highest average machine milk yield in purebred ewes of the Lacaune breed (332.70 ± 12.312 mL), and conversely, the lowest average machine milk yield in the monitored population was found in purebred ewes of the Tsigai breed (207.60 ± 12.004 mL).

Table 4 shows that, as expected, we found the largest average total milk yield in purebred Lacaune ewes (524.69 ± 13.571 mL), and conversely, the

lowest average value in this indicator was found in purebred Tsigai ewes at (278.53 ± 13.208 mL). The highest average machine stripping was again found in purebred ewes of the Lacaune breed (194.51 ± 7.875 mL), and conversely, the lowest average value in this indicator was found in purebred ewes of the Tsigai breed (73.39 ± 7.656 mL). The highest average machine stripping was found in purebred ewes of the Lacaune breed, up to (37.69 ± 1.357%), and vice versa, the lowest average machine stripping ratio was found in crossbreeds IV x SDB (25% SDB) at the level (23.09 ± 2.442%). In the indicator of the milk yield ratio in 30 s, we found the highest average value in purebred ewes of the Tsigai breed (64.03 ± 1.540%) and, vice versa, the lowest in purebred ewes of the Lacaune breed (42.95 ± 1.582%). In the indicator of the milk yield ratio in 60 s, we found the highest average value in purebred ewes of the Improved Valachian breed, namely (75.21 ± 1.607%), and vice versa, the lowest average value was found in purebred ewes of the Lacaune breed (58.93 ± 1.492%). Another factor considered to affect milk production and the milkability of ewes is the "lactation order" factor.

Table 5 shows that the factor "lactation order" had a statistically highly significant effect ($p < 0.001$) on the indicators of the machine stripping, the machine stripping ratio and the milk yield ratio in 60 s. We found a statistically significant effect ($p < 0.01$) in the indicators of the milk yield in 30 s and 60 s and machine mild yield. The influence of the lactation order on the indicators of the milk yield in 10 s and the time of machine milk yield was not statistically significant. The differences between the ewes on the 1st to 3rd lactation were statistically insignificant for the indicators of the milk yield in 10 s and the time of machine milk yield, except for the indicators of the milk yield in 30 s and 60 s and machine milk yield ($p < 0.01$). The best ejection of milk in the first 10 s, 30 s and 60 s and the highest average machine milk yield had ewes on the 1st lactation. Total milk yield was not statistically significantly affected by the "lactation order" factor. The ewes in the first lactation had the largest total milk yield, and the milk yield ratio in 30 s and 60 s. On the contrary, the machine stripping ratio gradually increased, reaching the highest average value (30.77 ± 1.148%) in ewes on the 3rd lactation. The influence of the factor "lactation stage" on individual indicators of milk production and milkability of ewes was statistically highly evident in the indicators of total milk yield ($p < 0.001$) and the milk yield ratio in 30 s ($p < 0.01$) and, vice versa, inconclusive for machine stripping, the machine stripping ratio, the time of machine milk yield, the milk yield in 10 s and 30 s and the milk yield ratio in 60 s.

Discussion

In dairy ewes, 25% of the total milk yield for the entire lactation is produced during the first month (Folman et al., 1966; Ricordeau et al., 1962). This is primarily due to the fact that milk production is increasing from parturition to about 24 days of lactation when the peak milk production is attained. To complicate matters, ruminants have the highest probability of mastitis during the first 45 days post-partum (Hamann, 2000). Generally, milk yield and length of lactation in sheep vary across breeds (i.e., dairy and non-dairy breeds). The East Friesian breed is widely reported as the highest milk producer with around 3100 g/day (at peak lactation) and 500–700 kg total milk yield, with the longest lactation length (around 240 days) compared with non-dairy breeds (90–150 days) (Green et al., 2016). Boyazoglu (1991) reviewed the results of experiments that evaluated the East Friesian in countries of the Mediterranean region. In all countries, the pure East Friesian was found to be unacceptable due to high incidence of respiratory disease and poor adaptability to high environmental temperatures. Only in Israel, a cross of the East Friesian with the local Awassi breed was found to result in a more productive animal than the local breed (Gootwine and Goot, 1996). East Friesian ewes also have been reported to have some undesirable milking characteristics relative to the Lacaune. Bruckmaier et al. (1997) reported that East Friesian ewes had a greater proportion of the udder cistern located below the exit into the teat channel, delayed oxytocin release and milk iniciation, slower milk flow rates during milking, and longer milking times compared with Lacaune ewes. Macuhova et al. (2007) found in 80 ewes of the breeds Improved Valachian, Tsigai, Lacaune and their crossbreeds that 28% of the ewes initiated milk during the first 10 seconds of machine milking.

According to Menzies et al. (2013), the total milk production in sheep is dependent on the shape of the lactation curve, which deals with the time and height of peak milk production (maximum daily milk yield during lactation) and the length of lactation. However, the length of lactation and peak milk production are influenced by breed, photoperiod (daylight length), nutrition, multiplicity of lactacion (first- or second-time lactation), stress and pain at milking, milking frequency and presence of intramammary infections (Pollott and Gootwine, 2004). Some studies have demonstrated that milk production is associated with litter size, i.e., in twin- and triplet- bearing ewes, thereby production is about 20 litres of milk per lactation and a 1% increase in lactation persistency than in single-bearing ewes. This was recorded in some Assaf dairy breed in Israel where the animals were kept under an intensive management system, and surprisingly, the lambs were weaned at birth (and reared artificially) on the premise of accurate measurement of the ewes' milk production (Pollott and Gootwine, 2004). A similar effect is possible in non-dairy breeds, but some differences may occur because they produce lower quantity of milk

(averagely 47–103 litres) compared with the dairy breeds which produce about 234–354 litres of milk per lactation (Shrestha et al., 2008). Nieto et al. (2018) have reported a 30% reduction in milk yield of merino ewes bearing single lambs compared with the twin-bearing ewes, and there was no effect of production in the dams suckling ewe lambs or ram lambs. This impact of milk production was further explained –there was a consistently higher milk production in twin-bearing ewes than the single-bearing ones, and with a 33% and 28% decline from days 28 and 56 for the single- and twin-bearing ewes respectively. Meanwhile, the sharp decline from day 56 to 70 (57% for the singles and 42% for the twins) was associated with a lambs' decreasing dependence on milk. However, the milk yield between parturition and day 28 was not given in the study, which may be in order not to compromise the growth and development of the lambs; hence the ewes were milked near their peak lactation period (Bencini et al., 1992; Bencini and Purvis, 1990). In addition, multiparous ewes have higher peak milk production and lactation persistency than the primiparous ewes. It has been further observed in some studies (Bencini and Pulina, 1997; Paten et al., 2017; Snowder and Glimp, 1991) that heavier ewes (multiparous ewes) produced more milk than their lighter counterparts, i.e., primiparous ewes. This may be because the multiparous ewes are usually older and more matured than the primiparous, which are still undergoing physiological development.

Conclusions

Based on our results, we propose to use the indicators of the machine milk yield and the machine yield ratio in the selection of sheep for better milkability. Optionally also some others are recommended. In accordance with the trend in all sheep-developed countries, we propose to include them in the routine performance control and later in the genetic evaluation of dairy sheep in Slovakia.

Acknowledgments

M. Nagy was supported by project KEGA č. 002PU-4/2021 „Univerzitná výučba genetiky inovovanými formami a metódami."

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Received 10 March 2022
Accepted 15 June 2022

Neurobehavioral and Biochemical Toxicity of Atrazine in Chicks

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Keywords: *Atrazine; neurobehavioral; chicks; toxicity.*

Abstract. *The aim of the study was to examine the neurobehavioral and biochemical toxicity of atrazine herbicides in chicks. This is a model of poisoning we explored recently to further elucidate the toxic action of atrazine. The acute oral LD50 of atrazine was determined by the Dixon method; acute toxic symptoms of atrazine were recorded. The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (liver enzymes), as well as blood glucose, were determined, and the effects of atrazine on open field activity and body weight were also examined. Atrazine's LD_{50} was 1435.25 mg/kg when given orally. The oral administration of atrazine at doses of 1300 mg/kg and 1000 mg/kg to chicks resulted in the appearance of acute poisoning symptoms such as depression, dyspnea, frequent defecation, lacrimation, ataxia (which appeared in both treatments at 100%), as well as profuse salivation, nasal discharge followed by tremors, convulsions, recumbency, and death (which appeared in the first treatment at a percentage higher than the second treatment). The oral dosing of atrazine at 574 mg/kg and 861 mg/kg caused a significant increase in AST, ALT, and glucose levels in the plasma of treated chicks after 24 hours compared with the control group and with treated groups after 4 hours. The oral administration of atrazine at doses 71.75 mg/kg, 143.5 mg/kg, and 287 mg/kg, twice a week for 2 weeks, led to an increase in motor activity through the significant increase in squares crossed and jumping frequency, and decrease in the bodyweight of chick. Our results conclude that atrazine has toxic effects on chicks through its effects on liver enzymes, body weight, and neurobehavioral activity.*

Introduction

The atrazine herbicide is commonly used to control broadleaf and grass weeds in farming around the world because of its low cost and effectiveness in agricultural crops protection (Konstantinou et al., 2006; Adams, 2017). Because of its ability to accumulate in the environment, detection of atrazine in both communities and humans has been recorded (Foradori et al., 2011; Campos-Pereira et al., 2012). Atrazine is stored in many organs and tissues in case of chronic exposure (Foradori et al., 2013; Liu et al., 2017). Several studies have shown that atrazine had adverse effects on animals and humans (Li et al., 2014; 2015; Ma et al., 2015). An increase in the number of workers affected by Parkinson's disease has been recorded in areas exposed to atrazine contamination (Priyadarshi et al., 2000; Brown et al., 2005). Atrazine causes neurotoxic effects by alteration in the neurochemical transmitters (Coban & Filipov, 2007), as well as disturbance function of hypothalamic and pituitary-ovarian (Cooper et al., 2000). Atrazine causes a decrease in the level of dopamine suggesting atrazine-induced neurodegenerative disorder (Rodriguez et al., 2013). It is metabolized by CYP450s and excreted in urine and feces (Dutheil et al., 2008). Atrazine has an elimination half-life of about 31 hours in humans (Campbell et al., 2016). In mammals, atrazine causes endocrine imbalance, such as increased corticosterone levels in rats (Good, 1961; Laws et al., 2009), leading to a reduction in the level of luteinizing hormones in rats (Foradori et al., 2011). Atrazine causes immunotoxicity through oxidative stress and calcium hemostasis imbalance (Gao et al., 2016).

The central nervous system is one of the main target sites of atrazine, in addition to the atrazine effects on the reproductive function (Giusi et al., 2006; Foradori et al., 2009). Also, in rodents, atrazine has toxic effects on developmental and immune system activity as well as causes Parkinson disease-like symptoms (Stoker et al., 2000; Rowe et al., 2007). In addition, several studies have reported changes in neurobehavioral and sensorimotor functions in mice (Belloni et al., 2007).

Previous findings of rodents have shown that atrazine causes alteration in brain dopamine, and serotonin balance and possible change the metabolism of tyrosine and tryptophan (Coban & Filipov, 2007; Rodr´ıguez et al., 2013). Atrazine exposure has also been linked to the progression of neurological diseases, including Parkinson's disease (Ascherio et al., 2006). The United States Environmental Protection Agency has classified atrazine as endocrine-disrupting herbicides (Morales-Pe´rez et al., 2016). Atrazine has different toxic effects on plants, animals, and humans (Simranjeet et al., 2018). The mechanism of action of

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atrazine in plants is carried out through impairing the process of photosynthesis (Simranjeet et al., 2018). Young chicks have been used as a model to assess the toxicity of xenobiotics (Al-Zubaidy & Mohammed 2013; Alrawe & Alzubaidy, 2022). Due to the limited information on the toxicity of atrazine in chicks, the aim of the present study was to examine the acute toxicity of atrazine in chicks through investigating the effects on the central nervous system and liver function, after determining the median lethal dose.

Materials and Methods

Animals

The broiler chicks (type Ross) were treated between the ages of 10 and 14 days, with a body weight (B.Wt) of 125–295 g. They were placed in the animal house at laboratory conditions with a 12/12-hour light-dark cycle at a temperature of 35°C and provided with water and food ad libitum. All of the procedures were carried out in accordance with institutional guidelines for animal use in biomedical research. The protocol of this study was evaluated and approved by the Scientific Committee of the College of Veterinary Medicine, University of Mosul, Iraq, as of 27-11-2019.

Dose preparation

Different concentrations of a commercial wettable powder of the insecticide atrazine (50% Lizz Agrochem, China) were dissolved in freshly made distilled water each day before dosing, with a volume of administration of 10 mL/kg of body weight given orally via a gavage needle.

1. Determination of the oral median lethal dose (LD_{50}) of atrazine in chicks by Dixon method.

Seven chicks weighing between 125 g and 153 g were used, and atrazine was dosed orally at 750 mg/kg based on previous studies and trials; the result was read as death (X) or survival (O) after 24 hours, and the quantity of the dose increased or decreased at a constant rate of 250 mg/kg according to the Dixon method (Dixon, 1980). The following equation was used to calculate the LD_{50} value:

LD50 =xf +Kd

where xf – last dose, K – tabular value at a variance of 0.56, d – the values of a dose increase or decrease.

2. The acute toxic effects of atrazine in chicks

Twenty chicks were tested, with body weights ranging from 170 g to 295 g. Groups of chicks were orally dosed, and acute toxic symptoms of atrazine were recorded for 4 hours following oral dosage.

In this experiment, the chicks were divided into two groups of 10 birds each and treated with 90% and 70% of the LD_{50} of atrazine. The first group was given 1300 mg/kg body weight (B.W.) of atrazine orally. The second group was administered 1000 mg/kg B.W. orally.

3. The effects of different toxic doses of atrazine at different times on levels of glucose, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) in plasma of treated chicks.

We used 42 chicks at the age of 14 days. Their body weight ranged within 121–205 g, and they were dosed orally by using a gavage needle with different doses as follows.

a) The effects of 40% of LD_{50} of atrazine after 4 and 24 hours of treatment on glucose, AST, and ALT levels

Chicks were divided into three groups, 7 chicks each. The first group (control) was dosed with distilled water, the second group was dosed with atrazine at 574 mg/kg B.W. and killed after 4 hours, and the third group was dosed with the same dose but killed after 24 hours.

b) The effects of 60% of LD_{50} of atrazine after 4 and 24 hours of treatment on glucose, AST, and ALT levels:

Chicks were divided into 3 groups of 7 birds each. The first group (control) was dosed with distilled water; the second group was dosed with atrazine at 861 mg/kg B.W. and killed after 4 hours, and the third group was dosed with the same dose and killed after 24 hours.

Glucose, AST, and ALT were measured by using specific kits (Biolabo, France) with a colorimetric method by using a spectrophotometer for absorption measuring and then make calculations by using specific equations to find concentrations.

4. Neurobehavioral effects of many low doses of atrazine in chicks

We used 28 chicks 7 days aged; their body weight ranged within 93–135 g; they were treated orally twice a week for 2 weeks. Chicks were divided into 4 groups, 7 chick each. The first group (control) was dosed with distilled water, the second group was dosed with atrazine at 71.75 mg/kg B.W. (5%) LD_{50}, the third group was dosed with atrazine at 143 mg/kg B.W. (10%) LD_{50}, and the last group was dosed with atrazine at 28 7mg/kg B.W. (20%) LD_{50}. When 2 weeks of treatments ended, all chicks were subjected to neurobehavioral tests.

Chicks were subjected to the open-field activity test by using a 60 x 60 x 30 cm box. The arena of the box was divided into 16 equal squares, and each chick was placed in the middle of the arena to record the latency time (time spent moving from the center of arena), the number of squares crossed, jumping, defecation, as well as watching pecking and vocalization scores within 3 minutes (Al-Zubaidy & Mohammed, 2013; Al-Zubaidy, 2021; Alrawe & Alzubaidy, 2022) .

The tonic immobility response test measures the sensorimotor activity; after completing the open-field activity test, the same chick was subjected to tonic immobility response test (Hennig et al., 1984).

Statistical Analysis

The parametric data were statistically analyzed using the one-way analysis of variance (ANOVA)

test, followed by the least significant difference test using the SPSS (version 16). The nonparametric data were statistically examined using the Mann-Whitney U test, with a significance level of $p < 0.05$.

Results

1. Determination of the oral median lethal dose LD_{50} of atrazine in chicks by the Dixon method

Atrazine's oral LD_{50} was 1435.25 mg/kg body weight, resulting in toxic symptoms which included depression, dyspnea, salivation, lacrimation, defecation, recumbency, tremors, convulsions, and death at high toxic doses (Table 1).

The following equation was used to calculate the LD_{50} value:

LD50 =xf +Kd

where xf – last dose; K – tabular value at a variance of 0.56; d – the value of a dose increase or decrease.

2. Acute toxic effects of high doses of atrazine in chicks

In chicks, oral administration of atrazine at doses of 1300 mg/kg and 1000 mg/kg B.W. resulted in the appearance of acute poisoning symptoms such as depression, dyspnea, defecation, lacrimation, ataxia (which appeared in both treatments at 100%), profuse salivation, nasal discharge accompanied by tremors, convulsions, recumbency, and death (which appeared in the first treatment; Table 2).

3. The effects of different toxic doses of atrazine with different times on levels of glucose, aspartate aminotransferase and alanine aminotransferase in plasma of treated chicks

Table 1. Atrazine LD_{50} and toxic symptoms in chicks

Measurements	Result
Atrazine LD50	1435.25 mg/kg orally
Doses range	750–1500 mg/kg
First dose	750 mg/kg
Last dose	1250 mg/kg
Up and down dose	250 mg/kg
No. of chicks	7 (OOOXOXO)
Onset of toxic symptoms	29–40 minutes

O: a chick still alive during 24 hours, X: a chick dead during 24 hours.

a) The effects of 40% of LD_{50} of atrazine after 4 and 24 hours of treatment on glucose, AST, and ALT levels

The oral dosing of atrazine at 574 mg/kg causes a significant increase in AST, ALT, and glucose levels in the plasma of treated chicks after 24 hours compared with the control group and with a treated group at 574 mg/kg after 4 hours (Table 3).

b) The effects of 60% of LD_{50} (861 mg/kg) of atrazine after 4 and 24 hours of treatment on glucose, AST, and ALT levels

The oral dosing of atrazine at 861 mg/kg after 24 hours causes a significant increase in AST, ALT, and glucose levels in the plasma compared with the control group, and with the treated group at 861 mg/kg after 4 hours (Table 4).

4. Neurobehavioral effects of low doses of atrazine in chicks

After the end of the second week of the treatment, chicks were subjected to neurobehavioral tests and behavior changes were measured, which were represented by a significant prolongation in time spent by the chick to move from the center square

Table 2. Acute toxic symptoms induced by atrazine doses of 1300 mg/kg and 1000 mg/kg PO in chicks

Symptoms of toxicosis	1300 mg/kg %	1000 mg/kg %
Depression	100%	100%
Dyspnea	100%	100%
Ataxia	100%	100%
Defecation	100%	100%
Lacrimation	100%	100%
Salivation and nasal discharge	60%	20% *
Tremor	40%	10% *
Convulsions	40%	10% *
Recumbence	40%	10% *
Dead after 24 hours	40%	10% *
Onset of symptoms time	31.8 ± 0.71 minutes	36.8 ± 0.67 minutes*

Values are mean ± SE for 10 chicks /group.
* The value significantly different from the group treated with 1000 mg/kg atrazine at $p < 0.05$.

Table 3 Plasma levels of AST, ALT, and glucose of atrazine at a dose of 574 after 4 h and 24 h

Treatments	AST activity IU/L	ALT activity IU/L	Glucose level mg/dL
Control group (distilled water)	106.55 ± 1.7	78.98 ± 3.16	163.12 ± 4.6
Atrazine at a dose of 574 mg/kg after 4 h	137.14 ± 2.02 *a	123.88 ± 5.38 *a	222.95 ± 4.75 *a
Atrazine at a dose of 574 mg/kg after 24 h	143.57 ± 2.55*	155.29 ± 3.38 *	304.76 ± 5.12*

Values are mean ± SE for 7 chicks /group.
* The value significantly different from the control group at $p < 0.05$.
a the value is significantly different compared with the same dose with 24 h at $p < 0.05$.

Table 4. Plasma levels of AST, ALT, and glucose of atrazine at a dose of 862 mg/kg after 4 h and 24 h

Treatment	AST activity IU/L	ALT activity IU/L	Glucose level Mg/dL
Control group (distilled water)	108.79 ± 1.58	78.98 ± 3.16	164.88 ± 3.5
Atrazine at a dose of 861 mg/kg after 4 h	150.38 ± 1.59*	183.53 ± 3.40 *	263.12 ± 4.72 *
Atrazine at a dose of 861 mg/kg after 24 h	177.17 ± 5.03* a	237.48 ± 11.39 * a	371.25 ± 12.88 * a

Values are mean ± SE for 7 chicks /group.
* The value significantly different from the control group at $p < 0.05$.
a – the value significantly different compared with the same dose with 4 h at $p < 0.05$.

Table 5. Neurobehavioral measures of 71.75 mg/kg, 143 mg/kg and 287 mg/kg of atrazine

Variables	Treatments			
	Control	71.75 mg/kg	143 mg/kg	287 mg/kg
Latency (seconds)	5.71 ± 0.77	19.85 ± 2.34*	34.71 ± 2.46*[a]	38.29 ± 2.31*[a]
Squares crossed / 3min	15.0 ± 0.92	17.0 ± 1.35	22.42 ± 2.44*[a]	27.0 ± 1.96*[a]
Jumping times / 3 min	0.28 ± 0.18	0.57 ± 0.29	1.57 ± 0.61*	0.85 ± 0.34
Pecking scores / 3 min	2.00 ± 0.3	1.5 ± 0.22*	1.28 ± 0.18	1.57 ± 0.42*
Vocal scores / 3 min	1.42 ± 0.2	1.85 ± 0.34	2.0 ± 0.37[a]	1.71 ± 0.35[ab]
Defecation / 3 min	1.29 ± 0.28	0.57 ± 0.29	0.71 ± 0.28	0.29 ± 0.18*
Tonic immobility (seconds)	4.0 ± 0.43	14.57 ± 1.63*	32.85 ± 2.66*[a]	51.57 ± 3.94*[ab]
Chick weight at end of the experiment	469.6 ± 27.2	350.0 ± 26.9*	326.0 ± 12.7*	335.5 ± 12.9*

Values are mean ± SE for 7 chicks /group.
* The value significantly different from the control group at $p < 0.05$.
a – the value significantly different from the group treated with 5% of LD_{50}.
b – the value significantly different from the group treated with 10% of LD_{50}.

of the arena (latency) in comparison with the control group and with other groups treated with atrazine at 71.75 mg/kg, 143 mg/kg and 287 mg/kg. The open-field activity showed a significant increase in squares crossed compared with the control group and with 5% of the LD_{50} treated group. The jumping time shows a significant increase for the group treated with 10% of LD_{50} in comparison with the control group. The defecation time decreased only in 20% of the LD_{50} treated group compared with the control group. The tonic immobility time was significantly prolonged in comparison with the control group, and with 5% and 10% of the LD_{50} treated groups. The chick weight was decreased significantly in comparison with the control group.

Discussion

Atrazine is one of the widely used herbicides in Iraq and many countries of the world. Due to the lack of behavioral toxicological assessment of atrazine toxicity in poultry, our current study revealed the neurobehavioral and biochemical toxicity in chicks. The median lethal dose of atrazine was 1435.25 mg/kg orally with the appearance of toxic symptoms, which were represented by depression, dyspnea, salivation, lacrimation, defecation, tremors, convulsions, recumbency and eventually death at a high toxic dose. Based on our current results, atrazine is a moderately toxic herbicide, which is in agreement with the guidelines of British Columbia Ministry of Agriculture (2017).

The significantly higher liver marker (ALT and AST) activities in chicks dosed with atrazine at 571 mg/kg and 861 mg/kg orally are attributed to the leaking of AST enzymes from injured liver cells when compared with the control group. These findings are consistent with those of other investigations (Campos-Pereira et al., 2012; Fowler et al., 2012). Because ALT is found mostly in the cytosol of the liver and in modest amounts in other tissues, it is regarded to be more selective for hepatic damage (Zilva et al., 1988). The increase in ALT in the blood plasma of the current investigation was explicitly ascribed to the harmful effect of atrazine on liver cells (Campos-Pereira et al., 2012; Fowler et al., 2012). The increased serum AST is thought to be caused by mitochondrial dysfunction. Atrazine may induce damage by the reactive oxygen species (Grasiela et al., 2012). Our research with chicks given 574 mg/kg and 861 mg/kg of atrazine orally after 4 and 24 hours showed a significant increase in glucose level plasma. The hepatotoxic action of atrazine, which inhibits the activity of important glyconeogenesis enzymes such as hexokinase, glycogen synthase, and glucokinase, could be responsible for this effect (Curic et al., 1999; Gluzczak et al., 2006). The decrease in the body weight

of the chicks in our study is in accordance with other findings (Gluzczak et al., 2006; Dinesh et al.,2014) on fish and mice exposed to ATZ. They noticed a decrease in glycogen and an increase in lipids of the liver (Gluzczak et al., 2006; Dinesh et al., 2014). Current findings provide evidence that exposure to low doses of atrazine in chicks can cause behavioral alteration, which is represented by a significant delay in the latency to move in the open–field arena, which could be attributed to the effect of the toxicant on the curiosity of the chick to move in the novel environment, however, the chicks manifested hyperactivity which could have been resulted from a direct effect of toxicants on the CNS (Belloni et al., 2007; 2011). The disturbances in the neurobehavioral activity of atrazine–treated chicks could be due to the ability of atrazine to induce neurochemical changes in the brain regions (Giusi et al., 2006; Walters, 2014).

Conclusion

This study used chicks as a model of poisoning that we explored recently to further elucidate the acute toxic action of atrazine. Our results show that atrazine had toxic effects in a chick model represented by LD_{50}, liver enzyme activity, neurobehavioral test, and body weight. Further studies are needed to explain the precise neurotoxic mechanism of atrazine.

Conflict of interest

There are no conflicts of interest declared by the author.

Acknowledgment

The University of Mosul in Mosul, Iraq and the College of Veterinary Medicine collaborated on this study.

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Received 8 February 2022
Accepted 30 May 2022

The Fatty Acids Profile of Intramuscular Fat in the Muscle Tissue of Large White and Landrace Pig Breeds

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Keywords: *pigs, muscle tissue, fatty acids, iodine value, atherogenic index.*

Abstract. *The purpose of this study was to compare the fatty acid profile of intramuscular fat (IMF) in semimembranosus, longissimus dorsi, ventral serratus, rectus abdominis, costal part of the diaphragm, and trapezius muscles in Large White and Landrace pigs.*

Samples were taken from pair carcasses of pigs at the Taurian Bacon slaughterhouse of ZAO "Freedom Farm Bacon", Kherson. The fatty acid composition of the muscles of the Large White and Landrace pig breeds is similar to each other, especially in m. longisimus dorsi. The greatest differences in fatty acid composition at the interbreed level were noted only for m. semimembranosus ($p > 0.05$, difference is not statistically significant). The lack of statistically significant differences in the composition of fatty acids in different muscles may be due to the insufficient sample size and genetic relatedness of the selected breeds. The largest, statistically significant differences were found in the content of fatty acids in the muscles of different groups, which is associated with the peculiarities of their structure and metabolism. Meat of Large White and Landrace pigs, grown in ZAO "Freedom Farm Bacon" in terms of iodine value met the requirements of processing technology, and the ratio of PUFA/SFA in muscle tissues exceeded the recommended norms by 2 times with overestimated values of the atherogenic index in m. semimembranosus.

Introduction

Animal muscle tissue is the main human food, due to the high content of complete proteins, essential amino acids, fats, vitamins A, E, D, F and a number of trace elements. Pork and products made from it are traditionally used as food by the population living on the territory of Ukraine, which is an important element of national food traditions (Makedonskyi & Babaiev, 2009). At present, the requirements of the modern market are not only related to the sale of products of high palatability and manufacturability, but also specific properties related to human health, in which the content and the profile of fatty acids (FA) are important factors.

Animal fats (with the exception of fish oil), including pig lard, generally contain a higher proportion of saturated fatty acids (SFA) than most vegetable oils, which are predominantly unsaturated (UFA) fatty acids. It has been shown that excessive consumption of SFA in humans is associated with an increase in low-density lipoprotein cholesterol and can potentially lead to obesity, insulin resistance, liver steatosis, the development of coronary heart disease and oncological diseases (colon, breast and prostate cancer) (Rubio, Martinez et al., 2008). On the contrary, the introduction of a balanced amount of UFA into the daily diet reduces the risk of developing atherosclerosis and coronary heart disease (Picklo, Idso et al., 2017). Therefore, an important factor in assessing the dietary value of meat products is to determine the balance of saturated and unsaturated fatty acids UFA/SFA, as well as to calculate the potential health risk, i.e., the atherogenic index (AI), which takes into account those fatty acids that affect changes in cholesterol levels (Stajića, Zivkovića et al., 2011).

The process of obtaining "dietically healthy meat" is possible by introducing unsaturated fatty acids of plant origin into the diet of pigs (Coates & Ayerza, 2009). However, significant problems arise in the processing of such products, since negative correlations have been established between a high content of UFA and organoleptic indices of meat, its shelf life and other technological parameters (Bryhni, Kjos et al., 2002).

The main standard for a comprehensive assessment of the meat products quality is the chemical composition of *m. longissimus dorsi* in animals. With the development of industrial technologies for pork processing, changes in the quality parameters of meat (decrease in the content of intramuscular fat, marbling of meat) in connection with selection aimed at reducing the lard content pig carcasses, it has become necessary to study the quantitative and qualitative composition of fatty acids of various muscles in pigs (Kouba, Enser, et al., 2003; Puig-Oliveras, Ramayo-Caldas, et al., 2014). In its turn, the amount and composition of subcutaneous and intramuscular fat (IMF) and fatty acids (FA) are important characteristics for the quality indices of processed and fresh meat products (Zappaterra, Gioiosa, et al., 2021).

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However, the composition of fatty acids in different groups of animal muscle tissue differs significantly in different species (monogastric, ruminant, poultry) (Smith, Fletcheer et al., 1993; Wood, Enser et al., 2008). For pigs, the fatty acid composition of different muscle tissues has been relatively studied, but the results obtained are very controversial (Zappaterra, Gioiosa et al., 2021). The deposition of lipids in different muscles of pigs is influenced by a whole range of factors, among which the key ones are gender, age, live weight at slaughter (Minelli, Macchioni et al., 2019), feeding and maintenance features (Puig-Oliveras, Ramayo-Caldas et al., 2014), genetic characteristics of breeds and direction of their productivity (Wood, Enser et al., 2008; Piedrafita, Christian et al., 2001).

Thus, the purpose of this study is to clarify the composition of fatty acids in various pig muscles of two breeds – Large White and Landrace – in order to select further strategies for improving carcass meatiness and palatability of meat through selection and correction of diets. Promising fields for further research are the assessment of the negative impact of fatty acid composition of different pig muscle groups on human health when they are consumed, as well as the role of artificial selection of pigs in modifying the structure and biochemical processes in their muscle tissues.

Materials and Methods

We studied the muscle tissue of pigs of two breeds – Landrace (n = 5) and a Large White breed (n = 5) of English selection (neuter boars). When choosing experimental animals, the rule of pairs of analogues was observed, and fattening pigs were kept in the same conditions (group pens) in accordance with the minimum standards for their protection. Animals were fed with standard complete feeds, taking into account the nutritional and energy requirements for fattening pigs, with the inclusion of premixes from the English company FRANK WRIGHT. Pigs were slaughtered when they reached a live weight of 96–112 kg at the age of 5.5–6 months. Samples of muscle tissues were taken from pair carcasses of pigs at the "Taurian Bacon" slaughterhouse of ZAO "Freedom Farm Bacon", Kherson. All animals were slaughtered in accordance with the technical conditions and instructions approved by the enterprise, followed by veterinary control. Pig muscles studied were *longisimus dorsi, serratus ventralis, pars costalis diafragmatis, rectus abdominis, trapezius, and semimembranosus*.

The study was carried out in accordance with the "Rules for the use and keeping of animals for experiments and other purposes".

To determine the content of fatty acids in muscle tissue, we used the method in our own developed modification. A Crystal 2000M chromatograph was used with an HP FFAP 50 m × 0.32 mm × 0.2 μm capillary column loaded with a phthalates-modified PEG-20M stationary phase. Carrier gas was nitrogen, column temperature was 210°C, detector temperature was 250°C, and evaporator temperature was 220°C. To identify the results obtained, substances with a known fatty acid composition were used, namely butter and coconut oil, sunflower oil and methyl stearate. Quantification was performed using internal normalization.

The sum of all components' peaks in the test sample was taken as 100%. To extract lipids, muscle tissue was crushed and dried to an air-dry state. A sample weighing 0.5 g was taken, 3 mL of hexane was added, thoroughly shaken for 2 minutes, and the mixture was heated to boiling. It was centrifuged for 5 minutes at 2000 rpm. 2 mL of the extract was decanted, 0.5 mL of 10% sodium methoxide was added in methanol and shaken vigorously for 2 minutes. After settling (5 min), a sample of the upper transparent layer with the volume of 3 μL was taken, which was introduced into the chromatograph evaporator for analysis using a microsyringe.

Fatty acids were divided into the following categories: saturated fatty acids (SFA), unsaturated fatty acids (UFA), including monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA).

Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) with Excel software. The statistical characteristics of the sample (arithmetic mean – M, standard error – SE) were calculated using the Excel software. Significance was determined at $p < 0.05$ and $p < 0.01$.

The content of fatty acids in various muscles of pigs associated with the formation of cholesterol when consumed by humans was calculated using the atherogenic index (AI) according to the formula (Vesely, Krizova et Al., 2009):

$$AI = \frac{C12 + 4 \times C14 + C16}{\sum UFA}$$

where: UFA – unsaturated fatty acids; C12 – lauric acid; C14 – myristic acid, C16 – palmitic acid.

The mean values of the fatty acids' content were used in the calculations. The ratio of polyunsaturated and saturated (PUFA/SFA), unsaturated and saturated (UFA/SFA) fatty acids was determined for each of the studied pig muscles of both breeds.

The iodine value (IV) of intramuscular fat in each of the studied pig muscles was calculated by the formula (Lo Fiego, Minelli et al., 2016):

$$IV = 85.703 + [C14:0] \times 2.740 - [C16:0] \times \times 1.085 - [C18:0] \times 0.710 + [C18:2] \times 0.986,$$

where C14:0 is myristic acid, C16:0 is palmitic acid, C18:0 is stearic acid, C18:2 is linoleic acid.

Results

When comparing the results of the analysis on the percentage of fatty acids in various muscles and the value of individual SFA, UFA, MUFA and PUFA in intramuscular fat (IMF) of large white pigs, a significant trend was found (Table 1).Thus,

Table 1. Fatty acids content of the different muscles of Large White pig breed (means ± SE, n = 5)

Fatty acids	*m. longisimus dorsi*	*m. serratus ventralis*	*pars costalis diafragmatis*	*m. rectus abdominis*	*m. trapezius*	*m. semimem-branosus*	Significance
C 8:0	0.034 ± 0.007	0.049 ± 0.001	0.087 ± 0.008	0.071 ± 0.001	0.059 ± 0.001	0.094 ± 0.008	**
C 10:0	0.250 ± 0.041	0.194 ± 0.001	0.221 ± 0.014	0.260 ± 0.001	0.227 ± 0.002	0.357 ± 0.010	**
C 12:0	0.146 ± 0.018	0.122 ± 0.001	0.129 ± 0.005	0.162 ± 0.001	0.132 ± 0.001	0.381 ± 0.041	**
C 14:0	2.015 ± 0.175	1.771 ± 0.002	1.723 ± 0.016	2.062 ± 0.001	1.892 ± 0.002	2.596 ± 0.026	**
C 16:0	30.669 ± 1.500	27.911 ± 0.057	28.868 ± 0.150	30.471 ± 0.020	28.472 ± 0.029	34.892 ± 0.143	NS
C 18:0	11.026 ± 1.037	13.709 ± 0.011	17.061 ± 0.030	13.438 ± 0.034	12.461 ± 0.017	8.861 ± 0.052	**
C 20:0	0.094 ± 0.042 (n = 2)	0.112 ± 0.007	0.115 ± 0.000 (n = 1)	–	0.095 ±0.005	–	NS
SFA	44.176 ± 1.412	43.868 ± 0.062	48.112 ± 0.160	46.463 ± 0.035	43.337 ± 0.023	47.180 ± 0.142	NS
C 16:1	5.233 ± 0.540	3.444 ± 0.006	2.691 ± 0.030	3.648 ± 0.020	4.167 ± 0.006	5.875 ± 0.099	**
C 18:1	46.039 ± 1.114	44.085 ± 0.007	40.291 ± 0.157	41.771 ± 0.030	45.649 ± 0.038	43.075 ± 0.145	NS
C 18:2	4.140 ± 0.388	7.530 ± 0.021	8.278 ± 0.073	7.366 ± 0.010	6.078 ± 0.026	3.566 ± 0.206	**
C 18:3	0.384 ± 0.087 (n = 3)	0.500 ± 0.006	0.533 ± 0.006	0.438 ± 0.0063	0.322 ± 0.003	–	*
C 20:1	0.452 ± 0.042 (n = 2)	0.573 ± 0.039	0.479 ± 0.000 (n = 1)	0.313 ± 0.029	0.446 ±0.024	–	**
UFA	55.824 ± 1.412	56.132 ± 0.062	51.888 ±0.160	53.537 ± 0.035	56.663 ± 0.023	52.820 ± 0.142	NS
MUFA	51.724 ± 1.018	48.102 ± 2.524	46.39 ±1.884	45.732 ±1.980	50.511 ± 3.545	48.950 ± 2.431	NS
PUFA	4.524 ± 0.472	8.030 ± 0.415	8.811 ± 0.626	7.804 ± 0.698	6.400 ± 0.557	3.566 ± 0.206	**
UFA/SFA	1.263	1.280	1.078	1.152	1.307	1.120	
PUFA/SFA	0.102	0.183	0.183	0.168	0.148	0.076	
IV	54	58	55	56	57	52	
AI	0.70	0.63	0.70	0.73	0.64	0.86	

NS: non-significant differences ($p > 0.05$).
* Mean value in rows differ at $p \leqslant 0.05$.
** Mean value in rows differ at $p \leqslant 0.01$.

for saturated fatty acids (SFA) within the compared muscles, the difference was statistically significant ($p \leqslant 0.01$), with the exception of palmitic acid, the value of which ranged from 27.911% in *m. serratus ventralis* up to 34.892% in *m. semimembranosus*. In animals of the Large White breed, no significant ($p > 0.05$) differences were found in the content of C20:0, C18:1, SFA, UFA and MUFA in the studied muscles. The results showed a statistically significant difference between the content of other fatty acids in the studied muscle groups ($p < 0.05$). The highest content of saturated fatty acids (C8:0, C10:0, C12:0, C14:0) was observed in *m. semimembranosus* and was statistically significantly different from other muscles ($p < 0.01$). The largest amount of stearic acid (C18:0) was noted in the *pars costalis diafragmatis* at the level of 17.061%, with the minimum value of this parameter in m. *semimembranosus* – 8.861% ($p < 0.05$).

The largest contribution to the value of monounsaturated fatty acids in intramuscular pork fat is made by oleic acid, the values of which for the Large White breed ranged from 40.291% pars *costalis diafragmatis* to the maximum of 46.039% in *m. longisimus dorsi* ($p > 0.05$). For fatty acid 20:1, the differences in values in the muscles of different groups were statistically significant ($p \leqslant 0.01$) and ranged within 0.313–0.573% with the complete absence of its content in *m. semimembranosus*. Statistically significant differences in the concentration of polyunsaturated fatty acids – linoleic (C18:2) and linolenic (C18:3) – were noted within the compared muscle groups ($p \leqslant 0.01$, $p < 0.05$, respectively). We separately note the absence of the important essential linolenic acid in *m. semimembranosus* of Large White pig breed.

The highest concentration of polyunsaturated fatty acids (PUFA) necessary for human health was found in the *pars costalis diafragmatis* – 8.811%, while the most optimal values of PUFA/SFA and AI were found in *m. serratus ventralis* – 0.183% and 0.626%, respectively. It should be noted that intramuscular fat m was characterized by the highest absolute value of the atherogenicity index in *m. semimembranosus*, i.e. 0.864, with the lowest iodine value among the studied muscle groups being 52.183.

Table 2 shows the percentage of fatty acids in various muscles and the proportions of individual SFA, UFA, MUFA and PUFA in intramuscular fat (IMF) of Landrace pigs.

As a result of the analysis of the results obtained, in most cases, similar patterns were observed in the absence of significant ($p > 0.05$) differences in the content of C16:0, C20:0, C18:1, SFA, UFA and MUFA in the studied muscles of Landrace pigs. The highest content of saturated fatty acids (C8:0, C10:0, C14:0 at $p < 0.01$, C12:0 at $p < 0.05$) was observed in *m. semimembranosus*. The maximum C18:0 values were also found for *pars costalis diafragmatis* at the concentration similar to that of the Large White muscle, 17.119%.

The nature of the distribution of monounsaturated fatty acids in different muscle groups of Landrace animals was somewhat different in the absence of statistically significant patterns: the maximum value of oleic acid (C18:1) was found in *m. serratus ventralis* (45.086%) with the minimum of this index for *m. semimembranosus* – 41.228%. By analogy with the Large White breed, in *m. semimembranosus* landrace C20:1 was not found.

It should be noted that within the compared muscles, the content of the essential polyunsaturated fatty acid C18:3 differed statistically significantly ($p < 0.01$), and its highest concentration, 0.795%, was recorded in *m. semimembranosus* of Landrace pigs in the absence of this fatty acid in the similar muscle in individuals of the Large White breed ($p > 0.05$).

When comparing the calculated UFA/SFA, PUFA/SFA, AI indices for different muscles in representatives of the Large White and Landrace breeds (Fig. 1), similar

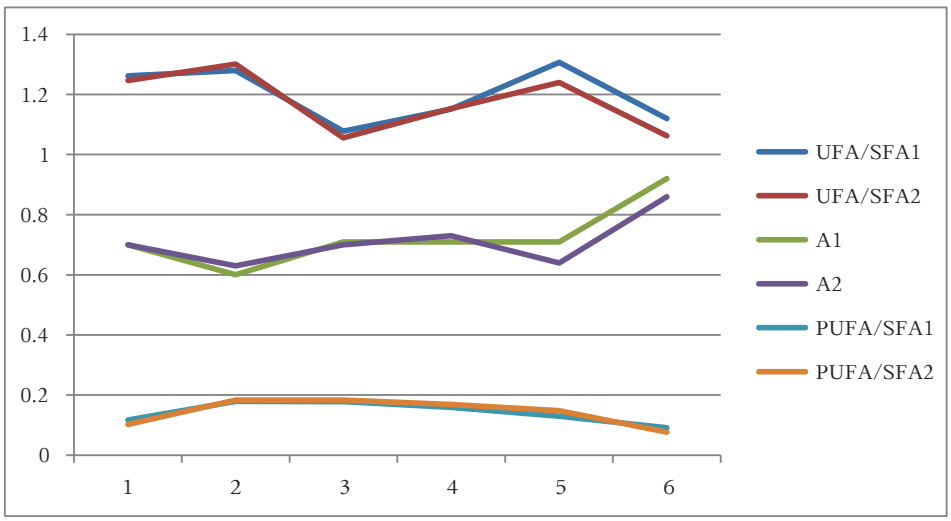

Fig. 1. Comparative characteristics of the values in the indices UFAs/SFA, PUFAs/SFA, AI in various muscles of the Large White (1) and Landrace (2) animals. Along the abscissa: 1. *m. longisimus dorsi*; 2. *m. serratus ventralis*; 3. *pars costalis diafragmatis*, 4. *m. rectus abdominis; 5. m. trapezius*; 6. *semimembranosus*.

Table 2. Fatty acids content in different muscles of Landrace pig breed (means ± SE, n = 5)

Fatty acids	*m. longisimus dorsi*	*m. serratus ventralis*	*pars costalis diafragmatis*	*m. rectus abdominis*	*m. trapezius*	*m. semimem-branosus*	Significance
C 8:0	0.041 ± 0.014	0.051 ± 0.001	0.055 ± 0.001	0.053 ± 0.001	0.081 ± 0.001	0.100 ± 0.002	**
C 10:0	0.248 ± 0.061	0.167 ± 0.001	0.169 ± 0.001	0.213 ± 0.002	0.260 ± 0.002	0.458 ± 0.007	**
C 12:0	0.166 ± 0.046	0.106 ± 0.001	0.108 ± 0.001	0.130 ± 0.001	0.145 ± 0.001	0.223 ± 0.033	*
C 14:0	2.087 ± 0.204	1.594 ± 0.002	1.655 ± 0.003	1.935 ± 0.002	1.961 ± 0.006	2.704 ± 0.030	**
C 16:0	30.479 ± 1.390	27.535 ± 0.022	29.469 ± 0.022	30.299 ±0.022	30.379 ± 0.037	36.254 ± 0.285	NS
C 18:0	11.427 ± 1.368	13.851 ± 0.010	17.119 ± 0.009	13.812 ± 0102	11.821 ± 0.020	8.924 ± 0.146	**
C 20:0	0.158 ± 0.047 (n = 2)	0.149 ± 0.006	0.119 ± 0.009 (n = 3)	–	–	–	NS
SFA	44.511 ± 0.795	43.452 ± 0.023	48.647 ± 0.015	46.442 ± 0.032	44.647 ± 0.041	48.663 ± 0.411	NS
C 16:1	5.148 ± 0603	3.115 ± 0.014	2.553 ± 0.007	3.483 ± 0.023	4.301 ± 0.006	5.670 ± 0.049	**
C 18:1	44.982 ± 0.535	45.086 ± 0.017	39.735 ± 0.050	42.320 ± 0.034	44.995 ± 0.052	41.228 ± 0.376	NS
C 18:2	4.869 ± 0.701	7.345 ± 0.011	8.131 ± 0.005	6.983 ± 0.020	5.418 ± 0.025	3.645 ± 0.094	**
C 18:3	0.347 ± 0.080 (n = 4)	0.441 ± 0.009	0.507 ± 0.008	0.408 ± 0.013	0.342 ± 0.009	0.795 ± 0.070	**
C 20:1	0.530 ± 0.138 (n = 2)	0.561 ± 0.048	0.427 ± 0.038	0.363 ± 0.011	0.296 ± 0.032	–	**
UFA	55.489 ± 0.795	56.548 ± 0.023	51.353 ± 0.015	53.558 ± 0.032	55.353 ± 0.041	51.676 ± 0.411	NS
MUFA	50.660 ± 0.457	48.762 ± 2.509	42.695 ± 2.605	46.166 ± 2.050	49.592 ± 1.534	46.898 ± 1.486	NS
PUFA	5.216 ± 0.761	7.786 ± 0.173	8.638 ± 0.688	7.391 ± 0.464	5.760 ± 0.232	4.440 ± 0.435	**
UFA/ SFA	1.247	1.301	1.056	1.153	1.240	1.062	
PUFA/SFA	0.117	0.179	0.178	0.159	0.129	0.091	
IV	55	58	54	55	55	51	
AI	0.70	0.60	0.71	0.71	0.71	0.92	

NS: non-significant differences ($p > 0.05$).
* Mean value in rows differ at $p \leqslant 0.05$.
** Mean value in rows differ at $p \leqslant 0.01$.

trends were noted: in terms of the UFA/SFA ratio, the obtained values almost coincided even in absolute mathematical values. Some differences were found only in PUFA/SFA and AI for *m. semimembranosus*, in breeds Large White and Landrace; however, the difference between them was not statistically significant ($p > 0.05$). It should be noted that the atherogenic index had the maximum value for this particular muscle (0.864% and 0.915% for the Large White and Landrace, respectively). The most optimal ratio of PUFA/SFA of the compared muscle groups of two breeds – Large White and Landrace – in terms of the degree of negative impact on the health of consumers was noted by us for *m. serratus ventralis* (0.183% and 0.179%, respectively).

Discussion and Conclusions

Animal fat and fatty acids, both in adipose tissue and in muscles, make an important contribution to various aspects of meat quality and play a central role in its nutritional value. A sufficiently proven fact is the established correlation between the content of fatty acids in the diet and the deposition of fat in monogastric animals, which include pigs. Thus, the technological and palatability of meat can be controlled by saturating pig diets with unsaturated vegetable fats and antioxidants (Wood, Enser et al., 2008). The exploration of fatty acids composition in different muscle groups of pigs in this study showed that their distribution is significantly different, especially in SFA, with the exception of the dominant C16:0. The content of fatty acids in *m. longissimus dorsi* was the same ($p > 0.05$) in pigs of the compared breeds. In this study, the concentration of caprylic acid (C8:0) in *m. serratus ventralis* and *m. trapezius*, arachidic acid (C20:0) in *m. serratus ventralis* and capric acid (C10:0) in *m. semimembranosus* ($p < 0.05$) was significantly higher in Landrace pigs than in Large White ones. However, the content of some fatty acids in *m. serratus ventralis* (C12:0, C20:1), *pars costalis diafragmatis* (C8:0, C10:0, C16:1), *m. rectus abdominis* (C8:0, C10:0), *m. trapezius* (C20:1) and *m. semimembranosus* (C12:0) was higher in Large White pigs ($p < 0.05$).

The qualitative composition of fatty acids in some muscles of Landrace pigs differed from their distribution in Large White pigs. Arachidic acid (C20:0) and gondoic acid (C20:1) in *pars costalis diafragmatis* have been found in Landrace pigs. But arachidonic acid (20:0) was found in *m. trapezius* in Large White pigs only.

Of greatest interest was a comparative study on the distribution of polyunsaturated fatty acids in the intramuscular fat of Large White pigs and Landrace – linoleic and linolenic. As for C18:2, its content in the muscle tissue of most farm animals is insignificant, and a distinctive species feature of pigs is that this acid is obtained exclusively from the diet with subsequent deposition in subcutaneous and intramuscular fat, and as a result of our own research, its higher content is in *pars costalis diafragmatis* compared with other muscles.

α-Linolenic acid (18:3n-3) is an important essential fatty acid. Due to the low possibility of inclusion into the structure of phospholipids, its content in the muscle tissue of pigs is small and can differ significantly in different types of muscles (Wood, Enser et al., 2008). Note that the highest concentration of linolenic acid was found in the *pars costalis diafragmatis* of the Large White breed (0.533%), with its complete absence in *m. semimembranosus*, while in Landraces it was in this muscle that the maximum concentration of C18:3 was recorded – 0.795% ($p > 0.05$)

It is known that PUFA more often cause oxidative effects in muscles (Zappaterra, Gioiosa et al., 2021). Our study has shown that *m. rectus abdominis* is the most sensitive to oxidative processes in pigs of both breeds, and *m. semimembranosus* is half less susceptible to them. Thus, the uneven distribution of fatty acids that we found in various muscles of pigs can be explained from the standpoint of the metabolic hypothesis (red and white muscles). Muscles of the oxidative type (red) contain more phospholipids and cholesterol, and, accordingly, biochemical metabolism differs in different types of muscle fibers (Alasnier, Re'mignon et al., 1996).

Differences in the lipid metabolism of pig intramuscular fat can also be associated with the fact that PUFA are deposited mainly in the outer layer of the subcutaneous adipose tissue (Alasnier, Re'mignon et al., 1996; González-Domínguez, Sayago et al., 2020). It should be noted that in connection with breeding for meat, in most specialized pig breeds (Duroc, Landrace, Pietrain), there was a change in the structure of muscle fibers to their hypertrophy (changes in red fibers to white ones). At the same time, there is a change in the nature of the course of oxidative processes, especially in the muscles of the lumbar region, limbs and chest (Chizzolini, Zanardi et al., 1991). Apparently, this can explain the most significant difference in the distribution of fatty acids between the Large White and Landrace breeds in *m. semimembranosus*.

The differences in the distribution of fatty acids in different muscles of pigs of the two breeds that we noted in the course of our studies had minor differences that were not statistically significant. This phenomenon can be explained by the fact that, in the historical aspect, the Large White breed and Landrace are genealogically related, since Large White pigs were used as the mother breed in the creation of Landrace (Zeven, 1998). On the other hand, due to the intensive selection of both these breeds to reduce the lard content of carcasses, increase meatiness, growth intensity and multifetation of sows, the selection vector for the main complex of genes for quantitative traits was similar, which led to an analogy in the passage of the main biochemical processes in muscle tissues.

The quality of the adipose tissue is one of the components of quality indices of meat. One of the technological parameters of fat quality is the iodine number, which reflects the degree of its saturation with polyunsaturated fatty acids, and for most meat processing plants in the leading countries of the world, its boundary value is 70–73. Significant negative correlations were established between the iodine value and the visual color, elasticity and marbling of meat. Thus, according to the iodine value calculated in various muscles of pigs, one can judge the potential palatability of meat and the products obtained from them (Minelli, Macchioni et al., 2019).

As a result of our research, the iodine number, calculated from the ratio of fatty acids of different muscles, ranged within 52–58 for the Large White and 51–58 for the Landrace breed. At the same time, maximum IV at the most negative PUFA/SFA ratio was noted by us for *m. serratus ventralis* for pigs of both breeds. Thus, IV for the muscles of both pig breeds was significantly lower than the limiting technological value, which indicates the absence of possible defects in the quality of meat and its high ability to heat treatment without loss of taste.

In recent years, there has been a steady trend to limit the consumption of the products that can have a potentially negative impact on health. Such products, due to their high fat content, include, first of all, beef, lamb and pork. Since the most dangerous for the state of the human cardiovascular system is the consumption of products that increase the concentration of cholesterol in the blood, the negative impact of animal fats on the health of consumers can be controlled using the atherogenic index (Vesely, Krizova et al., 2009).

On average, the atherogenic index for pork is estimated at 0.6, while this index can vary significantly both in different muscle groups and in representatives of different breeds. As a result of our own study, we showed AI variation within 0.63–0.87 and 0.60–0.92 for muscles of different groups in Large White and Landrace pigs, respectively. At the same time, fat in *m. semimembranosus* and the most optimal value of the atherogenic index were calculated for *m. serratus ventralis*.

In accordance with the recommendations of nutritionists, the prevention of excess unsaturated fatty acids in food, associated with an increase in plasma cholesterol, reflects the ratio of PUFA/SFA with values of more than 0.4–0.5 (Ansorena & Astiasaran, 2004). Only pars *costalis diafragmatis* and *m. serratus ventralis* in pigs of both breeds had maximum values of PUFA/SFA in our experiment, but they were almost by 2 times lower than the recommended threshold value.

In conclusion, it is important to note that due to the increase in pork prices, for the population of most European countries, including Ukraine, the share of this meat product consumption in the diet is decreasing. Thus, the prevention of cardiovascular diseases should be aimed at balancing the diet, saturating it with fiber and antioxidants, and changing lifestyle towards increasing physical activity.

The prospect of research in the field of fat deposits control lies in the field of breeding genetics aimed at reducing the subcutaneous and visceral fat of pigs while regulating intramuscular fat to an optimal level corresponding to quality and nutritional requirements. To overcome negative genetic correlations and increase the heritability coefficient of such an important selection index as intramuscular fat, it is important to understand gene interactions at the level of biochemical metabolism of their products, primarily fatty acids.

It can be concluded that the fatty acid composition in the muscles of the Large White and Landrace pig breeds is similar to each other, especially in *m. longisimus dorsi*. The greatest differences in fatty acid composition at the interbreed level were only noted for *m. semimembranosus*.

The observed trends in the uneven distribution of individual fatty acids in different muscles of the Landrace and Large White breeds were not statistically significant, which can be explained by the insufficient number of animals in the sample, as well as the genetic relationship of the selected breeds. The largest, statistically significant differences were found in the content of fatty acids in the muscles of different groups, which is associated with the peculiarities of their structure and metabolism. Breeding for meat of Large White and Landrace pigs and the technology of their fattening at ZAO "Freedom Farm Bacon" in this experiment did not show a technological decrease in the quality of meat according to the calculated iodine value indices, but from the point of view of the consumer, it led to an unfavorable ratio of PUFA/SFA in muscle tissues and overestimated indices of the atherogenic index. The prospect of further research may be to find the best ways to improve the quality of meat, both by correcting diets and by methods of breeding improvement in pigs of specialized meat breeds.

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Received 19 April 2022
Accepted 24 May 2022

Short communication: *In vitro* Evaluation of Resistance of *Rhipicephalus* (*Boophilus*) *microplus* against Three Widely Used Ixoidicides

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Keywords: *ticks, cattle, resistance, acaricides.*

Abstract. *The deficiency of tick control causes large economic losses for the health of animals and the risk of transmission of zoonotic diseases. Their control is based on the use of acaricides such as organophosphates, synthetic pyrethroids, amidines and macrocyclic lactones; however, its inappropriate use has favoured the presence of populations resistant to the main families of acaricides. This study was conducted with the objective to evaluate the resistance of Rhipicephalus (Boophilus) microplus against three widely used ixoidicides in Nicaragua, namely coumaphos, amitraz and cypermethrin, applied in the concentrations indicated by the manufacturer. For this, the techniques of Adult Immersion Test (AIT) and Larval Package Test (LPT) were applied, for which ticks were collected from farms in Western Nicaragua. The results obtained through the AIT show that coumaphos has a higher percentage of oviposition inhibition of 86%, while cypermethrin only demonstrated an oviposition of 12.9%. Mortality in adults treated with coumaphos was 97.72%, and in those treated with amitraz it was 48.8%, while cypermethrin only provided a mortality of 14.71%. In this study, the Rhipicephalus (Boophilus) microplus ticks collected from cattle were determined to have elevated levels of resistance to cypermethrin but low resistance to coumaphos.*

Introduction

The common cattle tick *Rhipicephalus (Boophilus) microplus* is one of the most important tick species in the world, causing animal mortality and high costs related to the treatment and prevention of infectious diseases in cattle (Vargas-Cuy *et al.,* 2019). In Latin America, losses associated with the production of milk, meat and skins are reported (Bock *et al.*, 2004). During the parasitism stage in cattle, each adult tick can feed with 1 mL of blood, leading to a loss of 1 g of body weight and 8.9 mL of milk reduction (Narladkar, 2018). Ticks can cause indirect damage to cattle through the infectious agents they can transmit, primarily *Babesia bovis*, *B. bigemina* and *Anaplasma marginale*, during infestation, which can kill them (Rodriguez-Vivas *et al.*, 2018)

Synthetic acaricides are the primary method of tick control; however, the presence of ticks resistant to commercial acaricides is a major concern in areas with tropical and subtropical climates (Maya-Delgado *et al.*, 2020). Animals affected by *R. microplus* in Nicaragua are treated with knapsack sprays using pyrethroid-based acaricides and amitraz or with systemic treatments such as Fipronil and Ivermectin (Barrios *et al.*, 2022). The excessive use of conventional products and the lack of implementation of tick control strategies have promoted the development of drug resistance, a serious problem, since it leaves fewer options for its control (Canul-Ku *et al.*, 2012; Grace, 2015). If ticks are regularly exposed to chemical agents, they can develop resistance to the point that acaricides lose their effectiveness over time, and therefore the product rotation process can lead to the inability to use all active ingredients (Coles & Dryden, 2014). This study was conducted with the objective of evaluating the resistance of *Rhipicephalus (Boophilus) microplus* against three widely used ixoidicides, namely coumaphos, amitraz and cypermethrin, using the dose recommended by the manufacturing laboratory. These data will allow knowing the state of the resistance of ticks in bovines against the most widely used acaricides in Nicaragua.

Materials and methods

A cross-sectional study was carried out in a population of 471 cattle that belonged to 15 farms in western Nicaragua. From this population, a sample size of 60 cattle was calculated considering an accepted error of 10%, a level of confidence of 95% and an unknown prevalence (50%). The selection of each animal was carried out consecutively numbering the population and later a simple random sampling

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was carried out. From each selected animal, at least 5 engorged female ticks were collected.

Sample collection and transport

Engorged ticks of the species *Rhipicephalus (Boophilus) microplus* were collected between 6:00–7:00 am. The specimens were collected directly from the bovines. They were immediately placed in test tubes covered with moist cotton to provide oxygen. The samples were transported in a thermos to the parasitology laboratory of the Centro Veterinario de Diagnóstico e Investigación (CEVEDI).

Management of ticks in the laboratory

The engorged female ticks were separated to be observed with a stereoscope to carry out the taxonomic identification. Later they were washed and weighed and placed on a Petri dish. In addition, wet cotton was placed as a source of humidity, and oxygen, incubated in low light at 33°C. The ticks of the control group were incubated for 18 days until they began their oviposition (Figure 1). Once the eggs occurred, they were weighed and placed in test tubes for 21 more days until the day of hatching. The larvae obtained were placed in Petri dishes for 18 days until they reached the maturity required to apply the larval pack test (LPT).

Determination of efficacy in adults

It was carried out based on the Adult Immersion Test (AIT), using 5 repetitions per treatment in groups of 10 ticks per replica. The specimens were submerged for 3 minutes in the solutions of coumaphos (200 ppm), amitraz (200 ppm), cypermethrin (200 ppm) and water as control to each group containing 10 mL of the solutions prepared (Drummond *et al.*, 1973). They were subsequently dried and placed in Petri dishes (5.5 × 1.5 cm) to be incubated at a temperature of 27–28°C, and humidity of 70–80%. Mortality was recorded for 18 days based on three specific signs and characteristics: (i) increased darkness of the cuticle, (ii) leg without movement, when the ticks were inverted under a stereomicroscope and tested with a paintbrush and (iii) haemorrhagic skin lesions that were identified by observation under stereoscopes, as previously described (Pirali-Kheirabadi & Teixeira da Silva, 2011).

Fig. 1. Females of *R. microplus* in oviposition

Determination of efficacy in larvae

The previously described Larval Pack Test (LPT) was applied (Shaw, 1966). Solutions were prepared at the recommended concentration of 200 ppm for each acaricide; Whatman number 1 paper packets impregnated with the solutions and containing 90–100 larvae were used, and each packet was incubated at 33°C for 24 hours using 5 repetitions per group. Packages in the control group were impregnated only with distilled water. The mortality percentage was estimated by quantifying the dead larvae with the help of a stereoscope.

Statistical analysis

Percentages of mortality and oviposition in adults are described with means and their respective standard deviations (SDs). To compare the groups, the one way ANOVA F test was applied, and significant differences were considered when $p < 0.05$.

Results and discussion

Research on resistance has been scarce in Nicaragua; however, in other countries, it has been documented that the dosage and inadequate application of acaricides have increased resistance levels, mainly in cattle of small producers (Ravindran *et al.*, 2018).

In this study, significant differences (one way ANOVA, $p < 0.05$) were observed in the percentage of inhibition of oviposition in *R. microplus* when comparing the different acaricides most used in cattle farming in Nicaragua. It was found that coumaphos at a concentration of 200 ppm was the acaricide that provided a higher percentage of oviposition inhibition of 86% (SD = 8.40), although this is a lower result than that found by another investigation carried out in Mexico, in which they report a 100% inhibition of oviposition when the product is applied at a concentration of 50 ppm (Ravindran *et al.*, 2018). In the application of amitraz (200 ppm), an inhibition of 47.00% (SD = 7.60) was found, and the results were similar to those found in India, where they report inhibition of 78.35%; however, in that study, they applied a concentration of 1000 ppm (Dutta *et al.*, 2017). The inhibition of oviposition by cypermethrin was the lowest of the three acaricides studied with 12.90% (SD = 5.20), (Figure 2), a finding similar to that found in Colombia, where they found a low effectiveness of this pyrethroid for the control by *R. microplus* (Diaz-Rivera *et al.*, 2019).

The AIT revealed that mortality in adults treated with coumaphos was 97.72% (SD = 12.60), unlike

females treated with amitraz in which only a 48.8% mortality was observed, and in those treated with cypermethrin, the mortality was 14.71%. (SD = 2.80), (one way ANOVA, $p < 0.05$), (Figure 3).

The elevated level of resistance to cypermethrin in adult ticks is similar to that found in India, where the overall prevalence of pyrethroid-resistant *R. (B.) microplus* was found to be 66.6%. Cypermethrin resistance was detected in 16 areas and 96% of the *B. microplus* tick population was resistant to cypermethrin, this being the least effective product (Sharma *et al.*, 2012). The mechanism of action of pyrethroids is the modification of the sodium channels that are responsible for maintaining the nerve impulse and generate muscle paralysis (Stone *et al.*, 2014). These compounds cause membrane depolarization by slowing the gating kinetics of activation and inactivation of sodium channels, induction of a large, slow decaying tail current associated with membrane repolarization and in the delayed and prolonged opening of single sodium channels (Silver *et al.*, 2014). However, the low cost and high availability of this product in the region have increased its indiscriminate use, selective pressure and the population of *R. microplus* resistant to cypermethrin, in which it has been identified that the modification of the site acaricide binding occurs due to point mutations in domains II and III in the sodium channel-related gene (Diaz-Rivera *et al.*, 2019). However, studies have shown that pyrethroids can still show some efficacy when combined with other products, including a combination of cypermethrin, chlorpyrifos, and fenthion obtaining a 95% *in vitro* efficacy against *R. microplus* (Rodrigues *et al.*, 2018).

The LPT showed significant differences between

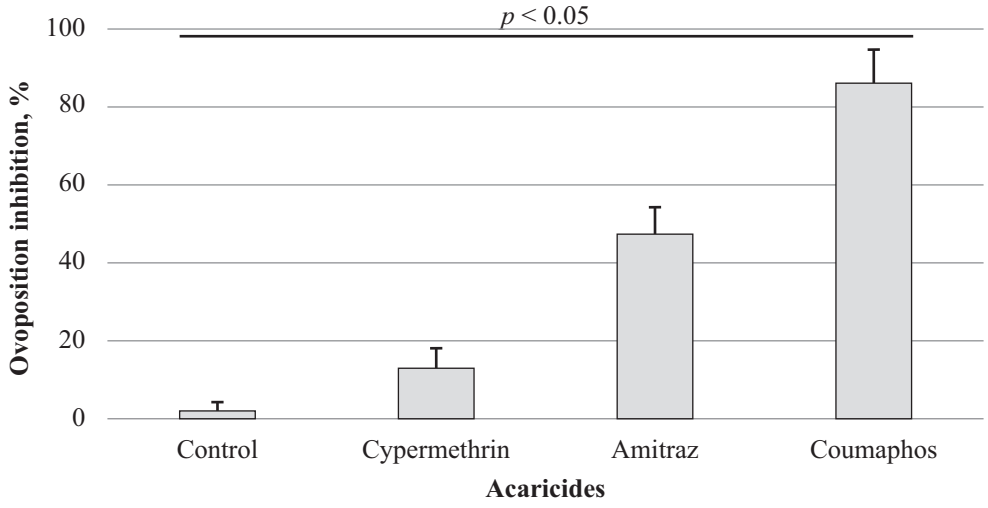

Fig. 2. Percentage of inhibition of oviposition in females of R. microplus treated with the different acaricides.
*: Significance value based on one way ANOVA test.
The upper horizontal bar represents the comparison between the four study groups.

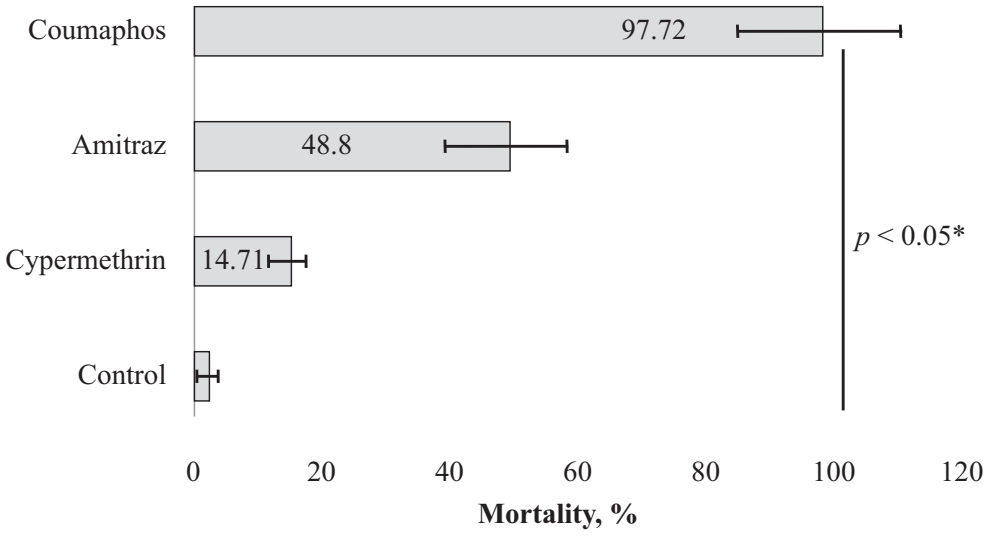

Fig. 3. Percentage of mortality in adult females of R. microplus treated with the different acaricides.
*: Significance value based on one way ANOVA test.
The vertical bar represents the comparison between the four study groups.

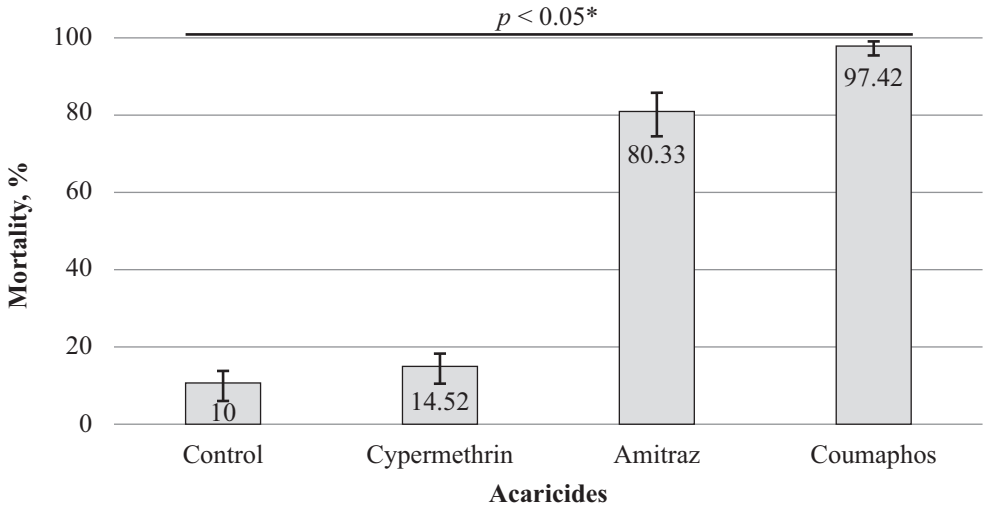

Fig. 4. Mortality percentage in R. microplus larvae treated with the different acaricides.
*: Significance value based on one way ANOVA test.
The vertical bar represents the comparison between the four study groups.

the acaricidal effectiveness (one way ANOVA, $p < 0.05$). It was found that coumaphos provides a percentage of mortality of 97.43% vs. only 14.52% observed in ticks treated with cypermethrin, with higher percentages of larval mortality in the packages impregnated with coumaphos with 97.43%, followed by amitraz with 80.33% (Figure 4). According to this study, amitraz is more effective against tick larvae than against adult ticks, so its use should be recommended during the period when tick larvae are most active in the cattle herd. The low mortality obtained with cypermethrin can be attributed to its low cost and indiscriminate use, which facilitated its rapid development in different parts of the Nicaraguan territory (Grace, 2015).

The resistance of ticks to acaricides is conferred mainly by two important mechanisms: increased activity of metabolic enzymes and genetic mutations that modify the sites of action (Rodrigues *et al.*, 2018). For example, single nucleotide polymorphism (SNP) in the first transmembrane region (TM1) of β-adrenergic-like (β-AL) has been identified in populations of amitraz-resistant *R. microplus* in Australia and indicating that this amino acid substitution could be a cause of resistance to amitraz (Corley *et al.*, 2013). However, in order to identify these mechanisms, molecular studies are required to determine the type of resistance that ticks are showing at the national level.

Conclusion

The most effective treatment against *Rhipicephalus (Boophilus) microplus* was coumaphos, with a higher control percentage compared with amitraz and cypermethrin. It was determined that coumaphos presented the highest mortality in adults, the highest percentage of inhibition of oviposition and the highest mortality in larvae.

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Received 11 April 2022
Accepted 30 May 2022

Analysis of Feed as an Ecological Factor of Influence on the Organism of a Productive Animal under Conditions of Increased Anthropogenic Load on Agroecosystems

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Keywords: *toxic metals, cadmium, lead, copper, zinc, mineral elements.*

Abstract. *Feed for farm animals is an environmental factor that has a significant impact on various physiological functions, productivity, health, and homeostasis of the body. It is the subject of research in many sciences, including ecology. Scientists in different countries of the world have begun to pay special attention to the study of the ecological safety of animal feed, which is associated with the urbanization of settlements, the development of industry, oil and gas production, accidents on main gas pipelines, mining of coal and gas, the placement of agricultural land by large cars, and livestock farms not far from the developed industrial center of metallurgical complex enterprises, etc., which leads to soil pollution with toxic heavy metals, and then migration and accumulation of elements in plants from the produced feed included in the rations. The aim of the research was to establish the quality and biological completeness of feed rations of dairy cows in enterprises located near environmentally unfavorable anthropogenic objects. The object of research included norms of rations, corn silage, alfalfa haylage, corn turf, fodder beets, grain hay, cereals and legumes hay, alfalfa hay. To conduct a scientific experiment on the production of cow's milk, plant-based feed from four farms located around an industrial city and not far from sources of anthropogenic pollution was selected. Laboratory analysis of selected average feed samples for the content of mineral elements, including toxic metals Cd and Pb, was carried out by the atomic absorption method. Statistical data processing was carried out using the STATISTICA software package version 10.0. The analysis of feed shows not only an increased concentration of heavy metals, but also a low content of vital essential macro- and microelements in plants (feed) against this background, which was typical for all farms. In such conditions, the problem of producing high-quality biologically valuable ecologically safe milk arises. Therefore, in case of local contamination of agroecosystems and ration feeds with heavy metals, especially Cd and Pb, it is necessary to take into account the actual nutritional value of feed, ensure the rationing of essential and non-essential elements in the diet and develop new feed additives, mineral and vitamin premixes of antidote action, homeostasis of an intoxicated organism, increasing the productivity of cows, improving their health status and obtaining environmentally friendly milk. Further research is aimed at constant environmental monitoring of various mineral elements, primarily toxic heavy metals in the feed of farms specializing in milk production in different regions of Ukraine, forest-steppe and Polissya.*

Introduction

Feed is the subject of research in many sciences, including ecology. The feed eaten by animals is considered as a link in the biotic circulation and as an integral part of the biogeochemical trophic chain in the agroecosystem. In a narrower essence, feed is a biogeocenosis factor that affects the animal population, species, individual, their organs, tissues, cells and subcellular structures. Many microminerals play an important role in the organism as cofactors for enzymes involved in controlling free radicals in the organism and are vital for antioxidant capacity. These same minerals, when consumed in excess, can become prooxidants in the body, generating destructive free radicals. Complex interactions between minerals can jeopardize the effectiveness of feeding in promoting health and improving performance of a dairy cow (Jesse, 2018). Today, the ecological study of animal feeding is to a certain extent based on data on feed production and feeding (trophology), since feed is the basic object of research in these sciences.

Ecology and feeding pay great attention to increasing the efficiency of bioconversion, i.e., converting the organic matter of plants into zoo mass (meat, milk, eggs, wool, etc.). In this case, migration during bioconversion of heavy metals, especially cadmium, lead, copper and zinc, plays an important role.

Scientists (Hejna et al., 2018) point to the need to develop new approaches to the ecology of animal feeding containing heavy metals and to constantly monitor environmental pollution by pollutants, especially in those industries related to animal husbandry. Controlling the intake of various mineral elements, including toxic metals from animal feed, can be an effective strategy to reduce health risks

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for humans consuming animal products, as well as soil contamination with animal waste. According to scientists (Hejna et al., 2018), legal restrictions on the content of certain pollutants in feed will also have a positive effect on their distribution in the components of the biosphere and migration in the food chain.

Feed and diet terms are certainly not identical, but they have a lot in common. Their commonality lies in the fact that both feed and diet are objects of animal feeding. During the period when dangerous toxicants of ecocidal origin such as Cd, Pb, Cu, and Zn enter the body of animals, attention to the structure of diets and their composition and nutritional value increases significantly, which is due to the production of environmentally friendly products (milk) containing xenobiotics within the established permissible levels. Therefore, experts in the animal husbandry industry (zoological engineers, veterinarians and production technologists) make up rations, balancing them in terms of energy content, dry matter, feed units, digestible protein, that is, proteins, fats, carbohydrates and other components of macro- and microelements. Scientists (Song Ren-ju et al., 2015) indicate that the relationship between micronutrient content in feed and milk can provide a theoretical basis for dairy farming, which would be beneficial to increase milk yield and quality of milk and dairy products produced.

The diet of feeding is a complex ecological factor, consisting of various substances, each of which has specific properties in terms of the nature of the impact and, therefore, is an independent ecological factor. The feed factors of the diet affect the animal organism not in isolation from each other, but in aggregate and in the corresponding interdependence, where antagonistic and synergistic properties are manifested, for example, between macro- and microelements, which is very important in case of intoxication of the body with contaminated feed that gets into the diet with time of feeding the animals.

It has been established that feed has a significant effect on animals, their productivity, quality and environmental safety of products, not to mention reproductive ability, population resistance or its sensitivity to various diseases, which may be accompanied by the consumption of feed containing toxic metals such as cadmium and lead and other elements in concentrations exceeding permissible levels. Scientists from China (Hui Wang et al., 2013) have examined 360 samples of feed and manure collected from 150 livestock farms in Jiangsu province, an area of intensive livestock farming, and analyzed them for the content of heavy metals and various mineral elements. Any food containing any chemical element above the maximum permissible concentration in the diet will serve as a limiting factor that will lead to the depression of the animal's condition and the production of low-quality products, and possibly even the disease of the animal due to loss of appetite and live weight, hypo-, hypervitaminosis and hypo-, hyper-, macro-, microelementosis, accompanied by general dystrophy, impaired hair growth, decreased skin elasticity, pallor of mucous membranes, the development of anemia, etc.

The aim of research is to establish the quality and biological value of feed, which are part of the diet of dairy cows at various agricultural enterprises located around an industrial city and near other ecologically unfavorable anthropogenic objects.

Material and methods

To conduct scientific and economic experience in the production of ecologically safe cow's milk and approbation of premixes and a biological product antidote to the action of toxic metals, samples of various forages of plant origin were taken, which were part of the main diet of animals in four commercial farms: a specialized agricultural enterprise "Druzhba", agricultural production cooperative "Khoroshkivsky", agricultural limited liability company "Svitanok", and agricultural limited liability company "Udai". Agricultural lands are located around the industrial city and near ecologically harmful anthropogenic objects of influence on agroecosystems, i.e. highways with increased traffic of motor transport Kyiv – Kharkiv – Dovzhanskyi, natural gas production fields and gas condensate enterprises, main oil and gas pipelines, enterprises for the production of asphalt concrete.

According to the ecological-meteorological-topographic scheme of research using the terrain map in Google, the Udai enterprise is located within a radius of 15.3 km from the main sources of pollution of the industrial city, "Druzhba" 8 km, "Svitanok" 13.9 km, "Khoroshkivsky" 25.7 km, respectively. Agricultural lands are located at a distance of several kilometers from the highway Kyiv – Kharkiv – Dovzhanskyi in the farms "Udai" and "Druzhba". In the farm "Khoroshkivsky", agroecosystems are located at a distance of 10 km from the gas condensate plant, and in the farms "Druzhba" and "Udai", they are near the main gas pipeline.

In the first farm, 36 heads of cows of Ukrainian black-spotted and red-spotted dairy breed with silage-root type of feeding were selected for experiment; 195 heads of cows of black-spotted dairy breed with silage-hay type of feeding were selected in the second; 63 dairy cows of the Ukrainian red-spotted dairy breed with silage-haylage type of feeding were selected in the third; and 126 heads of red-spotted breed with silage-haylage-concentrate type of feeding of cows were selected in the fourth farm.

Average samples of feed were taken according to the method generally accepted in zootechnical practice from places of storage of feed – reinforced concrete trenches (pits) with silage and haylage, storage facilities for storing hay and straw, piles with fodder beets, storage facilities for storing skins, mixed feed, etc. Sampling was carried out once in the autumn at

the beginning of the experiment and stall keeping animals. Samples of feeds included in the feeding rations of the animals in the experimental conditions were taken: cereal and bean hay, wheat straw, corn silage, alfalfa haylage, fodder beet, and corn grits in the "Druzhba" agricultural enterprise; alfalfa hay, wheat straw, corn silage, alfalfa haylage, fodder beet, and barley grits in "Khoroshkivsky"; alfalfa hay, cereal hay, corn silage, alfalfa haylage, oatmeal, and peagrass in "Svitanok"; alfalfa hay, cereal and legume hay, corn silage, alfalfa haylage, corn husk and pea husk in "Udai". The samples for the study were sent to the laboratory of the Institute of Animal Husbandry of the Ukrainian Academy of Sciences to establish their nutritional value, the content of macro- and microelements, including heavy metals.

Biochemical analysis of the samples of plant origin (feed) for the content of macro-, microelements, toxic metals, etc. was carried out by the method of atomic absorption spectrophotometry (spectrophotometer AAS-30) (Praice, 1972). The deficiency of macro- and microelements in feed was established relative to the average nutritional value of feed determined in detailed norms (Kalashnikov et al., 1985). The content of Ca, P, Mg, K, S, microelements Fe, Cu, Zn, Co, Mn, J, as well as the concentration of toxic heavy metals Pb and Cd were determined in the average feed samples among macronutrients.

Statistical data processing was carried out in the STATISTICA 10.0 software package for the Windows 7 operating system. The average values of concentrations of mineral elements in 1 kg of feed were determined.

Result

The quality and safety of livestock products, including milk, directly depends on the quality of feed and their biological usefulness. In this regard, in each of the four experimental farms, average samples of feed were taken.

The content of Cd in the feed of the first farm, included in the diet of dairy cows, exceeded the established permissible norms by an average of 2.1–3.2 times, Pb by an average of 2.4–5.7 times, Cu by an average of 1.4–2.3 and Zn by an average of 1.2–2.4 times, respectively. The greatest excess of the norm for Cd and Pb was found in cereal-legume hay (3.2 and 5.7 times), for Cu in corn turf (2.3 times), and for Zn in wheat straw (2.4 times) (Fig. 1).

The concentration of heavy metals in the feed of other experimental farms fluctuated, which was due to the different content of mobile forms of toxicants in the soil and the location of the land where the plants were grown, depending on the distance to the industrial center, highways, natural gas viewing sites, gas condensate enterprises, etc. In the fodder of the second farm, the content of Cd, Pb, Cu, and Zn in excess of the permissible norm was found in fodder beet, respectively, by 2.5, 3.4, 3.8, and 4.1 times. Plants to a greater extent accumulate heavy metals in the root system, that is, in the part that is in the soil, and slightly less pollutants enter the vegetative system; therefore, of all feeds, it was the fodder beet that had the highest level of contamination for all the studied elements in comparison with other feeds (Fig. 2).

In the feed grown on the agricultural lands of the third farm, in addition to exceeding the norm for the

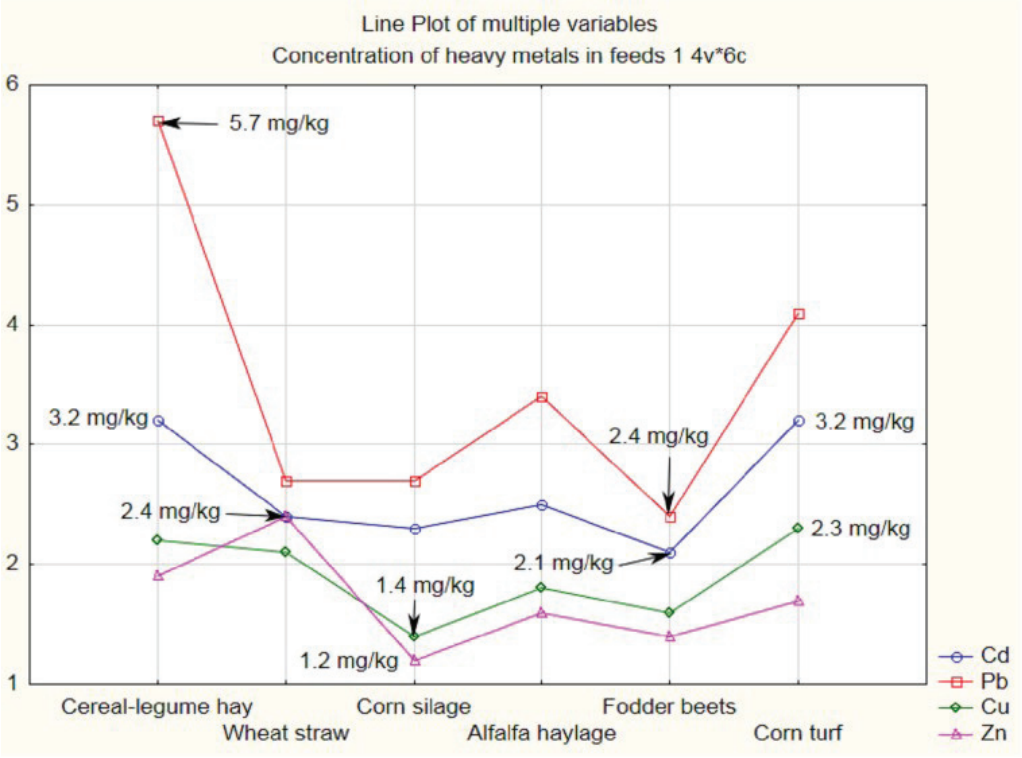

Fig. 1. The concentration of Cd, Pb, Cu and Zn in the feed of the main ration of the first tested farm, mg/kg.

content of Cd, Pb, Cu, and Zn compared with other farms, a high content of zinc was recorded in feed and, in particular, in oat and pea grain on average by 6.3–6.8 times. The highest content of cadmium and lead among other feeds was observed in corn turf, and the highest content of copper was observed in cereal-legume hay (3.9 times) (Fig. 3).

Among all four farms, the fodder of the fourth had the highest contamination of lead by 7.3 times, zinc by 7.8 times and copper by 4.1 times. In terms of feed contamination with cadmium, the farm ranks last along with the second farm. The highest content of cadmium, lead and copper among the ration feeds was found in cereal-legume hay, while corn accumulated more zinc (Fig. 4).

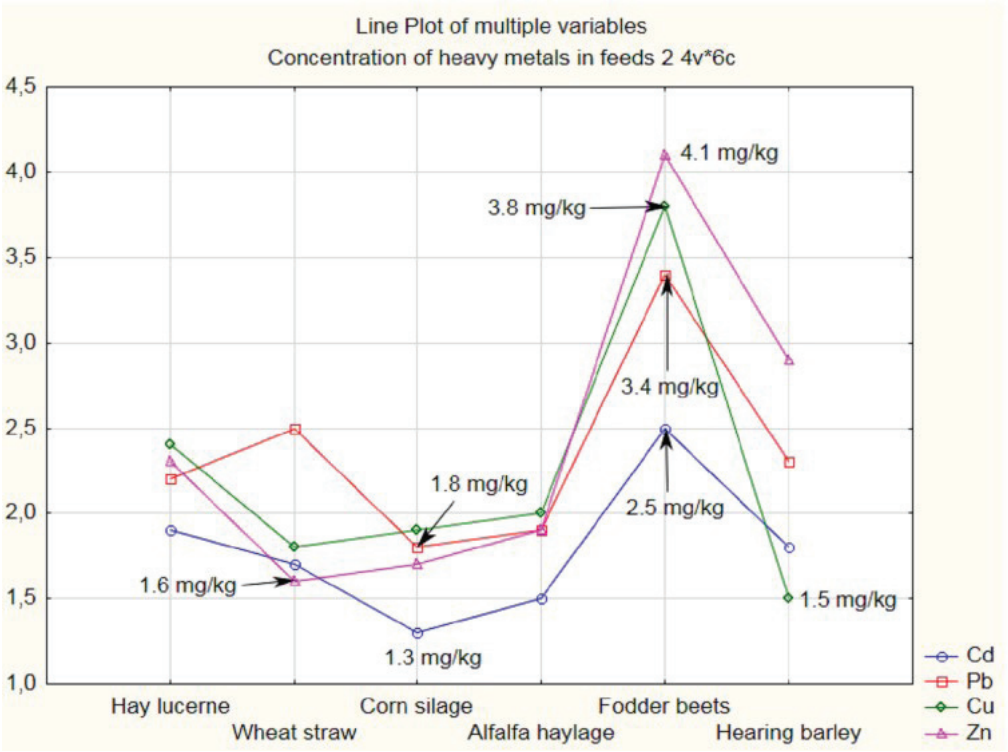

Fig. 2. The concentration of Cd, Pb, Cu and Zn in the feed of the main ration of the second tested farm, mg/kg.

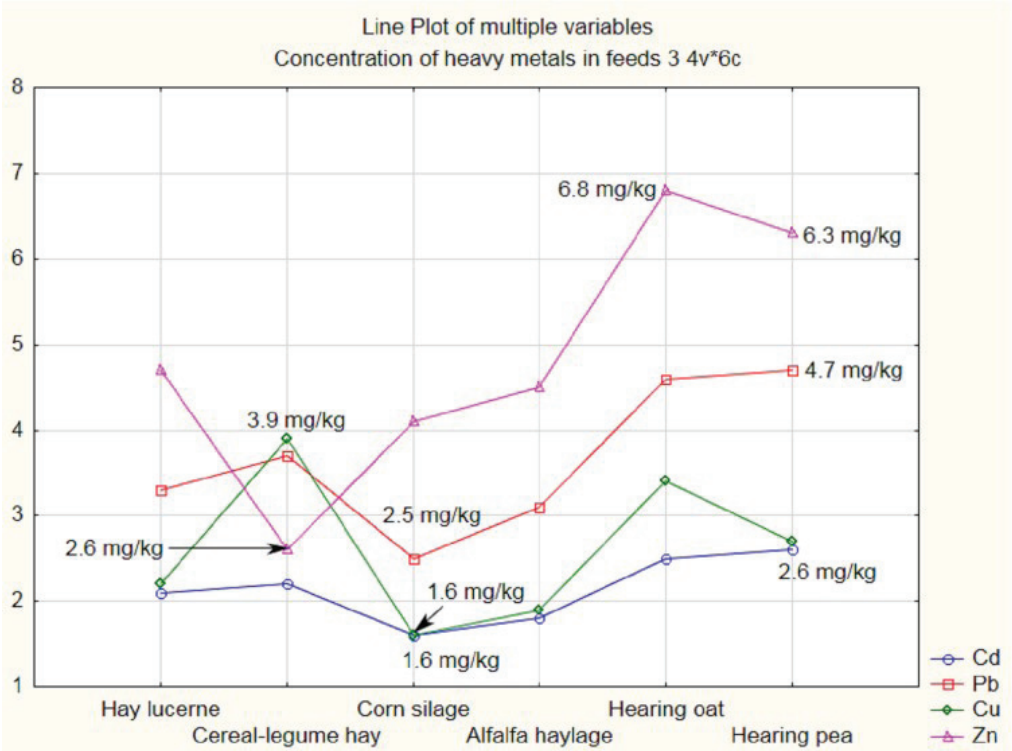

Fig. 3. The concentration of Cd, Pb, Cu and Zn in the feed of the main ration of the third tested farm, mg/kg.

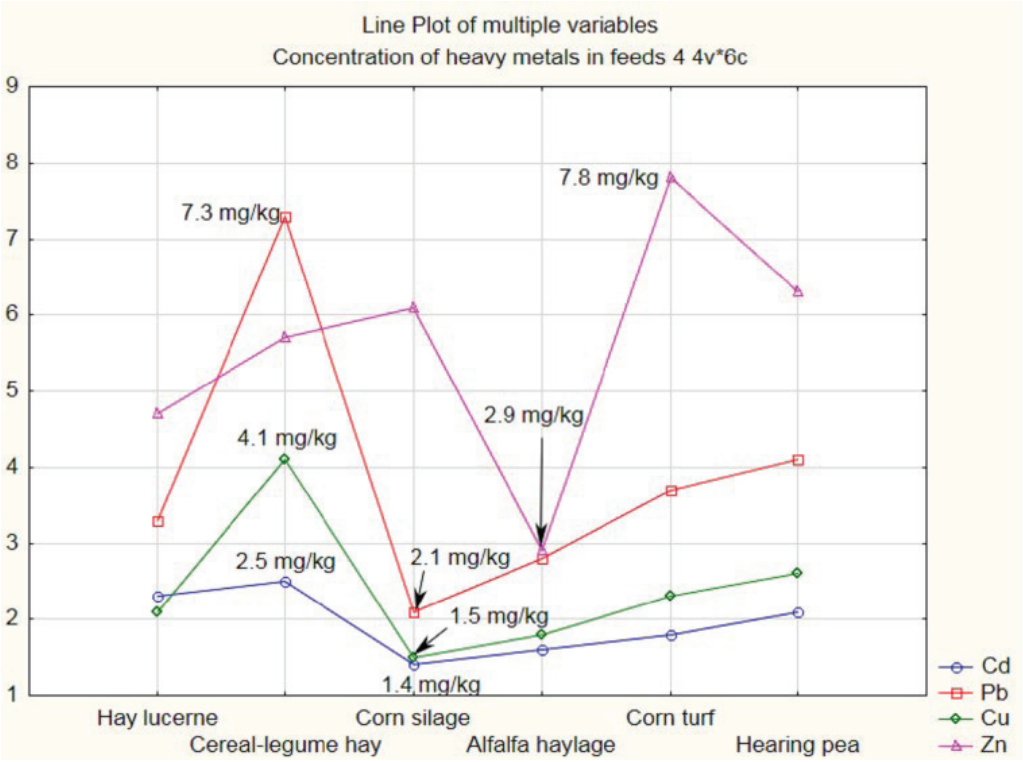

Fig. 4. Concentration of Cd, Pb, Cu and Zn in the feed of the main ration of the fourth tested farm, mg/kg.

Therefore, according to the level of fodder contamination, farms can be ranked as follows: cadmium contamination (in descending order) No. 1 → No. 3 → No. 2 → No. 4; lead contamination (in descending order) No. 4 → No. 1 → No. 3 → No. 2; copper contamination (in descending order) No. 4 → No. 3 → No. 2 → No. 1; zinc contamination (in descending order) No. 4 → No. 3 → No. 2 → No. 1. Copper and zinc are essential elements involved in various biochemical processes vital for the animal organism: hormonal, enzymatic, etc. In a certain amount, they must enter the animal's body. No less important role in the body is played by other essential macro- and microelements, and we carefully studied their content in the feed of farms.

The chemical analysis of the feed shows not only an excess of heavy metals, but also a low content of essential macro- and microelements in plants – antagonists of cadmium, lead, copper and zinc. As evidenced by the results of laboratory analysis of feed for all farms, a low content of essential elements against the background of an excess of heavy metals, especially cadmium and lead, is characteristic. In such conditions, rationing and balancing of rations becomes more difficult.

There is a problem of producing ecologically safe high-quality milk containing heavy metals within the limits of permissible concentrations, as well as containing essential elements important for the human body: calcium, phosphorus, potassium, magnesium, sulfur, iron, iodine, cobalt, vitamins, etc. bringing dairy raw materials into the category of biologically valuable products.

Discussion

Scientists from China (Hui Wang et al., 2013) analyzed a large number of feed and manure samples for heavy metals, collected from 150 livestock farms in Jiangsu Province (China), unlike in our case, where we collected samples from four farms. In the study from China, Zn and Cu concentrations in animal feed were approximately 15.9–2041.8 and 392.1 mg/kg, respectively, while Hg, As, Pb, Cd and Cr in all feeds were below 10 mg/kg. The concentrations of Cu, Zn, and Cr in animal manure were 8.4–1726 mg/kg, 39.5–11 379 mg/kg, and 1.0–1602 mg/kg, respectively, and As, Cd, Hg, and Pb were < 10 mg/kg. The concentration of Cu, Zn, As, and Cr in animal feed and manure demonstrated a positive correlation ($p < 0.001$), but Cd, Hg, and Pb did not statistically correlate with the content in feed and manure. Concentrations of Cu and Zn were highest in pig feed and manure, followed by poultry and lactating animals. During 1990–2008, the content of Cu, Zn, As, Cr, and Cd increased by 771%, 410%, 420%, 220% and 63% in pig manure, by 212%, 95%, 200%, 791% and 63% in dairy animal manure, and by 181%, 197%, 1500%, 261 and 196% in poultry manure. According to scientists, the majority of the increase occurred from 2002 to 2008, indicating the widespread use of feed additives after 2002. In contrast, the levels of Pb and Hg in manure declined steadily from 1990 to 2008. Research results indicate that the content of heavy metals in animal manure has increased significantly over more than 18 years, which will accordingly increase their entry into the soil.

Research on the content of heavy metals in feed is of interest to scientists from different countries and continents of the planet. Scientists (Nicholson et al., 1999) from England examined 183 samples of livestock feed and 85 samples of animal manure collected from commercial farms in England, including Wales. The scientists determined the content of zinc, copper, nickel, lead, cadmium, arsenic, chromium and mercury. Concentrations of zinc and copper in pig feed ranged within 150–2920 mg/kg dry matter for zinc and within 18–217 mg/kg for copper, respectively, depending on the age of the pigs. In poultry feeds, concentrations ranged within 28–4030 mg/kg for zinc and 5–234 mg/kg for copper, while laying hen feeds tended to have higher levels of heavy metals than broiler feeds. Concentrations of Zn and Cu in dairy and meat feeds of cattle were significantly lower than in feeds for pigs and poultry. Pig pus typically contained about 500 mg/kg of zinc and about 360 mg/kg of copper, indicating the metal concentration in the feed. Typical concentrations in poultry manure were 400 mg/kg of zinc and 80 mg/kg of copper, and in cattle manure, there was 180 mg/kg of zinc and 50 mg/kg of copper. The dry matter content of cattle and pig waste has been a useful indicator of the concentration of heavy metals in natural matter.

Scientists from Bashkortostan (Kuramshina et al., 2014) have studied the process of migration of heavy metals from soil to plants (feed) and animals in the area of oil and ore deposits in order to biologically indicate the state of the ecosystem and assess the environmental safety of livestock production in different agricultural areas of Bashkortostan. The authors carefully studied the central part of the republic with the dominant agricultural complex as a background area. Concentrations of elements in soil and forages were determined, coefficients of transition of heavy metals from forages to organisms of animals were established, influence of anthropogenic factors on environmental pollution by heavy metals was estimated. The conclusions made by the scientists in this situation are extremely important from a practical point of view: the more elements entered the environment, polluting agro-ecosystems, plants (feed), the more they were consumed by horses.

Scientists from China (Song Ren-ju et al., 2015) note that feed intake by productive animals plays a key role and has a significant impact on milk yield and milk quality. The researchers have also carried out a laboratory analysis of feed for the content of various mineral elements, including heavy metals, and their transfer into milk of cows. Using similar techniques, in particular, atomic absorption spectrophotometry, they have found the content of Pb, Cd, As, Cu, Mg, Ca, Fe and Zn in different feed and milk. The content of Pb, Cd, and As was determined using an AAS graphite furnace, and the content of Cu, Mg, Ca, Fe, and Zn was determined by flame atomic absorption spectrometry. Research results showed that Pb, Cd, As and Cuwere present in feed, but Pb, Cd and As were poorly detected in milk samples, while Cu was not detected at all. The Mg content in the concentrated feed was lower than in other feeds. However, the Mg content in milk when feeding concentrated feed was higher than when feeding other types of feed, which indicates a more intensive absorption of Mg from the concentrated feed of the diet. The behavior of the concentration of Ca and Zn was opposite to that of Mg. The assimilation of Ca and Zn in the body from other feeds was higher than from concentrated ones. We also observed significant fluctuations in the content of macro- and microelements both in different feeds and in different farms, including milk, which is natural. The problem is that the content of these elements did not correspond to the average nutritional value of feed according to detailed norms of animal feeding. Scientists from China (Song Ren-ju et al., 2015) have not revealed significant changes in the behavior of Fe in the study of feed and milk samples. They come to the conclusion that the relationship between the content of trace elements in feed and milk can become an important theoretical basis for dairy farming, increasing milk yield, improving its quality and milk products. We also agree with that.

Researchers from Peru (Doris Maritza Chirinos-Peinado & Jorge Isaac Castro-Bedriñana, 2020) point to a significant health problem in humans, especially infants, due to the heavy metal content in milk. The concentration levels of lead and cadmium in the blood of animals and the transition of toxic elements into milk of cows in rural areas near the metallurgical complex in Peru (La Oroya Metallurgical Complex in Peru), which has been emitting into the air for more than 90 years, have been investigated and evaluated. The samples were analyzed in the same way as in our research (Mamenko & Portiannik, 2019; Mamenko & Portiannik, 2021). The results of the analysis showed that the levels of Pb in blood and milk in mg/kg were 0.38 ± 0.041 and 0.58 ± 0.018, respectively; the Pb level in milk was 54% higher than in blood ($p < 0.01$). The concentration of Cd, in mg/kg, in blood and milk was 0.016 ± 0.002 and 0.02 ± 0.007, respectively; milk contained 28% more Cd than blood ($p < 0.05$). The results of the Pb content in milk were compared with the Codex Alimentarius standard (0.002 mg/kg); the average concentration of the element in milk was 29 times higher than the permissible norm, and the average concentration of Cd was 2 times higher than the permissible norm of the Romanian standard (0.01 mg/kg). The scientists explain the result by the influence of environmental pollution by waste products of the enterprise. In the state standard for milk in Ukraine, the norm for cadmium is 0.03 mg/L, and for dairy raw materials used for the production of baby food, it is 0.02 mg; for lead, respectively, 0.1 mg/L and 0.05 mg/L. The Romanian standard is more stringent in terms of milk safety than the Ukrainian one. In Peru, as

the scientists note, there are no norms for maximum values of Pb and Cd at all, so the researchers propose to establish the norms for the maximum permissible concentration of these elements in cow's milk. Ukrainian scientists (Slivinska et al., 2021) have also conducted research in conditions of increased man-made pollution of the Lvov-Volyn coal basin on dairy cows of black-and-white breed from the influence of toxic metals cadmium and lead on the physiological state of animals. As a result, it was found that protein metabolism in cows from the contaminated area was characterized by hypoproteinemia (32.5% of cows), hypoalbuminemia (30%), hypogammaglobulinemia; 77.5% of cows increased the activity of aspartate aminotransferase and 66.5% increased the activity of alanine aminotransferase. The concentration of urea and creatinine increased in the serum of cows, depending on the distance of keeping animals 3–5 km from the mines. It is obvious that the intoxication of the cows' organisms occurred both as a result of the consumption of feed containing heavy metals, and as a result of their intake from the atmospheric air.

All these studies by scientists from around the world indicate that pollution of the environment with heavy metals leads to their accumulation in plants (feed), which are used in animal feeding and then enter milk or simply accumulate in various organs and tissues, which can have a negative impact on the health of people consuming the product. Therefore, it is important to constantly monitor the pollution of ecosystems with heavy metals, environmental control of polluting sectors of the economy in different countries of the world, including Ukraine, the development of new more effective technological methods for the production of ecologically safe high-quality milk, or their improvement.

Conclusion

In conditions of local pollution of agroecosystems with heavy metals, it is unacceptable to balance rations without taking into account the concentration of pollutants, especially toxic heavy metals such as cadmium and lead in feed, and also without taking into account the actual nutritional value of feed. The production of ecologically safe cow's milk in such farms is a technologically very difficult task and it will be impossible to solve the problem without rationing these mineral elements in the diet or using special antidotes in the form of mineral and vitamin premixes of other feed additives. Food, as an environmental factor, will lead to disruption of the homeostasis of the organism of a productive animal, which can lead to a decrease in productivity and deterioration of health.

Further research is aimed at environmental monitoring of the content of toxic metals and various essential and non-essential mineral elements in feed for productive animals in different regions of Ukraine, forest-steppe and Polissya.

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Received 17 February 2022
Accepted 16 June 2022