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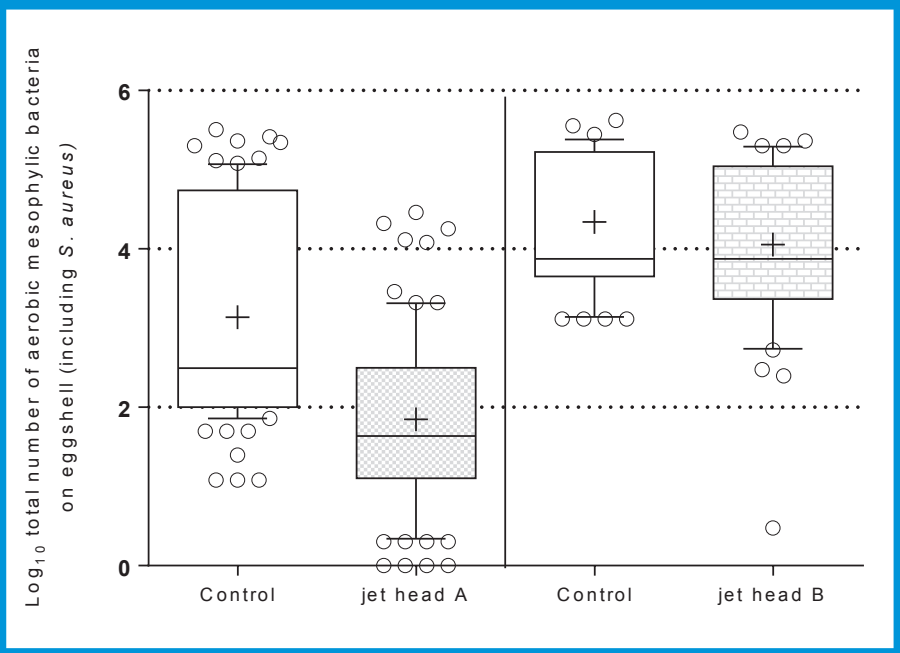

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Original Scientific Article

EFFECTS OF OREGANO ESSENTIAL OIL, GRAPEFRUIT SEED EXTRACT AND THEIR COMBINATION ON THE GROWTH AND SURVIVAL OF *Salmonella* Typhimurium AND *Listeria monocytogenes* IN POULTRY FILLETS UNDER MODIFIED ATMOSPHERE PACKAGING

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Summary: The antimicrobial effect of oregano essential oil (OEO), grapefruit seed extract (GSE) and their combination on the growth and survival of foodborne pathogens (*Salmonella* Typhimurium and *Listeria monocytogenes*) were determined in poultry fillets under modified atmosphere packaging (30% CO_2/70% N_2). In a preliminary experiment, OEO and GSE were used at concentrations of 0.05%, 0.1%, 0.5%, 0.8%, 1.0%, 1.5%, 2.0% and of 0.01%, 0.02%, 0.04%, 0.08%, 0.1%, respectively. Paper disc diffusion testing showed that OEO at 0.05%, 0.1% and GSE at 0.01%, 0.02%, 0.04%, 0.08% had weak antibacterial activity. In addition, due to the very strong odour and taste, poultry samples treated with OEO at 1.0%, 1.5%, 2.0% and the combinations were assessed with scores below the limit of acceptance. Thus, the levels of 0.5% and 0.8% of OEO and 0.1% of GSE were further used in poultry fillets. In this study, the pathogens were affected by OEO and GSE. *L. monocytogenes* was the most sensitive pathogen. In conclusion, the results of this study confirmed the possibility of using natural products with MAP in food production to prevent the growth of foodborne bacteria.

Key words: oregano essential oil; grapefruit seed extract; poultry; foodborne pathogens

Introduction

Poultry is a very popular food commodity worldwide, and its consumption has increased over the last decades in many countries due to its relatively low cost of production, low fat content and its high nutritional value (1). Increased demand for fresh poultry and a desire to transport to more distant markets have increased the need to extend the shelf life of poultry products (2).

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Nowadays, consumers increasingly demand the use of natural products as alternative preservatives in foods, as the safety of synthetic additives has been questioned. The most abundant groups of natural compounds are represented by essential oils and plant extracts (3). Oregano and Grapefruit Seed Extract (GSE) have frequently been used successfully for food preservation (4, 5). The practical application of several essential oils in foods is limited due to the strong flavour they impart to foods and also to their interaction with some food ingredients. The preservative effect of essential oils and extracts may be achieved by

using lower concentrations of essential oils in combination with other preservation technologies, such as modified atmosphere packaging (MAP) (6).

The aim of this study was to determine the effect of oregano essential oil (OEO), GSE and their combinations on the survival of pathogens (*S.* Typhimurium and *L. monocytogenes*) in poultry fillets under MAP.

Material and methods

Extraction of OEO and Preparation of GSE

Oregano (*Origanum vulgare*) leaves from Aegean part of Turkey, samples were air dried at room temperature (23±1 °C), and their essential oils were obtained by continuous steam distillation, using a Clevenger-type apparatus for 3 h. The essential oil was collected, dried over anhydrous sodium sulphate and stored at 4 °C until analysis (7).

GSE (Nutribiotic, Lakeport, USA) was dissolved in double distilled water with 0.05% (v/v) Tween-80 (Merck 822187, Darmstadt, Germany) as a surfactant. A 10% (v/v) stock solution prepared and filter sterilized through with a 0.22 µm filter.

Gas Chromatography – Mass Spectroscopy (GC-MS) Analysis

GC-MS analysis was conducted in MERLAB, Research Institute of Istanbul University. The analysis was performed using a Trace GC Ultra (Thermo Electron Corporation, Milan, Italy) equipped with MS DSQ II detectors (30 m × 0.25 mm, Zebron) with 0.25 µm film thickness was used. For GC-MS detection, the sample was concentrated at 43 °C/1 min with the rotavapor and then was flushed. OEO in the amount of 0.4 µl was subjected, and electron ionization energy of 70 eV was used. The temperature program, starting from 60 °C for 8 min and then gradually increased to 240 °C at 3 °C/min, held for 10 min and finally raised to 325 °C at 10 °C/min. The injector, interface and ion source temperature were 200 °C, 275 °C, and 200 °C, respectively. Helium was used as a carrier gas, the flow through the column was 1.0 ml/min, and the split ratio was set to 100:1. The components were identified with the comparison of retention time (RT) and mass spectra with standard compounds (carvacrol, ρ-cymene, thymol, linalool, caryophyllene, cineole, α-pinene, y-terpinene, borneol, phellandrene, and 4-terpineol) by using NIST and the Wiley mass spectral library of the GC-MS system and literature data.

Reference Bacteria

S. Typhimurium (ATCC 14028) and *L. monocytogenes* (ATCC 7644) strains were obtained from Microbiologics® (Minnesota, USA). All strains were maintained in glycerol (30%) at -80 °C. They were streaked on Tryptone Soya Agar (Oxoid CM131, Basingstoke, England) plates and incubated at 35 °C overnight (18–24 h). Working cultures of the selected strains were made by inoculation from stock cultures into 10 ml Tryptone Soya Broth (TSB; Oxoid CM129) and incubating for 20 h at 37 °C.

Antibacterial Assay Using the Disc Diffusion Method

OEO, GSE, and their combination were tested for antibacterial activity with the paper disc diffusion method. Sterilized filter paper discs (Whatman No 1, 0.6 cm in diameter) were placed on the surface of Nutrient Agar (Oxoid CM309) that *S.* Typhimurium and *L. monocytogenes* were individually seeded by spreading 0.1 ml from TSB incubated at 37 °C. Fifteen microliters of dilutions of OEO (0.05%, 0.1%, 0.5%, 0.8%, 1.0%, 1.5%, and 2.0%) and GSE (0.01%, 0.02%, 0.04%, 0.08%, and 0.1%) were applied to sterile filter paper discs. The inhibition zone diameter was measured after 24 h (8). All analyses were performed in triplicate.

Poultry Inoculation

S. Typhimurium and *L. monocytogenes* strains individually were prepared in 10 ml TSB and incubating at 30 °C for 24 h. Strains were sub-cultured twice in TSB before use. The strains were centrifuged (8000 x g) at 4 °C for 10 min, washed with sterile phosphate buffered saline (PBS) and serially diluted with PBS to a concentration capable of giving approximately 10^4 CFU/g of poultry samples.

Poultry breast meat (totalling 30 kg) was supplied from a poultry (broiler) processing plant within 12 h after slaughter. Immediately after delivery, the meat was filleted in small pieces (20 g). The poultry fillets were divided into three equal groups

and portion was placed in a polyethylene sachet. Samples in the first group were contaminated with only *S.* Typhimurium and the second group was contaminated with only *L. monocytogenes*. The non-inoculated group was used for sensory analysis. Poultry fillets were placed in stomacher bags and inoculated with single-strain pathogens by dipping. The inoculated samples were manually massaged for 10 min at room temperature (23±1 °C) to ensure proper distribution of the pathogen. Prior to the inoculation, oils and fillets were also examined for any contamination by tested pathogens.

Following homogenization, the inoculated and non-inoculated groups (*S.* Typhimurium, *L. monocytogenes*, and no bacterial cultures) were treated with six different applications. Treatments were (1) the addition of OEO at 0.5%, (2) the addition of OEO at 0.8%, (3) the addition of GSE at 0.1%, (4) additions at the combinations of 0.5% OEO plus 0.1% GSE, (5) additions at the combinations of 0.8% OEO plus 0.1% GSE, and (6) untreated control. OEO at 0.05%, 0.1% and GSE at 0.01%, 0.02%, 0.04%, and 0.08% were not examined for screening because of weak antibacterial activity against pathogens. In addition, OEO at 1.0%, 1.5%, 2.0%, and their combinations were not used because of unacceptable organoleptic properties in the poultry meat.

Immediately after treatment, all subgroups were individually (300-350 g) packaged under MAP conditions (30% CO_2/70% N_2). MAP was carried out using Ponapack (VTK 40 SC) packaging machine (Ponapack, Istanbul, Turkey) in low O_2 permeable (8–12 $cm^3/m^2/24$ h at STP) polystyrene/ethyl vinyl alcohol (EVOH)/polyethylene (PE) trays and were over-wrapped with oxygen permeable (6000–8000 $cm^3/m^2/24$ h at STP) polyvinyl-chloride film (Wrap Film Systems Ltd., Shropshire, England). Packages were prepared by placing samples into 400 mm^3 inner volume trays to obtain 1:1 (v/v) headspace ratios and were stored at 4 °C for 7 days. Sampling was carried out at predetermined time intervals: 0, 1, 3, 5 and 7 days of storage for microbiological and sensory analysis. On each sampling date, six packs from each group were examined.

Gas Analysis of Package Atmospheres

Gas analyses of the internal package atmosphere were done in duplicate at 1, 3, 5 and 7 days of storage. Analyses for CO_2, O_2, and N_2 within the packages were monitored by injecting 0.5 ml of gas removed from the headspace with a syringe (B. Braun, Melsungen, Germany) into a PDI gas chromatograph (PBI-Dansensor A/B, Ronnedevaj 18, Ringsted, Denmark) fitted with a thermal conductivity detector.

Microbiological Analysis

Samples (25 g) were combined with 225 ml buffered peptone water (Oxoid CM509) in sterile stomacher bags (Seward, Worthing, England) and homogenized for 2 min in a stomacher (Interscience, St. Nom la Breteche, France). Following homogenization, 10-fold serial dilutions were made in sterile Maximum Recovery Diluents (Oxoid CM317) and sample dilutions (0.1 ml) were streaked onto Xylose Lysine Deoxycholate Agar (Oxoid CM469) and Chromogenic Listeria Agar (Oxoid CM1080) supplemented with Listeria Selective Supplement (Oxoid SR227) and Listeria Differential Supplement (Oxoid SR228) for enumeration of inoculated *S.* Typhimurium and *L. monocytogenes*, respectively (9, 10). Microbiological analyses were carried out in triplicate.

Sensory Evaluation of Poultry Fillets

Non-inoculated poultry fillets were examined, and sensory analysis was used only for determining the concentration in terms of acceptability by consumers. Sensorial attributes were evaluated by eight well-experienced panelists, ranging in age between 26 and 45 years (2 females and 6 males), trained according to ISO 1993 (11). Prior to the analysis, vocabularies of the sensory attributes (odour-odour intensity (sour, sweet, and spicy) and taste-flavour intensity (spicy taste, salty taste, sweet taste, and acidic taste)) were developed by the panelists, using a standardized procedure (12).

The panel members were seated in individual booths in a temperature and light-controlled room (fluorescent lighting of 2000 lx; Philips 40W Cool White), receiving a set of six samples in a completely randomized order. Before evaluation, poultry samples were wrapped in aluminium foil and cooked individually in an oven (220 °C) for 20 min. Each sample was served warm in dishes coded with three-digit code numbers. Unsalted crackers and water were served to panelists to freshen their mouth between each sub-samples assessment.

Statistical Analysis

Analysis of variance was conducted for each variable to investigate the effect of the antibacterial activities of OEO, GSE, and their combination during storage time. The trial was performed in triplicate, and the General Linear Model procedure (PROC GLM) of SPSS 13.0 was used to analyse the data (13). The microbiological analysis and sensory characteristics were evaluated, and significant differences were defined as $P < 0.05$. Microbial counts were expressed as log CFU/g and mean separations was obtained using Duncan's multiple range tests.

Results

The main volatile components of OEO used in poultry fillets were characterized by prominent (>1%) concentrations of carvacrol (68.97%), ρ-cymene (11.47%), thymol (4.85%), linalool (2.21%), caryophyllene (2.85%), cineole (1.14%), α-pinene (1.04%), y-terpinene (2.37%) and 4-terpineol (1.57%). In the present study, carvacrol was detected as a major constituent. The composition of OEO may be due to the variety of the plant, origin, extraction modality and agronomic practices (14).

The antibacterial activities of OEO, GSE and the combination determined by the paper disc diffusion are shown in Table 1.

Table 1: Inhibition zones of OEO and GSE against the pathogens (mm)

	Different concentrations of oils and extracts														
Test Bacteria	OEO (%)							GSE (%)				OEO (%) + GSE (%)			
	0.05	0.1	0.5	0.8	1.0	1.5	2.0	0.01	0.02	0.04	0.08	0.1	0.5+0.08	0.5+0.1	0.8+0.1
S. Typhimurium	11	17	22	24	26	28	30	ND	3	8	14	15	23	25	28
L. monocytogenes	12	19	24	26	27	28	29	3	6	10	15	19	25	27	28

OEO: Oregano Essential Oil; GSE: Grapefruit Seed Extract; ND: Not Detected.

Table 2: Effect of OEO and GSE on *S.* Typhimurium in poultry fillets under MAP (log CFU/g)

Storage Days	Control	OEO (%)		GSE (%)	OEO (%) + GSE (%)	
		0.5%	0.8%	0.1%	0.5% +0.1%	0.8% + 0.1%
0	4.32*±0.04^{aE**}	3.98±0.05^{bA}	3.94±0.06^{bcA}	4.24±0.07^{aA}	3.93±0.06^{bcA}	3.90±0.07^{cA}
1	4.36±0.10^{aD}	3.60±0.12^{cB}	2.81±0.15^{eB}	4.15±0.11^{bB}	3.52±0.10^{dB}	2.75±0.12^{eB}
3	5.05±0.14^{aC}	2.98±0.14^{cC}	1.07±0.10^{eC}	3.65±0.23^{bC}	2.08±0.15^{dC}	1.00±0.10^{eC}
5	5.15±0.13^{aB}	2.22±0.27^{cD}	ND^{eD}	3.50±0.22^{bD}	1.40±0.21^{dD}	ND^{eD}
7	5.22±0.19^{aA}	1.30±0.22^{cE}	ND^{eD}	3.47±0.17^{bE}	1.12±0.20^{dE}	ND^{eD}

a, b, c, d, e: Means with different lowercase letters in the same row are significantly different (P < 0.05)
A, B, C, D, E: Means with different capital letters in the same column are significantly different (P < 0.05)
OEO: Oregano Essential Oil; GSE: Grapefruit Seed Extract; ND: Not Detected
*log CFU/g
** Standard Error (S.E.).

Table 3: Effect of OEO and GSE on *L. monocytogenes* in poultry fillets under MAP (log CFU/g)

Storage Days	Control	OEO (%)		GSE (%)	OEO (%) + GSE (%)	
		0.5%	0.8%	0.1%	0.5% +0.1%	0.8% + 0.1%
0	4.61*±0.83^{aE**}	4.20±0.05^{bcA}	4.18±0.12^{bcA}	4.38±0.15^{abA}	4.14±0.15^{bcA}	4.08±0.14^{cA}
1	4.71±0.23^{aD}	3.56±0.14^{bB}	3.21±0.15^{cB}	4.05±0.21^{aB}	3.02±0.80^{cdB}	2.98±0.25^{dB}
3	4.83±0.27^{aC}	3.02±0.10^{cC}	2.12±0.12^{dC}	3.56±0.23^{bC}	2.16±0.22^{dC}	1.75±0.21^{eC}
5	5.42±0.20^{aB}	2.35±0.28^{cD}	ND^{dD}	2.60±0.20^{bD}	ND^{dD}	ND^{dD}
7	5.61±0.28^{aA}	ND^{cE}	ND^{cD}	2.18±0.13^{bE}	ND^{cD}	ND^{cD}

a, b, c, d, e: Means with different lowercase letters in the same row are significantly different ($P < 0.05$)
A, B, C, D, E: Means with different capital letters in the same column are significantly different ($P < 0.05$)
OEO: Oregano Essential Oil; GSE: Grapefruit Seed Extract; ND: Not Detected
*log CFU/g
** Standard Error (S.E.).

Table 4: Headspace gas compositions of packages during storage time

Group	Storage time (day)											
	1			3			5			7		
	O_2	CO_2	N_2	O_2	CO_2	N_2	O_2	CO_2	N_2	O_2	CO_2	N_2
Control	0.13	29.80	70.07	0.55	28.20	71.25	0.75	27.60	71.65	0.92	26.40	72.68
0.5% OEO	0.12	29.80	70.08	0.55	28.30	71.15	0.76	27.80	71.44	0.88	26.45	72.67
0.8% OEO	0.11	29.75	70.14	0.54	28.10	71.36	0.75	27.45	71.80	0.87	26.40	72.73
0.1% GSE	0.10	29.80	70.10	0.50	28.50	71.00	0.78	27.85	71.37	0.93	26.35	72.72
0.5% OEO + 0.1% GSE	0.12	29.70	70.18	0.56	28.00	71.44	0.78	27.70	71.52	0.90	26.30	72.80
0.8% OEO + 0.1% GSE	0.12	29.85	70.03	0.59	28.10	71.31	0.79	27.70	71.51	0.91	26.50	72.59

OEO: Oregano Essential Oil; GSE: Grapefruit Seed Extract

The essential oils and plant extracts showed strong activity by producing a clear inhibition zone ≥ 20 mm (8). In this study, *S.* Typhimurium and *L. monocytogenes* were inhibited by OEO at 0.5%, 0.8%, 1.0%, 1.5%, and 2.0%. Our results also showed that *L. monocytogenes* were the most susceptible microorganism. In contrast, it was detected that GSE at 0.1% had moderate activity (inhibition zone < 12-20 mm) against bacteria. Therefore, GSE was used in combination with OEO.

Sensory properties (odour and taste) of treated fillets with OEO at 0.5%, 0.8% and GSE at 0.1% were assessed by the panelists with scores above ($P < 0.001$) the rejection limit (score of 5) whereas samples treated with OEO at 1.0%, 1.5%, 2.0% and the combinations were assessed with scores below the rejection limit ($P < 0.001$). Based on sensory scores, OEO at 0.5%, GSE at 0.1% and the combinations of 0.5% OEO plus 0.1% GSE had the highest acceptability scores between 6 and 8 during storage. The odour and taste of poultry fillets treated with OEO at 0.8% and OEO at 0.8% plus 0.1% GSE was found distinctive but pleasant, and scores were slightly higher than the acceptable limit. Due to the very strong odour and taste of OEO at the concentration of 1.0%, 1.5%, 2.0% and the combinations had lowest scores for sensory evaluation. According to Skandamis and Nychas (15), OEO at 1.0% in beef gave a more acceptable odour and colour as compared to the untreated samples. However, Chouliara *et al.* (1) and Solomakos *et al.* (16) stated that OEO at 1.0% and 0.9% gave adverse organoleptic properties in chicken and beef, respectively.

The inhibitory effects of OEO, GSE, and combinations on *S.* Typhimurium and *L.*

monocytogenes in poultry fillets under MAP are shown in Tables 2 and 3.

Discussion

There is a relationship between the chemical composition of the tested oil and the antimicrobial activity (17). The phenolic compounds particularly thymol and carvacrol widely reported to possess high levels of antimicrobial activity (18). In another study by Govaris *et al.* (19), the sum of thymol, carvacrol, ρ-cymene, and y- terpinene were found to be important for screening antibacterial activity. Baranauskiene *et al.* (20) found that the bacteriostatic properties of OEO are suspected to be associated with the carvacrol content. Our results were supported by this aspect.

The measured mean headspace compositions of the packaging at 7 days of storage were 72.7 ± 3.1% N_2, 26.4 ± 1.5% CO_2 and 0.9 ± 0.4% O_2 (Table 4). The gas compositions of each package were almost constant during storage. It may be the result of the permeability of packaging material and respiration of the product. It was reported that reduction in CO_2 in packages was due to the solubility of CO_2 in the poultry meat aqueous phase (21).

Extracts of volatile compounds from plants are widely used in the food industry because of their antimicrobial properties for the inhibition of growth and reduction in numbers of the foodborne pathogens (22). In this study, the tested pathogens were affected by OEO and GSE ($P < 0.001$). The use of OEO and GSE resulted in a reduction in *S.* Typhimurium population ($P < 0.001$). The concentrations of 0.8% OEO and 0.8% OEO + 1.0% GSE completely inhibited the *S.* Typhimurium at 5 days of storage. At 7 days, the population of *S.* Typhimurium was reduced by 2.68 log CFU/g (0.5% OEO), 0.77 log CFU/g (0.1% GSE) and 2.81 log CFU/g (0.5% OEO + 0.1% GSE) ($P < 0.001$). The similar results are in accordance with those of Ahn *et al.* (23) and Xu *et al.* (24).

Our results showed that *L. monocytogenes* was the most sensitive pathogen. Our results are in agreement with the results of Shelef (25) and Marino *et al.* (26), who reported that gram-negative bacteria are less sensitive to OEO than gram-positive bacteria due to their cell wall composition. Conversely, Kotzekidou *et al.* (22) indicated that gram-positive bacteria are more resistant. The volume of inocula, culture media, detection method and pH of the medium can be considered to be the reason for the difference.

The addition of OEO, GSE and their combinations with MAP showed a significant effect on the reduction and inhibition of *L. monocytogenes* ($P < 0.001$). All OEO concentrations inhibited the growth of *L. monocytogenes*; while 0.1% GSE resulted in a reduction by 2.20 log CFU/g. The efficacy of OEO against *L. monocytogenes* has been shown on beef (16) and cheese (19). OEO between 0.25% and 0.8% was used by these authors. Conversely, Ting and Deibel (27) reported that 1.0% OEO on meat did not reduce the *L. monocytogenes*. The differences could be attributed to several factors including the composition of OEO, bacterial strain, pH of food and storage temperature. In contrast and, Ahn *et al.* (23) and Xu *et al.* (24) examined the GSE against *L. monocytogenes*, and significant reductions were determined ($P < 0.01$). Jayaprakasha *et al.* (28) stated that GSE would be mainly effective against gram-positive bacteria, with gallic acid as the main active component.

The results of this study revealed the possibility of using OEO, GSE, and their combinations against foodborne pathogens on poultry meat under MAP stored at 4 °C. The usage of OEO and GSE concentrations in foods as preservatives is limited by the adverse sensorial properties. Further studies are needed to explore the efficacy of suitable concentrations of OEO and GSE in foods.

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References

1. Chouliara E, Karatapanis A, Savvaidis IN, et al. Combined effect of oregano essential oil and modified atmosphere packaging on shelf-life extension of fresh chicken breast meat, stored at

4 °C. Food Microbiol 2007; 24: 607–17.

2. Jimenez SM, Salsi MS, Tiburzi MC, et al. Spoilage microflora in fresh chicken breast stored at 4 ˚C influence of packaging methods. J Appl Microbiol 1997; 83: 613–8.

3. Burt S. Essential oils: their antibacterial properties and potential applications in foods-a review. Int J Food Microbiol 2004; 94: 223–53.

4. Aureli P, Costantini A, Zolea S. Antimicrobial activity of some plant essential oils against *Listeria monocytogenes*. J Food Prot 1992; 55: 344–8.

5. Reagor I, Gusman J, Mccoy I, et al. The effectiveness of processed grapefruit-seed extract as an antibacterial agent: I. an in vitro agar assay. J Altern Complement Med 2002; 8: 325–32.

6. Mastromatteo M, Conte A, Del Nobile MA. Combined use of modified atmosphere packaging and natural compounds for food preservation. Food Eng Rev 2010; 2: 28–38.

7. Baydar H, Sagdic O, Ozkan G, Karadogan T. Antibacterial activity and composition of essential oils from *Origanum*, *Thymbra* and *Satureja* species with commercial importance in Turkey. Food Control 2004; 15: 169–72.

8. Rota C, Carraminana JJ, Burillo J, Herrera A. In vitro antimicrobial activity of essential oils from aromatic plants against selected foodborne pathogens. J Food Prot 2004; 67: 1252–6.

9. BS EN ISO 6579:2002. Microbiology of food and animal feeding stuffs. Horizontal method for the detection of *Salmonella* spp. London : International Standardization Organization, 2002.

10. ISO 11290-1:2005. Horizontal method for the detection and enumeration of *Listeria monocytogenes*. Part: 1 Detection method. Geneva : International Standardization Organization, 2005.

11. ISO 8586-1:1993. Sensory analysis. General guidance for the selection, training and monitoring of assessors. Part 1: Selected assessors. Geneva : International Standardization Organization, 1993.

12. ISO 8589:1998. Sensory analysis – General guidance for design of test rooms. Geneva : International Standardization Organization, 1998.

13. Statistical Package for the Social Sciences (SPSS): Release 13.0. IL, Chicago: SPSS Inc., 2001.

14. Siroli L, Patrignani F, Montanari C, et al. Characterization of oregano (*Origanum vulgare*) essential oil and definition of its antimicrobial activity against *Listeria monocytogenes* and *Escherichia coli* in vitro system and on foodstuff surfaces. Afr J Microbiol Res 2014; 8: 2746–53.

15. Skandamis PN, Nychas GJE. Effect of oregano essential oil on microbiological and physico-chemical attributes of minced meat stored in air and modified atmospheres. J Appl Microbiol 2001; 91: 1011–22.

16. Solomakos N, Govaris A, Koidis P, et al. The antimicrobial effect of thyme essential oil, nisin and their combination against *Listeria monocytogenes* in minced beef during refrigerated storage. Meat Sci 2008; 25: 120–7.

17. Dimitrijevic SI, Mihajlovski KR, Antonovic DG, et al. A study of the synergistic antilisterial effects of a sub-lethal dose of lactic acid and essential oils from *Thymus vulgaris* L., *Rosmarinus officinalis* and *Origanum vulgare* L. Food Chem 2007; 104: 774–82.

18. Friedman M, Henika PR, Mandrell RE. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella enterica*. J Food Prot 2002; 65: 1545–60.

19. Govaris A, Botsoglou E, Sergelidis D, et al. Antibacterial activity of oregano and thyme essential oils against *Listeria monocytogenes* and *Escherichia coli* O157:H7 in feta cheese packaged under modified atmosphere. Lebensm Wiss Technol 2011; 44: 1240–4.

20. Baranauskiene R, Venskutonis PR, Dewettinck K, et al. Properties of oregano (*Origanum vulgare* L.), citronella (*Cymbopogon nardus* G.) and marjoram (*Majorana hortensis* L.) flavors encapsulated into milk protein-based matrices. Food Res Int 2006; 3: 413–25.

21. Seydim AC, Acton JC, Hall MA, et al. Effects of packaging atmospheres on shelf-life quality of ground ostrich meat. Meat Sci 2006; 73: 503−10.

22. Kotzekidou P, Giannakidis P, Boulamatsis A. Antimicrobial activity of some plant extracts and essential oils against foodborne pathogens in vitro and on the fate of inoculated pathogens in chocolate. Lebensm Wiss Technol 2008; 41: 119–27.

23. Ahn J, Grun IU, Mustapha A. Effects of plant extracts on microbial growth, color change and lipid oxidation in cooked beef. Food Microbiol 2007; 24: 7–14.

24. Xu W, Qu W, Huang K, et al. Antibacterial effect of grapefruit seed extract on food-borne pathogens and its application in the preservation

of minimally processed vegetables. Postharvest Biol Tec 2007; 45: 126–33.

25. Shelef LA. Antimicrobial effects of spices. J Food Saf 1983; 6: 29–44.

26. Marino M, Bersani C, Comi G. Impedance measurements to study the antimicrobial activity of essential oils from Lamiacea and Compositae. Int J Food Microbiol 2001; 67: 187–95.

27. Ting EWT, Deibel KE. Sensitivity of *Listeria monocytogenes* to spices at two temperatures. J Food Safy 1992; 12: 129–37.

28. Jayaprakasha GK, Selvi T, Sakariah KK. Antibacterial and antioxidant activities of grape (*Vitis vinifera*) seed extracts. Food Res Int 2003; 36: 117–22.

UČINKI ETERIČNEGA OLJA ORIGANA, IZVLEČKA SEMEN GRENIVKE TER NJIHOVE KOMBINACIJE NA RAST IN PREŽIVETJE *Salmonelle* Typhimurium IN *Listerie monocytogenes* V FILEJIH PERUTNINE V SPREMENJENEM ATMOSFERSKEM PAKIRANJU

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Povzetek: V perutninskih filejih pakiranih v spremenjenih atmosferskih pogojih (30% CO_2 / 70% N_2), smo ugotavljali protimikrobne učinke eteričnega olja origana (OEO), izvlečka semen grenivke (GSE) ter njihove kombinacije na rast in preživetje patogenov, ki se prenašajo s hrano (*Salmonella* Typhimurium in *Listeria monocytogenes*). V poprejšnjem poskusu je bil OEO uporabljen v koncentracijah 0,05 %, 0,1 %, 0,5 %, 0,8 %, 1,0 %, 1,5 %, 2,0 %, GSE pa v koncentracijah 0,01 %, 0,02 %, 0,04 %, 0,08 %, 0,1 %. Testiranje s pomočjo difuzije s papirnatih diskov je pokazalo, da OEO pri koncentracijah 0,05 %, 0,1 % in GSE pri koncentracijah 0,01 %, 0,02 %, 0,04 %, 0,08 % slabo antibakterijsko delujeta. Poleg tega so bili zaradi zelo močnega vonja in okusa vzorci perutnine obdelani z OEO 1,0 %, 1,5 %, 2,0 % ter v kombinacijah ocenjeni z rezultati pod mejo organoleptične sprejemljivosti. Zato smo v nadaljnjih raziskavah uporabili koncentracije OEO 0,5 % in 0,8 % ter GSE 0,1 %. V tej raziskavi sta na patogene mikrorganizme vplivala tako OEO kot GSE, *L. monocytogenes* pa je bila občutljiv patogen kot *S.* Typhimurium. Rezultati te raziskave so potrdili možnost uporabe naravnih izdelkov pri proizvodnji hrane in pakiranju v spremenjenih atmosferskih pogojih za preprečevanje rasti bakterij, ki se prenašajo s hrano.

Ključne besede: eterično olje origana; ekstrakt semen grenivke; perutnina; patogeni, ki se prenašajo s hrano

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Original Scientific Article

ARTERIAL SUPPLY OF THE CEREBRAL CORTEX IN CATTLE (*Bos primigenius f. dom.*)

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Summary: Studies of the vascularization of the cerebrum in cattle were performed on 60 cerebral hemispheres received from a meat-processing plant in Bydgoszcz, Poland. The sex of the cattle was not determined. It was found that the middle cerebral artery is the strongest vessel supplying blood to the cerebrum. The artery is divided into ten permanent branches. Two olfactory arteries supply the region of the cerebrum located on the border between the old and the new cortex. The other eight are divided into three branches heading towards the frontal lobe of the brain, two branches heading towards parietal lobe, and three temporal branches heading towards the temporal lobe, that supply the region of the new cortex. The frontal, parietal and temporal branches descended independently from the main trunk of the middle cerebral artery or formed a common trunk. Common trunks for the respective groups of branches have been described as the rostral, dorsal and caudal middle cerebral arteries. The present research show that the division of the middle cerebral artery into the same branches or their groups observed in cattle, like in the other mammalian species investigated thus far, is a result of genetic limitations.

Key words: brain arteries; cattle

Introduction

In the literature concerning telencephalon vascularity in cattle, Hofman (1900), Jenke (1919), Chomiak and Walento (1967) report on the anatomy and the variability of the brain base arteries, while Godynicki (1972) does so on the system of blood supply to the brain. In the literature, there seem to be no paper on the cortical branches of the middle cerebral artery in cattle, which is the main blood vessel supplying the cerebrum. There are publications in which authors describe the cortical branches of the middle cerebral artery; see Chadzypanagiotis (1975), Wiland (1991) on the cortical branches of some predatory species, Skoczylas and Wiland (1999) of wild boar.

Regarding biungulates, descriptions of the cortical branches of the middle cerebral artery in domestic pig (Walinczus, 1973), bison (Węgrzyn et al, 1983), roe deer (Jabłoński, Roskosz 1997), elk (Jabłoński et al, 1999), goat (Brudnicki et al, 2005) and in fallow deer (Skoczylas et al, 2011) have been made.

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It was found that the cortical branches of the middle cerebral artery in these species come to the same areas of the telencephalon. The differences occur in the pattern of descent and division of respective cortical branches of the middle cerebral artery. The pattern of division of the middle cerebral artery is affected by how the species has been classified and the pattern of the groove-coverage of the cortex. In mammals, there is a different pattern of sulci on the surface of the cortex, which can affect the structure of the cortical branches of the middle cerebral artery (Brauer, Schober 1970). Considering the discrepancy resulting from respective description and considering new studies, it was decided that the pattern, the division and variation of cortical branches of the middle cerebral artery in cattle should be investigated and the results compared with the data reported by other authors.

Materials and methods

The research was performed on 30 brains in cattle: a total of 60 cerebral hemispheres received from meat processing plant in Bydgoszcz. The sex of the samples was not determined. The animal heads were cut off at the height of the 3rd–4th cervical vertebrae. The arteries were filled with black latex introduced with medical syringe into the common carotid artery. The heads were fixed in a 5% formalin solution for 3 months, and then decalcified in hydrochloric acid; the skull cavity was opened and brains were removed. The cerebral hemispheres were photographed, and the following are described: the anatomy, the division pattern, and the course of cortical branches of the middle cerebral artery.

Results

In cattle, blood is supplied to the brain with internal carotid arteries. Its intracranial segment is regenerated from the rostral epidural rete mirabile. The extracranial section of the internal carotid artery undergoes atrophy after birth. Having passed the dura mater, the internal carotid artery (Fig. 1a) is divided into the rostral cerebral artery (Fig. 1b) and the caudal communicating artery that, together with the symmetrical vessels, form the arterial circle of the brain. At the height of the rostral border of the optic chiasma, the rostral cerebral artery gives off a thick arterial vessel: the middle cerebral artery (Fig. 1-Y, 2-Y). The middle cerebral artery is the most powerful vessel supplying blood to the telencephalon. The initial section of the main trunk of the middle cerebral artery runs along the ventral surface of the optic tract; the section then bends around the piriform lobe and runs in front of its rostral border. Further on, it runs towards the lateral rhinal sulcus and then, having passed it, it is divided. From the initial section of the main trunk of the middle cerebral artery, minor central branches supplying blood to olfactory tracts and the piriform lobe descend. The main trunk of the middle cerebral artery is divided into a number of cortical branches that run towards the specific region of the cerebral hemisphere, supplying blood to specific regions of the brain.

The first permanent branches of the middle cerebral artery, which supply both the old and the new cortex, are olfactory arteries.

The rostral olfactory artery (Fig. 1-1), having separated from the main trunk of the middle cerebral artery, runs towards the rostral part of

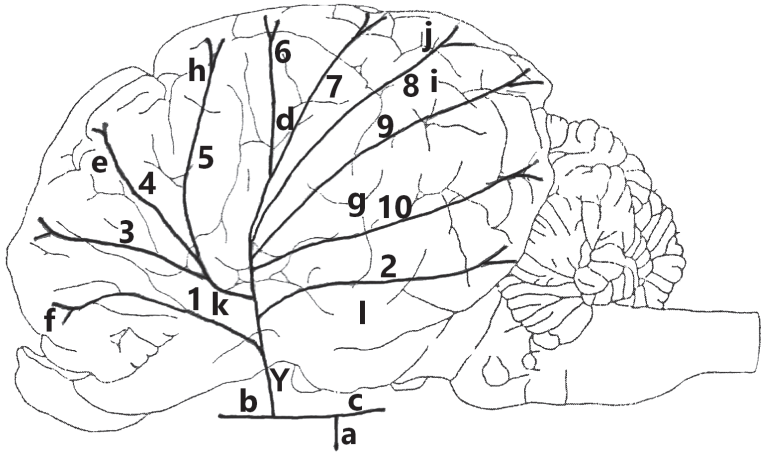

Figure 1: Diagram of the division of the middle cerebral artery on the surface of the cortex in cattle

1 – rostral olfactory artery, 2 – caudal olfactory artery, 3 – orbital branch, 4 – ventral frontal branch, 5 – dorsal frontal branch, 6 – rostral parietal branch, 7 – caudal parietal branch, 8 – dorsal temporal branch, 9 – middle temporal branch, 10 – ventral temporal branch, a – internal carotid artery, b – rostral cerebral artery, c- caudal communicating artery, d – Sylvian fissure, e – diagonal sulcus, f - Presylvian sulcus, g - caudal ectosylvian sulcus, h - middle Suprasylvian sulcus, i- caudal Suprasylvian sulcus, j - ectomarginal sulcus, k - rostral lateral rhinal sulcus, l - caudal lateral rhinal sulcus, Y - middle cerebral artery

the lateral rhinal sulcus and can descend into in various places. Its terminal branches can also appear again from under the lateral rhinal sulcus, and they ascend under the surface of the cortex.

The caudal olfactory artery (Fig. 1-2) ascends into the caudal part of the lateral rhinal sulcus, and its terminal branches also supply blood to the area of the cortex located over that sulcus. On the cortex, towards the frontal lobus, three thick branches spread.

The orbital branch descends first (Fig. 1-3); it supplies blood to the area of the cortex situated over the presylvian sulcus and below the diagonal sulcus. Its terminal branches reach the coronary sulcus.

The ventral frontal branch (Fig. 1-4) runs towards the diagonal sulcus, and then one of its branches ascends into that sulcus; the others spread on the surface of the cortex between the ansiform sulcus and the middle suprasylvian sulcus.

The dorsal frontal branch (Fig. 1-5), having descended from the main trunk of the middle cerebral artery, ascends into the initial section of the middle suprasylvian sulcus. Its terminal branches supply blood to the upper part of the medial surface of the frontal lobus.

Another vessel that runs towards the parietal lobus, ascending to the Sylvian fissure onto the surface of the cortex; after a short course, it bifurcates into two branches.

The rostral parietal branch (Fig. 1-6) and caudal parietal branch (Fig. 1-7) run towards the middle suprasylvian sulcus. Having passed the sulcus, the vessels spread medially, reaching the marginal sulcus. The lateral-caudal surface of the hemisphere is supplied by the branches of the middle cerebral artery, which descend at various heights; they have been referred to as temporal branches.

The dorsal temporal branch (Fig. 1-8), having descended from the Sylvian fissure, goes towards the caudal suprasylvian sulcus. Then, having passed the marginal sulcus, its terminal branches reach the internal marginal sulcus. They do not cross any more.

The middle temporal branch (Fig. 1-9) descends in a short distance from the dorsal temporal branch; it runs over the caudal ectosylvian sulcus. The terminal branches run towards the terminal section of the caudal suprasylvian sulcus and arrive on the surface of the occipital lobus.

The ventral temporal branch (Fig. 1-10), having separated from the main trunk of the middle cerebral artery on the surface of the cortex, supplies blood to the area between the lateral rhinal sulcus and the caudal ectosylvian sulcus. Its terminal branches participate in the supply of a part of the occipital lobus.

Considering the presented general pattern of the distribution of cortical branches of the middle cerebral artery, it should be noted that respective sections of those branches can be located inside respective sulci, sometimes undergoing further divisions, but always going in the direction of the area of the cortex described.

Factoring in the pattern of descent of cortical branches of the middle cerebral artery in the individual cattle , it was found that one independent vessel descended in all the cases from the rostral cerebral artery: the middle cerebral artery. Among them, on 6 (10.0%) hemispheres from the main trunk of the middle cerebral artery, a common trunk descended rostrally for the rostral olfactory artery, for the orbital branch, and for the ventral and dorsal frontal branches. The main trunk caudally separated an independent caudal olfactory artery with a common descent for the ventral and the middle temporal branches. The main trunk, having ascended into the Sylvian fissure, brought a common descent for the dorsal temporal branch and parietal branches onto the surface of the cortex.

In 6 (10.0%) cases, an independent rostral olfactory artery descended rostrally from the main trunk, followed by a common descent for the orbital branch and for ventral frontal branch. Caudally, from the main trunk of the middle cerebral artery, the common departure for the middle, ventral and dorsal temporal branches separated as well as for the caudal olfactory artery. The main trunk, having ascended into the Sylvian fissure, brought a common descent for rostral and caudal parietal branches as well as the dorsal temporal branch into the surface of the cortex.

On another 9 (15.0%) hemispheres, the independent rostral olfactory artery separated rostrally from the main trunk of the middle cerebral artery, followed by a common descent for the orbital branch and the ventral frontal branch. Caudally, from the main trunk of the middle cerebral artery, the independent caudal olfactory artery and the common departure for the ventral, middle and dorsal temporal branches separated.

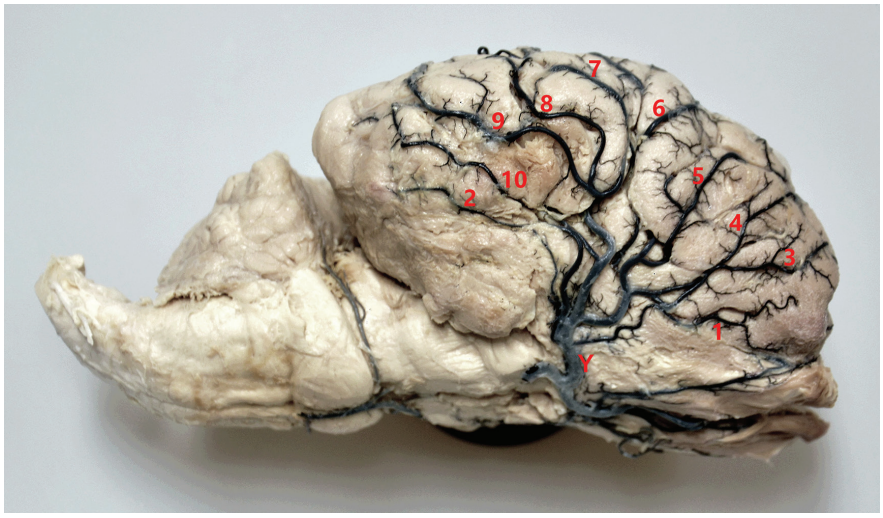

Figure 2: A single trunk of the middle cerebral artery that give rise to the specific cortical branches

1 - rostral olfactory artery, 2 - caudal olfactory artery, 3 - orbital branch, 4 - ventral frontal branch, 5 - dorsal frontal branch, 6 - alterior parietal branch, 7 - caudal parietal branch, 8 - dorsal temporal branch, 9- middle temporal branch, 10 - ventral temporal branch, Y - middle cerebral artery.

The main trunk, having ascended into the Sylvian fissure, brought a common descent for the dorsal frontal branch as well as the rostral and caudal parietal branches.

On 3 (5.0%) hemispheres rostrally from the main trunk of the middle cerebral artery, the rostral olfactoral artery descended independently, then the common trunk for the orbital branch as well as the ventral and dorsal frontal branch. Caudally from the main trunk, with a common descent, the ventral and the middle temporal branches separated as did the caudal olfactory artery. The main trunk, having ascended into the Sylvian fissure, brought a common descent for the dorsal temporal branch and for the rostral and caudal parietal branches onto the surface of the cortex.

In 6 (10.0%) cases, the independent caudal olfactory artery and a common trunk for the orbital branch rostrally descended, followed by a common descent for rostral and caudal parietal branches. Having ascended into the Sylvian fissure, a common trunk for rostral and caudal parietal branches came to the surface of the cortex. Caudally from the main trunk of the middle cerebral artery, with a common descent, the ventral, dorsal and the middle temporal branches separated, while the caudal olfactory artery descended independently from the main trunk of the middle cerebral artery.

In the other 9 (15%) hemispheres, from the main trunk, rostrally with a common departure the orbital branch, the ventral frontal branch and the rostral olfactory artery descended. The main trunk of the middle cerebral artery caudally separated the dorsal frontal branch with a common descent for rostral and caudal parietal branches as well as the dorsal, middle and ventral temporal branches. The caudal olfactory artery descended independently from the main trunk of the middle cerebral artery.

In another 6 (10%) cases, a common departure for the caudal olfactory artery, the orbital branch, and the ventral frontal branch descended rostrally. The caudal bifurcation was a common trunk for the ventral temporal branch and the caudal olfactory artery. The main trunk came to the surface of the cerebral cortex from the Sylvian fissure and formed a common descent for the dorsal frontal branch, rostral and caudal parietal branches, as well as the dorsal and middle temporal branches.

In another 9 (15%) cases, from the main trunk, the following separated rostrally with a common trunk: the orbital branch, the ventral and dorsal frontal branch and the rostral olfactory artery. Caudally, the following separated with a common descent from the main trunk of the middle cerebral artery: the rostral and caudal parietal branches; the ventral, dorsal and the middle temporal branches, while the caudal olfactory artery descended independently from the main trunk of the middle cerebral artery.

On another 3 (5%) cerebral hemispheres, the common trunk for the rostral olfactory artery and for the orbital branch departed rostrally from the main trunk of the middle cerebral artery, followed by a common departure for the ventral and dorsal frontal branches. Caudally from the main trunk, the ventral olfactory artery descended through the common trunk with the ventral temporal branch. The main trunk, having descended into the

Sylvian fissure, came to the surface of the cortex with a common descent for the rostral and caudal parietal branches as well as the middle and dorsal temporal branch.

In another 3 (5%) cases, an independent rostral olfactory artery and a common trunk descended rostrally for the orbital branch as well as the interior and dorsal frontal branches. Caudally from the main trunk of the middle cerebral artery, the caudal olfactory artery descended through the common trunk with the ventral temporal branch and a common trunk for the middle and dorsal temporal branches. The main trunk, having descended into the Sylvian fissure, came to the surface of the cortex with a common descent for the rostral and caudal parietal branches (Fig. 2).

Discussion

In cattle, the middle cerebral artery supplies the same areas of the brain as in the mammalian species studied thus far. The discrepancies concern mostly its division into respective branches. Chadzypanagiotis (1975), describing the cortical branches in cats, differentiated between the branches supplying the old cortex, the branches on the border of the old and the new cortex as well as the branches for the new cortex. In cattle, the arteries supplying the old cortex are minor branches onto the piriform lobe and olfactory tracts. On the border of the old and the new cortices, the rostral and caudal olfactory arteries are found. In cattle, in 45% of the cases, the rostral olfactory artery was a vessel that descended independently from the rostral cerebral artery. In 5% of the cases, a common descent with the orbital branch was demonstrated. On 25% of the cerebral hemispheres, one of the branches descended from the common trunk of the middle cerebral artery, which gave rise to the orbital branch and the ventral frontal branch. In the other 25% of the hemispheres, the rostral olfactory artery demonstrated a common descent with the orbital branch as well as the ventral and dorsal frontal branches.

The caudal olfactory artery, in contrast, was a vessel that descended independently from the main trunk of the middle cerebral artery in 55% of the cases. In 20% of the cases, the caudal olfactory artery separated with a common descent with the ventral temporal branch. On 15% of the cerebral hemispheres, it was one of the branches descending from the common trunk of the middle cerebral artery, which gave rise to the middle and dorsal temporal branches. In another 10% of the cases, it was one of the branches of the common trunk for the ventral, middle and dorsal temporal branches. The other cortical branches of the middle cerebral artery can be divided into a group of frontal, parietal and temporal branches. In cattle, similarly as in other Ruminantia species, there are eight main vessels that supply blood to the area of the new cortex of the cerebrum. Moreover, respective cortical branches can descend from the main trunk of the middle cerebral artery with a common descent. Such cases of descent were reported by Wiland (1991), Skoczylas et al. (2012) as the rostral, dorsal and caudal middle cerebral arteries. In cattle, the rostral middle cerebral artery has been presented as a common trunk for frontal branches in 20% of the cases investigated; the dorsal middle cerebral artery was described as a common trunk for parietal branches, which was observed in 15% of the cases. The caudal middle cerebral artery as a common trunk for temporal branches was found in 25% of the cases.

In cattle, the dorsal middle cerebral artery occurred as the lowest percentage of the cases; however, in these cases the caudal middle cerebral artery dominated. Making a comparison of the present results with those reported by Skoczylas et al. (2012) in otters, it can be stated that the dorsal middle cerebral artery was reported in the lowest percentage of the cases. In cattle, similarly as in the other Artiodactyla, the parietal branches have developed the poorest. On the surface of the cerebrum, the temporal branches of the middle cerebral artery are the best developed.

From the description of the structure of the middle cerebral artery in the publications by Jabłoński and Roskosz (1997), Brudnicki et al. (2005), Skoczylas et al. (2011) in roe deer, goat and fallow deer, respectively, usually a single vessel descending from the rostral cerebral artery can be observed. The vessel, having passed the lateral rhinal sulcus, is divided along its course into respective branches and its main trunk heads towards the fornix. In the material investigated, such a pattern of division of the middle cerebral artery was found in 100% of the cases.

This research shows that the division of the middle cerebral artery into the same branches or their groups, observed in cattle, is a result of genetic limitations, as in the other mammalian species investigated thus far (Wiland, 1980).

References

1. Brudnicki W, Jabłoński R, Nowicki W, et al. Cortical branches of the middle cerebral artery in goat (*Capra hircus*). Prace Kom Nauk Rolniczych Biol BTN Bydgoszczy 2005; B56: 29–32.

2. Chadzypanagiotis D. Arteries on the surface of the cerebral hemisphere in the cat. Fol Morphol 1975; 32: 385–99.

3. Chomiak M, Welento T. The brain arteries in calf. Pol Vet Arch 1967; 2: 185–94.

4. Godynicki S. The comparative morphology of the main arteries of the head in (Artiodactyla). Ann AR Poznań 1972; 36: 1–60.

5. Hofmann M. Zur vergleichenden Anatomie der Gehirn und Ruckenmarksarterien der Vertebraten. Z Morphol Anthropol 1900; 2: 247–322.

6. Jabłoński R, Roskosz T. Middle cerebral artery, a. cerebri media in roe-deer (*Capreolus capreolus L.*). Ann Warsaw Univ Agric SGGW Anim Sci 1997; 20: 35–41.

7. Jabłoński R, Skoczylas B, Wiland C. 1999. The main branches of the middle cerebral artery in elk (*Alces alces*). Electron J Pol Agric Univ Vet Med 1999; 2(2): 1(5p.). http://www.ejpau.media.pl/volume2/issue2/veterinary/art-01.html (March, 2016)

8. Jenke TW. Die Gehirnarterien des Pferdes, Hundes, Rindes und Schweines verglichen mit denen des Menschen. Dissertation. Dresden, 1919.

9. Skoczylas B, Wiland C. Cortical branches of the middle cerebral artery in the wild boar (*Sus scrofa L.*). Electron J Pol Agric Univ Vet Med 1999; 2(1): 1(6 p.). http://www.ejpau.media.pl/volume2/issue1/veterinary/art-01.html (March, 2016)

10. Skoczylas B, Brudnicki W, Nowicki W, et al. Cortical branches of the middle cerebral artery in fallow deer (*Dama dama*). Electron J Pol Agric Univ Vet Med 2011; 14(4): 9(6 p.). http://www.ejpau.media.pl/volume14/issue4/art-09.html (March, 2016)

11. Skoczylas B, Brudnicki W, Nowicki W, et al. The cortical branches of the middle cerebral artery in the otter (*Lutra lutra*). Vet Med 2012; 57 (6): 282–6.

12. Sobociński M. Układ nerwowy u zwierząt domowych . PWN : Wrocław, 1973.

13. Walinczus J. Sredniaja mozgowaja arterja swini. Uczenyje Zapiski Witebskowo Weterinarnowo Instituta 1973; 26: 123–7.

14. Węgrzyn M, Roskosz T, Mazowiecka M. Brain arteries of the European bison, *Bison bonasus L.* 1758. Ann Warsaw Univ Agric SGGW Anim Sci 1983: 11: 9–16.

15. Wiland C. Factors affecting the variability of the brain base arteries in mammals. Zool Rev 1974; 18: 400–16.

16. Wiland C. Comparative studies of the cortical branches of the middle cerebral artery in carnivores (Carniviora). Zesz Nauk ATR Bydgoszcz 1991; 44: 1–52.

ARTERIJSKA OSKRBA MOŽGANSKE SKORJE PRİ GOVEDU (*Bos primigenius f. dom.*)

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Povzetek: Študije prekrvavitve možganov pri govedu so bile izvedene na 60 možganskih hemisferah, prejetih iz mesnopredelovalnega obrata v Bydgoszczu na Poljskem. Spol živine ni bil določen oziroma upoštevan. V raziskavi je bilo ugotovljeno, da je srednja cerebralna arterija najmočnejša žila, pomembna pri dovajanju krvi v možgane. Arterija je razdeljena na deset stalnih vej. Dve vohalni arteriji oskrbujeta področje v možganih na meji med staro in novo skorjo. Ostalih osem arterij je razdeljenih v tri veje, ki se nadaljujejo v smeri proti prednjemu režnju možganov, dve veji sta usmerjeni proti parietalnemu režnju, tri senčnične veje pa proti senčnemu režnju, ki oskrbuje področje nove skorje. Prednja, parietalna in senčnična veja se spustijo neodvisno od glavnega debla srednje možganske arterije ali pa oblikujejo skupno deblo. Skupna debla za posamezne omenjene skupine vej so bila opisana kot rostralne, dorzalne in kavdalne srednje možganske arterije. Pričujoča raziskava kaže, da je delitev srednje možganske arterije v iste veje ali njihove skupine, opažena pri govedu, tako kot tudi pri drugih doslej raziskanih vrstah sesalcev posledica genetskih omejitev.

Ključne besede: možganske arterije; govedo

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Original Scientific Article

COMMON CARP RESPONSE TO THE DIFFERENT CONCENTRATION OF LINSEED OIL IN DIET

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Summary: Common carp (*Cyprinus carpio* L.) fingerlings were fed five diets in triplicate containing 0, 2, 3, 4 and 5% of linseed oil for 75 days to determine the effect of the different concentration of linseed oil on growth parameters, as well as on the proximate and fatty acid composition of the fish flesh. The fish had the average initial weight of 200 g and were stocked in 15 cages. The fish which were fed with the diets supplemented with 3, 4 and 5% linseed oil had significantly better growth parameters compared to the fish fed with the diets supplemented with 0 or 2% linseed oil. The lipid content in muscle increased from 1.25% in the control group without the addition of linseed oil, to 1.46%; 1.56%; 1.94% and 2.37% in the groups of fish fed with the diets supplemented with 2, 3, 4 and 5% linseed oil, respectively. The fatty acid profiles in the muscle tissue reflected the diet concentrations with significant increases ($p < 0.05$) in 18:3n-3 in the fish fed with the diets supplemented with 2, 3, 4 and 5% of linseed oil. Other fatty acids which increased significantly ($p < 0.05$) in the muscle tissue, of fish fed with the diets supplemented with linseed oil, were: eicosatrienoic acid (C20:3n-3), eicosapentaenoic acid (EPA, C20:5n-3), docosapentaenoic acid (DPA, C22:5n-3), docosahexaenoic acid (DHA, C22:6n-3), as well as polyunsaturated fatty acids (PUFA), total n−3 fatty acids, and the n-3/n-6 ratio. The fatty acids which decreased significantly ($p < 0.05$) were monounsaturated fatty acids (MUFA) as well as linoleic acid (LA, C18:2n-6). The fish fed with the diet supplemented with 5% of linseed oil had three times more the n−3 fatty acids (24.02%) than those from the control group (7.5%). The n-3/n6 ratio ranged from 0.59 in fish from the control group to 4.14 in fish fed with the diet supplemented with 5% of linseed oil, mainly due to the difference in content of the alfa-linolenic acid (ALA, C18:3n-3). The inclusion of 5% linseed oil in diets showed the most favourable effects on the content of essential fatty acids in the tissue of carp as well as on other tested parameters.

Key words: *Cyprinus carpio*; cages; chemical composition; fatty acid; growth parameters; nutrition

Introduction

The n-3 highly unsaturated fatty acids (n-3 HUFA) are well known to have numerous beneficial effects on human health (1) and, undoubtedly, fish meat represents the best source of these nutrients in the human diet (2, 3). Moreover, the consumption of fish meat is encouraged due to the high protein content, the presence of essential amino acids, minerals and vitamins (4). Dietary lipids have an important role in aquafeeds as they are the source of energy and fatty acids which are essential for normal growth and development of fish. Furthermore, lipids can spare protein in the diets from being used as the source of energy (5) and additionally they can decrease ammonia production (6). Fish oil (FO) represents the main source for lipid in fish feed, especially for carnivorous fish species. The fish feed industry uses approximately 87% of the total produced FO, from which 66% is specifically used by salmon

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species. However, the significance of cyprinid and other warm-water fish species, when it comes to FO consumption, cannot be ignored due to their high share in the total world aquaculture production (7). Merino et al. (8) predicted that the price of FO will rise significantly in the future. An important concern is the possibility of contamination of the FO with persistent organic pollutants (9), which emphasizes the increasing international demand for safe and high quality fish feeds. Because of this, there is a growing need for sustainable alternatives to FO for aquafeeds, representing a considerable challenge for the future development of aquaculture. The replacement of FO with vegetable oils (VO) should be as such, where the catabolism of eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) is minimized while the deposition of linoleic acid (LA, C18:2n-6) has to be avoided (10). Some vegetable oils are considered to be alternatives to FO, such as rapeseed oil, soybean oil and linseed oil (LO), and have been tested as an alternative lipid source in different fresh water cyprinid fish species (11, 12, 13, 14, 15) and particularly in common carp (5, 16) one of the most important fish species worldwide. LO is rich in C18:2n-6 and especially rich in α-linolenic acid (ALA, C18:3n-3) (17), so the addition of this oil could increase the amount of C22:6n-3 and C20:5n-3 in fish meat, regarding the fact that fresh water fish species could synthesize n-3 HUFA from dietary C18:3n-3 (12). Feed with an addition of different content of LO, a vegetable oil rich with n-3 polyunsaturated fatty acids (PUFA) and especially with C18:3n-3, as an alternative lipid source, seems to produce a more similar fatty acid profile to FO than other vegetable oils in some cyprinids, without compromising fish growth or feed utilization (13, 15, 18). Enhancement in fish meat n-3 fatty acids causes a more convenient n-3/n-6 ratio which may improve the health value of cultured fresh water fish, whereas a modern diet contains high levels of n-6 and low levels of n-3 PUFAs (19). High dietary lipid content can also decrease feed consumption and reduce the growth of some fish species (11, 12) and it can lead to an increase in the lipid deposition in the body of fish (20) and consequently affect the nutritive quality of fish meat. Hence, the optimal dietary lipid level and source should to be investigated and carefully evaluated. Therefore, the aim of the present work was to investigate the effects of the different levels of linseed oil in diets on the growth performance, proximate composition and tissue fatty acid composition in common carp, which is one of the most commercially important fish species worldwide.

Materials and methods

Fish and facilities

The cage platform with 15 cages was set up in a 650 m² fishpond at a fish farm in Grabovo (Croatia). Water was added after the fishpond had been disinfected with 160 kg of Ca $(HCO_3)_2$. For the purpose of this trial, triplicate groups of common carp, were distributed in 15 cages. Approximately 60 one-year-old fish, with an initial mean weight of 200 g, were randomly distributed in each cage. All fish were reared under variable natural atmospheric conditions.

Experimental diets and feeding

Five comercial extruded diets were formulated to provide 32% of protein. Experimental diets were further prepared by an addition of 0% (control group – C), 2% (group L2), 3% (group L3), 4% (group L4) or 5% (group L5) of LO (Table 1). The daily feed amount was given in three sessions at 8.00, 13.00, and 17.00 h; by automatic feeders. The feed was provided in an amount of 1–2% with respect to fish biomass and depending on the water temperature, and water saturation with oxygen. The diet samples were collected at the beginning of the trial. Prior to the feeding trial, the fish were fed with commercial extruded feeds for one month. The trial lasted for 75 days, from April until June. Growth performance indicators such as specific growth rate (SGR), feed conversion ratio (FCR), weight gain (WG) and the survival rate (SR) were calculated (Table 3).

The water content in the diets and fish flesh was estimated by drying at 103 ± 2°C until their weight was constant for 24 hours. The level of crude protein (N × 6.25) was assessed with the Kjeldahl method (Manual Book, Kjeltec Auto 1030 Analyzer; Tecator, Höganäs, Sweden), and the total ash was determined after the combustion at 550 ± 25°C overnight. Crude fat from the diets samples and fish flesh was extracted with a Soxhlet extractor.

Lipid extraction and fatty acid analysis

The fatty acid composition analysis of experimental diets and fish flesh was performed as described previously (21). Briefly, the fatty acids in the experimental samples were determined following the extraction of total lipids by means of accelerated solvent extraction (ASE) on Dionex ASE 200. The mixture of n-hexane and isopropanol (60:40, v/v) was used for lipid extraction at 100°C and nitrogen pressure of 10.3 MPa in two static cycles lasting a total of 10 minutes. Fatty acid methyl esters were separated on a polar cyanopropyl aril column HP-88 (column length 100 m, diameter 0.25 mm, film thickness 0.20 µm; Agilent, Santa Clara, USA), in a programmed temperature range, on a capillary gas chromatograph (Shimadzu 2010; Shimadzu, Kyoto, Japan), with a flame ionisation detector. The temperature of the injector was 250°C and the detector temperature was 280°C. The carrier gas was nitrogen, with a flow rate of 1.33 ml min and a split ratio 1:50. The identification of fatty acid methyl esters was based on comparing their retention times with the standard, Supelco 37 Component FAME Mix (Supelco, Bellefonte, USA). The content of each fatty acid was expressed as the percentage of the total fatty acid content.

Statistical analyses

All data is shown as means ± SD (n=3). Statistical analyses were conducted by using a statistical software program Statistica 12 for Windows (Statistica Version 12.0; StatSoft Inc., Tulsa, USA), to determine if variables differed between treatments. Significant effects were further explored using analysis of variance (one-way ANOVA) with repeated measurements, as well as Tukey's post-hoc multiple range test. A significance level of $p < 0.05$ was used.

Results

Diet composition

The oil increased concentrations of linseed oil led to an increasing content of crude fat in the experimental diets, as well as increasing content of dry matter, while protein and ash content remain constant (Table 1). The oil concentration in the diets showed a significant effect on the dietary fatty acid compositions (Table 2). The C diet was free of external lipid addition and depended on the internal lipid contents of the diet ingredients. The C diet contained 21% of saturated fatty

Table 1: Composition and proximate analysis of the extruded formulated diet

Ingredients (%)	Diet for C	Diet for L2	Diet for L3	Diet for L4	Diet for L5
Soybean meal	50	50	50	50	50
Sunflower meal	18	18	18	18	18
Brewery yeast	5	5	5	5	5
Linseed oil	0	2	3	4	5
Wheat flour	10.6	10.6	10.6	10.6	10.6
Corn	12	12	12	12	12
Methionin	0.1	0.1	0.1	0.1	0.1
Lysine L	0.3	0.3	0.3	0.3	0.3
Vitamin mix[1]	2	2	2	2	2
Mineral mix[2]	2	2	2	2	2
Chemical analysis (%)					
Dry matter	89.91	90.15	90.27	90.38	90.5
Crude protein	32.49	32.33	32.25	32.17	32.09
Crude fat	1.56	3.46	4.4	5.36	6.31
Crude ash	4.49	4,46	4.45	4.44	4.43
NFE[3]	61.45	59.74	58.88	58.03	57.17

[1]C-control group; L2-group with addition of 2% of linseed oil; L3- group with addition of 3% of linseed oil; L4-group with addition of 4% of linseed oil; L5-group with addition of 5% of linseed oil; [1]Vitamin mix(mg/kg of diet): vitamin B1, 15; vitamin B2, 10; vitamin B6, 20; vitamin B12, 0,15; vitamin K3, 15; inositol, 250; Ca-pantothenic acid, 80; nicotinic acid, 100; folic acid, 1; vitamin H (biotin), 1; vitamin E, 140; vitamin C, 500; vitamin A, 20 000 IU; vitamin D3, 6 000 IU; choline chloride, 1 800, and cellulose was used as a carrier.
[2]Mineral mix (mg/kg of diet): Cu 20, Fe 40, Mn 30, Se 0.4, Zn 125, and cellulose was used as a carrier
[3]NFE, nitrogen-free extract, g/kg DM = 100 – (CP + CF + CA)

Table 2: Fatty acid composition of control diet (C) and experimental diets supplemented with 2% (L2), 3% (L3), 4% (L4) and 5% (L5) linseed oil (LO)

Fatty acid (%)	Diet for C	Diet for L2	Diet for L3	Diet for L4	Diet for L5
Myristic acid, C14:0	0.45	0.5	0.5	0.5	0.5
Pentadecylic acid, C15:0	0.1	0.1	0.1	0.1	0.1
Palmitic acid, C16:0	15.2	14.2	14.1	14.2	13.6
Palmitoleic acid, C16:1	0.62	0.4	0.4	0.4	0.5
Stearic acid, C18:0	4.67	3.5	3,6	3.5	3.4
Oleic acid, C18:1cis-9	29.5	32.9	31.7	31.7	28.5
Vaccenic acid, C18:1cis-11	1.2	0.5	0.5	0.5	0.6
Linoleic acid, C18:2 n-6	40.4	23.1	19.5	16.9	15.9
γ- linolenic acid, C18:3 n-6	0.15	0.19	0.13	0.15	0.1
α-linolenic acid, C18:3 n-3	5.56	22.3	27.5	30.1	33.9
Arachidic acid,C20:0	0.5	0.3	0.3	0.25	0.4
Eicosenoic acid, C20:1	0.24	0.15	0.25	0.37	0.3
Behenic acid, C20:2 n-6	0.1	0.2	0.2	0.2	0.2
Dihomogammalinolenic acid, C20:3 n-6	0.84	0.76	0.70	0.69	0.6
Eicosatrienoic acid, C20:3 n-3	0.2	0.2	0.2	0.2	0.2
Arachidonic acid, C20:4 n-6	0.2	0.1	0.1	0.1	0.1
Eicosapentaenoic acid, C20:5 n-3	0	0	0	0	0
Docosapentaenoic acid, C22:5 n-3	0	0	0	0	0
Docosahexaenoic acid, C22:6 n-3	0	0	0	0	0
SFA	20.92	18.6	18.6	18.55	18.1
MUFA	31.56	33.95	32.85	32.97	29.9
PUFA	47.45	46.85	48.33	48.34	51
∑ n -6	41.69	24.35	20.63	18.04	16.9
∑ n -3	5.76	22.5	27.7	30.3	34.1
n-3/n-6	0.14	0.92	1.34	1.65	2.02
n-6/n3	7.24	1.08	0.74	0.6	0.5
PUFA/SFA	2.27	2.52	2.6	2.34	2.82
USFA/SFA	3.78	4.35	4.36	4.38	4.47

C-control group; L2-group with addition of 2% of linseed oil; L3- group with addition of 3% of linseed oil; L4-group with addition of 4% of linseed oil; L5-group with addition of 5% of linseed oil; SFA-saturated fatty acids, MUFA-monounsaturated fatty acids; PUFA-polyunsaturated fatty acids of n-3 and n-6 series; USFA-unsaturated fatty acids Proximate composition of the fish and fish feed

Table 3: Growth performance of experimental fish

Variable	C	L2	L3	L4	L5
Initial number of fish	180	180	180	180	180
IBW(g)	201.67±1.53	203.67±2.52	201±1	201±2.65	201±1
FBW (g)	$484.3±4.04^{a}$	$492.33±4.93^{ab}$	$502±2^{ab}$	$508±2^{b}$	$519.67±2.52^{b}$
Final number of fish	141	147	150	147	153
Survival rate (%) SR	$78.33±2.3^{a}$	$81.67±2.1^{a}$	$83.33±1.9^{ab}$	$81.67±2.4^{a}$	$85±2.4^{b}$
WG (gfish^{-1})	$282.67±2.51^{a}$	$288.67±2.42^{ab}$	$301±1.73^{ab}$	$307±3.61^{bc}$	$318.67±3.5^{c}$
DGR (g day^{-1})	$3.77±3.2^{a}$	$3.84±3.1^{a}$	$3.99±3.2^{ab}$	$4.01±2.8^{b}$	$4.2±3.1^{b}$
SGR (%·day^{-1})	$1.17±1.29^{a}$	$1.18±0.03^{ab}$	$1.22±0.01^{ab}$	$1.24±0.02^{b}$	$1.27±0.01^{b}$
FCR (g·g^{-1})	$2.16±0.04^{a}$	$1.93±0.03^{b}$	$1.76±0.01^{c}$	$1,79±0.03^{c}$	$1,59±0.02^{d}$

C-control group; L2-group with addition of 2% of linseed oil; L3- group with addition of 3% of linseed oil; L4-group with addition of 4% of linseed oil; L5-group with addition of 5% of linseed oil; All values are mean ± SD (n = 3). Groups with different letter indexes in the same row statistically significantly different ($P < 0.05$); IBW, initial body weight; FBW, final body weight; SR, survival rate = (Final fish number/initial fish number) X 100; WG, weight gain = [Final body weight (g) - initial body weight (g)] X100 X [initial body weight (g)]$^{-1}$; DGR, Daily growth rate = [final body weight (g) - initial body weight (g)] X time^{-1} of rearing (days); SGR, specific growth rate = 100 X [ln final body weight (g) - ln initial body weight (g)] X time^{-1} of rearing (days); FCR, feed conversion ratio = total food supply (g) X [final fish biomass (g) - initial fish biomass (g)]$^{-1}$.

Table 4: Proximate composition of experimental fish

Parameters (%)	C	L2	L3	L4	L5
Moisture content (%)	79.16±0.07^{a}	78.87±0.16^{ab}	78.61±0.07^{b}	78.53±0.03^{b}	77.84±0.15^{c}
Protein content (%)	17.13±0.08^{a}	17.99±0.14^{b}	18.24±0.02^{b}	18.62±0.03^{c}	18.87±0.1^{c}
Fat content (%)	1.25±0.12^{a}	1.46±0.23^{a}	1.56±0.1^{a}	1.94±0.05^{b}	2.37±0.28^{c}
Ash content (%)	0.99±0.03	0.99±0.03	0.98±0.03	0.96±0.01	1.01±0.05

C-control group; L2-group with addition of 2% of linseed oil; L3- group with addition of 3% of linseed oil; L4-group with addition of 4% of linseed oil; L5-group with addition of 5% of linseed oil; Values are means ± SD (n = 3); Values in the same row with different letter notation statistically significantly differ at $p < 0.05$

Table 5: Fatty acid composition in fillets of experimental fish

Fatty acid (%)	C	L2	L3	L4	L5
C14:0	0.93±0.07	0.92±0.1	0.95±0.02	0.91±0.02	0.94±0.06
C15:0	0.16±0.03^{a}	1.17±0.01^{b}	1.17±0.03^{b}	1.33±0.01^{bc}	1.63±0.01^{c}
C16:0	21.29±3.58	21.25±2.03	21.07±0.16	21.36±0.49	21.12±0.6
C16:1	5.33±0.58^{a}	4.96±0.4^{b}	4.88±0.51^{b}	4.79±0.75^{b}	4.85±0.51^{b}
C17:0	0.27±0.04	0.29±0.03	0.28±0.02	0.28±0.04	0.26±0.01
C18:0	4.67±1.66	4.6±0.83	4.58±0.15	4.52±0.37	4.54±0.36
C18:1cis-9	38.29±2.67^{a}	35.27±1.94^{b}	35.34±2.81^{b}	36.23±0.73^{b}	33.20±0.78^{b}
C18:1cis-11	5.12± 0.17^{a}	1.73± 0.24^{b}	1.65± 2.86^{b}	1.53±0.22^{b}	0.21±0.00^{c}
C18:2 n-6	9.56±0.29^{a}	1.12±0.33^{b}	1.73± 0.08^{c}	1.86±0.05^{c}	1.75±0.19^{c}
C18:3 n-6	0.15±0.02	0.19±0.06	0.13±0.01	0.15±0.04	0.19±0.09
C18:3 n-3	5.42±3.32^{a}	14.93 ±0.44^{b}	15.21±0.72^{c}	15.46±0.05^{c}	17.72±1.19^{d}
C20:0	0.21±0.03	0.27±0.05	0.26±0.01	0.25±0.02	0.20±0.01
C20:1	2.54±0.24	2.38±0.15	2.44±0.25	2.31±0.37	2.31±0.37
C20:2 n-6	0.46±0.05	0.45±0.07	0.49±0.02	0.45 ±0.09	0.44±0.09
C20:3 n-6	0.84±0.16^{a}	0.86±0.14^{a}	0.70±0.03^{b}	0.69±0.07^{b}	0.62±0.06^{b}
C20:3 n-3	0.21±0.04^{a}	0.4±0.07^{b}	0.5±0.01^{b}	0.62±0.07^{bc}	0.73±0.04^{c}
C20:4 n-6	1.70±0.48^{a}	2.57±0.07^{b}	2.65±0.06^{b}	2.76±0.04^{bc}	2.8±0.01^{c}
C20:5 n-3	0.18±0.02^{a}	1.22±0.01^{b}	1.25±0.01^{b}	1.28±0.01^{b}	1.31±0.02^{b}
C22:5 n-3	0.16±0.02^{a}	1.21±0.02^{b}	1.27±0.02^{b}	1.30±0.01^{bc}	1.36±0.03^{c}
C22:6 n-3	1.54±0.26^{a}	2.67±0.1^{b}	2.68±0.02^{b}	2.79±0.02^{c}	2.9±0.07^{d}
SFA	27.52±5.25^{a}	28.5±2.86^{b}	28.31±0.33^{b}	28.65±0.53^{b}	28.69±0.3^{b}
MUFA	51.28±3.42^{a}	44.34±2.49^{b}	44.31±0.4^{b}	44.86±0.96^{b}	40.57±0.69^{c}
PUFA	20.22±3.42^{a}	25.62±0.35^{b}	26.61±0.86^{bc}	27.35±1.02^{c}	29.82±1.26^{d}
∑ n -6	12.71±3.05^{a}	5.19±0.33^{b}	5.7±0.81^{bc}	5.91±0.92^{c}	5.8±1.44^{c}
∑ n -3	7.51±0.4^{a}	20.43±0.34^{b}	20.91±0.07^{b}	21.45±0.12^{c}	24.02±0.18^{d}
n-3/n-6	0.59±0.01^{a}	3.94±0.02^{b}	3.67±0.01^{b}	3.63±0.01^{b}	4.14±0.02^{c}
n-6/n3	1.69±0.02^{a}	0.25±0.03^{b}	0.27±0.05^{b}	0.28±0.02^{b}	0.24±0.03^{b}
PUFA/SFA	0.73±0.3^{a}	0.90±0.22^{b}	0.94±0.26^{b}	0.95±0.32^{bc}	1.04±0.34^{c}
USFA/SFA	3.90±0.38^{a}	2.45±0.26^{b}	2.51±0.21^{b}	2.52±0.34^{b}	2.46±0.38^{b}

C-control group; L2-group with addition of 2% of linseed oil; L3- group with addition of 3% of linseed oil; L4-group with addition of 4% of linseed oil; L5-group with addition of 5% of linseed oil; Values are means ± SD (n = 3); Values in the same row with different letter notation statistically significantly differ at $p < 0.01$. SFAsaturated fatty acids; MUFAmonounsaturated fatty acids; PUFApolyunsaturated fatty acids from the n3 (n3 PUFA) and n6 (n6 PUFA) families.

acids (SFA) of which approximately two thirds (15.2%) was palmitic acid (C16:0) and 31.56 % monounsaturated fatty acids (MUFA), whereas the content of oleic acid (OA, C18:1) was 29.5%. The C diet contained 41.7 % n-6 PUFA, predominantly C18:2n-6 (40.4%) and 5.76 % n-3 PUFA, with 5.56% C18:3n-3. Increasing oil content resulted in a decreased level of SFA, which was 18.1 % in the L5 diet and the content of MUFA was almost the same among the diets. The inclusion of the increased content of LO in the experimental diets resulted in a decreased content of C16:0 and arachidonic acid (AA, C20:4n-6), and in a slightly increased content of eicosanoic acid (C20:1). The n-3/n-6 ratio increased from 0.14 in the C diet to 2.02 in the L5 diet, mainly due to the increasing content of n-3 (especially C18:3n-3) from 5.8% in C diets to 34% in L5 diets. The total content of n-6 PUFA was decreased from 41.7% in the C diet to 16.9 in L5 diet.

Production performance

There were no significant differences in the initial mean weights of the fish (Table 3). Following the 75 days of trial, the mean weight was between 484 g in the C and 520 g in the L5 group. The statistically significant effect of oil concentration was observed in the final body weight, and the highest body weight was observed in the L4 and L5 groups. Moreover, the significant effect of the above mentioned factor was identified in the growth parameters (SGR, DGR and WG). The feed conversion ratio (FCR) was satisfactory for all treatments and ranged from 1.59 to 2.16. One-way ANOVA showed a significant effect of oil concentration on the FCR.

Proximate composition of common carp filets

A significant effect of treatments was observed in the content of crude protein and lipid in the common carp fillets (Table 4). Moisture content was significantly lower in the carp fillets comparing to groups which received diets with higher concentrations of LO which were accompanied with a higher content of fat in muscle tissue. The ash content was the same in all the analysed groups. Oil content showed a significant effect on the proximate composition of the carp fillets, and also resulted in significant changes in the fillets fatty acid compositions (Table 5).

Fatty acid composition of common carp fillets

The proportions of the analysed fatty acid groups were significantly affected by the used diet. The fatty acid compositions of the carp muscle tissue are shown in Table 5. The addition of linseed oil significantly affected the muscle fatty acid composition. However, the significant effect regarding oil concentration in the diets was observed only for a few fatty acids. It was observed that the content of C18:1 decreased slightly, the total MUFA decreased approximately by 14%, the C18:2n-6 decreased to a 7-fold and the total n-6 PUFA 2-fold in the flesh of common carp from groups with an addition of linseed oil compared to the flesh of the carp from C group. On the other hand, the content of saturated fatty acids (SFA) slightly increased, C20:4n-6 increased 1.4-fold, C18:3n-3 3-fold, eicosatrienoic acid (C20:3n-3) from 2 to 3 fold, C20:5n-3 6-fold, docosapentaenoic acid (DPA, C22:5n-3) 6 – fold, C22:6n-3 1.7-fold and n-3/n-6 ratio 6-fold in the flesh of carp from groups L1, L2, L3, L4 and L5 compared to C group. It is interesting that the content of C22:6n-3 was higher in the muscle tissue of the carp fed with all the experimental diets compared to the content of C22:6n-3 in the diets. The L5 group contained the highest levels of SFA (28.69%), while the lowest was measured in the C group (27.52%); the most prominent differences concerned the pentadecylic acid (C15:0), which was almost ten times higher in the L5 than in the C group (1.63% vs 0.16%). The content of MUFA in the fillets of the common carp from the C group was significantly higher than in the other groups (51.28% vs 40.57–44.86%). The contents of C18:2n-6 and C18:3n-3 in the common carp fillets were lower than determined in the diets analysed, and the contents of C20:4n-6, C20:5n-3, C22:5n-3 and C22:6n-3 were higher than the content in the diets. Increased levels of the intermediates eicosadienoic acid (C20:2n-6) and C20:3n-3 in fish that were fed with LO diets were detected. Regarding the C20:4n-6, its content in the fish fillets from the groups fed with an addition of linseed oil was higher (2.57-2.8%) than in those fed with the C diet (1.7%). However, statistically significant differences were noted in the contents of C20:4n-6, C20:5n-3, or C22:6n-3 in the fillets of common carp fed with the diets supplemented with different concentrations of LO ($P < 0.05$; Table 5). The ratio of n-3/n-6 ranged from 4.1 (L5 group) to 0.6 (C group) ($P < 0.05$).

Discussion

Production performance

Several studies have shown that the use of vegetable oils, including LO in diets of cyprinid fish species, has no negative effects on fish growth and growth parameters (13, 15, 18, 22). Growth performance was significantly affected by an addition of increased concentrations of LO in fish diets. It should be noted that the growth rate of carp fed diets supplemented with LO in this study was significantly faster than that of fish fed with diets without the supplementation of LO. The concentration of added oil also showed significant influence on the growth performance. This could indicate that increased fat contents in the diets, which is in the present study LO, has a favorable impact on the growth of common carp. Similar results were reported for catfish (23) and for tench (13). The results of the present research agreed with some reports that by increasing the dietary lipid content, the growth of the fish may improve (24, 25). On the other hand, some authors have reported that a high dietary lipid level (more than 7%) could reduce fish growth (24, 25) which could be due to the low ability to digest and absorb high lipid, the reduction in feed intake and fatty acid imbalance in the diet.

Proximate composition of common carp fillets

The increase of dietary oil levels herein is usually associated with an increase in fish flesh lipid content. A positive correlation between the dietary lipid levels and the total lipid levels in muscle tissue was also observed previously in other species (24, 26). On the contrary, the increase of dietary oil levels led to a decrease in the fish flesh moisture content. A negative correlation between the lipid and moisture contents in the flesh of the carp was also previously shown (27, 28). This is in accordance with previous studies (15, 20) which also showed that the chemical composition of carp flesh was influenced by the experimental diets, especially the content of fat. There were no adverse effects of oil addition on the proximate composition of the common carp fillets, after 75 days of feeding. Our findings are in agreement with previous studies in cyprinid species (goldfish, *Carassius auratus*; common carp, *C. carpio*; grass carp, *Ctenopharyngodon idella*; tench, *Tinca tinca*) in which the addition of VO in feed did not negatively affect the chemical composition of these fish over either short or long-term periods (13, 15, 29, 30).

Fatty acid composition of common carp fillets

The fatty acid composition of muscle lipids of common carp is known to be highly influenced by dietary fatty acids (15, 16) and a linear correlation exists between individual fatty acids in muscle lipid and their concentration in dietary lipid. In the present study, the differences in the muscle tissue fatty acid composition resulted from the different levels of oil in the diets. It seems that common carp also utilize C18:2n-6 and C18:3n-3 as a source of metabolic energy. Lower levels of the above mentioned fatty acids in the muscle tissue, regardless of the dietary treatment, might designate highly active mitochondrial enzymes oxidising fatty acids as previously noted (31). This can further be related to the fact that mitochondrial β-oxidation is of specific significance to muscle tissues (32). Higher levels of C18:2n-6 and C18:3n-3 in muscle tissue of freshwater fish species fed diets with different VO were also previously noted (5, 15, 18). The intermediates C20:2n-6 and C20:3n-3 acid were detected in the carp fillets in the LO groups. Since VO are mainly deprived of these fatty acids and they are an important part of the biosynthetic pathways of n-6 and n-3 HUFA, this result highlights the adjustable attempts to alleviate HUFA deficiencies. When the level of C18:3n-3 or C18:2n-6 in the diets was increased, C20:3n-3 or C20:2n-6 level in muscle was also increased. A similar phenomenon was previously observed in tench (15) and in common carp (5). Previous studies have shown that grass carp (11, 12), tench (15) and common carp (15) can synthesise n-3 HUFA from dietary C18:3n-3. In the present trial, although experimental diets contained no HUFA, including C20:5n-3 and C22:6n-3, the mentioned fatty acids were found in the muscle tissues of common carp fed with experimental diets (both control diet and diets supplemented with LO) for 75 days, preventing essential fatty acids (EFA) deficiency and also suggesting that the common carp was able to synthesise n-3 HUFA from the C18:3n-3 in the diets. Besides that, in the muscle

tissue of groups, the presence of C20:4n-6 formed from C18:2n-6 upholds this theory. In the present experiment, increasing levels of C22:5n-3 and C22:6n-3 in the common carp fillets relative to their respective dietary levels were observed and they induced the bioconversion of C20:5n-3 to C22:6n-3. A similar pattern of selective storage was observed in n-6 fatty acids in which increased retention was noted in C20:4n-6. The changes in muscle fatty acid composition suggest selective utilisation or deposition of individual fatty acids as previously reported for cyprinid omnivorous fish species (5, 13, 14, 15, 18, 22, 30). It should be emphasized that the main consequence of higher C18:3n-3, C20:5n-3, C22:5n-3 and C22:6n-3 and lower C18:2n-6 contents in the fillets of the common carp was the increased value of the n-3/n-6 ratio compared to the levels of this ratio in the diets. However, in the case of the C group, this value was significantly lower than that in the groups fed diets supplemented with LO. The abundance of C18:3n-3 apparently fulfils the essential fatty acids needs of the common carp in this study. This is presumably due to the ability of common carp to selectively retain C22:6n-3 and bioconvert C18:3n-3 to C20:5n-3 and C22:6n-3; C20:5n-3 to C22:6n-3, and C18:2n-6 to C20:4n-6. Results obtained in the present study suggest that common carp have high tolerance to diets that differ significantly in lipid content and fatty acid composition. In the present research, n-6/n-3 ratios of all experimental groups were below 4.0, which is in accordance with the recommendation of Simopoulos (19) for human nutrition. The PUFA/SFA ratio was the highest in Diet L5 (2.82), mostly due to the very high content of C18:3n-3, which was 33.9 % in this sample. The lowest level of PUFA/SFA ratio recommended by WHO and FAO is 0.4 (33) and all experimental diets comply with this recommendation. The mentioned differences are even more obvious in the fatty acid composition of carp meat. All groups fed with the experimental diets supplemented with LO had a better n-6/n-3 ratio than the control group, and the common carp fed with diet L5 showed the best results with a n-6/n-3 ratio of 0.24. In this group, C20:5n-3 content increased about seven times in comparison to the control group (1.31% vs. 0.18 %), while no significant differences were observed between the common carp fed diets supplemented with LO. C22:6n-3 content increased 1.9 times (2.9% vs. 1.54%) and significant differences were observed between groups fed diets with different levels of LO. It can be concluded that the addition of higher concentrations in fish feed (4 or 5%) gave more favorable fatty acid composition of carp meat than the addition of LO in lower concentrations (2 or 3%) and especially more than the control group without the addition of LO. Based on the obtained results, we conclude that LO in the diet in concentrations of 4 or 5% leads to satisfactory production performance, as well as favorable proximate composition of muscles and lipid quality regarding fatty acid composition of common carp. The significance of the present study also relies on the fact that the study was conducted in natural atmospheric conditions, while the majority of the previous studies (11, 12, 13, 14) were conducted in indoor closed tanks; so the results of this study are more applicable to the aquaculture industry.

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Reference

1. Balk EM, Lichtenstein AH, Chung M, Kupelnick B, Chew P, Lau J. Effects of omega-3 fatty acids on serum markers of cardiovascular disease risk: a systematic review. Atherosclerosis 2006; 189: 19–30.

2. Topic Popovic N, Kozacinski L, Strunjak - Perovic I, et al. Fatty acid and proximate composition of bluefin tuna (*Thunnus thynnus*) muscle with regard to plasma lipids. Aquac Res 2012; 43: 722–9.

3. Sarma D, Akhtar MS, Das P, et al. Nutritional quality in terms of amino acid and fatty acid of five coldwater fish species: implications to human health. Natl Acad Sci Lett 2013; 36: 385–91.

4. Hathwar SC, Rai AK, Modi VK, Narayan B. Characteristics and consumer acceptance of healthier meat and meat product formulations: a review. J Food Sci Technol 2012; 49: 653–64.

5. Ljubojević D, Radosavljević V, Puvača N, et al. Interactive effects of dietary protein level and oil source on proximate composition and fatty acid composition in common carp (*Cyprinus carpio* L.) J Food Compost Anal 2015; 37: 44–50.

6. Mungkung R, Aubin J, Prihadi TH, Slembrouck J, van der Werf HM, Legendre M. Life cycle assessment for environmentally sustainable aquaculture management: a case study of combined aquaculture systems for carp and tilapia. J Clean Prod 2013; 57: 249–56.

7. Tacon AG, Metian M. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: trends and future prospects. Aquaculture 2008; 285: 146–58.

8. Merino G, Barange M, Blanchard JL, et al. Can marine fisheries and aquaculture meet fish demand from a growing human population in a changing climate? Glob Environ Change 2012; 22: 795–806.

9. Bell JG, Dick JR, Strachan F, Guy DR, Berntssen MH, Sprague M. Complete replacement of fish oil with a blend of vegetable oils affects dioxin, dioxin-like polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) in 3 Atlantic salmon (*Salmo salar*) families differing in flesh adiposity. Aquaculture 2012; 324: 118–26.

10. Thanuthong T, Francis DS, Manickam E, Senadheera SD, Cameron - Smith D, Turchini GM. Fish oil replacement in rainbow trout diets and total dietary PUFA content: II. Effects on fatty acid metabolism and in vivo fatty acid bioconversion. Aquaculture 2011; 322: 99–108.

11. Du ZY, Liu YJ, Tian LX, He JG, Cao JM, Liang GY. The influence of feeding rate on growth, feed efficiency and body composition of juvenile grass carp (*Ctenopharyngodon idella*). Aquac Int 2006; 14: 247–57.

12. Du ZY, Clouet P, Huang LM, Degrace P, Zheng WH, He JG. Utilization of different dietary lipid sources at high level in herbivorous grass carp (*Ctenopharyngodon idella*): mechanism related to hepatic fatty acid oxidation. Aquac Nutr 2008; 14: 77–92.

13. Zakęś Z, Jankowska B, Jarmolowicz S, Zmijevski T, Partyka K, Zakęś DK. Effects of different dietary fatty acids profiles on the growthperformance and body composition of juvenile tench (*Tinca tinca* L.). Rev Fish Biol Fisheries 2010; 20: 389–401.

14. Sun S, Ye J, Chen J, Wang Y, Chen L. Effect of dietary fish oil replacement by rapeseed oil on the growth, fatty acid composition and serum non-specific immunity response of fingerling black carp, *Mylopharyngodon piceus*. Aquac Nutr 2011; 17: 441–50.

15. Ljubojević D, Ćirković M, Novakov N, et al. Comparison of meat quality of tench, *Tinca tinca*, reared in extensive and semi-intensive culture systems. J Appl Ichthyol 2014; 30: 50–7.

16. Steffens W, Wirth M. Influence of nutrition on the lipid quality of pond fish: common carp (*Cyprinus carpio*) and tench (*Tinca tinca*). Aquac Int 2007; 15: 313–9.

17. Łukaszewicz M, Szopa J, Krasowska A. Susceptibility of lipids from different flax cultivars to peroxidation and its lowering by added antioxidants. Food Chem 2004; 88: 225–31.

18. Turchini GM, Moretti VM, Mentasti T, Orban E, Valfre F. Effects of dietary lipid source on fillet chemical composition, flavour volatile compounds and sensory characteristics in the freshwater fish tench (*Tinca tinca* L.). Food Chem 2007; 102: 1144–55.

19. Simopoulos AP. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Exp Biol Med 2008; 233: 674–88.

20. Keshavanath P, Manjappa K, Gangadhara B. Evaluation of carbohydrate rich diets through common carp culture in manured tanks. Aquac Nutr 2002; 8: 169–74.

21. Spirić A, Trbović D, Vranić D, Đinović J, Petronijević R, Matekalo - Sverak V. Statistical evaluation of fatty acid profile and cholesterol content in fish (common carp) lipids obtained by different sample preparation procedures. Anal Chim Acta 2010; 672: 66–71.

22. Kukačka V, Chalopkova L, Fialova M, Kopp R, Mareš J. The influence of linseed oil and fish oil supplements to the fatty acid spectrum of common carp (*Cyprinus carpio* L.) muscle. Acta Univ Agric Silvic Mendel Brun 2009; 57 (5): 193–2.

23. Bogut I, Has - Schön EM, Čačić M, Milaković Z, Novoselić D, Brkić S. Linolenic acid supplementation in the diet of European catfish (*Silurus glanis*): effect on growth and fatty acid composition. J Appl Ichthyol 2002; 18: 1–6.

24. Pei Z, Xie S, Lei W, Zhu X, Yang Y. Comparative study on the effect of dietary lipid level on growth and feed utilization for gibel carp (*Carassius auratus* gibelio) and Chinese longsnout catfish (*Leoiocassis longirostris* Günther) Aquac Nutr 2004; 10: 209–16.

25. El-Marakby M. Effect of dietary sources and levels of lipids on growth performance and feed utilization of fry Nile tilapia, *Oreochromis ni-*

loticus (L.) (Teleostei: Perciformes). J Fish Aquat Sci 2006; 1: 117–25.

26. Chou BS, Shiau SY. Optimal dietary lipid level for growth juvenile hybrid tylapia, *Oreochromis niloticus* X *Oreochromis aureus*. Aquaculture 1996; 143: 185–95.

27. Ljubojević D, Ćirković M, Đorđević V, et al. Fat quality of marketable fresh water fish species in the Republic of Serbia. Czech J Food Sci 2013; 31: 445–50.

28. Ljubojević D, Ćirković M, Novakov N, et al. Productivity and meat nutrient in fish: the diet effect. Kafkas Univ Vet Fak Derg 2013; 19: 43–9.

29. Steffens WM, Rennert B. Effects of adding variousoils to the diet on growth, feed conversion and chemicalcomposition of carp (*Cyprinus carpio*). Arch Anim Nutr 1995; 47: 381–9.

30. Pozernick M, Wiegand MD. Use of canola oil in the feed of larval and juvenile goldfish, *Carassius auratus* (L.). Aquac Res 1997; 28: 75–83.

31. Torstensen BE, Lie O, Froyland L. Lipid metabolism and tissue composition in Atlantic salmon (*Salmo salar* L.): effects of capelin oil, palm oil, and oleic acid-enriched sunflower oil as dietary lipid sources. Lipids 2000; 35: 653–64.

32. Nanton DA, Vegusdal A, Rora AMB, Ruyter B, Baeverfjord G, Torstensen BE. Muscle lipid storage pattern, composition, and adipocyte distribution in different parts of Atlantic salmon (*Salmo salar*) fed fish oil and vegetable oil. Aquaculture 2007; 265: 230–43.

33. FAO/WHO. Report of the WHO/FAO Joint Expert Consultation. Diet nutrition and the prevention of chronic diseases. Geneva : World Health Organization, 2003. (Technical Report Series, No. 916)

POVPREČNA ODZIVNOST KRAPOV NA RAZLIČNE KONCENTRACİJE LANENEGA OLJA V HRANİ

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Povzetek: Mladice krapa (*Cyprinus carpio* L.) so bile krmljene 75 dni s petimi različnimi krmili v treh ponovitvah, ki so vsebovala 0, 2, 3, 4 ali 5 % lanenega olja, z namenom določanja učinka različnih koncentracij lanenega olja na rastne parametre kakor tudi na sestavo neposredne in maščobno-kislinske sestave mesa rib. Ribe so imele povprečno začetno težo 200 g in so bile nastanjene v 15 kletkah. Ribe, ki so bile krmljene s krmili, dopolnjenimi s 3, 4 ali 5 % lanenega olja, so imele bistveno boljše kazalnike rasti v primerjavi z ribami, ki so prejemale krmila, dopolnjena z 0 ali 2 % lanenega olja. Vsebnost maščob v mišicah se je povečala z 1,25 % v kontrolni skupini brez dodatka lanenega olja na 1,46 %, 1,56 %, 1,94 % in 2,37 % v skupinah rib, krmljenih s krmo, dopolnjeno z 2, 3, 4 oziroma 5 % lanenega olja. Maščobno-kislinski profili mišičnega tkiva so pokazali znatno povečanje ($p < 0,05$) vsebnosti maščobnih kislin 18:3n-3 pri ribah, krmljenih s krmili, ki so vsebovala 2, 3, 4 oz. 5 % lanenega olja. Druge maščobne kisline, ki so se v mišičnem tkivu rib, krmljenih s krmili, dopolnjenimi z lanenim oljem, znatno povečale ($p < 0,05$) so bile: eikozanojska kislina (C20: 3n-3), eikozapentanojska kislina (EPA, C20: 5n-3), dokozanojska kislina (DPA, C22: 5n-3), dokoheksanojska kislina (DHA, C22: 6n-3) kakor tudi polinenasičene maščobne kisline (PUFA), skupne n-3 maščobne kisline, spremenilo pa se je tudi razmerje maščobnih kislin n-3 : n6. Maščobne kisline, katerih vsebnost se je bistveno zmanjšala ($p < 0,05$) so mononenasičene maščobne kisline (MUFA), pa tudi alfa linolne kisline (LA C18: 2n-6). Ribe, ki so bile krmljene s krmili s 5 % lanenega olja, so imele trikrat več n-3 maščobnih kislin (24,02 %) kot ribe iz kontrolne skupine (7,5 %). Razmerje n-3/n6 je bilo ugotovljeno v razponu od 0,59 pri ribah iz kontrolne skupine do 4,14 pri ribah, krmljenih s krmili s 5 % lanenega olja, predvsem zaradi razlike v vsebnosti alfa-linolenske kisline (ALA, C18: 3n-3). Vključitev 5 % lanenega olja v krmi je pokazala najugodnejše učinke na vsebnost esencialnih maščobnih kislin v mesu krapov, pa tudi drugih testiranih parametrov.

Ključne beside: *Cyprinus carpio*; gojenje; kemična sestava mišic; maščobne kisline; parametri rasti; prehrana

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Original Scientific Article

ANTIBACTERIAL PROPERTIES OF A NON-THERMAL, ATMOSPHERIC, OPENAIR®, PLASMA JET IN SURFACE DECONTAMINATION OF EGGS IN SHELL

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Summary: In the European Union, eggs may not be washed or cleaned. So, in order to reduce food safety risks, several techniques for eggs in shell decontamination have been developed. The current study was undertaken to determine the potential of the Non-thermal, Atmospheric, Openair® Plasma Jet for surface decontamination of eggs in shell. In the experiment Polyethylene Theraphtalate plates and the eggshells of table eggs were exposed to single or multiple treatments with a Non-thermal, Atmospheric, Openair® Plasma Jet. This resulted in up to > 3 log reduction of *Staphylococcus aureus* (*S. aureus*) (NCTC 8325) on Polyethylene Theraphtalate and 1.8–2.5 log reduction of a common number of native aerobic mesophylic bacteria and S. aureus (NCTC 8325) on eggshells in a treatment time of 10–60 seconds. Ionising gas of the plasma jet was obviously not harmful to eggshell cuticle, since no significant alterations to plasma-treated eggs were found, or to physico-chemical properties of the contents of plasma-treated eggs, such as: air cell height, pH, the whole weight of eggs, or height of the thickness of egg white, which did not significantly differ from the untreated eggs during 54 days of aging. The results of the experiment indicate that the treatment of eggshells with the plasma jet has the potential for egg in shell decontamination with no side effects on egg quality, which is important as far as food safety and quality characteristics that are acceptable to consumers.

Key words: egg in shell; non-thermal; openair; atmospheric plasma jet; aerobic mesophylic bacteria; *S. aureus*; decontamination

Introduction

Salmonella, Listeria, *Escherichia coli* and *Campylobacter* are the most often found food contaminants (1, 2, 3, 4, 5, 6). Contaminated food can pose a considerable risk. For this reason, besides obligatory bio-security measures in the food industry, new approaches, additional, and alternative methods for food decontamination are still developing (7, 8). Among numerous methods of decontamination, plasma is one of the most important innovations for preventing food contamination (9, 10, 11). The main function of the plasma decontamination activity is in its diffusion of highly energetic reactive species (OH radicals and NO), oxygen atoms, and UV photons in a bacterial cell, resulting in irreversible damages to DNA and vital cell macromolecules (1, 12, 13, 14). Therefore, novel methods for plasma generation, and above all the development of technology by which the plasma is generated at atmospheric pressure in ambiental temperatures (15, 16, 17), are contributing to new approaches in safe

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and environmental-friendly food processing. Due to the demands for absolute cleanliness also in food processing plants, different types of plasma were examined as alternatives to chemical and certain biological decontamination procedures and decontaminants. For these purposes plasma, as a non-chemical agent, used without water or other solvents, leaving no residue, has an important potential in terms of food safety and environmental protection (15, 18, 19). Whereas, the use of plasma was primarily directed on the surface decontamination of food premises and packing material, some recent investigations have been directed to using plasma directly on foods. Some studies of the non-thermal plasma surface treatment of chicken meat and chicken skin, in order to prevent *Campylobacter* contamination, have been already conducted (20, 21, 22, 17). Similar investigations have been made for inactivation of *Listeria innocua* in ready-to-eat meat products (22), meanwhile investigations, concerning the advantages and weaknesses of plasma used directly on food matrices, especially for decontamination of *Salmonella* spp., *Campylobacter, Streptococci*, *E.coli*, and *Lactobacillus*, were conducted as well (6, 21, 22, 10, 1). Nevertheless, investigations of plasma used for food decontamination, and above the all, for decontamination of commercial table egg in shell (14), are not numerous. This is a lack of important information, because until the beginning of the last decade, table eggs in shell were among the most important *Salmonella*-contaminated foods in the EU and USA. Therefore, in order to protect consumers from *Salmonella*, an integrated approach to food safety from the farm to the fork has been adopted in last five years in EU. However, the risk of contamination still exists, which is why some recent investigations focused on searching for innovative methods of table egg decontamination (23). For example given the ban of hen battery cages rearing in the EU (European Union Council Directive 1999/74/EC), several studies (RESCAPE project) have been conducted, searching how to diminish risk factors for potential egg contamination (24) by introducing the alternative egg-laying hen rearing systems. In recent years some innovative methods for eggshell decontamination, which do not include washing or chemical sterilization (25, 26), were investigated. Among the different methods, also non-thermal plasma for eggshell decontamination was explored, to ensure safer egg handling, and to prevent the penetration of (not necessary pathogenic) micro flora from the eggshell to its contents (27). However, only a few studies in this field have achieved this goal. Amongst them, only Ragni *et al.* (2010) (28), and Vaninni *et al.* (29) reported about eggshell decontamination (*Salmonella enteritidis*) with the Resistive Barrier Discharge (RBD) gas plasma under atmospheric conditions. So, with respect to the lack of information in this field, the aim of our study was to establish possible implications of the Non-thermal, Atmospheric, Openair® Plasma Jet (Plasmatreat) for egg in shell decontamination. The most important goal of this research was to determine whether it is possible to decontaminate eggshells in ambiental conditions without damaging of the eggshell cuticle, and contemporary retaining all the characteristics of high-quality table eggs in shell.

Materials and methods

Non-thermal, Atmospheric, Openair® Plasma Jet and surface treatments

A Non-thermal, Atmospheric, Openair® Plasma Jet (Plasmatreat) (AOPJ) is a special type of highly energized plasma (30, 16, 17, 31, 27) generated in the process of compressed gas discharging in high electricity voltage and in a pulsed electric arc. This type of plasma is especially useful in industrial processes where absolute surface purity is needed, such as micro-cleaning of plastic, metal, glass, ceramic, and other materials (30). An AOPJ is generated in the neutral atmospheric air pressure at room temperature inside the reaction chamber between the electrode and dielectric barriers, producing a non-equilibrium discharge (normally 5–10 kV; in our experiment 1 kV) at a working frequency of 21 kHz, and at a power range from 500 to 1000 W. The plasma jet carrier is compressed (6 bar), oil free (max. concentration of oil in air: 0.1 mg/m^3 at 20°C), and filtered air (99.9% particles reduction in diameter up to <0.03 μm), which streams on the surface as a jet through the reaction chamber of the jet head (Figure 1). Consequently, the plasma jet treatment results in strong activation of material surface (30), so, in the present experiment an AOPJ was used as a source of plasma jet energy for surface eggshell decontamination.

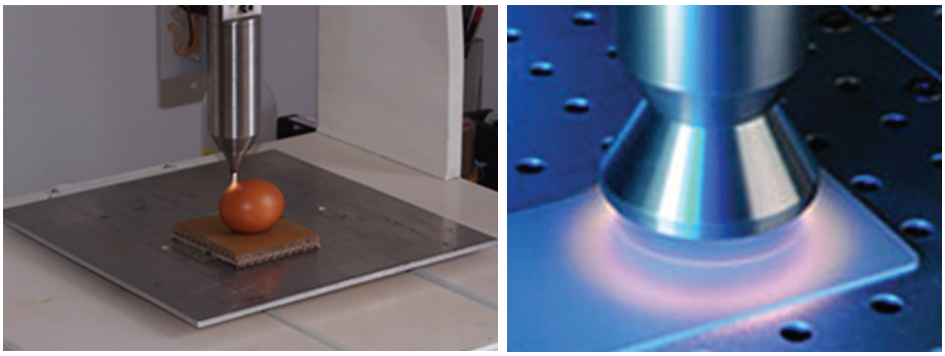

Figure 1: AOPJ head A (Left); OPJ head B (Right) (Plasmatreat, 2014)

Two types of AOPJ head nozzles were used in experiment. AOPJ head A is a static (firm) nozzle intended for the linear surface treatment in the width of 8–16 mm, which depends on the distance between the nozzle and treated surface (from 4 to 20 mm) (Figure 1, left). AOPJ head B is a rotary nozzle which rotates at 2000 RPM, forming a ring of spinning plasma jet in an outlet angle of 25°, enabling a circular surface treatment in the width of 40 mm, regarding the distance between the nozzle and treated surface (from 5 up to 20 mm) (Figure 1, right).

AOPJ heads A and B were constructed to move above the treated surfaces, following the component geometry with exact precision, driven by the software programmed robot. In the experiment they were regulated to follow the curve line of experimental eggs in shell surface (Figure 1, left) and/or the flat shape (Figure 1, right) of experimental Polyethylene Theraphtalate (PET).

In order to find the optimal decontamination efficiency, an AOPJ was tested in different experimental conditions, e.g. with regard to: distances of the heads A and B to the treated surface (10 mm, 15 mm, 20 mm), head speeds (5 cm/s and 10 cm/s, which is equivalent to 1/50 s/mm and 1/100 s/mm), and treatments recurrences (single or triple). Treatment times were in range from 10 – 60 s depending on the width of the AOPJ stream and area of tested surfaces. Therefore, with head A, PET and eggshells need to be treated in 5 parallel lines, or in 3 parallel lines when head B was employed.

An antimicrobial test of an AOPJ was performed in a preliminary test on PET, and in an experiment on eggs in shell. The antimicrobial efficiency of an AOPJ was experimentally tested on native aerobic mesophylic microorganisms on eggshells, and on a test microorganism *S. aureus* (NCTC 8325). The reasoning behind this is in farm egg production *S. aureus* is among the most common eggs contaminants (32). Thus *S. aureus* was used as the test microorganism on PET, wherein irrespective to the variability in quantity of native aerobic mesophylic microorganisms on eggshells from egg to egg, part of the eggs were additionally contaminated with the suspension of *S. aureus* of a known concentration (10^6 CFU/ml) in order to get a more accurate AOPJ antibacterial efficiency evaluation. So, the common number of bacteria on eggshells consists of common counts of native aerobic mesophylic microorganisms and *S. aureus*.

Preliminary test of AOPJ treatment of Polyethylene Theraphtalate (PET)

In order to investigate the potential antimicrobial activity of an AOPJ on PET, flat surfaces of PET plates (6 cm x 3 cm) were covered with suspension of *S. aureus* of known concentration (10^6 CFU/ml) and treated with head A and head B. *S. aureus* was chosen as a reference material for aerobic mesophylic bacteria. For this purpose, 65 PET plates were artificially coated with *S. aureus* on the surface of 18 cm^2. Thirty PET plates were treated with head A, and 30 with head B, while 5 PET plates were used as a control (Table 1). The results were presented as a total count of *S. aureus* (TC *S.a.*).

AOPJ treatment of eggs in shell

The antibacterial efficiency of AOPJ on the medium-sized table eggs (54-62 g) in shell from the same age stable and rearing technology was analysed. So, eggshell surfaces of naturally contaminated eggs with native aerobic mesophylic

bacteria and eggs additionally coated with *S. aureus* were treated with head A and head B (Table 1). In order to perform tests on 120 eggs, two test surfaces (each 4 cm^2) were marked on each egg on the opposite sides of the eggshell (direction east-west) (Figure 2). One side of the egg was AOPJ-treated, while the opposite side was not, and served as a control. Immediately after an AOPJ treatment, swabs were taken (test surfaces 4 cm^2) from both sides of egg for microbiological analysis. The results of the total number of native aerobic mesophylic microorganisms were presented as a total viable count (TVC). Meanwhile, when *S. aureus* was additionally coated, the common counts of bacteria on eggshells were presented as the sum of native aerobic mesophylic microorganisms and *S. aureus* (TVC + TC *S.a.*). The experiment was performed in conditions of the air temperature 22°C and 55% relative humidity, which were monitored using Testo 350 M/XL 454. Temperatures of the treated surfaces were monitored using an infra-red camera (Testo 881).

Microbiological and physico-chemical properties of eggs during the period of 54 days (in 7 days intervals starting from day the 4th) after AOPJ treatment

For determining the differences in microbial and physico-chemical properties of AOPJ-treated and untreated eggs, eggshell surfaces of naturally contaminated, medium-sized table eggs (n=189) with native aerobic mesophylic bacteria, and eggs (n=19) additionally coated with *S. aureus* (were treated with head A (Table 2). Sampling was performed in 7 days intervals, starting from 4th day thus after the treatment, the TVC on eggshells was analysed on the 4th, 11th, 18th, 25th, 32nd, and 54th day, meanwhile the TVC + TC *S.a.* in egg contents was determined on the 18th and 32nd day.

Table 1: AOPJ treatment of PET and eggshell and microbiological analysis

AOPJ surface treatment	No. of samples (PET/egg)	Tested surface	Head A	Head B	Aerobic mesophylic bacteria analysis	Additional coating and *S. aureus* analysis
PET test surface	30	18 cm^2	yes			yes
	30	18 cm^2		yes		yes
PET control surface	5	18 cm^2	no	no		yes
Eggshell						
test surface	50	4 cm^2	yes	no	yes	no
control surface	50	4 cm^2	no		yes	no
Eggshell						
test surface	30	4 cm^2	yes	no	no	yes
control surface	30	4 cm^2	no			yes
Eggshell						
test surface	40	4 cm^2	no	yes	no	yes
control surface	40	4 cm^2		no		yes

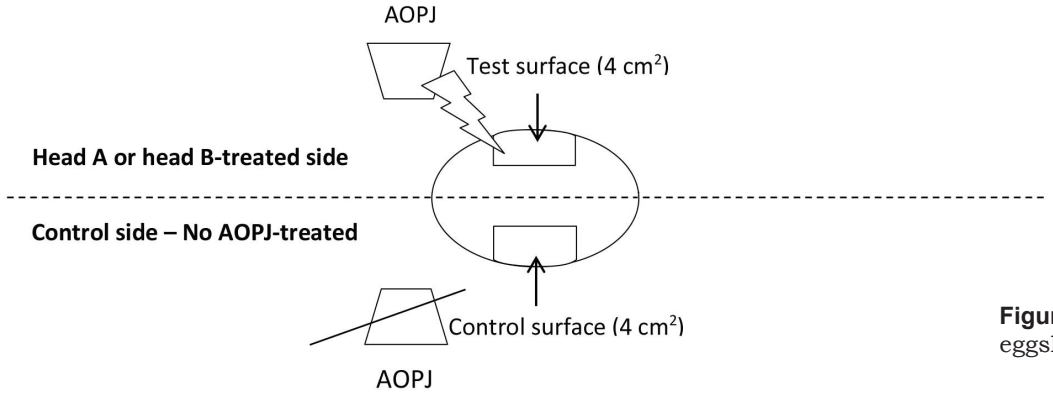

Figure 2: AOPJ treatment of eggshell

Table 2: AOPJ treatments (head A) of eggshell and tests for 54 days after treatment

Head A eggshell surface treatment	No. of eggs for TVC on eggshell	No. of eggs with additional coating *S. aureus*	No. of eggs for TVC+ TC *S.a.* in egg content	No. of eggs for egg weight	No. of eggs for egg pH values	No. of eggs for air cell height	No. of eggs for thick egg white height
test eggs	80	19	33	10	6	30	30
control eggs	80	19	33	10	6	30	30

Eggs were weighed on an analytical balance (XP 205, Mettler Toledo, accuracy of 0.1 mg weekly one the 4th, 11th, 18th, 25th, 32nd, and 54th day after treatment. The air cell height of eggs (mm) were determined weekly at 4th, 11th, 18th, 25th, and 32nd day after treatment, using the air cell measurer and bright light to 'candle' the egg. For the analysis of the egg contents we cracked the eggs under sterile conditions. The pH values of albumen were measured using a pH meter (PHM210 MeterLab, Radiometer analytical, and accuracy of 0.01) on the 32nd day of experiment. The height (mm) of a thick egg white was measured using a micrometer at 4th, 11th, 18th, 25th, and 32nd day after treatment. During the experiment eggs were kept in conditions with a constant temperature of 22°C and a relative humidity of 60%.

Diagnostics of aerobic mesophylic bacteria (TVC)

Samples for aerobic mesophylic bacteria enumeration were taken as swabs from the PET plates (18 cm^2) and the eggshell test surfaces (4 cm^2), and in egg contents (20 ml) by egg cracking in sterile conditions. Laboratory analysis of mesophylic microorganisms and *S. aureus* (TVC + TC *S.a.*) were performed with the same procedures in accordance with the ISO standard 4833-1:2013, while a results interpretation was performed in accordance with the ISO standard 7218:2007/ A1:2013. The TVC was calculated as CFU/cm^2 or CFU/g, depending on the sampling matrix.

Scanning electron microscopy (SEM) of eggshell

Since a plasma jet contains highly energetic species which can be potentially harmful for the eggshell cuticle, the scanning electron microscopy (Field emission SEM microscope JEOL 7600F) was used to screen the surface of the eggshell. Prior to the SEM investigation the surface of the eggs was coated with 3nm thick layer of carbon using a Precision Etching Coating System (Gatan, model 682). The microscope was operating at 10 kV and working distances were from 8.0 to 4.5 mm. For images taken at low magnification (LM-from 25 to 80000 times), a lower secondary detector for lower resolved secondary electrons (LEI) was used, while an upper secondary detector (SEI) was used for images taken at higher magnifications. Cuticle surface damage was estimated by the appearance of differentiation among eggshell images of the AOPJ-treated and untreated (control) eggs at x400, x1000 and x20,000 magnifications. The evaluation was based on the density of visible cuticle cracks, their width, and the edges sharpness, while at high magnifications the density and distribution of visible Ca, Mg, P spherules (80–300 nm in diameter) were evaluated.

Results evaluation

Statistical evaluation of results was carried out by ANOVA, t-test, and correlation analyses using the GraphPad Prism 6 computer programme (GraphPad Software, Inc., USA, 2014). The Pearson product-moment correlation and linear regressions ABS versus time were accepted for $r > 0.95$, and values of the slopes less than $P<0.05$ were considered statistically significant. Counts of the mean TVC were calculated in common logarithms ($\log_{10}$) ± SD, while the percentage (%) of TVC reduction was calculated in the absolute numbers of TVC. The term 'log reduction' is used as the total reduction of microorganisms determined by the following formula: log reduction = $\log_{10}$ initial population – $\log_{10}$ final population (e.g. 3 log reductions = 99.9% kill rate).

Results

The antibacterial effects of the AOPJ treatments on Polyethylene Theraphtalate (PET)

The results depicted in Figure 3 and Table 3 show the bactericidal activity of the AOPJ head A and B treatments on the PET. In a mutual comparison head A showed a 24% higher mean bactericidal efficiency than head B. The median values of the total count (log_{10}) of *S. aureus* (TC *S.a.*) showed significant (P<0.0001) difference (1.73±0.89, r=0.15) in bactericidal activity between treatments with head A and head B (Figure 3). The highest bacterial efficiency of 3.15 log reductions was achieved using head A at a distance of 20 mm from the surface, with a speed of 5 cm/s and triple successive treatments (Table 3).

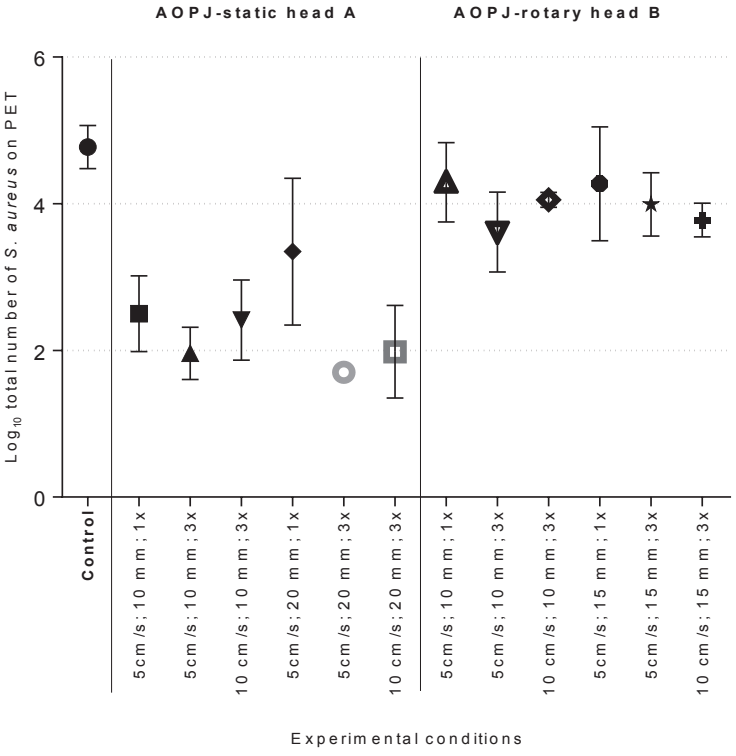

Figure 3: Mean of total number (with SD in error bars) of the TC *S.a.* (log_{10} CFU/cm^2) after head A and B treatments of PET plates coated with *S. aureus* with regard to the untreated PET plates (control) in different experimental conditions

Table 3: Mean differences in total number of the TC *S.a.* (log_{10} CFU/cm^2) after jet head A and B treatments of PET plates with regard to untreated PET plates (control), considering the mean of experimental conditions and in experimental conditions in which the highest differences of *S. aureus* were recorded and the log reduction of the TC *S.a*

PET surface AOPJ to control	head A	head B	head A	head B
Experimental conditions	mean	mean	5 cm/s; 20 mm; 3×	5 cm/s; 10 mm; 3×
Mean differences in TC *S.a.*	-2.27±0.49, r=0.35, P=0.0005	-0.48±0.58, r=0.10, P=0.14	-3.07±0.29, P<0.0001	-1.16±0.48, r=0.44, P=0.006
TC *S.a.* log reduction	1	<1	3.15	1
TC *S.a.* reduction (%)	98.2	74	99.93	89.4

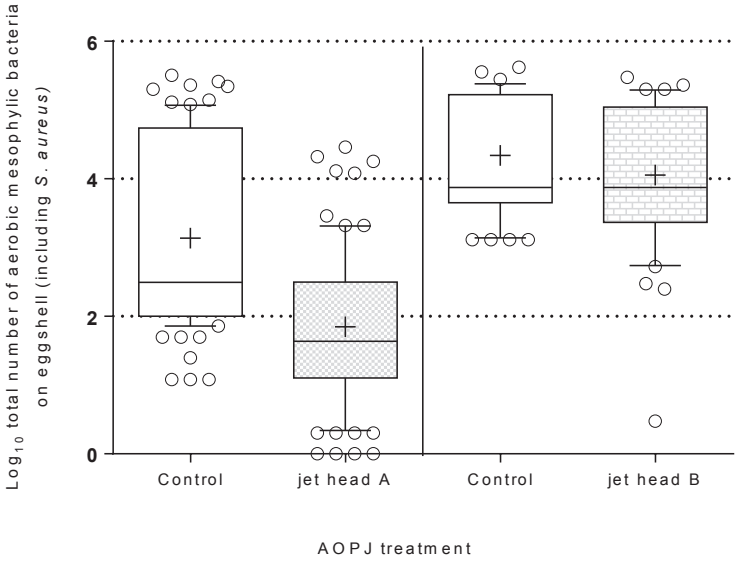

Figure 4: Boxplot of the number of TVC + TC *S.a.* colonies (log_{10} CFU/cm^2) on the eggshells after the head A and B treatments considering the mean of experimental conditions, with regard to the untreated (control) eggshells

Comparisons are presented separately with respect to the treatment with head A or head B. The median value of each distribution is shown with a horizontal line within each box, while the + marks the mean. The boundary of the box closest to zero indicates the 25th percentile, and the boundary farthest from zero indicates the 75th percentile. Whiskers (error bars) above and below the box indicate the 90th and 10th percentiles. The outliers are shown as dots.

Table 4: Mean differences in the total number of the TVC + TC *S.a.* (log_{10} CFU/cm^2) after head A and B treatments of AOPJ-treated eggshells with regard to the untreated (control) eggshells considering the mean of experimental conditions and the log reduction of TVC and TC *S.a.* on the eggshells

Eggshell surface AOPJ to control	head A		head A	head B
Experimental conditions	mean		mean	mean
Mean differences in TVC + TC *S.a.*	-1.29±0.90, r=0.73, P<0001	mean differences in TC *S.a.*	-1.62±0.62, r=0.62, P<0001	-0.28±0.75, r=-0.72, P=0.02
TVC + TC *S.a.* log reduction	1.36	TC *S.a.* log reduction	1.33	< 1
TVC + TC *S.a.* reduction (%)	95.69	TC *S.a.* reduction (%)	95.35	29.1

The antibacterial effects of the AOPJ treatments (using head A and B) on the TVC on an eggshell surface

The results presented in Figure 4 show the bactericidal activity of the AOPJ treatments (head A, head B) on the eggshell surfaces. The median number (log_{10}) of the TVC and TC *S.a.* colonies from eggs treated with head A and head B was significantly (P<0.0001) different (2.12±1.64, r=-0.22), wherein head A had a 66% higher antibacterial efficiency as was attained by the treatment with head B. Thus, in further assays only head A was still tested with regard to significant higher bactericidal efficiency, as was depicted by head B.

In Figure 5 and Table 5 the mean differences (log10 CFU/cm2) and the log reduction of TVC + TC S.a. after head A treatments in different experimental conditions were presented. Most significant 1.8 - 2.5 log reductions were depicted in experimental conditions when eggshells were head A-treated in triple successive treatments (Table 5).

Owing to head A treatments, the contact maximum temperatures (T_{max}) of the eggshells varied in the range 53-80°C (average 66°C) for not more than 1/50 or 1/100 sec, but never exceeded 80°C.

Microbiological and physico-chemical properties of the eggs during the 54 days after head A treatment

In the analysis of egg properties during the 54 days, the mean of TVC + TC *S.a.* on the eggshells after treatment (5 cm/s; 20 mm; 3x), was significantly (r=0.38, P<0.0001) 99.54% lower (0.72±0.64) than the number (1.85±1.05) on eggshells on plasma untreated (control) group of eggs. Furthermore, the mean number of TVC + TC *S.a.* in egg contents of plasma treated and untreated (control) group of eggs

was under the limit of confidentiality, meanwhile the results of TVC were negative or less than 40 CFU/ml, thus comparisons were not possible. The mean of differences (0.4 g) in whole weight between head A-treated eggs (58.66 g) and control eggs (59.08 g) was insignificant (-0.41±3.66, r=0.28, P=0.38). The mean of differences of air cell height values between the group of treated (4.27mm) and control group of eggs (4.35 mm) were insignificant as well (0.07±1.16, r=0.67, P=0.73), wherein almost no difference (-0.005±0.05, r=0.2, P=0.8) (mean = 9.31) was depicted in pH values between the egg contents of plasma-treated and untreated eggs. The height (mm) of a thick egg white was barely different (0.006±0.58, r=0.91, P=0.95) between treated (3.28 mm±0.26) and untreated eggs (3.27 mm±1.45).

Scanning electron microscopy (SEM) of the plasma (AOPJ) treated eggshell cuticle

In general, after the AOPJ head A treatment (5 cm/s; 20 mm; 3x), the eggshell surfaces examined by Scanning Electron Microscopy (SEM) looked slightly cleaner and more polished (Figure 6). By SEM estimating at x400 magnification, 25% more cracks on the treated eggshell cuticle were noticed (image B) than on the untreated (control) eggs (image A). At x1000 magnification it was depicted that the cracks sharpness and width (0.25–0.3 μm) of edges did not differ between the plasma-treated (image D) and control eggs (image C). Individual grains of Ca, Mg, P spherules (80–300 nm) were observed at the highest magnification

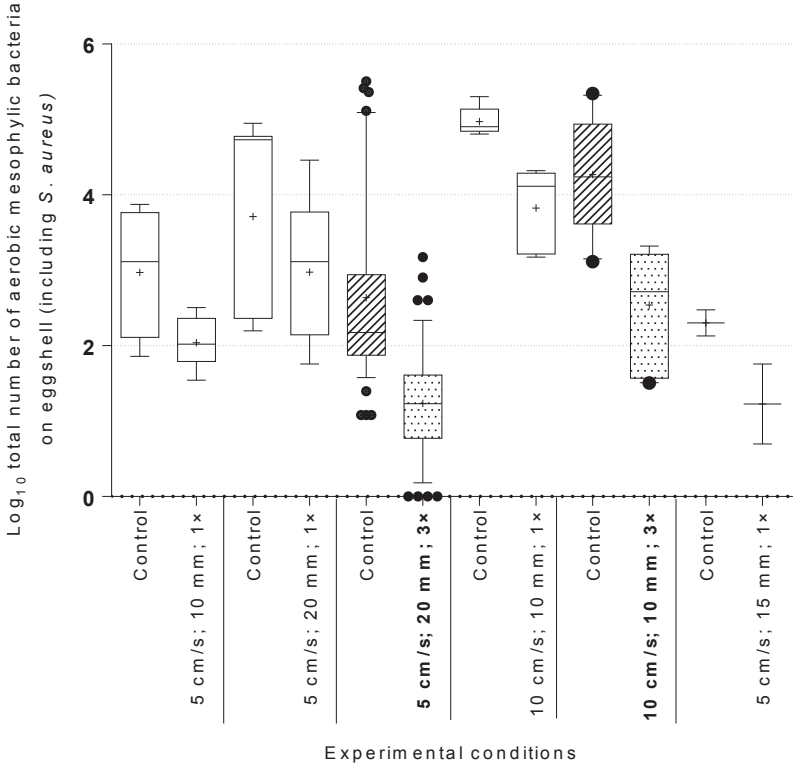

Figure 5: Boxplot of the number of TVC + TC *S.a.* colonies (log_{10} CFU/cm^2) on the eggshells after head A treatments in different experimental conditions with regard to the untreated (control) eggshells.

Comparisons are presented separately for the each experimental condition. Boxes with pattern present experimental conditions, where the most significant reductions of bacteria, were depicted. The median value of each distribution is shown with horizontal line within each box, while the + marks the mean. The boundary of the box closest to zero indicates the 25th percentile, and the boundary farthest from zero indicates the 75th percentile. Whiskers (error bars) above and below the box indicate the 90th and 10th percentiles. The outliers are shown as dots.

Table 5: Mean differences in total number of the TVC + TC *S.a.* (log_{10} CFU/cm^2) after head A treatments of AOPJ-treated eggshells with regard to the untreated (control) eggshells in different experimental conditions and the log reduction of the TVC and TC *S.a.* on the eggshells

Eggshell surface jet head A to control	5 cm/s; 10 mm; 1×	5 cm/s; 20 mm; 1×	5 cm/s; 20 mm; 3×	10 cm/s; 10 mm; 1×	10 cm/s; 10 mm; 3×	5 cm/s; 15 mm; 1×
Exposition time (s)	20	20	60	10	30	20
Mean differences in TVC + TC *S.a.*	-0.93±0.63, r=0.68, P=0.002	-0.73±0.68, r=0.86, P=0.01	-1.40±1.05, r=0.56, P<0.0001	-1.14±0.50, r=0.43, P=0.00	-1.73±0.36, r=0.88, P<0.0001	-1.07±0.99, P=0.36
TVC + TC *S.a.* log reduction	1.3	1	2.5	1	1.8	1.5
TVC + TC *S.a.* reduction (%)	92.4	85.2	99.7	89.4	98.4	85.6

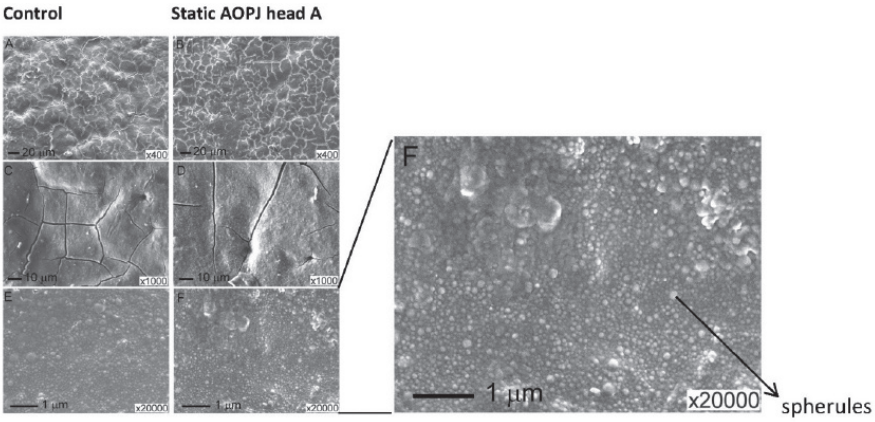

Figure 6. SEM images of AOPJ head A treated (5 cm/s; 20 mm; 3x) (images B, D, F) and plasma untreated eggshells (control) (images A, C, E) taken at magnifications x400, x1000, and x20,000

(x20,000) which are of approximately same density on the treated (image F), as was on the untreated (control) eggs (image E) (Figure 6).

Discussion

Assuring the most effective conditions enabling the highest antimicrobial effectiveness of an AOPJ, and to prevent side effects on the eggshell cuticles, the antimicrobial activity of static (head A) and rotary (head B) heads in different experimental conditions was compared. Already from our experiments on PET it has been ascertained that head A had a significantly higher antibacterial efficiency than head B, whose antibacterial efficiency did not exceed 1 log reduction. Actually, the reduction of bacteria (*S. aureus*) on the PET treated with head A was in the range of 1 – 3.15 log reduction, and was achieved in 20–60 seconds. The main reason for such difference is presumably on the concentration of the plasma jet streaming out of the head A nozzle, directly targeted to the treated surface, forming an 90°angle, meanwhile head B forms a wider 25° angle, which presumably reduces the energy of the plasma jet on the treated surface. Similar experiments were made by Noeske *et al.* (15) and Lommatzsch *et al.* (31), who tested the physical functionality of a plasma jet on polymers and polyethylene surfaces, and by Noriega *et al.* (19) who investigated the antimicrobial properties of cold atmospheric gas plasma-pen in desinfection of membrane filters establishing more than 3 log reductions of *L. innocua* in 10 seconds. According to the results of the experiment on PET, it was shown that the bactericidal effectiveness of an AOPJ strongly depended on influential experimental conditions. Besides the type of jet heads, the distance between the surface substrate and the exposition time are crucial for the optimal antimicrobial efficiency (16). We also showed that prolonging the exposition time and lowering the distance resulted in higher bactericidal activity, but it was also demonstrated higher risks for the eggshell cuticle alterations induced by the plasma's temperature, and the energy of ionised gas (reactive species OH radicals and NO). Therefore, in our experiment, the speed of the jet head was lowered presently with an increasing distance (10, 15, 20 mm) and *vice versa*, so the contact temperatures (T_{max}) of treated areas were in the average of 66°C. Baier *et al.* (33) reported about similar experiences in the experiment of decontamination efficiency of an atmospheric pressure plasma jet on fresh meats at distances of 13 and 18 mm, where the T_{max} never exceeded 25°C. Also, Liu *et al.* (34) studied an atmospheric plasma jet for the sterilisation of *S. aureus* on glass, where the surface temperature did not exceed 35°C. We showed in our experiment that neither surface temperatures nor the ionising gas of the plasma jet were obviously harmful to eggshell cuticle. Those findings were similar to experiments of Hyun *et al.* (3) who investigated a plasma jet for the inactivation of *Listeria monocytogenes* on agar and processed meat, at a distance of 40 mm, and have not reported about harmful effects on treated surfaces. The energy of plasma on surfaces can be moderated with shorter, but multiple plasma treatments, as was reported by Laroussi (35), who discussed the potential use of cold plasma on medical applications. Also, in our experiment it was found that for the successful reduction of bacteria with an AOPJ, multiple treatments were obligatory, since, in any experimental condition with a single application, the reduction

of bacteria was not greater than 1 log. This means that the sufficient treatment time for the AOPJ antimicrobial operation is neededd, although the surface of eggs should not be continuously exposed to a plasma operation due the high intensity of plasma jet. Thus, we showed an intermediate time for cooling of the eggshell surface is needed. Owing to that, in our experiment, to improve antimicrobial efficiency, an AOPJ was applied in short, intermittent multiple treatments, which has been repeated in at least 20 second intervals, to avoid side effects on the treated surfaces. We also showed that the enlarging of the distances of the AOPJ head to the treated surfaces, in combination with the high AOPJ head speeds, can predict immoderate raisings of the surface temperatures. The same statements were also considered by Rod *et al.* (21), who tested the antimicrobial effects of cold atmospheric plasma on deli meat in multiple 10 minutes intervals. In addition, plasma's high excitation frequencies are responsible for the higher plasma energy and stability (36). Thus, in order to protect the eggshell cuticle against excessive energy of the plasma jet, a relatively low frequency (21 kHz) of AOPJ was used in our experiment. In other similar experiments, plasma was used in a higher frequency range of 30 − 38 kHz (22, 20).

Therefore, the results of our experiment on the antibacterial properties of the AOPJ in surface decontamination of eggs in shell are represented in the <1−2.5 log reduction (29.1 – 99.7%) of TVC + TC *S.a.* with regard to untreated eggshells, and was achieved in 10−60 seconds, depending on the experimental conditions. Those results are similar to the studies of Ragni *et al.* (28), in which the eggshells were treated with non-thermal RBD (Resistive Barier Discharge) plasma (15 kV), and being found that the number of TVC was reduced in a range from 1 to 1.6 log reduction within an exposition time of 10−20 minutes, and even a 5.5 to 6.5 log reduction, although within 90 minutes of exposition. In addition, Liu *et al.* (34) reported about a 100% *S. aureus* reduction after an atmospheric non-thermal plasma jet treatment on a glass slide in 120 seconds, at the electro discharge of 18 kV. In both experiments a higher electric voltage and at least double exposition time for plasma jet treatments were used, as in our experiment. However, in our experiment the highest reduction of bacteria (up to 2.5 log) on eggshells was achieved with an AOPJ an electro discharge of 1 kV, within a treatment time of 60 seconds. Owing to a SEM analyses of eggshell cuticles (37), we assumed that the plasma jet treatment did not leave significant changes on elemental composition of the eggshell (38). From SEM images it can be seen that the appearance of the surface of the AOPJ treated eggshells seemed more polished, which is logical considering the cleansing properties of an AOPJ. However, no significant microscopic damages to the eggshell cuticle were evidenced during the experiment, since no significant alterations were found, with the exception of a slightly higher number of cuticle cracks on plasma-treated *vs.* untreated eggshells, but this did not affect the aging or higher contamination of eggs contents after 54 days. Similar statements was also confirmed by Ragni *et al.* (28) and Vannini *et al.* (29), who did not find significant changes on cuticle after plasma treatment of eggshells. No significant side effects to chicken meat or skin exposed to cold atmospheric gas plasma was determined in the experiment of Noriega *et al.* as well (19). Supposing an AOPJ alters the functionality of the eggshell cuticle, as the first line of defence against soil and bacterial penetration, more microorganisms can penetrate the eggshell, and so could be found in egg contents (38, 39). However in the experiment, during 54 days, the total numbers of bacteria in the egg contents of both: AOPJ treated and untreated eggs, were negative or less than 40 CFU/ml, which is under the limit of confidentiality. This result is evidence of unchanged cuticles of AOPJ-treated eggs. Another indication that an AOPJ did not influence the cuticle egg protective properties was evident in the investigated physico-chemical properties of the AOPJ-treated eggs as air cell height, pH, whole weight of eggs, or height of the thick of egg white (40), which did not significantly differ from the untreated eggs during aging. Indeed, a slightly higher whole weight and lower air cell height of AOPJ treated eggs were obtained, meanwhile negligible differences among the other investigated physico-chemical egg properties were established. This is important due to the stability of the mechanical properties of the cuticle responsible for resisting water transmission, bacterial penetration, and CO_2 losses, which slow down the natural decline of egg internal quality, and indicates an unchanged functional operation of the cuticle after the AOPJ treatment (41). So, no important influence of the AOPJ on the functional operation of cuticle was established, considering the experimental conditions in which eggshells were treated.

Conclusion

The results of the experiment of antibacterial properties of AOPJ in surface decontamination of eggs in shell demonstrated anitimicrobial efficiency in short operating time with no significant side effects on eggs quality and that is the main advantages of an AOPJ in decontamination of table eggs in shell. The running system should be developed in further investigations; meanwhile the experiment contributes to the knowledge of new approaches on how to diminish contamination of table eggs, and thus on improving food safety.

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References

1. Fernandez A, Thompson A. The inactivation of *Salmonella* by cold atmospheric plasma treatment. Food Res Int 2012; 45: 678–84.

2. Gurol C, Ekinci FY, Aslan N, et al. Low temperature plasma for decontamination of *E. coli* in milk. Int J Food Microbiol 2012; 157: 1–5.

3. Hyun JL, Heeso J, Wonho C, et al. Inactivation of *Listeria monocytogenes* on agar and processed meat surfaces by atmospheric pressure plasma jets. Food Microbiol 2011; 28: 1468–71.

4. Xu G, Zhang G, Shi X, et al. Bacteria inactivation using DBD plasma jet in atmospheric pressure argon. Plasma Sci Technol 2009; 11: 83–8.

5. Oyarzabal O. Reduction of *Campylobacter* spp. by commercial antimicrobials applied during the processing of broiler chickens: a review from the United States perspective. J Food Prot 2005; 68: 1752–60.

6. Kim B, Yun H, Jung S, et al. Effect of atmospheric pressure plasma on inactivation of pathogens inoculated onto bacon using two different gas compositions. Food Microbiol 2011; 28: 9–13.

7. Higgins SE, Wolfenden AD, Bielke LR, et al. Application of ionized reactive oxygen species for disinfection of carcasses, table eggs and fertile eggs. J Appl Poultry Res 2005; 14: 716–20.

8. Potts HE, Diver DA, Everest PC. Plasma decontamination of sealed packages. In: 30th International Conference on Phenomena of Ionized Gases, ICPIG. Belfast, 2011: D16 topic number.

9. Shama G, Kong MG. Prospects for treating foods with cold atmospheric gas plasmas. In: Machala Z, Hensel K, Akishev Y, eds. Plasma for bio-decontamination, medicine and food security: NATO science for peace and security series A: chemistry and biology. Dordrecht : Springer, 2012: 33–43.

10. Afshari R, Hosseini H. Atmospheric pressure plasma technology: a new tool for food preservation. In: International Conference on Environment, Energy and Biotechnology. Singapore: IPCBEE, 2012: 275–8.

11. Suckewer S. Excitation and ionisation of atoms and ions in a non-thermal plasma. I. populations of excited levels. J Phys B 1970; 3: 380–9.

12. Muranyi P. Novel decontamination technologies for fresh cut industry. J Food Process Technol 2012; 3: e108 (2 p.). http://www.omicsonline.org/novel-decontamination-technologies-for-fresh-cut-industry-2157-7110.1000e108.php?aid=7908 (Aug. 2015)

13. Perni S, Shama G, Hobman JL, et al. Probing bactericidal mechanisms induced by cold atmospheric plasma with *Escherichia coli* mutants. Appl Phys Lett 2007; 90(7): e073902 (15 p.) https://dspace.lboro.ac.uk/dspace-jspui/handle/2134/3476 (Aug. 2015)

14. Misra NN, Tiwari BK, Rahavarao KSMS, Cullen P. Nonthermal plasma inactivation of foodborne pathogens. Food Eng Rev 2011; 3: 159–70.

15. Noeske M, Degenhardt J, Strudthoff S, et al. Plasma jet treatment of five polymers at atmospheric pressure: surface modifications and the relevance for adhesion. Int J Adhes Adhes 2004; 24: 171–7.

16. Fridman A, Chirokov A, Gutsol A. Non-thermal atmospheric pressure discharges. J Phys D Appl Phys 2005; 38: R1–R24.

17. Loretz M, Stephan R, Zweifel C. Antimicrobial activity of decontamination treatments for poultry carcasses: a literature survey. Food Control 2010; 21: 791–804.

18. Senat Commission on Food Safety. Opinion on the Use of Plasma Processes for Treatment of Foods. Bonn: SKLM, 2012: 1–14.

19. Noriega E, Shama G, Laca A, et al. Cold atmospheric gas plasma disinfection of chicken meat and chicken skin contaminated with *Listeria*

inncua. Food Microbiol 2011; 28: 1293–300.

20. Dirks BP, Dobrynin D, Fridman G, et al. Treatment of raw poultry with non-thermal dielectric barrier discharge plasma to reduce *Campylobacter jejuni* and *Salmonella enterica*. J Food Prot 2012; 1: 22–8.

21. Rod KS, Hansen F, Leipold F, et al. Cold atmospheric pressure plasma treatment of ready-to-eat meat: inactivation of *Listeria innocua* and changes in product quality. Food Microbiol 2012; 30: 233–8.

22. Manfreda G, Cevoli C, Lucchi A, et al. Hot air treatment for surface decontamination of table eggs experimentally infected with *Salmonella, Listeria, and Escherichia coli*. Vet Res Commun 2010; 34: 179–82.

23. Editorial team. Reducing salmonella in European egg-laying hens: EU targets now set. Euro Surveill 2006; 11(32): e3021 http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=3021 (Aug. 2015)

24. Galis A, Marcq C, Marlier D, et al. Control of salmonella contamination of shell eggs- preharvest and postharvest methods: a review. Compr Rev Food Sci Food Saf 2013; 12: 155–82.

25. Messens W, Leleu S, De Preter S, et al. Effect of egg washing on the cuticle of table eggs. In: 19th European Symposium on the Quality of Poultry Meat & 13th Symposium on the Quality of Eggs and Egg Products. Turku : World Poultry Science Association, 2009: EO12 (10 p.)
http://www.cabi.org/animalscience/worlds-poultry-science-association-wpsa/wpsa-finland-2009 (Aug. 2015)

26. Nys Y, Rehault - Godberg S, Gautron J, et al. Strategies for reinforcing hygienic quality of table eggs. Lucr Ştiinţ Ser Zoot 2011; 55: 257–62.

27. Moreau M, Orange N, Feuilloley MGJ. Non-thermal plasma technologies: new tools for bio-decontamination. Biotechnol Adv 2008; 26: 610–7.

28. Ragni L, Berardinelli A, Vannini L, et al. Non-thermal atmospheric gas plasma device for surface decontamination of shell eggs. J Food Eng 2010; 100: 125–32.

29. Vaninni L, Montanari C, Berardinelli A, et al. Assessment to the efficacy of a low-temperature gas plasma prototype for superficial decontamination of table eggs. In: 19th European Symposium on the Quality of Poultry Meat & 13th Symposium on the Quality of Eggs and Egg Products. Turku : World Poultry Science Association, 2009: EO 29 (8 p.)
http://www.cabi.org/animalscience/worlds-poultry-science-association-wpsa/wpsa-finland-2009 (Aug. 2015)

30. Melamies IA. Atmospheric plasma replaces chemicals. Plasmatreat. JOT Special J Oberflachentech 2011; (Nov): e1–7. http://www.plasmatreat.com/downloads/english/11-11_JOT-special_eco-friendly-treatment.pdf (Aug. 2015)

31. Lommatzsch U, Pasedag D, Baalmann A, et al. Atmospheric pressure plasma jet treatment of polyethylene surfaces for adhesion improvement. Plasma Process Polym 2007; 4: S1041–5.

32. Stepien-Pysniak D, Marek A, Rzedzicki J. Occurence of bacteria of the genus Staphylococcus in table eggs descended from different sources. Pol J Vet Sci 2009; 12: 481–4.

33. Baier M, Gorgen M, Frohling A, et al. Fresh produce decontamination by an atmospheric pressure plasma jet. In: Proceedings of the 11th International Congress on Engineering and Food ICEF: vol. III. Potsdam, 2011: 1643–4.

34. Liu X, Hong F, Guo Y, et al. Sterilisation of *Staphylococcus aureus* by an atmospheric non-thermal plasma jet. Plasma Sci Technol 2013; 15: 439–42.

35. Laroussi M. Low-temperature plasmas for pedicine. IEEE Trans Plasma Sci 2009; 37: 714–25.

36. Walsh J, Kong MG. Sharp bursts of high-flux reactive species in sub-microsecond pulsed atmospheric pressure glow discharges. Appl Phys Lett 2006; 89: e231503 (3 p.)
http://scitation.aip.org/content/aip/journal/apl/89/23/10.1063/1.2397570 (June 2015)

37. Fraser AC, Cusack M. Investigation of partridge eggshell cuticle using SEM and EDX. Microsc Anal 2002; 88: 15–6.

38. Leleu S, Messens W, De Reu K, et al. Effect of egg washing on the cuticle quality of brown and white table eggs. J Food Prot 2011; 74: 1649–154.

39. Mohammad Q, Al-Natour AR, Akram RA, et al. *Escherichia coli* 0157:H7 faciliates the penetration of *Staphylococcus aureus* into table eggs. J Food Sci 2012; 71: 29–34.

40. Scott TA, Silversidest FG. The effect of storage and strain of hen on egg quality. Poultry Sci 2000; 79: 1725–9.

41. Rodriguez-Navarro A, Domingues - Gasca N, Munoz A, et al. Change in the chicken eggshell cuticle with hen age and egg freshness. Poultry Sci 2013; 92: 3026–35.

ANTIBAKTERIJSKE LASTNOSTI HLADNE ATMOSFERSKE PLAZME S CURKOM PRI POVRŠINSKI DEKONTAMINACIJI JAJC V LUPINI

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Povzetek: V državah Evropske unije konzumnih kokošjih jajc pred oddajo v prodajo ni dovoljeno prati ali jih mehanično čistiti, zato se za zmanjšanje tveganj glede varnosti živil razvija več tehnik za dekontaminacijo jajc v lupini. V poskusu smo testirali potencialno učinkovitost hladne atmosferske plazme za površinsko dekontaminacijo jajc v lupini. Jajčne lupine konzumnih jajc smo izpostavili enkratnemu ali večkratnemu vplivu atmosferskega plazemskega curka za 10 - 60 sekund. Zmanšanje prisotnosti *Staphylococcus aureus* (NCTC 8325) na ploščicah iz polietilen teraftalata je znašala > 3 log stopnje, medtem, ko je zmanjšanje prisotnosti števila aerobnih mezofilnih bakterij in *S. aureus* na površini s plazmo tretiranih jajčnih lupin znašala med 1,8 do 2,5 log stopnje. Povrhnjica jajčnih lupin s plazmo obdelanih jajc je ostala funkcionalno nepoškodovana, kljub fizikalnim in ionizirajočim lastnostim plina v plazmi. S plazmo obdelana jajca niso bila spremenjena glede senzoričnih in fizikalno-kemijskih lastnosti, tudi procesi staranja so bili enaki kot pri neobdelanih jajcih. Rezultati poskusa kažejo, da tretiranje jajčne lupine s curkom atmosferske plazme pozitivno vpliva na dekontaminacijo jajc v lupini in nima negativnih vplivov na kakovost in staranje jajc, kar je pomembno z vidika varnosti in kakovosti živil.

Ključne beside: jajca v lupini; atmosferska hladna plazma; aerobne mezofilne bakterije; *S. aureus*; dekontaminacija

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Original Scientific Article

THE PREVALENCE AND ANTIMICROBIAL RESISTANCE OF *Salmonella* SPECIES ISOLATED FROM CAPTIVE REPTILES AT LJUBLJANA ZOO

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Summary: Cloacal swabs from 74 healthy reptiles at Ljubljana Zoo were examined for the presence of salmonellae. Thirty nine reptiles underwent at least one antimicrobial treatment 24 - 48 months before sample collection. The identification of salmonellae was performed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry, and positive strains were serotyped. Salmonellae were found in 29.7% of all reptiles investigated, in 55.6% of reptiles kept with regularly direct contact with zoo visitors, and in 26.2% of reptiles kept strictly in terraria. The isolation prevalence was 38.6%, 18.2% and 12.5% in snakes, lizards and chelonians, respectively. *Salmonella enterica* subspecies *enterica* was the most common (63.6%) followed by subspecies *diarizone* (31.8%) and subspecies *arizonae* (4.5%). The *Salmonella enterica* subsp. *enterica* serotypes Infantis 6,7,14:r:1,5 and Uzaramo 1,6,14,25:z_4,z_{24}- were detected in 27.3% and 36.4% of *Salmonella* positive samples, respectively. Resistance to antimicrobial agents was found in 9% of strains. A high percentage (63.6%) of *Salmonella* positive reptiles at Ljubljana Zoo shed serotypes that are known to be causative agents of human salmonellosis. This is the first documented isolation of *Salmonella* enterica subsp. *diarizonae*, serotype IIIb 57:k:e,n,x,z_{15} from captive reptiles.

Key words: captive reptiles; salmonellosis; antibiotic resistance; MALDI-TOF MS

Introduction

As a source of educational programs many zoos and rescue centres traditionally provide opportunities to visitors for direct contact and handling with certain captive reptiles. Reptiles are important reservoirs of salmonellae as they harbour these bacteria without showing any clinical signs (1, 2). Carriage rates of *Salmonella* spp. in captive reptiles vary between 50% and 86% (3, 4, 5). Factors associated with poor husbandry, systemic viral infections and intestinal parasitism in reptile collections may lead to reactivation of clinical salmonellosis and bacterial shedding (6, 7, 8). Cases of human salmonellosis associated with pet reptiles are known and have been well documented (9, 10, 11). The infection can be transmitted to humans by direct contact or through an environment contaminated with salmonellae (2, 12).

Salmonellae are often resistant to ampicillin, tetracycline, colistin sulphate, streptomycin, sulfamethoxazole/trimethoprim and nalidixic acid (5, 13, 14). For that reason care must be taken with the use of antibiotics as it may result in the development of antibiotic resistance (6).

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The purpose of this study was to investigate the prevalence and antimicrobial resistance of salmonellae isolated from captive reptiles at Ljubljana Zoo.

Materials and methods

Animals

Cloacal swabs from 74 clinically healthy reptiles at Ljubljana Zoo, Slovenia, were examined for the presence of salmonellae. Sixty-seven reptiles (19 lizards, 42 snakes, six chelonians) were captive bred, and seven reptiles (three lizards, two snakes, two chelonians) were rescued from the wild. In the period of sample collection all reptiles were kept at the Ljubljana Zoo for more than one year. From the 74 reptiles, 39 underwent at least one five-day fluoroquinolone treatment against salmonellae 24 - 48 months before the study. Nine reptiles have been used for teaching purposes and have had contact with visitors, whereas the remaining 65 reptiles did not have any contact with visitors.

Sample collection

Each animal was manually restrained and a sterile cotton swab (Copan Italia S.p.A, Italy) was gently inserted and turned inside the reptile's cloaca. Samples were transported in Amies transport medium (Copan Italia S.p.A, Italy) and kept in 5 °C until processed, 48–120 hours after collection.

Cultivation, identification and serotyping of salmonellae strains

Cloacal swabs were placed into 5 ml of buffered peptone water (BPW) (Oxoid Ltd., UK) and incubated overnight at 37 °C. Upon incubation, 0.1ml of BPW was dropped onto modified semisolid Rappaport-Vassiliadis agar (Oxoid Ltd., UK), incubated at 41.5 °C and inspected for typical growth (halo zone around the inoculated drop) at 24 and 48hrs. In parallel, Xylose lysine deoxycholate agar (Oxoid Ltd., UK) was inoculated with the positive samples and incubated at 37 °C overnight to select *Salmonella* isolates. Suspect *Salmonella* colonies were cultured on blood agar, incubated at 37 °C for 24 hours, and then confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS, Microflex LT, Bruker Daltonics, Germany). *Salmonella* isolates were serotyped by slide agglutination assay using commercial O and H antisera (BioRad, France; Denka Seiken, Japan). The identification of serotypes was carried out according to Kauffmann-White-Le Minor scheme (15).

Antimicrobial susceptibility test

Salmonella isolates were tested for susceptibilities to amoxicillin/clavulanic acid (30 μg), ampicillin (10 μg), ceftazidime (30 μg), chloramphenicol (30 μg), tetracycline (30 μg), colistin sulphate (10 μg), streptomycin (10 μg), neomycin (30 μg), gentamicin (10 μg), sulfonamide compounds (300 μg), sulfamethoxazole/trimethoprim (25 μg), ciprofloxacin (5 μg), enrofloxacin (5 μg) and marbofloxacin (5 μg) (Oxoid Ltd., UK), using antibiotic disk diffusion method (16).

Results

Salmonellae were found in 29.7% of all reptiles investigated (Table 1), in 55.6% of reptiles kept with regularly direct contact with zoo visitors, and in 26.2% of reptiles kept without direct contact with visitors. The prevalence of salmonellae in reptiles with previous antimicrobial treatment and without antimicrobial treatment was 35.9% and 62.9% respectively. Snakes had the highest prevalence (38.6%) of salmonellae. *Salmonella enterica* subspecies *enterica* was the most common (63.6%), followed by the subspecies *diarizone* (31.8%) and subspecies *arizonae* (4.5%). In each positive individual one serotype was isolated. The *Salmonella enterica* subsp. *enterica* serotypes Infantis 6,7,14:r:1,5 and Uzaramo 1,6,14,25:z_4,z_{24}- were detected in 27.3% and 36.4% of *Salmonella* positive samples, respectively.

Resistance to antimicrobial agents was found in 9% of *Salmonella* strains. Resistance to ampicillin and amoxicillin/clavulanic acid was detected in one strain of *Salmonella enterica* subsp. *enterica* serotype Infantis 6,7,14:r:1,5 isolated from a *Zamenis longissimus*, and resistance to streptomycin was detected in one strain of *Salmonella enterica* subsp. *diarizonae* serotype IIIb 57:k:e,n,x,z_{15} isolated from a *Vipera ammodytes*. Both snakes were previously treated with fluoroquinolones. Strains of *Salmonella enterica* subsp. *enterica* serotype Infantis 6,7,14:r:1,5,

Table 1: The prevalence of *Salmonella* species and serotypes isolated from reptiles at Ljubljana Zoo

Reptile species	Number of samples collected	Samples positive for *Salmonella* spp. (%)	*Salmonella* serotypes
Lizards	**22**	**4 (18.2%)**	
Pogona vitticeps	5	0	
Chlamydosaurus kingii	2	0	
Lacerta viridis	1	0	
Lacerta agilis	7	2	Infantis 6,7,14:r:1,5
Furcifer pardalis	2	0	
Iguana iguana	1	1	IIIb 65:z_{10}:e,n,x,z_{15}
Ophisaurus apodus	4	1	IIIb 57:k:e,n,x,z_{15}
Snakes	**44**	**17 (38.6%)**	
Python regius	2	0	
Python bivittatus	2	0	
Pantherophis guttatus	10	4	Infantis 6,7,14:r:1,5*, IIIb 53:z_{10}:z_{35}, IIIa 53:z_4,z_{23}:-
Elaphe quatuorlineata	3	1	IIIb 53:z_{10}:z_{35}
Zamenis longissimus	3	2	Infantis 6,7,14:r:1,5, Uzaramo 1,6,14,25:z_4,z_{24}-
Pantherophis emoryi	6	3	Uzaramo 1,6,14,25:z_4,z_{24}-*, IIIb 53:z_{10}:z_{35}
Lampropeltis triangulum	1	0	
Thamnophis sirtalis	6	3	Uzaramo 1,6,14,25:z_4,z_{24}-
Natrix tessellata	2	0	
Natrix natrix	4	1	Infantis 6,7,14:r:1,5
Vipera berus bosniensis	2	1	IIIb 53:z_{10}:z_{35}
Vipera ammodytes	2	2	IIIb 57:k:e,n,x,z_{15}, Uzaramo 1,6,14,25:z_4,z_{24}-
Vipera aspis francisciredi	1	0	
Chelonians	**8**	**1 (12.5%)**	
Geochelone sulcata	2	0	
Emys orbicularis hellenica	1	0	
Emys orbicularis orbicularis	4	0	
Trachemys scripta scripta	1	1	Uzaramo 1,6,14,25:z_4,z_{24}-*
Total	**74**	**22 (29.7%)**	

* Serotypes isolated from reptiles with direct contact with zoo visitors

Salmonella enterica subsp. *enterica* serotype Uzaramo 1,6,14,25:z_4,z_{24}-, *Salmonella enterica* subsp. *diarizonae* serotype IIIb 53:z_{10}:z_{35} and *Salmonella enterica* subsp. *diarizonae* serotype IIIb 57:k:e,n,x,z_{15} isolated from a *Pantherophis guttatus*, *Zamenis longissimus*, *Pantherophis emoryi* and *Ophisaurus apodus* respectively, were intermediate susceptible to streptomycin.

Discussion

Despite modern zoos make efforts to provide suitable conditions for captive reptiles, the enclosure design would hardly replicate the reptile's natural habitat. Keeping reptiles in groups leads to intense competition for basking spots and food items, sexual frustration and increased exposure to faeces (17). In comparison with the results of Martínez Barreda et al. (3), Geue and Löschner (4) and Corrente et al. (5) who assumed shedding rates up to 50% in captive reptiles, a relatively low prevalence of *Salmonella* spp. (29.7%) was found in our study. This difference may be due to the fact that the animals in the present study were only sampled once, and the excretion of *Salmonella* spp. is intermittent (18, 19). The higher frequency of salmonellae isolation in snakes than in lizards and chelonians is in agreement with previously published data (4, 14, 20).

In the present study *Salmonella* subspecies *enterica* was the most common (63.6%) followed by the subspecies *diarizone* (31.8%) that was isolated mainly in snakes with a history of previous antimicrobial treatment.

Mitchell and Shane (6) and Johnson-Delaney (8) remarked that antimicrobials may merely suppress the excretion of salmonellae without their complete elimination, and antimicrobial treatment in reptiles without any clinical symptoms of salmonellosis can promote the emergence of antimicrobial resistant strains. This statement is accordance with our finding because more than one third of reptiles treated with fluoroquinolones were found positive for salmonellae. Generalized use of antimicrobial agents in reptiles or in their environment is not recommended (21).

A high percentage (63.6%) of *Salmonella* spp. positive reptiles at Ljubljana Zoo shed serotypes that are known to be causative agents of clinical salmonellosis. *Salmonella enterica* subsp. *enterica* serotype Infantis 6,7,14:r:1,5 belongs to the top ten *Salmonella* serotypes that may cause gastroenteritis in humans (22), and multidrug-resistant clones of this serotype have been already detected (23). *Salmonella enterica* subsp. *enterica* serotype Uzaramo 1,6,14,25:z_4,z_{24}- has been related to a case of reptile-associated human salmonellosis (24). To our knowledge this study is the first to document isolation of *Salmonella enterica* subsp. *diarizonae* serotype IIIb 57:k:e,n,x,z_{15} from captive reptiles.

Results of this study could help to improve knowledge about the prevalence of *Salmonella* spp. in zoo reptilian collections. It is clear that proper hygiene practices are needed in order to minimize the infectious risk for zoo personnel and visitors. Recommendations for reducing the risk of transmission of *Salmonella* to humans from reptiles include the following: (i) Veterinarians and pet store owners should provide information to clients about the risk of acquiring salmonellosis from reptiles. (ii) People with immature or weakened immune system, including children, pregnant women, elderly people and immunocompromised persons should avoid contact with reptiles. (iii) People should wash their hands after handling these animals or their environments. (iv) Kitchen sinks should not be used to bath reptiles or to wash their dishes, cages or aquariums. (v) Reptiles should not be allowed to roam freely throughout a home. (vi) Other household pets, such as dogs and cats, should not be in contact with reptiles, their cages, faeces or feed to reduce spread of transmission of salmonellae. (vii) Zoos and exhibits should be equipped with adequate hand-washing facilities, and food and drinking should not be allowed in animal-contact areas. (viii) Good care of household reptiles should be given to reduce stress, which may cause the excretion of salmonellae (25).

Future studies will be extended to identify sources of infection and transmission routes of salmonellae in reptiles at Ljubljana Zoo.

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References

1. Marschang RE, Chitty J. Infectious diseases. In: Girling, SJ, Raiti P, eds. Manual of reptiles. Gloucester : British Small Animal Veterinary Association, 2004: 330–45.

2. Jacobson ER. Bacterial diseases of reptiles. In: Jacobson ER, eds. Infectious diseases and pathology of reptiles. Boca Raton : CRC Press, 2007: 462–526.

3. Martínez Barreda C, Gallegos Antúnez DC, Bär W, Márquez De Bär G, Fernández Cano R, Ruiz Reyes G. Pet reptiles: a potential source of *Salmonella*. Enf Infec Microbiol 1999; 19: 266–9. http://www.medigraphic.com/pdfs/micro/ei-1999/ei996b.pdf (June 2015)

4. Geue L, Löschner U. *Salmonella enterica* in reptiles of German and Austrian origin. Vet Microbiol 2002; 84: 79–91.

5. Corrente M, Madio A, Friedrich KG, et al. Isolation of *Salmonella* strains from reptile faeces and comparison of different culture media. J Appl Microbiol 2004; 96: 709–15.

6. Mitchell MA, Shane SM. *Salmonella* in reptiles. Semin Avian Exot Pet Med 2001; 10: 25–35.

7. Lamberski N, Seurynck K, Gregory C, Moore TA, Pfaff CS, Hines R. Recovery of a pathogenic *Salmonella arizona* from four snakes at the Riverbanks Zoo. J Herp Med Surg 2002; 12: 17–22.

8. Johnson - Delaney CA. Reptile zoonoses and threats to public health. In: Mader DR, eds. Reptile medicine and surgery. 2nd ed. St. Louis : Saunders Elsevier, 2006: 1017–30.

9. Woodward DL, Khakhria R, Johnson WM. Human salmonellosis associated with exotic pets. J Clin Microbiol 1997; 35: 2786–90.

10. Cyriac J, Wozniak ER. Infantile salmonella meningitis associated with gecko keeping. Commun Dis Public Health 2000; 3: 66–7.

11. CDC. Reptile-associated salmonellosis-selected states, 1998–2002. Morbid Mortal Wkly Rep 2003; 52: 1206–9.

12. Friedman CR, Torigian C, Shillam PJ, et al. An outbreak of salmonellosis among children attending a reptile exhibit at a zoo. J Pediatr 1998; 132: 802–7.

13. Gopee NV, Adesiyun AA, Caesar K. Retrospective and longitudinal study of salmonellosis in captive wildlife in Trinidad. J Wildl Dis 2000; 36: 284–93.

14. Chen CY, Chen WC, Chin SC, et al. Prevalence and antimicrobial susceptibility of salmonellae isolates from reptiles in Taiwan. J Vet Diagn Invest 2010; 22: 44–50.

15. Grimont PAD, Weill FX. Antigenic formulae of the *Salmonella* serovars. 9th ed. Paris: WHO Collaborating Centre for Reference and Research on *Salmonella* ; Institut Pasteur, 2007: 166 p.

16. CLSI. Performance standards for antimicrobial disc and dilution susceptibility tests for bacteria isolated from animals; Second Informational Supplement, Vet01-S2. Wayne, PA : Clinical and Laboratory Standards Institute, 2013: 74 p.

17. Scheelings TF, Lightfoot D, Holtz P. Prevalence of *Salmonella* in Australian reptiles. J Wildl Dis 2011; 47: 1–11.

18. Ward L. *Salmonella* perils of pet reptiles. Commun Dis Public Health 2000; 3: 2–3.

19. Hydeskov HB, Guardabassi L, Aalbaek B, Olsen KEP, Nielsen SS, Bertelsen MF. Dynamic *Salmonella* shedding in a collection of zoo reptiles. In: Proceedings of the American Association of Zoo Veterinarians, AAZV Annual Conference. Oakland, 2012: 234.

20. Clancy MM, Valitutto M, Sykes JM. Evaluation of *Salmonella* in reptiles at the wildlife conservation society´s Bronx Zoo (2000–2012). In: Proceedings of the American Association of Zoo Veterinarians Annual Conference, AAZV. Salt Lake City, 2013: 91.

21. Mitchell MA, Shane SM, Nevarez J, et al. Establishing a *Salmonella*-free iguana, *Iguana iguana* model using enrofloxacin. In: Proceedings of the Association of Reptilian and Amphibian Veterinarians. Seattle, 2001: 189–90.

22. Samiullah S. *Salmonella* Infantis, a potential human pathogen has an association with table eggs. Int J Poult Sci 2013; 12: 185–91.

23. Nógrády N, Tóth A, Kostyák A, Pászti J, Nagy B. Emergence of multidrug-resistant clones of *Salmonella* Infantis in broiler chickens and humans in Hungary. J Antimicrob Chemother 2007; 60: 645–8.

24. Paton JH, Mirfattahi MB. *Salmonella* meningitis acquired from pet snakes. Arch Dis Child 1997; 77: 91.

25. Centers for Disease Control and Prevention. Reptile-associated salmonellosis-selected states, 1998–2002. Morb Mortal Wkly Rep 2003; 52: 1206–9.

POJAVLJANJE VRST *Salmonella* IN NJIHOVA ODPORNOST PROTI PROTIMIKROBNIM SREDSTVOM V IZOLATIH PLAZILCEV V ZOO LJUBLJANA

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Povzetek: Kloakalni brisi 74 zdravih plazilcev so bili v ljubljanskem živalskem vrtu pregledani na prisotnost salmonel. Devetintrideset plazilcev je bilo zdravljenih z vsaj enim protimikrobnim zdravilom 24 - 48 mesecev pred zbiranjem vzorcev. Določanje salmonel je bilo opravljeno s pomočjo masne spektrometrije MALDI (ionizacija v matriksu z lasersko desorpcijo) ter s pomočjo serotipizacije pozitivnih sevov. Salmonele so bile ugotovljene v 29,7 % vseh preiskanih plazilcev, in sicer pri 55,6 % plazilcev, ki so v rednem neposrednem stiku z obiskovalci živalskega vrta, in pri 26,2 % plazilcev, ki so nastanjeni izključno v terarijih. Okuženih je bilo 38,6 % kač, 18,2 % kuščarjev in 12,5 % želv. Najpogosteje je bila izolirana *Salmonella enterica*, podvrsta *enterica* (63,6 %), sledita pa podvrsti *diarizone* (31,8 %) in *arizonae* (4,5 %). *Salmonella enterica*, subsp. *enterica* serotipa Infantis 6,7,14: r: 1,5 je bila odkrita pri 27,3 %, seroptipa Uzaramo 1,6,14,25: z4, z24- pa v 36,4 %. Odpornost proti protimikrobnim sredstvom je bila ugotovljena pri 9 % sevov. Visok odstotek (63,6 %) na salmonelo pozitivnih plazilcev v ljubljanskem živalskem vrtu je bil okužen z enim izmed serotipov, ki so znani povzročitelji salmoneloze pri ljudeh. To je prva dokumentirana izolacija *Salmonella enterica* subsp. *diarizonae*, serotip IIIb 57: K: E, n, x, Z15 pri plazilcih v ujetništvu.

Ključne beside: plazilci v ujetništvu; salmoneloza; odpornost proti antibiotikom; MALDI-TOF MS

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Original Scientific Article

STUDY OF TRACE AND ULTRATRACE ELEMENTS IN SILAGE INTENDED FOR CATTLE NUTRITION

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Summary: Grass silage and maize silage are important sources of trace and ultratrace elements in cattle nutrition. Data regarding the function, metabolism and deposition, content in feed and animal requirements, as well as some parts of risk assessment, are still needed. To study the presence of selected elements (Al, As, Ba, Be, Cd, Cr, Co, Cu, B, Fe, Pb, Li, Mn, Hg, Mo, Ni, Se, Ag, Sr, Sn, Sb, V, Tl, Ti and Zn) in silage, the appropriate analytical procedures for their determination were introduced and validated in our laboratory. After closed-vessel microwave digestion, inductively coupled plasma mass spectrometry (ICP-MS) was used to analyse samples. Grass silage and maize silage samples from three important cattle-producing regions of Slovenia were analysed and statistically evaluated. The selected elements were found in both types of silage in a wide range of concentrations, with the exception of Tl in maize silage, where all results were below the limit of detection. Statistically significant differences were found for 9 elements in grass and maize silage from three different regions and for 23 elements when comparing grass silage and maize silage. The data suggest that cattle fed Slovenian grass silage or maize silages should receive routine mineral supplementation, of which the most important trace elements needed are Cu and Se. Cattle fed Slovenian maize silage also should receive supplementary Zn and Co. Based on research in other countries, though not tested here, an I supplement is advisable too.

Key words: trace elements; ultratrace elements; grass silage; maize silage; ICP-MS

Introduction

Feedstuffs contain a wide range of elements, either naturally occurring, or added on purpose, as well as by adventitious contamination. These mineral elements usually are classified as nutritionally essential major and trace elements, and those regarded as toxic, or with an essential/toxic duality (1). Since 1970, the term 'ultratrace element' has been used for elements with estimated dietary requirements that usually are <1mg kg-1, and often <50µg kg-1 dry matter (DM) (2). In order to distinguish between trace and ultratrace elements, data on their function, metabolism and deposition, content in feed (feed material and compound feedstuffs) and animal requirements (including allowances and their use in practice), as well as some risk assessments, are still needed (3).

Normal functioning of almost all biochemical processes in the body requires trace elements. They are necessary to maintain body function, to optimize growth and reproduction and to stimulate the immune response; therefore, they determine health status (4). Furthermore, there is evidence that ultratrace elements, given in an appropriate amount, can evoke pharmacological responses in animals (2). The necessary amounts of trace elements are difficult to establish and

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most estimates are based on the minimum level required to alleviate the symptoms of deficiency, and not necessarily to promote productivity. When estimating the need for mineral supplements, the quantity and type of feed ingredients and their inherent element content, the processing of the diet, the storage and environmental conditions as well as the inclusion and content of other elements must be considered (4). Most, if not all, elements, including essential trace elements, can cause toxic effects to animals and humans if present at excessive levels (5). The values of trace and ultratrace elements in grass and maize silage, however, remain poorly documented (3, 5–9).

In Slovenia, grass and maize comprise the main fodders used for ensiling. The estimated annual quantity (t/year) of prepared silage in Slovenia is 1.5M for grass silage and 1.2M for maize silage. Silage has been made for more than 100 years, but ensiling was used widely after the 1970s, when ensiling machinery and silos became more accessible to farmers. The use of hay as winter-feed declined gradually as silage making increased. In recent times, silage has become important as summer-feed for cattle and 25% of Slovenian dairy farms now use silage exclusively as a summer forage feed for milking cows (10).

Analytical methods based on inductively coupled plasma mass spectrometry (ICP-MS) after closed-vessel microwave digestion is the most recent and advanced tool to determine the levels of trace and ultratrace elements in feedstuffs. Its features are low limits of detection (LOD), multi-elemental capabilities, high sensitivity, a wide linear dynamic range, high sample throughput, and the ability to discriminate between isotopes (11). Several articles report the use of different methods for the determination of trace and ultratrace elements in plant material (12–17), cereals (18–25) and different food matrices (11, 26–32), mainly connected with human consumption. To the best of our knowledge, there have been no reports until now on the use of this multi-elemental technique for the determination of trace and ultratrace elements in silage. For this reason, and according to the European Food Safety Authority (EFSA) (3), a procedure for the determination of 25 selected elements in silage was introduced and validated in our laboratory. These elements are: aluminum (Al), arsenic (As), barium (Ba), beryllium (Be), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), boron (B), iron (Fe), lead (Pb), lithium (Li), manganese (Mn), mercury (Hg), molybdenum (Mo), nickel (Ni), selenium (Se), silver (Ag), strontium (Sr), tin (Sn), antimony (Sb), vanadium (V), thallium (Tl), titanium (Ti) and zinc (Zn). The validation was performed according to Regulation (EC) No 882/2004 (33) and Decision 2002/657/EC (34). Using the validated procedure, samples of grass and maize silage taken at farms in three important cattle-producing regions of Slovenia were analysed and statistically evaluated.

Materials and methods

Sample collection

Samples of grass silage and maize silage (n=30 each), grown on Slovenian farms, were collected from three different regions of Slovenia. These three regions: Murska Sobota (16 samples), Maribor (25 samples), and Celje (19 samples), are important cattle-producing areas. Samples were dried in an UFE 500 oven (Memmert, Schwabach, Germany) at 60°C overnight, and then ground and stored in glass containers at temperatures below 14°C until analysis.

Reagents and materials

All solutions were prepared using high purity deionised water obtained by a Milli-Q water purification system (Bedford, MA). Suprapur nitric acid (HNO_3, 65%) and hydrogen peroxide (H_2O_2, 30%) were purchased from Merck (Darmstadt, Germany). Stock standard solution for the calibration of the system VAR-TS-MS and an internal standard solution Var-IS-1, containing 100µg L-1 of ^{6}Li, ^{45}Sc, ^{115}In, ^{89}Y, ^{159}Tb and ^{209}Bi, were purchased from Inorganic Ventures (Lakewood, VA).

ICP multi-element stock standard solution XVI containing As, Be, Cd, Co, Cr, Cu, Fe, Li, Mn, Mo, Ni, Pb, Sb, Se, Sr, Ti, Tl, V and Zn, with a concentration of each element of 100mg L^{-1}, and separate stock standard solutions containing Ba, Al, Ag, B and Sn, with the concentration of each element of 1000mg L^{-1}, were purchased from Merck (Darmstadt, Germany). A working standard solution of Hg was prepared from a stock standard solution containing 100mg L^{-1} of Hg (Inorganic Ventures, Lakewood, VA) and was added to the working standard solutions separately.

Two standard reference materials ERM-CD 281

Table 1: Instrumental operating conditions for ICP-MS

	Operating conditions	
Nebuliser	Glass concentric, Micromist	
Spray chamber	Scott-type, Peltier-cooled (3°C)	
RF power (kW)	1.40	
Interface	Nickel sampler and skimmer cones for the CRI 820-MS	
	Normal mode	CRI mode
Plasma gas flow (L min^{-1})	18.50	17.00
Auxiliary gas flow (L min^{-1})	1.85	1.70
Sheath gas flow (L min^{-1})	0.14	0.25
Nebuliser gas flow (L min^{-1})	0.94	0.90
CRI skimmer gas flow (L min^{-1})	-	H_2, 70
Scan mode	Peak hopping	Peak hopping
Scans/replicate	20	20
Replicate/sample	5	5
Attenuation mode	Automatic	None

Table 2: Values of elements in samples of grass silage (*n*=30) and maize silage (*n*=30)

	Grass silage				Maize silage			
	Median	Mean	S.E.M.	Range	Median	Mean	S.E.M.	Range
Al	480	857	172	60.7–4204	46.0	68.0	12.3	23.0–353
Fe	468	744	137	113–3489	58.3	76.9	9.3	45.7–275
Mn	71.8	79.0	5.8	26.3–159	23.2	25.0	1.7	12.5–49.6
Zn	27.7	30.6	1.9	20.9–77.5	23.1	23.9	0.9	13.3–35.9
Ti	19.0	29.5	5.1	5.89–143	4.09	4.69	0.48	2.17–14.6
Ba	15.4	21.2	2.3	6.71–62.4	2.67	2.98	0.28	1.04–8.29
Sr	11.7	13.2	1.1	5.96–28.4	3.07	3.38	0.18	2.17–6.11
Cu	7.98	8.05	0.29	5.15–11.1	5.36	5.53	0.15	4.27–7.53
B	7.13	8.04	0.65	4.02–17.0	5.78	5.75	0.20	4.10–8.78
Ni	2.20	2.57	0.40	0.802–12.2	0.395	1.32	0.52	0.179–15.6
Cr	1.24	1.78	0.28	0.241–7.15	0.366	0.412	0.034	0.193–0.848
Mo	1.02	1.17	0.14	0.272–2.92	0.283	0.327	0.031	0.096–0.747
V	1.01	1.70	0.34	0.145–9.16	0.092	0.158	0.026	0.057–0.723
Li	605	1024	203	107–5174	58.3	89.5	15.2	33.1–458
Pb	553	1060	328	159–10067	135	146	13.2	58.9–384
Co	270	380	63	74.7–1645	24.3	44.6	7.5	14.1–150
As	210	418	101	60.4–2852	51.9	53.6	6.8	<7.90–126
Cd	90.9	130	19	19.8–518	59.2	68.0	7.0	22.6–182
Sn	82.2	120	20	40.3–493	47.2	49.1	2.1	27.8–77.7
Se	52.7	65.9	7.4	<32.5–176	51.6	55.0	4.5	<9.70–65.8
Hg	33.0	40.1	8.0	<7.40–56.1	58.2	58.2	-	<7.40–58.2
Be	32.2	56.0	10.2	11.3–229	9.27	9.93	0.64	5.02–22.0
Tl	27.9	37.6	8.3	<1.90–143	<1.90	<1.90	–	–
Sb	22.1	27.8	2.8	11.3–65.2	17.6	23.2	7.3	<9.8–124
Ag	11.9	13.0	1.8	<1.10–30.6	10.6	10.3	0.9	<1.10–13.9

Notes: Values are reported in mg kg^{-1} DM for elements from Al to V and in µg kg^{-1} DM for elements from Li to Ag.

Table 3: Medians and *p*-values of elements in samples of silage according to region[a]

	Grass silage				Maize silage			
	Median 1	Median 2	Median 3	p^b	Median 1	Median 2	Median 3	p^b
Al	1133	396	480	0.209	42.4	50.7	42.0	0.376
Fe	972	360	461	0.218	53.9	65.8	59.4	0.383
Mn	116	74.0	50.9	<0.05	27.4	20.7	19.7	0.107
Zn	24.2	29.5	30.0	0.082	21.7	23.5	26.1	0.093
Ti	33.2	22.7	14.5	<0.05	3.84	4.78	3.63	0.165
Ba	24.1	16.6	15.4	0.603	2.80	3.71	2.13	<0.05
Sr	13.2	9.00	13.3	0.216	3.44	3.08	2.63	0.414
Cu	7.46	7.26	9.62	0.107	5.26	5.43	5.26	0.519
B	6.86	6.47	10.6	<0.05	5.38	6.05	5.98	0.518
Ni	2.33	1.87	2.13	0.985	0.253	0.424	0.903	<0.05
Cr	2.11	1.24	1.05	0.226	0.339	0.355	0.450	0.476
Mo	0.369	1.38	1.27	<0.05	0.159	0.366	0.339	<0.05
V	2.01	0.860	1.11	0.238	0.076	0.096	0.099	0.184
Li	1234	494	599	0.184	54.0	75.1	58.9	0.800
Pb	747	533	594	0.485	133	143	124	0.517
Co	377	180	266	0.179	24.2	23.9	33.7	0.905
As	344	174	262	0.511	46.4	52.2	51.9	0.293
Cd	82.7	76.3	122	0.063	51.7	61.1	72.7	0.359
Sn	104	80.2	80.6	0.341	46.7	47.6	49.9	0.993
Se	47.3	48.9	56.1	0.056	51.6	<32.5	56.5	0.084
Hg	31.1	<24.8	44.6	0.476	<7.40	<7.40	58.2	<0.05
Be	58.6	28.9	35.7	0.272	9.09	9.22	10.2	0.349
Tl	20.7	29.3	26.5	0.260	<1.90	<1.90	<1.90	-
Sb	39.9	19.9	22.5	<0.05	17.9	12.2	17.8	0.615
Ag	13.1	8.56	15.3	0.230	11.4	8.51	10.6	<0.05

Notes:
[a]Medians of elements concentrations within regions are reported in mg kg^{-1} DM for elements from Al to V and in µg kg^{-1} DM for elements from Li to Ag.
[b]*p*-values for elements in grass silage and maize silage samples from three regions. $p<0.05$, indicate significant differences between three regions.

(rye grass), purchased from IRMM (Geel, Belgium), and SRM 1570a (spinach leaves), purchased from NIST (Gaithersburg, MD, USA), were included in the validation of the analytical procedure.

Microwave digestion

A total of 500mg of dry sample was weighed in a 100mL Teflon vessel and 5mL of 65% HNO_3 and 1mL of 30% H_2O_2 were added. After being left at laboratory temperature for 60 min, the samples were digested in the closed 12-vessel microwave system CEM MARS 5 (Matthews, NC) at the power of 1600W following a three-step program. In the first 15min, the temperature was raised to 200ºC and held at the temperature for the next 20min. After digestion was completed, the samples were cooled for 20min in a stream of air. After complete cooling, samples were quantitatively transferred to 100mL volumetric flasks and made up with Milli-Q water.

Instrumental analysis

Measurements were performed with a Varian 820-MS system (Mulgrave, Australia) equipped with Varian's ICP-MS Expert software for the system control and data processing.

A Collision Reaction Interface (CRI) was used for measurements of ^{75}Se and ^{78}As to reduce common polyatomic interferences. The isotopes ^{7}Li, ^{9}Be, ^{11}B, ^{27}Al, ^{47}Ti, ^{51}V, ^{53}Cr, ^{55}Mn, ^{57}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{88}Sr, ^{95}Mo, ^{107}Ag, ^{111}Cd, ^{118}Sn, ^{121}Sb, ^{137}Ba, ^{202}Hg, ^{205}Tl, $^{206+7+8}Pb$ were selected as analytical

masses in the ICP-MS normal sensitivity mode. The instrumental settings are given in Table 1.

Statistical analysis

Data from the grass silage and maize silage samples were analysed using the Statistical Package for Social Sciences (SPSS, version 12, November 2003). Arithmetic mean, median and ranges (minimum and maximum) of element values were calculated. If measured values were below the limit of quantification (LOQ), the value equal to LOQ/2 was taken for the calculations (35). Shapiro-Wilk's test was used to test normality. Because the distributions were not normal ($p<0.05$), the non-parametric Kruskal-Wallis test was used to evaluate differences between silage samples from three different regions. A non-parametric Mann-Whitney test was used to evaluate differences between grass and maize silage. The Spearman's correlation analysis was used to determine correlations between element values.

Results and discussion

Analysis of silage samples

Table 2 summarises the data for the elements in farm samples of grass silage and maize silage (n=30 each). The table shows the median, arithmetic mean, S.E.M. and ranges (minimum–maximum). All results for elemental values, discussed below, are given on a dry matter (DM) basis.

Mean DM in grass- and maize- silage was 47.9% (range 30.6–69.8%) and 38.6% (range 31.3–47.3%), respectively. The selected elements were found in both types of silage, except for Tl in maize silage, where all Tl values were below LOD (1.9µg kg^{-1}). Hg (range 31.1–56.1µg kg^{-1}) was present in three samples of grass silage and in only one sample of maize silage (58.2µg kg^{-1}).

Our results are similar to those reported globally. Our values for Cu, Mo, Zn, Mn, Co, and Se can be compared with values in grass silage from Iceland (8) and with values in grass silage and pasture herbage from Ireland (36).

Cu values (mg kg^{-1}) were similar in Slovenian grass silage and Icelandic forage (mean 8.05, range 5.16–11.1) and (mean 8.0, range 4–16), respectively, but were lower than in Irish grass silage (mean 10.36, range 2.8–39.7) (36). Mean Cu values in Slovenian and Icelandic grass silage are marginally deficient (<10mg kg^{-1}). Therefore routine supplementation with Cu is advisable.

Mo is a proven Cu-antagonist in cattle. Mo values (mg kg^{-1}) in grass silage showed the biggest differences: Slovenia (mean 1.17, range 0.272–2.92), Iceland (mean 0.23, range 0.0043–2.37) and Ireland (mean 1.48, range 0.1–18.3, n=1505). Mo-induced copper deficiency in cattle usually occurs on forages with Mo values >5mg kg^{-1} and/or forage Cu:Mo ratios <3:1. Such values were uncommon in grass silage in Slovenia and Iceland. However, clinical Mo-induced copper deficiency is common in unsupplemented Irish cattle at pasture. Herbage in Irish pasture has higher Mo values (mean 2.49, range 0.1–52.0, n=1658) and lower Cu values (mean 9.22, range 1.6–23.7, n=1741) than those in Irish grass silage. However, even Irish grass silage had many Mo values >5mg kg^{-1} (36). Marginal Cu and elevated Mo in Irish herbage and grass silage mean that Cu supplementation is used routinely in Irish herds (38).

Se values (µg kg^{-1}) in Slovenian grass silage (mean 65.9, range 32.5–176) agree with those reported by Pick et al. (9) (mean 65.3, range 17.6-180). These means are slightly higher than the mean (50.2) reported previously by Žust et al. (6) but were considerably lower than in Irish grass silage (mean 93, range 2–232, n=1507) and Irish pasture herbage (mean 93, range 1–250, n=1459), reported by Rogers & Murphy (36). Mean Se values (µg kg^{-1}) in maize silage (55.0 in our case, 28.6 according to Pick et al. (9) and 38.7 in the study by Žust et al. (6)) were even lower than in grass silage.

Our data confirm that the content of Se in Slovenian grass silage and maize silage is low and does not meet the requirements of ruminants. The data also confirm the conclusion of Pick et al. (9), which stated the need to update the spreadsheets from the German Agricultural Society (DLG), since most of the results were <90–130µg kg^{-1}, the recommended value in both cases. Indeed, the optimum Se value recommended in cattle diets in Ireland is higher still (>200µg kg^{-1}) (37). From those data, cattle fed Slovenian forages need a substantial Se supplement.

Mean Zn values (mg kg^{-1}) in grass silage in Slovenia, Iceland and Ireland (36) were 30.6 (range 20.9–77.5), 35 (range 14.1–85.0) and 29.7 (range 10–94), respectively. Zn values for Irish pasture herbage were very similar to those in Irish grass silage (mean 30.8, range 13–84, n= 928) (36).

Some values in all countries were <25mg kg^{-1} (the minimum Zn value recommended for cattle feed) but the means were all normal and the risk of simple Zn deficiency arising on such feeds is low. Rogers (38) reported that he had failed to confirm simple Zn deficiency in Irish herds, but had confirmed a few cases of secondary Zn deficiency, induced by excessive Ca values in feed.

Mean Mn values (mg kg^{-1}) in grass silage in Slovenia, Iceland and Ireland (36) were 79.0 (range 26.3–159), 125 (range 40–550) and 103.5 (range 2–477), respectively. Values in Irish pasture herbage were 119.8 (range 10–693, n=1872) (36). As the mean values greatly exceeded the minimum Mn level recommended in cattle feed (25mg kg^{-1}) and there were few values in Ireland below that, the risk of simple Mn deficiency in cattle is very low in all three countries. Rogers (38) reported that he had failed to confirm simple clinical Mn deficiency in Irish herds, but an Irish colleague (Dr. John Mee, Moorepark Dairy Research Centre) had seen a few cases of achondroplastic/dwarf calves attributed to it.

Mean Co values (µg kg^{-1}) in grass silage in Slovenia and Iceland were 380 (range 74.7–1645) and 317 (range 41–2010), respectively. These values exceeded the minimum Co recommended for cattle feed (100µg kg^{-1}). However, because soil Co values greatly exceed values in forage tissue, the elevated Co values probably reflect soil contamination.

Evidence for significant soil contamination when harvesting grass silage comes from the massive differences between Co, Al and Fe values in grass silage versus maize silage. Co in grass silage was circa 9-times higher than in maize silage (mean 44.6, median 24.3, range 14.1–150). Al values (mg kg^{-1}) usually found in pasture are <100 are but can exceed 1000 under unfavorable conditions, because of soil contamination. Al values in grain products are usually 5–68 (3). Al values in Slovenian grass silage (mean 857, median 480, range 60.7–4204) was circa 13-times higher than in maize silage (mean 68.0, median 46.0, range 23.0–353) and Fe in grass silage (mean 744, median 468, range 113–3489) was circa 10-times higher than in maize silage (mean 76.9, median 58.3, range 45.7–275). Though cattle on Slovenian grass silage may not need a Co supplement, those on maize silage probably need one because the mean values in maize silage were <50% of the minimum Co recommended for cattle feed (100µg kg^{-1}).

In some studies (8), samples with Fe values >1000mg kg^{-1} were considered to be contaminated by soil and these samples were excluded from further processing. As for the other elements, the processes of harvesting herbage for conservation as hay or silage often result in soil being picked up with crops, and the elevated values of elements may reflect spurious contamination (5). This would explain the wide ranges of selected elements present in the analysed samples and the differences between mean and median values. However, in our opinion, the exclusion of these samples would not provide an accurate reflection of the values of elements in silage fed to animals on Slovenian farms.

Values for I in Slovenian grass silage and maize silage are not included in this paper. However, in humans and animals, I deficiency is the most prevalent trace element deficiency globally (4, 8). It can be associated with common clinical problems in cows (infertility, late abortion, high postnatal calf mortality (goiter in calves, stillbirth, weak calf syndrome), retained placenta, etc) (37–40). Mean I values (µg kg^{-1}) in Irish grass silage and pasture herbage were very similar (36), namely, 269 (range 4–980, n=627) and 261 (range 5–1000, n=777), respectively. National Advisory Recommendations on minimal I requirement of cattle and on optimum I supplementation levels differ greatly between countries. In Ireland, a routine supplement of 30–60mg I/cow/day is advised from circa 1 month prepartum to circa 4 months postpartum. If I deficiency is suspected as a cause of late abortions in cows at pasture, 30–60mg I/cow/d (via the trough water supply) is advised (37). This far exceeds the I intake from Irish forage (circa 2.6–4.2mg/cow/day assuming a mean forage DM input of 10–16kg/cow/day and a mean forage I value of 265µg kg^{-1}. This approach may also be advisable in Slovenia.

Li et al. reported values of Zn, Cu, Cr, Cd, Pb and As in maize silage fed to Wisconsin dairy herds (7).

Zn values (mg kg^{-1}) in maize silage in Slovenia and Wisconsin (mean 23.9, median 23.1, range 13.3–35.9) versus (mean 27, median 24, range 17–77) were similar but the range in Slovenia was narrower than in Wisconsin. Maize silage in both surveys was marginal in Zn because the minimum Zn recommended for cattle feed is 25mg kg^{-1} DM.

Mean Cu values in Slovenia (5.53mg kg^{-1}, range 4.27–7.53mg kg^{-1}) were slightly higher than in Wisconsin (4.0mg kg^{-1}, range 2–6.4mg kg^{-1}). Maize silage in both surveys was very low in Cu because the minimum Cu recommended for cattle feed is 10mg kg^{-1} DM.

Mn values in Slovenian silages (mg kg^{-1}) are

comparable with values reported previously (3). The reported values of Mn in grass and maize silage were 98 and 28, respectively. Our mean Mn values were slightly lower: 79 in grass silage and 25 in maize silage.

Except for Cu, the maximum values of all investigated elements were 2–10 times lower in Slovenian maize silage than in Wisconsin maize silage.

Cr values (mg kg^{-1}) in Slovenian maize silage (mean 0.412, range 0.194–0.848) were slightly lower than in Wisconsin (0.519, range 0.207–1.702). Mean Cd values (µg kg^{-1}) in Slovenia and Wisconsin, 62 and 68, respectively, were very similar. However, mean Pb value (µg kg^{-1}) in Slovenia was half of that in Wisconsin (146 versus 260, respectively), and mean As (µg kg^{-1}) was much less than in Wisconsin (53.6 versus 201, respectively).

Ni mean values (mg kg^{-1}) in Slovenian maize silage (mean 1.32, range 0.179–15.6) agree well with 1.28 mg kg^{-1} reported by Van Paemel et al. (3).

Until now, data were lacking for the values of elements in feed materials that were considered to be non-essential for animals. Forage and grains are generally rich Li sources, but the Li values vary with the soil on which the plants are grown (3). The range of B value of terrestrial plants is 2–95mg kg^{-1}. Reported Sr values of hay were 9.4mg kg-1, but there are no data available for grass silage. The accumulation by terrestrial plants of Ag from soils is low, even from soils fertilised with Ag-containing sewage sludge. Generally, land plants have Ag values of 60µg kg^{-1}. Sn values in pastures were reported to range from 0.3–0.4mg kg^{-1}. However, no data are available on V values in feed materials in the main literature sources (3). Therefore, we cannot compare our results for these elements.

Regional differences between elements and differences between grass silage and maize silages

Analysis of variance found significant differences in 9 element values in silage between three regions in Slovenia (Table 3). There were significant regional differences ($p<0.05$) between B, Ti, Mn, Mo and Sb in grass silage samples and between Ni, Mo, Ba and Hg in maize silage samples. Median Mo values in grass silage and Ni in maize silage showed the biggest regional differences. Significant differences between grass silage and maize silage were found for most of the elements, except for Ag and Hg.

Conclusions

Marginal or low values were found for some essential trace elements in grass silage, and especially in maize silage in Slovenia. We suggest that cattle fed grass silage or maize silages should receive routine mineral supplementation, of which the most important trace elements needed are Cu and Se. Cattle fed maize silage should also receive supplementary Zn and Co. Though we did not test for I values, the research and conclusions from other countries suggests that I supplementation is also advisable.

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References

1. Ihnat M. A survey of methods of analysis for minerals in feedstuffs. J Anim Sci 2003; 81: 3218–25.

2. Nielsen FH. The importance of diet composition in ultratrace element research. J Nutr 1985; 115: 1239–47.

3. Van Paemel M, Dierick N, Janssen G, Fievez V, De Smet S. Selected trace and ultratrace elements: biological role, content in feed and requirements in animal nutrition: elements for risk assessment. Ghent: Technical report submitted to EFSA, 2010. (online) http://www.efsa.europa.eu/en/supporting/pub/68e.htm (24. 1. 2012)

4. López - Alonso M. Trace minerals and livestock: not too much not too little. ISRN Vet Sci 2012; 2012: e704825 (18 p.) http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3671743/ (27. 12. 2013)

5. Fink - Gremmels J. Animal feed contamination: effects on livestock and food safety. Cambridge: Woodhead Publishing Limited, 2012.

6. Žust J, Hrovatin B, Šimundić B. Assessment of selenium and vitamin E deficiencies in dairy herds and clinical disease in calves. Vet Rec 1996; 139: 391–4.

7. Li Y, McCrory DF, Powell JM, Saam H, Jackson-Smith D. A survey of selected heavy

metal concentrations in Wisconsin dairy feeds. J Dairy Sci 2005; 88: 2911–22.

8. Jóhannesson T, Eiríksson T, Gudmundsdóttir KB, Sigurdarson S, Kristinsson J. Seven trace elements in Icelandic forage: their value in animal health and with special relation to scrapie. Icel Agric Sci 2007; 20: 3–24.

9. Pick D, Leiterer M, Einax JW. Reduction of polyatomic interferences in biological material using dynamic reaction cell ICP-MS. Microchem J 2010; 95: 315–9.

10. Mohorko M, Ambrožič I, Šturbej E, Verbič J. Legislative requirements for using silage additives at agricultural holdings. In: Proceedings of the 18th international scientific symposium on nutrition of domestic animals »Zadravec-Erjavec days«. Radenci: KGZS Zavod Murska Sobota, 2009: 148–61.

11. Cubadda F. Inductively coupled plasma-mass spectrometry for the determination of elements and elemental species in food: a review. J AOAC Int 2004; 87: 173–204.

12. Zhou H, Liu J. The determination of rare earth elements in plant foods by ICP-MS. At Spectrosc 1997; 18: 192–4.

13. Wu S, Feng X, Wittmeier A. Microwave digestion of plant and grain reference materials in nitric acid or a mixture of nitric acid or a mixture of nitric acid and hydrogen peroxide for the determination of multi-elements by inductively coupled plasma mass spectrometry. J Anal At Spectrom 1997; 12: 797–806.

14. Lustig S, Zang S, Michalke B, Schramel P, Beck W. Platinum determination in nutrient plants by inductively coupled plasma mass spectrometry with special respect to the hafnium oxide interference. Fresen J Anal Chem 1997; 357: 1157–63.

15. Feng X, Wu S, Wharmby A, Wittmeier A. Microwave digestion of plant and grain standard reference materials in nitric and hydrofluoric acids for multi-elemental determination by inductively coupled plasma mass spectrometry. J Anal At Spectrom 1999; 14: 939–46.

16. Rodushkin I, Ruth T, Huhtasaari Å. Comparison of two digestion methods for elemental determinations in plant material by ICP techniques. Anal Chim Acta 1999; 378: 191–200.

17. Shimbo S, Zhang ZW, Watanabe T, et al. Cadmium and lead contents in rice and other cereal products in Japan in 1998–2000. Sci Total Environ 2001; 281: 165–75.

18. Lintschinger J, Fuchs N, Moser H, et al. Uptake of various trace elements during germination of wheat, buckwheat and quinoa. Plant Food Hum Nutr 1997; 50: 223–37.

19. Crews HM, Owen LM, Langford N, et al. Use of the stable isotope ^{106}Cd for studying dietary cadmium absorption in humans. Toxicol Lett 2000; 112/113: 201–7.

20. Cubadda F, Baldini M, Carcea M, Pasqui LA, Raggi A, Stacchini P. Influence of laboratory homogenization procedures on trace element content of food samples: an ICP-MS study on soft and durum wheat. Food Addit Contam 2001; 18: 778–87.

21. Al-Dayel OAF. Uranium contents in wheat samples of Saudi Arabia. Asian J Chem 2002; 14: 1307–10.

22. Branch S, Burke S, Evans P, Fairman B, Briche CSJW. A preliminary study in determining the geographical origin of wheat using isotope ratio inductively coupled plasma mass spectrometry with 13C, 15N mass spectrometry. J Anal At Spectrom 2003; 18: 17–22.

23. Cubadda F, Raggi A, Marconi E. Effects of processing on five selected metals in the durum wheat food chain. Microchem J 2005; 79: 97–102.

24. Hongxing Z, Yu-Kui R. Determination of trace elements, heavy metals and rare earth elements in corn seeds from Beijing by ICP-MS simultaneously. E-J Chem 2011; 8: e782–6. http://www.hindawi.com/journals/jchem/2011/152713/abs/ (27. 12. 2013)

25. Hongxing Z, Yu-Kui R. Determining mineral elements in four kinds of grains from Beijing market by ICP-MS simultaneously. J Saudi Chem Soc 2012; 16: 31–3.

26. Sánchez-Castillo CP, Dewey PJS, Reid MD, de Lourdes Solano M, James WPT. The mineral and trace element content of Mexican cereals, cereal products, pulses, and snacks: preliminary data. J Food Compost Anal 1997; 10: 312–33.

27. Zhang ZW, Watanabe T, Shimbo S, Higashikawa K, Ikeda M. Lead and cadmium contents in cereals and pulses in north-eastern China. Sci Total Environ 1998; 220: 137–45.

28. Cubadda F, Raggi A, Testoni A, Zanasi F. Multielemental analysis of food and agricultural matrixes by inductively coupled plasma-mass spectrometry. J AOAC Int 2002; 85: 113–21.

29. Cubadda F, Raggi A, Zanasi F, Carcea M. From durum wheat to pasta: effect of technological processing on the levels of arsenic, cadmium,

lead and nickel: a pilot study. Food Addit Contam 2003; 20: 353–60.

30. Cubadda F, Raggi A. Determination of cadmium, lead, iron, nickel and chromium in selected food matrices by plasma spectrometric techniques. Microchem J 2005; 79: 91–6.

31. Bilandžić N, Sedak M, Đokić M, et al. Comparative study of iron, magnesium and zinc and daily intakes in certain meats and meat products. Slov Vet Res 2013; 50: 103–10.

32. Bilandžić N, Sedak M, Đokić M, et al. Content of macro- and microelements in the milk of Croatian coldblood mares during lactation. Slov Vet Res 2014; 51: 171–7.

33. Regulation (EC) No 882/2004 of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules. Off J EU 2004; L191: e1–52. http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=URISERV:f84005 (24. 1. 2012)

34. Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Off J EU 2002; L221: e8–36. http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32002D0657 (24. 1. 2012)

35. Waegeneers N, Hoenig M, Goeyens L, De Temmerman L. Trace elements in home-produced eggs in Belgium: Levels and spatiotemporal distribution. Sci Total Environ 2009; 407: 4397–402.

36. Rogers P, Murphy W. Levels of dry matter, major elements (Ca, Mg, N, P, K, Na and S) and trace elements (Co, Cu, I, Mn, Mo, Se and Zn) in Irish grass, silage and hay. Wexford : Teagasc, 2000. (online) http://homepage.eircom.net/~progers/0forage.htm#silcompt (17. 4. 2015)

37. Rogers P, Gately T. Control of mineral imbalances in cattle and sheep: a reference manual for advisers and vets. Wexford : Teagasc (Irish Agriculture and Food Development Authority), 2000. (online) http://homepage.eircom.net/~progers/3control.htm (17. 4. 2015)

38. Rogers P. Dublin: personal communication, 2015.

39. Rogers P. Iodine supplementation of cattle: end of project report No. 4381. Dunsany : Teagasc, Grange Research Centre, Ireland, 1999. (online) http://homepage.eircom.net/~progers/i_report.htm (17. 4. 2015)

40. Rogers P. Copper, iodine and selenium status in Irish cattle: end of project report No. 4382. Dunsany : Teagasc, Grange Research Centre, Ireland, 2001. (online) http://homepage.eircom.net/~progers/abattoir.htm (17. 4. 2015)

ŠTUDIJA ELEMENTOV V SLEDOVIH V SILAŽI ZA PREHRANO GOVEDI

K. Pavšič Vrtač, B. Jakovac Strajn, J. Salobir, K. Šrimpf, G. Tavčar Kalcher

Povzetek: Travna in koruzna silaža sta v prehrani goved pomemben vir elementov v sledovih. Podatki o bioloških vlogah, metabolizmu in odlaganju teh elementov v živalskih tkivih, vsebnostih v krmi, potrebah živali in ocenah tveganja se še vedno zbirajo. Za preučevanje vsebnosti izbranih elementov v silažah (Al, As, Ba, Be, Cd, Cr, Co, Cu, B, Fe, Pb, Li, Mn, Hg, Mo, Ni, Se, Ag, Sr, Sn, Sb, V, Tl, Ti in Zn) smo vpeljali in validirali ustrezne analizne postopke. Zaradi nizkih vsebnosti teh elementov v silažah smo uporabili najsodobnejšo metodo indukcijsko sklopljene plazme z masno detekcijo (ICP-MS). Za pripravo vzorcev smo uporabili razklop v zaprtem mikrovalovnem sistemu z dušikovo kislino in vodikovim peroksidom. Vzorce silaž, odvzetih v treh pomembnih govedorejskih regijah v Sloveniji, smo analizirali in dobljene podatke statistično obdelali. Izbrani elementi so prisotni v obeh vrstah silaž v širokih koncentracijskih območjih, razen Tl v koruzni silaži, kjer so vsi rezultati pod mejo določanja. Statistično značilne razlike za travno in koruzno silažo iz treh različnih regij smo ugotovili za 9 elementov. Pri primerjavi vzorcev travnih in koruznih silaž pa so se statistično značilne razlike pokazale za 23 elementov. Rezultati študije kažejo, da bi moralo govedo, hranjeno s slovensko travno in koruzno silažo, dobivati tudi ustrezne mineralne dodatke. Najpomembnejša elementa, ki jih je potrebno dodajati, sta Cu in Se. Govedo krmljeno s slovensko koruzno silažo, bi potrebovalo tudi dodatke Zn in Co. Glede na raziskave v drugih državah, bi bilo potrebno dodajati tudi I.

Ključne besede: elementi v sledovih; travna silaža; koruzna silaža; ICP-MS

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