SHORT TERM SCIENTIFIC MISSION (STSM)
SCIENTIFIC REPORT

This report is submitted for approval by the STSM applicant to the STSM coordinator

Action number: CA15224
STSM title: Assessment of keel bone damage of laying hens infected with mycoplasma (*Mycoplasma synoviae*) after tetracycline antimicrobial and dietary essential oil therapy
STSM start and end date: 25/10/2020 to 06/11/2020
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PURPOSE OF THE STSM:

(max.200 words)

The keel bone damage is a serious issue confronting the laying hen industry because of the probable pain managing to laying hens welfare and the possibility for decreased production. Many articles indicate that damage, while highly variable and likely dependent on a host of factors, extends to all production systems, genetic lines, and management types.

The most consumed table eggs, from laying hens treated with antibiotics can produce eggs contaminated with antibiotic residues. Residues of antibiotics may present a risk for consumer health. Keeping in mind that laying hens flocks are almost always suffer from Mycoplasma (*Mycoplasma synoviae*), for which they are treated with antibiotics, high-quality egg production is even more challenging.

Having in mind that in most cases battery cages, furnished cages, and non-cage systems, genetic lines, and management styles was investigated regarding the occurrence of keel bone damage of laying hens and their influence on hens welfare and overall laying hens productive parameters.

The purpose of the suggested STSM was to assess the keel bone damage of laying hens infected with mycoplasma after tetracycline antimicrobial and dietary essential oil therapy in the spotlight of veterinary prescribing antibiotics therapy and their possible natural alternatives on keel bone damage.

DESCRIPTION OF WORK CARRIED OUT DURING THE STSMS

(max.500 words)

The keel bone damage is an essential obstacle for industrial laying hens, worldwide and it is advised that it is one of the most crucial issues facing the poultry industry. The critical interest stems from the pain hens expected to experience following keel bone damage, which has significant consequences for welfare. The investigation regarding the post-application of different antibiotics and dietary essential oil on keel bone damage and laying hens overall welfare have been conducted at Section of Veterinary Science and Animal Production, University of Bari “Aldo Moro”, Valenzano, Bari, Italy. According to Toscano personal observations the keel is a pronounced bone that extends from the sternum and runs axially over the midline. It is situated ventral to the heart where it anchors the muscles used for wing motion, the *pectoralis* major and *pectoralis* minor. The length of a keel bone from the *Carina apex* to the caudal tip along the ventral surface is approximately 9 to 12 cm, though this varies with genetic line, age, and other factors. Also, the height of the keel measured from the Carina apex to the dorsal surface is approximately 30 to 33 mm. So basically, damage of keel bone can generally be divided into fractures and deviations. Fractures are characterized by sharp bends, shearing, or fragmented sections of the keel bone. In contrast, the deviations of the keel bone as a bone with an abnormally shaped structure that has not resulted from a fracture but contains section(s)

that vary from a theoretically perfect 2-dimensional straight plane in either the transverse or sagittal planes. Additionally, indentations along the ventral surface can also be classified as a deviation. In this proposed STSM the technique of ultrasonography was used in assessment of keel bone damage of laying hens infected with mycoplasma after tetracycline antimicrobial and dietary essential oil therapy. Using ultrasound waves, ultrasonography captures the reflection of sound from structures within the body yielding images of various tissues. A significant benefit of ultrasound is that neither the human operator or hen are exposed to ionizing radiation and thus the technique is much safer than the other approaches. The method has been used to assess keel fractures in adult hens which perfectly fits having in mind that the hens were over 45 weeks old in the time of assessment. In this method, the associated probe was run along the ventral ridge of the keel bone and the recorded image is observed for fractures. Depending on the breast muscle mass, the probe can also be run along the lateral surface of the keel to obtain additional information on deviations, which was accessible having in mind the hens average body weight was between 1.78 and 1.81 kg during the experimental period. Nevertheless, the biggest challenge in using ultrasonography in determining the appropriate size and shape of the probe to best detect fractures. The week feather cover around the breast, as well as breast muscling, did not have significant impact on the efficacy in using this technology of assessment.

DESCRIPTION OF THE MAIN RESULTS OBTAINED

Having in mind proposed thematic of this STSM "**Assessment of keel bone damage of laying hens infected with mycoplasma (*Mycoplasma synoviae*) after tetracycline antimicrobial and dietary essential oil therapy**", biological experiments with laying hens, antibiotics and essential oils already have been performed. The aim of the suggested STSM was to assess the keel bone damage of laying hens infected with mycoplasma (*Mycoplasma synoviae*) after tetracycline antimicrobial and dietary essential oil therapy in the spotlight of veterinary prescribing antibiotics therapy and their possible natural alternatives on keel bone damage.

The experiment with laying hens was conducted under the principles of the European Union Strategy for the Protection and Welfare of Animals. A total of 20,000 Lohmann Brown hens aged 42 weeks were divided into four different treatment-diets supplemented with 100 mg/kg tea tree essential oil (TT) as a control treatment, followed by 100 mg/kg of each tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC), respectively. The tea tree essential oil and antibiotics were applied in laying hens' treatments trough the feed. Each treatment consisted of 5000 laying hens, respectively. The hens were housed in an environmentally controlled facility with a constant temperature of 22 °C. The environmental conditions in the facility were in line with hybrid specifications. All laying hens showed clinical signs of mycoplasmosis as nasal discharge and tracheal rales. In each treatment, 120 hens were marked and nasal swabs were collected aseptically. Marked hens were examined daily for 5 days after the application of the essential oil and antibiotics. The criterion for recovery was the absence of any nasal discharge when pressure was applied to the paranasal sinus. Recovery percentage was calculated from the incidence of nasal discharge within the application days and after application. All the laying hens that had recovered were examined weekly for 4 weeks. Any hen that recovered but showed clinical signs in subsequent examination was classified as a relapse. The long-term cure rate for each treatment was calculated by subtracting the number of relapses to 33 days after treatment from the number of recovered hens recorded on day 5 after application Gas chromatography (GC) and gas chromatography–mass spectrometric (GC–MS) analyses of the applied tea tree essential oil was performed using an Agilent 7890A GC equipped with an inert 5975C XL EI/CI mass spectrometer detector (MSD) and flame ionization detector (FID) connected by a capillary flow technology 2-way splitter with make-up. An HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm) was used. The GC oven temperature was programmed from 60 °C to 300 °C at a rate of 3 °C min^{-1} and held for 15 min. Helium was used as the carrier gas at 16.255 psi (constant pressure mode). An auto-injection system (Agilent 7683B Series Injector) was employed to inject 1µL of the sample. The sample was analyzed in the splitless mode. The injector temperature was 300 °C and the detector temperature 300 °C. MS data were acquired in the EI mode with scan range 30–550 m/z, a source temperature of 230 °C and a quadrupole temperature of 150 °C; the solvent delay was 3 min. A total of 200 egg samples, 50 eggs from each treatment, were randomly selected. From each treatment 10 eggs were minced, making 5 samples each and then stored in polypropylene bottles at −20 °C until analyzed. The aliquot of 2.0 g of each sample was weighed into a 50 mL polypropylene tube, with 8 mL of acetonitrile added and the sample was homogenized. The sample was centrifuged and the supernatant was decanted into a 15 mL polypropylene tube. The volume of the extract was brought up to 10 mL with distilled water and 0.3 g of dispersive C18 was added to it. The supernatant was shaken for 1 min and then centrifuged for 5 min. Afterward, a 1 mL aliquot was diluted with 1 mL of distilled water and the extract was passed through a PTFE membrane filter. The final extract corresponded to a 0.1 g/mL sample equivalent. LC-MS/MS was performed with a spiking level of 0.1 mg/kg and the test data were evaluated based on recovery percentage (%) and relative standard deviation (SD). The obtained

results of the recovery treatments after the application of the tea tree essential oil and the antibiotics at periods of 1 to 33 days after the finished application of the essential oil and the antibiotics was recorded. All treatments showed a significantly high cure rate, without any statistically significant differences ($p > 0.05$) among the treatments. The cure rate of the laying hens was detected as soon as one day after the application of feed with 100 mg/kg tea tree essential oil (TT), tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC), which was maintained for 33 days, respectively. High long-term cure rates were achieved. The values of the calculated hens' recovery and pooled standard error (SEp) varied from 66% to 89% (SEp 0.992) for TT, 73% to 100% (SEp 1.134) for TC, 71% to 88% (SEp 0.878) for OTC and 74% to 96% (SEp 0.937) for the CTC treatment, respectively. According to Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding MRLs in foodstuffs of animal origin, the maximum allowed residues of used antibiotics is 200 µg/kg (0.2 mg/kg). Our findings showed statistically significant differences ($p < 0.05$) in the residual antibiotics in table eggs. Nevertheless, the TT treatment served as a control treatment with the addition of the tea tree essential oil in the dietary amount of 100 mg/kg of hen weight. This treatment recorded 0.00 mg/kg of residual antibiotic concentration. The highest concentration of residual antibiotics in eggs (0.15 mg/kg) was recorded in the TC treatment with significant differences ($p < 0.05$) compared to the OTC treatment (0.08 mg/kg), but without significant differences ($p > 0.05$) when compared to the CTC treatment (0.11 mg/kg) treatment. Based on the aforementioned results of the laying hens' recovery percentage, our results indicated that the tea tree essential oil could be usefully used in cure treatments for laying hens infected with *M. synoviae*, but without any adverse effects on table eggs. Moreover, we cannot neglect the positive effects of the used antibiotics with MRLs in eggs, which follow the Commission Regulation (EU) No 37/2010. These eggs can be safely used in human nutrition, keeping in mind the limits of antibiotics, but from the consumer point of view, these eggs are "not antibiotic-free", which makes the tea tree essential oil much more desirable. Tea tree essential oil can be also used in green agriculture and organic agricultural production as a natural antibiotic and natural remedy for poultry illness and as a natural enhancer of production parameters.
Based on our previous findings, we concluded that the tea tree (*M. alternifolia*) essential oil, compared to different antibiotics, administered through the feed to naturally infected laying hens with *M. synoviae*, showed positive results. The used antimicrobial therapy with tetracycline, oxytetracycline and chlortetracycline demonstrated the presence of antibiotic residues in eggs, which were within the average range of the recommended maximum residue limits (MRLs). The eggs from the tea tree essential oil treatment were free from the antibiotics completely.

Within this STSM I have expecting that after the assessment of keel bone damage of laying hens infected with mycoplasma after tetracycline antimicrobial and dietary essential oil therapy, positive results and improved hens welfare with reduced injuries of keel bone which can give new insight in a possible dietary prevention measure or veterinary treatment in reducing the pain in hens caused by keel bone dame and increased the comfort of hens which could bring in the increased overall welfare of laying hens.

THE MAIN RESULTS OBTAINED DURING STSM

A total of 20,000 Lohmann Brown hens aged 42 weeks were previously divided into four different treatment-diets supplemented with 100 mg/kg tea tree essential oil (TT) as a control treatment, followed by 100 mg/kg of each tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC), respectively. The tea tree essential oil and antibiotics were applied in laying hens' treatments through the feed. Each treatment consisted of 5000 laying hens, respectively. Detecting keel bone damage in live hens has been problematic, as the bone must either be palpated, which is a measure of low inaccuracy, or dissected, in which case the hen must be sacrificed. However, the use of imaging technology is increasing in experimental studies. In this STSM we aim to determine if ultrasonography can be used to detect keel bone damage. Ultrasonography showed high accuracy for detecting fractures. Having in mind that the keen bone damage of the hens was not initially planned and performed at the beginning of the experiment, this STSM was designed to assess the keel bone damage of laying hens infected with mycoplasma (*Mycoplasma synoviae*) after tetracycline antimicrobial and dietary essential oil therapy. As initially experiment was designed to have four treatments (one with the essential oil dietary addition and three with the supplementation of three different antibiotics from the group of tetracyclines) within a total of 20,000 hens and 5000 hens in each treatment. For the assessment of keel bone damage, we have used the same hens from a previously conducted experiment. A total of 40 hens was examined (10 per each treatment), respectively. All 40 hens were ultrasonographed by a trained, licensed veterinarian using a SonoSite Edge II, with images obtained using a 5–8 MHz transducer. The probe was passed along the ventral aspect of the keel in the sagittal plane, and the resulting image was scored as indicating the presence or absence of fractures, deviations, and tip fractures. Hens had not entered rigor mortis at the time of the ultrasonography procedure. To obtain "control treatment", we have also performed the palpation procedure on all 40 hens, which were ultrasonographed. The one trained and experienced palpator was used in hens keel bone palpation. Before the start of palpation, the assessor

was trained on normal keel bone anatomy. To palpate, assessor were instructed to run his thumb and forefinger against the hen along the sagittal axis of the keel bone no more than twice to feel for the presence or absence of deviations or deformations, fractures, and keel bone tip fractures. Assessor performed the palpation of each hen before checking the hens identifying the treatment group and recorded the score. Hens were palpated, after ultrasonography. Data of ultrasonography were compared to palpable keel bone abnormalities, respectively. All gain and further analyses will be performed using statistical software STATISTIC for all statistical testing. The preliminary results have shown high accuracy for detecting fractures and damages of hens keel bone. Results of ultrasonography and palpation for keel bone deviations, fractures, and tip fractures between the treatments were different. Ultrasonography have shown the lowest percentage (<50%) deviations in treatment with tea tree essential oil (TT), increased percentage of fractures >77% was recorded in treatment oxytetracycline (OTC), while the highest percentage of tip fractures (>93%) was recorded in treatment chlortetracycline (CTC), respectively. Regarding the palpation method the similar percentages were recorded. All the obtained data will be statistically analyzed and prepared as an article for publication.
Results will be formed in the form of an article and submitted for publishing in the journal such as Agriculture or special issue of the journal Sustainability as the STSM results of COST Action CA15224.

FUTURE COLLABORATIONS (if applicable)

Future collaboration with the University of Bari could be beneficial. Due to the recent increase in reports of the prevalence of keel bone damage in laying hens, this has become a topic of welfare concern. Keel bone damage is often in the form of a fracture, which may compromise the hen's welfare. Having in mind not only the poultry welfare, but the welfare of other food animals as well, including honey bees and fishes, there has been discussion and significant interest for preparing mutual new COST Action proposal regarding the animal welfare and creating the European network with the ultimate guidelines for animal welfare.

Regarding keel bone, further research is necessary to confirm the findings in a larger sample, including participants from all European countries and near neighbor countries as well.
This STSM was useful in getting new knowledge and insights into using essential oils and antibiotics and their influence on keel bone damage in laying hens after the treatment of mycoplasma (*Mycoplasma synoviae*). Besides, this STSM can bring to a fruitful and new exciting COST Action regarding animal welfare and their wellbeing.