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Combination of a feeding trial with *in-vivo* and *in-vitro* modelling to evaluate the suitability of a phytonutrient as a supplement for laying hens in the late laying period

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Abstract

A prolongation of the hens' laying cycle whilst stabilizing laying persistency is a major aim in egg producing industry. Decreasing performance as well as impaired mechanical eggshell represent a limiting factor of the duration of a laying cycle. Dietary supplements like phytonutrients with metabolic antioxidant activity may counteract these negative implications. The phytonutrient was evaluated by a combination of *in-vitro* testing (determination of inflammatory gene expression and cytokine concentration in THP-1 cells), a novel *in-vivo* model using fruit flies (determination of intestinal barrier function in *D. melanogaster*) and a feeding trial with commercial laying hens during the late stage of production (production parameters and eggshell quality). The results from the feeding trial indicate an improvement of egg number, egg mass, egg weight, feed conversion, and eggshell breaking strength in aged layers by phytonutrient supplementation. It can be assumed that these results are due to an improved intestinal health by strengthened gut barrier integrity and reduced inflammation as shown in the *in-vitro* and *in-vivo* trials.

Introduction

In modern layer breeds, the egg production and egg quality decline in the end of the laying cycle which is an economically limiting factor leading to the replacement of the animals when they reach 80 to 85 weeks of age. The decrease in performance and egg quality may be related to the aging of oviducts, as the age increases oxidative stress, while the efficiency of the antioxidant acting system declines, with close connection to chronic inflammatory responses compared to young layers (Zhang et al. 2015; Liu et al. 2018, Xie et al. 2019). Strategies are required to improve the egg production performance and egg quality in the late laying period. This will extend the laying cycle and increase the breeding efficiency of laying hens. Our hypothesis is that the supplementation of a phytonutrient containing wood lignans (agromed®ROI agromed Austria GmbH) known to have anti-inflammatory properties, could be a helpful tool in prolonging the laying cycle while maintaining a high laying persistency. This contributes to an improved efficiency and economy of egg production, with implications for more sustainable livestock production. As the factor sustainability has a close connection to animal welfare, the target in evaluating novel feed supplements should be the replacement of animal trials, for a reduction of test animals and for refinement, which aims to minimize the stress, pain and suffering for our animals. Therefore, we combined a feeding trial under controlled environment with laying hens to test the impact of the feed supplement on the performance with *in-vitro* and *in-vivo* models to get further information about a plausible mode-of-action.

Materials and methods

a) Layer Feeding Trial

60 laying hens (Lohmann LSL) were allocated randomly to 2 treatments á 10 replicates comprising 3 hens per pen under controlled environment. Laying hens were offered the laying diet without supplementation (control group) or with a phytonutrient supplement (400 mg/kg feed; agromed®ROI agromed Austria GmbH) from wk 61 to wk 76 of age (16-wk or 112-d experimental period) after a 7-d adaptation period. Diets were isoenergetic and isonitrogenous, formulated according to the nutrient levels recommended from Lohmann Tierzucht (2023), with reduced Vitamin E levels (18 mg/kg). The further trial design, measured parameters, as well as statistical evaluation followed the description from Hirtenlehner et al. (2022).

b) Determination of Inflammatory Gene Expression in THP-1 Cells

The phytonutrient supplement was extracted as described by Heckmann et al. (2022). The extract was also used for *D. melanogaster* experiments. The THP-1 (DSMZ) cell line were differentiated THP-1 cells and cultured under standardised conditions. Cells were grown and differentiated in 6-well plates (5 × 105 cells/mL). for 24 h. Treatments: a) Negative control (NC), no supplement; b) Positive Control (LPS), stimulated with Lipopolysaccharide (LPS; 250 ng/mL) as stressor; c) Phytonutrient extract (ROI). The following day, the supernatants were collected, centrifuged (200 g, 4 min, room temperature) and frozen in 3 × 500 µL aliquots at −80 ◦C for cytokine secretion analysis. The cells were lysed, and the RNA was isolated. The RNA samples were stored at −80 ◦C until RT-qPCR analysis. The mRNA expression levels of the genes IL-6 and TNFα were measured quantitatively via RT-qPCR. For detailed description of materials and methods see Heckmann et al. (2022).

c) Determination of Intestinal Barrier Function in *D. melanogaster*

The assay was executed as described in Heckmann et al. (2022). Principle: the fruit flies are treated with a chemical stressor (dextran sodium sulphate, DSS) which increases intestinal permeability and triggers a leaky gut syndrome in the intestinal tract. When flies feed on blue coloured food (Brilliant Blue FC dye), the colour molecules penetrate the permeable gut wall into the haemolymph and consequently into all body compartments, giving the fly a bluish appearance (so called "Smurf-flies"). Insofar as feed additives strengthen the intestinal integrity of these flies, they are indistinguishable compared to healthy and unaffected flies. Treatments: a) Negative control (NC), no supplement; b) Positive Control (DSS), stimulated with DSS as stressor; c) Phytonutrient extract (ROI).

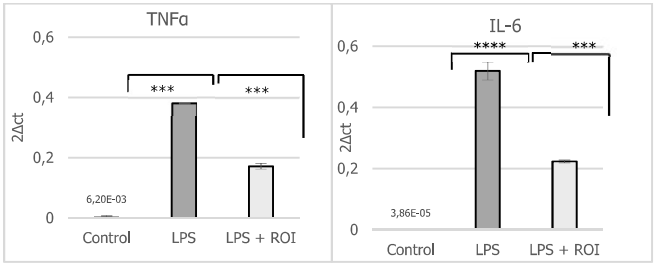
Results

Hens fed the diet supplemented with phytonutrient consumed significantly less feed compared to the control diet, although no differences in body weight appeared (table 1). Egg number, egg mass and feed to-egg-mass ratio were improved significantly. The percentage of broken eggs tended to be reduced by the supplement. Eggshell stability, expressed as the average force required to break the eggs of 10 eggs per data point, was improved by the supplementation with agromed®ROI. Data for blood profile measured at the end of the trial (75 weeks of age) were in a physiological range. Although not significant, agromed®ROI caused a numerical reduction of TBARS (thiobarbituric acid-reactive substances, which are substances formed as by-products during the oxidative degradation of fat and lipids) from 4.8 nmol/ml in the control group to 4.2 for agromed®ROI. The determination of pro-inflammatory gene expression in THP-1 cells shows significant upregulation of IL-6 and TNFα levels in response to LPS administration (figure 1). The phytonutrient supplementation significantly decreased the mRNA expression in comparison to the control.

Table 1: Performance data of laying hens (mean values ± standard deviation)

	Control	Phytonutrient
Body weight start (g)	1,985.3 ± 79.5	1,985.7 ± 97.2
Body weight end (g)	2,187.5 ± 87.2	2,216.4 ± 105.1
Body weight change (g)	202.1 ± 33.6	230.7 ± 31.4
Cumulative feed intake (kg)	**14.53 ± 0.33^{a}**	**13.68 ± 0.58^{b}**
Daily feed intake (g)	**129.7 ± 2.9^{a}**	**122.1 ± 5.2^{b}**
Egg number (n)	**102.8 ± 1.5^{a}**	**104.9 ± 1.2^{b}**
Egg weight (g)	65.2 ± 0.9	65.8 ± 1.2
Total egg mass (g)	**6.71 ± 0.15^{a}**	**6.90 ± 0.14^{b}**
Broken egg rate (%)	0.74 ± 0.44	0.48 ± 0.40
Feed conversion ratio (rel. egg mass)	**2.166 ± 0.064^{a}**	**1.983 ± 0.078^{b}**
Egg shell stability (N)	**38.26 ± 1.96^{x}**	**40.22 ± 1.96^{y}**

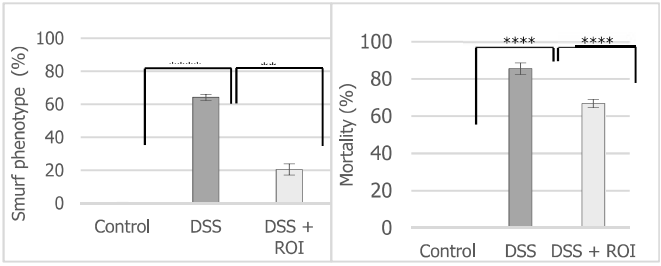
^{a,b} significant difference $p < 0.05$; ^{x,y} significant difference $0.05 < p < 0.1$



** p < 0.01, **** p < 0.0001

Figure 1: The mRNA expression levels of IL-6 and TNFα in LPS-stimulated THP-1 cells

The results for the assay to determine the intestinal barrier function in *D. melanogaster* showed that the treatment with the phytonutrient significantly decreased the total number of Smurfs (Figure 2 left), as well as mortality in DSS-challenged *D. melanogaster* (figure 2 right).



** p < 0.01, **** p < 0.0001

Figure 2: Mean fractions of Smurf flies (left) and the mortality of experimental flies (%) after 7 days of DSS challenge.

Discussion

In elder hens the declining laying performance as well as an impaired eggshell stability with progressing life age are limiting factors that forces egg producers to replace laying hens early due to economic reasons. A long-term egg production causes an accumulation of oxidative stress and is a main cause for a decline of zootechnical performance and egg quality during the late laying period (Liu et al. 2018). The aging process disrupts the balance between reactive oxygen species generation and antioxidants because of gradually decreased levels of antioxidants. This is closely connected with increasing inflammation (Subramanian and James, 2010). The *in-vitro* results suggest that the phytonutrient exhibits anti-inflammatory activity by inhibiting proinflammatory cytokine expression and secretion in LPS-stimulated macrophages. This is in line with the findings of Xie et al. (2019) who associated this

and an additional enhanced antioxidant capacity with a suppression of systemic inflammation which resulted in an improved production performance an egg quality in aged layers. The reduction of TBARS in blood of layers supplemented with the phytonutrient indicates an improved antioxidative capacity and suggest a higher stress resilience compared to birds of the control group (table 1). The results from the *in-vivo* testing showing reduced fraction of Smurf flies challenged by DSS, indicate protective effects of the phytonutrient on intestinal barrier function. This may have contributed to the improved hens performance, as an impaired barrier function is closely related to leaky gut, enteritis and therefore inflammation (Adedokun and Olojede, 2019).

Conclusion

The presented results indicate that the supplementation compensated for impairment of performance and eggshell stability and suggests that the phytonutrient beneficially affects the physiology of old laying hens via both, nutritional and functional properties. The combination of the a classical controlled feeding trial with *in-vitro* and *in-vivo* evaluation using cell culture and fruit flies has proven suitable for testing the efficiency of the phytonutrient, particularly with regard to the mode of action, and can serve as a model for reducing animal experiments in the interests of sustainability.

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