Theses of doctoral (PhD) dissertation

Investigation into the anti-inflammatory effects of metabolites from fungal and plant origin employing chicken-derived *in vitro* models

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Introduction

The poultry industry faces significant challenges in maintaining health standards and managing diseases, particularly in growing consumer demand for affordable and high-quality meat products. A major concern is the overuse of antibiotics, which has led to the development of antimicrobial resistance (AMR), threatening both animal and human health. Efforts to mitigate AMR have shifted towards alternative solutions such as probiotics, prebiotics, and plant-derived compounds. The excessive stress placed on poultry, particularly in intensive farming environments, further exacerbates the potential for infections and poor animal welfare. This stress can compromise the immune system, causing gastrointestinal disruptions, decreased feed efficiency, and reduced overall growth.

Bacterial and viral infections affect not just health but also the productivity of chickens. The gastrointestinal tract (GIT), the body's largest surface exposed to the external environment, plays a critical role in immune defense. Under physiological conditions, the gut tolerates feed and commensal microorganisms; however, a pro-inflammatory immune response is triggered upon pathogen invasion. This response is initiated in the GIT, where pathogen-associated molecular patterns (PAMPs) activate the innate immune system, particularly through Toll-like receptors (TLRs) present on various gut cells such as enterocytes, dendritic cells, and intraepithelial lymphocytes. Gastrointestinal diseases disrupting the enterocytes like runting-stunting syndrome (RSS), necrotic enteritis and dysbiosis damage the intestinal barrier and the so-called "leaky gut" allows PAMPs, other pathogen byproducts (e.g. toxins) and further pro-inflammatory molecules [e.g. cytokines, damage-associated molecular patterns (DAMPs)] to reach the liver via the portal vessels and eventually the systemic circulation. The liver cells, hepatocytes and Kupffer cells engage in pathogen filtration and production of pro- and anti-inflammatory cytokines and acute-phase proteins. Dysregulation of the cellular crosstalk leads to severe oxidative stress, cellular damage, and chronic inflammation, ultimately reducing poultry growth, productivity, and immune competence. This complex interplay between the gut and liver highlights the need for interventions targeting gastrointestinal and hepatic health. Moreover, viral infections such as RSS and infectious bursal disease (IBD) can significantly compromise the immune competence of poultry, even in vaccinated flocks, thereby predisposing animals to secondary infections.

Medicinal fungi and plant secondary metabolites have received considerable attention for their therapeutic potential due to their diverse bioactive compounds, which exhibit anti-inflammatory, antioxidant, and cytoprotective properties. Among the most important fungal secondary metabolites, polysaccharides, terpenoids, and alkaloids can be listed, and they have been confirmed to modulate immune responses, reduce oxidative stress, and enhance cellular repair mechanisms. Similarly, plant-derived secondary metabolites, including phenolic compounds, alkaloids, and terpenes, regulate inflammatory pathways, balance redox homeostasis, and protect cells from damage. The present thesis focuses on two phenolic compounds, a flavonoid and a phenolic acid, as phytochemicals and a nucleoside derivative of fungal origin, possibly contributing to immune modulation and cellular resilience.

The overproduction of cytokines and reactive oxygen species (ROS) exacerbates tissue damage, hindering the ability of the animal to maintain physiological growth and health. In light of the current health issues related to traditional antibiotics (e.g. AMR) and the limitations of antiviral drugs, there is growing interest in natural remedies for controlling infections and reducing inflammation in humans and animals. Chicoric acid (CA), luteolin, and cordycepin, three potent natural compounds, have shown promising results in modulating immune responses in mammalian cell lines and animal models. However, little is known about their effects on poultry. CA, known for its anti-inflammatory and antioxidant properties, has been shown to reduce cytokine production and oxidative stress both *in vitro* and *in vivo*. This phenolic acid, typically found in certain members of the Asteraceae family (e.g. common chicory and coneflower species), has shown promise due to its immunomodulatory and presumed antiviral effect, CA and plant extracts containing high levels of it show promise and warrant thorough investigation: CA can be a natural agent in supporting poultry health in the face of viral challenges, offering a safer and more affordable alternative to conventional antiviral drugs. Luteolin, a flavonoid found in various vegetables and herbs, has anti-inflammatory, antioxidant, and immunomodulatory effects. It has been reported to suppress pro-inflammatory cytokine production. The potential of luteolin in poultry lies in its ability to modulate systemic inflammation and oxidative damage in the liver. Cordycepin, derived from the medicinal fungus *Cordyceps militaris*, has demonstrated antiviral and anti-inflammatory properties. It inhibits RNA synthesis during viral replication, making it particularly valuable in combating viral infections. Furthermore, cordycepin acts on several points of the immune system, exerting its effect by acting on multiple stages of the inflammatory response, which may reduce inflammation in the gastrointestinal tract, thereby enhancing overall poultry health and productivity.

Aims of the study

In the poultry industry, there is growing evidence that the enhanced performance previously obtained with the subtherapeutic application of antibiotics is attainable with natural feed additives of plant origin. The scientific literature on specific plant- and fungus-derived metabolites is detailed and comprehensive. However, studies using mammalian models dominate existing academic sources. The broiler industry operates on tight profit margins, making it highly sensitive to even minor increases in production costs. As the broiler industry is one of the most intensive sectors in food production, I believe any approach aimed at enhancing the quantity and quality of the final product should be welcomed, given its particular significance from both economic and public health perspectives.

In vitro studies are inevitable to obtain beneficial feed additives and paramedical products. By screening potential pro-inflammatory substances [flagellin, polyinosinic-polycytidylic acid (poly I:C) and LPS] on chicken primary hepatocyte – non-parenchymal cell co-cultures, it was aimed to set and characterize an inflammatory chicken hepatic cell model suitable for challenging substances with antioxidant and immunomodulatory effects. However, according to the literature, the cell-cell interactions within the gut are even more complex to replicate. Explant systems comply with the 3R principles of laboratory animal experiments since they enable the preparation of multiple cultures from a single animal while providing a tissue-specific response within a regulated environment. Nevertheless, preventing anoxia-induced damage to the explants is challenging. Moreover, the number of uniform and size-standardized explant cultures remains limited in the literature. This work led to the development and characterization of an *ex vivo* explant model of the chicken ileum meeting the abovementioned criteria.

The objectives of the PhD thesis are presented below:

Ad 1, to test the potential pro-inflammatory effect of LPS, flagellin and poly I:C on the previously characterized chicken primary hepatocyte – non-parenchymal cell co-culture model of chicken origin.

Ad 2, to establish and characterize a uniform and size-standardized chicken ileal explant culture and to test the potential pro-inflammatory effect of LTA, flagellin and poly I:C on it.

Ad 3, to investigate the presumed anti-inflammatory and protective effect of chicoric acid on the chicken primary hepatocyte – non-parenchymal cell co-culture exposed to poly I:C.

Ad 4, to investigate the presumed anti-inflammatory and protective effect of luteolin on the chicken primary hepatocyte – non-parenchymal cell co-culture exposed to flagellin.

Ad 5, to investigate the presumed anti-inflammatory and protective effect of *Cordyceps militaris* extract on the chicken ileal explant culture exposed to poly I:C.

To carry out our experiments with the abovementioned goals, the following study plan has been followed.

	Model	**Investigated compounds**	**Cell viability parameters**	**Inflammatory parameters**	**Redox parameters**
Preliminary Study I.	Chicken primary hepatocyte – non-parenchymal cell co-culture	LPS, flagellin, poly I:C	Metabolic activity and extracellular LDH activity	IL-6 and IL-8 concentrations	-
Study I.A	Chicken primary hepatocyte – non-parenchymal cell co-culture	poly I:C, chicoric acid, N-acetylcysteine	Metabolic activity and extracellular LDH activity, caspase-3 level	IL-6, IL-8, IL-10, IFN-α, IFN-γ, M-CSF concentrations	MDA level
Study I.B	Chicken primary hepatocyte – non-parenchymal cell co-culture	flagellin, luteolin	Metabolic activity and extracellular LDH activity	IL-6, IL-8, IL-10, IFN-α, IFN-γ, concentrations	H_2O_2, MDA levels
Preliminary Study II.	Chicken ileal explant	LTA, flagellin, poly I:C	Metabolic activity and extracellular LDH activity	IL-2, IL-6, IL-8, IL-10, IFN-α, IFN-γ, RANTES concentrations	-
Study II.	Chicken ileal explant	poly I:C, *Cordyceps militaris* extract	Metabolic activity and extracellular LDH activity	IL-1β, IL-6, IFN-γ, RANTES concentrations	H_2O_2, MDA levels

Materials and methods

Cell isolation and preparation of the explants

The cells for the chicken primary hepatocyte – non-parenchymal cell co-cultures and the ileal explants were isolated from a 3-week-old male Ross-308 broiler chicken. The animals were raised and fed following the guidelines of the breeder. The experiments were executed in conformity with institutional policies, approved by the Government Office of Zala County (number of permission: GK-419/2020; approval date: May 11, 2020). For each experiment a single chicken was anesthetized with carbon dioxide and then slaughtered via decapitation. Then access to the liver through the gastropancreaticoduodenal vein of the hepatic portal system was provided and after the perfusion of the liver, the cell fractions were obtained after differential centrifugation. The cell suspensions were mixed in the ratio of 6:1 (hepatocytes to non-parenchymal cells) and then seeded onto 24-well and 96-well plates previously coated with collagen type I.

In case of the ileal explant studies a 15 cm long ileal segment was carefully removed, cleaned of adipose tissue, thoroughly washed with PBS + Pen-Strep solution, and processed further on a sterile surface:

1. One intestinal segment was placed, mucosa side up, on a sterile, chilled glass, and then the segment was gently and evenly pressed against the glass tile using glass slides.
2. The surface of the intestinal samples was rinsed frequently with ice-cold PBS + Pen-Strep solution using a syringe during the excision of the explants to keep the tissue moist.
3. Explant removal was performed with a biopsy punch perpendicular to the glass plate, moving the punch in a circular motion.
4. The excised specimens were transferred directly into the prepared wells with the medium by the plunger of the instrument.
5. Peyer's patches were avoided, and explant replicates were sectioned in a row for each experimental group to minimize the potential difference between groups.

The measured parameters in case of each study are listed in the table on page 6.

Preliminary study I.: Establishment of a hepatic inflammatory model

The medium of the cultures was supplemented with 0 (control), 10 or 50 µg/mL LPS from *Escherichia coli* (O55:B5) for 24 h, and in a separate plate cell cultures were exposed to culture media supplemented with 0 (control), 100 or 250 ng/mL *Salmonella* Typhimurium derived flagellin, further with 50 or 100 µg/mL poly I:C for 24 h. To achieve the re-annealing the poly I:C was heated at 50 °C for 3 min then cooled down before added to cell culture media.

Study I.A: Effects of chicoric acid on the hepatic model

The cells were isolated and cultured according to the protocol of the chicken hepatic co-culture. After 24 h, the cultured cells were treated with culture media which was supplemented with 0 (control) or 50 µg/mL poly I:C to mimic virus-induced inflammation. Additionally, cells of the treatment groups were treated with 100 or 200 µg/mL N-acetylcysteine (NAC) and 10 or 100 µg/mL CA. The remaining treatments consisted of combinations of 50 µg/mL poly I:C and 100 or 200 µg/mL NAC or 50 µg/mL poly I:C together with 10 or 100 µg/mL CA.

Study I.B: Effects of luteolin on the hepatic model

The cells were isolated and cultured according to the protocol of the chicken hepatic co-culture. After 24 h of incubation, cells were cultured in medium supplemented as detailed in chapter 2.1 but without FBS and further supplemented with flagellin derived from *S.* Typhimurium at the concentrations of 0 (control) and 250 ng/mL, with 0, 4 or 16 µg/mL luteolin, or with the combination of flagellin (250 ng/mL) and luteolin (4 or 16 µg/mL). Flagellin and luteolin stock solutions were freshly prepared with pure medium.

Preliminary study II.: Establishment of a gut explant inflammatory model

The explants were obtained according to the protocol of the ileal explant model. In preliminary study II.1, the viability, cell membrane damage and histology of 1 and 2 mm diameter explant cultures were assessed. Six 1 and 2 mm diameter explants were fixed instantly with buffered formalin on biopsy sponges at room temperature for further evaluation (H&E stain). The remaining explants were directly deposited into the cell culture plate wells. 1 mm explants were cultured on 96-well plates in 100 µl, and the 2 mm explants were placed in the wells of 24-well plates in 400 µl media proportionately to the volume of the explant to make the biological response of 1 and 2 mm explants comparable. The wells were allocated to have n=6 separate explants for 24 h histology (H&E stain and pan-cytokeratin immunohistochemistry), metabolic activity and lactate dehydrogenase (LDH) measurements (H&E stain and pan-cytokeratin immunohistochemistry) from both the 1 and the 2 mm diameter explants. The media of the explants was changed after 1 hour and then every 12 h and a CCK-8 assay was performed. The removed medium samples were stored for LDH measurement.

Based on the results of Preliminary Study II.1 and for practical reasons, the smallest obtainable and manageable, 1.5 mm diameter explants were excised in Preliminary study II.2. Five explants were incubated on 96 well plates in separate wells filled with 200 µl of medium. The explants were left untreated for 12 h, fixed with formalin on biopsy sponges, and stored for H&E staining and pan-cytokeratin immunohistochemistry.

Based on the results of Preliminary Study II.1 and II.2, 1.5 mm diameter explants were obtained in Preliminary Study II.3. The samples (n=5/group) were placed in 200 µl of medium on 96-well plates. The medium was changed after 1 hour for the treatment media supplemented with the following: 10 or 50 µg/mL LTA from *Staphylococcus aureus* or 100 and 250 ng/mL *Salmonella* Typhimurium derived flagellin, or with 50 and 100 µg/mL poly I:C for 12 h, respectively. The concentrations were selected based on the results of the hepatic preliminary study. The culture media were collected after 12 h and the viability of the cultures was tested at this time point using CCK-8 method.

Study II.: Effects of *Cordyceps militaris* extract on the gut explant model

The explants were obtained and cultured according to the protocol of the ileal explant model and Preliminary Study II.2 and II.3. The treatment groups were established as follows. The experimental groups received either 0.12% (m/m) or 0.6% (m/m) *Cordyceps miltaris* extract (CME) formulated with dextrose. According to the analytical examination (LC-MS/MS), the powder contains 5264 ng/g cordycepin, 4592 beauveriolide I and 9912 ng/g beauveriolide III, and approximately 1‰ β-glucan. Respectively, the control groups were treated with 0.12% (m/m) dextrose and 0.6% (m/m) dextrose. To induce inflammation, explants were treated with 50 µg/mL poly I:C in combination with either 0.12% (m/m) or 0.6% (m/m) dextrose. Furthermore, combined treatment groups were established, in which explants received 0.12% (m/m) or 0.6% (m/m) CME formulated with dextrose and 50 µg/mL poly I:C.

Statistical analysis

All statistical analysis was performed in R v. 4.0.3 or R v. 4.0.4 (R Core Team, 2020). The curves or ELISAs and colorimetric assays were fitted and concentrations were calculated with the free arigo GainData® platform. The yielded concentrations of caspase-3 and MDA and also the cytokine levels in the explant experiments were corrected to the protein content of the cell/explant suspension samples. The distribution of the data was examined using a Q-Q plot. In all the experiments the difference was considered significant between two groups if the p-value was 0.05 or less.

Results and discussion

Preliminary study I.: Establishment of a hepatic inflammatory model

The liver with resident tissue macrophages is the site of vivid innate immunity, activated also by PAMPs leaking through the intestinal barrier. The present study aimed to establish a proper hepatic inflammatory model by comparing the action of different PAMPs from poultry pathogens on chicken 2D primary hepatocyte—non-parenchymal cell co-cultures. Poly I:C depressed the metabolic activity at 50 and 100 μg/mL concentrations. Concerning the extracellular LDH activity significant elevation was detected after 50 ng/mL poly I:C treatment. The IL-6 concentration was significantly elevated in the culture medium after 250 ng/mL flagellin and 50 ng/mL poly I:C treatments. 50 μg/mL poly I:C challenge significantly increased the concentration of IL-8. The O55:B5 serotype *Escherichia coli* LPS failed to trigger proinflammatory cytokine production. The inadequate IL response is possibly related to the deficient TLR4 signal transduction in chicken and the lack of this additional route which phenomenon is investigated by other research groups also. Although this concept is still controversial, the use of other TLR agonists is hardly suggested. Based on these data flagellin and poly I:C can be considered as potent candidates to induce inflammation in chicken primary hepatic cell cultures

Study I.A: Effects of chicoric acid on the hepatic model

Virus induced damage triggered by excessive inflammation and free radical production is a major threat in the poultry industry, leading to low productivity even in vaccinated flocks. The purpose of the study was to induce inflammation with the viral double-stranded RNA analog poly I:C on chicken primary hepatocyte - non-parenchymal cell co-cultures to investigate the immunomodulatory and cell protectant effects of CA in comparison to NAC. Poly I:C significantly elevated the activity of the cell damage marker, LDH and the concentration of inflammatory cytokines (IL-6, IL-8, IFN-α, IFN-γ and M-CSF) in the culture medium and decreased cellular metabolic activity. CA significantly reduced the elevated LDH and cytokine levels, moreover, the higher, 100 μg/mL concentration of CA even elevated the level of metabolic activity when applied on the chicken primary hepatocyte – non-parenchymal cell co-culture exposed to poly I:C. Therefore, it proved to be not only anti-inflammatory - detectable in the decrease of IL-8, IFN-α (10 and 100 μg/mL) and IL-6, M-CSF levels (100 μg/mL) - but also protective regarding the cell viability. The reference molecule, 10 μg/mL NAC treatment decreased the level of each inflammatory cytokine but did not rectify cell damage or metabolic depression. The results indicate, that CA, present in common plants of the Asteraceae family, proves to be a beneficial hepatoprotective, and along with NAC, an immunomodulatory supplement *in vitro* under a stimulus mimicking viral infection. CA showed similar effect comparable to sylibin on a human hepatic damage cellular model and found exceptionally cell compatible in a study carried out with 85 other phytochemicals tested on BV2 murine microglia cells. This cell protectant nature is in line with our findings. Extracellular LDH activity after poly I:C exposure was in strong positive correlation with pro-inflammatory cytokines (IL-6, IL-8, M-CSF). The possible pyroptosis-related cell damage in the present study could be further supported by the notable but not significant caspase-3 level decrease under poly I:C exposure recovered in the CA treatment group.

Study I.B: Effects of luteolin on the hepatic model

The present research aimed to investigate the proinflammatory effect of flagellin released from the bacterial flagellum of *Salmonella* Typhimurium and to attenuate the induced inflammation with luteolin as a plant-derived flavonoid on a chicken primary hepatocyte-non-parenchymal cell co-culture. Flagellin significantly increased the concentration of the proinflammatory cytokine IL-8 and the ratio of IFN-γ/IL-10, while it decreased the level of IL-10, indicating that the model served adequate to study inflammation *in vitro*. Luteolin treatment at 4 µg/mL did not prove to be cytotoxic, as reflected by metabolic activity and extracellular LDH activity, and significantly reduced the flagellin-triggered IL-8 release of the cultured cells. Further, it had a diminishing effect on the concentration of IFN-α, H_2O_2 and MDA and restored the level of IL-10 and the ratio of IFN-γ/IL-10 when applied in combination with flagellin. These results suggest that luteolin at lower concentrations may protect hepatic cells from an excessive inflammatory response and act as an antioxidant to attenuate oxidative damage. The effect of luteolin on cell cultures of chicken origin was investigated for the first time to the best of our knowledge. The liver is the major organ to respond to flagellin-like products in contrast to bacterial LPS, and the flagellin induced inflammation has a major role in the pathogenesis of Salmonellosis. Moreover, the liver is one designated place of paratyphoid S. enterica multiplication in chicken, which further accentuates the importance of the topic.

Preliminary Study II.: Establishment of a gut explant inflammatory model

Gastrointestinal inflammation leads to maldigestion and systemic diseases in poultry. To tackle the problem of the industry and to search for therapeutic candidates *in vitro* models are inevitable. Explant systems comply with the 3R principles of laboratory animal experiments since they enable the preparation of multiple cultures from a single animal while providing a tissue-specific response within a regulated environment. Animal gut explant studies were done on a large scale to investigate the effects of mycotoxins and pathogens and to assess the impact of specific dietary components (eg. gliadin in coeliac disease). Both immersion and air-liquid interface explant models are available, although there is limited information on the size-dependent applicability and response to different PAMPs in the case of these model systems and most studies apply gut explants with large and inconsistent size. The study aimed to compare the morphology and viability of miniature chicken gut explant cultures obtained with a biopsy punch to examine the size-dependent change over time. To verify the applicability of the model, PAMPs: flagellin, LTA and poly I:C were applied to induce inflammation. The 2 mm diameter explants showed a decrease in metabolic activity measured by CCK-8 assay after 12 h and a significantly higher extracellular LDH activity indicating cellular damage compared to the 1 mm explants, supported by histological differences after 24 h of culturing. After 12 h of incubation, the 1.5 mm explants retained columnar epithelial lining with moderate damage of the lamina propria (H&E and pan-cytokeratin staining). Exposure to 100 µg/mL poly I:C reduced the metabolic activity of the 1.5 mm explants. LTA and poly I:C increased IFN-γ concentration at both applied doses (10, 20, and 50, 100 µg/mL respectively) and IFN-α and IL-6 concentration was elevated by 50 µg/mL and RANTES by 100 µg/ml poly I:C treatment. Flagellin administration in 100 ng/ml raised IL-6 and IL-10, while 250 ng/ml increased the concentration of IL-10 and RANTES. 10 µg/mL LTA exposure was the treatment solely inducing IL-2 elevation. IFN-γ/IL-10 ratio, the indicator of gut inflammatory homeostais was increased by the higher LTA and both poly I:C concentrations.

Effects of *Cordyceps militaris* extract on the gut explant model

The key marker molecule of the orange caterpillar fungus (*Cordyceps militaris*) is cordycepin, which exhibits antiviral and anti-inflammatory potential via different mechanisms. Additionally, cordycepin targets the adenosine receptor, which may activate the repair mechanisms of cells under conditions of excessive inflammation or ischemia. To alleviate the elevated cytokine production induced by poly I:C, co-treatment groups were also exposed to CME extract formulated with dextrose with 2,3 µg/g cordycepin content. In the case of IFN-γ, both the 0.6% (m/m%) and 0.12% CME treatments showed a reducing effect compared to the inflammatory control. Regarding RANTES, the 0.12% CME treatment decreased the cytokine level, while for IL-1β, the 0.6% CME treatment was found to be anti-inflammatory compared to the poly I:C-treated group. The present study substantiates that the application of CME even in moderate concentrations can trigger a therapeutic effect *in vitro*. Cordycepin exerts its anti-inflammatory effects primarily by inhibiting NF-κB signal pathway, but additional anti-inflammatory effect elicited on MAPK signaling pathways or A2A/A3 adenosine receptors. The antiviral effect of the molecule is mediated by its RNA chain-terminating nucleotide nature, but additionally it can enhance type I interferon production via the intracellular pattern recognition receptor RIG-I. Therefore, literature data and the results of the present study substantiate the outstanding potential of *Cordyceps militaris* and cordycepin in broiler farming facing profound economic loss because of virus-induced diseases.

Conclusions

The present thesis has successfully characterized and refined *in vitro* and *ex vivo* inflammatory models employing chicken hepatic co-cultures and ileal explants, thereby providing a platform for the evaluation of presumed immunomodulatory agents. The chicken primary hepatocyte – non-parenchymal cell co-culture model, responded clearly to inflammatory triggers like flagellin and poly I:C. This allowed for careful measurement of changes in cell metabolism, membrane integrity, and cytokine production. Similarly, the gut explant system, optimized for explant size and incubation time, proved sensitive to PAMP-induced inflammatory responses, reflecting the complexity of mucosal immunity.

Among the compounds tested, CA, a plant-derived metabolite, showed strong anti-inflammatory and cell-protective effects. Luteolin, a plant derived flavonoid, also reduced key markers of inflammation and oxidative stress, while restoring anti-inflammatory cytokine balance, although decreased the metabolic activity of the cells in the higher applied concentration. The fungal CME containing cordycepin, significantly lowered pro-inflammatory cytokine levels of the ileal explant model, suggesting it could help regulate intestinal immune responses.

Overall, these results highlight the value of advanced chicken tissue models for studying how bioactive compounds work and for screening new candidates. The clear benefits of CA, luteolin, and CME observed *in vitro* suggest that plant and fungal metabolites could become effective, natural alternatives to growth promoters, anti-inflammatory drugs and antibiotics in poultry health management. This approach supports the move toward more sustainable, antibiotic-free animal production, while also improving food safety. With further research, these natural compounds and the plants and fungi that contain large amounts of these substances could play an important role in future feed additives.

New scientific results

Ad 1,

An inflammatory model based on the chicken primary hepatocyte – non-parenchymal cell co-culture was established with the application of flagellin and poly I:C. Flagellin at 250 ng/mL concentration and poly I:C at 50 μg/mL concentration demonstrated pro-inflammatory effects indicated by the elevation of pro-inflammatory interleukin levels (IL-6 and IL-6, IL-8 respectively). Additionally, poly I:C reduced metabolic activity and moderately increased extracellular LDH activity.

Ad 2,

Chicoric acid significantly reduced the elevated IL-8, IFN-α (10 and 100 μg/mL), IL-6 and M-CSF (100 μg/mL) inflammatory cytokine levels of chicken primary hepatocyte – non-parenchymal cell co-culture exposed to poly I:C. Moreover, chicoric acid at 100 μg/mL concentration even elevated the metabolic activity and at 10 and 100 μg/mL concentration decreased the LDH activity in contrast to the reference molecule, N-acetylcysteine.

Ad 3,

Luteolin treatment at 4 μg/mL did not exhibit cytotoxicity and significantly reduced the flagellin-triggered IL-8 release of the chicken primary hepatocyte – non-parenchymal cell co-culture. Further, it had an attenuating effect on the concentrations of IFN-α, H_2O_2, and MDA, and restored the level of IL-10 as well as the ratio of IFN-γ/IL-10 when applied in combination with flagellin.

Ad 4,

The viability of chicken ileal explant cultures is size dependent, and 1.5 mm diameter explants can be maintained for 12 h. Chicken ileal explants stimulated with LTA and poly I:C produced excessive IFN-γ at 10, 20, and 50, 100 μg/mL concentrations. 10 μg/mL LTA exposure induced IL-2 elevation. IFN-α and IL-6 concentrations were elevated by 50 μg/mL, and RANTES by 100 μg/ml poly I:C treatments. Flagellin administration in 100 ng/ml raised IL-6 and IL-10, while 250 ng/ml increased the concentration of IL-10 and RANTES. IFN-γ/IL-10 ratio the indicator of gut inflammatory homeostasis was increased by the higher LTA and both poly I:C concentrations.

Ad 5,

Cordyceps militaris extract shows significant anti-inflammatory effects on miniature chicken gut explant cultures. The IFN-γ level decreased in case of 0.12% and 0.6% treatment concentrations, and the former decreased the RANTES and the latter the IL-1β level compared to the poly I:C inflammatory control.

Own scientific publications

Publications related to the topic of the present dissertation

Full text papers in peer-reviewed journals:

Sebők C, Tráj P, Vörösházi J, Mackei M, Papp M, Gálfi P, Neogrády Z, Mátis G (2021) **Two Sides to Every Question: Attempts to Activate Chicken Innate Immunity in 2D and 3D Hepatic Cell Cultures**. Cells 10:1910. https://doi.org/10.3390/cells10081910

Tráj P, Herrmann EM, Sebők C, Vörösházi J, Mackei M, Gálfi P, Kemény Á, Neogrády Z, Mátis G (2022) **Protective effects of chicoric acid on polyinosinic-polycytidylic acid exposed chicken hepatic cell culture mimicking viral damage and inflammation.** Vet Immunol Immunopathol 250:110427. https://doi.org/10.1016/j.vetimm.2022.110427

Tráj P, Sebők C, Mackei M, Kemény Á, Farkas O, Kákonyi Á, Kovács L, Neogrády Z, Jerzsele Á, Mátis G (2023) **Luteolin: A Phytochemical to Mitigate S. Typhimurium Flagellin-Induced Inflammation in a Chicken *In Vitro* Hepatic Model.** Animals 13:1410. https://doi.org/10.3390/ani13081410

Mátis G, Sebők C, Horváth DG, Márton RA, Mackei M, Vörösházi J, Kemény Á, Neogrády Z, Varga I, Tráj P. (2025) **Miniature chicken ileal explant culture to investigate the inflammatory response induced by pathogen-associated molecular patterns.** Front Vet Sci. 12:1484333. https://doi.org/10.3389/fvets.2025.1484333

Tráj P, Neogrády Z, Varga K, Bohár G, Gálfi P, Mátis G (2025) **Az állatgyógyászatban alkalmazható lehetséges antivirális és immunmoduláló hatással bíró gyógyhatású anyagok: a cikóriasav és a cordycepin.** MÁL. Közlésre elfogadva.

Poster presentations at international conferences

Tráj P, Sebők C, Mackei M, Márton RA, Vörösházi J, Lakatos B, Pápay M, Bohárné Varga K, Bohár G, Kemény Á, Mátis G Mátis, Neogrády Z. **Anti-inflammatory Effect of Cordyceps Militaris Extract in *Ex Vivo* Chicken Ileum Culture,** 17th International Scientific Conference On Probiotics, Prebiotics, Gut Microbiota And Health, Prága, 2024.06.18-20.

Oral presentations at Hungarian national conferences

Tráj P, Herrmann E, Sebők C, Vörösházi J, Mackei M, Márton R A, Gálfi P, Kemény Á, Neogrády Zs és Mátis G (2023) **A cikóriasav hatása virus RNS analóg molekulával kezelt csirke máj eredetű primer ko-kultúrán**, Akadémiai Beszámolók 2022. évi 49. füzet

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