Investigation of certain antimicrobial peptides in novel in vitro chicken and porcine gut-liver axis models

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Final report

1. Introduction

Poultry and swine, the most important meat-producing livestock species kept today, are highly exposed to various enteric infections, which can be exacerbated by stressors such as suboptimal housing conditions or transportation. The majority of these diseases are of bacterial origin and are proving increasingly difficult to treat since the spread of antimicrobial resistance (AMR). Antibiotics are becoming less and less effective and the use of agents to which pathogens are still susceptible is increasingly restricted by the need to protect human life. The demand for natural and synthetic alternatives for antibiotics is therefore growing.

Living organisms have various ways to defend themselves against harmful effects, for example by secreting biologically active substances. Antimicrobial peptides (AMPs), also known as host defense peptides (HDPs) are an important part of this system, produced by almost all organisms, and are promising candidates in the treatment of bacterial and inflammatory diseases. They are composed of up to 100 amino acids and are considered to have a direct antimicrobial effect as part of the innate immune system and to be able to modulate the immune response. Although their discovery is mainly due to their direct antimicrobial property, it has subsequently been demonstrated that in vertebrates, including humans, they can protect against pathogens by stimulating the body's self-defense and by suppressing harmful inflammatory processes. Their direct antimicrobial effect is poorly observed *in vivo* which is the reason why the term HDP is nowadays often used in the literature to refer to their protective function in assisting the immune system.

According to our hypothesis, based on their wide spreading immunomodulatory action, AMPs may be innovative candidates for novel anti-infective therapies in the health management of food producing animals. Hence, the main aim of this project was to investigate the effects of certain AMPs, such as cathelicidin-2, IDR-1002, cecropin A and Pap12-6 on the immune and oxidative stress response of the liver and the intestinal mucosa. Primary intestinal and hepatic cell culture models provide good tools for studying the cellular mechanisms of the inflammation and the redox homeostasis. Two *in vitro* models, established by our research group, were applied for this purpose: a primary hepatocyte – non-parenchymal (NP) cell co-culture and an intestinal

explant model, both of chicken and porcine origin. The reason behind choosing these models was that most of the infections affecting chickens and pigs are of intestinal origin and are often either caused by pathogenic bacteria or by an imbalance in the gut bacteriome. Therefore, it is beneficial to investigate the effects of such promising compounds on the immune response of the intestinal wall. Moreover, the liver is the first organ to interact with molecules absorbed from the intestines, therefore it has an especially important role in the fight against infections and pro-inflammatory agents entering the body.

2. Aims, materials and methods of the studies

Ethic statement

The hepatocytes and NP cells for the hepatic cell cultures and the intestinal explants were isolated from a 3-week-old male Ross-308 broiler chicken or from a post-weaning pig of the Hungarian great white breed weighing 15 kg in case of each experiment. The animals were fed according to the actual technology and all efforts were made to support the welfare of the animals. All experiments were in accordance with European Union laws, institutional guidelines, confirmed by the Local Animal Welfare Committee of the University of Veterinary Medicine Budapest, and permitted by the Government Office (number of permission: GK-419/2020; approval date: 11 May 2020).

Reagents and chemicals

Unless stated otherwise, all chemicals were purchased from Merck (Darmstadt, Germany).

Overview of the studies performed

To fulfill the goals of the project, the following *in vitro* studies have been designed and performed (**Table 1**).

Table 1. Overview of the performed studies.

Study No	Applied cell culture	Topic of the study
Study 1.	**Chicken primary hepatocyte – NP cell co-culture**	**The effects of AMPs on the inflammatory and oxidative stress response in the liver**
Study 1.1.		Preliminary study on pro-inflammatory compounds (*LPS, LTA, flagellin, enterotoxin, PMA, poly I:C*)
Study 1.2.		Testing the effects of *cathelicidin-2*

Study 1.3.		Testing the effects of *IDR-1002*
Study 1.4.		Testing the effects of c*ecropin A*
Study 1.5.		Testing the effects of *Pap12-6*
Study 2.	**Chicken primary hepatocyte – NP cell co-culture**	**The effects of plant derived bioactive compounds on the inflammatory and oxidative stress response of the liver**
Study 2.1.		Testing the effects of *chicoric acid*
Study 2.2.		Testing the effects of *luteolin*
Study 3.	**Chicken primary hepatic 3D spheroid co-culture**	**The effects of mycotoxins on the inflammatory and oxidative stress response of hepatic 3D spheroids**
Study 3.1.		Testing the effects of *T-2 toxin*
Study 3.2.		Testing the protective effects of *baicalin* under *DON* exposure
Study 4.	**Chicken primary intestinal explant culture**	**The effects of AMPs on the inflammatory and oxidative stress response of intestinal explant cultures**
Study 4.1.		Establishment and characterization of intestinal explant cultures from chicken
Study 4.2.		Preliminary study on pro-inflammatory compounds (*LTA, flagellin, poly I:C*)
Study 4.3.		Testing the effects of c*athelicidin-2*
Study 4.4.		Testing the effects of *IDR-1002*
Study 5.	**Porcine primary hepatic and intestinal cell culture**	**The effects of AMPs on the inflammatory and oxidative stress response of hepatic and intestinal cell cultures**
Study 5.1.		Preliminary study on pro-inflammatory compounds (*LPS, LTA, flagellin, poly I:C*)
Study 5.2.		Testing the effects of IDR-1002

Study 1. The effects of AMPs on the inflammatory and oxidative stress response in the liver

Aims

In the first part of the project, several AMPs were aimed to be tested on the chicken derived primary hepatic co-culture comprised of hepatocytes and NP cells, which has been previously established and characterized by our research group (Mackei et al., 2020). To monitor the immunomodulatory role of AMPs, at first numerous pro-inflammatory candidates had to be screened to achieve proper compounds for mimicking hepatic inflammation. Hence, a preliminary study (Study 1.1) with potential pro-inflammatory molecules was carried out as the first step, followed by the series of studies involving different AMPs (Studies 1.2-1.5).

Cell isolation and culturing

As mentioned above, hepatic cells were isolated from the liver of a 3-week-old male Ross-308 broiler chicken. Slaughter of the chicken was done under CO_2 anesthesia by decapitation. After opening the body cavity and accessing the liver, the portal system was cannulated through the *gastropancreaticoduodenal* vein (**Figure 1**). In order to remove the blood and disrupt the connective tissue of the organ, a three-step perfusion was performed with 150 ml of 0.5 M EGTA containing Hanks' Balanced Salt Solution (HBSS) buffer, 150 ml EGTA-free HBSS and in the final step, 100 ml HBSS supplemented with 100 mg collagenase type IV, 7 mM $CaCl_2$ and 7 mM $MgCl_2$ (Nordmark, Uetersen, Germany) as described previously by Mackei et al. (2020). After removal of the liver and the disruption of the Glisson's capsule, the cells were suspended in 50 mL ice cold bovine serum albumin (BSA, 1.25 g) containing HBSS buffer and filtered through three layers of sterile gauze. To prevent cell aggregation, this cell suspension was then kept on ice for 45 minutes. Following this, the suspension was centrifuged three times at 100x g for 3 minutes. The pellet containing hepatocytes was resuspended in Williams Medium E supplemented with 0.22% $NaHCO_3$, 50 mg/ml gentamycin, 0.5 µg/mL amphotericin B, 2 mM glutamine, 4 µg/l dexamethasone, 20 IU/l insulin, and 5% fetal bovine serum (FBS). The FBS was only present in the medium for the first 24 hours after seeding.

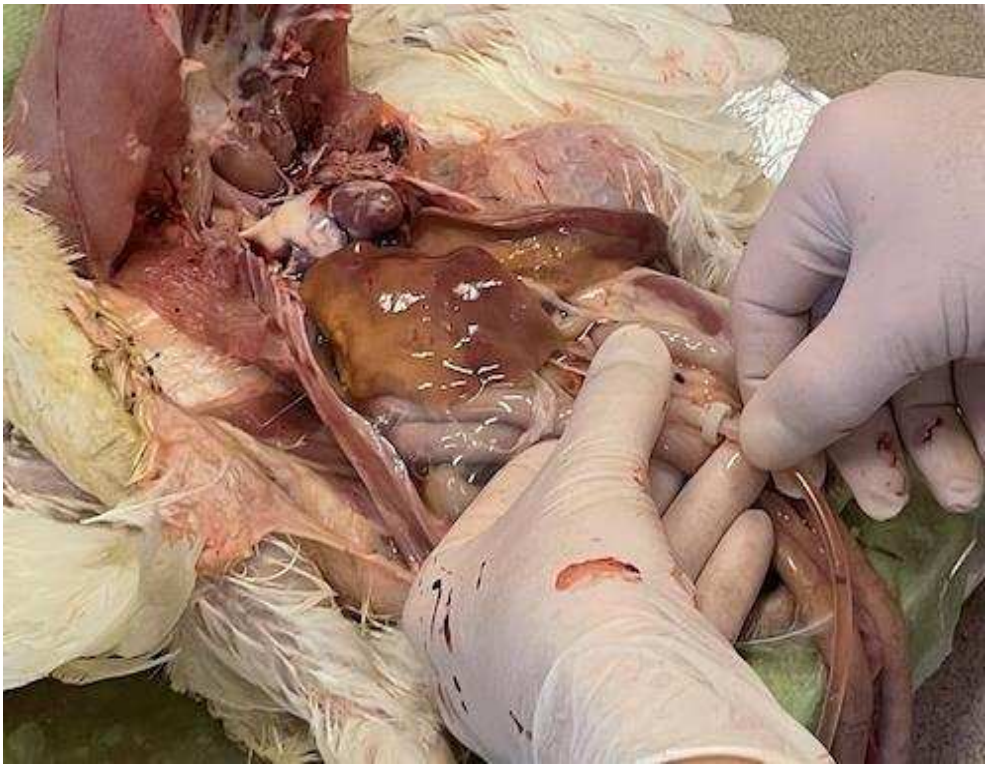


Figure 1. Perfusion of the chicken liver with collagenase-containing buffer solution.

To guarantee that any remaining hepatocytes, cell debris, and erythrocytes were sedimented, the supernatant containing NP cells was centrifuged once more at 350x g for 10 minutes. Thereafter, the supernatant was centrifuged at 800x g for 10 minutes, and the pellet was resuspended in Williams Medium E. Viability of the isolated hepatocytes and NP cells was confirmed by trypan blue exclusion test. The cell suspensions were diluted to $8.5*10^5$ cells/ml in the hepatocyte-enriched fraction and to $1.5*10^5$ cells/ml in the NP cell-containing fraction. Both hepatocyte and NP cell-enriched fractions have been previously characterized by flow cytometry and immunofluorescent detection of specific markers for hepatocytes and macrophages (Mackei et al., 2020).

After mixing the cell suspensions in the ratio of 6:1 (hepatocytes to NP cells), the hepatocyte – NP cell co-cultures were seeded onto 96-well plates (Greiner Bio-One Hungary Kft., Mosonmagyaróvár, Hungary) previously coated with collagen type I (10 µg/cm^2). The seeding volume was 100 µl/well. The cell cultures were incubated at 37°C in humid atmosphere with 5% CO_2. Culture media were changed after 4 hours and confluent co-cultures were gained after 24 hours (**Figure 2**). Culture medium contained 5% FBS only in the first 24 hours of culturing. Other supplements added to the medium during the experiment were the same as in the medium used for the seeding.

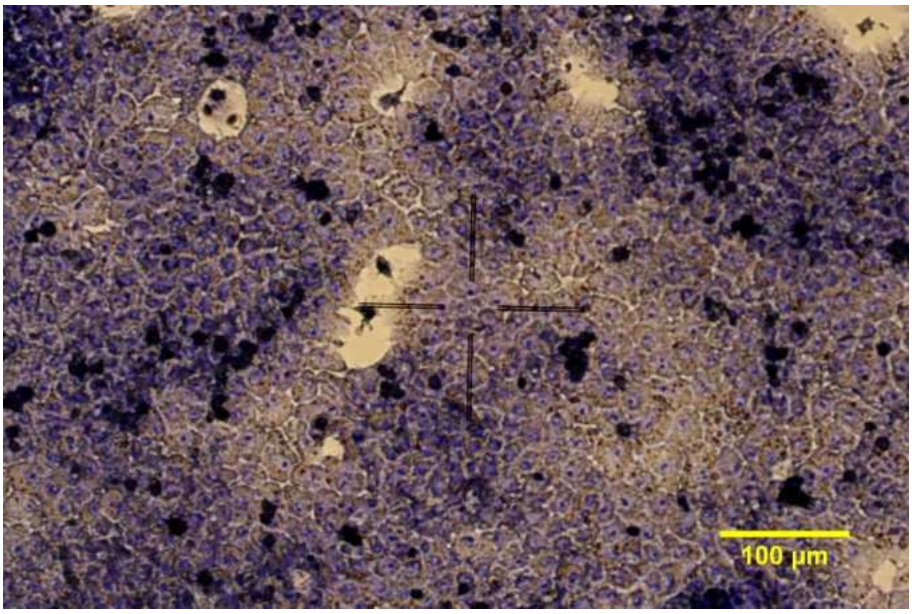


Figure 2. Giemsa staining of chicken hepatocyte – NP cell co-cultures (bar line = 100 µm)

Study 1.1. Preliminary study on pro-inflammatory compounds

Treatment of cell cultures

In the first part of the study, the medium of the cell cultures was supplemented with 0 (control), 10 or 50 µg/mL LPS from *Escherichia coli* (O55:B5), and with 10 or 50 µg/mL LTA from *Staphylococcus aureus* (n=6). In the second part of the study, cells were exposed to culture media supplemented with 0 (control), 20 or 50 µg/ml B subunit of the heat-labile enterotoxin derived from *Escherichia coli* (ETxB), 100 or 250 ng/mL *Salmonella* Typhimurium derived flagellin, 100 or 1000 ng/ml phorbol myristate acetate (PMA), further with 50 or 100 µg/ml polyinosinic polycytidylic acid (poly I:C) (n=5, except for metabolic activity, where n=10). 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. Cells were incubated with the treatment solutions for 24 hours.

Laboratory measurements

The metabolic activity of the cells was measured on 96-well plates by CCK-8 assay (Cell counting Kit-8, Dojindo Molecular Technologies, Rockville, USA), detecting the amount of $NADH+H^+$ and $NADPH+H^+$produced in the catabolic pathways. The test was performed according to the manufacturer's protocol. Extracellular lactate dehydrogenase (LDH) activity was measured from the cell culture media of 24-well plates using an enzyme kinetic photometric assay (Diagnosticum Ltd., Budapest, Hungary). The LDH activity was estimated by measuring the absorbance six times in one-minute increments while incubating the mixture at 37°C and averaging the changes between the consecutive time points.

The concentrations of interleukin-6 (IL-6) and chicken chemotactic and angiogenic factor (CXCLi2, also known as chicken interleukin-8 [IL-8]) were measured in the culture media by chicken specific ELISA kits (MyBioSource, San Diego, CA, USA) following the manufacturer's protocol.

Study 1.2. Testing the effects of cathelicidin-2

Aims

The major goal of this study was to assess the immunomodulatory role of certain AMPs under hepatic inflammatory conditions, at first that of the chicken-derived cathelicidin-2.

Cell isolation and culturing

In Study 1.2., the preparation of hepatocyte – NP cell co-cultures was carried out following the methodology explained in the Preliminary study (Study 1.1.). After isolation of the cells from a 3-week-old Ross-308 broiler chicken, cell cultures were prepared using 24-well and 96-well culture plates (Greiner Bio-One, Frickenhausen, Germany) pre-coated with collagen type I. On the 24-well plates, the seeding quantity was 400 µL/well, while 100 µL/well on the 96-well plates.

Treatment of cell cultures

After the 24-hour incubation time of seeded cell cultures, the media was removed and replaced by culture media containing the investigated agents. Cells were treated with Cath-2 in 5 nmol/mL and 10 nmol/mL concentrations (Isca Biochemicals, Exeter, Devon, UK). *Staphylococcus aureus*-derived LTA (Sigma-Aldrich, Darmstadt, Germany) was used in 50 µg/ml, and PMA (Sigma-Aldrich, Darmstadt, Germany) in 1000 ng/mL concentrations. The Control group received only Williams Medium E, without any supplementation. The treatment groups are presented in **Table 2** (n=6/group). After another 24 hours of incubation, samples were taken from the cell culture media and stored at -80°C until further measurements. 96-well plates were used for the measurement of the metabolic activity of the cells. Treatment groups are summarized in **Table 2**.

Table 2. Treatment groups in Study 1.2.

Treatment group	Cathelicidin-2	LTA	PMA
Control	-	-	-
Cath-5	5 nmol/ml	-	-
Cath-10	10 nmol/ml	-	-
LTA	-	50 µg/ml	-
LTA+Cath-5	5 nmol/ml	50 µg/ml	-
PMA	-	-	1000 ng/ml
PMA+Cath-5	5 nmol/ml	-	1000 ng/ml

Laboratory measurements

The metabolic activity and membrane damage were monitored with the CCK-8 and LDH leakage assay, respectively, as described in Study 1.1.

Chicken specific ELISA kits (MyBioSource, San Diego, CA, USA) were employed following the manufacturer's instructions to measure the CXCLi2 concentrations from the cell culture media of the 24-well plates. Absorbance values were obtained with a Multiskan GO 3.2 reader at 450 nm.

Luminex xMAP technology was used to determine the following protein concentrations, performing Milliplex Chicken Cytokine/Chemokine Panel (Cat.Nr.: GCYT1-16K, Merck KGaA, Darmstadt, Germany) according to the instructions of the manufacturer: IFN-γ, IL-10, and macrophage colony-stimulating factor (M-CSF) concentrations were measured.

Briefly, all samples were thawed and tested in a blind-fashion and in duplicate. 25 ml volume of each sample, standard, control, and assay buffer was added to a 96-well plate (provided with the kit). An additional 25 µl of the distinctly colored, capture antibody-coated bead sets were added to each well. After overnight incubation, biotinylated detection antibody mixture and streptavidin phycoerythrin were added to the plate following appropriate washing steps. After the last washing step, 150 ml drive fluid was added to the wells, the beads were resuspended for an additional 5 minutes on a plate shaker and read on the Luminex MAGPIX® instrument. Luminex xPonent 4.2 software was used for data acquisition. Five-PL regression curves were generated to plot the standard curves for all analytes by the Milliplex Analyst 5.1 (Merck Millipore, Darmstadt, Germany) software calculating with bead median fluorescence intensity (MFI) values.

The fluorometric Amplex Red method (Thermo Fisher Scientific, Waltham, MA, USA) was used to detect extracellular H_2O_2 content in the culture medium. A Victor X2 2030 fluorometer (Perkin Elmer, Waltham, MA, USA) was used to detect fluorescence (λex = 560 nm, λem = 590 nm) after a 30-minute incubation of 50 µl freshly prepared Amplex Red (100 µM) and HRP (0.2 U/mL) containing working solution with 50 µl culture medium at room temperature (21°C).

A specific colorimetric assay was used to detect MDA concentration as a marker of lipid peroxidation in cell culture medium. 300 µl of freshly prepared thiobarbituric acid stock solution was mixed with 100 µl cell culture medium according to the protocol. After 1 hour of incubation at 95°C, the solutions were cooled on ice for 10 minutes. A Multiskan GO 3.2 reader was used to measure absorbance at 532 nm.

Study 1.3. Testing the effects of IDR-1002

Aims

The major goal of this study was to assess the immunomodulatory role of IDR-1002, which is a synthetic derivate of the cattle-derived bactenecin.

Cell isolation and culturing

In Study 1.3., the preparation of hepatocyte – NP cell co-cultures was carried out following the methodology explained in the Preliminary study (Study 1.1.). After isolation of the cells from a 3-week-old Ross-308 broiler chicken, cell cultures were prepared using 24-well and 96-well culture plates (Greiner Bio-One, Frickenhausen, Germany) pre-coated with collagen type I. On the 24-well plates, the seeding quantity was 400 µl/well, while 100 µl/well on the 96-well plates.

Treatment of cell cultures

After 24 h culturing, cells were exposed to the following substances for 24 h. IDR-1002 (Isca Biochemicals, Exeter, Devon, UK) was added in concentrations of 10, 30, and 90 µg/ml (IDR-low, IDR-medium, and IDR-high, respectively) alone and in combination with 50 µg/ml LTA from *Staphylococcus aureus*. The Control group received only Williams Medium E, without any supplementation. The treatment groups are presented in **Table 3** (n=6/group). After sampling from the cell culture media of 24-well plates, cells were lysed by applying 50 µl/well Mammalian Protein Extraction Reagent (M-PER) lysis buffer and scraped from the surface. Culture media and cell lysate samples were stored at -80°C until further processing. 96-well plates were used for the measurement of the metabolic activity of the cells.

Table 3. Treatment groups in Study 1.3.

Treatment group	**IDR-1002**	**LTA**
Control	-	-
IDR-low	10 µg/ml	-
IDR-medium	30 µg/ml	-
IDR-high	90 µg/ml	-
LTA	-	50 µg/ml
LTA + IDR-low	10 µg/ml	50 µg/ml
LTA + IDR-medium	30 µg/ml	50 µg/ml

LTA + IDR-high	90 µg/ml	50 µg/ml

Laboratory measurements

The metabolic activity and membrane damage were monitored with the CCK-8 and LDH leakage assay, respectively, as described in Study 1.1.

Chicken specific ELISA kits (MyBioSource, San Diego, CA, USA) were applied following the manufacturer's instructions to assess the concentrations of CXCLi2, IL-6, and Nuclear factor erythroid 2-related factor 2 (Nrf2) in the culture media, and protein carbonyl (PC) in the cell lysate samples of the 24-well plates. Absorbance values were obtained with a Multiskan GO 3.2 reader at 450 nm.

Luminex xMAP technology was used to determine the following protein concentrations, performing Milliplex Chicken Cytokine/Chemokine Panel (Cat.Nr.: GCYT1-16K, Merck KGaA, Darmstadt, Germany) according to the instructions of the manufacturer: IL-16, IFN-γ, IL-10, M-CSF and RANTES (Regulated And Normal T-cell Expressed and Secreted) concentrations were measured as explained in case of Study 1.2.

Similarly to Study 1.2, the extracellular H_2O_2 concentration was determined with the Amplex Red assay, while MDA levels were assessed with the specific colorimetric method. Total protein concentrations were measured from the cell lysate samples with the Pierce™ Bicinchoninic Acid (BCA) Protein Assay (Thermo Fisher Scientific, Waltham, MA, USA) as indicated by the manufacturer, with BSA as standard. 25 µL of sample was added to 200 µL of reagent mixture, and absorbance was measured after 30 min incubation at 37°C at 562 nm with a Multiskan GO 3.2 reader. The values were used to standardize the PC values which were measured from the same cell lysate samples.

Study 1.4. Testing the effects of cecropin A

Aims

The major goal of this study was to assess the immunomodulatory role of cecropin A as a natural insect AMP under Poly I:C-evoked inflammation.

Cell isolation and culturing

In Study 1.4., the preparation of hepatocyte – NP cell co-cultures was carried out following the methodology explained in the Preliminary study (Study 1.1). After isolation of the cells from a 3-week-old Ross-308 broiler chicken, cell cultures were prepared using 24-well

and 96-well culture plates (Greiner Bio-One, Frickenhausen, Germany) pre-coated with collagen type I. On the 24-well plates, the seeding quantity was 400 µL/well, while 100 µL/well on the 96-well plates.

Treatment of cell cultures

Treatment of the cells was accomplished according to **Table 4**. The control was created by adding only cell culture media to the cells. To evoke inflammation, Poly I:C was added at a concentration of 50 µg/ml. The effects of CecA were examined solely and in Poly I:C-induced inflammation, respectively, at five different concentrations, being 1, 3.125, 6.25, 12.5, and 25 µg/ml.

Table 4. Treatment groups in Study 1.4.

Treatment group	Cecropin A	Poly I:C
Control	—	—
Cec-1	1 µg/ml	—
Cec-2	3.125 µg/ml	—
Cec-3	6.25 µg/ml	—
Cec-4	12.5 µg/ml	—
Cec-5	25 µg/ml	—
PI:C	—	50 µg/ml
PI:C+Cec-1	1 µg/ml	50 µg/ml
PI:C+Cec-2	3.125 µg/ml	50 µg/ml
PI:C+Cec-3	6.25 µg/ml	50 µg/ml
PI:C+Cec-4	12.5 µg/ml	50 µg/ml
PI:C+Cec-5	25 µg/ml	50 µg/ml

Following 24 h treatments, cell culture media samples of 24-well microplates were taken and centrifuged (5 min, 4,000×g), and aliquots were frozen at −80°C until the below-mentioned measurements.

Laboratory measurements

The viability of cultured cells was monitored based on the membrane damage assessed with the LDH leakage assay as described in Study 1.1.

Based on our results regarding cellular viability, inflammatory and redox markers continued to be examined only in treatment groups containing CecA at concentrations of 1, 3.125 and 6.25 µg/ml, respectively. IL-8 (often referred to as CXCLi2 in chicken) and transforming growth factor-ß1 (TGF-ß1) were measured in the culture media by chicken-specific ELISA kits (MyBioSource Inc., San Diego, CA, USA), according to the manufacturer's instructions and absorbance values were read by a Multiscan GO 3.2 reader at 450 nm.

Levels of IL-6, IL-10 and interferon-γ (IFN-γ) were assayed by Luminex xMAP technology, using Milliplex Chicken Cytokine/Chemokine Panel (Merck KGaA Cat.Nr.: GCYT1-16K) as described before in Study 1.2.

Similarly to Study 1.2, the extracellular H_2O_2 concentration was determined with the Amplex Red assay, while MDA levels were assessed with the specific colorimetric method.

Study 1.5. Testing the effects of Pap12-6

Aims

The major goal of this study was to assess the immunomodulatory role of Pap12-6 (PAP), which is a 12-meric synthetic AMP, derived from the N-terminal end of the natural papiliocin found in swallowtail butterfly (*Papilio xuthus*) larvae.

Cell isolation and culturing

In Study 1.5., the preparation of hepatocyte – NP cell co-cultures was carried out following the methodology explained in the Preliminary study (Study 1.1.). After isolation of the cells from a 3-week-old Ross-308 broiler chicken, cell cultures were prepared using 24-well and 96-well culture plates (Greiner Bio-One, Frickenhausen, Germany) pre-coated with collagen type I. On the 24-well plates, the seeding quantity was 400 µL/well, while 100 µL/well on the 96-well plates.

Treatment of cell cultures

Chicken hepatocyte – NP co-cultures were treated according to **Table 5**, using the previously mentioned supplemented Williams' Medium E culture media, however, without the use of FBS. Two different inflammatory conditions were evoked by the addition of LTA (50 µg/ml) and Poly I:C (50 µg/ml). PAP was applied in three different concentrations (5, 25 and 50 µg/ml) solely and together with LTA and Poly I:C, respectively. Cells receiving only Williams' Medium E were considered as Control. After 24 hours of treatment, cell culture media

samples were taken and frozen to -80°C until further measurements. Thereafter, to gain lysate samples, culture plates were washed first with 300 µl/well of phosphate-buffered saline (PBS) solution, followed by the addition of 50 µl/well of M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA). Thereafter, cells were scraped from the bottom of the wells and frozen to -80°C until further processing.

Table 5. Treatment groups in Study 1.5.

Treatment group	PAP	LTA	Poly I:C
Control	—	—	—
PAP-1	5 µg/ml	—	—
PAP-2	25 µg/ml	—	—
PAP-3	50 µg/ml	—	—
LTA	—	50 µg/ml	—
LTA+PAP-1	5 µg/ml	50 µg/ml	—
LTA+PAP-2	25 µg/ml	50 µg/ml	—
LTA+PAP-3	50 µg/ml	50 µg/ml	—
PI:C	—	—	50 µg/ml
PI:C+PAP-1	5 µg/ml	—	50 µg/ml
PI:C+PAP-2	25 µg/ml	—	50 µg/ml
PI:C+PAP-3	50 µg/ml	—	50 µg/ml

Laboratory measurements

The viability of cultured cells was monitored based on the membrane damage assessed with the LDH leakage assay as described in Study 1.1.

To investigate the influence of PAP on the immune response, the levels of IL-6, IL-8, IL-10, interferon (IFN)-γ and regulated upon activation, normal T cell expressed, and secreted (RANTES) were measured in the cell culture media. IL-8 (often referred to as CXCLi2 in chicken) was examined with chicken-specific ELISA (MyBioSource Inc., San Diego, CA, USA, Cat. Nr.: MBS289628), using a sandwich technique, following the manufacturer's instructions. Absorbance values were measured with a Multiscan GO 3.2 reader, at 450 nm. The concentrations of IL-6, IL-10, IFN-γ and RANTES were determined by Luminex xMAP Technology, using Milliplex Chicken Cytokine/Chemokine Panel 1 – Immunology Multiplex Assay (Cat. Nr.: GCYT1-16K).

Similarly to Study 1.2, the extracellular H_2O_2 concentration was determined with the Amplex Red assay. Levels of Nrf2 were assayed using a Chicken NFE2L2 (Nuclear Factor, Erythroid Derived 2 Like Protein 2) ELISA Kit (MyBioSource Inc., San Diego, CA, USA, Cat. Nr.: MBS8807992), following the protocol provided by the manufacturer. Absorbance values were determined with a Multiscan GO 3.2 reader, at 450 nm. Total protein concentrations were measured from the cell lysate samples with the Pierce™ Bicinchoninic Acid (BCA) Protein Assay (Thermo Fisher Scientific, Waltham, MA, USA) as indicated in Study 1.3.

Study 2. The effects of plant derived bioactive compounds on the inflammatory and oxidative stress response of the liver

Aims

Beside assessing the immunomodulatory action of different AMPs as the core scope of the project, it was also aimed to investigate plant-derived bioactive compounds, such as chicoric acid (CA) and luteolin on the already applied hepatocyte – NP cell co-culture of chicken origin.

Study 2.1. Testing the effects of chicoric acid

Cell isolation and culturing

In Study 2.1., the preparation of hepatocyte – NP cell co-cultures was carried out following the methodology explained in Study 1.1. After isolation of the cells from a 3-week-old Ross-308 broiler chicken, cell cultures were prepared using 24-well and 96-well culture plates (Greiner Bio-One, Frickenhausen, Germany) pre-coated with collagen type I. On the 24-well plates, the seeding quantity was 400 µl/well, while 100 µl/well on the 96-well plates.

Treatment of cell cultures

After 24 h, the cultured cells were treated with Williams' Medium E supplemented with 0 (control) or 50 µg/ml poly I:C to mimic virus-induced inflammation. Additionally, cells of the treatment group were treated with 10 or 100 µg/ml CA and 100 or 200 µg/mL N-acetylcysteine (NAC) as a control agent. The other treatment groups consisted of combinations with 50 µg/ml poly I:C together with 10 or 100 µg/ml CA, or 50 µg/mL poly I:C and 100 or 200 µg/ml NAC. The overview of the applied treatment of the cells can be found in **Table 6**.

Table 6. Treatment groups in Study 2.1.

Group	Poly I:C	CA	NAC
Control	-	-	-
C1	-	10 μg/ml	-
C2	-	100 μg/ml	-
N1	-	-	100 μg/ml
N2	-	-	200 μg/ml
P	50 μg/ml	-	-
PC1	50 μg/ml	10 μg/ml	-
PC2	50 μg/ml	100 μg/ml	-
PN1	50 μg/ml	-	100 μg/ml
PN2	50 μg/ml	-	200 μg/ml

Laboratory measurements

The viability of cultured cells was monitored based on the metabolic activity assayed with the CCK-8 test, and on the membrane damage assessed with the LDH leakage assay as described in Study 1.1.

The measurements of IL-6 and IL-8 concentration were carried out with chicken specific ELISA kits (MyBioSource, San Diego, CA, USA) according to the manufacturer's instructions, and the absorbance was measured at 450 nm with a Multiskan GO 3.2 reader.

Luminex xMAP method was used to measure the protein concentrations of IFNα, IFN-γ, IL-10 and M-CSF, performing Milliplex Chicken Cytokine/Chemokine Panel (Cat.Nr.: GCYT1-16K, Merck KGaA, Darmstadt, Germany) following the instructions of the company as described above.

Study 2.2. Testing the effects of luteolin

Cell isolation and culturing

In Study 2.2., the preparation of hepatocyte – NP cell co-cultures was carried out following the methodology explained in Study 1.1. After isolation of the cells from a 3-week-

old Ross-308 broiler chicken, cell cultures were prepared using 24-well and 96-well culture plates (Greiner Bio-One, Frickenhausen, Germany) pre-coated with collagen type I. On the 24-well plates, the seeding quantity was 400 µl/well, while 100 µl/well on the 96-well plates.

Treatment of cell cultures

After 24 hours of incubation, cells were cultured in FBS-free Williams' Medium E supplemented with flagellin derived from *Salmonella* 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) for 24 hours.

The culture medium of cells in 24-well plates was then collected, and they were lysed in Mammalian Protein Extraction Reagent (M-PER) lysis buffer. Samples of cell lysate and culture medium were stored at -80 °C until the measurements.

Laboratory measurements

The viability of cultured cells was monitored based on the metabolic activity assayed with the CCK-8 test, and on the membrane damage assessed with the LDH leakage assay as described in Study 1.1. H_2O_2 concentrations were measured with the fluorimetric Amplex Red method, MDA levels were assessed with the specific colorimetric assay as mentioned in Study 1.2.

The measurements of IL-6 and IL-8 concentration were carried out with chicken specific ELISA kits (MyBioSource, San Diego, CA, USA) according to the manufacturer's instructions, and the absorbance was measured at 450 nm with a Multiskan GO 3.2 reader.

Study 3. The effects of mycotoxins on the inflammatory and oxidative stress response of hepatic 3D spheroids

Aims

Following the monitoring of several AMPs, it was also aimed to assess the deteriorative action of certain mycotoxins, such as the trichotecenes T-2 toxin and deoxynivalenol (DON) on hepatic cell cultures originated from chicken. Further, novel candidates as protective agents against the cellular effects of mycotoxins (such as the plant-derived bioactive molecule baicalin) were also tested in order to restore normal cell function. For these studies, a primary

three dimensional (3D) spheroid co-culture was prepared from the freshly isolated hepatocytes and NP cells, enabling prolonged maintenance to study subacute or chronic toxin exposures.

Cell isolation and culturing

In Studies 3.1-3.2., the isolation of hepatocyte and NP cell enriched fractions was carried out following the methodology explained in Study 1.1. After isolating and mixing the two cell fractions in 6:1 ratio, hepatocytes to NP cells, 3D spheroid were prepared with the magnetic bioprinting technology. All the needed equipment and chemicals for the preparation of magnetic 3D cell cultures were purchased from Greiner Bio-One Hungary Ltd (Mosonmagyaróvár, Hungary). In order to magnetize the cells, 800 µl of magnetic nanoparticles (NanoShuttle^{TM}-PL) were added to 8 ml of hepatocyte – NP cell co-culture suspension. The cells were then seeded onto a 96-well cell repellent plate and were incubated at 37°C for 1 h. During this time, the magnetic nanoparticles bound to the cell membrane. Afterwards, the plate was placed on top of a magnetic drive with small magnets under each well of the plate (Spheroid Drive) and was incubated for 48 h at 37°C in humid atmosphere with 5% CO_2. The culture medium was changed to serum-free medium after 24 h with the use of a Holding Drive.

Study 3.1. Testing the effects of T-2 toxin

Treatment of cell cultures

After 48 h incubation, the 3D cell cultures were exposed to culture media supplemented with 0 (control), 100, 500 or 1000 nM T-2 toxin for 24 h. Samples were collected at the end of the incubation from the cell culture media, and the cells were lysed by intermittent sonication (1/second) in 40 µl of M-PER buffer for 5 seconds using a Bandelin Sonopuls HD 2200 homogenizer (Bandelin Electronic GmbH & Co. KG, Berlin, Germany). The samples were stored at -80°C until further analysis. The control, untreated spheroids have been fixed in 10% buffered formalin solution and after embedding and sectioning the slides they were stained with haematoxylin and eosin to examine the spheroid morphology.

Laboratory measurements

The viability of cultured cells was monitored based on the metabolic activity assayed with the CCK-8 test, and on the membrane damage assessed with the LDH leakage assay as described in Study 1.1.

All measurements concerning stress and inflammatory markers were performed using chicken specific ELISA kits (MyBioSource, San Diego, CA, USA) according to the manufacturer's instructions. To investigate the oxidative stress, the concentration of a lipid peroxidation marker, malondialdehyde (MDA) was measured from the cell culture media. The protein damage caused by oxidative stress was detected by the determination of protein carbonyl (PC) content from the cell lysates. In order to determine the effects of T-2 toxin on cellular inflammation, the concentrations of two pro-inflammatory cytokines, IL-6 and IL-8 were assayed from cell-free supernatants. Endoplasmic reticulum stress was evaluated by measuring the concentration of glucose regulated protein 78 (GRP78) and heat shock protein 27 (HSP27) from the media.

Metabolome analysis of the samples was carried out using the AbsoluteIDQ p180 Kit (Biocrates Life Science AG, Innsbruck, Austria) in accordance with the manufacturer's instructions. This kit detects and quantifies up to 188 metabolites belonging to 5 separate compound classes: acylcarnitines, proteinogenic and modified amino acids, glycerophospho- and sphingolipids (phosphatidylcholines, lysophosphatidylcholines, sphingomyelins), biogenic amines and hexoses.

Study 3.2. Testing the effects of DON and baicalin

Treatment of cell cultures

After 48 h of cell culturing, cells were treated with three concentrations of baicalin (5, 15 and 45 µg/ml) and two concentrations of DON (2 and 20 µg/ml) or combinations of them for 24 and 48 h as shown in **Table 7**. At the end of the incubation times, the cell culture media was collected and after 48 h, the cells were lysed by intermittent sonication (1/second) in 40 µl M-PER (Mammalian Protein Extraction Reagent, Thermo Fisher SSC, Budapest, Hungary) buffer using Bandelin Sonopuls HD 2200 homogenizer (Bandelin Electronic GmbH & Co. KG, Berlin, Germany). The samples were then stored at -80°C until further use.

Table 7. Setting of the treatment groups. DON: deoxynivalenol, BAI: baicalin.

Group	**DON**	**BAI**
Control	-	-
BAI5	-	5 µg/ml
BAI15	-	15 µg/ml
BAI45	-	45 µg/ml

DON2	2 µg/ml	-
DON20	20 µg/ml	-
DON2+BAI5	2 µg/ml	5 µg/ml
DON2+BAI15	2 µg/ml	15 µg/ml
DON2+BAI45	2 µg/ml	45 µg/ml
DON20+BAI5	20 µg/ml	5 µg/ml
DON20+BAI15	20 µg/ml	15 µg/ml
DON20+BAI45	20 µg/ml	45 µg/ml

Laboratory measurements

The viability of cultured cells was monitored based on the metabolic activity assayed with the CCK-8 test, and on the membrane damage assessed with the LDH leakage assay as described in Study 1.1.

Oxidative parameters and inflammatory cytokines were quantified using chicken-specific ELISA kits (MyBioSource, San Diego, CA, USA) following the manufacturer's instructions. For the investigation of oxidative stress, NRF-2 was measured from cell culture medium and the level of 8-OHdG was determined from the cell lysates. To monitor the inflammatory processes, the concentrations of IL-6 and -8, as well as IFN-γ were assessed from the cell culture medium.

Study 4. The effects of AMPs on the inflammatory and oxidative stress response of intestinal explant cultures

Aims

Following the experiments carried out on hepatic models, a chicken-derived primary small intestinal explant culture was established and characterized in order to study the inflammatory and oxidative stress response of the avian gut, as well as the modulatory effects of certain AMPs. Therefore, a novel method was applied to make chicken ileal immersion explant cultures of defined diameter comparable in size to organ spheroids. Reusable biopsy punches with plungers were used to puncture the gut to obtain explants. Furthermore, these miniature explants were cultured to assess their hypothetically size-dependent viability and microscopic morphology, and to select the ideal explant size. Afterwards the ileal explants of the selected size were stimulated with TLR agonists and selected AMPs to investigate their inflammatory response.

Study 4.1. Establishment and characterization of intestinal explant cultures from chicken

Explant isolation and culturing

Similarly to the preparation of hepatic cell cultures, a 3-week-old male Ross-308 broiler chicken was sacrificed for the isolation of intestinal explants in case of each experiment. Before the aseptic opening of the coelomic cavity, the animal was decapitated under CO_2 anesthesia and restrained in a dorsal position. Then 15 cm long ileal segment was excised 10 cm distally from the Meckel's diverticulum. The adipose tissue was removed from this intestinal section by hand. The outer side was washed with phosphate buffered saline (PBS) + Pen-Strep solution (1%) and the removed section was flushed from both directions using a stainless-steel feeding needle. The intestinal section was cut longitudinally on the mesenteric side and washed three consecutive times in PBS + Pen-Strep solution. This step might be repeated until no physical contaminants are visible. Thereafter, it was cut lengthwise into four pieces, which were placed in ice cold fresh PBS + Pen-Strep solution and were further handled on ice. For culturing 24- and 96-well plates (Greiner Bio-One Hungary Kft. in Mosonmagyaróvár, Hungary) coated with collagen type I ($10 g/cm^2$) were applied. Before excising the explants, the wells of the required culture plates were filled with culture medium (24-well plates – 400 µL medium, 96-well plates – 100 µL medium). The medium used throughout the experiment was Dulbecco's Minimal Essential Medium-F12 supplemented with FBS (fetal bovine serum) 2.5%, glutamine 1%, Pen-

Strep 1% and one package of HCMTM SingleQuotsTM Kit. The explants were cultured at 37°C under 5% CO_2.

The excision of the explants was carried out as follows. 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. The surface of the intestinal samples was rinsed with ice-cold PBS + Pen-Strep solution using a syringe every few minutes during the excision of the explants to keep the tissue moist. Explant removal was performed with a biopsy punch perpendicular to the glass plate, moving the punch in a circular motion. The excised specimens were transferred directly into the prepared wells with the medium by the plunger of the instrument. Peyer's patches were avoided, and explant replicates were sectioned in a row for each experimental group at a time to minimize the potential difference between groups.

Characterization of explant cultures

The viability, cell membrane damage and histology of 1 and 2 mm diameter gut tissue samples were assessed. Six 1 and 2 mm diameter explants were fixed instantly with formaldehyde solution on biopsy sponges at room temperature for further evaluation (H&E stain). The rest of the explants were directly deposited into the cell culture 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 shape of the 1 and 2 mm explants were cylinder-like with the same height, therefore the volume was calculated and compared according to the formula of the volume of a cylinder. Volumes were kept at a low minimum to ease the diffusion of gases to the explants. 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 measurement (H&E stain and pan-cytokeratin immunohistochemistry) from both, the 1-and the 2 mm diameter explants. The media of the explants was changed every 12 hours with storing the removed medium samples for LDH measurement in case of the separate LDH groups of explants and a CCK assay was performed with a concomitant medium change of the separate CCK groups. In case of the 24h histology groups a simple change of the medium has happened. The 2 mm explants for the CCK and LDH tests were macroscopically disrupted after approximately 48 hours of culturing, therefore the measurements were terminated after 36 hours. 1 mm samples were maintained longer with regular changes of the media every other day. After 9 days the study was concluded and the 1 mm explants were stained according to Giemsa.

Laboratory measurements

The viability of cultured cells was monitored based on the metabolic activity assayed with the CCK-8 test after 1, 12, 24 and 36 h of culturing, and further, based on the membrane damage assessed with the LDH leakage assay at the same time points (1, 12, 24 and 36 h) as described in Study 1.1.

The explants directly after excision from the gut and after 24 hours culturing were fixed on foam biopsy pads. After 24 hours of fixation at room temperature in formaldehyde solution, samples were trimmed and dehydrated with a series of ethanol and xylene in an automatic tissue processor. The dehydrated tissue samples were embedded in paraffin blocks and 3-4 μm thin sections were cut manually and mounted onto Superfrost+ adhesion slides (Thermo Fisher Scientific, Waltham, USA). The unstained sections were deparaffinized and rehydrated in xylene and alcohol, respectively. Routine H&E staining was performed in an automatic staining instrument.

Pan-cytokeratin immunohistochemistry was applied to evaluate the integrity of intestinal epithelium via the assessment of cytokeratin intermediate filaments. Sections were deparaffinized in xylene (2×15min) and rehydrated in 96% and absolute ethanol for 5-5 minutes. This was followed by a 2x rinse in distilled water for a few minutes. Antigen retrieval was performed in EnVision FLEX target retrieval solution, High pH 9 (50x) in a microwave (800 Watts for 5 minutes, then 180 Watts for 10 minutes), then the sections were flushed with PBS 2 times. Thereafter EnVision FLEX Peroxidase-Blocking reagent was added for 5 minutes which was followed by a PBS rinse 3x (EnVision Flex Wash Buffer 20x). The primary antibody (Monoclonal Mouse Anti-Human Cytokeratin, clones AE1/AE3, Agilent Technologies) was added and incubated at a 1:200 dilution in a wet chamber for 30 minutes at room temperature. The slides were rinsed 2x in PBS before and after the 20-minute incubation of the secondary antibody (Dako, EnVision Flex HRP). The staining was displayed with DAB Chromogen (Dako Envision Flex DAB + Chromogen 1 drop + 1ml Envision Flex Substrate Buffer) for 1-3 minutes and thereafter the sections were rinsed 2x in PBS. The slides were counterstained with hematoxylin according to GILL II for 10-15 seconds, bluing was performed in PBS for 5 minutes. After dehydration in ethanol and xylene (3-3min in 96% and absolute ethanol, and 2×3min in xylene), the slides were covered with coverslips.

Study 4.2. Preliminary study on pro-inflammatory compounds (LTA, flagellin, poly I:C)

Explant isolation and culturing

Small intestinal explants were isolated and cultured as mentioned in Study 4.1. Based on the viability and histological data in Study 4.1 and for technical reasons 1.5 mm diameter explants were prepared for the testing of various pro-inflammatory candidates. The samples (n=5/group) were placed in 200 µl of medium on 96-well plates.

Treatment of explant cultures

The medium of explants was changed 1 hour after seeding for the treatment media supplemented with 10 or 50 µg/mL LTA from *Staphylococcus aureus* or 100 or 250 ng/ml *Salmonella* Typhimurium-derived flagellin, or further with 50 or 100 µg/ml poly I:C for 12 h. The concentrations were selected based on preliminary results and previous studies with chicken cell cultures (Study 1.1). The culture media were collected after 12 h and stored at -80°C until further analyses. The viability of the cultures was tested at this time point using CCK-8 method. The untreated control explants were fixed with formalin on biopsy sponges and stored for H&E staining and pan-cytokeratin immunohistochemistry.

Laboratory measurements

Following the 12 h incubation time, the CCK-8 assay and the measurement of the extracellular LDH activity were carried out as described in Study 1.1. Routine H&E staining and immunohistochemical detection of pan-cytokeratin were conducted as described in Study 4.1.

IL-8 concentrations were determined from medium samples using a chicken-specific sandwich ELISA kit (MyBioSource, San Diego, CA, USA) according to the manufacturer's instructions. The Milliplex Chicken Cytokine/Chemokine Panel (Cat.Nr.: GCYT1-16 K, Merck KGaA, Darmstadt, Germany) medium was used to assess the protein concentrations of IFN-α, IFN-γ, IL-2, IL-6, IL-10 and RANTES in samples of supernatants as mentioned in Study 1.2.

Study 4.3. Testing the effects of cathelicidin-2

Explant isolation and culturing

Small intestinal explants with the diameter of 1.5 mm were isolated and cultured as mentioned in Study 4.1. The samples (n=6/group) were placed in 200 µl of medium on 96-well plates.

Treatment of explant cultures

After the 2-hour incubation, cell culture medium was removed from the wells and fresh medium substituted with the treatment solutions was added. The treatment solutions included: 5, 10 and 25 nmol/mL concentrations of chicken cathelicidin-2 (Cath-low, Cath-medium and Cath-high, respectively), 10 µg/mL *Staphylococcus aureus* derived LTA (LTA), and the combination of the former (LTA+Cath-low, LTA+Cath-medium and LTA+Cath-high). Control group received only fresh cell culture medium. Explants were incubated with the solutions for 12 hours. After incubation, samples were taken from the cell culture medium and stored at -80°C until further processing. Treatment groups are explained in **Table 8**.

Table 8. Treatment groups of the study. First column indicates the name of the groups (also seen on Figures), second column contains the given concentrations of chicken cathelicidin-2 (Cath-2) and third column the concentrations of lipoteichoic acid (LTA).

Treatment group	Cath-2	LTA
Control	-	-
Cath-low	5 nmol/ml	-
Cath-medium	10 nmol/ml	-
Cath-high	25 nmol/ml	-
LTA	-	10 µg/ml
LTA+Cath-low	5 nmol/ml	10 µg/ml
LTA+Cath-medium	10 nmol/ml	10 µg/ml
LTA+Cath-high	25 nmol/ml	10 µg/ml

Laboratory measurements

Following the 12 h incubation time, the CCK-8 assay and the measurement of the extracellular LDH activity were carried out as described in Study 1.1 to assess the viability of

explants. CxCLi2/IL-8 concentrations were determined from medium samples using a chicken-specific sandwich ELISA kit (MyBioSource, San Diego, CA, USA) according to the manufacturer's instructions. The Milliplex Chicken Cytokine/Chemokine Panel (Cat.Nr.: GCYT1-16 K, Merck KGaA, Darmstadt, Germany) medium was used to assess the protein concentrations of IFN-γ, IL-2, IL-6 and IL-10 in samples of supernatants as mentioned in Study 1.2.

Study 4.4. Testing the effects of IDR-1002

Explant isolation and culturing

Small intestinal explants with the diameter of 1.5 mm were isolated and cultured as mentioned in Study 4.1. The samples (n=6/group) were placed in 200 µl of medium on 96-well plates.

Treatment of explant cultures

After the 2-hour incubation, the medium was removed from the wells and replaced with treatment solutions using the medium as a solvent. The explants were treated with three different concentrations (10, 30, and 90 µg/ml) of IDR-1002, 10 µg/ml LTA derived from *Staphylococcus aureus*, and the combinations of them. Treatment groups with concentrations are shown in **Table 9**. Each group contained 6 explants, which were incubated for 12 hours with the treatment solutions in the same conditions as before.

Table 9. Treatment groups of the study. First column indicates the name of the groups (also seen on Figures), second column contains the given concentrations of chicken IDR-1002 and third column the concentrations of lipoteichoic acid (LTA).

Treatment group	**IDR-1002**	**LTA**
Control	-	-
IDR-low	10 µg/ml	-
IDR-medium	30 µg/ml	-
IDR-high	90 µg/ml	-
LTA	-	10 µg/ml
LTA + IDR-low	10 µg/ml	10 µg/ml
LTA + IDR-medium	30 µg/ml	10 µg/ml
LTA + IDR-high	90 µg/ml	10 µg/ml

Laboratory measurements

Following the 12 h incubation time, the CCK-8 assay and the measurement of the extracellular LDH activity were carried out as described in Study 1.1 to assess the viability of explants. CxCLi2/IL-8 concentrations were determined from medium samples using a chicken-specific sandwich ELISA kit (MyBioSource, San Diego, CA, USA) according to the manufacturer's instructions. The Milliplex Chicken Cytokine/Chemokine Panel (Cat.Nr.: GCYT1-16 K, Merck KGaA, Darmstadt, Germany) medium was used to assess the protein concentrations of IFN-γ, IL-2, IL-10 and RANTES in culture media samples as mentioned in Study 1.2. To detect the amount of extracellular H_2O_2 in the culture medium, the fluorometric Amplex Red method (Thermo Fisher Scientific, Waltham, MA, USA) was used as described in Study 1.2.

Study 5. The effects of AMPs on the inflammatory and oxidative stress response of hepatic and intestinal cell cultures

Aims

Beside poultry farming, the breeding and fattening of swine plays also key role in food production. Since antibiotic resistance is an emerging issue in the pig sector as well, following the experiments carried out on chicken-derived models, porcine primary hepatic co-cultures and small intestinal explant cultures were also established in an attempt to study the inflammatory and oxidative stress response of pigs. Further, the immunomodulatory effects of certain AMPs were also aimed to be tested in the frame of the project and in future studies.

Hepatic cell isolation and culturing

Cells were isolated from a post-weaning Hungarian great white pig of 15 kg body weight, originated from Dunahyb Kft. (Szekszárd, Hungary). The hepatic lobe *processus caudatus* was excised from the abdominal cavity and transported to the cell laboratory chilled on ice in sterile phosphate-buffered saline (PBS) supplemented with 1% penicillin-streptomycin (Pen-Strep, Gibco [Waltham, MA, USA]). Cell isolation was performed by 3-step *ex vivo* perfusion, with buffer solutions being passed directly into the lobe through the visible large vessels of the incision plate. The buffer solutions, pre-warmed to 37ºC and bubbled with Carbogen (95% O_2, 5% CO_2), were injected into the liver lobe at a rate of 100 ml/min. In the first step, 500 ml of Hanks' Balanced Salt Solution (HBSS) solution containing ethylenediamine-tetraacetic acid (EDTA, 1 g/l) was used, followed by 600 ml of EDTA-free

buffer, and finally the liver structure was digested with 300 ml of HBSS supplemented with type IV collagenase (1 g/l ,7 mM $CaCl_2$ and 7 mM $MgCl_2$[Nordmark, Uetersen, Germany]).

Cell harvesting and purification then continued under sterile conditions with continuous ice cooling. Once the Glisson's capsule has been excised, the digested liver parenchyma was filtered through a 100 µm pore size sterile gauze sheet to remove cell aggregates. The resulting primary cell suspension was centrifuged three times (50g, 75 seconds, 4ºC), resuspending the sediment in Williams' Medium E (supplemented with 0.22% $NaHCO_3$, 50 mg/ml gentamycin, 2 mM glutamine, 4 µg/L dexamethasone, 20 IU/L insulin, 0.5 µg/ml amphotericin-B and 5% fetal bovine serum [FBS]), to obtain a cell suspension rich in liver cells at the end of the differential centrifugation process. Trypan blue exclusion test was used to determine cell viability and cell count. The cell concentration was adjusted to 4×10^5/ml after cell counting using the Bürker's chamber.

The cell suspension was seeded on 24-well culture dishes (Greiner Bio-One, Frickenhausen, Germany) (300 µl/well) pre-coated with type I collagen and incubated for 4 hours at 37°C with 5% CO_2. The first medium exchange was performed 4 hours after seeding with the appropriately supplemented Williams' Medium E. To monitor the metabolic activity of the cells, the cell suspension was seeded in 96-well plates (Greiner Bio-One, Frickenhausen, Germany) (100 µl/well) and the cells were incubated in the same way as the 24-well dishes. After 24 hours, confluent monolayer cultures were formed.

Isolation and culturing of intestinal explants

Intestinal explants were isolated from the same animals as mentioned in case of the hepatic model. After opening of the abdominal cavity, a section of ileum at least 15-20 cm long was excised and washed with PBS after removal of adipose tissue. The segment of the intestine was cut lengthwise and placed in a sterile glass container after rinsing both sides four times with PBS. The next steps described were carried out under sterile conditions with continuous ice cooling. After the washing steps described above, the ileum was stretched and 1.5 mm diameter pieces of intestinal epithelium were cut from the intestinal wall using biopsy punches (MDE GmbH; Heidelberg, Germany) and placed one by one in a 96-well culture dish, and 200 µl of DMEM-F12 medium was measured per well on the explants. The medium used was Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (supplemented with 2.5% FBS [heat inactivated], 1% L-glutamine; 1% penicillin/streptomycin; Growth factor mixture supplement

a singular dose of HCM™ SingleQuots™ Kit (Lonza-Biocenter [Szeged, Hungary]). After two hours incubation (37°C, 5% CO_2) and a single medium exchange, the tissue cultures could be treated.

Study 5.1. Preliminary study with pro-inflammatory compounds (LPS, LTA, flagellin, poly I:C)

Treatments of cell cultures

The treatments were performed for 24 hours in case of the liver and 12 hours in case of the intestinal explants, prepared and maintained as mentioned above. After the incubation period has elapsed, samples of the 24-well culture dish medium and cell lysate were taken and stored at -80°C until further use. Nine treatment groups were formed, each group containing 6 wells. The culture medium of liver cell cultures and intestinal explants was supplemented with 10 or 50 µg/ml LPS from *Escherichia coli* (O55:B5), 10 or 50 µg/ml of LTA from *Staphylococcus aureus*, flagellin from *Salmonella* Typhimurium at concentrations of 100 or 250 ng/ml, and 50 or 100 µg/ml of poly I:C, similarly to the chicken studies.

Laboratory measurements

Following the 12 h incubation time, the CCK-8 assay and the measurement of the extracellular LDH activity were carried out as described in Study 1.1 to assess the viability of explants. The Milliplex Porcine Cytokine/Chemokine Panel (Cat.Nr.: GCYT1-16 K, Merck KGaA, Darmstadt, Germany) medium was used to assess the protein concentrations of GM-CSF, IFN-γ, IL-4, IL-6, IL-8, IL-10 and TNF-α in culture media samples as mentioned in Study 1.2.

Study 5.2. Testing the effects of IDR-1002

Cell isolation and culturing

Primary porcine hepatocyte cultures were prepared and maintained as described above in the previous study.

Treatment of explant cultures

After the 24-hour incubation, the medium was removed from the wells and replaced with treatment solutions using the medium as a solvent. Hepatic cell cultures were treated with

three different concentrations (10, 30, and 90 µg/mL) of IDR-1002, 10 µg/mL LPS from *Escherichia coli* (O55:B5), and the combinations of them. Treatment groups with concentrations are shown in **Table 10**. Each group contained 6 wells, which were incubated for 24 hours with the treatment solutions under the same conditions as before.

Table 10. Treatment groups of the study. First column indicates the name of the groups (also seen on Figures), second column contains the given concentrations of IDR-1002 and third column the concentrations of lipopolysaccharide (LPS).

Treatment group	**IDR-1002**	**LPS**
Control	-	-
IDR-low	10 µg/mL	-
IDR-medium	30 µg/mL	-
IDR-high	90 µg/mL	-
LPS	-	10 µg/mL
LPS + IDR-low	10 µg/mL	10 µg/mL
LPS + IDR-medium	30 µg/mL	10 µg/mL
LPS + IDR-high	90 µg/mL	10 µg/mL

Statistics

All statistical analyses were performed in R v. 4.0.3 (R Core Team, 2020). The normal distribution of samples was assessed with Shapiro-Wilk's test. In case of normal distribution, one-way ANOVA was applied with post-hoc tests for pairwise comparisons, or two-sampled Student's t-test was applied in certain cases. Pairwise comparisons were performed using Wilcoxon signed-rank test if some of the treatment groups showed non-normal distribution. We have considered a difference statistically significant if the p-value was less than 0.05. Graphs were generated using GraphPad Prism 9 software (GraphPad Software Inc., San Diego, CA, USA) or MS Excel.

3. Results of the studies

Study 1. The effects of AMPs on the inflammatory and oxidative stress response in the liver

Study 1.1. Preliminary study with pro-inflammatory compounds

No alteration was found in the metabolic activity of cell cultures after LPS or LTA exposure compared to controls (**Figure 3/A**). There was a significant increase after 20 and 50 µg/mL enterotoxin treatment ($p<0.001$, $p=0.0232$, respectively), and a significant decrease after applying 100 and 1000 ng/mL PMA ($p=0.0041$, $p=0.0041$, respectively), as well as when using 50 and 100 µg/mL poly I:C ($p<0.001$, $p=0.0041$, respectively) (**Figure 3/B**).

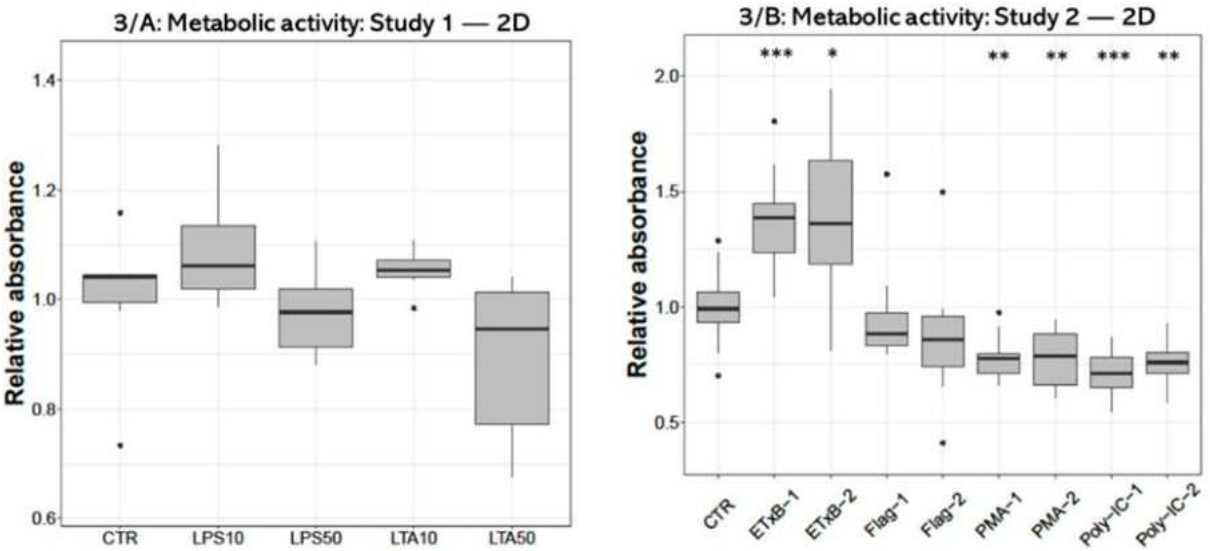


Figure 3. Boxplots showing the metabolic activity of hepatocyte—NP cell co-cultures (**A**) and (**B**) indicated by the CCK-8 assay. Relative absorbances were calculated by considering the mean value of control cultures as 1. The "CTR" refers to control cells that received none of the treatments. The treatments were: LPS10 and LPS50 = 10 and 50 µg/mL lipopolysaccharide (LPS) from *Escherichia coli*, LTA10 and LTA50 = 10 and 50 µg/mL lipoteichoic acid (LTA) from *Staphylococcus aureus*, ETxB-1 and -2 = 20 and 50 µg/mL subunit B of heat-labile enterotoxin of *Escherichia coli*, Flag-1 and -2 = 100 and 250 ng/mL flagellin from *Salmonella* Typhimurium, PMA-1 and -2 = 100 and 1000 ng/mL phorbol myristate acetate (PMA), poly-IC-1 and -2 = 50 and 100 µg/mL polyinosinic polycytidylic acid (poly

I:C). Asterisks over the boxes refer to significant differences compared to "CTR" cells within the same cell culture model and the same study. * p<0.05, ** p<0.01, *** p<0.001.

Concerning the extracellular LDH activity significant increase was found after both (10 and 50 µg/mL) LTA concentrations (p=0.0260, p=0.0043, respectively) (**Figure 4/A**). Significant elevation was detected after 50 ng/mL poly I:C treatment (p=0.0318) (**Figure 4/B**).

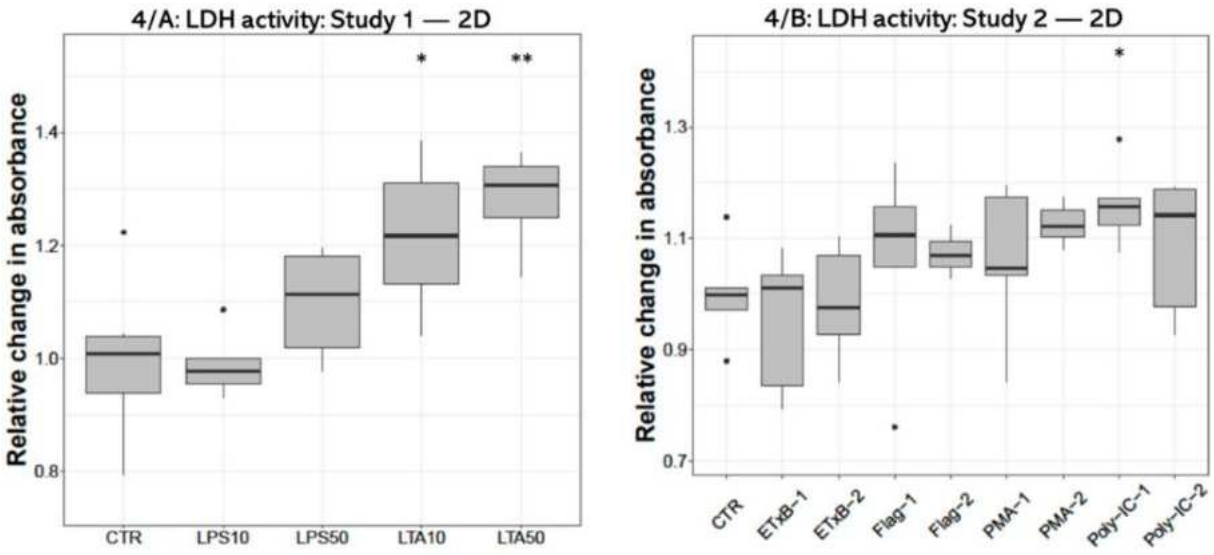


Figure 4. Boxplots showing the cell viability of hepatocyte—NP cell co-cultures (**A**) and (**B**) indicated by the measurement of the extracellular LDH activity. Relative chenges in absorbances were calculated by considering the mean value of control cultures as 1. The "CTR" refers to control cells that received none of the treatments. The treatments were: LPS10 and LPS50 = 10 and 50 µg/mL lipopolysaccharide (LPS) from *Escherichia coli*, LTA10 and LTA50 = 10 and 50 µg/mL lipoteichoic acid (LTA) from *Staphylococcus aureus*, ETxB-1 and -2 = 20 and 50 µg/mL subunit B of heat-labile enterotoxin of *Escherichia coli*, Flag-1 and -2 = 100 and 250 ng/mL flagellin from *Salmonella* Typhimurium, PMA-1 and -2 = 100 and 1000 ng/mL phorbol myristate acetate (PMA), poly-IC-1 and -2 = 50 and 100 µg/mL polyinosinic polycytidylic acid (poly I:C). Asterisks over the boxes refer to significant differences compared to "CTR" cells within the same cell culture model and the same study. * p<0.05, ** p<0.01.

The IL-6 concentration was significantly elevated in the culture media of cell cultures after 250 ng/mL flagellin (p=0.0195), 1000 ng/mL PMA (p=0.0286) and 50 ng/mL poly I:C treatments (p=0.0195) (**Figure 5/B**), while both LPS and LTA could not influence the cytokine level (**Figure 5/A**). The concentrations of IL-8 were significantly increased by 50 µg/mL LTA

treatment (p=0.0133) (**Figure 6/A**). The 1000 ng/mL PMA (p=0.0286) and 50 µg/mL poly I:C challenges significantly increased the concentration of IL-8 (p=0.036).

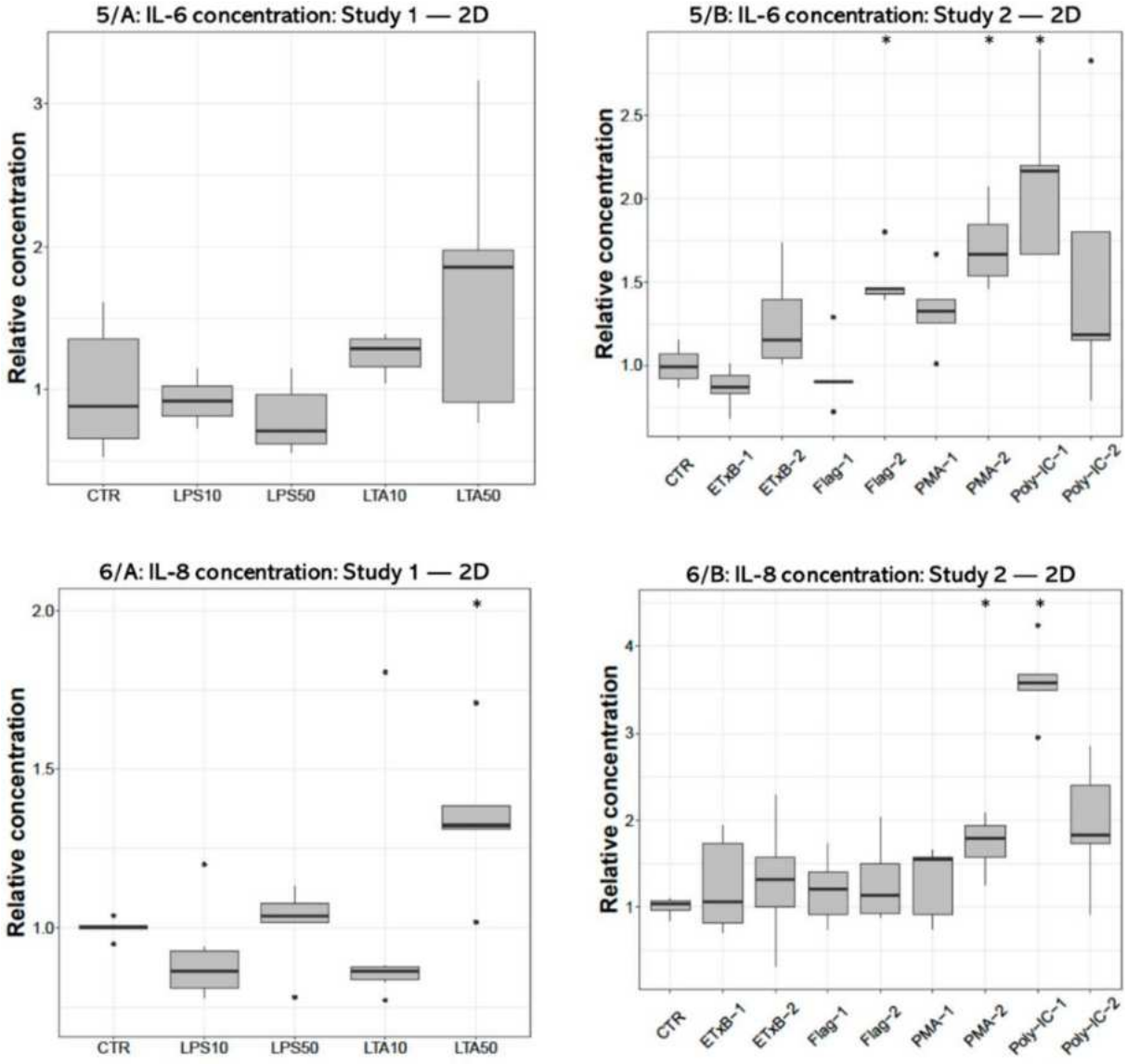


Figure 5-6. Boxplots showing the extracellular IL-6 concentration as measured from the cell culture media of hepatocyte—NP cell co-cultures. Relative concentrations were calculated by considering the mean value of control cultures as 1. The "CTR" refers to control cells that received none of the treatments. The treatments were: LPS10 and LPS50 = 10 and 50 µg/mL lipopolysaccharide (LPS) from *Escherichia coli*, LTA10 and LTA50 = 10 and 50 µg/mL lipoteichoic acid (LTA) from *Staphylococcus aureus*, ETxB-1 and -2 = 20 and 50 µg/mL subunit B of heat-labile enterotoxin of *Escherichia coli*, Flag-1 and -2 = 100 and 250 ng/mL flagellin from *Salmonella* Typhimurium, PMA-1 and -2 = 100 and 1000 ng/mL phorbol myristate acetate (PMA), poly-IC-1 and -2 = 50 and 100 µg/mL

polyinosinic polycytidylic acid (poly I:C). Asterisks over the boxes refer to significant differences compared to "CTR" cells within the same cell culture model and the same study. * $p<0.05$.

Study 1.2. Testing the effects of cathelicidin-2

Metabolic activity was significantly decreased in the samples treated with both concentrations of Cath-2 (Cath-5 and Cath-10) compared to the Control, as seen in Hiba! A hivatkozási forrás nem található.**7/A** ($p=0.004$, $p<0.001$, respectively). LTA or PMA exposure did not affect significantly the metabolic activity of the cells when compared to the Control. Cells exposed to LTA or PMA and treated with the higher dose of Cath-2 (LTA+Cath-10, PMA+Cath-10) showed significantly lower metabolic activity than the cells that were exposed to only LTA or PMA ($p=0.003$, $p=0.002$, respectively; **Figure 7/A**).

The extracellular LDH activities were increased in the medium of the cells treated with both 5 nmol/mL and 10 nmol/mL Cath-2 (Cath-5 and Cath-10) compared to Control ($p<0.001$ for both cases, **Figure 7/B**). LDH activity in the cultures that were exposed to LTA showed no significant change compared to the Control, while PMA decreased the LDH activity ($p=0.045$). With regards to the LTA-exposed cells that were treated with Cath-2, only the treatment with the lower dose of the peptide (LTA+Cath-5) showed a significant increase in LDH levels when compared to the LTA-only condition ($p=0.045$). Both concentrations of Cath-2 (PMA+Cath-5, PMA+Cath-10) increased the LDH activity compared to the group that was treated only with PMA ($p<0.001$ in both cases; **Figure 7/B**).

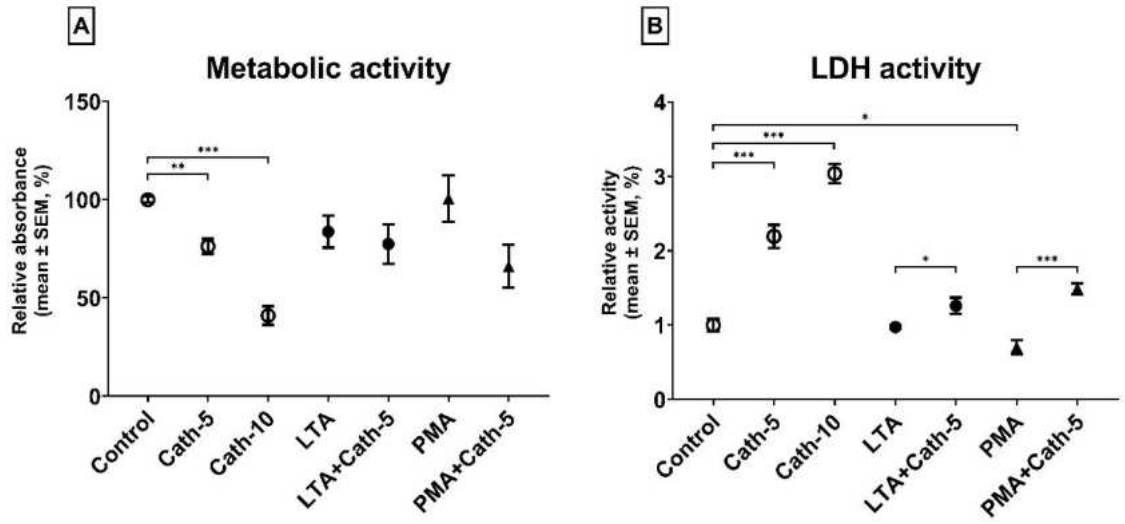


Figure 7. Meanplots showing the changes in metabolic activity (A) and extracellular lactate dehydrogenase (LDH) activity (B) of the hepatic cell cultures. Results are expressed as mean ± SEM in relative values where 100% is the mean of the control group. Cath-5/10 = 5 and 10 nmol/mL

concentrations of chicken cathelicidin-2; LTA = 50 μg/mL concentration of lipoteichoic acid from Staphylococcus aureus; PMA = 1000 ng/mL concentration of phorbol myristate acetate. Control groups received none of the treatment. n = 6. * $p<0.05$; ** $p<0.01$; *** $p<0.001$

There was no significant elevation in IFN-γ levels when treated with the lower concentration of Cath-2 (Cath-5), however, as seen in **Figure 8/A**, cells treated with the higher dose of the peptide (Cath-10) showed higher levels of IFN-γ than the Control ($p<0.001$). Compared to the Control, the cultures exposed to LTA showed higher levels of IFN-γ ($p<0.001$). The LTA-exposed cells treated with the lower dose of Cath-2 (LTA+Cath-5) showed a decrease in IFN-γ levels in comparison with the LTA-only condition ($p=0.001$). PMA did not affect the IFN-γ production of the cells, and when administered together, the higher dose of Cath-2 (PMA+Cath-10) increased the levels of IFN-γ compared to the PMA group ($p=0.001$; **Figure 8/A**).

As seen in **Figure 8/B**, both Cath-2 concentrations (Cath-5 and Cath-10) significantly decreased the levels of M-CSF ($p=0.031$, $p<0.001$, respectively) compared to the Control. LTA also showed a depressing effect on the concentrations of this molecule ($p=0.004$) compared to the Control, and the lower dose of Cath-2 (LTA+Cath-5) further decreased the levels of M-CSF compared to the group that only received LTA ($p=0.003$). There was a significant increase in the PMA-treated cultures ($p=0.003$) compared to the Control, and in comparison with this group, both concentrations of Cath-2 (PMA+Cath-5 and PMA+Cath-10) decreased the concentration of M-CSF ($p=0.001$, $p<0.001$, respectively; **Figure 8/B**).

Cultures treated with the lower (Cath-5) and the higher dose (Cath-10) of Cath-2 had significantly increased levels of CXCLi2 compared to the Control ($p=0.028$, $p<0.001$, respectively, Figure 11/C). LTA or PMA did not significantly alter the CXCLi2 levels. In the LTA+Cath-5 and LTA+Cath-10 groups, the CXCLi2 concentrations did not differ from the LTA group significantly, however, in the PMA+Cath-5 and PMA+Cath-10 groups, the CXCLi2 levels were significantly elevated compared to the group that only received PMA ($p=0.002$, $p<0.001$, respectively; **Figure 8/C**).

IL-10 cytokine levels followed patterns mostly similar to CXCLi2 (**Figure 8/D**). Compared to the Control, cells treated with only the higher concentration of Cath-2 (Cath-10) showed an increase in IL-10 levels ($p<0.001$). There were no significant differences in IL-10 levels between the control and the cell cultures exposed to only LTA or PMA. However, a significant decrease in IL-10 levels was observed in the LTA-exposed cells that were treated with the lower dose of Cath-2 (LTA+Cath-5) compared to the LTA group ($p=0.033$), and a

significant increase in PMA-exposed cells with the higher dose of the peptide (PMA+Cath-10) compared to the PMA group (p=0.002; **Figure 8/D**).

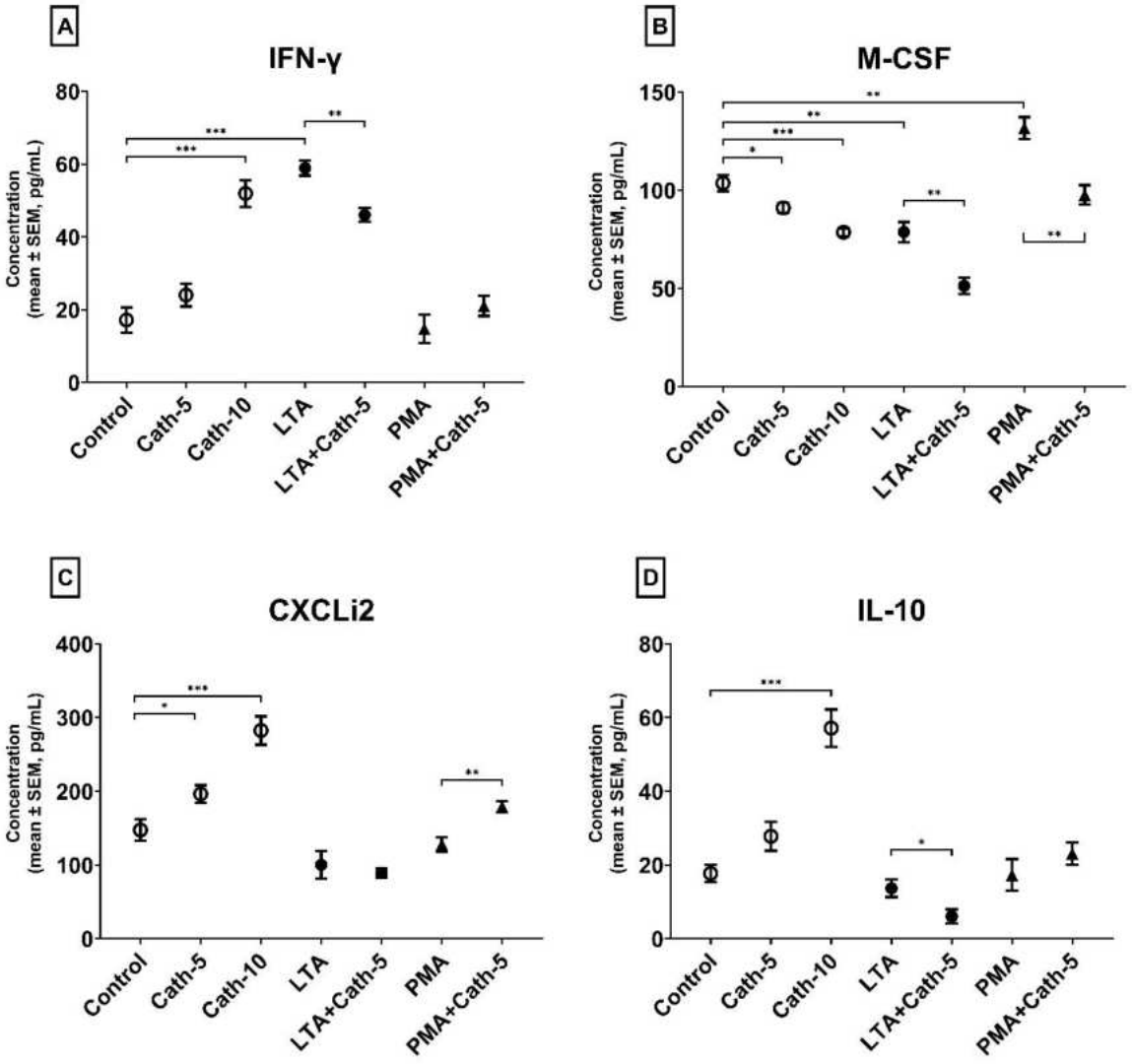


Figure 8. Meanplots showing the changes in concentrations of interferon (IFN)-γ (A), macrophage colony-stimulating factor (M-CSF, B), CXCLi2 (C), and interleukin (IL)-10 (D) of the hepatic cell cultures. Results are expressed as mean ± SEM in pg/mL. Cath-5/10 = 5 and 10 nmol/mL concentrations of chicken cathelicidin-2; LTA = 50 µg/mL concentration of lipoteichoic acid from Staphylococcus aureus; PMA = 1000 ng/mL concentration of phorbol myristate acetate. Control groups received none of the treatment. n = 6. * $p<0.05$; ** $p<0.01$; *** $p<0.001$

The H_2O_2 concentrations were elevated after both Cath-2 concentrations (Cath-5 and Cath-10) (p=0.001, $p<0.001$, respectively). LTA also significantly increased the H_2O_2 concentrations ($p<0.001$), and the lower concentration of Cath-2 (LTA+Cath-5) decreased it

compared to the LTA group (p=0.002). The H_2O_2 concentration was increased after the PMA treatment (p=0.009), and it was significantly greater following the treatment with the higher dose of Cath-2 (PMA+Cath-10) supplementation compared to the PMA group (p<0.001; **Figure 9/A**).

The higher dose of Cath-2 (Cath-10) decreased the concentration of MDA (p=0.006). Neither LTA nor PMA changed the MDA levels significantly, however, when LTA was administered together with the higher concentration of Cath-2 (LTA+Cath-10), the MDA concentration was significantly lower compared to the LTA group (p=0.042, **Figure 9/B**).

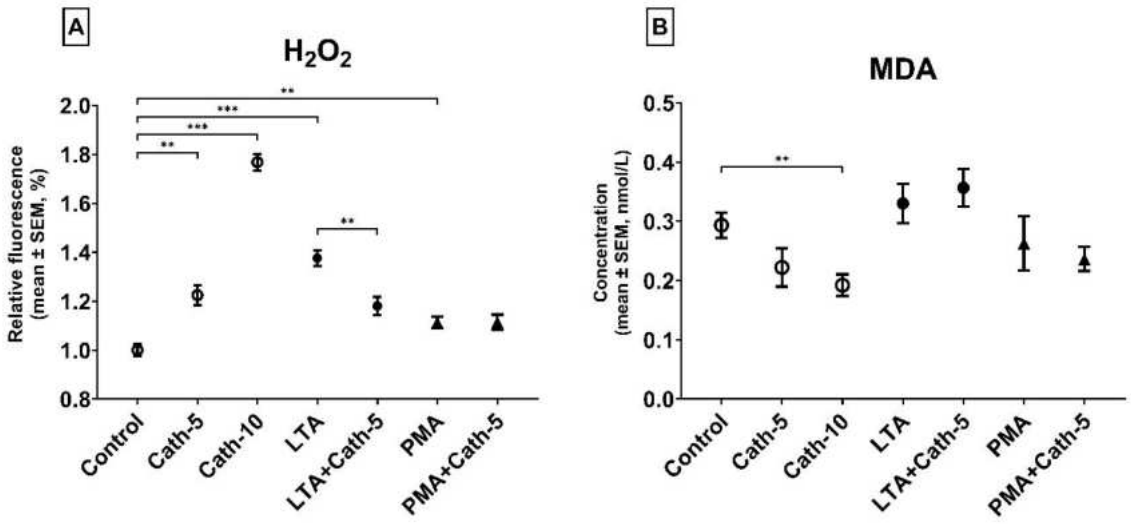


Figure 9. Meanplots showing the changes in extracellular H_2O_2 levels (A) and malondialdehyde (MDA) concentrations (B) of the hepatic cell cultures. Results are expressed as mean ± SEM, in the case of H_2O_2 levels in relative values where 100% is the mean of the control group and in nmol/mL in the case of MDA. Cath-5/10 = 5 and 10 nmol/mL concentrations of chicken cathelicidin-2; LTA = 50 µg/mL concentration of lipoteichoic acid from Staphylococcus aureus; PMA = 1000 ng/mL concentration of phorbol myristate acetate. Control groups received none of the treatment. n = 6. ** p<0.01; *** p<0.001

Study 1.3. Testing the effects of IDR-1002

No significant changes in metabolic activity were observed either after IDR-1002, LTA, or combination treatments (**Figure 10/A**). IDR-1002 or LTA alone did not alter the extracellular LDH activity, however a significant increase was detected after treatment with LTA and 90 µg/mL concentration of IDR-1002 together compared to the LTA group (p=0.015, Figure 10/B). Based on these results, we can conclude that none of the treatments caused a change in cell viability. However, the high concentration of IDR-1002 treatment in the presence of an inflammatory stimulus can cause cell membrane damage.

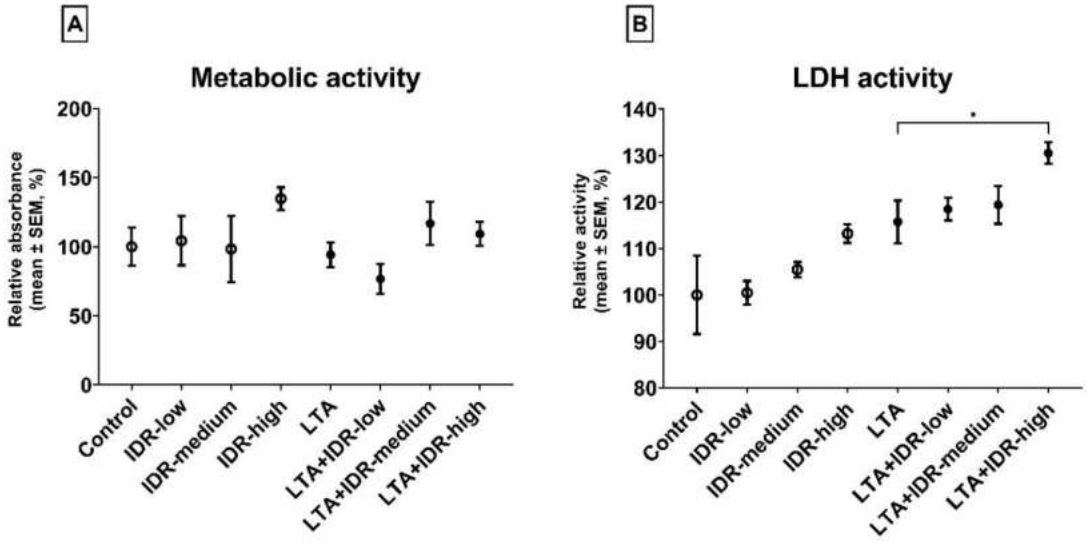


Figure 10. Meanplots showing the changes in metabolic activity (A) and extracellular lactate dehydrogenase (LDH) activity (B) of the hepatic cell cultures. Results are expressed as mean ± SEM in relative values where 100% is the mean of the control group. IDR-low/medium/high = 10, 30 and 90 µg/mL concentrations of innate defense regulator-1002; LTA = 50 µg/mL concentration of lipoteichoic acid from *Staphylococcus aureus*. Control groups received none of the treatment. n = 6. * p<.0.05

After IDR-1002 treatment, IL-6 levels were decreased (IDR-low: p=0.030, IDR-medium: p=0.032, IDR-high: p=0.004, **Figure 11/A**), as well as IL-10 concentrations (IDR-low: p=0.009, IDR-high: p=0.002, **Figure 11/B**).

LTA increased the concentrations of CXCLi2 (p=0.027, **Figure 11/C**), IL-6 (p=0.016, **Figure 11/D**), and IFN-γ (p=0.009, **Figure 11/E**). These elevations were then decreased by IDR-1002, in the case of CXCLi2 by 30 and 90 µg/mL (p=0.019, p = 0.016, respectively, **Figure 11/C**), IL-6 by 10, 30 and 90 µg/mL (p=0.004, p=0.016, p=0.004, respectively, **Figure 11/A**), and IFN-γ by 30 and 90 µg/mL of IDR-1002 (p=0.015, p=0.002, respectively, **Figure 11/E**).

IL-10 and IL-16 concentrations were decreased compared to LTA after treatment with 30 and 90 µg/mL IDR-1002 (IL-10: p=0.002 in both cases, **Figure 11/B**, IL-16: p=0.002, p=0.005, respectively, **Figure 11/D**).

M-CSF levels were elevated after treatment with 10, 30, 90 µg/mL of the peptide (p=0.015, p=0.004, p=0.009, respectively, **Figure 11/G**), and RANTES levels after treatment with 90 µg/mL (p=0.002, **Figure 11/F**). LTA also increased RANTES concentrations (p=0.002), and IDR-1002 further elevated it compared to LTA (30 and 90 µg/mL: p=0.002 in both cases, **Figure 11/F and G**). M-CSF levels were also increased in the combination groups compared to LTA (LTA+IDR-medium: p=0.009, LTA+IDR-high: p=0.004, **Figure 11/G**).

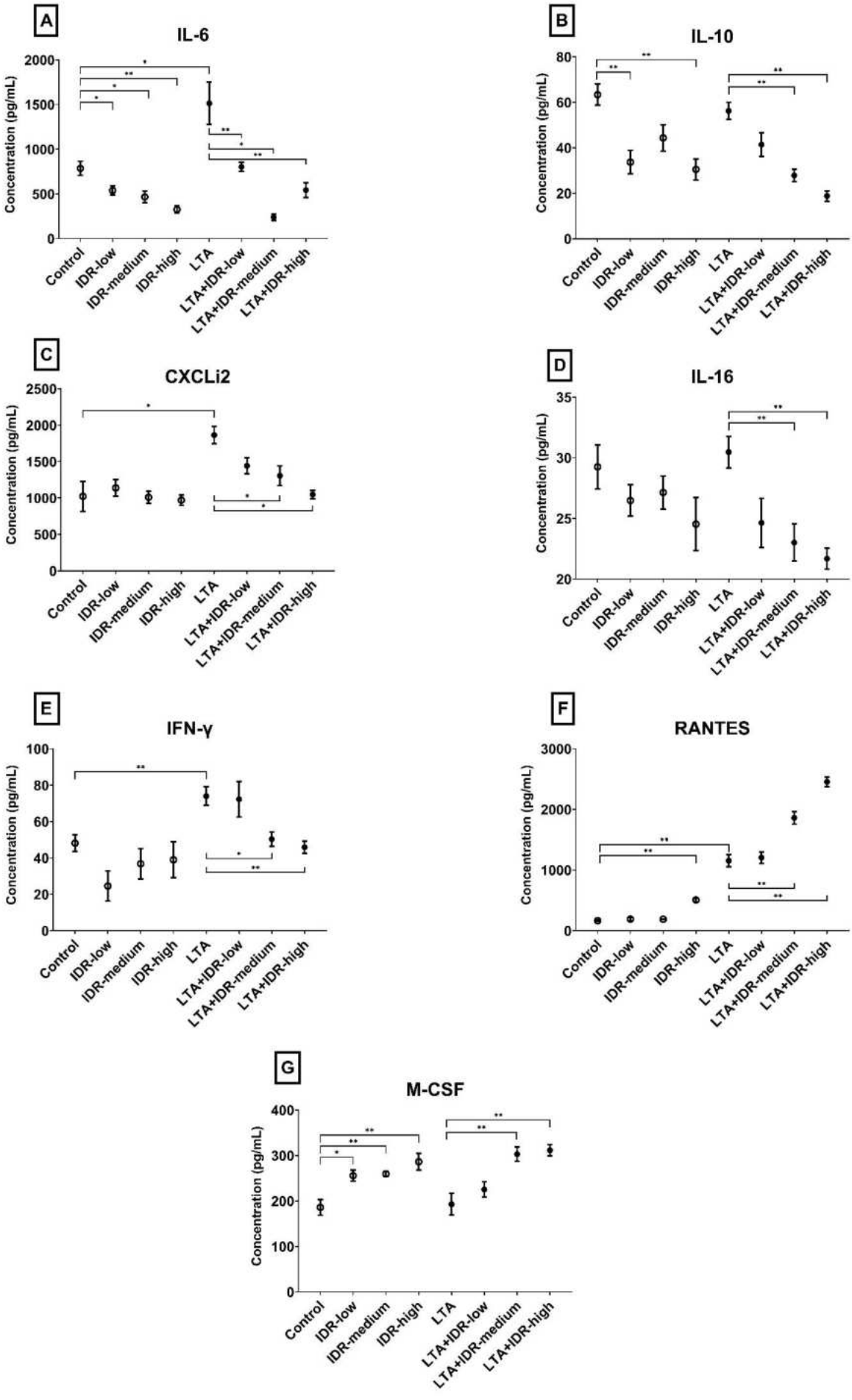

A
IL-6
Concentration (pg/mL)
2000
1500
1000
500
0
B
IL-10
80
60
40
20
C
CXCLi2
2500
D
IL-16
35
30
25
20
E
IFN-γ
100
F
RANTES
3000
G
M-CSF
400
300
200
100
Control
IDR-low
IDR-medium
IDR-high
LTA
LTA+IDR-low
LTA+IDR-medium
LTA+IDR-high

Figure 11. Meanplots showing the changes in concentrations of interleukin (IL)-6 (A), IL-10 (B), CXCLi2 (C), IL-16 (D), interferon (IFN)-γ (E), RANTES (F) and macrophage colony stimulating factor (M-CSF, G) of the hepatic cell cultures. Results are expressed as mean ± SEM in pg/mL. IDR-low/medium/high = 10, 30 and 90 μg/mL concentrations of innate defense regulator-1002; LTA = 50 μg/mL concentration of lipoteichoic acid from *Staphylococcus aureus*. Control groups received none of the treatment. n = 6. * p<.0.05, **p<0.01

Regarding the redox homeostasis, 30 μg/mL IDR-1002 decreased the H_2O_2 concentration (p=0.03, **Figure 12/B**), and all three concentrations of the peptide elevated the Nrf2 concentrations (p=0.014, p=0.032, p=0.016, respectively, **Figure 12/A**). Protein carbonyl levels were also increased after treatment with all concentrations (p=0.004 in all cases, **Figure 12/C**). LTA similarly elevated Nrf2 (p=0.016), and in combination, 10 μg/mL IDR-1002 decreased it (p=0.032). H_2O_2 levels were also decreased by the LTA+IDR-high treatment compared to LTA (p=0.026, **Figure 12/A**).

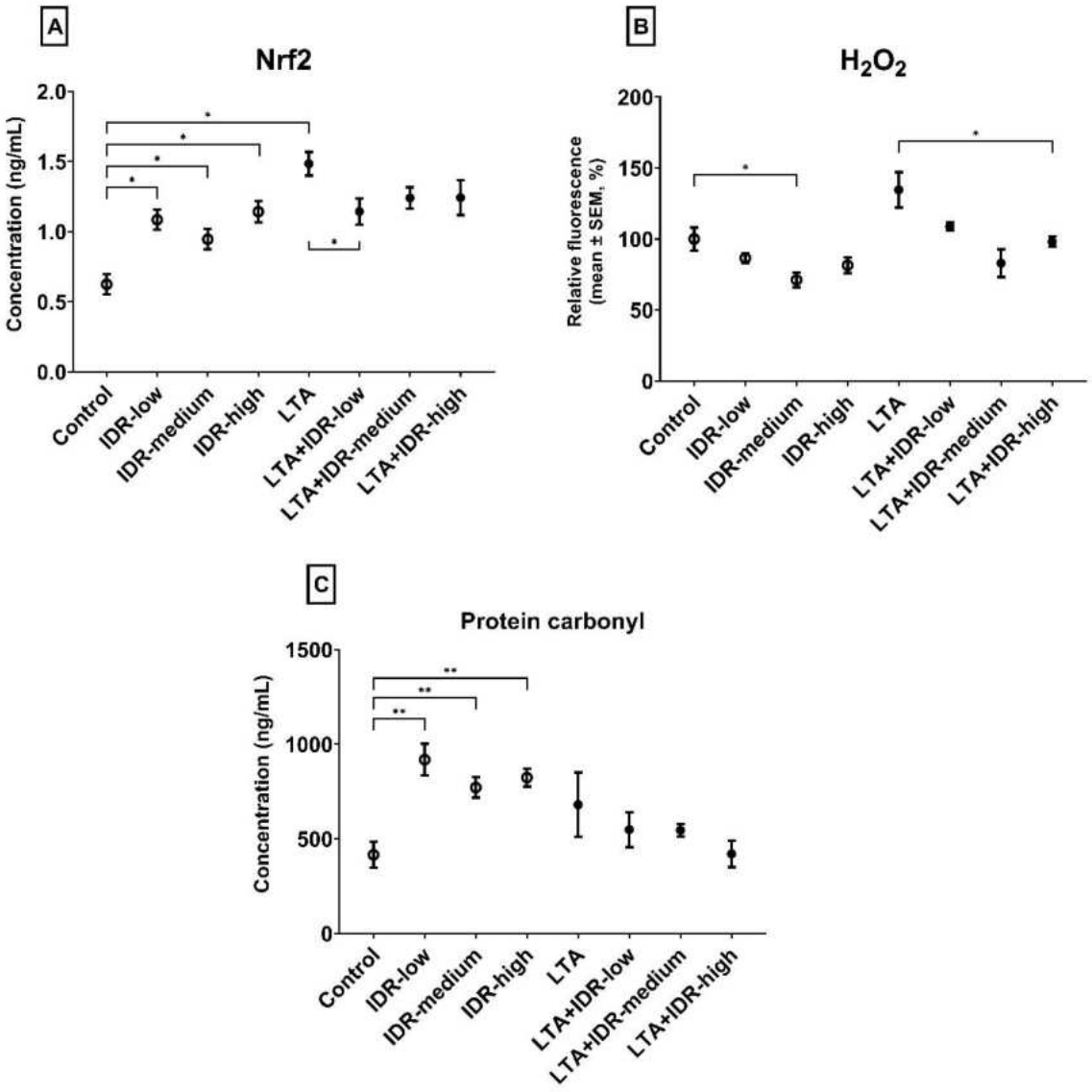


Figure 12. Meanplots showing the changes in nuclear factor erythroid 2-related factor (Nrf2, A) concentrations, H_2O_2 levels (B), and protein carbonyl concentrations (C) of the hepatic cell cultures. Results are expressed as mean ± SEM as relative values where 100% is the mean of the control group in the case of H_2O_2 levels, and in pg/mL in the case of Nrf2 and protein carbonyl. IDR-low/medium/high = 10, 30 and 90 μg/mL concentrations of innate defense regulator-1002; LTA = 50 μg/mL concentration of lipoteichoic acid from *Staphylococcus aureus*. Control groups received none of the treatment. n = 6. * p<.0.05, **p<0.01

Study 1.4. Testing the effects of cecropin A

To assess cell membrane damage, extracellular LDH activity was determined. Cells treated only with CecA showed a significant increase of enzyme activity at the two highest concentrations (12.5 and 25 μg/ml) of the peptide (p=0.0159 and p=0.0381, respectively), whereas the 1, 3.125, and 6.25 μg/ml concentrations did not seem to affect the cell membrane

integrity. Poly I:C-induced inflammation exerted a significant increase in LDH activity compared to the control group (p=0.0095), which was affected by neither of the applied concentrations of CecA (**Figure 13**).

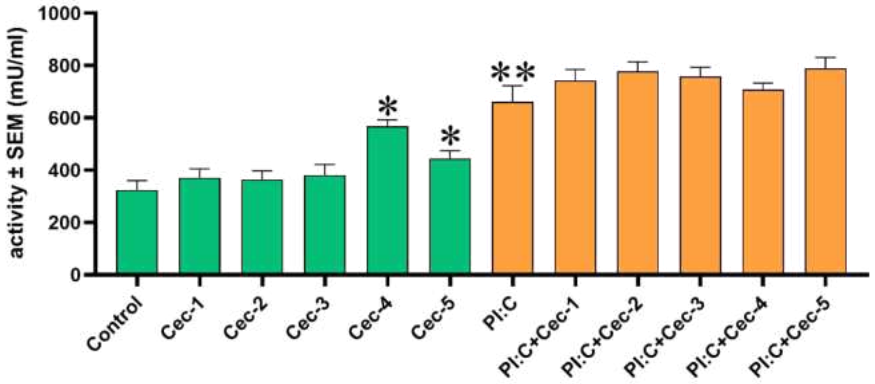
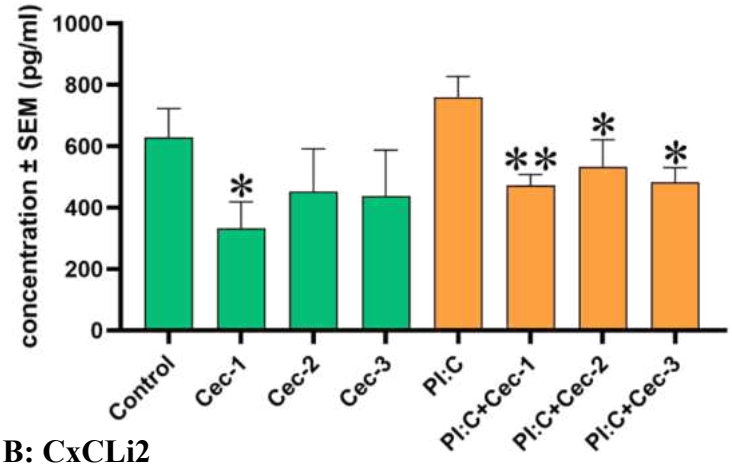


Figure 13. Bar graph showing extracellular lactate dehydrogenase (LDH) activity measured by colorimetric assay**.** Chicken hepatocyte – NP cell co-cultures were treated with five different concentrations of cecropin A (CecA) alone and in combination with polyinosinic-polycytidylic acid (Poly I:C). Columns represent means ± SEM (n=6 / treatment group). Cec-1 = 1 µg/ml CecA, Cec-2 = 3.125 µg/ml CecA, Cec-3 = 6.25 µg/ml CecA, Cec-4 = 12.5 µg/ml CecA, Cec-5 = 25 µg/ml CecA, PI:C = 50 µg/ml Poly I:C. Cells receiving none of the treatments are considered as Control. Asterisks indicate significant differences between treatment groups. Groups Cec-1, Cec-2, Cec-3, Cec-4, Cec-5 and PI:C were compared to Control, whereas combinations of Poly I:C and CecA (PI:C+Cec-1, PI:C+Cec-2, PI:C+Cec-3, PI:C+Cec-4, PI:C+Cec-5) were compared to the group PI:C. *p<0.05, **p<0.01

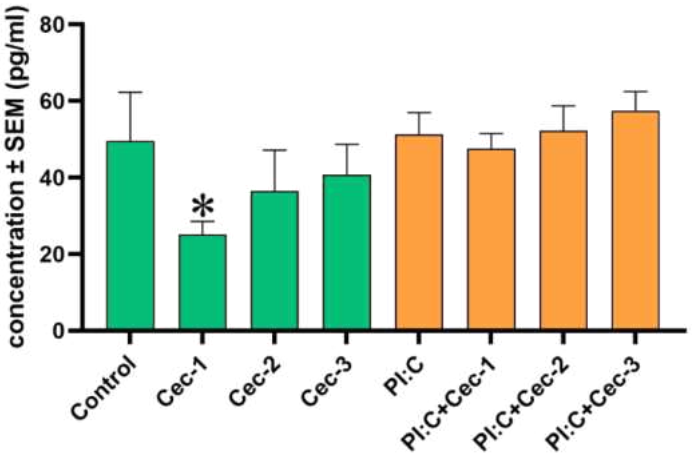
In order to investigate the impact of CecA on the immune response, the levels of IL-6, IL-8, IFN-γ, IL-10 and TGF-ß1 were determined. When measuring IL-6, CecA alone at 1 µg/ml was able to decrease the level of the cytokine (p=0.0381). Compared to the inflammation evoked by Poly I:C, concentrations of 1, 3.125, and 6.25 µg/ml of CecA attenuated the production of IL-6 (p=0.0022, p=0.0411 and p=0.0152, respectively) (**Figure 14/A**).

In the case of CxCLi2 (earlier IL-8), solely applied CecA in 1 µg/ml contributed to a significant decrease (p=0.0381), whereas at concentrations of 3.125 and 6.25 µg/ml, no significant changes were observed. Neither Poly I:C alone, nor the combined treatments of Poly I:C and the different concentrations of CecA affected the amount of CxCLi2 (**Fig. 14/B**).

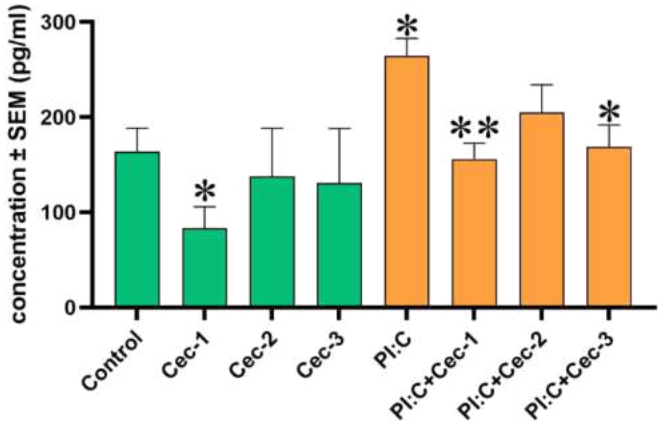
A: IL-6



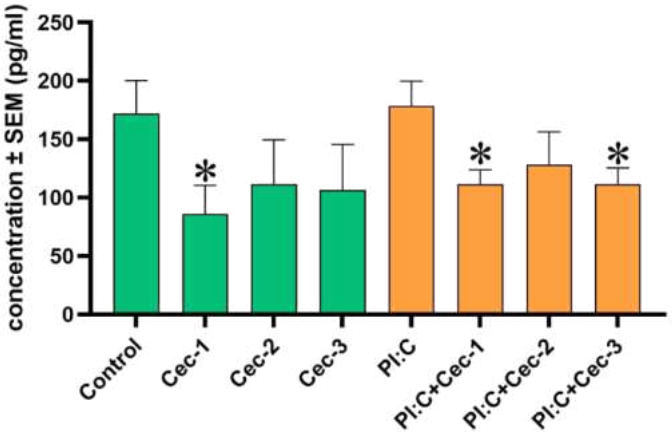
B: CxCLi2



C: IFN-γ



D: IL-10



E: TGF-1β

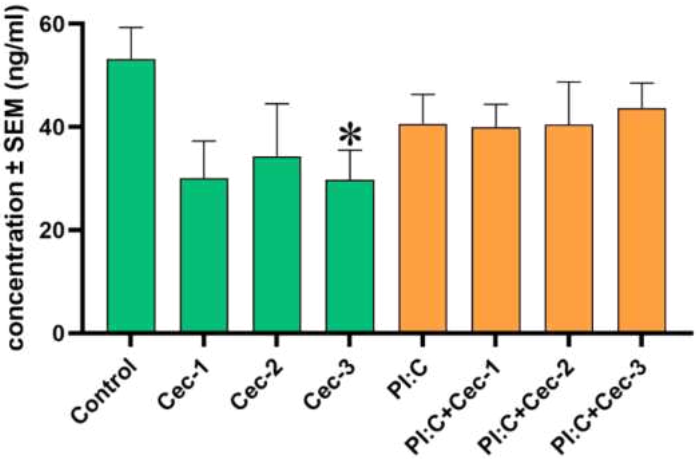


Figure 14. Bar graphs showing IL-6 (**A**), CxCLi2 (**B**), IFN-γ (**C**), IL-10 (**D**) and TGF-ß1 (**E**) concentrations measured by Luminex assay or by chicken-specific ELISA (CxCLi2). Chicken hepatocyte – NP cell co-cultures were treated with three different concentrations of cecropin A (CecA) alone and in combination with polyinosinic-polycytidylic acid (Poly I:C). Columns represent means ±

SEM (n=6 / treatment group). Cec-1 = 1 µg/ml CecA, Cec-2 = 3.125 µg/ml CecA, Cec-3 = 6.25 µg/ml CecA, PI:C = 50 µg/ml Poly I:C. Cells receiving none of the treatments are considered as Control. Asterisks indicate significant differences between treatment groups. Groups Cec-1, Cec-2, Cec-3 and PI:C were compared to Control, whereas combinations of Poly I:C and CecA (PI:C+Cec-1, PI:C+Cec-2, PI:C+Cec-3) were compared to the group PI:C. *p<0.05, **p<0.01

Regarding IFN-γ, the sole administration of CecA at 1 µg/ml diminished the level of the cytokine (p=0.0381). On the contrary, inflammation exerted by Poly I:C significantly elevated the level of IFN-γ (p=0.0191), which was attenuated by CecA at 1 and 6.25 µg/ml (p=0.0022 and p=0.0152, respectively) (**Fig. 14/C**). The level of IL-10 was found to be decreased only by the lowest dose (1 µg/ml) of solely applied CecA (p=0.0381). Combinations of Poly I:C and the 1 µg/ml, as well as the 6.25 µg/ml concentrations of CecA also contributed to a significant reduction of the amount of the cytokine (p=0.0260 and p=0.0411, respectively) (**Figure 14/D**). In addition, when measuring TGF-ß1, solely applied CecA in 6.25 µg/ml contributed to a significant decrease in its level (p=0.0416), whereas at concentrations of 1 and 3.125 µg/ml, no significant changes were observed. Neither Poly I:C alone, nor the combined treatments of Poly I:C and the different concentrations of CecA affected the amount of TGF-ß1 (**Figure 14/E**).

For the examination of the effect of CecA on redox homeostasis, the level of EC H_2O_2 and the MDA concentration indicating lipid peroxidation were measured. In the case of the H_2O_2 level, the highest administered dose of CecA (6.25 µg/ml) was observed to enhance the amount of the oxidative marker (p=0.0191), whereas the other applied concentrations of the peptide did not seem to affect it. When evoking inflammation, Poly I:C alone significantly increased the level of H_2O_2 (p=0.0095), which elevation was further enhanced by CecA at a concentration of 6.25 µg/ml (p=0.0043), however, no significant changes were observed concerning the treatment groups with concentrations of 1 or 3.125 µg/ml of the HDP (**Figure 15/A**).

Regarding MDA, neither of the solely applied concentrations of CecA influenced the marker of lipid peroxidation. On the other hand, in Poly I:C-induced inflammation, CecA contributed to a significant elevation at its concentration of 3.125 µg/ml (p=0.0123) (**Figure 15/B**).

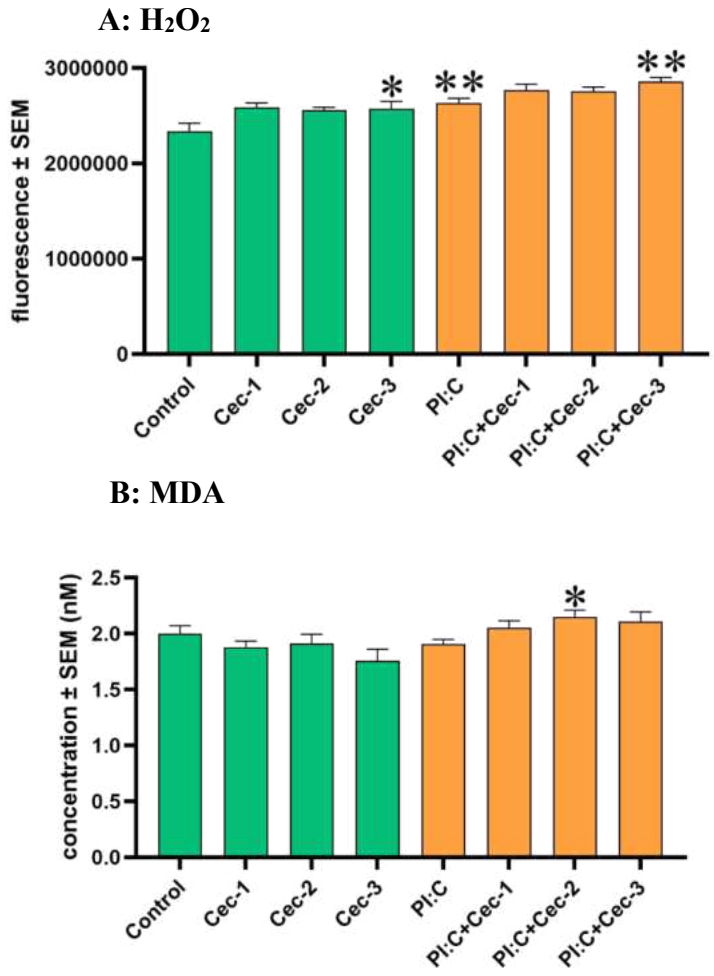


Figure 15. Bar graph showing extracellular H_2O_2 level (**A**) assessed by the fluorimetric Amplex Red method, MDA concentration (**B**) measured by colorimetric assay. Chicken hepatocyte – NP cell co-cultures were treated with three different concentrations of cecropin A (CecA) alone and in combination with polyinosinic-polycytidylic acid (Poly I:C). Columns represent means ± SEM (n=6 / treatment group). Cec-1 = 1 µg/ml CecA, Cec-2 = 3.125 µg/ml CecA, Cec-3 = 6.25 µg/ml CecA, PI:C = 50 µg/ml Poly I:C. Cells receiving none of the treatments are considered as Control. Asterisks indicate significant differences between treatment groups. Groups Cec-1, Cec-2, Cec-3 and PI:C were compared to Control, whereas combinations of Poly I:C and CecA (PI:C+Cec-1, PI:C+Cec-2, PI:C+Cec-3) were compared to the group PI:C. *$p<0.05$, **$p<0.01$

Study 1.5. Testing the effects of Pap12-6

With regards to the EC LDH activity, the solely applied highest concentration of PAP (50 µg/ml) contributed to a significant decrease ($p=0.026$). In addition, Poly I:C-evoked inflammation led to a significant elevation ($p=0.002$), which was significantly reduced by PAP at its concentration of 50 µg/ml ($p=0.002$) (**Figure 16**).

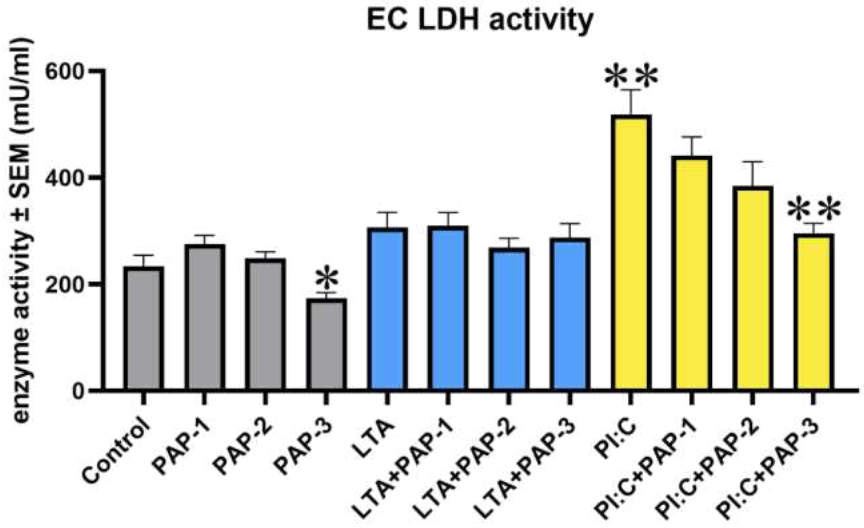


Figure 16. Bar graph showing extracellular lactate dehydrogenase (LDH) activity measured by colorimetric assay. Chicken hepatocyte – NP cell co-cultures were treated with three different concentrations of Pap12-6 (PAP) solely and in combination with lipoteichoic acid (LTA) or polyinosinic-polycytidylic acid (Poly I:C). Grey color refers to treatment groups without the addition of LTA or Poly I:C, blue color refers to treatment with LTA, whereas yellow color refers to treatment with Poly I:C. Columns represent means ± SEM (n=6 / treatment group). PAP-1 = 5 µg/ml PAP, PAP-2 = 25 µg/ml PAP, PAP-3 = 50 µg/ml PAP, LTA = 50 µg/ml LTA, PI:C = 50 µg/ml Poly I:C. Asterisks indicate significant differences between treatment groups. Groups PAP-1, PAP-2, PAP-3, LTA and PI:C were compared to Control, whereas combinations of LTA and PAP (LTA+PAP-1, LTA+PAP-2 and LTA+PAP-3) were compared to the group LTA, and the combinations of Poly I:C and PAP (PI:C+PAP-1, PI:C+PAP-2 and PI:C+PAP-3) were compared to the group PI:C. *p <0.05, **p <0.01

When measuring IL-6 concentrations, Poly I:C was found to exert a significant increasing effect (p=0.002), which was significantly reduced by the concomitant application of PAP at its 50 µg/ml concentration (p=0.004) (**Figure 17/A**). In the case of IL-10, the level of the cytokine was changed only by the sole Poly I:C exposure, causing a significant increase (p=0.002) (**Figure 17/B**). IL-6/IL-10 ratio was found to be heightened in the case of inflammation evoked by Poly I:C (p=0.002), which elevation was decreased by the addition of PAP at its concentrations of 25 and 50 µg/ml (p=0.002 in both cases) (**Figure 17/C**).

In the case of CXCLi2 (IL-8 in chickens), the levels were found to decrease by the sole application of PAP at its 50 µg/ml concentration (p=0.004) and increase by LTA (p=0.026), whereas the combinatory treatment of Poly I:C and the highest concentration of PAP (50 µg/ml) exerted a significant reducing effect (p=0.030) (**Figure 17/D**). Concentrations of RANTES

were elevated by both LTA (p=0.002) and Poly I:C (p=0.002), the latter of which was significantly decreased by the highest concentration (50 μg/ml) of PAP (p=0.015) (**Figure 17/E**). Regarding the production of IFN-γ, the only change observed was the significant elevation caused by Poly I:C (p=0.002) (**Figure 17/F**).

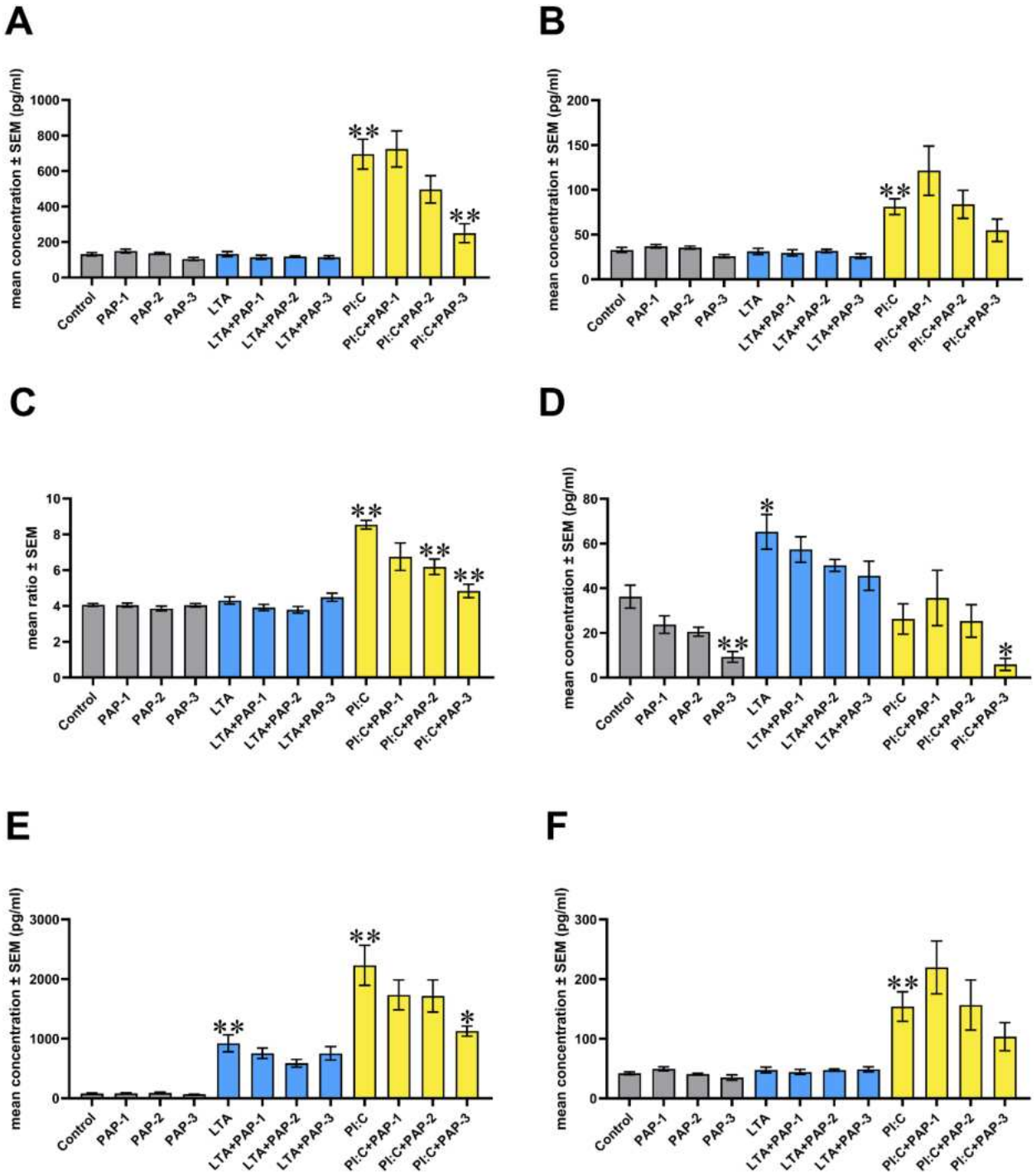


Figure 17. Bar graphs showing the concentrations of different inflammatory markers examined: (A) IL-6, (B) IL-10, (C) IL-6/IL-10 ratio, (D) IL-8, (E) RANTES, (F) IFN-γ. Chicken hepatocyte – NP cell co-cultures were treated with three different concentrations of Pap12-6 (PAP) solely and in combination with lipoteichoic acid (LTA) or polyinosinic-polycytidylic acid (Poly I:C). Grey color refers to treatment

groups without the addition of LTA or Poly I:C, whereas blue color refers to treatment with LTA, and yellow color refers to treatment with Poly I:C. Columns represent means ± SEM (n = 6 / treatment group). PAP-1 = 5 µg/ml PAP, PAP-2 = 25 µg/ml PAP, PAP-3 = 50 µg/ml PAP, LTA = 50 µg/ml LTA, PI:C = 50 µg/ml Poly I:C. Cells receiving none of the treatments are considered as Control. Groups PAP-1, PAP-2, PAP-3, LTA and PI:C were compared to Control. Combinations of LTA and PAP (LTA+PAP-1, LTA+PAP-2 and LTA+PAP-3) were compared to the group LTA. Combinations of Poly I:C and PAP (PI:C+PAP-1, PI:C+PAP-2 and PI:C+PAP-3) were compared to the group PI:C. Asterisks indicate significant differences between the above-mentioned treatment groups.*p <0.05, **p <0.01.

When measuring EC H_2O_2 levels, Poly I:C alone contributed to a significant increase (p=0.002), which was lessened by the addition of PAP at its 25 and 50 µg/ml concentrations (p=0.041 and p=0.002, respectively) (**Figure 18/A**). In the case of Nrf2, PAP at 50 µg/ml alone resulted in a significantly decreased level (p=0.015). Furthermore, LTA was found to exert a significant elevating effect (p=0.009), whereas the change caused by LTA was significantly reduced by PAP treatment at its 25 µg/ml concentration (p=0.015). In addition, at its concentrations of 25 and 50 µg/ml, PAP was able to reduce the Nrf2 level when applied together with Poly I:C (p=0.015 and p=0.002, respectively) (**Figure 18/B**).

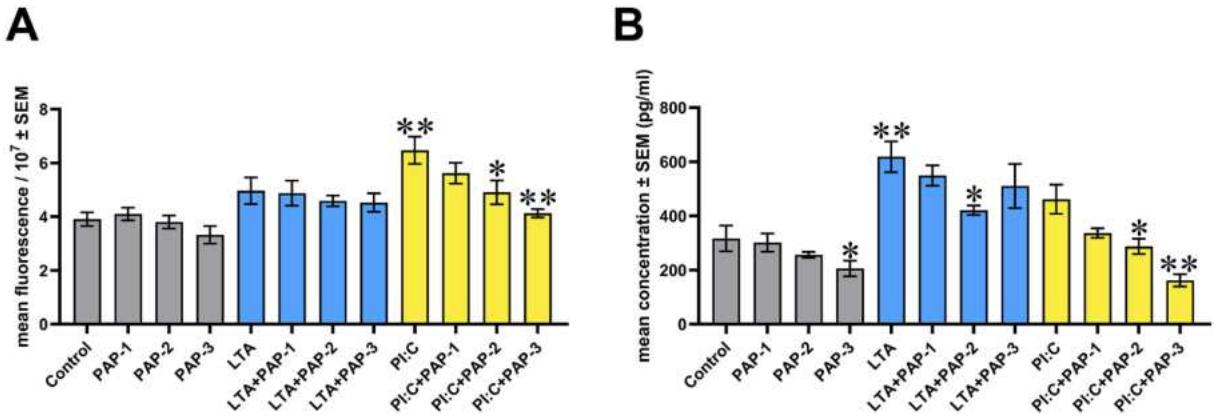


Figure 18. Bar graphs showing the concentrations of different redox markers examined: (**A**) EC H_2O_2 levels, (**B**) Nrf2 levels. Chicken hepatocyte – NP cell co-cultures were treated with three different concentrations of Pap12-6 (PAP) solely and in combination with lipoteichoic acid (LTA) or polyinosinic-polycytidylic acid (Poly I:C). Grey color refers to treatment groups without the addition of LTA or Poly I:C, whereas blue color refers to treatment with LTA, and yellow color refers to treatment with Poly I:C. Columns represent means ± SEM (n = 6 / treatment group). PAP-1 = 5 µg/ml PAP, PAP-2 = 25 µg/ml PAP, PAP-3 = 50 µg/ml PAP, LTA = 50 µg/ml LTA, PI:C = 50 µg/ml Poly I:C. Cells receiving none of the treatments are considered as Control. The treatment groups were compared to one another using Wilcoxon sign-ranked tests. Groups PAP-1, PAP-2, PAP-3, LTA and PI:C were compared to Control. Combinations of LTA and PAP (LTA+PAP-1, LTA+PAP-2 and LTA+PAP-3) were compared to the group

LTA. Combinations of Poly I:C and PAP (PI:C+PAP-1, PI:C+PAP-2 and PI:C+PAP-3) were compared to the group PI:C. Asterisks indicate significant differences between the above-mentioned treatment groups. *p <0.05, **p <0.01.

Study 2. The effects of plant derived bioactive compounds on the inflammatory and oxidative stress response of the liver

Study 2.1. Testing the effects of chicoric acid

The results show that NAC treatment resulted in a significant decrease of metabolic activity (N1: p=0.002, N2: p=0.004). Moreover, a significant decrease of metabolic activity was observed in those cells treated with Poly I:C (p=0.009) compared to the control. The other applied treatments did not influence this parameter except of poly I:C + 100 µg/mL CA (p=0.013), which was able to restore the metabolic activity depressed by Poly I:C. (The visualization of metabolic activity data is not included in the report, they can be found in the appropriate paper published, Tráj et al., 2022.)

The results of the extracellular LDH activity indicating the level of cytotoxicity, reveal that the treatment with Poly I:C increased the membrane permeability of the cultured cells (p=0.002), while the combined exposure to Poly I:C and CA showed a significant reduction in LDH activity compared to the cells only treated with Poly I:C (PC1, PC2: p=0.002). Correspondingly compared to the absolute control, C2 treatment significantly decreased the LDH activity (p=0.025). In contrast, combined Poly I:C and 200 µg/mL NAC treatment resulted in a slightly, but significantly increased cytotoxic effect (p=0.041).

The application of CA induced significant decrease in IL-6 (C1, C2: p=0.004; **Figure 19**) and IL-8/CxCLi2 (C1: p=0.008, C2: p=0.004; **Figure 20**) concentration compared to control. Furthermore, the concentrations of IL-6 and IL-8 as proinflammatory cytokines showed a significant increase in the presence of poly I:C stimuli (IL-6: p=0.004; IL-8: p=0.002). Application of poly I:C and 100 µg/mL CA the IL-6 (p=0.041) and IL-8 (p=0.002) concentrations were reduced compared to the sole poly I:C exposure. The administration of poly I:C together with 10 µg/mL CA also had a significant reducing effect on IL-8 concentration (p=0.002). Additionally, the treatment with low NAC concentration (100 µg/mL) was able to decrease the concentration of IL-6 (p=0.002) and IL-8 (p=0.004) significantly when combined with poly I:C.

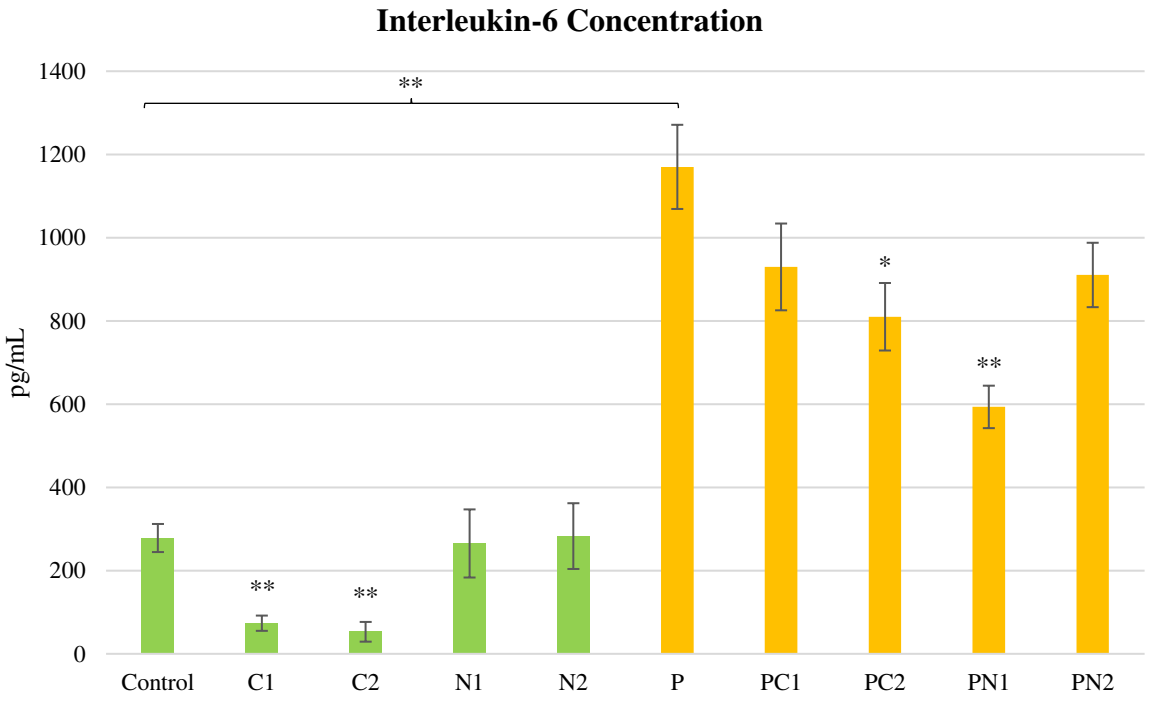


Figure 19. Measurements of IL-6 concentration using chicken specific ELISA. C1=chicoric acid (10 µg/mL), C2=chicoric acid (100 µg/mL), N1=N-acetylcysteine (100 µg/mL), N2=N-acetylcysteine (200 µg/mL), P=poly I:C (50 µg/mL), PC1=poly I:C (50 µg/mL) and chicoric acid (10 µg/mL), PC2=poly I:C (50 µg/mL) and chicoric acid (100 (50 µg/mL) and N-acetylcysteine (200 µg/mL). Mean±SEM, * p≤0.05, **p<0.01. The groups C1, C2, N1, N2 and P were compared to Control, while groups with combined poly I:C + CA/NAC treatments (PC1, PC2, PN1 and PN2) were compared to group P.

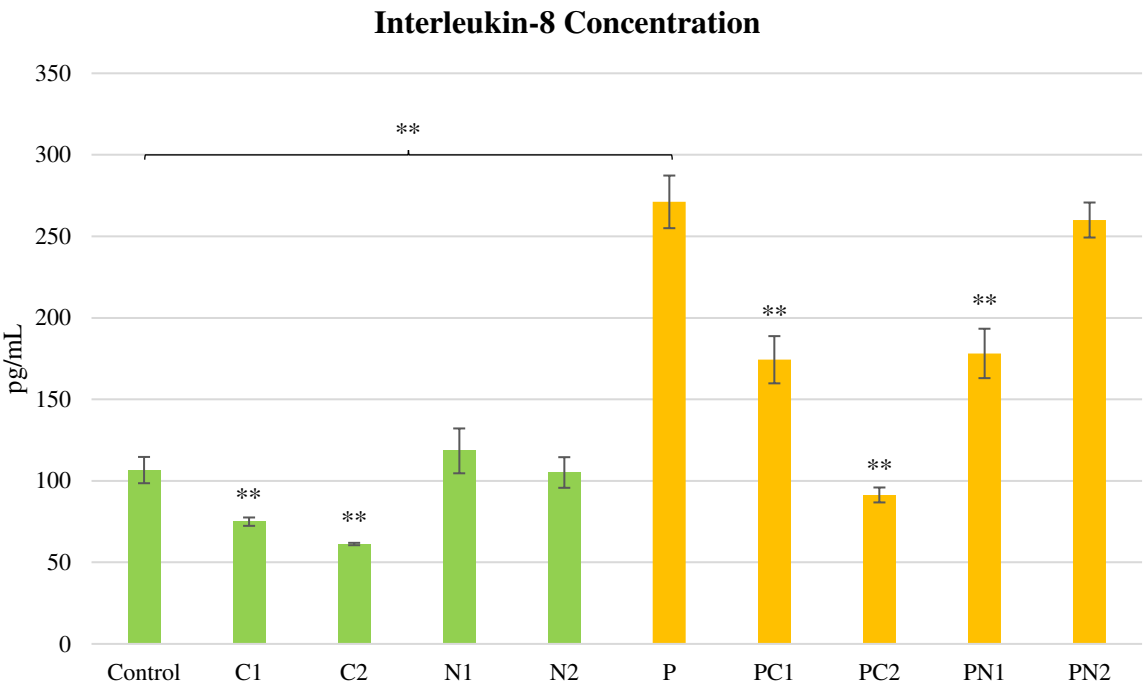


Figure 20. Measurements of IL-8 concentration using chicken specific ELISA. C1=chicoric acid (10 µg/mL), C2=chicoric acid (100 µg/mL), N1=N-acetylcysteine (100 µg/mL), N2=N-acetylcysteine (200 µg/mL), P=poly I:C (50 µg/mL), PC1=poly I:C (50 µg/mL) and chicoric acid (10 µg/mL), PC2=poly I:C (50 µg/mL) and chicoric acid (100 µg/mL), PN1=poly I:C (50 µg/mL) and N-acetylcysteine (100 µg/mL), PN2=poly I:C (50 µg/mL) and N-acetylcysteine (200 µg/mL). Mean±SEM, **$p<0.01$. The groups C1, C2, N1, N2 and P were compared to Control, while groups with combined poly I:C + CA/NAC treatments (PC1, PC2, PN1 and PN2) were compared to group P.

The anti-inflammatory cytokine, IL-10 showed lower concentrations following 200 µg/mL NAC administration (p=0.036; **Figure 21**) compared to the control group. Combined supplementation of poly I:C with lower concentration of NAC resulted in a decrease in the IL-10 concentration (p=0.030) compared to poly I:C exposed group. Meanwhile, the application of CA had no significant effect on IL-10 concentration.

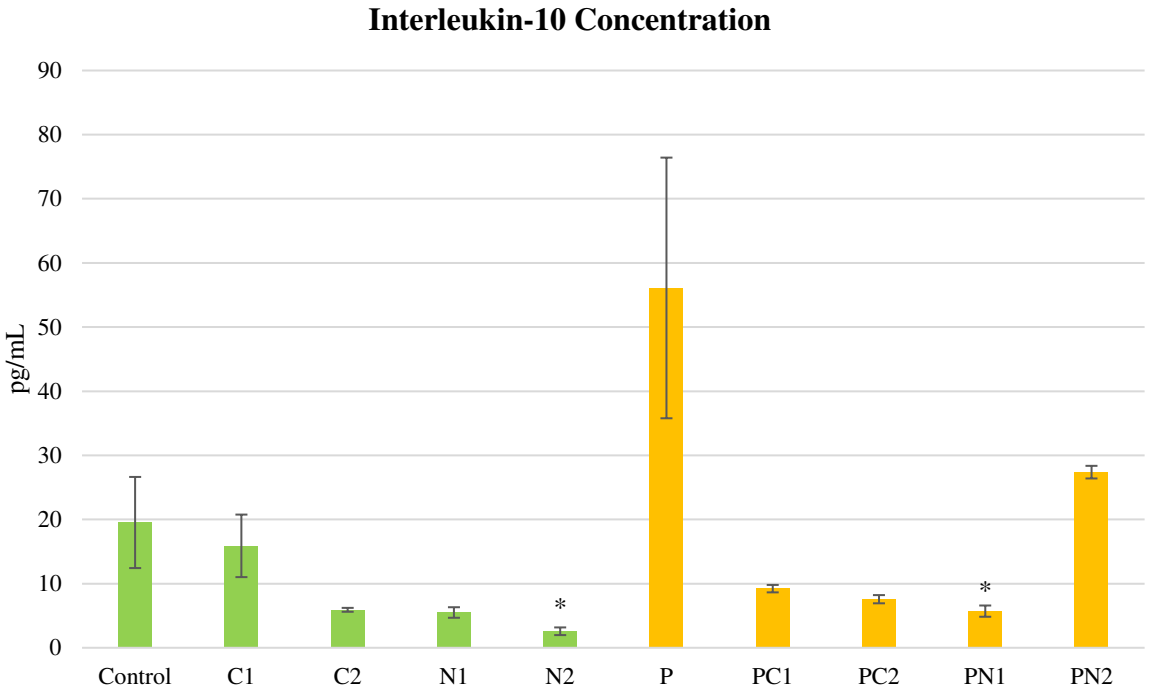


Figure 21. Measurements of IL-10 concentration using Luminex-based Milliplex immunoassay method. C1=chicoric acid (10 µg/mL), C2=chicoric acid (100 µg/mL), N1=N-acetylcysteine (100 µg/mL), N2=N-acetylcysteine (200 µg/mL), P=poly I:C (50 µg/mL), PC1=poly I:C (50 µg/mL) and chicoric acid (10 µg/mL), PC2=poly I:C (50 µg/mL) and chicoric acid (100 (50 µg/mL) and N-acetylcysteine (200 µg/mL). Mean±SEM, *p≤0.05. The groups C1, C2, N1, N2 and P were compared to Control, while groups with combined poly I:C + CA/NAC treatments (PC1, PC2, PN1 and PN2) were compared to group P.

The results of the M-CSF measurements revealed a significant decrease in its concentration in those cell cultures exposed to NAC (N1: p=0.002, N2: p=0.009; **Figure 22**) and 100 µg/mL CA (p=0.025) compared to control. Furthermore, a significant increase in M-CSF concentration was shown after poly I:C application (p=0.025). Combined administration of poly I:C and 100 µg/mL CA as well as 100 µg/mL NAC induced a decrease in the level of M-CSF (PC2: p=0.002, PN1: p=0.002).

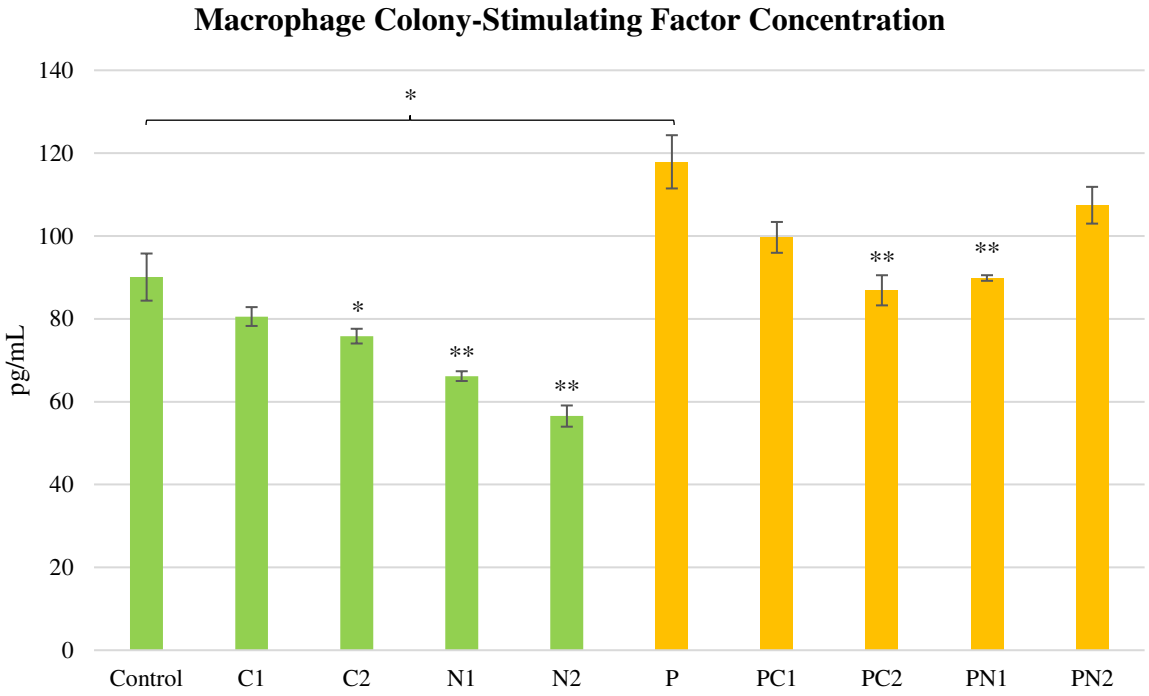


Figure 22. Measurements of M-CSF concentration using Luminex-based Milliplex immunoassay method. C1=chicoric acid (10 µg/mL), C2=chicoric acid (100 µg/mL), N1=N-acetylcysteine (100 µg/mL), N2=N-acetylcysteine (200 µg/mL), P=poly I:C (50 µg/mL), PC1=poly I:C (50 µg/mL) and chicoric acid (10 µg/mL), PC2=poly I:C (50 µg/mL) and chicoric acid (100 (50 µg/mL) and N-acetylcysteine (200 µg/mL). Mean±SEM, * p≤0.05, **p<0.01. The groups C1, C2, N1, N2 and P were compared to Control, while groups with combined poly I:C + CA/NAC treatments (PC1, PC2, PN1 and PN2) were compared to group P.

To trace the antiviral response of the cultures, the concentrations of type I (IFN-α) and II (IFN-γ) interferons were measured. The results indicate that poly I:C significantly increased the IFN-α (p=0.009; **Figure 23**) and IFN-γ (p=0.050; **Figure 24**) concentration in comparison to the control. Furthermore, after treating the cell cultures with poly I:C and CA, IFN-α concentration was reduced (PC1: p=0.015, PC2: p=0.007). The combined application of poly I:C and 100 µg/mL NAC also resulted in a significant decrease of IFN-α concentration (p=0.044). Although significant difference between poly I:C exposed and control group was observed, comparing the level of IFN-γ in PC1, PC2, PN1 group to the absolute controls, no significant difference could be found, indicating the restoration of the level of this cytokine (p

values belonging to comparisons with the absolute controls: PC1: p=0.114, PC2: p=0.234, PN1: p=0.314). However, neither CA, nor NAC application resulted in significant change compared to the solely poly I:C exposed group.

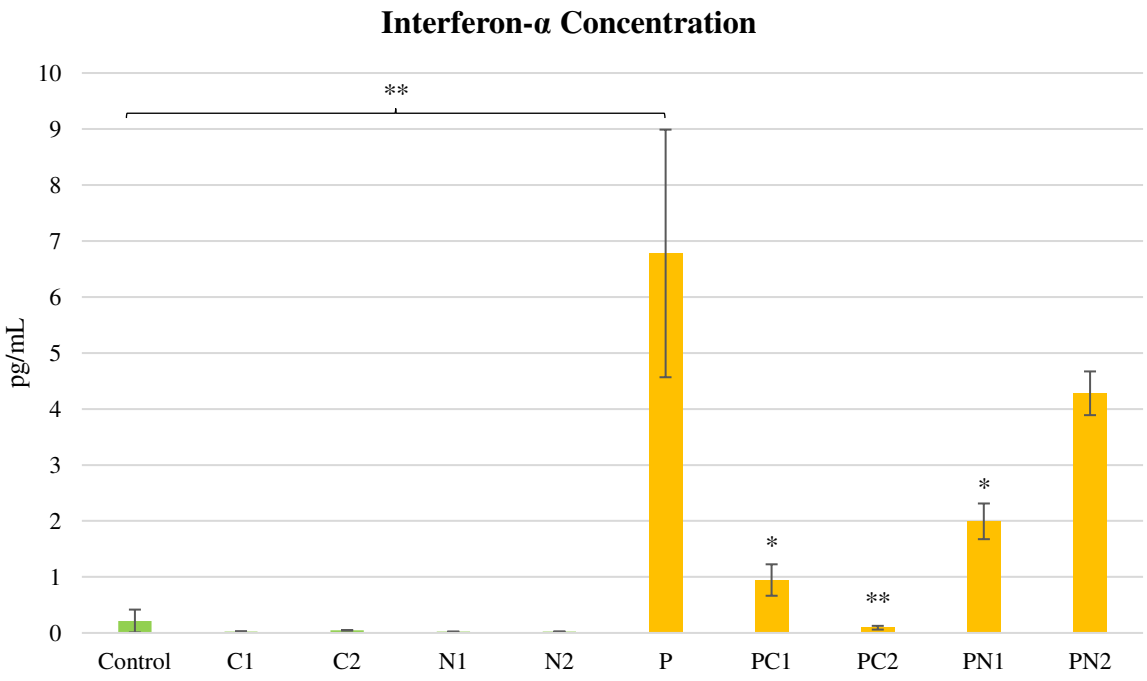


Figure 23. Measurements of IFN-α concentration using Luminex-based Milliplex immunoassay method. C1=chicoric acid (10 µg/mL), C2=chicoric acid (100 µg/mL), N1=N-acetylcysteine (100 µg/mL), N2=N-acetylcysteine (200 µg/mL), P=poly I:C (50 µg/mL), PC1=poly I:C (50 µg/mL) and chicoric acid (10 µg/mL), PC2=poly I:C (50 µg/mL) and chicoric acid (100 (50 µg/mL) and N-acetylcysteine (200 µg/mL). Mean±SEM, * p≤0.05, **p<0.01. The groups C1, C2, N1, N2 and P were compared to Control, while groups with combined poly I:C + CA/NAC treatments (PC1, PC2, PN1 and PN2) were compared to group P.

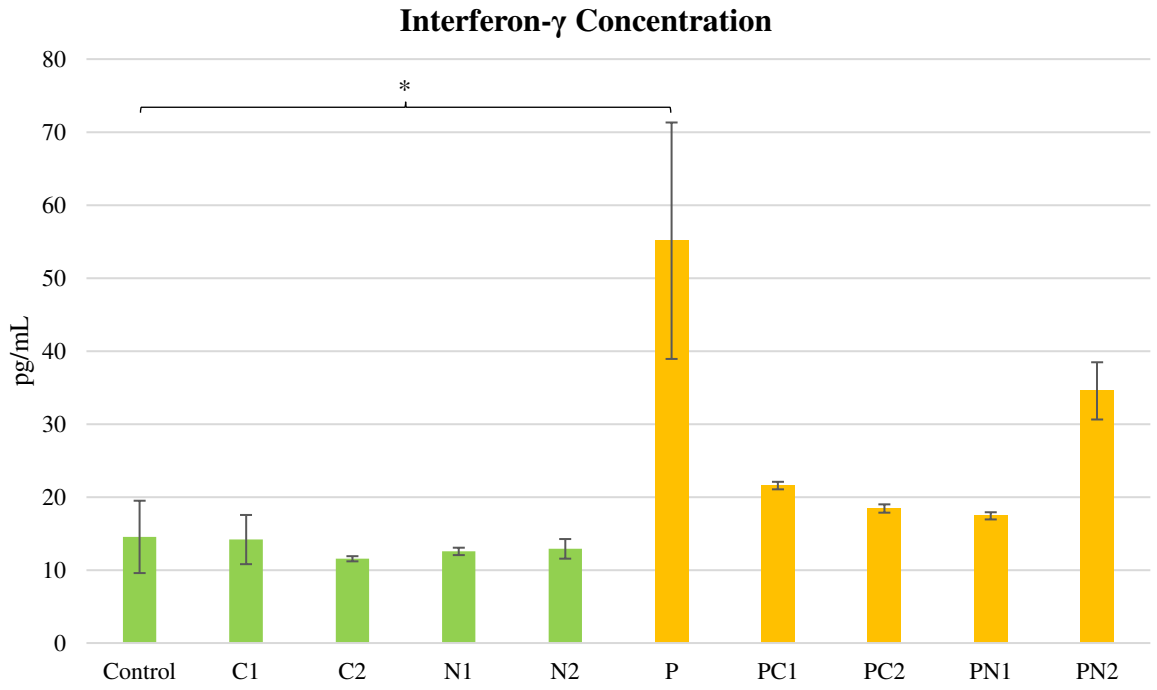


Figure 24. Measurements of IFN-γ concentration using Luminex-based Milliplex immunoassay method. C1=chicoric acid (10 µg/mL), C2=chicoric acid (100 µg/mL), N1=N-acetylcysteine (100 µg/mL), N2=N-acetylcysteine (200 µg/mL), P=poly I:C (50 µg/mL), PC1=poly I:C (50 µg/mL) and chicoric acid (10 µg/mL), PC2=poly I:C (50 µg/mL) and chicoric acid (100 (50 µg/mL) and N-acetylcysteine (200 µg/mL). Mean±SEM, * p≤0.05, **p<0.01. The groups C1, C2, N1, N2 and P were compared to Control, while groups with combined poly I:C + CA/NAC treatments (PC1, PC2, PN1 and PN2) were compared to group P.

Study 2.2. Testing the effects of luteolin

The higher applied dose of luteolin (L2, 16 µg/mL) significantly (p = 0.002) reduced the metabolic activity of the cells (Fig. 1.A). This effect of the high luteolin concentration was also observed in combination with flagellin (FL2, p = 0.004). However, flagellin (F) or luteolin (L1) at a concentration of 4 µg/mL – both alone and in combination (FL1) – did not significantly alter the metabolic activity of the cells. The activity of lactate dehydrogenase released into the medium in response to cell membrane damage was significantly reduced by luteolin at 16 µg/mL both in combination with flagellin (FL2, p = 0.030) and alone (L2, p = 0.009) (**Figure 25**). Since luteolin was found to be cytotoxic at a concentration of 16 µg/ml indicated by the decrease of the metabolic activity and the lactate dehydrogenase activity, the L2 and FL2 groups were excluded from the subsequent measurements.

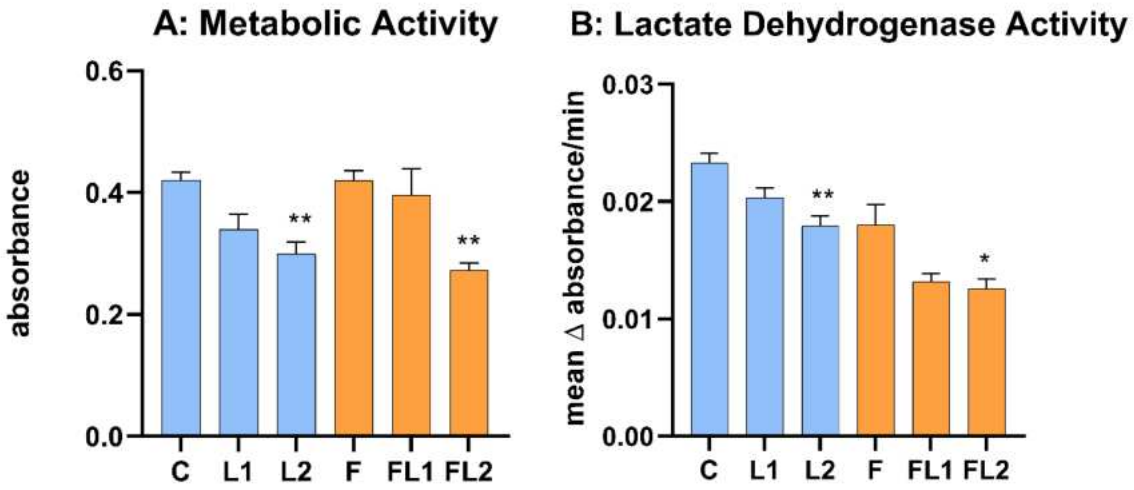


Figure 25. Metabolic activity measured by CCK-8 assay (A) and extracellular lactate dehydrogenase activity measured by an enzyme kinetic photometric assay (B). C=control, L1=luteolin (4 µg/mL), L2=luteolin (16 µg/mL), F=flagellin (250 ng/mL), FL1=flagellin (250 ng/mL) and luteolin (4 µg/mL), FL2=flagellin (250 ng/mL) and luteolin (16 µg/mL). Mean (n = 6/group) ± SEM, *p < 0.05, **p < 0.01. Group L1, L2 and F were compared to C, while group FL1 and FL2 were compared to group F.

The decrease in extracellular H_2O_2 concentrations in response to luteolin (p = 0.002) was revealed by Amplex Red measurements both in the sole luteolin (L1) exposure group and in the combined flagellin-luteolin treatment group (FL1, p = 0.002) (**Figure 26/A**). Similarly, as observed for H_2O_2 levels, MDA, a parameter indicative of membrane lipid peroxidation showed a significant decrease (p = 0.016) in the luteolin and flagellin combined treatment group (FL1) compared to the level observed in the case of flagellin exposed cells (F) (**Figure 26/B**).

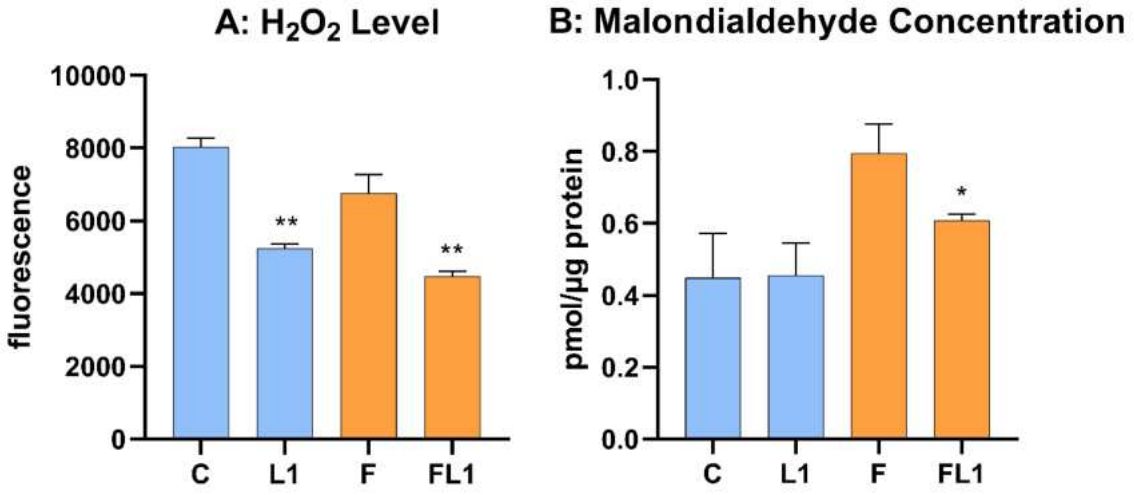


Figure 26. H_2O_2 level of the cell culture medium measured by Amplex Red fluorimetric assay (A) and MDA concentration of the cell lysate measured by a colorimetric assay (B). C=control, L1=luteolin (4 µg/mL), F=flagellin (250 ng/mL), FL1=flagellin (250 ng/mL) and luteolin (4 µg/mL). Mean (n = 6/group) ± SEM, *p < 0.05, **p<0.01. Group L1 and F were compared to C, while group FL1 was compared to group F.

No significant difference in IL-6 concentration was observed between any of the investigated treatment groups (**Fig. 27/A**). IL-8/CxCLi2 concentration was significantly increased by flagellin treatment (F, p = 0.016) compared to the absolute control group (C). This increase was attenuated (p = 0.016) by the concomitantly applied luteolin (FL1) (**Figure 27/B**).

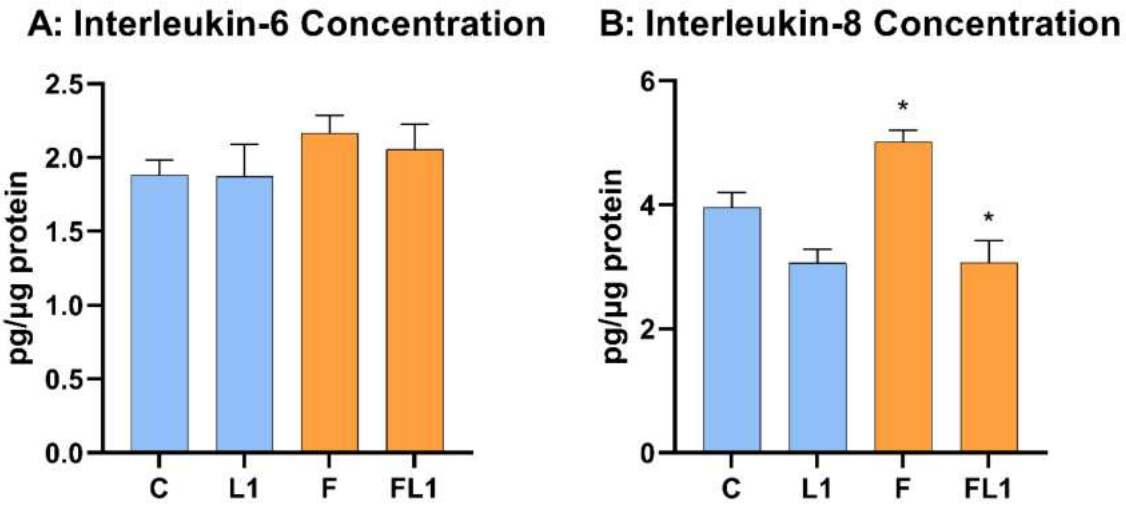


Figure 27. IL-6 (A) and IL-8 (B) concentration of the cell culture medium measured by chicken specific ELISA. C=control, L1=luteolin (4 µg/mL), F=flagellin (250 ng/mL), FL1=flagellin (250 ng/mL) and luteolin (4 µg/mL). Mean (n = 6/group) ± SEM, *p < 0.05. Group L1 and F were compared to C, while group FL1 was compared to group F.

The concentration of IFN-α was significantly decreased in the luteolin-treated groups (L1, FL1) compared to the control (C, $p = 0.002$) and the flagellin-exposed group (F, $p = 0.041$), respectively (**Figure 28/A**). No significant change has been observed in the level of IFN-γ (**Figure 28/B**). The concentration of IL-10 decreased in the case of sole flagellin exposure (F, $p = 0.015$); meanwhile, the level of the luteolin-cotreated (FL1) cells showed no significant difference in comparison with the control group (C) (**Figure 28/C**). Further, the ratio of IFN-γ/IL-10 was elevated in the case of the flagellin group (F, $p = 0.025$) compared to the control (C) (**Figure 28/D**).

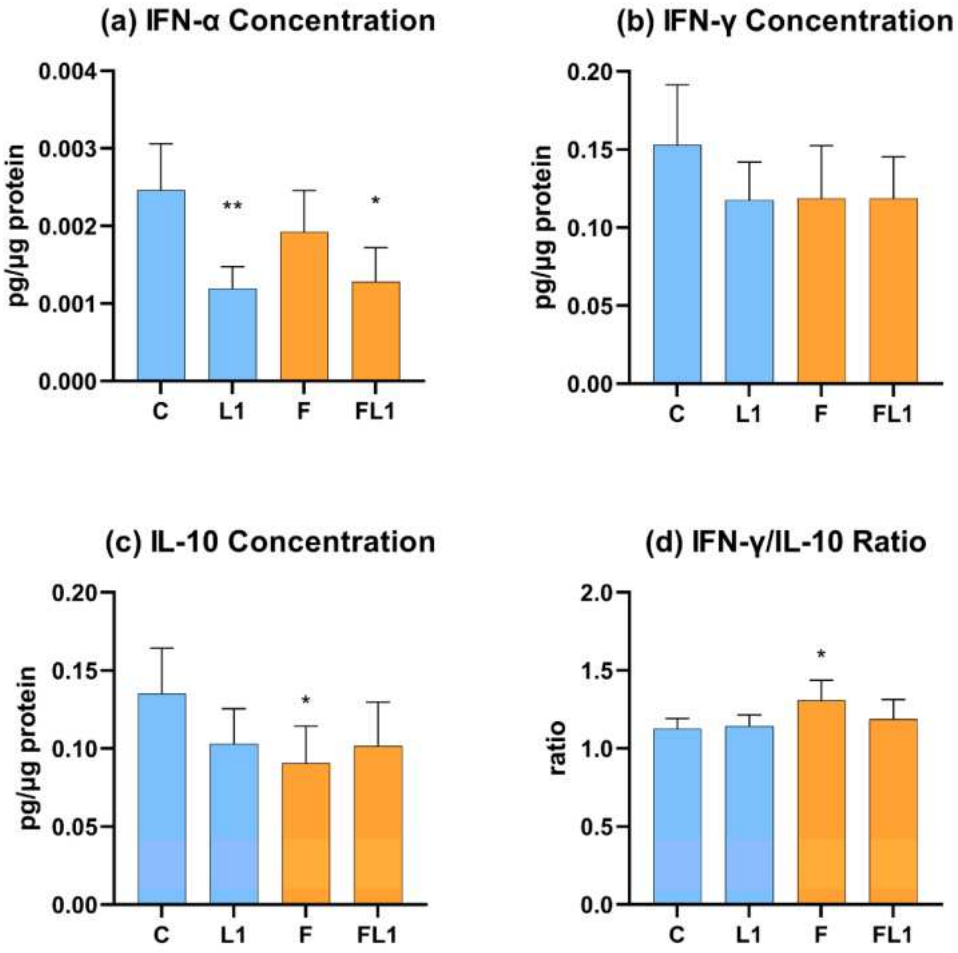


Figure 28. IFN-α (**A**), IFN-γ (**B**), IL-10 (**B**) concentration of the cell culture medium measured by chicken-specific Luminex MAGPIX Panel and the IFN-γ/ IL-10 ratio (**D**). C = control, L1 = luteolin (4 µg/mL), F = flagellin (250 ng/mL), FL1 = flagellin (250 ng/mL) and luteolin (4 µg/mL). Mean (n = 6/group) ± SD, * $p < 0.05$, ** $p < 0.01$. Group L1 and F were compared to C, while group FL1 was compared to group F.

Study 3. The effects of mycotoxins on the inflammatory and oxidative stress response of hepatic 3D spheroids

Study 3.1. Testing the effects of T-2 toxin

A formalin fixed paraffin embedded (FFPE) slide of a hepatic spheroid after haematoxylin and eosin (H&E) staining is presented in **Figure 29**. Morphologically intact hepatocytes were observable in high abundance in loose connection with each other as well as other NP cells with smaller nucleus. Severe degenerative changes were not visible.

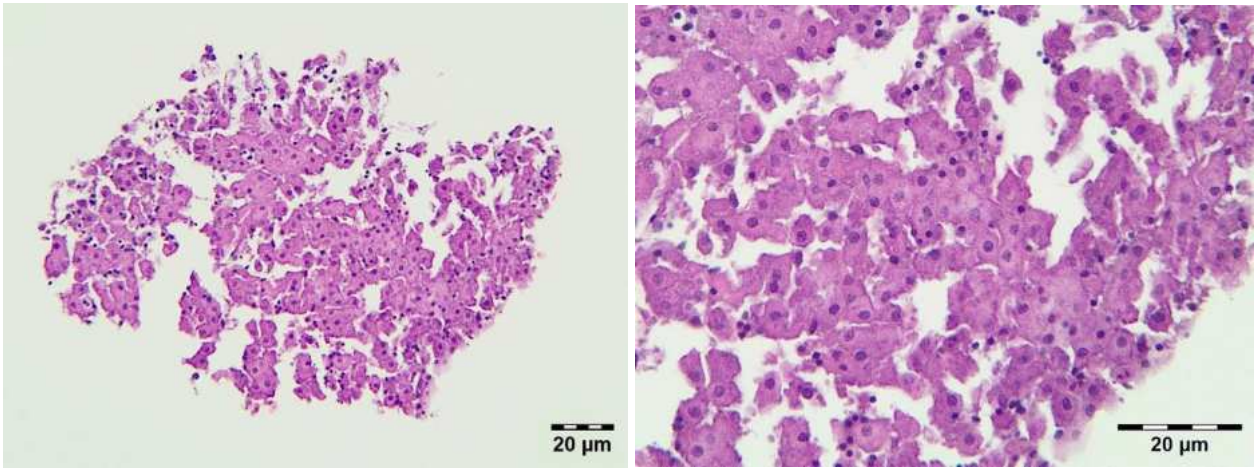


Figure 29. Primary 3D hepatic spheroids from chicken origin. H&E staining, bar: 20 µm.

T-2 toxin significantly decreased ($p < 0.001$) the metabolic activity in every treatment group compared to the control (**Figure 30**).

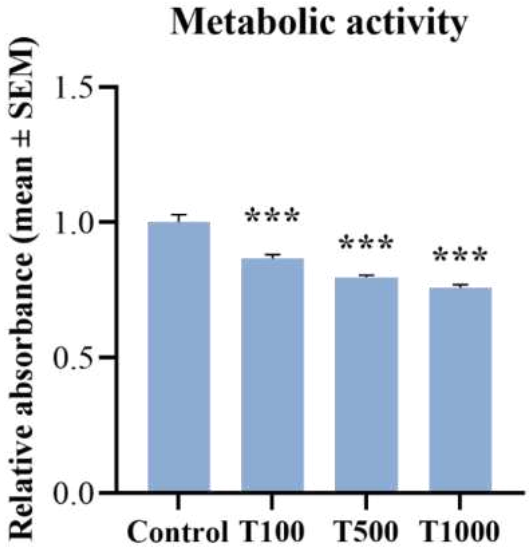


Figure 30. Effects of 24 h T-2 toxin treatment on the metabolic activity of primary hepatic 3D cell co-cultures of chicken origin assessed by CCK-8 test. Control: cells without T-2 toxin exposure; T100: 100 nM, T500: 500 nM, T1000: 1000 nM T-2 toxin treatment. Relative absorbances were calculated by considering the mean value of the Control group as 1. Results are expressed as mean ± SEM. *** $p < 0.001$.

The 500 nM T-2 toxin concentration significantly increased ($p = 0.038$) the LDH activity of the cells in comparison with the control group (**Figure 31**).

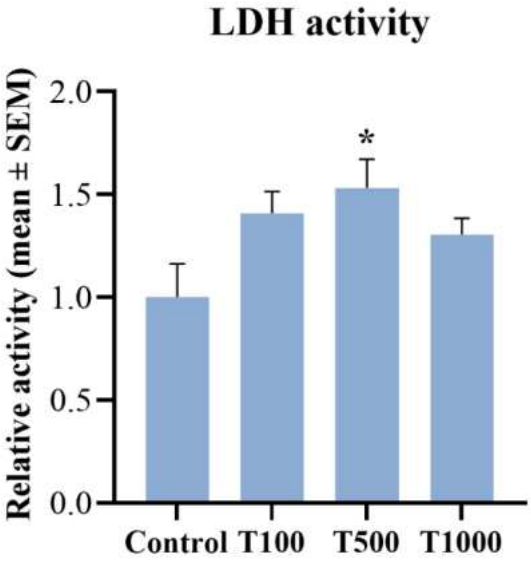


Figure 31. Effects of 24 h T-2 toxin treatment on the LDH activity of primary hepatic 3D cell co-cultures of chicken origin. Control: cells without T-2 toxin exposure; T100: 100 nM, T500: 500 nM, T1000: 1000 nM T-2 toxin treatment. Relative activity was calculated by considering the mean value of the Control group as 1. Results are expressed as mean ± SEM. * $p < 0.05$.

The MDA level was significantly decreased ($p = 0.030$) in the 1000 nM treatment group after 24 h (**Figure 32.A**). The higher levels of T-2 toxin (500 nM and 1000 nM) significantly decreased ($p = 0.007$, $p = 0.001$, respectively) the intracellular PC concentration of the cultured cells compared to the control group (**Figure 32.B**).

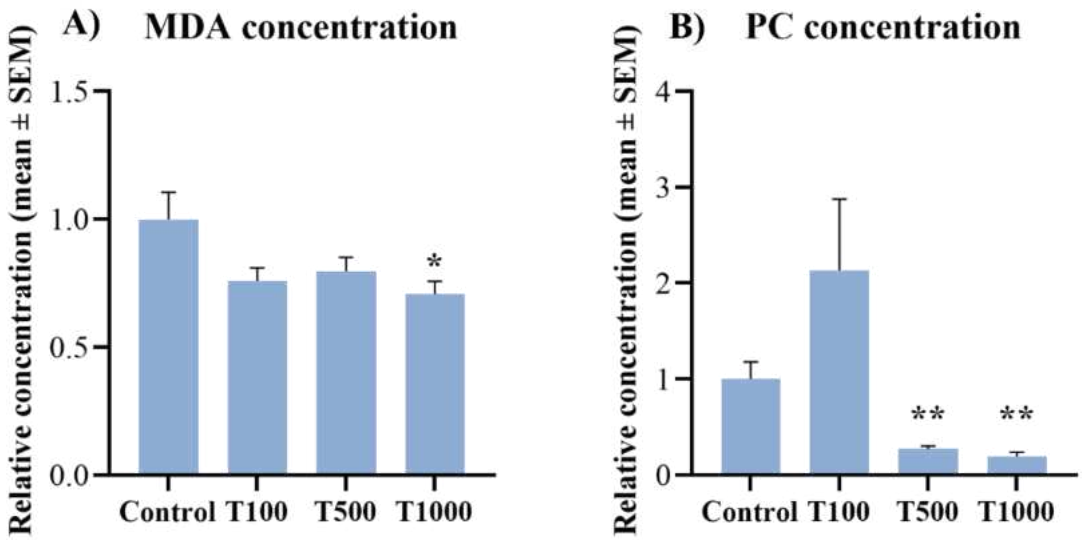


Figure 32. Effects of 24 h T-2 toxin treatment on the A) MDA and B) PC concentrations of primary hepatic 3D cell co-cultures of chicken origin assessed by chicken specific ELISA test. Control: cells without T-2 toxin exposure; T100: 100 nM, T500: 500 nM, T1000: 1000 nM T-2 toxin treatment.

Relative concentrations were calculated by considering the mean value of the Control group as 1. Results are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$.

The extracellular concentration of IL-6 was significantly lowered ($p = 0.026$) after 24 h by the 100 nM T-2 toxin treatment when compared to that of the control cells (**Figure 33.A**). The IL-8 concentration was significantly increased ($p = 0.025$) after 24 h in the 100 nM T-2 toxin treatment group (**Figure 33.B**).

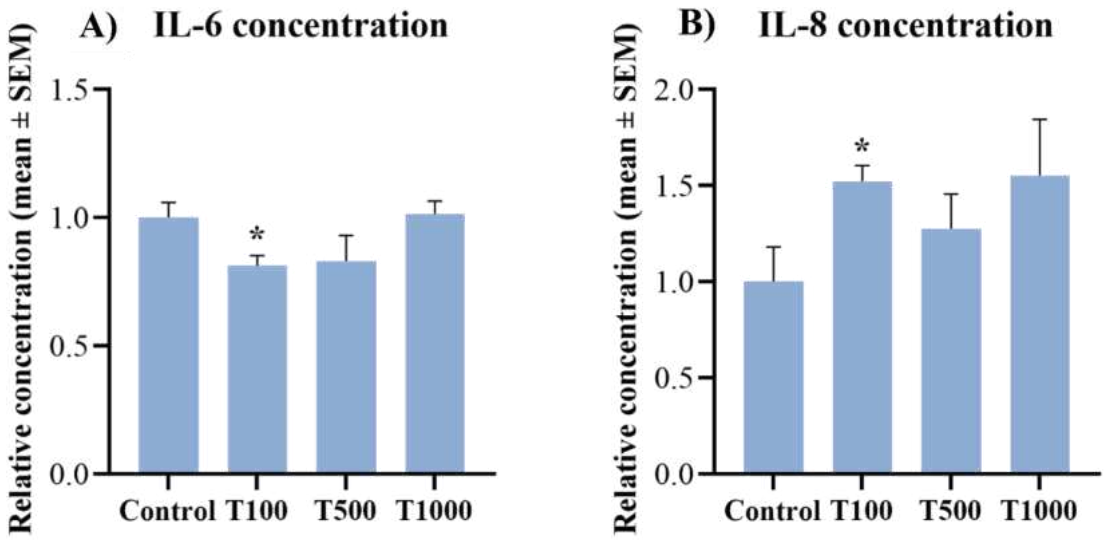


Figure 33. Effects of 24 h T-2 toxin treatment on the A) IL-6 and B) IL-8 concentrations of primary hepatic 3D cell co-cultures of chicken origin assessed by chicken specific ELISA test. Control: cells without T-2 toxin exposure; T100: 100 nM, T500: 500 nM, T1000: 1000 nM T-2 toxin treatment. Relative concentrations were calculated by considering the mean value of the Control group as 1. Results are expressed as mean ± SEM. * $p < 0.05$.

The GRP78 and HSP27 contents were assessed after 24 hours of T-2 toxin treatment by chicken specific ELISA tests. The applied toxin exposures did not influence significantly the concentrations of these two parameters (**Figure 34.A-B**).

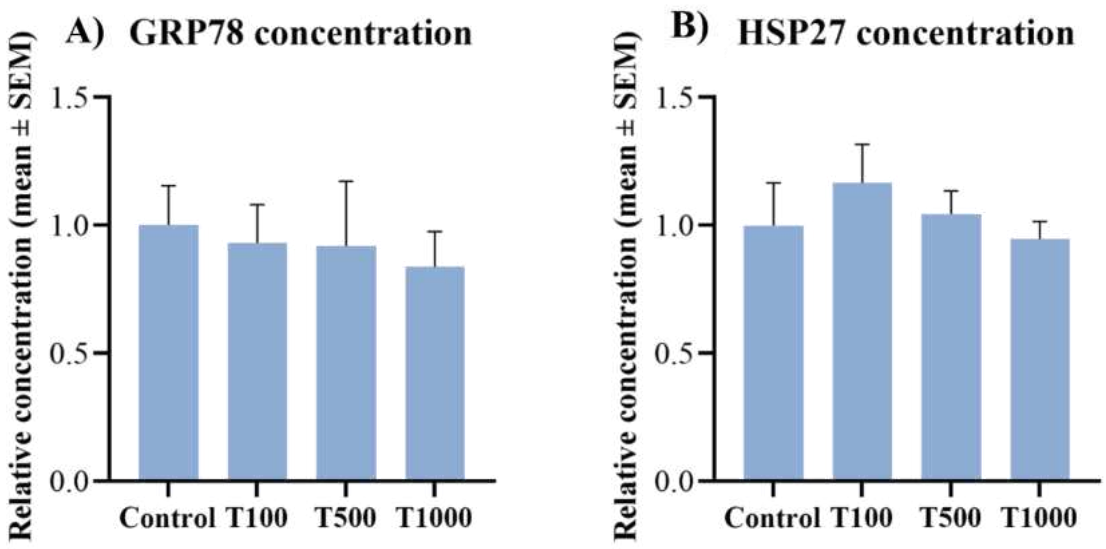


Figure 34. Effects of 24 h T-2 toxin treatment on the A) GRP78 and B) HSP27 concentrations of primary hepatic 3D cell co-cultures of chicken origin assessed by chicken specific ELISA test. Control: cells without T-2 toxin exposure; T100: 100 nM, T500: 500 nM, T1000: 1000 nM T-2 toxin treatment. Relative concentrations were calculated by considering the mean value of the Control group as 1. Results are expressed as mean ± SEM.

Figure 35. shows the overview of the 11 metabolites {alanine (Ala), proline (Pro), glutamine (Gln), lysine (Lys), citrulline (Cit), alpha-aminoadipic acid (alpha-AAA), lysophosphatidylcholine with acyl residue (lysoPC a) C24:0, phosphatidylcholine with dyacyl residue sum (PC aa) C34:1 and C38:4, phosphatidylcholine with acyl-alkyl residue sum (PC ae) C42:4 and C38:1) significantly being influenced by T-2 toxin exposure, visualised on a heatmap. As reflected by the colour scheme of the heatmap, most of the significantly changed metabolites showed T-2 toxin triggered increased abundance, while the concentrations of 4 metabolites (Gln, Lys, PC aa C34:1, PC aa C38:4) were found to be decreased after the T-2 toxin treatment.

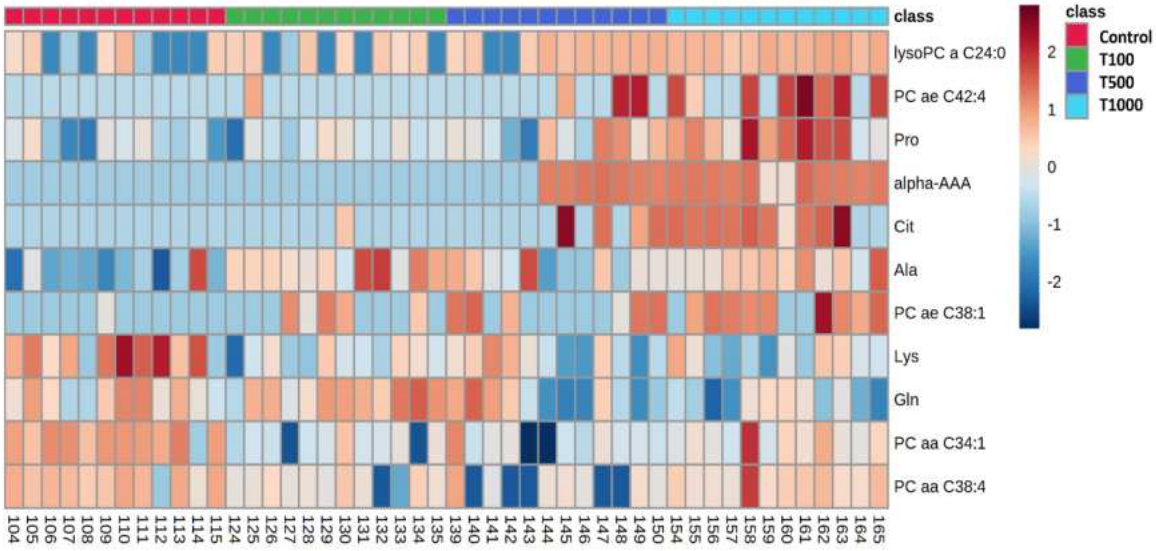


Figure 35. Heatmap visualising the contentrations of 11 metabolites with significantly changed abundance in response to 24 h T-2 toxin treatment. The heatmap is based on the data obtained after normalisation. Control: cells without T-2 toxin exposure; T100: 100 nM, T500: 500 nM, T1000: 1000 nM T-2 toxin treatment.

Study 3.2. Testing the effects of DON and baicalin

No significant differences were observed between the metabolic activity of control groups of different incubation times ($p = 0.364$). In the case of the 24 h incubation, all three baicalin concentrations significantly increased ($p < 0.001$ in all cases) the cellular viability compared to control (**Figure 36/a**). In contrast, DON decreased ($p = 0.042$; $p = 0.044$, respectively) the metabolic activity at both used concentrations (**Figure 36/a**). When combined with the 2 µg/ml DON treatment, all three baicalin treatments elevated (in the case of all three $p < 0.001$) the viability of the cells compared to the cultures solely exposed to DON (**Figure 36/c**). The same increasing trend (in the case of all three $p < 0.001$) was observed when baicalin was combined with 20 µg/ml DON treatment (**Figure 36/e**).

After the 48 h incubation, the 5 µg/ml and 45 µg/ml baicalin significantly increased ($p = 0.001$; $p < 0.001$, respectively) the cellular viability in comparison with the absolute controls (**Figure 36/b**), while it was not affected by DON exposure. All three baicalin treatments in combination with 2 µg/ml DON elevated (in the case of all three $p < 0.001$) the metabolic activity of cells compared to the DON-exposed controls (**Figure 36/d**). Similarly, all three baicalin concentrations increased ($p = 0.017$; $p < 0.001$; $p < 0.001$, respectively) the cellular viability in combination with the 20 µg/ml DON treatment (**Figure 36/f**).

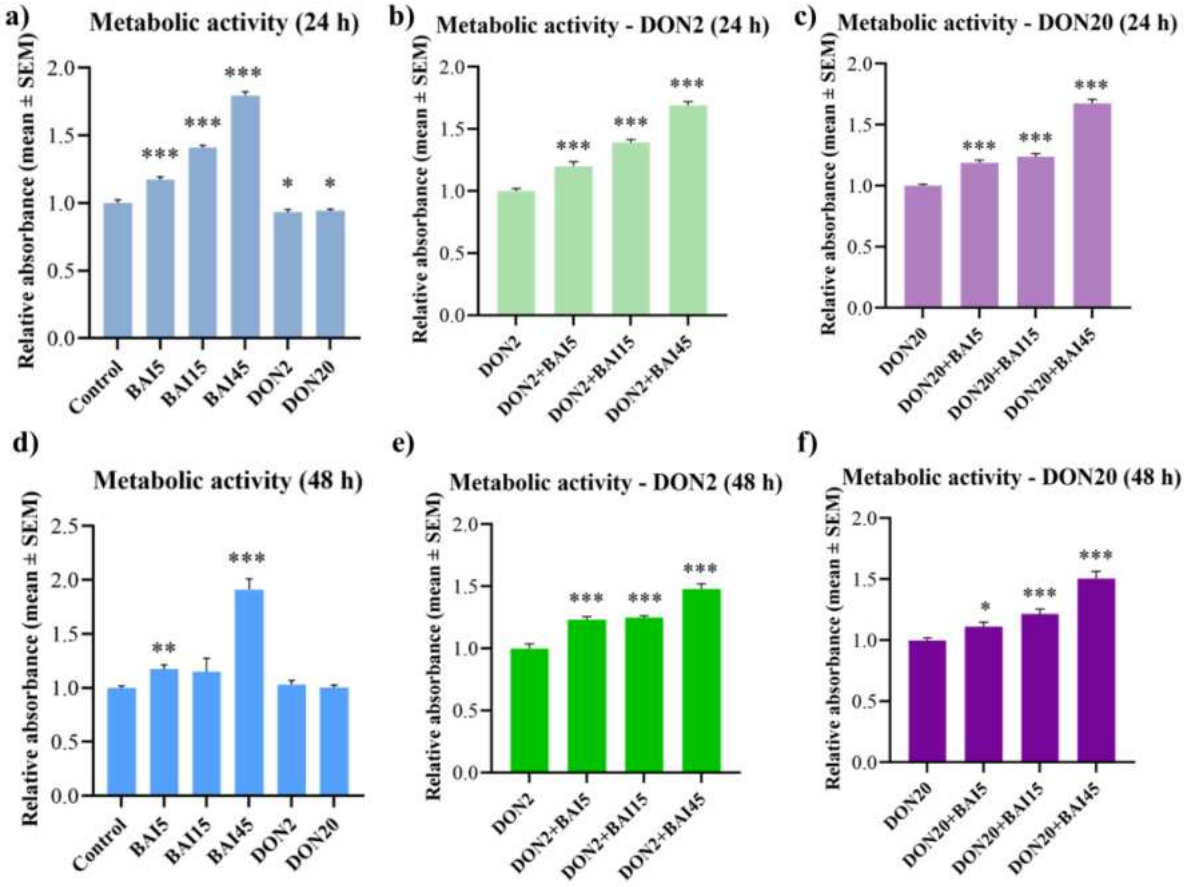


Figure 36. Effects of 24 and 48 h of deoxynivalenol (DON) and baicalin (BAI) treatment on the metabolic activity of primary hepatic 3D cell co-cultures of chicken origin assessed by CCK-8 test. BAI and DON-treated cells were compared to the control group after a) 24 h and d) 48 h. Baicalin and 2 µg/ml DON-treated cells were compared to the 2 µg/ml DON group after b) 24 h and e) 48 h. Baicalin and 20 µg/ml-DON treated cells were compared to the 20 µg/ml DON group after c) 24 h and f) 48 h. Relative absorbances have been calculated according to these comparisons, considering the mean values of control, DON2 or DON20 groups as 1. Control: cells without DON or BAI exposure; BAI: cells treated with BAI; DON: cells treated with DON; DON+BAI: cells treated with the combination of DON and BAI. See Table 7 for detailed concentrations. Results are expressed as mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Concerning the extracellular LDH activity, upon incubation for 24 h, all three baicalin concentrations decreased ($p = 0.017$; $p < 0.001$; $p = 0.010$, respectively) and the 20 µg/ml DON increased ($p = 0.003$) the LDH leakage (**Fig 37/a**). In addition, the 45 µg/ml baicalin treatment attenuated ($p = 0.014$) this DON-triggered elevated LDH activity (**Figure 37/c**).

For the 48 h samples, both DON concentrations lowered ($p = 0.005$; $p = 0.003$) the LDH release of the cells (**Figure 37/d**). Additionally, 45 µg/ml baicalin further decreased ($p < 0.001$) the LDH activity when applied together with 2 µg/ml DON (**Figure 37/e**).

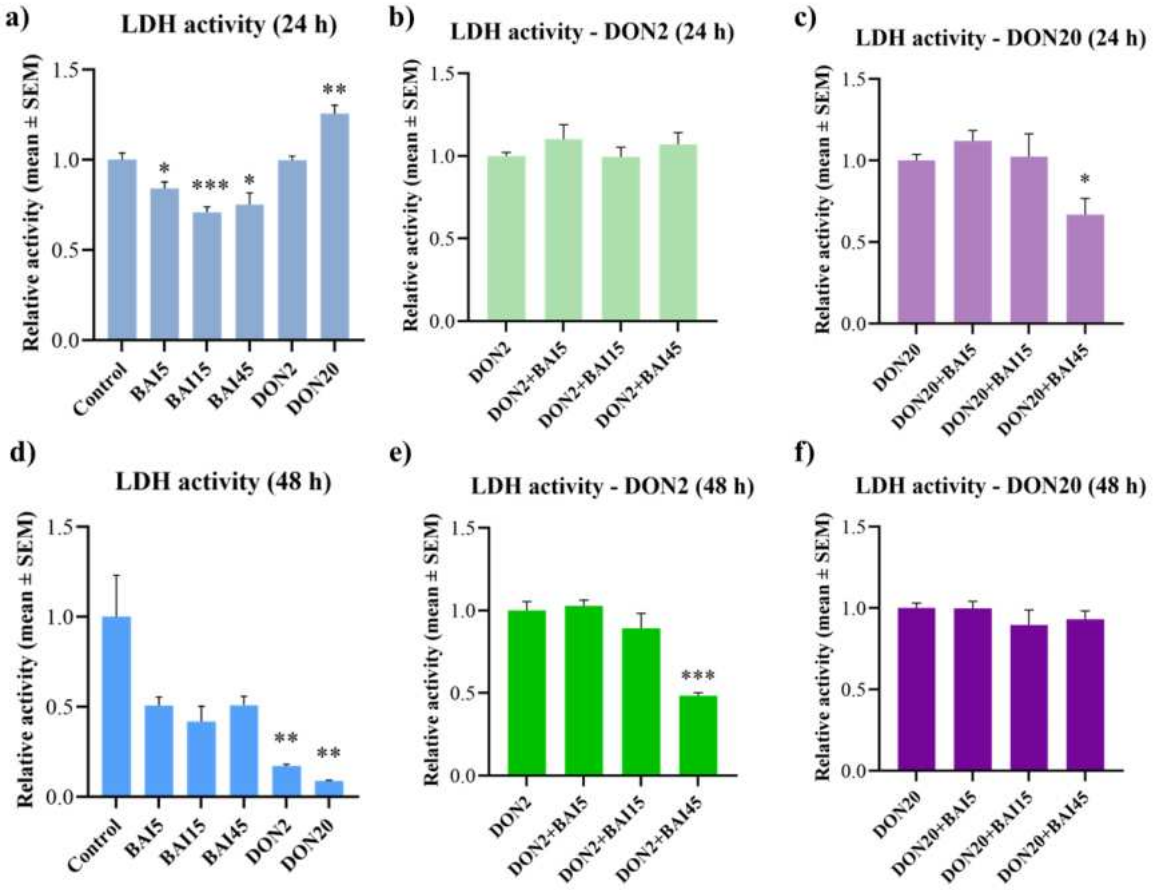


Figure 37. Effects of 24 and 48 h of deoxynivalenol (DON) and baicalin (BAI) treatment on the LDH activity of primary hepatic 3D cell co-cultures of chicken origin. BAI and DON-treated cells were compared to the control group after a) 24 h and d) 48 h. Baicalin and 2 µg/ml DON-treated cells were compared to the 2 µg/ml DON group after b) 24 h and e) 48 h. Baicalin and 20 µg/ml DON-treated cells were compared to the 20 µg/ml DON group after c) 24 h and f) 48 h. Relative activities have been calculated according to these comparisons, considering the mean values of control, DON2 or DON20 groups as 1. Control: cells without DON or BAI exposure; BAI: cells treated with BAI; DON: cells treated with DON; DON+BAI: cells treated with the combination of DON and BAI. See Table 7 for detailed concentrations. Results are expressed as mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

After 24 h of treatment, 45 µg/ml baicalin decreased ($p < 0.001$) and 20 µg/ml DON increased ($p = 0.029$) the NRF-2 content (**Figure 38/a**). In addition, in combination with 2 µg/ml DON treatment, 15 µg/ml baicalin lowered ($p = 0.007$) the amount (**Figure 38/b**). The

elevated levels of NRF-2 by 20 µg/ml DON were attenuated (p = 5.52×10^{-4}; p = 0.003, respectively) by the 15 µg/ml and 45 µg/ml baicalin treatment (**Figure 38/c**). In contrast, after 48 h, no significant changes were observed (**Figure 38/d-f**).

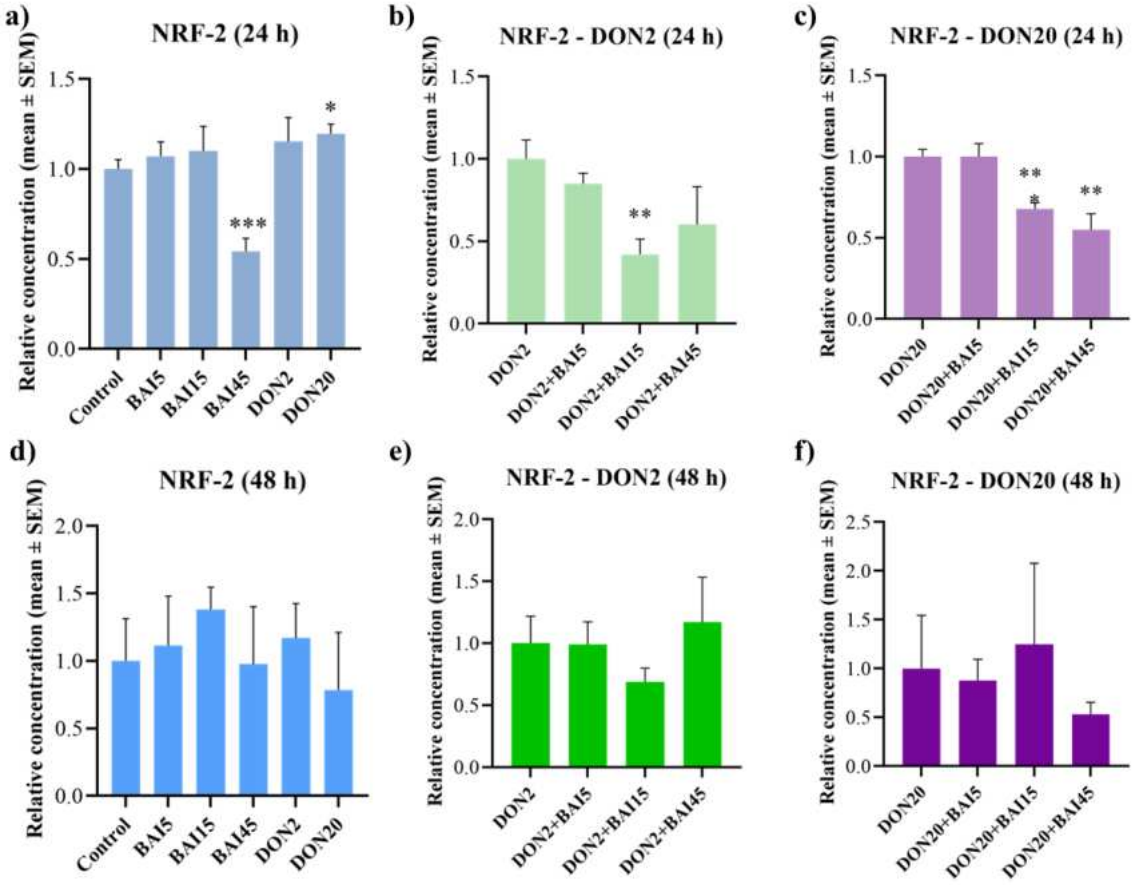


Figure 38. Effects of 24 and 48 h of deoxynivalenol (DON) and baicalin (BAI) treatment on the NRF-2 content of primary hepatic 3D cell co-cultures of chicken origin assessed by chicken-specific ELISA test. BAI and DON-treated cells were compared to the control group after a) 24 h and d) 48 h. Baicalin and 2 µg/ml DON-treated cells were compared to the 2 µg/ml DON group after b) 24 h and e) 48 h. Baicalin and 20 µg/ml-DON treated cells were compared to the 20 µg/ml DON group after c) 24 h and f) 48 h. Relative concentrations have been calculated according to these comparisons, considering the mean values of control, DON2 or DON20 groups as 1. Control: cells without DON or BAI exposure; BAI: cells treated with BAI; DON: cells treated with DON; DON+BAI: cells treated with the combination of DON and BAI. See Table 7 for detailed concentrations. Results are expressed as mean ± SEM. * p < 0.05; ** p < 0.01; *** p < 0.001.

The concentration of 8-OHdG was measured from cell lysate after 48 h. The 45 µg/ml baicalin and both applied DON concentrations reduced (p = 0.049; p < 0.001; p < 0.001, respectively) the 8-OHdG concentration compared to the control group (**Figure 39/a**).

Furthermore, in combination with the 20 µg/ml DON treatment, the 5 and 45 µg/ml baicalin increased (p = 0.005; p = 0.004, respectively) the levels of 8-OHdG (**Figure 39/c**).

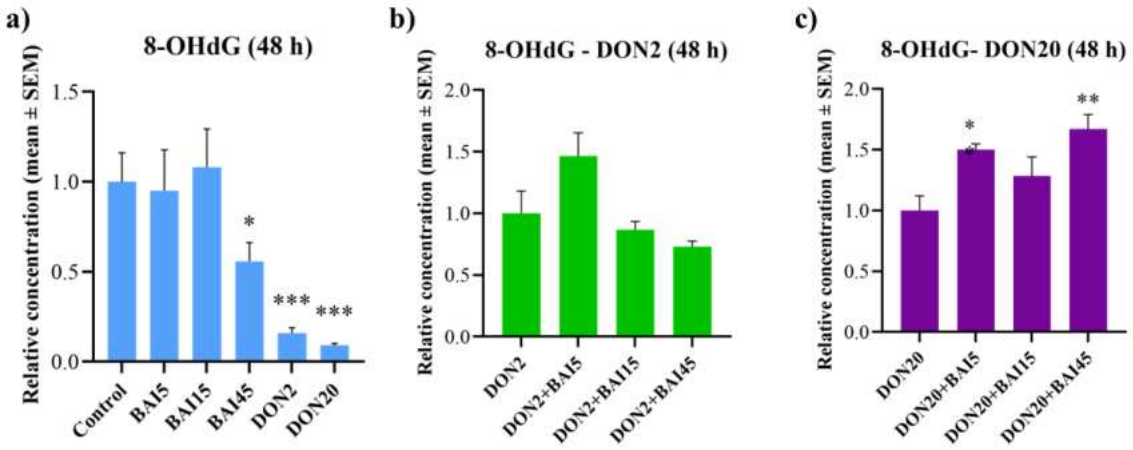


Figure 39. Effects 48 h of deoxynivalenol (DON) and baicalin (BAI) treatment on the 8-OHdG content of primary hepatic 3D cell co-cultures of chicken origin assessed by chicken-specific ELISA test. a) BAI and DON-treated cells compared to the control group. b) BAI and 2 µg/ml DON-treated cells compared to the 2 µg/ml DON. c) BAI and 20 µg/ml DON-treated cells compared to the 20 µg/ml DON group. Relative concentrations have been calculated according to these comparisons, considering the mean values of control, DON2 or DON20 groups as 1. Control: cells without DON or BAI exposure; BAI: cells treated with BAI; DON: cells treated with DON; DON+BAI: cells treated with the combination of DON and BAI. See Table 7 for detailed concentrations. Results are expressed as mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Regarding the inflammatory cytokines, DON did not affect the IL-6 levels after either incubation period. After 24 h of incubation, the 5 µg/ml baicalin in combination with the 2 µg/ml DON treatment increased (p = 0.014) the IL-6 levels (**Figure 40/b**). For the 20 µg/ml DON, the 5 and 15 µg/ml elevated (p = 0.039; p = 0.005, respectively) the amount of IL-6 (**Figure 40/c**). Furthermore, after 48 h of treatment, the solely applied 15 µg/ml BAI lowered (p = 0.047) the IL-6 concentration compared to the control group (**Figure 40/d**).

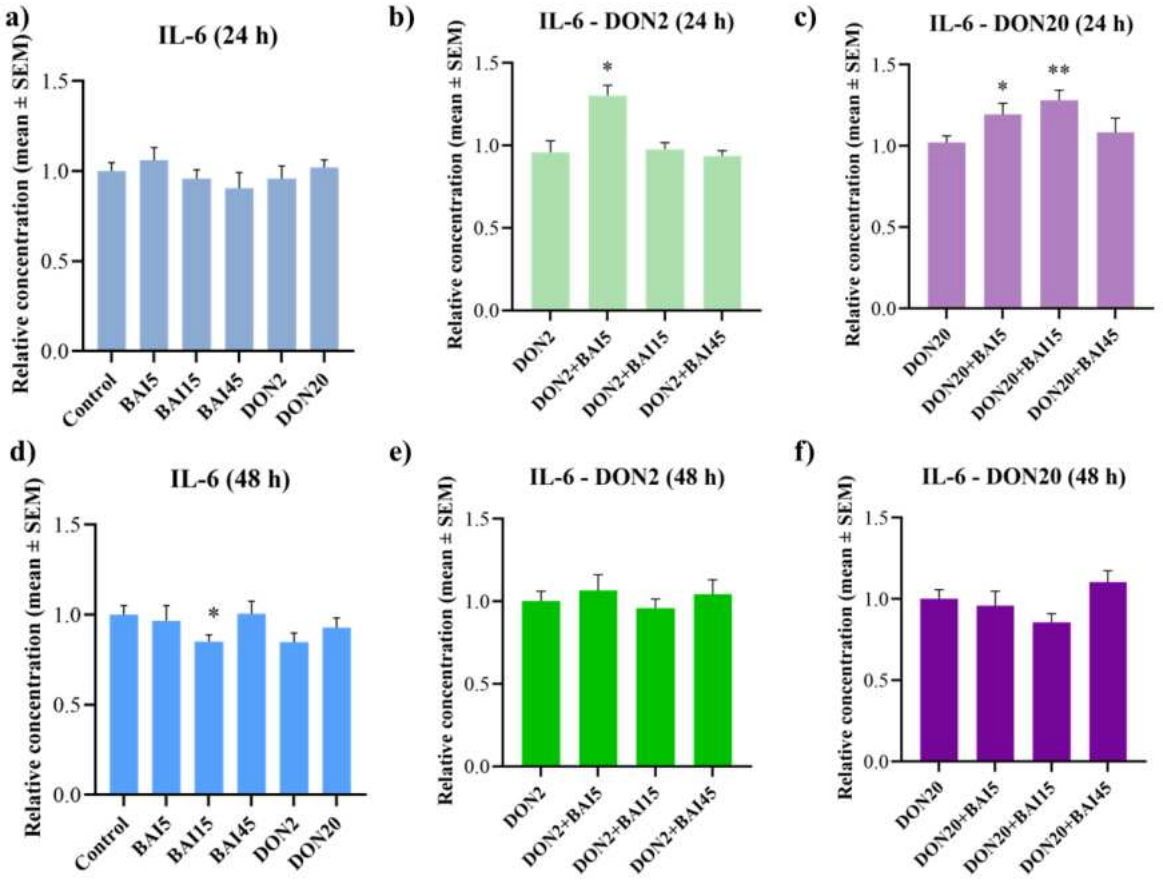


Figure 40. Effects of 24 and 48 h of deoxynivalenol (DON) and baicalin (BAI) treatment on the IL-6 content of primary hepatic 3D cell co-cultures of chicken origin assessed by chicken-specific ELISA test. BAI and DON-treated cells were compared to the control group after a) 24 h and d) 48 h. Baicalin and 2 µg/ml DON-treated cells were compared to the 2 µg/ml DON group after b) 24 h and e) 48 h. Baicalin and 20 µg/ml-DON treated cells were compared to the 20 µg/ml DON group after c) 24 h and f) 48 h. Relative concentrations have been calculated according to these comparisons, considering the mean values of control, DON2 or DON20 groups as 1. Control: cells without DON or BAI exposure; BAI: cells treated with BAI; DON: cells treated with DON; DON+BAI: cells treated with the combination of DON and BAI. See Table 7 for detailed concentrations. Results are expressed as mean ± SEM. * $p < 0.05$; ** $p < 0.01$.

In case of the IL-8 content, after 24 h, DON had no influence on IL-8 levels by itself. The 2 µg/ml DON treatment in combination with the 5 and 15 µg/ml baicalin reduced ($p = 0.024$; $p = 0.016$ respectively) the amount after 24 h (**Figure 41/b**). After the 48 h incubation, the 45 µg/ml baicalin and both applied DON reduced ($p = 0.016$; $p = 0.034$; $p = 0.024$, respectively) the levels of IL-8 compared to the control (**Figure 41/d**).

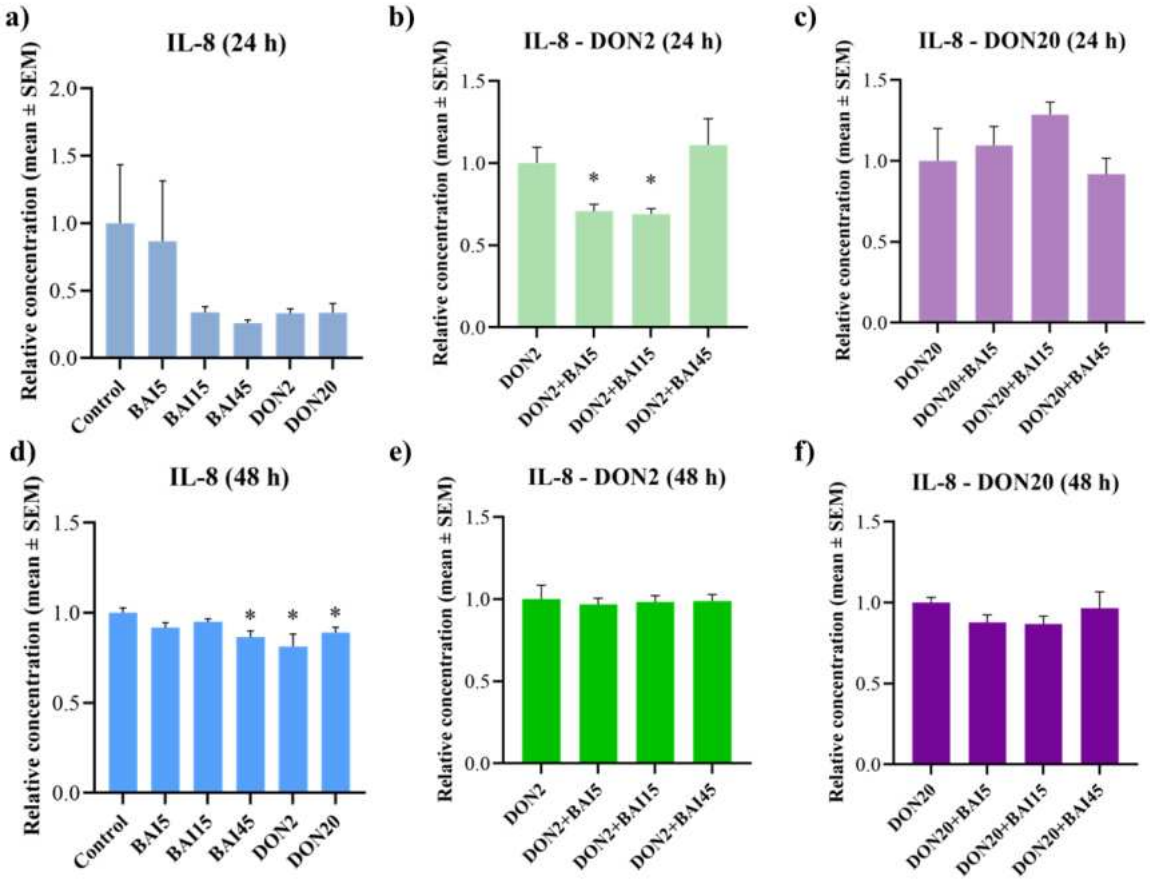


Figure 41. Effects of 24 and 48 h of deoxynivalenol (DON) and baicalin (BAI) treatment on the IL-8 content of primary hepatic 3D cell co-cultures of chicken origin assessed by chicken-specific ELISA test. BAI and DON-treated cells were compared to the control group after a) 24 h and d) 48 h. Baicalin and 2 µg/ml DON-treated cells were compared to the 2 µg/ml DON group after b) 24 h and e) 48 h. Baicalin and 20 µg/ml-DON treated cells were compared to the 20 µg/ml DON group after c) 24 h and f) 48 h. Relative concentrations have been calculated according to these comparisons, considering the mean values of control, DON2 or DON20 groups as 1. Control: cells without DON or BAI exposure; BAI: cells treated with BAI; DON: cells treated with DON; DON+BAI: cells treated with the combination of DON and BAI. See Table 7 for detailed concentrations. Results are expressed as mean ± SEM. * $p < 0.05$.

After 24 h, the 45 µg/ml baicalin reduced ($p = 0.033$) IFN-γ levels compared to the untreated cells (**Figure 42/a**). In case of the 2 µg/ml DON treatment, the concomitantly administered 15 and 45 µg/ml baicalin diminished ($p < 0.001$; $p = 0.005$, respectively) the IFN-γ conentrations (**Figure 42/b**). In addition, the 20 µg/ml DON in combination with the 15 µg/ml baicalin increased ($p = 0.023$) the amount of IFN-γ (**Figure 42/c**). No significant changes were detected after the 48 h treatment (**Figure 42/d-f**).

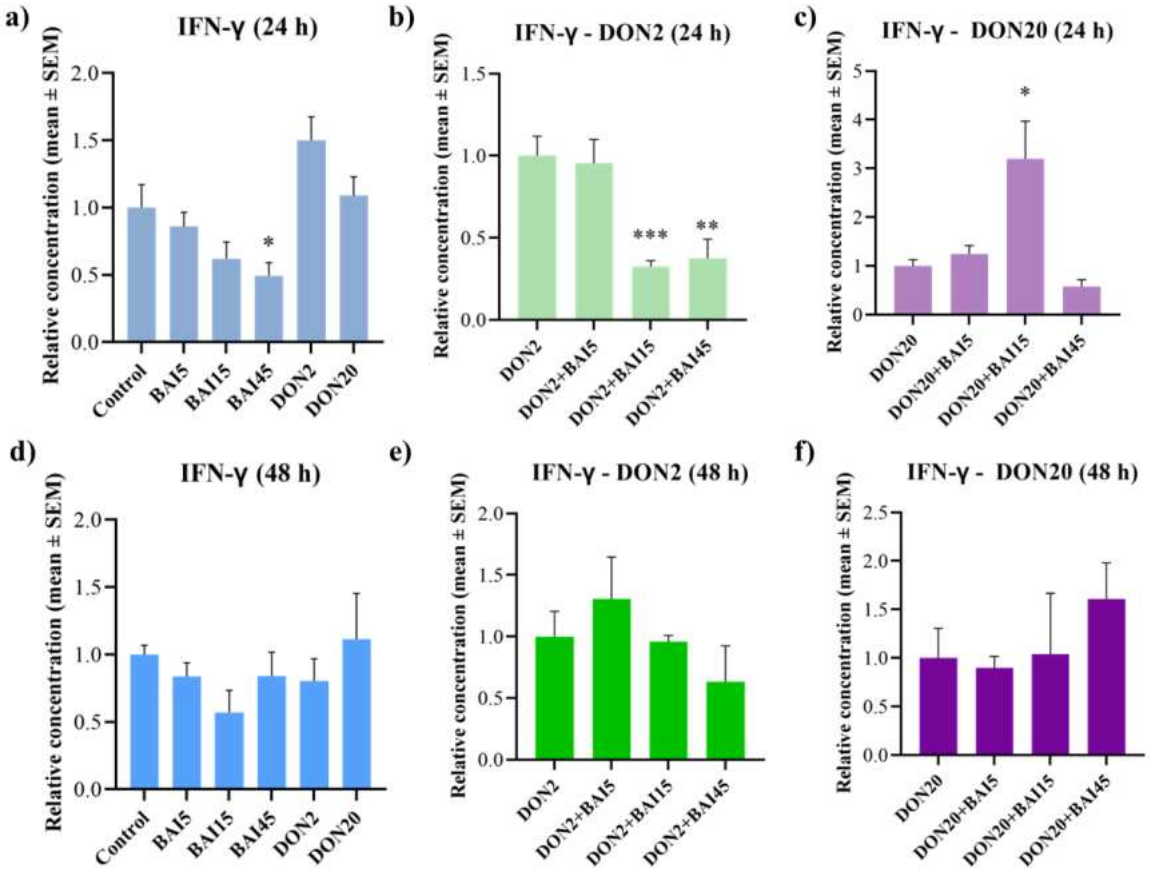


Figure 42. Effects of 24 and 48 h of deoxynivalenol (DON) and baicalin (BAI) treatment on the IFN-γ content of primary hepatic 3D cell co-cultures of chicken origin assessed by chicken-specific ELISA test. BAI and DON-treated cells were compared to the control group after a) 24 h and d) 48 h. Baicalin and 2 µg/ml DON-treated cells were compared to the 2 µg/ml DON group after b) 24 h and e) 48 h. Baicalin and 20 µg/ml-DON treated cells were compared to the 20 µg/ml DON group after c) 24 h and f) 48 h. Relative concentrations have been calculated according to these comparisons, considering the mean values of control, DON2 or DON20 groups as 1. Control: cells without DON or BAI exposure; BAI: cells treated with BAI; DON: cells treated with DON; DON+BAI: cells treated with the combination of DON and BAI. See Table 7 for detailed concentrations. Results are expressed as mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Study 4. The effects of AMPs on the inflammatory and oxidative stress response of intestinal explant cultures

Study 4.1. Establishment and characterization of intestinal explant cultures from chicken

The cellular metabolic and total LDH activities of the explants were measured in the first 36 hours of culturing. The initial metabolic activity of the 2 mm explants was remarkably higher than that of the 1 mm samples (p=0.002), but the value of the 2 mm explants decreased significantly by 12 h (p=0.001), hence there was no significant difference between the 1 and 2 mm explants from the 12 h time point on (**Figure 43**). Concerning the total LDH activity of the medium samples the levels of the 2 mm explants from 12 h time point on were found significantly higher than that of the 1 mm samples (12 h: p= 0.015, 24 h: p=0.006, 36 h: p=0.005) (**Figure 43**).

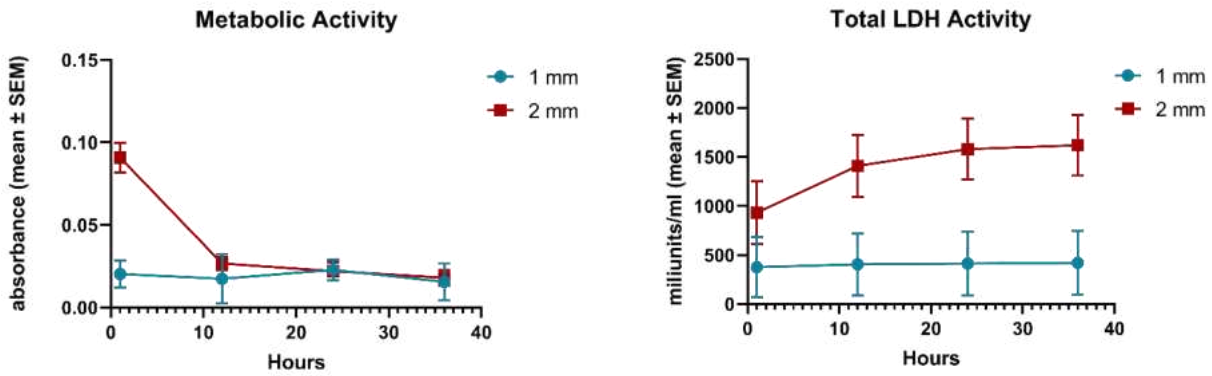


Figure 43. Cellular metabolic activity measured with CCK-8 assay and total lactate dehydrogenase (LDH) activity measured with an enzyme kinetic photometric assay of 1 and 2 mm explants. Mean (n = 5/group) ± SEM.

Histology showed that the ileal epithelial cells lost their typical columnar morphology, after 24 h the epithelium of 1 mm samples was rather cuboidal while that of the 2 mm explants was more flattened. Moreover, necrotic and/or apoptotic cell debris was observed in the crypts and the lamina propria mainly of 2 mm samples after 24 h of culturing (**Figure 44**).

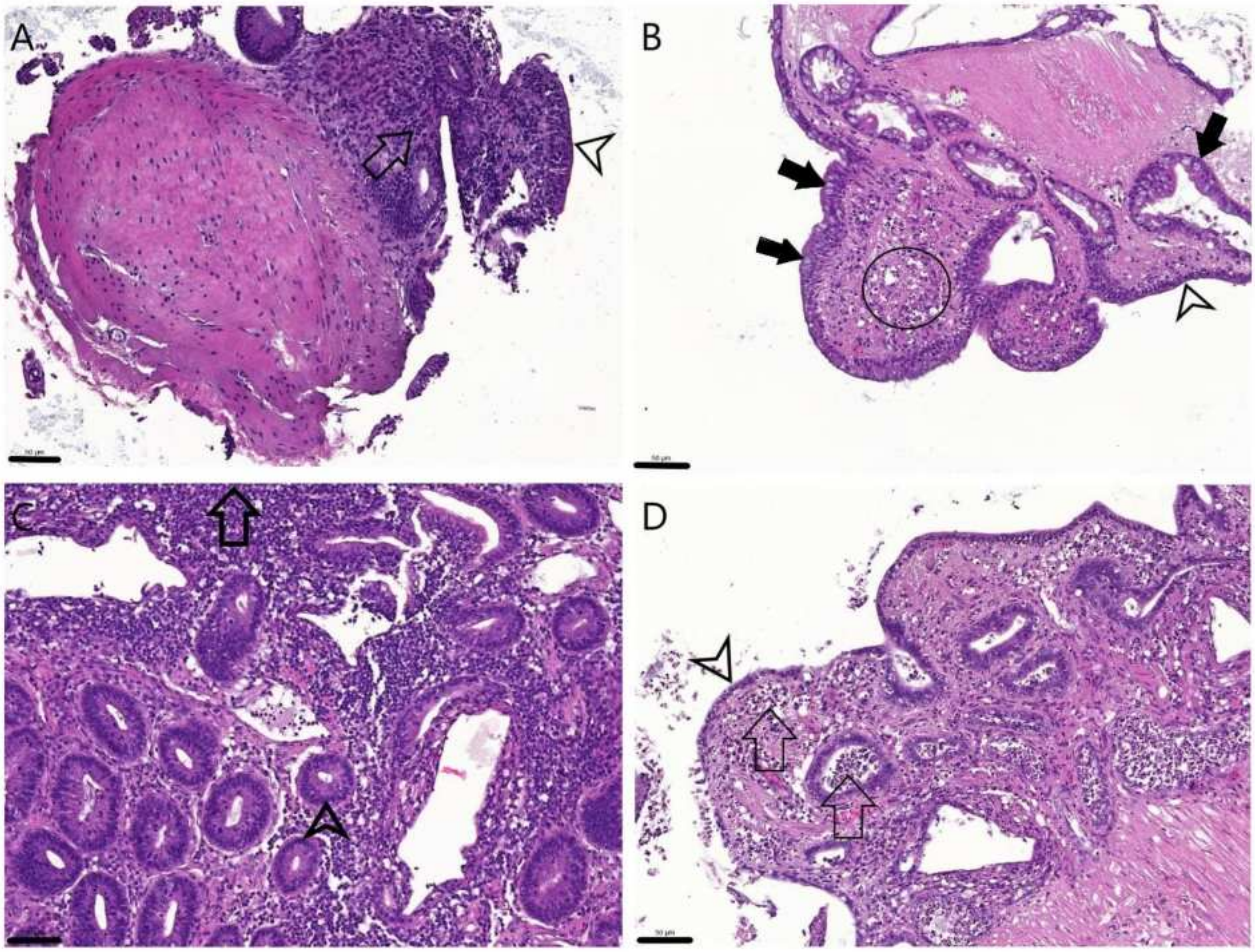


Figure 44. H&E stained sections of the explants. 0 h 1 mm (A) and 2 mm (C): The physiologic columnar epithelial morphology (arrowheads) is visible on the surface and within the crypts, and the proprial mononuclear infiltrate is well observable (arrows). 24 h 1 mm (B): The surface enterocytes are rather cuboidal (arrowhead), yet Goblet cells (full arrows) are still well appreciable both inside the crypts and on the surface. In the *lamina propria* apoptotic cell debris appears (circle). 24 h 2 mm (D): The surface enterocytes are frequently flattened if still present (arrowhead), cell debris is apparent in the lamina propria and also within the crypts (arrows). Bar line = 50 µm.

Pan-cytokeratin staining of explants after 24 h incubation highlighted presumably intact enterocytes and also Goblet cells in 1 mm explants. The Goblet cell counts apparently decreased in the 2 mm samples. While tissue debris was more evident in the lamina propria of 2 mm specimens, intact inflammatory cells were also visible at this location in the 1 mm explants (**Figure 45**). The disruption of the 2 mm explants was evident after 48 hours of culturing as they scattered into smaller pieces and cell debris which made impossible their further manipulation and culturing. Following 9 days of culturing when examined under inverse microscope, Giemsa staining of the 1 mm explants revealed that intact epithelial cells were apparently budding out from the original explant culture (**Figure 45/C,D**).

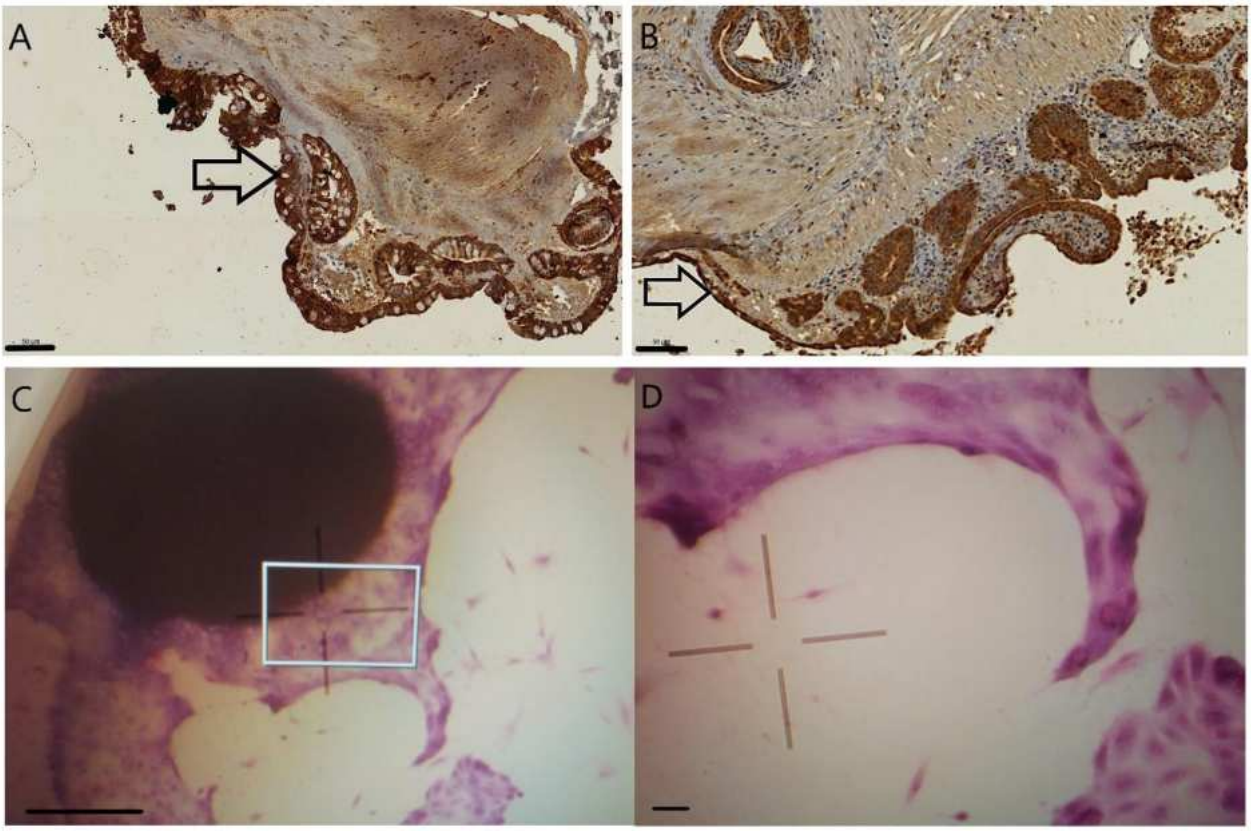


Figure 45. Pan-cytokeratin stained 24 h sections of 1 mm (A) and 2 mm (B) explants. The epithelial cells are marked in dark brown, Goblet cells with their unstained intracytoplasmic vacuoles are mostly seen in 1 mm explants (arrows). Giemsa staining of the 1mm explants after 9 days of culturing (C, D): epithelial cells budding from the original explant tissue. The indicated area of picture C (square) was magnified to gain picture D. Bar line = 50 µm (A, B, D), 500 µm (C).

Study 4.2. Preliminary study on pro-inflammatory compounds (LTA, Flagellin, Poly I:C)

The CCK-8 assay carried out with the explants immediately after 12 h PAMP (LTA, flagellin or poly I:C) exposures showed that the higher concentration of poly I:C treatment induced a significant decrease in the cellular metabolic activity compared to the control ($p = 0.032$; **Figure 46**). No significant change was observed between PAMP-exposed and control explants in case of the LDH activity (**Figure 46**). The after 12 h incubation showed retained villous architecture to a certain degree and an epithelial lining which was mostly columnar. Pan-cytokeratin staining highlighted presumably intact surface enterocytes, crypt epithelial cells and a few Goblet cells. A mild mononuclear infiltrate is visible in the *lamina propria* along with a small to moderate amount of tissue debris (**Figure 47**).

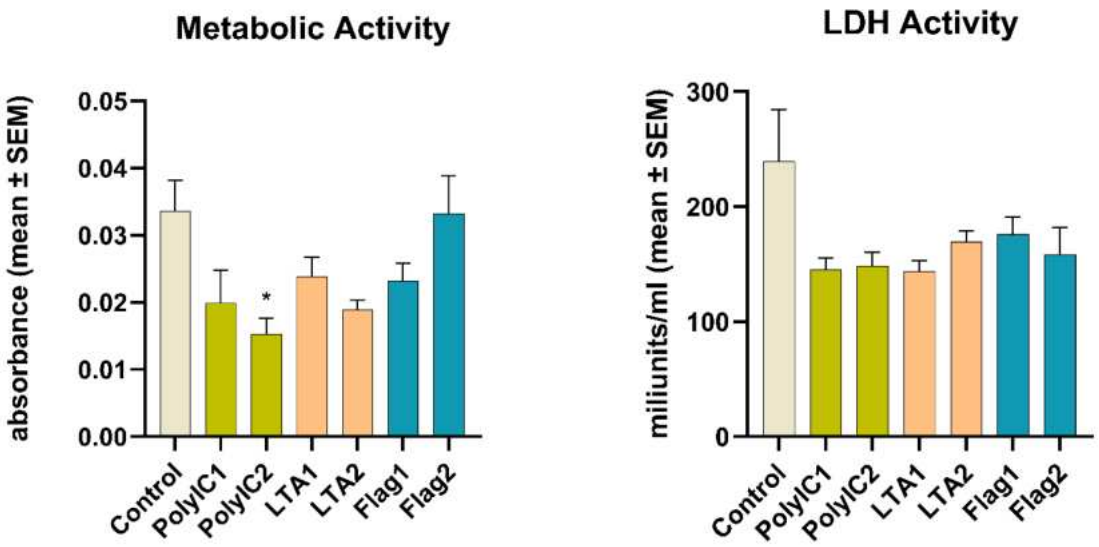


Figure 46. Metabolic activity measured by CCK-8 assay and extracellular lactate dehydrogenase (LDH) activity measured by an enzyme kinetic photometric assay of the 1.5 mm explants. PolyIC1 = 50 µg/ml, PolyIC2 = 100 µg/ml polyinosinic-polycytidylic acid, respectively; LTA1 = 10 µg/ml, LTA2 = 50 µg/ml *Staphylococcus aureus* lipoteichoic acid, respectively; Flag1 = 100 ng/ml, Flag2 = 250 ng/ml *Salmonella* Typhimurium flagellin, respectively. Mean (n =5/group) ± SEM.

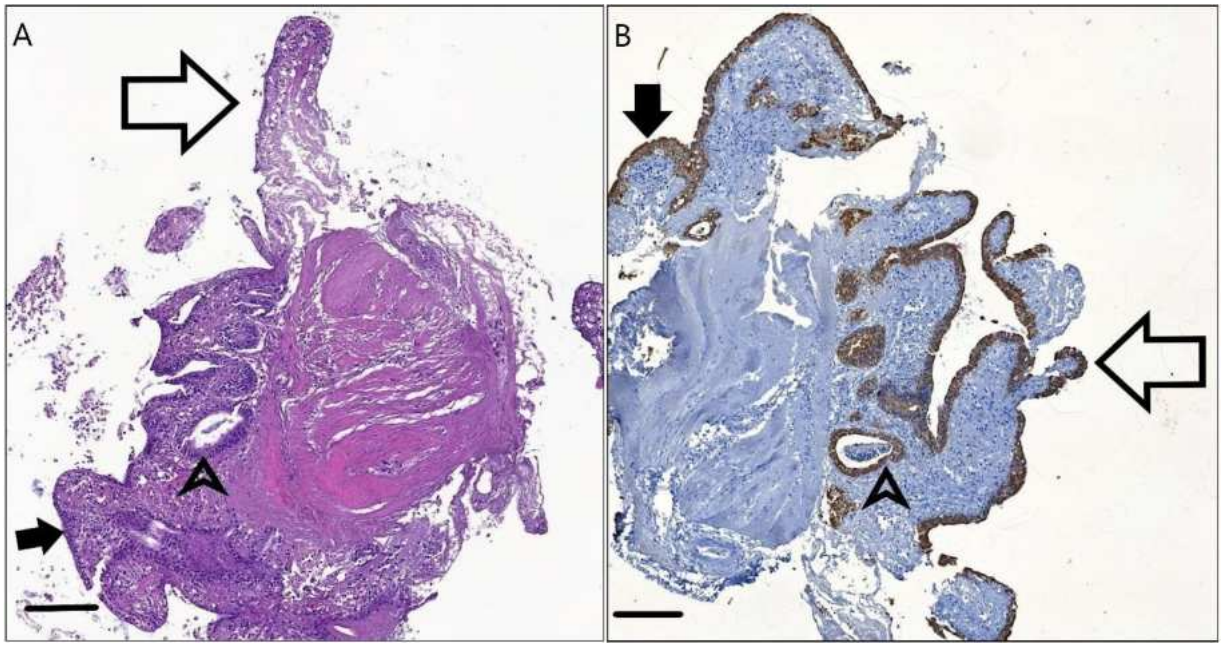


Figure 47. H&E stained (A) and Pan-cytokeratin stained (B) sections of the 1.5 mm explants after 12 h incubation. The physiologic columnar or cuboidal epithelial morphology (arrowheads) is visible on the

surface (full arrows) and within the crypts (arrowheads). Remnants of the intestinal villi are present (empty arrows.) Bar line = 100 μm.

IFN-α concentration was significantly increased by 50 μg/ml poly I:C treatment. IFN-γ was elevated by LTA and poly I:C at both applied concentrations. A significant increase was induced by LTA (10 μg/ml) for IL-2, whereas IL-6 was elevated by 100 ng/ml flagellin and 50 μg/ml poly I:C. In the case of flagellin treatment, IL-10 was up-regulated at both concentrations, while the higher concentration of poly I:C and flagellin increased the levels of RANTES (**Figure 48, Table 11**). The IFN-γ/IL-10 ratio was significantly increased by the higher applied concentration of LTA and in case of poly I:C in a concentration-dependent manner (**Figure 49, Table 11**). No significant change in the IL-8 concentration was induced by either of the applied treatments (**Figure 48, Table 11**).

Table 11. Significant changes with p values caused by the selected PAMPs compared to the control group.

Parameters	**Supplements**					
	Poly I:C (μg/mL)		**Lipoteichoic acid (μg/mL)**		**Flagellin (ng/ml)**	
	50	**100**	**10**	**50**	**100**	**250**
Metabolic activity		↓ 0.032				
IFN-α	↑ 0.024					
IFN-γ	↑ 0.014	↑ 0.015	↑ 0.015	↑ 0.013		
IL-2			↑ 0.019			
RANTES		↑ 0.016				↑ 0.016
IL-6	↑ 0.016				↑ 0.016	
IL-10					↑ 0.018	↑ 0.018
IFN-γ/IL-10 ratio	↑ 0.018	↑ 0.018		↑ 0.027		

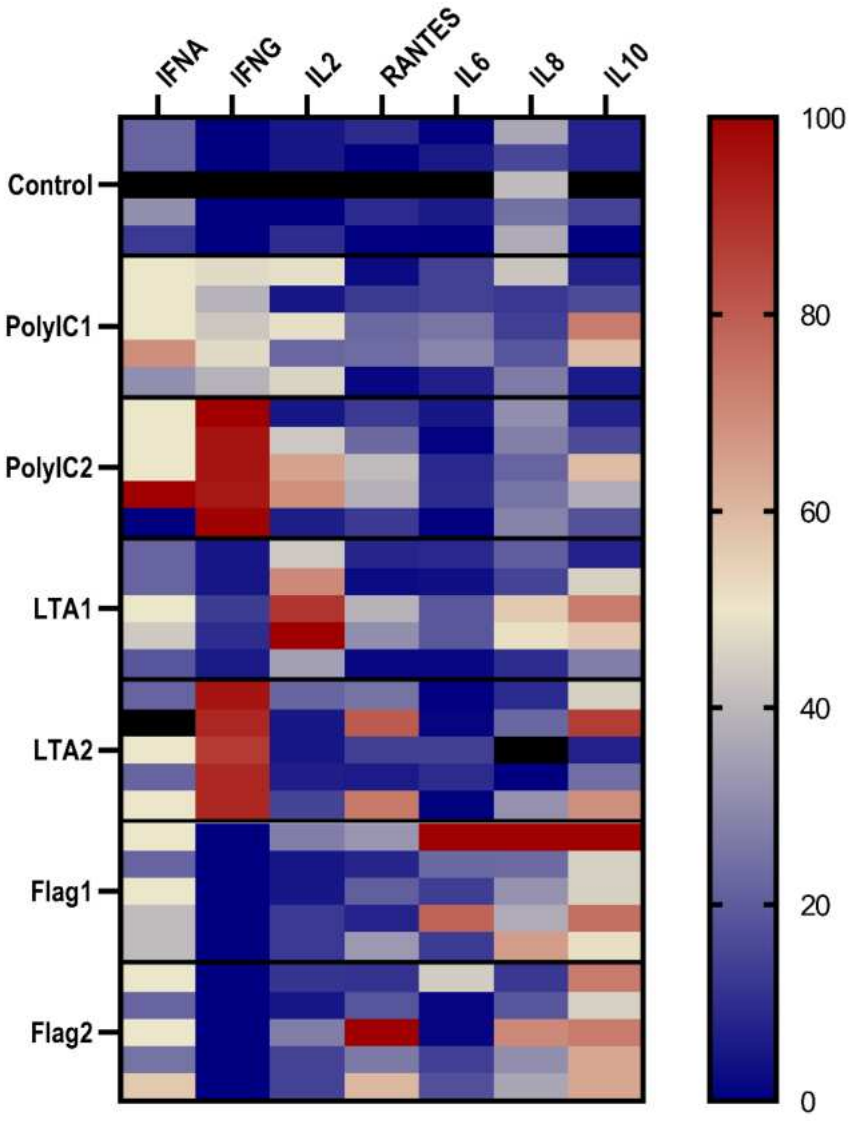


Figure 48. The heatmap presents the proportional change in the investigated cytokines. IFNA = interferon-α, IFNG = interferon-γ, RANTES = regulated on activation, normal T cell expressed and secreted, IL2 = interleukin-2, IL6 = interleukin-6, IL8 = interleukin-8, IL10 = interleukin-10, IL2 = interleukin-2, PolyIC1 = 50μg/ml polyinosinic-polycytidylic acid, PolyIC2 = 100μg/ml polyinosinic-polycytidylic acid, LTA1 = 10μg/ml *Staphylococcus aureus* lipoteichoic acid, LTA2 = 50μg/ml *Staphylococcus aureus* lipoteichoic acid, Flag1 = 100ng/ml *Salmonella* Typhimurium flagellin, Flag2= 250ng/ml *Salmonella* Typhimurium flagellin.

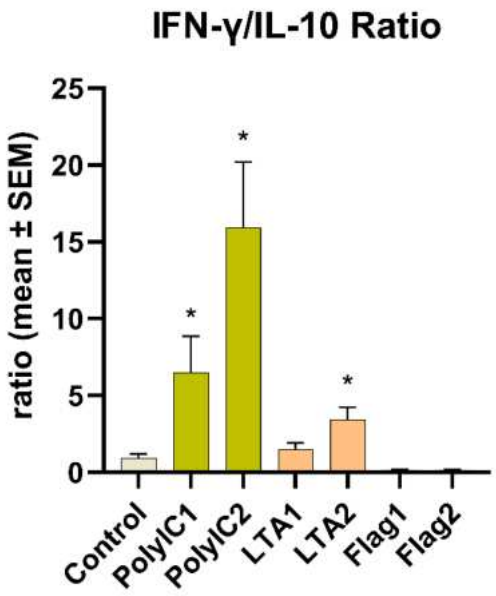


Figure 49. IFN-γ/IL-10 ratio. PolyIC1 = 50µg/ml polyinosinic-polycytidylic acid, PolyIC 2 = 100µg/ml polyinosinic-polycytidylic acid, LTA1 = 10µg/ml *Staphylococcus aureus* lipoteichoic acid, LTA2 = 50µg/ml *Staphylococcus aureus* lipoteichoic acid, Flag1 = 100ng/ml *Salmonella* Typhimurium flagellin, Flag2= 250ng/ml *Salmonella* Typhimurium flagellin. Mean (n = 5/group) ± SEM, * $p < 0.05$

Study 4.3. Testing the effects of cathelicidin-2

The metabolic activity of the explants was elevated after treatment with 25 nmol/mL Cath-2 (Cath-high; p=0.01). No other treatment influenced the metabolic activity or the LDH activity of the explants (data not shown).

IL-6 concentration increased following 10 nmol/mL concentration Cath-2 (Cath-medium), and LTA treatment (p=0.016; p=0.016, respectively, **Figure 50**) compared to Control. Addition of 25 nmol/mL concentration of Cath-2 (LTA+Cath-high) decreased IL-6 production compared to the LTA group (p=0.029, **Figure 50**).

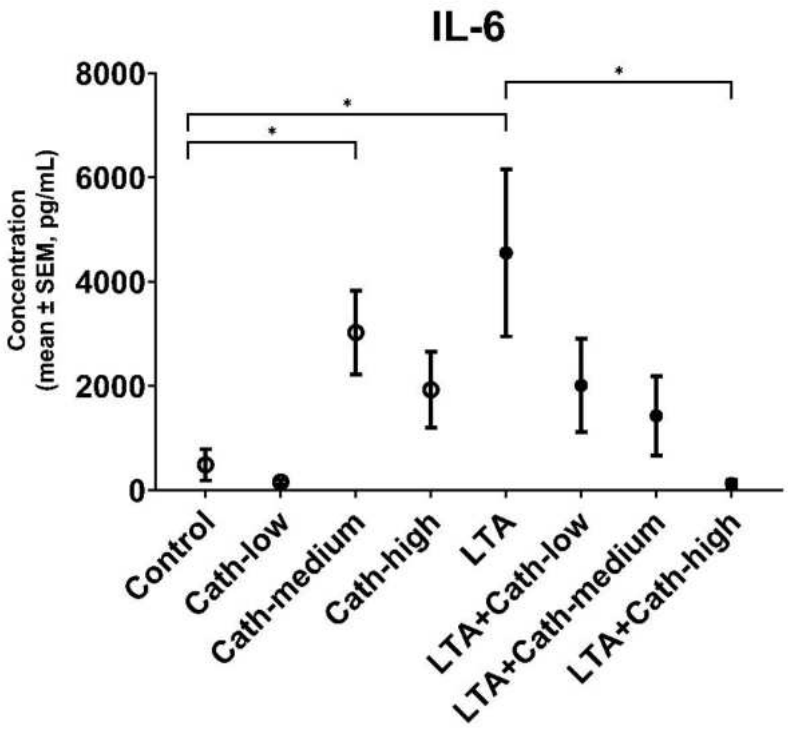


Figure 50. Interleukin (IL)-6 concentration of the cell culture medium after the treatment of the explants. Cath-low=5 nmol/mL of chicken cathelicidin-2, Cath-medium=10 nmol/mL of chicken cathelicidin-2, Cath-high=25 nmol/mL of chicken cathelicidin-2, LTA=10 µg/mL of *Staphylococcus aureus* derived lipoteichoic acid. Control group received none of the treatments. In each group: n=6. *p<0.05

CXCLi2/IL-8 levels were increased after treatment with 5, 10, 25 nmol/mL of Cath-2 (Cath-low, Cath-medium, and Cath-high), and LTA (p=0.019; p=0.027; p=0.014; p=0.016, respectively, **Figure 51**). Furthermore, 25 nmol/mL Cath-2 (LTA+Cath-high) lowered the concentration of CXCLi2 compared to the elevated production in the LTA group (p=0.016, **Figure 51**).

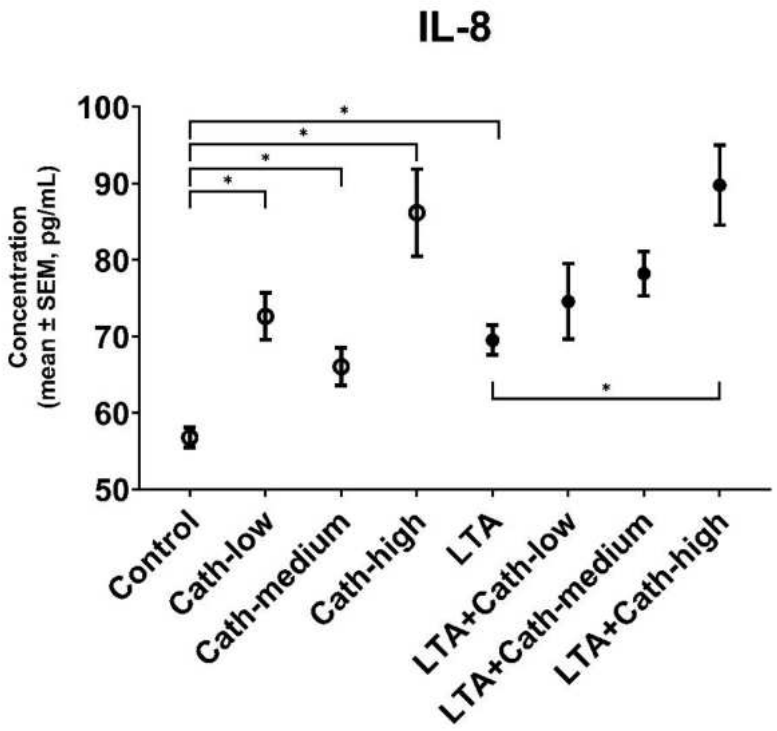


Figure 51. CXCLi2/IL-8 concentration of the cell culture medium after the treatment of the explants. Cath-low=5 nmol/mL of chicken cathelicidin-2, Cath-medium=10 nmol/mL of chicken cathelicidin-2, Cath-high=25 nmol/mL of chicken cathelicidin-2, LTA=10 µg/mL of *Staphylococcus aureus* derived lipoteichoic acid. Control group received none of the treatments. In each group: n=6. *p<0.05

25 nmol/mL Cath-2 (Cath-high), and LTA treatment increased the production of IL-2 (p=0.022; p=0.009, respectively, **Figure 52**). 10 and 25 nmol/mL concentrations of Cath-2 (LTA+Cath-medium and LTA+Cath-high) decreased IL-2 levels compared to the LTA group (p=0.013; p=0.013, respectively, **Figure 52**).

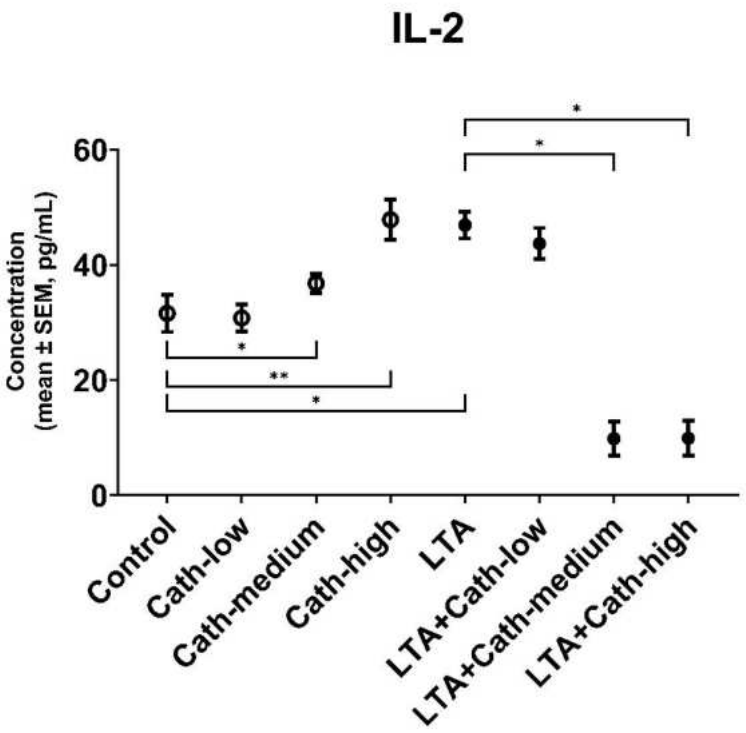


Figure 52. Interleukin (IL)-2 concentration of the cell culture medium after the treatment of the explants. Cath-low=5 nmol/mL of chicken cathelicidin-2, Cath-medium=10 nmol/mL of chicken cathelicidin-2, Cath-high=25 nmol/mL of chicken cathelicidin-2, LTA=10 µg/mL of *Staphylococcus aureus* derived lipoteichoic acid. Control group received none of the treatments. In each group: n=6. *p<0.05, **p<0.01

The concentration of IFN-γ was increased and IL-10 was decreased after LTA treatment (p=0.005; p=0.031; respectively, **Figure 53/A** and **53/B**), which then lead to an increase in the ratio of IFN-γ/IL-10 (p=0.036, **Figure 53/C**). 25 nmol/mL Cath-2 (Cath-high) elevated IL-10 levels compared to LTA (p=0.024, **Figure 53/B**), but had no influence on IFN-γ concentrations. However, the ratio of IFN-γ/IL-10 was decreased by 5, 10 and 25 nmol/mL of Cath-2 (Cath-low, Cath-medium, and Cath-high) compared to LTA (p=0.048; p=0.024; p=0.028, respectively, **Figure 53/C**).

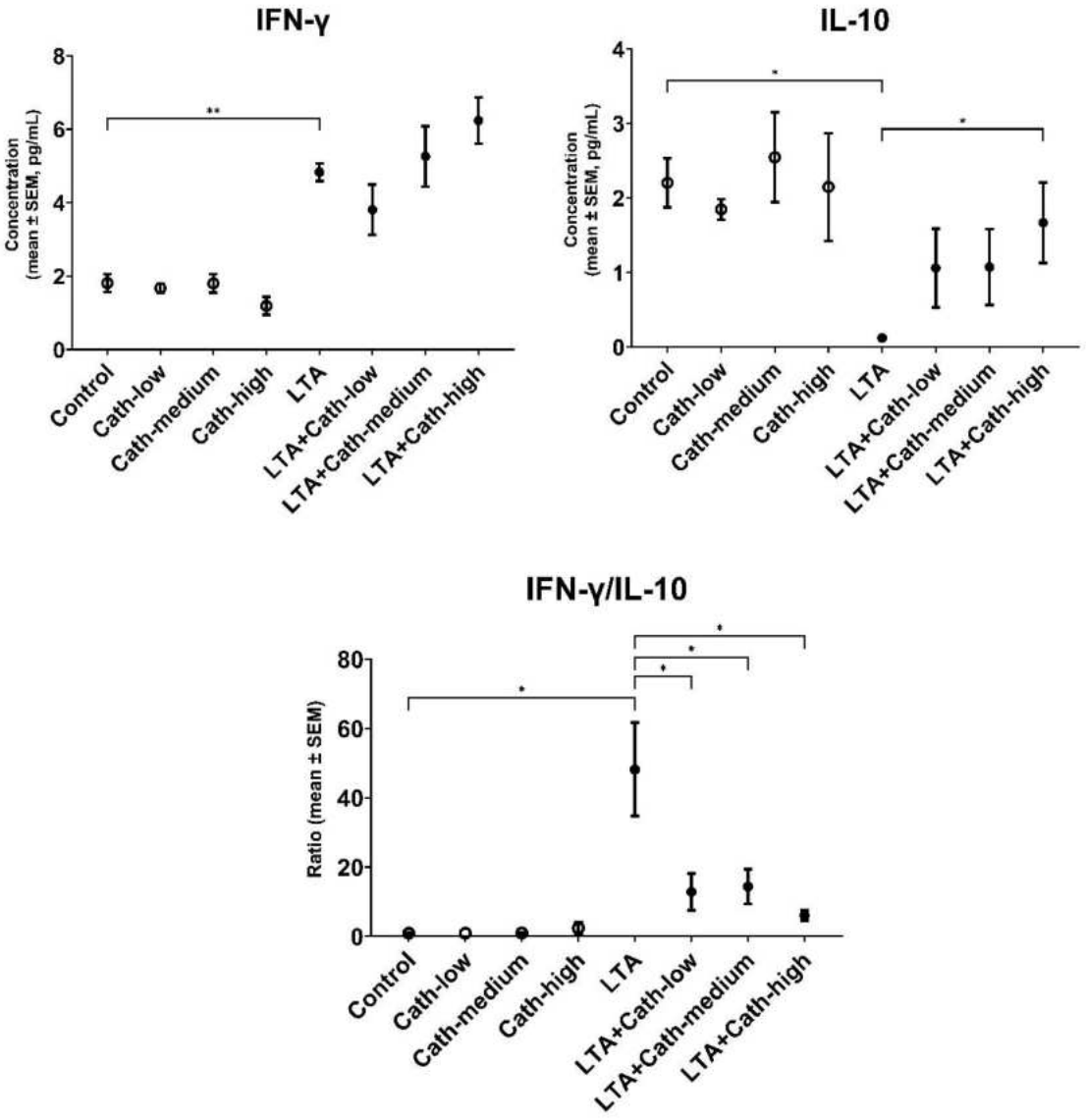


Figure 53. Interferon (IFN)-γ (A) and interleukin (IL)-10 (B) concentration, and the ratio of IFN-γ and IL-10 concentrations (C) of the cell culture medium after the treatment of the explants. Cath-low=5 nmol/mL of chicken cathelicidin-2, Cath-medium=10 nmol/mL of chicken cathelicidin-2, Cath-high=25 nmol/mL of chicken cathelicidin-2, LTA=10 µg/mL of *Staphylococcus aureus* derived lipoteichoic acid. Control group received none of the treatments. In each group: n=6. *p<0.05

Study 4.4. Testing the effects of IDR-1002

No significant changes were observed regarding the metabolic activity of the explants. LDH activity was decreased in the group treated with 30 µg/mL IDR-1002 compared to the control (IDR-medium; p=0.029; data not shown).

It was found that treatment with 10, 30, and 90 µg/mL of IDR-1002 (IDR-low, IDR-medium, IDR-high; p=0.035; 0.023; 0.036, respectively) and LTA (p=0.019) resulted in significantly elevated IL-2 levels compared to the control group. IDR-1002 applied with LTA

at a concentration of 10 µg/ml (LTA+IDR-low) significantly decreased (p=0.028) the IL-2 concentration of culture media compared to the group exposed to LTA alone (Figure 2).

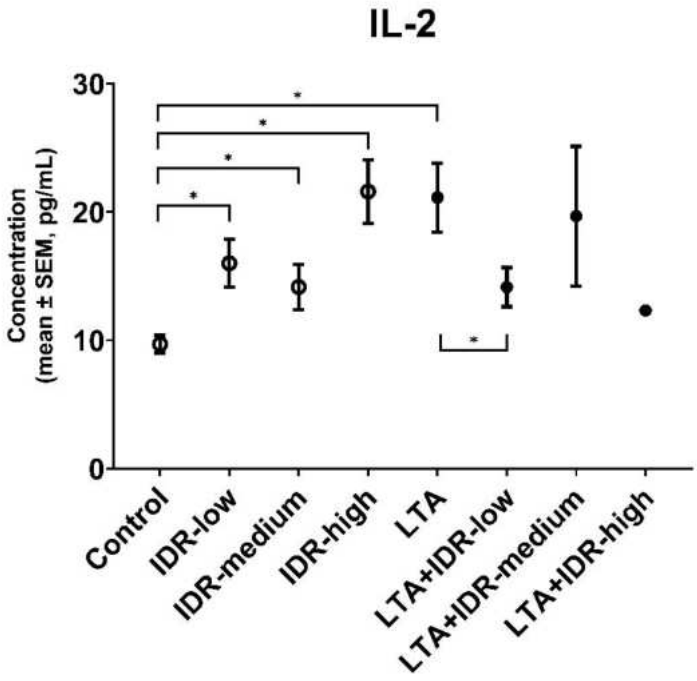


Figure 54. Interleukin (IL)-2 concentrations of chicken ileal explants from cell culture medium in response to IDR-1002 and LTA treatment. IDR-low = 10 µg/mL IDR-1002, IDR-medium = 30 µg/mL IDR-1002, IDR-high = 90 µg/mL IDR-1002, LTA = 10 µg/mL lipoteichoic acid from *Staphylococcus aureus* (n = 6/group). Cell cultures in Control group received none of the treatments. * $p<0.05$

The obtained results show that the sole treatment with 30 µg/mL IDR-1002 (IDR-medium) and with LTA resulted in elevated (p=0.006 in both cases) IL-8/ CXCLi2 levels compared to the control group. Further, all concentrations of IDR-1002 (10, 30, 90 µg/mL IDR-1002) in combination with LTA (LTA+IDR-low, LTA+IDR-medium, LTA+IDR-high) caused significantly lower (p=0.004; 0.002; 0.005, respectively) CXCLi2 concentrations compared to the group with LTA alone (**Figure 55**).

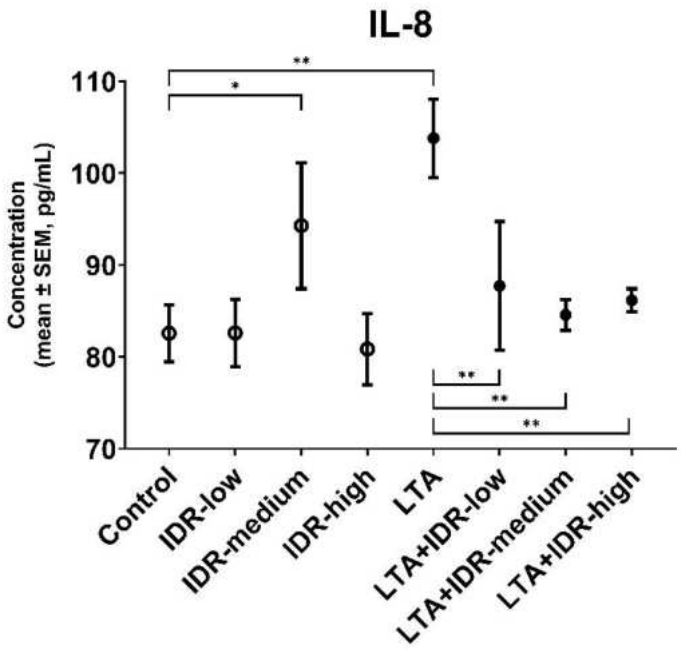


Figure 55. IL-8/CXCLi2 concentrations of chicken ileal explants from cell culture medium in response to IDR-1002 and LTA treatment. IDR-low = 10 µg/mL IDR-1002, IDR-medium = 30 µg/mL IDR-1002, IDR-high = 90 µg/mL IDR-1002, LTA = 10 µg/mL lipoteichoic acid from *Staphylococcus aureus* (n = 6/group). Cell cultures in Control group received none of the treatments. * $p<0.05$, ** $p<0.01$

Based on the obtained results LTA alone, and LTA supplemented with 90 µg/mL IDR-1002 (LTA+IDR-high) significantly increased ($p=0.01$, $p=0.041$, respectively) the measured RANTES concentrations compared to the control group. In addition, 10 and 30 µg/mL concentrations of IDR-1002 when administered together with LTA (LTA+IDR-low, LTA+IDR-medium) significantly decreased ($p=0.016$ and $p=0.029$, respectively) the levels of RANTES compared to explants treated with LTA (**Figure 56**).

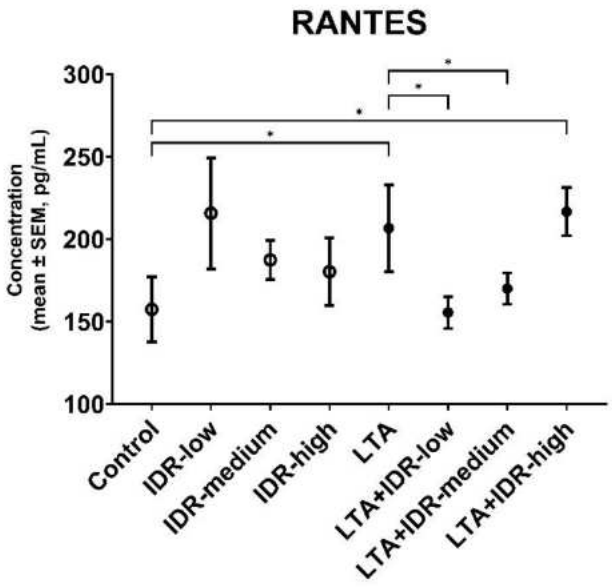


Figure 56. RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) concentrations of chicken ileal explants from cell culture medium in response to IDR-1002 and LTA treatment. IDR-low = 10 µg/mL IDR-1002, IDR-medium = 30 µg/mL IDR-1002, IDR-high = 90 µg/mL IDR-1002, LTA = 10 µg/mL lipoteichoic acid from *Staphylococcus aureus* (n = 6/group). Cell cultures in Control group received none of the treatments. * $p<0.05$

The treatment with LTA significantly increased ($p=0.017$) the concentration of IFN-γ produced by the explants compared to the control group, as well as all combination groups showed elevated IFN-γ concentrations (LTA+IDR-low, LTA+IDR-medium, LTA+IDR-high; $p=0.004$, $p=0.005$, $p=0.005$, respectively). Furthermore, a significant elevation ($p=0.009$) was observed when the LTA-exposed group was supplemented with IDR-1002 treatment at a concentration of 90 µg/mL (LTA+IDR-high) compared to the solely LTA-treated group ($p=0.019$; **Figure 57**).

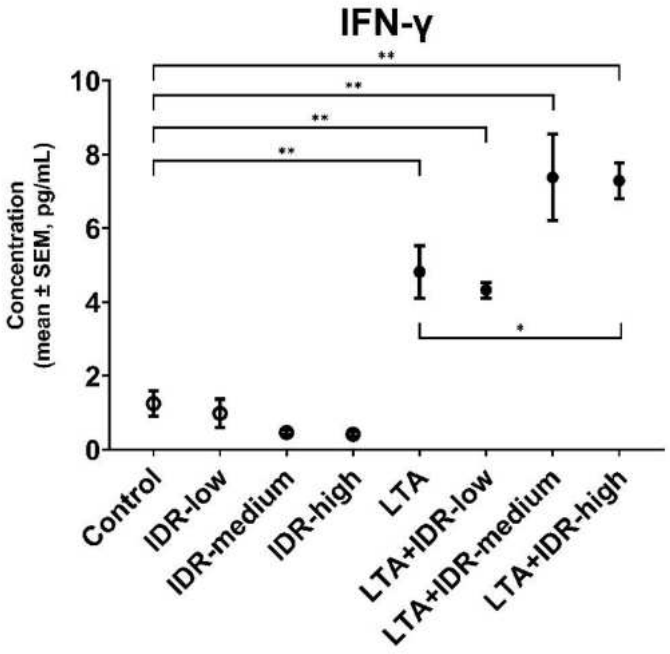


Figure 57. Interferon (IFN)-γ concentrations of chicken ileal explants from cell culture medium in response to IDR-1002 and LTA treatment. IDR-low = 10 µg/mL IDR-1002, IDR-medium = 30 µg/mL IDR-1002, IDR-high = 90 µg/mL IDR-1002, LTA = 10 µg/mL lipoteichoic acid from *Staphylococcus aureus* (n = 6/group). Cell cultures in Control group received none of the treatments. * $p<0.05$, ** $p<0.01$

IL-10 concentrations were decreased after treatment with LTA and 10 µg/mL IDR-1002 (LTA+IDR-low; p=0.011) and increased after treatment with LTA and 90 µg/mL of IDR-1002 (LTA+IDR-high; p=0.005) compared to control. Furthermore, supplementation of LTA with 90 µg/mL of IDR-1002 (LTA+IDR-high) elevated IL-10 concentrations compared to the LTA group (p=0.007; **Figure 58**).

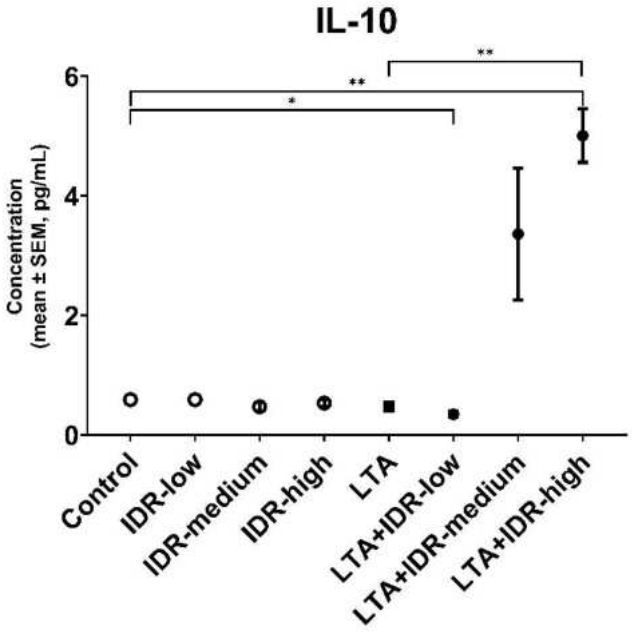


Figure 58. Interleukin (IL)-10 concentrations of chicken ileal explants from cell culture medium in response to IDR-1002 and LTA treatment. IDR-low = 10 µg/mL IDR-1002, IDR-medium = 30 µg/mL IDR-1002, IDR-high = 90 µg/mL IDR-1002, LTA = 10 µg/mL lipoteichoic acid from *Staphylococcus aureus* (n = 6/group). Cell cultures in Control group received none of the treatments. * $p<0.05$, ** $p<0.01$

The ratio of IFN-γ/IL-10 was elevated in the LTA-treated group (p=0.005) and also in the LTA group supplemented with 10 µg/mL IDR-1002 (LTA+IDR-low; p=0.011, p=0.007, respectively). The group with LTA and 90 µg/mL IDR-1002 (LTA+IDR-high) decreased the ratio compared to the LTA group (p=0.008; **Figure 59**).

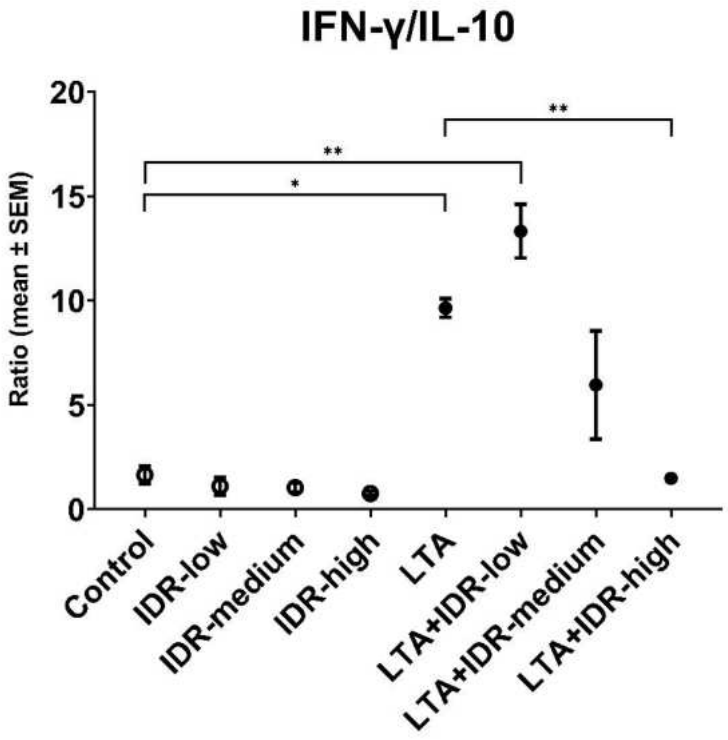


Figure 59. Interferon (IFN)-γ/Interleukin (IL)-10 ratio of chicken ileal explants from cell culture medium in response to IDR-1002 and LTA treatment. IDR-low = 10 µg/mL IDR-1002, IDR-medium = 30 µg/mL IDR-1002, IDR-high = 90 µg/mL IDR-1002, LTA = 10 µg/mL lipoteichoic acid from *Staphylococcus aureus* (n = 6/group). Cell cultures in Control group received none of the treatments. * $p<0.05$, ** $p<0.01$

Furthermore, our results show that extracellular H_2O_2 levels were decreased in groups containing 30 µg/mL IDR-1002 (IDR-medium), LTA, or LTA and 90 µg/mL IDR-1002 together (LTA+IDR-high; p=0.016, p=0.019, p=0.016, respectively; **Figure 60**).

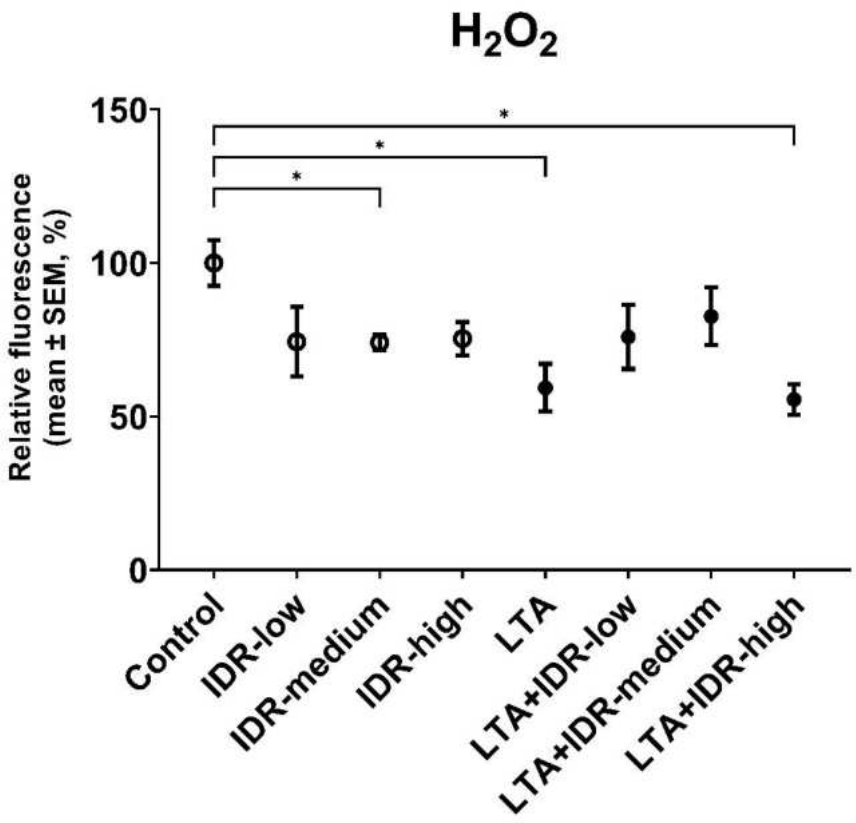


Figure 60. Extracellular H_2O_2 levels of chicken ileal explants from cell culture medium in response to IDR-1002 and LTA treatment. IDR-low = 10 µg/mL IDR-1002, IDR-medium = 30 µg/mL IDR-1002, IDR-high = 90 µg/mL IDR-1002, LTA = 10 µg/mL lipoteichoic acid from *Staphylococcus aureus* (n = 6/group). Cell cultures in Control group received none of the treatments. Results are displayed as percentage, where 100% is the mean value of control cultures. * $p<0.05$

Study 5. The effects of AMPs on the inflammatory and oxidative stress response of hepatic and intestinal cell cultures

Study 5.1. Preliminary study with pro-inflammatory compounds (LPS, LTA, flagellin, poly I:C)

Concerning the porcine hepatic cell cultures, the metabolic activity of the cells was significantly reduced by the higher level of poly I:C exposure ($P<0.001$), while the other treatments possessed no modulatory effects. The extracellular LDH activity was significantly increased by all treatments ($P<0.001$ in all cases) except for flagellin (data not shown).

The IL-4 concentration of the culture medium was elevated by both LPS treatments ($P<0.001$) and by the lower dose of LTA (10 µg/ml) (**Figure 61.A**). Further, the extracellular IL-6 level was remarkably stimulated by all pro-inflammatory candidates except for flagellin ($P<0.001$ in all cases, **Figure 61.B**).

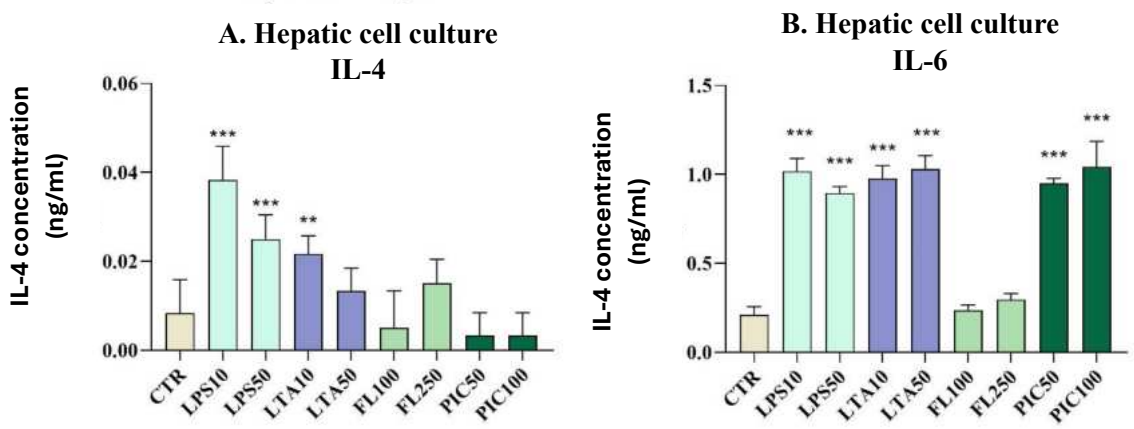


Figure 61. The concentration of IL-4 (**A**) and IL-6 (**B**) in the culture media of porcine hepatocyte mono-cultures after exposure to pro-inflammatory candidates. LPS10 = 10 μg/ml, LPS50 = 50 μg/ml *E. coli* lipopolysaccharide, respectively; LTA10 = 10 μg/ml, LTA50 = 50 μg/ml *Staphylococcus aureus* lipoteichoic acid, respectively; FL100 = 100 ng/ml, FL250 = 250 ng/ml *Salmonella* Typhimurium flagellin, respectively; PIC50 = 50 μg/ml, PIC100 = 100 μg/ml polyinosinic-polycytidylic acid, respectively. Mean (n =6/group) ± SEM. **P<0.01, ***P<0.001

The concentration of IL-8 was significantly elevated by both the lower and higher concentrations of LPS (P=0.039 and P<0.001, respectively) and by both LTA exposures (P=0.014 and P=0.017, respectively) (**Figure 62.A**). In contrast, IFN-γ levels were not increased by any exposures; however, significant reduction was observed following the higher flagellin and both poly I:C treatments (P<0.001 in all three cases) (**Figure 62.B**).

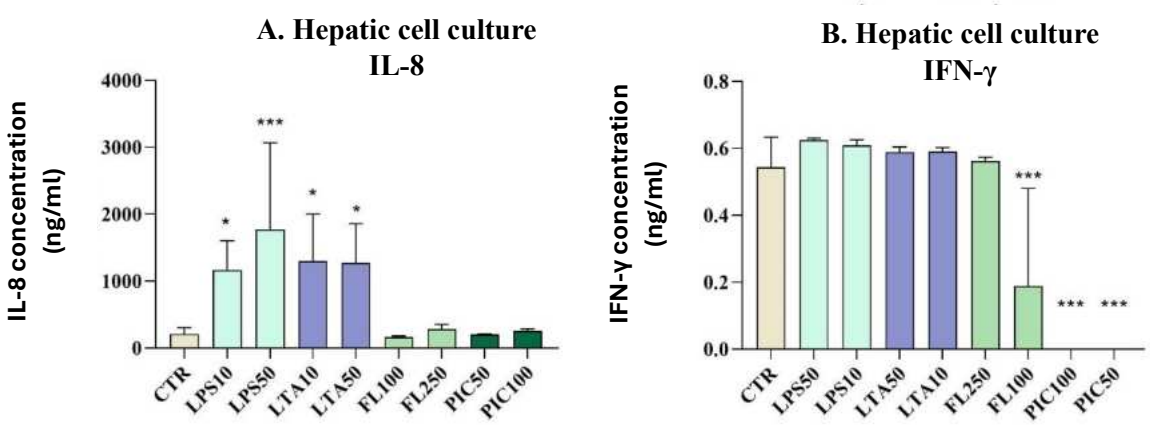


Figure 62. The concentration of IL-8 (**A**) and IFN-γ (**B**) in the culture media of porcine hepatocyte mono-cultures after exposure to pro-inflammatory candidates. LPS10 = 10 μg/ml, LPS50 = 50 μg/ml *E. coli* lipopolysaccharide, respectively; LTA10 = 10 μg/ml, LTA50 = 50 μg/ml *Staphylococcus aureus* lipoteichoic acid, respectively; FL100 = 100 ng/ml, FL250 = 250 ng/ml *Salmonella* Typhimurium

flagellin, respectively; PIC50 = 50 µg/ml, PIC100 = 100 µg/ml polyinosinic-polycytidylic acid, respectively. Mean (n =6/group) ± SEM. *P<0.05, ***P<0.001

The concentration of TNF-α was significantly elevated by all LPS and LTA treatments (P=0.001 for 10 µg/ml LTA and P<0.001 in all further cases), while it was not affected by flagellin and poly I:C (**Figure 63.A**). Further, GM-CSF level was decreased by both poly I:C exposures (P=0.006 for 50 µg/ml and P<0.001 for 100 µg/ml, respectively) (**Figure 63.B**).

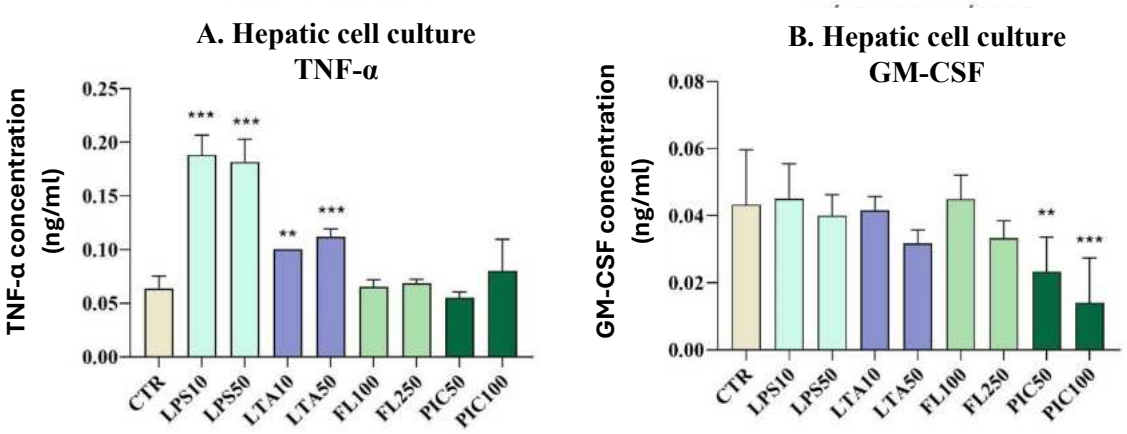


Figure 63. The concentration of TNF-α (**A**) and GM-CSF (**B**) in the culture media of porcine hepatocyte mono-cultures after exposure to pro-inflammatory candidates. LPS10 = 10 µg/ml, LPS50 = 50 µg/ml *E. coli* lipopolysaccharide, respectively; LTA10 = 10 µg/ml, LTA50 = 50 µg/ml *Staphylococcus aureus* lipoteichoic acid, respectively; FL100 = 100 ng/ml, FL250 = 250 ng/ml *Salmonella* Typhimurium flagellin, respectively; PIC50 = 50 µg/ml, PIC100 = 100 µg/ml polyinosinic-polycytidylic acid, respectively. Mean (n =6/group) ± SEM. **P<0.01, ***P<0.001

Regarding the inflammatory response of small intestinal explants, IL-6 concentration was significantly elevated following both doses of poly I:C exposure (P<0.001) and the lower dose of flagellin (P=0.033) (**Figure 64.A**). The IL-8 level of culture media was increased by both LPS concentrations (P=0.011 and P<0.001 for 10 and 50 µg/ml, respectively) (**Figure 64.B**). The concentrations of the other cytokines assessed were below the level of detection.

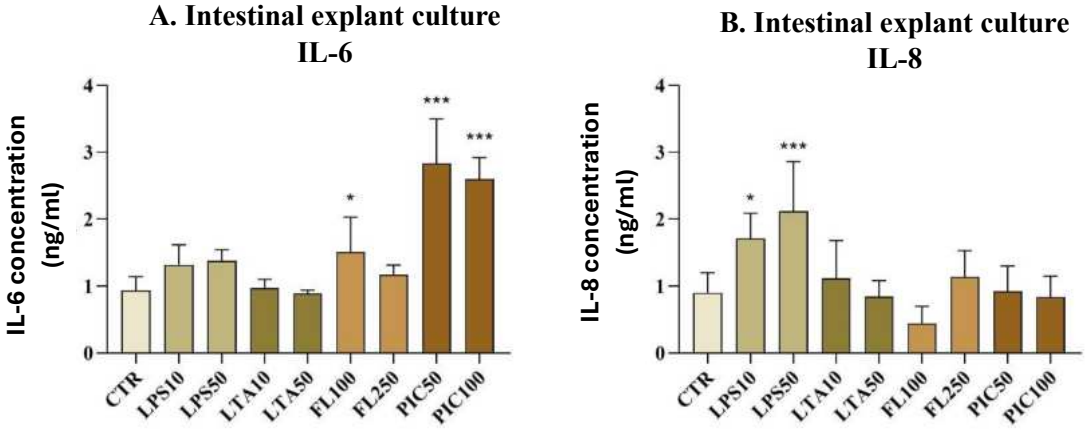


Figure 64. The concentration of IL-6 (**A**) and IL-8 (**B**) in the culture media of porcine small intestinal explant cultures after exposure to pro-inflammatory candidates. LPS10 = 10 μg/ml, LPS50 = 50 μg/ml *E. coli* lipopolysaccharide, respectively; LTA10 = 10 μg/ml, LTA50 = 50 μg/ml *Staphylococcus aureus* lipoteichoic acid, respectively; FL100 = 100 ng/ml, FL250 = 250 ng/ml *Salmonella* Typhimurium flagellin, respectively; PIC50 = 50 μg/ml, PIC100 = 100 μg/ml polyinosinic-polycytidylic acid, respectively. Mean (n =6/group) ± SEM. **P<0.01, ***P<0.001

Study 5.2. Testing the effects of IDR-1002

Based on the metabolic activity and extracellular LDH activity results, all three applied concentrations of IDR-1002 showed no cytotoxicity when applied on porcine hepatic cell cultures. The assessment of various cytokine concentrations (GM-CSF, IL-1β, IL-4, IL-6, IL-8, TNF-α) revealed that the level of most pro-inflammatory cytokines was elevated by LPS exposure. However, IDR-1002 could not alleviate the LPS-triggered cytokine release, but it had a further, concentration-dependent increasing action in combination with LPS. The data of this study are still under evaluation, the detailed results will be published soon.

4. Conclusions

To monitor the immunomodulatory action of certain AMPs on the applied primary cell cultures, it was essential to assess the pro-inflammatory action of certain candidates to establish hepatic and intestinal inflammatory models of chicken and swine origin. Based on the results obtained, it was concluded that LTA, PMA and poly I:C were suitable candidates to induce inflammatory response in the chicken liver cell cultures developed by our research group, and they can be used in future studies involving immunomodulatory agents. In case of chicken intestinal explants, LTA, poly I:C and flagellin were found to be effective in triggering inflammatory response.

Regarding the assessment of the pro-inflammatory action of various candidates on the applied porcine cell cultures, LPS, LTA and poly I:C possessed remarkable increasing action on the cytokine levels measured in hepatic cultures, while LPS and poly I:C were found to evoke inflammation in the intestinal explants, confirming their possible application in inflammatory models. It should be stressed that the LPS-provoked inflammatory response of both the liver and intestinal cells was absent in chicken, while it elicited a robust pro-inflammatory cytokine release in porcine cells.

Based on our trials studying the immunomodulatory role of different AMPs, it can be stated that cathelicidin-2 plays a substantial role in modulating the hepatic immune response with a multifaceted mode of action. It was found to have dose-dependent effects on metabolic activity, membrane integrity and ROS production, indicating that using it in excessively high concentrations can lead to cell damage. However, the lower applied dose was not found to elicit any remarkable deteriorative action on cultured liver cells. Our findings give evidence that this molecule can possess anti-inflammatory properties, reflected by the alleviation of the LTA-triggered IFN-γ surge, and as a potent immunomodulator it can also stimulate pro-inflammatory CXCLi2 release balanced by enhanced anti-inflammatory IL-10 production. Further, the complex interplay of endotoxins and AMPs was highlighted as cathelicidin-2 showed less pronounced effects in the presence of LTA due to its binding capability, also neutralizing the endotoxin-associated inflammatory response. In conclusion, cathelicidin-2 seems to be a promising candidate for future immunomodulating drug development with an attempt to reduce the application of antibiotics.

According to our further studies, IDR-1002 has a complex effect on the regulation of the immune system of the chicken liver. It showed to reduce the LTA-induced pro-inflammatory response (as seen for IFN-γ, IL-6, CXCLi2 and IL-16), suggesting that it may be a promising candidate for attenuating pathological inflammatory processes. Moreover, the measured

parameters suggested that macrophage activity was modulated to an intermediate state between anti- and pro-inflammatory forms, as IDR-1002 reduced both the concentration of pro-inflammatory cytokines (IFN-γ, IL-6) and the anti-inflammatory IL-10. This effect can be well explained by the increase in the level of RANTES (redirecting macrophages towards the pro-inflammatory M1 type) and M-CSF (stimulating differentiation towards the anti-inflammatory M2 type). In addition, we can conclude that IDR-1002 can also affect the oxidative status of cells by reducing the levels of extracellular H_2O_2, presumably due to its inductive effect on the Nrf2 signaling pathway. It was also able to attenuate LTA-induced H_2O_2 production accompanied by Nrf2 induction. In contrast, IDR-1002 did not elicit anti-inflammatory action in porcine hepatic cell cultures, but it was found to increase the LPS-triggered pro-inflammatory cytokine peak. Thus, it can be stated that IDR-1002 has a highly complex effect on the cellular immune response and, although further extensive studies are undoubtedly needed to elucidate its exact mechanism, it may be a promising candidate for the treatment of pathologies associated with inflammation of bacterial origin.

According to our results, cecropin A seems to have no harmful effects on the viability of hepatic cells when applied at its lower concentrations; however, the use of its higher concentrations might result in cell membrane damage. It can also be stated that cecropin A possesses a multifaceted impact on the host cells' immune response, as it was able to influence the levels of IL-6, IL-8, IFN-γ, IL-10, and TGF-ß1. Even though, based on our results, cecropin A cannot be considered purely anti-inflammatory, it is suggested to maintain the hepatic inflammatory homeostasis in Poly I:C-triggered immune response. In addition, the examination of the effects of cecropin A on the cellular redox state showed that the oxidative parameters were not affected in most cases of cecropin A exposure, even so, further studies are required to understand its action. To conclude, cecropin A offers more than a simple antibacterial effect, and it might be a promising candidate for the future design and development of antimicrobial agents, thereby contributing to the reduction of the use of conventional antibiotics and antibiotic resistance.

Based on our further trials, PAP12-6 seemed to exert no cytotoxic effects on chicken hepatic cells, suggesting its safe application in poultry. Moreover, PAP12-6 displayed a robust modulatory activity on the immune response as it was able to decrease the levels of IL-6, IL-8, and RANTES, as well as the IL-6/IL-10 ratio. Therefore, the peptide is suggested to provide beneficial effects to the host in Poly I:C-triggered and LTA-induced inflammatory conditions. Furthermore, the examination of the activity of PAP12-6 on extracellular H_2O_2 and Nrf2 levels showed that this AMP might act as an antioxidant and promote the elimination of ROS. Based

on our results, PAP12-6 possesses a highly potent immunomodulatory property, and it might be a promising candidate for replacing antibiotics, thereby contributing to the reduction of AMR in the future.

Based on the data gained from chicken intestinal cultures, cathelicidin-2 has a broad anti-inflammatory effect on chicken ileal explants as reflected by the alleviation of the LTA-triggered pro-inflammatory cytokine release, mostly at higher concentrations, without being cytotoxic. Moreover, the immunostimulant action of this AMP could also be observed, predominantly when administered to non-inflamed cells, which can prove to be an efficient mechanism to provide protection against infections. Therefore, cathelicidin-2 can be considered a suitable candidate for the treatment of bacterial diseases by maintaining the appropriate inflammatory homeostasis and with further *in vivo* studies may significantly contribute to the reduction of antibiotic use for enteric infections.

On the same model, IDR-1002 did not possess any negative effect on the viability of the explant models, it had a major impact on inflammatory processes. Our results propose that there are significant differences between the three concentrations considered here, suggesting that the effect of the peptide is not negligibly dependent on the dose used. Considering this, further studies are certainly needed to explore the differences between the different concentrations and to select the most optimal one. Nevertheless, it can be stated that the IDR-1002 AMP presented a potent immunomodulatory action on intestinal explants. The peptide alone enhanced the innate defense mechanisms by stimulating IL-2 and CXCLi2 production, while it alleviated the LTA-evoked pro-inflammatory cytokine peak (reflected by IL-2, CXCLi2, RANTES concentrations, and by the IFN-γ/IL-10 ratio), thus restoring the physiological inflammatory homeostasis. These data suggest that IDR-1002 might be a promising candidate in the multimodal treatment of inflammation caused by bacterial infections of intestinal origin in chicken.

Following the trials concerning the aforementioned AMPs, some plant-derived bioactive compounds as potential natural alternatives of antibiotics were also included in the project. These data pinpoint the antiviral and cell protectant nature of chicoric acid, a bioactive molecule of *Echinacea* spp. and roadside plants, like dandelion and chicory, as it had a robust and uniform anti-inflammatory effect on a broad scale of cytokines at the protein level in the present chicken primary hepatocyte – NP cell co-cultures induced with poly I:C. The antiviral properties of the molecule are hypothesized to be equally connected to its cell protectant nature, which manifested both with and without inflammatory stimuli, reflected by the metabolic activity and extracellular LDH activity alike. Bibliographic data and the present results suggest that chicoric

acid might alleviate the damage caused by viruses, improving poultry health and production. On the other hand, N-acetylcysteine failed to mitigate poly I:C induced cell damage but exhibited anti-inflammatory activity on the level of IL-6, IL-8, IL-10, IFN-α and IFN-γ genuinely under poly I:C induced inflammation.

Further, the applied dose of flagellin (250 ng/ml) was not cytotoxic, but it could provoke inflammation as indicated by the increased cellular IL-8 and decreased IL-10 production and by the elevated IFN-γ/IL-10 ratio, which were effectively restored by luteolin at the dose of 4 μg/ml. Besides its anti-inflammatory action, luteolin was also capable of remarkably decreasing the extracellular H_2O_2 and MDA concentrations indicative of oxidative stress and lipid peroxidation. Based on these data, it can be suggested that luteolin might be a potential natural candidate to maintain the physiological inflammatory and redox homeostasis of the liver in chicken, possibly mitigating the destructive action of flagellin-associated inflammation caused by enteric bacterial infection. Hence, the administration of luteolin or some other flavonoids might be promising tool to improve animal health and to reduce antibiotic application in poultry farming, which should be also addressed by further in vivo studies.

Beside studying the immunomodulatory role of different AMPs and plant-derived compounds, the cellular effects of mycotoxins and those of potential protective agents were also tested. T-2 toxin decreased the metabolic activity of cells and increased the extent of cell membrane damage, therefore had a negative effect on the viability of liver cells. Our results suggest that T-2 toxin may also alter the cellular oxidative homeostasis through the promotion of ROS production as well as the regulation of the Nrf2 transcription factor and may influence immune function including the release of pro-inflammatory cytokines such as IL-6 and IL-8. According to our results, lipid and amino acid metabolism were also affected by the toxin leading to remarkable alterations in the metabolome of cell cultures. In conclusion, several cellular processes, such as the inflammatory and oxidative stress response or the metabolic profile of hepatic spheroids were modulated by T-2 toxin exposure, and investigating the complex interactions of these processes is a good basis for future studies on the mechanism of action of T-2 toxin. Furthermore, our applied cell culture model can serve as a promising tool for the investigation of various further mycotoxins as well as other toxic agents in the future.

Furthermore, our results show that DON reduced cellular metabolic activity, but this effect was not found to be cytotoxic. Baicalin, on the other hand, had a positive influence on the viability of the cells and was able to counteract the negative effects of DON. The adverse effects of DON were probably mediated through the induction of oxidative stress, but baicalin was also able to alleviate this. The dual impact of DON on cellular immunity was also observed,

as well as a known anti-inflammatory influence of baicalin. However, a possible prooxidant activity of baicalin was also found after prolonged exposure. Furthermore, after 48 h, the cells adapted to the treatments, and defence mechanisms were triggered in order to restore cellular homeostasis. All this suggests that DON can affect cellular oxidative homeostasis and inflammatory processes, and baicalin may serve as a potential protective agent against these effects. However, it is also worth further investigation to understand the exact prooxidant activity of baicalin.

In conclusion, our studies demonstrate the diversified effects of AMPs, while showing their dependence on different biological factors. It can be concluded that a more substantial understanding of the cellular effects of AMPs is necessary before they can be introduced into clinical research. Our results can contribute to this goal considerably by providing valuable data on cell viability and immune response provoked by four important peptides, the cathelicidin-2, IDR-1002, cecropin A and Pap12-6 in hepatic and intestinal *in vitro* model systems.

As the resistance of infectious agents to the available chemotherapic drugs is getting increasingly serious, new ways have to be developed to combat these diseases. AMPs might provide an option to augment the use of antibiotics due to their wide range of effects on the immune system. AMPs show that utilizing the organism's own immune system to achieve this goal is an interesting approach in this search. While using the innate competence of the immune system to provide a possible noble solution to kill pathogenic bacteria, they have a wide range of abilities to combat harmful inflammation and to help the body regenerate more swiftly.

We hope that studies such as ours can provide invaluable basis for future research in this interesting field.

5. List of papers published in the frame of the project

Bibliographic data of the paper	Type	IF
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