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

Ph.D. thesis

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1 List of abbreviations

Abbreviation	Full Term
PI3K/Akt	Phosphoinositide 3-kinase/protein kinase B pathway
AMPK	AMP-activated protein kinase
AMR	Antimicrobial resistance
ATP	Adenosine triphosphate
CA	Chicoric acid
CCK-8	Cell Counting Kit-8
CD	Cluster of differentiation protein
CME	*Cordyceps militaris* extract
COX-2	Cyclooxygenase-2
CpG	Cytosine and guanine dinucleotide
DAMP	Damage-associated molecular pattern
DMEM-F12	Dulbecco's Minimal Essential Medium-F12
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
DSS	Dextran sodium sulfate
EGTA	Ethylene glycol tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
GALT	Gut-associated lymphoid tissue
H_2O_2	Hydrogen peroxide
H&E	Hematoxylin and eosin
HBSS	Hanks' Balanced Salt Solution
HO-1	Heme oxygenase-1
HOCl	Hypochlorous acid
IBD	Infectious bursal disease
ICAM-1	Intercellular adhesion molecule 1
IFN-α	Interferon alpha
IFN-γ	Interferon gamma
IL	Interleukin-(1β, 2, 6, 8, 18)
iNOS	Inducible nitric oxide synthase
IκB	Inhibitor of kappa B
JNK	c-Jun N-terminal kinase
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide

LTA	Lipoteichoic acid
M-CSF	Macrophage colony-stimulating factor
M-PER	Mammalian Protein Extraction Reagent
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial antiviral-signaling protein
MDA	Malondialdehyde
MyD88	Myeloid differentiation primary response 88
NAC	N-acetylcysteine
NAD(P)H+H$^+$	Reduced nicotinamide adenine dinucleotide
NADH+H$^+$	Reduced nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa B
NK	Natural killer (cell)
NLR	Nucleotide-binding oligomerization domain-like receptor
NLRP3	NLR family pyrin domain-containing 3
NOX	NADPH oxidase
Nrf2/Keap1	Nuclear factor erythroid 2–related factor 2/Kelch-like ECH-associated protein 1 pathway
PAMP	Pathogen-associated molecular pattern
poly I:C	Polyinosinic-polycytidylic acid
PRR	Pattern recognition receptor
PGE_2	Prostaglandin E_2
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSS	Runting-stunting syndrome
SD	Standard deviation
SEM	Standard error of mean
SOD	Superoxide dismutase
ssRNA	single-stranded ribonucleic acid
TGF-β	Transforming growth factor-beta
Th17	T helper 17
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TRIF	TIR-domain-containing adapter-inducing interferon beta
WM	Williams' Medium E

2 Summary

Since the use of antibiotics as growth promoters was banned in the European Union on 1 January 2006, veterinary medicine desperately needs effective antibiotic alternatives. There is a focus on prevention, good husbandry, hygiene and the use of alternative growth promoters to increase production and maintain animal health. Extensive scientific research is being carried out on the subject to tackle antimicrobial resistance, which is one of the most important current public health concerns. Poultry farming, the most intensive sector of animal production, urgently needs to identify potential antibiotic alternatives to meet growing consumption demands and reduce its significant contribution to the issue of antibiotic resistance.

Broiler chickens, characterized by an immature immune system and high stocking densities, are predisposed to the development of runting-stunting syndrome (RSS), dysbacteriosis, necrotic enteritis and further gastrointestinal diseases. Disruption of the intestinal integrity would lead to leakage of microbial toxins and byproducts through the epithelial barrier, leading to inflammation and remarkably repressing weight gain and thus productivity in poultry. Modeling these pathologies *in vitro* is relevant to finding potential molecules to counteract hepatic inflammatory and oxidative damage induced by gut-originated pathogen load. The gut-liver axis serves as the primary barrier for the gut-derived antigenic load. It protects the systemic circulation against both residual oxidative and pathogen burden originating from the gastrointestinal tract. Reactive oxygen species (ROS) are common byproducts of normal cellular metabolism. Therefore, numerous enzymes and molecules are incorporated in a rather intricate system protecting the redox status of a vital somatic cell. Concerning the whole organism, the gut mucous membrane barrier and the liver are also the key elements of the defense against oxidative stimuli. Nonetheless, the gut-liver axis exhibits a limited capacity against prooxidants and is particularly vulnerable to inflammation or oxidative stress provoked by pathogens. The goal of the present study was to model the complex inflammatory and oxidative responses triggered by bacteria and viruses *in vitro* using a chicken primary hepatocyte – non-parenchymal cell co-cultures and a novelly established and characterized miniature chicken ileal explant culture. The aim of the present thesis is to highlight the peculiar interactions between inflammation, oxidative stress, and cell damage caused by pathogen-associated molecular patterns (PAMPs), modeling the invasion of specific pathogens. Moreover, the present research was conducted with special attention to secondary

metabolites of plants or fungi with immunomodulatory, cytoprotective and antioxidant activity, although unexplored or unfamiliar to the sector. Specifically, chicoric acid (CA), a phenolic compound, and luteolin, a flavonoid, both present in leafy greens; and cordycepin, a nucleoside analog found in caterpillar fungi (*Ophiocordyceps sinensis* and *Cordyceps militaris*) of Asian folk medicine.

The primary aim of the author was to identify PAMPs that could induce inflammation on the chicken primary hepatocyte – non-parenchymal cell co-cultures previously characterized by our team. First of all, two PAMPs were tested against lipopolysaccharide (LPS) for their ability to elevate pro-inflammatory cytokine levels and to assess their cytotoxicity. Based on these results, flagellin and polyinosinic-polycytidylic acid (poly I:C) were deemed suitable. *In vitro* models of virus-induced inflammation are less common; therefore, the author favored poly I:C as a double-stranded viral ribonucleic acid (RNA) analog. In the other preliminary study, the authors aimed to compare the morphology and viability of miniature chicken gut explant cultures, obtained using a biopsy punch, to examine size-dependent changes over time. To verify the applicability of the model, the PAMPs - flagellin, lipoteichoic acid (LTA), and poly I:C - were applied to induce inflammation.

To counteract inflammation, oxidative stress and cell damage on the examined models, the effects of CA and luteolin were investigated in the chicken primary hepatocyte – non-parenchymal cell co-cultures, under inflammation induced by poly I:C and flagellin, respectively. Meanwhile, on the ileal explant model, the effects of a *Cordyceps militaris* extract, rich in cordycepin, were assessed under poly I:C exposure. The concept of this work was to develop simplified models for studying specific infectious diseases of chicken in each of the definitive studies. Viral hepatitis (caused by reovirus or other viruses inducing a prominent interferon response), Gram-negative septicemia and Salmonellosis-induced hepatitis and virus-induced ileal inflammation (similar to runting-stunting syndrome) may be modeled in the present thesis.

The parameter of cellular metabolic activity was assessed with the CCK-8 test, and membrane damage was monitored with the lactate dehydrogenase (LDH) leakage assay. Interleukin-6 and -8 (IL-6 and -8) concentrations were measured in cell culture medium with a chicken-specific ELISA or with Luminex xMAP method with further cytokines in some of the experiments.

The first study focused on the potential pro-inflammatory effects of flagellin and poly I:C in the chicken primary hepatocyte – non-parenchymal cell co-culture model. Poly I:C reduced metabolic activity and based on a moderate increase in extracellular LDH activity,

appeared to compromise cell membrane integrity. Both flagellin at its higher applied (250 ng/mL) and poly I:C in its lower applied concentration (50 µg/mL) demonstrated pro-inflammatory effects, as evidenced by elevated cellular IL-6 and in case of poly I:C also IL-8 release. These findings suggest that flagellin and poly I:C are potent candidates for inducing inflammation in chicken primary hepatic cell cultures. In contrast, LPS failed to stimulate pro-inflammatory cytokine production, possibly indicating the relatively high tolerance of avian liver cells to certain bacterial endotoxins.

The purpose of the subsequent study was to induce inflammation with the viral double-stranded ribonucleic acid (dsRNA) analog poly I:C on chicken primary hepatocyte – non-parenchymal cell co-cultures to investigate the immunomodulatory and cell protectant effects of CA in comparison to a reference drug, N-acetylcysteine (NAC). Poly I:C significantly elevated the activity of the cell damage marker, lactate dehydrogenase (LDH) and the concentration of inflammatory cytokines [IL-6, IL-8, interferon-α, γ (IFN-α, IFN-γ) and macrophage colony-stimulating factor (M-CSF)] in the culture medium and decreased cellular metabolic activity. CA significantly reduced the elevated LDH and cytokine levels in a dose-dependent manner, moreover, the higher (100 µg/mL) concentration of CA even elevated the level of metabolic activity. In contrast, 10 µg/mL NAC treatment decreased the level of each inflammatory cytokine but did not rectify cell damage or metabolic depression. Moreover, CA applied in 10 µg/mL in combination with poly I:C diminished the level of a marker of lipid peroxidation, malondialdehyde (MDA). The results indicate that CA, present in common plants of the Asteraceae family, proves to be a beneficial hepatoprotective, along with NAC, an immunomodulatory supplement *in vitro* under a stimulus mimicking viral infection.

The following study was aimed to investigate the pro-inflammatory effect of flagellin released from the bacterial flagellum of *Salmonella enterica* serovar Typhimurium and to attenuate the induced inflammation with luteolin as a plant-derived flavonoid on chicken primary hepatocyte – non-parenchymal cell co-cultures. Cells were cultured in a medium supplemented with 250 ng/mL flagellin and 4 or 16 µg/mL luteolin for 24 h. Cellular metabolic activity, LDH activity, IL-6, IL-8, IL-10, IFN-α, IFN-γ, hydrogen peroxide (H_2O_2) and MDA concentrations were determined. Flagellin significantly increased the concentration of the pro-inflammatory cytokine IL-8 and the ratio of IFN-γ/IL-10, while it decreased the level of IL-10, indicating that the model was satisfactory to study inflammation *in vitro*. Luteolin treatment at 4 µg/mL did not exhibit cytotoxicity, as reflected by metabolic activity and extracellular LDH activity, and significantly reduced the flagellin-triggered IL-8 release of the cultured cells. Further, it had a diminishing effect on the concentration of IFN-α, H_2O_2 and MDA and restored

the level of IL-10 and the ratio of IFN-γ/IL-10 when applied in combination with flagellin. These results suggest that luteolin at modest concentrations may protect hepatic cells from an inflammatory response and act as an antioxidant to attenuate oxidative damage.

The morphology and viability of miniature chicken gut explant cultures, in a previously unreported way, obtained with a biopsy punch, were examined to assess the size-dependent change over time in the second preliminary study. To confirm the applicability of the model, the following PAMPs were applied to induce inflammation. The previously described poly I:C and flagellin, as well as a PAMP, originated from the Gram-positive bacterial cell wall, lipoteichoic acid (LTA). The 2 mm diameter explants showed a decrease in metabolic activity measured by CCK-8 assay after 12 h and a significantly higher LDH activity, indicating cellular damage compared to the 1 mm explants, supported by histological differences after 24 h of culturing. After 12 h of incubation, the 1.5 mm explants selected by compromise retained columnar epithelial lining with moderate damage of the *lamina propria* (H&E and pan-cytokeratin staining). Exposure to 100 μg/mL poly I:C reduced the metabolic activity of the 1.5 mm explants. LTA and poly I:C increased IFN-γ concentration at all the applied concentrations further IFN-α and IL-6 concentration were elevated by 50 μg/mL and RANTES by 100 μg/ml poly I:C treatment. Flagellin administration in 100 ng/ml raised IL-6 and IL-10, while 250 ng/ml increased the concentration of IL-10 and RANTES. 10 μg/mL LTA exposure was the treatment solely inducing IL-2 elevation. IFN-γ/IL-10 ratio, the indicator of the gut inflammatory homeostasis was increased by the higher LTA and both poly I:C concentrations. According to the observations, the viability and integrity of the explants decreased with their size. Therefore, the 1.5 mm diameter miniature chicken ileal explants stimulated with the employed PAMPs and maintained for 12 h can be appropriate for mimicking *ex vivo* chicken infectious diseases involving tissue damage and inflammation.

Eventually, the effect of a dextrose-formulated *Cordyceps militaris* extract (CME) containing the active metabolite, cordycepin, administered at concentrations of 0.6% and 0.12% (w/w) in the culture medium, was assessed in intestinal explant cultures over a 12 h treatment period. The inflammatory response was induced using poly I:C at a 50 μg/mL concentration. After 12 h, the viability of the cultures was assessed, and samples were collected from the culture medium to measure LDH activity, along with the inflammatory cytokines IL-1β, IL-6, IFN-γ, and RANTES. Based on the results obtained, the CME demonstrated significant anti-inflammatory effects. This was evident even without an inflammatory challenge, as the 0.6% treatment significantly reduced IFN-γ cytokine levels in comparison to the untreated control. Compared to the poly I:C inflammatory control, the 0.6% and 0.12% CME treatments reduced

IFN-γ levels. Concerning RANTES, the 0.12% CME treatment was significantly effective, while for IL-1β, the 0.6% CME treatment exhibited anti-inflammatory properties in the presence of poly I:C. The results support the initial hypothesis that CME could effectively alleviate the inflammation in a chicken gut *ex vivo* model.

Összefoglalás

Az állatgyógyászat szükségszerűen természetes hozamfokozók alkalmazását sürgeti az antibiotikumok ezen célra történő felhasználásának 2006. január 1-jével bevezetett európai uniós tilalma miatt. A hangsúly a megelőzésen, a megfelelő tartáson, a higiénián, valamint alternatív növekedésserkentő készítmények alkalmazásán van annak érdekében, hogy növeljék a termelést és megóvják az állatok egészségét. Számos tudományos kutatás foglalkozik a témával, az antimikrobiális rezisztencia problémája központi kérdéssé vált, ez napjaink egyik legsúlyosabb közegészségügyi problémája. A baromfitartás, mint az állattenyésztés legintenzívebb ágazata, kell hogy nyisson az antibiotikum-alternatívák felé, hogy kielégítse a növekvő fogyasztói igényeket, és csökkentse a részét az antibiotikum-rezisztencia kialakulásában.

A brojlercsirkék éretlen immunrendszerük és a nagy állománysűrűség miatt hajlamosak a satnyaság és törpenövés szindróma [runting-stunting syndrome (RSS)], diszbakteriózis, elhalásos bélgyulladás és egyéb emésztőszervi betegségek kialakulására. A bélfal épségének felbomlása lehetővé teszi, hogy a mikrobák toxinjai és melléktermékei átjussanak a bélhámon, ami gyulladáshoz vezet, jelentősen visszafogva a súlygyarapodást és így a termelékenységet. E kórképek *in vitro* modellezése segíthet olyan potenciális molekulák azonosításában, amelyek képesek kivédeni a bél eredetű kórokozók okozta májgyulladást és az oxidatív stresszt. A bél-máj tengely az elsődleges védelmi vonal a bélből származó antigénterheléssel szemben, és védi a szervezetet a gyomor-bél traktusból származó reaktív vegyület és kórokozó terheléstől. A reaktív oxigén vegyületek [reactive oxygen species (ROS)] az anyagcsere gyakran nagyobb mennyiségben termelődő melléktermékei, melyekkel szemben számos enzim és molekula védi a sejtek redox egyensúlyát. Az élő szervezet egészét tekintve a bél nyálkahártyája és a máj is kulcsfontosságú szerepet tölt be az oxidatív ingerek elleni védekezésben. Ugyanakkor a bél-máj tengely védekezőképessége korlátozott a prooxidánsokkal szemben, érzékeny a kórokozók által kiváltott gyulladásra és oxidatív stresszre.

A jelen tanulmány célja az volt, hogy *in vitro* modellezze a baktériumok és vírusok által kiváltott összetett gyulladásos és oxidatív válaszokat csirke eredetű hepatocita–nem parenchimális sejt kokultúra, valamint egy újonnan kialakított és jellemzett miniatűr csirke ileum explant/explantátum kultúra segítségével. A dolgozat célja továbbá az volt, hogy bemutassa a gyulladás, az oxidatív stressz és a sejtkárosodás közötti sajátos kölcsönhatásokat, amelyeket patogén-asszociált molekuláris mintázatok (PAMP-ok) idéznek elő bizonyos kórokozók kiváltotta gyulladás, illetve sejt és szövetkárosodás esetén. A kutatás különös figyelmet fordított olyan növényi vagy gomba eredetű másodlagos anyagcseretermékek vizsgálatára, amelyek immunmoduláns, sejtvédő és antioxidáns hatással bírnak, de az ágazatban eddig kevéssé ismertek. Ilyen például a cikóriasav (CA), amely egy fenoloid, szerves sav, illetve a luteolin, egy flavonoid, amelyek kétszikű növényeinkben széles körben megtalálható. Továbbá a cordycepin, egy nukleozid-analóg, amely az ázsiai népi gyógyászatban használt hernyógombákban (*Ophiocordyceps sinensis* és *Cordyceps militaris*) fordul elő.

A tudományos munka további célja az volt, hogy azonosítsa azokat a PAMP-okat, amelyek gyulladást képesek kiváltani a korábban csoportunk által jellemzett csirke hepatocita–nem parenchimális sejt kokultúrán. A munka kezdeti szakaszában két PAMP került összehasonlításra az LPS-sel (lipopoliszacharid), hogy gyulladáskeltő hatásukról, illetve citotoxicitásukról pontos ismereteket kapjunk. Az eredmények alapján a flagellin és a poliinozin-policitidilsav (poly I:C) alkalmasnak bizonyultak, mint gyulladáskeltő anyagok. A vírus által kiváltott gyulladás *in vitro* ritkábban modellezett folyamat, ezért a szerző a poly I:C-t részesítette előnyben, mint virális duplaszálú RNS analógot. Egy későbbi módszertani előtanulmányban egy újonnan alkalmazott miniatűr csirke bél explant/explantátum modell került vizsgálatra,, hogy összevethető legyen a szövettani morfológiában és az életképesség változásban megmutatkozó, mérettől függő károsodás az idő előrehaladtával. A modell alkalmazhatóságát a gyulladásos válasz vizsgálatára a flagellin, a lipoteikolsav (LTA) és a poly I:C kezelésekkel sikerült igazolni.

A továbbiakban a CA, illetve a luteolin hatásának vizsgálata történt meg csirke hepatocita–nem parenchimális sejt ko-kultúrán, poly I:C, illetve flagellin által kiváltott gyulladásos válasz mellett. Az utolsó vizsgálat egy cordycepinben gazdag *Cordyceps militaris* kivonat csirke ileum explant/explantátumon való alkalmazása mellett zajlott, míg TLR agonistaként a poly I:C alkalmazása történt meg. Így összegezve az előbbieket a gyakorlat szempontjából egy vírusos májgyulladás és májkárosodás (reovírus vagy más, erős interferon-választ kiváltó vírusok okozta májgyulladás), egy Gram-negatív szeptikémia és *Salmonella*

okozta májgyulladás, valamint egy vírus által kiváltott vékonybélgyulladás (például RSS szindrómában) *in vitro* modellezése történt meg.

A sejtek metabolikus aktivitása CCK-8 teszttel, a membránkárosodás laktát-dehidrogenáz (LDH) aktivitás próbával, az interleukin-6 és -8 (IL-6 és IL-8) koncentrációja pedig csirke-specifikus ELISA-val vagy Luminex xMAP módszerrel került lemérésre, bizonyos kísérletekben további, az egyes szövetek szempontjából jelentős citokinek mellett.

A flagellin és a poly I:C potenciális gyulladáskeltő hatását tanulmányozó vizsgálatban a csirke hepatocita–nem parenchimális sejt kokultúra modellen a poly I:C csökkentette a metabolikus aktivitást, és a mérsékelten megnövekedett extracelluláris LDH-aktivitás alapján károsította a membrán integritását. Mind a flagellin, mind a poly I:C bizonyos koncentrációban gyulladáskeltő hatást mutatott, amit az emelkedett IL-6 koncentráció igazolt. Ezek az eredmények azt mutatják, hogy a flagellin és a poly I:C alkalmasak gyulladás kiváltására csirke primer máj eredetű kokultúrán. Ezzel szemben az LPS nem fokozta a proinflammatorikus citokin termelést, ami arra utal, hogy a madár májsejtek viszonylag jól tolerálnak bizonyos bakteriális endotoxinokat.

A CA hatásait vizsgáló kísérlet célja az volt, hogy a virális kettős szálú RNS analóg poly I:C-vel gyulladást idézzünk elő csirke primer hepatocita–nem parenchimális sejt kokultúrán, és vizsgáljuk a CA immunmoduláns és sejtvédő hatását, összehasonlítva egy referencia hatóanyaggal, az N-acetilciszteinnel (NAC). A poly I:C jelentősen növelte az LDH aktivitását, valamint az IL-6, IL-8, interferon-α és -γ (IFN-α, IFN-γ), illetve a makrofág kolóniastimuláló faktor (M-CSF) koncentrációját a tápfolyadékban, továbbá csökkentette a sejtek metabolikus aktivitását. A CA dózisfüggően csökkentette a megemelkedett LDH- és citokinszinteket, sőt, a magasabb (100 µg/mL) koncentrációban még a metabolikus aktivitást is növelte. Ezzel szemben a 10 µg/mL NAC kezelés minden gyulladásos citokin szintjét csökkentette, de nem mérsékelte a sejtkárosodást és a metabolikus aktivitás csökkenését. Emellett a 10 µg/mL CA a poly I:C-vel kombinált csoportban csökkentette a lipidperoxidációs marker, a malondialdehid (MDA) szintjét is. Az eredmények azt mutatják, hogy a CA gyulladáscsökkentő hatást mutat *in vitro* csirke máj eredetű tenyészetekben hasonlóan az NAC-hoz, azonban a referencia molekula NAC-cal szemben markáns májsejtvédő hatással is bír.

A luteolin hatásának vizsgálata szintén a máj eredetű kokultúrán történt. A kísérlet *Salmonella enterica* Typhimurium csillójából származó flagellin gyulladáskeltő hatásának vizsgálata mellett zajlott a csirke primer hepatocita–nem parenchimális sejt kokultúrán. A sejtek tenyésztése 250 ng/mL flagellint és 4 vagy 16 µg/mL luteolint tartalmazó tápfolyadékban

történt 24 órás kezelési idő mellett. A metabolikus aktivitás, az LDH-aktivitás, az IL-6, IL-8, IL-10, IFN-α, IFN-γ, hidrogén-peroxid (H_2O_2) és MDA koncentrációk kerültek lemérésre. A flagellin jelentősen növelte az IL-8 proinflammatorikus citokin szintjét és az IFN-γ/IL-10 arányt, miközben csökkentette az IL-10 szintjét, igazolva, hogy a modell alkalmas a gyulladásos válasz vizsgálatára *in vitro*. A 4 μg/mL luteolin kezelés nem volt citotoxikus, amit a metabolikus aktivitás és az extracelluláris LDH-aktivitás alakulása is alátámasztott, továbbá jelentősen csökkentette a flagellin által kiváltott IL-8 felszabadulást. Emellett mérsékelte az IFN-α, a H_2O_2 és az MDA koncentrációját, helyreállította az IL-10 szintjét és az IFN-γ/IL-10 arányt flagellinnel kombinálva. Ezek az eredmények arra utalnak, hogy az alacsonyabb koncentrációban alkalmazott luteolin megvédheti a májsejteket a túlzott gyulladásos választól, és segíthet az oxidatív károsodás mérséklésében.

A bél modellen elvégzett előkísérlet célja az volt, hogy a standardizált méretű miniatűr csirke bél explantátumok morfológiáját és életképességét összehasonlítsa, hogy vizsgálhatóak legyenek a méretfüggő változások az idő előrehaladtával. A modell alkalmazhatóságának igazolására a korábban leírt poly I:C és flagellin mellett a lipoteikolsav (LTA) Gram-pozitív bakteriális sejtfalalkotó hatását vizsgáltuk. A 2 mm átmérőjű explantátumok 12 óra után csökkent metabolikus aktivitást és szignifikánsan magasabb LDH-aktivitást mutattak, ami nagyobb sejtkárosodást jelez az 1 mm-es explantátumokhoz képest. Ezt a méretfüggő eltérést a 24 órás tenyésztés utáni szövettani vizsgálat is alátámasztotta. 12 óra inkubáció után az 1,5 mm átmérőjű explantátumok megőrizték a hengerhám borítást, mérsékelt *lamina propria* károsodással (H&E és pancitokeratin festés). A 100 μg/mL poly I:C kezelés csökkentette az 1,5 mm-es explantátumok metabolikus aktivitását. Az LTA és a poly I:C minden alkalmazott koncentrációban növelte az IFN-γ szintjét. Ezen túlmenően az 50 μg/mL poly I:C kezelés emelte az IFN-α és az IL-6 koncentrációját, míg 100 μg/mL poly I:C hatására a RANTES szintje iemelkedett meg. A flagellin 100 ng/mL koncentrációban az IL-6 és az IL-10, míg 250 ng/mL esetén az IL-10 és a RANTES koncentrációját növelte. Az LTA 10 μg/mL dózisban volt az egyetlen kezelés, amely az IL-2 koncentrációra emelő hatással volt. Az IFN-γ/IL-10 arány, amely a bél gyulladásos homeosztázisának indikátor értéke, a magasabb LTA és mindkét poly I:C koncentráció hatására megemelkedett. Az eredmények alapján az explantátumok életképessége és integritása a méret növekedésével csökken. A további vizsgálatokra legalkalmasabbnak így az 1,5 mm átmérőjű miniatűr csirke ileum explantátumokat találtuk, melyek a vizsgált PAMP-okkal stimulálva 12 h tenyésztés mellett szövetkárosodással és gyulladással járó betegségek *ex vivo* modelljei lehetnek.

Egy glükózzal formulázott *Cordyceps militaris* kivonat (CME), amely a cordycepin hatóanyagot is tartalmazza, 0,6% és 0,12% (m/m) koncentrációban került alkalmazásra a bél explantátum kultúrákon 12 órás kezelési idő mellett. A gyulladásos választ 50 µg/mL poly I:C-kiegészítés váltotta ki. 12 óra elteltével a kultúrák életképességét mértük, valamint mintákat gyűjtöttünk a tenyésztő közegből az LDH-aktivitás, illetve az IL-1β, IL-6, IFN-γ és RANTES citokinek meghatározására. Az eredmények alapján a CME jelentős gyulladáscsökkentő hatást mutatott. Ez már gyulladásos stimulus nélkül is megfigyelhető volt, mivel a 0,6%-os kezelés szignifikánsan csökkentette az IFN-γ citokin szintjét a kezeletlen kontrollhoz képest is. A gyulladásos kontrollhoz viszonyítva is mind a 0,6%, mind a 0,12% CME kezelés csökkentette az IFN-γ szintjét. A RANTES esetében a 0,12%-os CME kezelés volt képes mérsékelni a citokin szintjét, míg az IL-1β esetében a 0,6%-os CME kezelés mutatott gyulladáscsökkentő hatást a poly I:C jelenlétében. Az eredmények alátámasztják a kezdeti hipotézist, miszerint a CME jelentős gyulladáscsökkentő tulajdonsággal bírhat csirke eredetű szövettenyészteken is, az emlős *in vitro* vizsgálatokban tapasztaltakhoz hasonlóan.

3 Introduction and literature overview

3.1 Introduction

The poultry industry faces significant challenges in maintaining health standards and managing diseases, particularly in growing consumer demand for affordable and high-quality meat products (Connolly et al., 2022; Hedman et al., 2020; Hoerr, 2010). A major concern is the overuse of antibiotics, which has led to the development of antimicrobial resistance (AMR), threatening both animal and human health (Manyi-Loh et al., 2018). Efforts to mitigate AMR have shifted towards alternative solutions such as probiotics, prebiotics, and plant-derived compounds (Buchanan et al., 2008; Demir et al., 2003). The excessive stress placed on poultry, particularly in intensive farming environments, further exacerbates the potential for infections and poor animal welfare. This stress can compromise the immune system, causing gastrointestinal disruptions, decreased feed efficiency, and reduced overall growth (Hoerr, 2010).

Bacterial and viral infections affect not just the health but also the productivity of chickens (Hoerr, 2010; McNulty et al., 1991). The gastrointestinal tract, the body's largest surface exposed to the external environment, plays a critical role in immune defense (Takahashi and Kiyono, 1999). Under physiological conditions, the gut tolerates feed and commensal microorganisms; however, a pro-inflammatory immune response is triggered upon pathogen invasion. This response is initiated when pathogen-associated molecular patterns (PAMPs) activate the innate immune system, particularly through Toll-like receptors (TLRs) present on various gut cells such as enterocytes, dendritic cells, and intraepithelial lymphocytes (Mowat, 2003). Gastrointestinal diseases disrupting the enterocytes like runting-stunting syndrome (RSS), necrotic enteritis and dysbiosis damage the intestinal barrier and the so-called “leaky gut” allows PAMPs, other pathogen byproducts (e.g. toxins) and further pro-inflammatory molecules [e.g. cytokines, damage-associated molecular patterns (DAMPs)] to reach the liver via the portal vessels and eventually the systemic circulation (Bhatt et al., 2013; Rehman et al., 2018; Sharma et al., 2000; Wickramasuriya et al., 2022). The liver cells, hepatocytes and Kupffer cells engage in pathogen filtration and production of pro- and anti-inflammatory cytokines and acute-phase proteins. Dysregulation of the cellular crosstalk leads to severe oxidative stress, cellular damage, and chronic inflammation, ultimately reducing poultry growth, productivity, and immune competence. This complex interplay between the gut and

liver highlights the need for interventions targeting gastrointestinal and hepatic health (Brandl et al., 2017; Kiziltas, 2016; Rehman et al., 2018). Moreover, viral infections such as RSS and infectious bursal disease (IBD) can significantly compromise the immune competence of poultry, even in vaccinated flocks, thereby predisposing animals to secondary infections (Kang et al., 2012; Sharma et al., 2000).

Medicinal fungi and plant secondary metabolites have received considerable attention for their therapeutic potential due to their diverse bioactive compounds, which exhibit anti-inflammatory, antioxidant, and cytoprotective properties (Lull et al., 2005; Saleh et al., 2021). Among the most important fungal secondary metabolites, polysaccharides, terpenoids, and alkaloids can be listed, and they have been confirmed to modulate immune responses, reduce oxidative stress, and enhance cellular repair mechanisms (Lull et al., 2005). Similarly, plant-derived secondary metabolites, including phenolic compounds, alkaloids, and terpenes, regulate inflammatory pathways, balance redox homeostasis, and protect cells from damage (Ji et al., 1996; Pellegrini et al., 2019; Saleh et al., 2021). The present thesis focuses on two phenolic compounds, a flavonoid and a phenolic acid, as phytochemicals and a nucleoside derivative of fungal origin, possibly contributing to immune modulation and cellular resilience.

The overproduction of cytokines and reactive oxygen species (ROS) exacerbates tissue damage, hindering the ability of the animal to maintain physiological growth and health (McNulty et al., 1991; Rehman et al., 2018; Sharma et al., 2000). Considering the current health issues related to traditional antibiotics (e.g. AMR) and the limitations of antiviral drugs, there is growing interest in natural remedies for controlling infections and reducing inflammation in humans and animals. CA, luteolin, and cordycepin, three potent natural compounds, have shown promising results in modulating immune responses and mitigating inflammation associated with bacterial and viral infections. However, little is known about their effects on poultry. CA, known for its anti-inflammatory and antioxidant properties, has been shown to reduce cytokine production and oxidative stress both *in vitro* and *in vivo* (Lee and Scagel, 2013; Lis et al., 2019). This phenolic acid, typically found in certain members of the Asteraceae family (e.g. common chicory and coneflower species), has shown promise due to its immunomodulatory and presumed antiviral effect, CA and plant extracts containing high levels of it show promise and warrant thorough investigation: CA can be a natural agent in supporting poultry health in the face of viral challenges, offering a safer and more affordable alternative to conventional antiviral drugs (Healy et al., 2009; Lin et al., 1999; Pluymers et al., 2000; Saeed et al., 2018). Luteolin, a flavonoid found in various vegetables and herbs, has anti-inflammatory, antioxidant, and immunomodulatory effects (Lopez-Lazaro, 2009). It has been

reported to suppress pro-inflammatory cytokine production. The potential of luteolin in poultry lies in its ability to modulate systemic inflammation and oxidative damage in the liver (Ansari et al., 2020; Wang et al., 2021). Cordycepin, derived from the medicinal fungus *Cordyceps militaris*, has demonstrated antiviral, anti-inflammatory, anticarcinogenic and other beneficial properties (Tan et al., 2021). It inhibits ribonucleic acid (RNA)synthesis during viral replication, making it particularly valuable in combating viral infections (Ch et al., 2016; Daikopoulou et al., 2021). Furthermore, cordycepin acts on several points of the immune system, exerting its effect by acting on multiple stages of the inflammatory response, which may reduce inflammation in the gastrointestinal tract, still boosting antiviral immunity, thereby possibly enhancing overall poultry health and productivity (Ch et al., 2016; Du et al., 2021; Radhi et al., 2021).

3.2 Inflammation on the tissue level

Inflammation is a complex pathological process, a congenital, non-specific defense mechanism. Its purpose is to eliminate harmful effects that threaten the body, disrupt its balance, and damage cells, such as endogenous or exogenous agents, necrotic cells, and tissues. This response seeks to eliminate harmful effects and restore the balance, aiming to minimize damage (Cannon, W.B, 1929). At the tissue level, inflammation starts with resident cells, producing histamine and serotonin in response to injury, which then escape into the extracellular space. The resulting microvascular disruptions initiate a cascade system that triggers the release of more mediators, such as pro-inflammatory prostaglandins [e.g. prostaglandin E_2 (PGE_2)] various free radicals, serotonin, and leukotrienes. These substances increase the permeability of the blood vessel walls, leading to the leakage of plasma and proteins into the tissues, which constitutes the exudative phase of inflammation (Anwikar and Bhitre, 2010). In the subsequent cellular phase, white blood cells [neutrophils, basophils, eosinophils, monocytes, lymphocytes, mast cells, and natural killer (NK) cells] migrate to the site of inflammation via chemotactic migration. After exiting the circulatory system, they engage in phagocytosis within the tissues and initiate the production of chemokines and cytokines (James F. Zachary, 2016). These molecules attract more white blood cells to the site and regulate inflammation, the immune response, and anti-inflammatory processes through complex receptor-mediated signaling pathways (Czaja, 2014; Liu et al., 2016). The sequence of these processes leads to a coordinated local and systemic mobilization of immune, endocrine, and neurological mediators, which typically results in eliminating the insult and generally has a beneficial effect (Hannoodee and

Nasuruddin, 2021; Kumar et al., 2004). Conversely, excessive inflammation can cause organ damage. For example, inflammation of the liver can lead to hepatocyte injury, metabolic changes, ischemia-reperfusion trauma, and even irreversible hepatic impairment (Kiziltas, 2016; Seki and Brenner, 2008a).

3.3 Inflammation on the cellular level

3.3.1 Pathogen- and Damage-associated molecular patterns

In 1989, Charles Janeway hypothesized that the primary evolutionary role of the immune system is to defend the host against infectious pathogens, rather than harmless foreign antigens. He proposed that receptors on antigen-presenting cells of the innate or non-specific immunity recognize so-called "signal 0s"-now known as pathogen-associated molecular patterns (PAMPs). These molecules are generally conserved molecular motifs shared by pathogens and recognized as non-self (Tang et al., 2012). In other words, cells of the innate immune system, such as dendritic cells, macrophages and mucosal epithelial cells, recognize specific motifs of the potentially pathogenic invaders (Hug et al., 2018).

Principal PAMPs include microbial nucleic acids-such as deoxyribonucleic acid (DNA) with unmethylated CpG (cytosine and guanine dinucleotide) rich motifs, double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), and 5′-triphosphate RNA-as well as bacterial cell envelope components like peptidoglycans, lipoteichoic acid (LTA), lipopolysaccharide (LPS), and lipoproteins, and fungal surface components such as glycosylphosphatidylinositol-anchored proteins, glycoproteins, β-glucans and mannans (Tang et al., 2012). These molecular motifs are detected by TLRs and further distinct receptor families of pattern recognition receptors (PRRs): nucleotide-binding oligomerization domain-like receptors (NLRs), and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (Wicherska-Pawłowska et al., 2021).

TLRs are located on cell or endosome surfaces of immune or epithelial cells, determined by the properties of the respective pathogen. They initiate cellular signaling cascades such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) mediated and the mitogen-activated protein kinase (MAPK) pathways to induce the expression of pro-inflammatory cytokines [e.g. tumor necrosis factor-alpha (TNF-α) and interleukin (IL) -6 (IL-6)] and type I interferons (IFNs), precisely IFN-α and β (**Table 1.**) (Tang et al., 2012).

The cytosolic NLRs sense intracellular bacterial components (peptidoglycans, muramyl dipeptide) activating NF-κB and inflammasomes [e.g., NLR family pyrin domain-containing 3

(NLRP3)]. Inflammasomes are multiprotein complexes of the innate immune system responsible for activating interleukin-1β (IL-1β) and interleukin-18 (IL-18), and mediating an inflammatory programmed cell death, pyroptosis (**Table 1.**). RLRs detect cytoplasmic viral RNA (dsRNA, 5′-triphosphate RNA), signaling through mitochondrial antiviral-signaling protein (MAVS) to induce the production of type I and a type III interferon, IFN-λ, and localize perinuclearly in virally infected cells. Nonetheless the NF-κB pathway could be additionally activated in case of the NLR and RLR signaling, but its role is secondary in the final outcome (**Table 1.**) (Wicherska-Pawłowska et al., 2021).

Table 1. Characteristics of cellular inflammatory response induced by PRR receptor families (Bertheloot et al., 2021; Wicherska-Pawłowska et al., 2021). The explanations of the abbreviations can be found in the text.

Receptors	Key Pathogens	Main signaling pathways and the outcome	Characteristic cytokines
TLRs	Extracellular bacteria, viruses	NF-κB, pro-inflammatory response, possibly induce any type of regulated cell death	Interleukins, chemokines, colony-stimulating factors, type I IFNs (IFN-α/β), TNF-α
NLRs	Intracellular bacteria, DAMPs	Activation of caspase-1 and inflammasomes, pyroptosis	IL-1β/IL-18
RLRs	RNA viruses	MAVS, antiviral response, possibly induce apoptosis	Type I (IFN-α/β) and III IFNs

At sites of tissue injury, after the original term of Janeway, endogenous "signal 0" molecules initiate a sterile inflammatory response. Damage-associated molecular patterns (DAMPs) encompass a diverse array of intracellular or extracellular matrix-derived alarmins released during cellular injury, necrosis, or regulated cell death (e.g., apoptosis, pyroptosis), which function as danger signals to recruit immune cells and mediate tissue repair. Examples include heat shock proteins, hyaluronic acid fragments, adenosine triphosphate (ATP), uric acid crystals, heparan sulfate, extracellular RNA/DNA, and mitochondrial DNA. These DAMPs, often synergistically with PAMPs, modulate inflammatory pathways through the presented

PRRs, playing a pivotal role in the pathogenesis of gastrointestinal disorders such as inflammatory bowel disease (Boyapati et al., 2016; Tang et al., 2012).

3.3.2 Chicken Toll-like receptors

Altogether, thirteen TLRs have been identified in mammals, while birds possess ten TLRs, including orthologs of mammalian receptors as well as the avian-specific TLR15 and TLR21 (Abdel-Mageed et al., 2014; Brownlie and Allan, 2011). Chickens lack TLR8 but possess TLR7 with the same role, recognizing ssRNA, and is highly expressed in lymphoid (e.g., spleen, bursa of Fabricius) but scarcely in other organs (Philbin et al., 2005). TLR9 is also absent, but TLR21 performs the equivalent function by recognizing bacterial unmethylated CpG oligonucleotides (Brownlie et al., 2009). Membrane-bound TLR2 (TLR2A/B subtypes), TLR4, TLR5, and endosomal TLR3 are expressed in hepatic cells, monocytes, and epithelial cells, playing critical roles in sensing bacterial cell wall components and intracellular viral dsRNA respectively (He et al., 2007; Kannaki et al., 2010; Kogut et al., 2005; Sławińska et al., 2013). TLR expression varies genetically; commercial chicken lines exhibit higher TLR1 and TLR5 levels in the thymus and intestine than indigenous breeds (Sławińska et al., 2013). Infectious challenges (e.g., *Salmonella enterica* serovars., *Clostridium perfringens* infection) dynamically regulate TLR expression, influencing susceptibility to pathogens (Abasht et al., 2009; Ansari et al., 2018; Lu et al., 2009).

3.3.3 Cellular signaling pathway of Toll-like receptors

TLRs specified for extracellular pathogen recognition initially activate myeloid differentiation primary response protein 88 (MyD88) which further commences a downstream of several adaptor molecules. This process predominantly converges in the activation and translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) to the nucleus. Upon activation, the NF-κB signaling pathway promotes the transcription of pro-inflammatory genes. NF-κB functions as a heterodimer, typically composed of a p50 or p52 subunit and p65. In its inactive state, NF-κB is sequestered in the cytoplasm by binding to its inhibitor, IκB-α. Phosphorylation of IκB-α leads to its degradation, releasing NF-κB and allowing its translocation into the nucleus, where it binds to the promoters of target genes, such as cyclooxygenase-2 (COX-2) and various pro-inflammatory cytokines (e.g. IL-6, IL-12, IL-23, TNF-α), thereby inducing their expression. Eventually, the NF-κB transcription factor increases the transcription of pro-inflammatory cytokines (Kiziltas, 2016; Seki and Brenner, 2008a). Regarding interferon production, some alternative adaptor molecules can stimulate

interferon response factors via MyD88 dependent pathway triggered by TLR7, TLR8 and TLR9. However, primarily the myeloid toll/interleukin (IL)-1 receptor TIR-domain-containing adaptor-inducing interferon-beta (TRIF) related pathway, the exclusive pathway of TLR3 is commonly the activator of interferon response factors and ultimately leads to type I interferon gene expression (Gao et al., 2008; Kannaki et al., 2010; Seki and Brenner, 2008a). Apart from TLR ligands and generally PAMPs, many inducers of the NF-κB pathway have been described, including ROS, allergens, hypoxia, ultraviolet light, cytokines, intercellular adhesion molecule 1 (ICAM-1) and major histocompatibility complex I, (Baldwin, 1996; Dan Dunn et al., 2015; Guo et al., 2018; Mitchell et al., 2016; J. Wang et al., 2020). Furthermore, signals from TNF receptor, IL-1 receptor, metabolic, genotoxic and shear stresses are integrated by the inhibitor of NF-κB, IκB and the whole NF-κB signaling network to produce signal-specific, context-specific and cell-type-specific transcriptional responses (Mitchell et al., 2016).

3.3.4 TLR ligands

3.3.4.1 Components of the bacterial cell wall and the flagellum

The TLR ligand of the highest importance and clinical relevance is LPS. This molecule is a characteristic components of the Gram-negative bacterial cell wall (Dickson and Lehmann, 2019). Along with its Gram-positive bacterial counterpart, LTA, it can either modulate signal transduction through TLR activation or can be bound nonspecifically by type-I scavenger receptor in the liver (Gao et al., 2008; Kiziltas, 2016). The TLR activation is mediated by a cluster of differentiation protein (CD) 14 which is anchored to the leukocyte or enterocyte cell membrane or present as a soluble form secreted by monocytes, hepatocytes, or the gut epithelium (Gnauck et al., 2016). According to recent studies, LPS and LTA might play an equal role in septic shock, tissue damage, and multiorgan failure synergistically with other cell wall components like peptidoglycans of Gram-positive bacteria (Dickson and Lehmann, 2019; Ginsburg, 2002). However, LPS molecules show a certain level of specificity and activate the innate immune system in a dose- and pathogen-dependent manner. This specificity is interpreted by the acylation and phosphorylation of the lipid A moiety which determines the bonds between the LPS molecule and the coreceptor of TLR4, myeloid differentiation factor-2 and subsequently the structure and signal activity of the entire receptor complex. Therefore competition between different LPS types for the receptor and functional antagonism of TLR4 can spontaneously occur (Gnauck et al., 2016). Lipopolysaccharide from O55:B5 chicken pathogen *Escherichia coli* (*E. coli*) is the LPS most frequently used *in vitro* on chicken cell lines

and cultures (Kalaiyarasu et al., 2016; Zhang et al., 2017). *Staphylococcus aureus* LTA proved to induce oxidative burst via TLR activation in chicken heterophil granulocytes (Farnell et al., 2003)

Interestingly, much of the gene expression profile observed in human *Salmonella* enterocolitis could be attributed to the monomer of the bacterial organ, flagellum (Zeng et al., 2003). The protein monomer, flagellin, in 50-100 ng/mL concentration exerts an increase in cytokine gene expression and causes notable degranulation and oxidative burst in a chicken heterophil granulocyte culture (Gewirtz et al., 2004; Kogut et al., 2005). The presence of free bacterial flagellin in the living organism is hypothesized to result from the disintegration or the leaky assembly of the flagellum. Foreseeably, the conserved hidden core region of this motor protein induce the activation of the non-specific immune system via a PRR, TLR5 (Fliegmann and Felix, 2016). TLR5, even in chicken, is part of the requisite toolkit of monocytes and epithelial cells to activate inflammatory cascades (Kannaki et al., 2010). Wild S. Enteritidis trigger more severe histopathological changes in the liver, and more effective colonization and invasion of the caecum and the spleen in the first few days post-infection in chicken hatchlings than aberrant non-motile or non-flagellated mutants (Barbosa et al., 2017).

3.3.4.2 Double-stranded RNA

In addition to dsRNA viruses, dsRNA genomic structures are naturally produced by all virus families-either as replicative intermediates (in ssRNA and double-stranded DNA viruses) or as genomic secondary structures (such as in Orthomyxoviridae and Retroviridae). The accumulated virus-associated pattern serves as an IFN inducer outside of the infected cells and have a notable effect on the pathogenesis of viral acute phase response *in vivo* (Majde, 2000). Polyinosinic-polycytidylic acid (poly I:C) as an interferon inducer is yet another novel alternative to evoke inflammation in cell cultures. These molecules are similar to the viral double-stranded RNA genome, characteristic of Reoviridae and Birnaviridae virus families with abundant poultry-specific strains. The molecule exerts inflammatory cytokine synthesis via TLR3 receptors expressed on the endosomal surface both in mammalian and avian cells following the anticipated internalization of the macromolecular structure (Li et al., 2005; Matsumoto et al., 2003). This novel molecule showed a very potent effect both in splenocyte-derived leukocyte and oviduct-originated primary chicken cell cultures inducing high IL-1β, IL-6, IFN-α mRNA level elevation (Abdel-Mageed et al., 2014; Kamimura et al., 2017; Villanueva et al., 2011a).

3.3.5 Signaling pathways connected to inflammation and oxidative stress

To understand the pro-inflammatory cellular response, acquaintance with further signaling pathways directly connected to inflammation, oxidative homeostasis and cell death should be ensured.

The nuclear factor erythroid 2–related factor 2/Kelch-like ECH-associated protein 1 pathway (Nrf2/Keap1) is the master regulator of antioxidant defense, inducing enzymes such as heme oxygenase-1 (HO-1) and superoxide dismutase (SOD) to neutralize ROS. Nrf2/Keap1 also suppresses NF-κB and NLRP3 inflammasome activity, limiting the release of inflammatory cytokines. Nrf2 activation protects against oxidative damage and can contribute to cellular detoxification and cell survival (**Table 2.**) (Kupiec-Weglinski, 2024).

The phosphoinositide 3-kinase/protein kinase B pathway (PI3K/Akt) is a central cell survival, growth, and metabolism regulator. It inhibits apoptosis and enhances antioxidant defenses by activating Nrf2. In the context of inflammation, PI3K/Akt has a dual role: it can activate NF-κB to increase pro-inflammatory cytokine production like TNF-α and IL-6, particularly in chronic diseases, while also upregulating Nrf2 to reduce oxidative stress (**Table 2.**). This demonstrates its context-dependent function in balancing cell survival, inflammation, and antioxidant responses (Fruman et al., 2017).

The AMP-activated protein kinase pathway (AMPK) acts as a cellular energy sensor, inhibiting anabolic processes, activating autophagy, and supporting Nrf2-mediated antioxidant responses. AMPK also suppresses NF-κB and NLRP3 inflammasome activity, thereby reducing the level of pro-inflammatory cytokines such as IL-1β and IL-6, and promoting the clearance of ROS (**Table 2.**). Through these mechanisms, AMPK maintains energy homeostasis and contributes to the resolution of inflammation, especially in metabolic and age-related diseases (Xu et al., 2020).

The mitogen-activated protein kinase (MAPK) pathway has three distinct branches with distinct roles. Extracellular signal-regulated kinase (ERK) promotes cell proliferation and survival, while c-Jun N-terminal kinase (JNK) and p38 MAPK (p38) are primarily involved in apoptosis and inflammation, especially under stress conditions such as increased ROS or ultraviolet radiation. JNK and p38 can amplify oxidative stress-induced NLRP3 activation and apoptosis, whereas ERK may counteract these effects through crosstalk with PI3K/Akt (**Table 2.**). The balance between the MAPK pathways is crucial, as dysregulation can exacerbate tissue damage (Kyriakis and Avruch, 2012).

Table 2. Signal pathways connected to inflammation, oxidative stress and apoptosis (Fruman et al., 2017; Kupiec-Weglinski, 2024; Kyriakis and Avruch, 2012; Xu et al., 2020). The explanations of the abbreviations can be found in the text.

Pathway	Main Function	Inflammation	Oxidative Stress	Apoptosis / Cell Survival
PI3K/Akt	• Cell growth & metabolism • Anti-apoptotic	• Activates NF-κB → ↑ inflammation • Activates Nrf2 → ↓ inflammation	• Activates Nrf2	• Inhibits apoptosis • Promotes survival
AMPK	• Energy sensor • Activates autophagy	• Inhibits NF-κB, NLRP3 → ↓ inflammation	• Activates Nrf2	• Promotes autophagy • Inhibits apoptosis
Nrf2/Keap1	• Antioxidant master regulator • Detoxification	• Inhibits NF-κB, NLRP3 → ↓ inflammation	• ↑ HO-1, SOD • Enhances antioxidant defenses	• Promotes survival • Reduces oxidative apoptosis
MAPK	• ERK: proliferation • JNK/p38: stress/apoptosis	• JNK/p38 activates inflammasomes → ↑ inflammation	• JNK/p38 amplify the effects of ROS • ERK counters via PI3K/Akt	• ERK: promotes survival • JNK/p38: promotes apoptosis

3.4 Gut-liver axis

3.4.1 Physiology and diseases of the gut-liver axis

The primary function of the digestive system is the breakdown of food, the absorption of nutrients, and the facilitation of metabolic processes within the organism. Given its continuous interaction with the external environment, it also assumes a critical immunological role and plays a pivotal part in combating pathogenic microorganisms. Pathogenic bacteria have the potential to inflict damage on the host's health, either locally via receptors located on the intestinal epithelial cells or through the bloodstream following translocation from the gut (Baxter et al., 2019; Johansson et al., 2011). To augment the absorptive surface area of the small intestine, the mucosa forms finger-like projections towards the lumen, known as villi. These villi are covered by a monolayer of columnar epithelium featuring a brush border. The enterocytes that constitute this epithelium are primarily responsible for nutrient transport. Between these cells lie tight junctions, which impede the passage of pathogens between the

cells, preventing their entry into the body. Goblet cells are interspersed among the enterocytes, and the mucin they secrete forms a protective layer on the apical surface of the cells. This layer acts as a barrier between the lumen and the enterocytes, not only hindering pathogen invasion but also creating a conducive environment for the commensal microbiota residing within the gut. At the base of the villi, within the Lieberkühn crypts, reside the Paneth cells, which play a vital role in regulating the gut microbiome and defending against pathogens ingested with food. The presence of bacteria stimulates these cells to produce various digestive enzymes, such as lysozymes and phospholipase-A2, as well as antimicrobial peptides (AMPs), which subsequently damage the pathogens (Duangnumsawang et al., 2021).

In addition to the tight junctions between enterocytes and the enzymes secreted by the Paneth cells, lymphoid tissues within the mucosa also contribute to the defense against pathogens entering the gastrointestinal tract. In avian species, the gut-associated lymphoid tissue (GALT), diffusely distributed throughout the mucosa, is particularly well-developed. Lymphoid cells are found within the epithelial layer and *lamina propria*, as well as in Peyer's patches and the tonsils adjacent to the cecum. As observed in mammals, Peyer's patches in avian species are secondary lymphoid organs composed of aggregated lymphoid follicles. These patches are visible until two weeks of age, with their number steadily increasing as the animal matures. GALT constitutes the first line of immune defense against pathogens breaching through the mucosal barrier. While the immune system tolerates the host's microbiota, it can mount a rapid and effective response to pathogenic threats (Nochi et al., 2018).

The gastrointestinal tract represents the body's largest surface exposed to the external environment (Takahashi and Kiyono, 1999). The gut tolerates feed and commensal antigens but acts when a pathogen is detected. However, the impairment of the enterocyte layer enables molecules of extraneous origin to come into contact with the receptors of the innate immune cells. The alert with the cytokines spreads further and calls forth a pro-inflammatory response on the level of dendritic cells and the abundantly present intraepithelial lymphocytes (Mowat, 2003). In animals, one of the primary causes of dysbiosis and disruption of intestinal immunological homeostasis is an enteric infection caused by bacteria, viruses, or parasites. Several bacterial and viral pathogens can compromise the gut barrier in broiler chickens, leading to enteric diseases that impair health and performance. *Clostridium perfringens* causes necrotic enteritis by producing toxins that damage the intestinal lining, while *Escherichia coli* and *Salmonella* serovars contribute to colibacillosis and salmonellosis by invading the gut and disrupting epithelial integrity (Wickramasuriya et al., 2022). Viral agents such as chicken anemia virus induce immunosuppression, while infectious bursal disease virus, avian reovirus,

and other small non-enveloped viruses associated with runting-stunting syndrome (RSS) compromise the gut barrier by causing direct epithelial damage or immune suppression, thereby promoting secondary infections and gastrointestinal dysfunction (Bhatt et al., 2013; Rehman et al., 2018; Sharma et al., 2000; Wickramasuriya et al., 2022). The intestinal epithelium expresses most TLR types on the luminal surface; however, the majority of these TLRs are not intrinsically responsive to PAMPs. Nevertheless, pro-inflammatory cytokines such as IFN-γ and TNF-α could increase their abundance and reactivity (Abreu, 2003). The intestinal mucosa is a functional aggregation of neighborhoods and niches of roughly 20 types of immune and epithelial cells (Hickey et al., 2023). Growing evidence suggests that intestinal mucosal inflammation and barrier function are defined and regulated by the interaction of distinct cell types such as enteric glial cells, intraepithelial lymphocytes, and some epithelial cell types with different commitments and functions. Therefore, the structure determines the response (Liu and Yang, 2022; Randall et al., 2011).

The liver is the secondary organ barrier against gut-derived antigenic load and protects the systemic circulation against residual oxidative and pathogenic burdens from the gastrointestinal tract (Lauridsen, 2019). The liver receives much of its blood supply via the portal vein from the gut, containing bacterial products and dietary antigens. In contrast, the rest of its vascularization comes from the systemic circulation involving the hepatic artery (Gao et al., 2008). Blood from each source passes through liver sinusoids to eliminate invading pathogens and their byproducts with the help of endothelial cells and the most abundant resident macrophage population in the body, Kupffer cells (Ishibashi et al., 2009). The membrane-bound PRRs present on each Kupffer cell, hepatocyte, biliary epithelial cell, and sinusoidal endothelial cell are subject to microbial PAMPs. Therefore, these conserved pathogen degradation products and metabolites play a substantial role in hepatic diseases. Accordingly, hepatitis, liver fibrosis, cirrhosis, and ischemia are common pathologies associated with TLR-mediated signal perplexity and over-activation.

Nevertheless, a healthy liver has a relative immune tolerance against PAMPs. The upregulation of TLR gene transcription and the overactivation of PAMP triggered signal transduction implicate the breakdown of the tolerance and, therefore, leads to overexpression of pro-inflammatory cytokines (Gao et al., 2008; Kiziltas, 2016; Seki and Brenner, 2008a). Kupffer cells also produce inflammatory cytokines to induce acute phase protein secretion of hepatocytes or to regulate the local inflammatory response (Ishibashi et al., 2009). Materials escaping this barrier could be entrapped by opsonization with soluble PRRs and complement factors produced by hepatocytes. The liver, therefore, orchestrates the innate immune response

of the body via filtering out pathogens and producing soluble factors with systemic effect (Gao et al., 2008; Ishibashi et al., 2009).

3.4.2 Cytokine imbalances of the gut-liver axis

The gut-liver axis relies on a delicate balance of cytokines and signaling pathways to maintain homeostasis while occasionally mounting acute immune responses. IL-10, a potent anti-inflammatory cytokine, suppresses NF-κB activation in Kupffer cells and other macrophages, reducing the production of TNF-α, IL-1β, and IFN-γ during liver injury, thereby mitigating hepatocyte damage and fibrosis. IL-10 counteracts T helper 17 (Th17) cell-driven inflammation in the gut. Th17 cells, which produce IL-17, evolved to defend against fungal and bacterial infections at mucosal barriers but can drive autoimmune diseases when overactive. Their development is promoted by immune signals such as IL-6 and IL-23, tipping the balance toward inflammation (Kanai et al., 2012; Vujovic et al., 2023).

Paradoxically, type I IFNs, though central to antiviral defense, can exacerbate Th17-mediated pathology by amplifying IL-23/IL-17 signaling; yet they also suppress excessive inflammation by inducing IL-10 production. In the liver, Kupffer cells recruit neutrophils to sites of injury via IL-8, exacerbating oxidative damage via ROS production during oxidative burst. In the gut, IL-8 synergizes with RANTES, produced locally by intestinal epithelial cells and lymphocytes during inflammation, to enhance neutrophil infiltration and epithelial barrier breakdown. Similarly hepatic stellate cell-derived RANTES contributes to liver-specific fibrogenesis and leukocyte recruitment. IL-6, despite its role in Th17 differentiation, activates acute phase protein production in the hepatocytes. These acute phase proteins (e.g. C-reactive protein and serum amyloid A) could opsonize pathogens but perpetuate chronic inflammation when their release is dysregulated (Brandl et al., 2017; Gao et al., 2008; Seki and Brenner, 2008a, 2008a).

Macrophage colony-stimulating factor (M-CSF) further modulates this balance by polarizing macrophages toward an M2 phenotype, which secretes IL-10 and transforming growth factor-beta (TGF-β) to resolve inflammation but inadvertently activates hepatic stellate cells, linking acute repair to fibrosis. Meanwhile, M-CSF suppresses Th17 differentiation in the gut-liver axis, whereas M1 macrophages amplify it via IL-6 and IL-23, highlighting the dual roles of cytokines in balancing immune defense and tissue repair (Korn et al., 2009; Wynn and Vannella, 2016).

3.5 Inflammation and oxidative stress

Free radicals can be generated in the body during functional cellular metabolism but also due to external harmful stress or stimuli. Free radicals are molecules that possess an unpaired electron in their outer electron shell. Because of this characteristic, they are highly reactive molecules that can readily disrupt specific biochemical processes and cause cellular damage. Reactive compounds can be classified into three main groups based on the chemical elements they contain. The first group includes ROS containing an oxygen atom. An example is hydrogen peroxide, which can form hydroxyl or superoxide anion radicals. The second group consists of reactive nitrogen species, which include the nitrogen monoxide radical. The final group comprises free radicals containing chlorine atoms, known as reactive chlorine species, which include the hypochlorite radical derived from hypochlorous acid (Gray et al., 2013; Pham-Huy et al., 2008).

The cells fight against pathogens using ROS. An example of this can be observed on mucosal surfaces, which belong to the defense mechanisms of the innate immune response. Here, macrophages, monocytes, neutrophils, and eosinophils carry out phagocytosis to eliminate bacteria, other extracellular microbes and cellular debris from the body. In birds, heterophilic granulocytes function as non-oxidative phagocytes therefore macrophages and monocytes are of key importance (Harmon, 1998). The lysis of phagocytosed foreign materials occurs through the strong oxidative effect of superoxide, which is produced by the NADPH oxidase (NOX) enzyme in the cells (Babior, 2004). The production of superoxide is preceded by the uptake of large amounts of oxygen by the cells, which is not utilized during terminal oxidation. The cells generate hydrogen peroxide from superoxide, capable of reacting with halide ions in myeloperoxidase-catalyzed reactions. As a result, hypochlorous acid (HOCl) is primarily formed as a highly antibacterial substance. The various reactive species generated can also be released into the tissue during the degranulation of inflammatory cells, where they can oxidize the proteins and other macromolecules of the pathogens. This process is referred to as the respiratory burst. However, it is important to emphasize that excessive levels of HOCl and other ROS not only damage pathogens but also oxidize the proteins, nucleic acids, and lipid components of the host's cells within the inflammatory microenvironment (Thomas, 2017; Ulfig and Leichert, 2021).

Viruses, as intracellular pathogens, directly interfere with the cellular signaling processes to ensure the success of their replication. As a result, they disrupt the delicate and critical balance between pro- and antioxidant mechanisms within the cell. From another

perspective, the host cell and organism sacrifice the redox balance to eliminate the viral infection. Thought-provokingly, PRRs such as NLRs and RLRs depend on the membrane-bound, ROS-generating enzyme, NOX, and NOX-generated ROS for their activation during pathogen detection. However, excessive ROS production, as observed in viral infections like influenza, directly activates NF-κB and stress-responsive pathways like the p38 branch of the MAPK cascade. These pathways could trigger the hyperactivation of the immune system, manifesting in cytokine storms and tissue damage (M. Liu et al., 2017; Rehman et al., 2018).

Free radicals are also referred to as pro-oxidants. The harmful effects of pro-oxidants in the body are counteracted by various antioxidants. If there is an insufficient amount of antioxidants in the body to neutralize the prooxidants, oxidative stress occurs (Rahal et al., 2014). Oxidative stress is involved in the development of numerous diseases. The accumulated free radicals in the body can irreversibly oxidize DNA, proteins, and lipids, and also cause DNA strand breaks (Niki, 2016; Ohshima et al., 1998; van der Vliet and Janssen-Heininger, 2014). The oxidation of lipids is influenced by different factors such as the degree of unsaturated fatty acids, the position of fatty acids in the triacylglycerol molecules, lipid class, and the presence of antioxidants in lipids (Rehman et al., 2018). Polyunsaturated acyl chains of phospholipids, low-density lipoproteins, and polyunsaturated fatty acids like arachidonic acid and linoleic acid are especially susceptible to peroxidation. They degrade to highly reactive electrophilic aldehydes, such as malondialdehyde (MDA), ketones and peroxide adducts (Grimsrud et al., 2008; Rehman et al., 2018; Yang et al., 2020). These molecules disrupt the lipid bilayer and cause the inactivation of enzymes, proteins, and membrane-bound receptors, further increasing membrane permeability and resulting in cellular damage and cell death (Rehman et al., 2018; Yang et al., 2020).

3.6 Inflammation and cell death

Apoptosis is a programmed form of cell death triggered by harmful stimuli, involving caspases, which are proteases that cleave target proteins at aspartate residues. These enzymes exist as inactive proenzymes and become activated through cleavage, initiating two main pathways: the death receptor-mediated and mitochondrial pathways. Caspase-3 plays a central role in both pathways, being activated following receptor stimulation by molecules like TNF-α and Fas-associated death domain protein. Once activated, caspase-3 cleaves various substrates to execute apoptosis (Fu and Fan, 2002; Read et al., 2002; Wang et al., 2005).

Several pro-inflammatory cytokines can contribute to apoptosis. Amongst which RANTES, TNF-α and IFN-γ are of utmost importance. TNF-α, the central cytokine that induces inflammation and promotes the production of matrix metalloproteinases, thereby inducing the apoptosis of enterocytes through the digestion of the basement membrane (James F. Zachary, 2016). IFN-γ is a potent glycoprotein cytokine that influences various aspects of immune system function. It is produced by cells in response to viruses, virus-infected cells, and parasites, but its production is generally not TLR driven, but mediated mainly by cell-cell interactions and intermediary cytokines (Gajanayaka et al., 2021). It activates NK cells and regulates the activity of macrophages through both the adaptive and innate immune systems. IFN-γ enhances antigen presentation to T lymphocytes and is capable of both inducing and inhibiting apoptosis in cells. Upon binding to the IFN-γ receptor, it triggers responses in Th1 and Th17 lymphocytes. Numerous studies have demonstrated that IFN-γ can inhibit cancerous cell proliferation and, through the NF-κB signaling pathway, influence transcriptional processes within the cell, affecting cytokine production and the transcription of apoptotic genes (James F. Zachary, 2016; Kanai et al., 2012). RANTES chemokine is a classical chemotactic cytokine predominantly produced by CD8+ T lymphocytes and macrophages (Schall, 1991). This is a pro-inflammatory chemokine, with two known mechanisms of action. It exerts a chemotactic effect at low concentrations by binding to specific G-protein coupled transmembrane receptors. However, at higher concentrations, it associates with cell surface glycosaminoglycans, leading to the induction of apoptosis through the activation of caspase-9 and caspase-3, following the release of cytochrome C from the mitochondria into the cytoplasm (Appay et al., 1999; Bacon et al., 1996; Murooka et al., 2006; Nieto et al., 1997).

On the other hand, further cell death types could be specified, but because of the limitations of the present thesis, a pro-inflammatory form and lytic programmed cell death type, pyroptosis should be discussed in detail. Pyroptosis is highly connected to the inflammatory response at cellular and tissue level (L.-H. Lian et al., 2012). This inflammatory cell death is primarily mediated by NLRP3 and caspase-1, the converting enzyme of IL-1. It is a caspase-3 independent process with accelerated kinetics compared to apoptosis. The mechanism of action is not entirely elucidated, but pore formation is a hallmark of pyroptosis. Pores generated in the plasma membrane facilitate cell swelling, membrane rupture, and release of pro-inflammatory cytokines and lactate dehydrogenase (LDH). Bibliographic data is limited in avian species, but pyroptosis has been proven to occur in chicken primary hepatocyte cell cultures under copper-induced oxidative stress (Liao et al., 2019; Shi et al., 2017). The overactivation of this cell death mechanism is supposed to be highly detrimental and might accentuate damage in sepsis, acute

lung injury, disseminated intravascular coagulopathy and several liver pathologies (Kang et al., 2018; C. Wu et al., 2019; J. Wu et al., 2019).

3.7 Damage caused by chicken pathogens on cellular level

Thanks to the dedicated work of persistent researchers, the cellular-level effects of certain viral and bacterial infections have been elucidated. Understanding cellular pathomechanisms may help us appreciate the significance of processes occurring within individual cells in relation to the overall health of the living organism.

Current evidence suggests that IBD virus and chicken anemia virus can overcome the immune defense and cause immunosuppression by depleting the primary lymphoid organs, the Fabricius bursa and the thymus, through lymphocyte apoptosis and hematopoietic cell damage, often manifesting in chicks under 3 weeks old (Hoerr, 2010; Santen et al., 2004; Sharma et al., 2000). IBD virus is a highly contagious dsRNA virus belonging to the *Birnaviridae* family, which destroys immature B-lymphocytes in the bursa, causing immunosuppression and delayed thymic recovery, even in subclinical infections (He et al., 2021; Li et al., 2021; Meng et al., 2021; Shin et al., 2021). The bursal lymphocyte depletion and thymic atrophy are connected to disrupted cytokine signaling and caspase-3 activation, resulting in apoptosis (Rauf et al., 2012). Protecting flocks from severe disease outbreaks is solely possible with considered immune prophylaxis (Meng et al., 2021). Although, many vaccines do not provide sufficient immunity against IBD (Li et al., 2021).

Avian reoviruses, also possessing dsRNA nucleic acid, cause several disease conditions like arthritis/tenosynovitis, hepatitis, RSS and malabsorption syndrome, resulting in economic losses (Benavente and Martínez-Costas, 2007; Lin et al., 2007; Rehman et al., 2018). Avian reovirus infection leads to cell damage in several organs such as liver, bursa, intestines, pancreas, thymus and the spleen (Lin et al., 2007). Rehman et al. (2018) reported that it could increase lipid peroxidation, ROS level and supposedly induce DNA damage (Rehman et al., 2018). The agent of inclusion body hepatitis, a fowl adenovirus, can similarly cause high mortality in young broilers and thus lead to high economic losses. In this disease, whose main target organ is the liver, affected animals show depression, weakness, ruffled feathers, and yellowish watery diarrhea (Kiss et al., 2021; Mirzazadeh et al., 2020). A specific serotype of this virus, fowl adenovirus serotype 4—the causative agent of hepatitis-hydropericardium syndrome—leads to apoptosis, autophagy, and a severe inflammatory response, resulting in loss of liver integrity and function (Niu et al., 2018). ROS overproduction during viral infections

suppresses interferon signaling (M. Liu et al., 2017) but may amplify NF-κB-mediated inflammation (Rehman et al., 2018). Moreover, avian influenza virus increases nitric oxide production and reactive species leading to the disturbance of cellular redox homeostasis causing apoptosis and increased fatality associated with pneumonia and lung damage (Rehman et al., 2018).

Salmonella enterica serovar Typhimurium and Enteritidis are the primary causes of foodborne human salmonellosis worldwide (Hendriksen et al., 2011). A source of this infection can be contaminated poultry meat and egg. The infection after 3 days of age persists in chicken for 8-9 weeks of age without clinical symptoms, therefore surveillance and eradication of *Salmonella* serovars accountable for salmonellosis is a major objective of the poultry industry (Beal et al., 2004; Hugas and Beloeil, 2014). In other cases, following the invasion from the gastrointestinal tract, *Salmonella* Typhimurium multiplies in the liver and spleen and might cause systemic infection and high mortality in chickens infected early after hatching (Barrow, 2000). Emerging *Salmonella enterica* serovars and unconventional organic farms still manifest a human threat (BAILEY et al., 2020; Hendriksen et al., 2011; Melendez et al., 2010). In bacterial infections, not only the live, proliferating bacteria pose a threat to the organism, but also the cell wall components released from them. The release of cell wall constituents is particularly evident after a successful antibiotic treatment due to bacterial lysis, leading to a distinctive inflammatory cascade process triggered by bacterial infections. The damage caused by that mechanism cannot be mitigated by antibacterial treatment (Gnauck et al., 2016).

3.8 Secondary metabolites of plants and fungi

In the poultry industry, especially in broiler production, there is growing evidence that the enhanced performance previously obtained with the subtherapeutic application of antibiotics is attainable with natural feed additives of plant origin (Aljumaah et al., 2020; Buchanan et al., 2008; Demir et al., 2003). The beneficial effect of these substances on animal health and productivity could be mediated by their anti-inflammatory, cytoprotective and antioxidant properties (Alagawany et al., 2021; Rathee et al., 2009), which should be extensively investigated. In the poultry industry, flavonoids, essential oils, polyphenols, and organic acids, as prominent secondary metabolites, are among the most efficient feed additives currently used to substitute antibiotics as growth promoters (Ayalew et al., 2022; Wang et al., 2024).

Essential oils supply bioactive compounds such as carvacrol and thymol that have antimicrobial, anti-inflammatory, antioxidant and hepatoprotective properties leading to improved digestion and reduced pathogen load. These effects translate into enhanced production parameters in poultry (Aljumaah et al., 2020; Buchanan et al., 2008; Gholami-Ahangaran et al., 2022). Organic acids (e.g. fumaric, citric and butyric acid) produced by plants or fungal fermentation serve as natural preservatives and antimicrobials which could increase feed efficiency, along with anti-inflammatory benefits that support overall health, and possibly increase meat production (e.g. breast weight in broiler chicken) (Buchanan et al., 2008; Khan et al., 2022). Flavonoids in poultry feed could improve feed conversion ratio and carcass yield and other parameters of production mostly by enhancing nutrient absorption and gut health through the modulation of beneficial gut microbes (e.g. *Lactobacillus spp.*) and reduction of pathogenic bacteria in broiler chicken. They exhibit positive effect on the villi height and even on meat quality (Paredes-Lopez et al., 2025). One of the most commonly cited polyphenols in broiler and poultry production is curcumin. It decreases the expression of pro-inflammatory genes parallelled with antioxidant effect in poultry. Its inclusion in feed promotes better nutrient assimilation and has antimicrobial and antiparasitic potential, improving meat quality, growth rates and feed efficiency (Paredes-Lopez et al., 2025; Sureshbabu et al., 2023).

Generally, plant secondary metabolites modulate multiple signaling pathways involved in inflammation, acting at various stages of the inflammatory response. These compounds can inhibit the NFκB pathway and the MAPK signaling (including ERK, JNK, and p38), both of which are involved in the regulation of pro-inflammatory gene expression. Additionally, they can suppress the activity of COX enzymes (COX-1 and COX-2), reducing the production of pro-inflammatory prostaglandins. Many plant metabolites also possess antioxidant effects, neutralizing ROS and reducing oxidative stress, contributing to inflammation. Moreover, plant secondary metabolites can modulate adenosine receptors, $A2_A$ and A3, which regulate the release of pro-inflammatory cytokines. In addition, these compounds influence immune cell function, such as macrophage polarization, and inhibit inflammasome activation, which is responsible for activating pro-inflammatory cytokines like IL-1β. Due to their ability to target these diverse pathways, plant secondary metabolites hold promise as natural anti-inflammatory agents in both prevention and therapy (Ji et al., 1996; Pellegrini et al., 2019; Saleh et al., 2021).

The present thesis focused on plant and fungal metabolites with documented effects on animal health and productivity. On the other hand, the agents investigated are not yet fully validated. Furthermore, the evidence-based adoption of them in the health management of

broiler chicken is lacking despite the fact that their general health benefits are reported in wide range of species and the health claims are touched even in certain veterinary formularies.

Chicoric acid (CA) and N-acetylcysteine (NAC) are familiar bioactive agents in human and veterinary pharmacology. Both of them are present in common plants, nonetheless, NAC, an *Allium* genus metabolite, is synthesized artificially as a common mucolytic and an antidote for paracetamol toxicosis (Diniz et al., 2006; Riera et al., 2000). NAC is present for instance in *Allium cepa* (onion) along with other organosulphur molecules (Diniz et al., 2006). CA was first isolated from the herbaceous perennial *Cichorium intybus* (common chicory), a member of the *Asteraceae* family, but it is also present in the aerial parts of other medicinal and wild plants, including *Taraxacum officinale* (dandelion), *Melissa officinalis* (lemon balm), various aquatic plants, and especially in high concentrations in species of the *Echinacea* genus (**Figure 1.**) (Andary et al., 2013; Lee and Scagel, 2013; Saeed et al., 2018). CA is a phenolic compound consisting of a tartaric acid core in which both hydroxyl groups are esterified with caffeic acid moieties (**Figure 1.**) (Lee and Scagel, 2013). *Echinacea* extracts have demonstrated immunostimulatory effects in several studies. Their primary mechanisms involve enhancing phagocytosis and macrophage activity and inhibiting endogenous and exogenous hyaluronidase production, thereby immobilizing pathogens and inflammation. These effects are mainly attributed to the polysaccharides, which, together with other metabolites, stimulate cytotoxic T lymphocytes and increase the production of cytokines such as IL-1, TNF, and interferons (Pleschka et al., 2009; Saeed et al., 2018; Thompson, 1998). CA, a phenolic compound derived from Echinacea, is ranked among the top three least cytotoxic agents out of 85 plant-derived secondary metabolites tested on murine BV2 microglial cells, which fact underscores its favorable cellular compatibility (Mu et al., 2019). It exerts both anti-inflammatory and antiviral effects by directly interfering with viral replication processes (Healy et al., 2009; Lin et al., 1999; Pluymers et al., 2000). In experimental models of duck hepatitis B virus infection, CA significantly attenuates viral replication and hepatocyte injury via ROS scavenging, demonstrating antiviral efficacy comparable to lamivudine (Zhang et al., 2014). Through suppression of NF-κB/MAPK signaling pathways and pro-inflammatory cytokines such as TNF-α and IL-6, CA emerges as a promising multifunctional adjunctive agent, combining potent antiviral activity with a high degree of cellular safety (Lee et al., 2015). Furthermore, CAhas demonstrated neuroprotective, anti-inflammatory, and antioxidant effects in regard to various *in vivo* and *in vitro* models, including the suppression of pro-inflammatory cytokines and oxidative mediators, modulation of gut microbiota, and protection against chemically

induced neuroinflammation and colitis (Landmann et al., 2014; Lee et al., 2015; Wang et al., 2022).

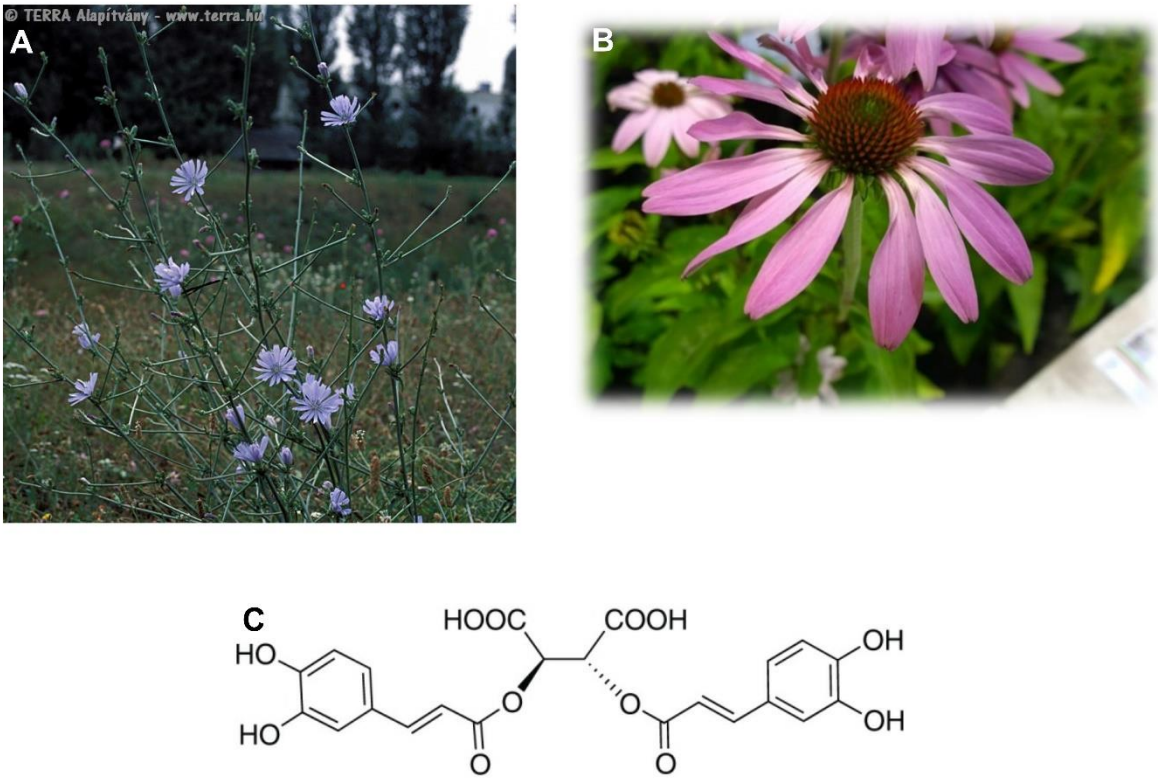

Figure 1. The aerial part of *Chicorium intybus* with bluish flowers (A), flowers of *Echinacea purpurea* (B) and the structure of chicoric acid (C).

Flavonoids as plant metabolites can influence mucosal and cellular immunity, modulate the endocrine response of the body and reduce oxidative damage caused by reactive compounds. In addition, the literature suggests that in chickens, some members of this molecular group have been shown to improve hematological parameters and attenuate increased inflammatory responses (Kamboh et al., 2018; Rathee et al., 2009). The use of flavonoids in both human and animal nutrition, including the foraging of broiler chicken, may be of great importance in the future to substitute for unnecessary antibiotic use (Kamboh et al., 2018; Lopez-Lazaro, 2009). Luteolin is a flavonoid found in most edible greens and vegetables, for instance, celery, rosemary, thyme, peppers, carrots, buckwheat and cabbage, but it was identified in the vast majority of *Magnoliophyta* families and lower phyla of plants as well (Lopez-Lazaro, 2009).

Luteolin has a molecular formula of $C_{15}H_{10}O_6$ with the structure depicted in **Figure 2.** (Ansari et al., 2020).

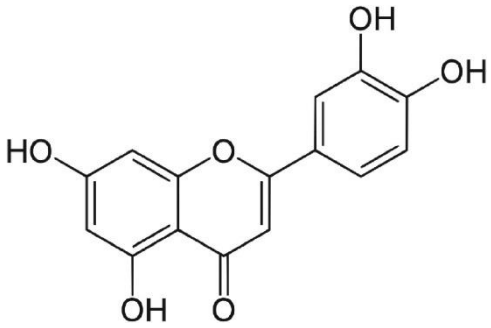

Figure 2. The structure of luteolin.

Medicinal fungi among others, *Lentinus edodes* (Shiitake) *Ganoderma lucidum* (Reishi), *Trametes versicolor* (Turkey tail) have been consumed for hundreds of years in East Asia and has been thoroughly investigated in the past 40 years because of their potential to stimulate the immune system and influence tumorigenesis (Mustafin et al., 2022). Most of these medicinal fungi contain glucans with varied biological activity based on the branching, the size and the water solubility of the molecule, according to which they target different immune cell receptors, like TLR2 and 4, complement receptor 3 and other scavenger receptors. The impact on different receptors of different cell populations contributes to a diverse biological response. The activation of antigen-presenting cells, cytokine production, change in T cell differentiation, and augmented antibody production could be attributable to the fungal polysaccharides (Lull et al., 2005). Therefore, some of them with specific health benefits has been characterized. Generally most medicinal fungi are recognized by their immunostimulant effect due to their varied polysaccharide (glucan, mannan or heteroglucan) content (Mustafin et al., 2022; Zhang et al., 2022). *Cordyceps militaris* is also rich in pharmacologically active agents. Glucans, sterols, fenoloids, and carotenoids can be listed, but the quality marker molecule of the fungus is an adenosine analog, cordycepin (Tuli et al., 2013). The molecule is an adenosine apart from the missing 3′ hydroxyl group, which is the most prominent characteristic of the molecule (**Figure 3.**) (Rabie, 2022). It is technically an RNA chain elongation terminator nucleoside (Tuli et al., 2013).

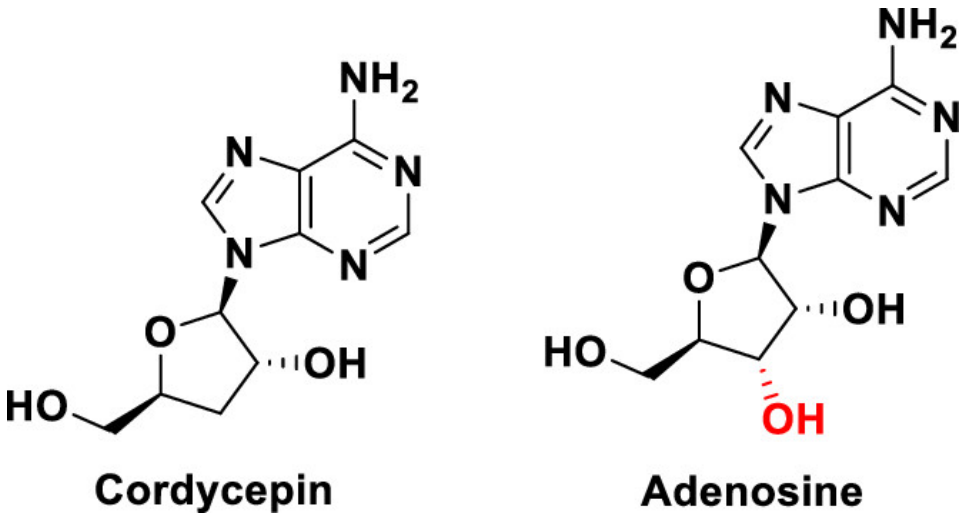

Figure 3. The structure of cordycepin (deoxyadenosine lacking the 3′ hydroxyl group) and adenosine. The 3' hydroxyl group of adenosine absent in cordycepin is highlighted with red.

The anti-inflammatory effects of cordycepin have been demonstrated in various cell lines and animal models. It is hypothesized that this effect is mediated through the inhibition of intracellular signaling mechanisms, leading to the cessation of pro-inflammatory cytokine production. Cordycepin particularly attenuates the transcription of inflammatory protein genes, mediated by NF-κB and MAPK, but it also targets the $A2_A$ and A3 adenosine receptors. These actions likely hinder the expression of pro-inflammatory cytokines and, additionally, can activate repair mechanisms in cells under excessive inflammation or ischemic conditions (Du et al., 2021; Tan et al., 2021). Furthermore, the interferon-inducing effect of cordycepin has also been described. These inflammatory mediators play a significant role in reducing viral replication *in vivo*, suggesting that cordycepin may activate the cytoplasmic RIG-I and, through the RIG-I signaling axis, potentially stimulate the production of type I interferons (Ch et al., 2016). Although certain signaling cascades influenced by cordycepin are known (e.g., NF-κB, MAPK), their precise downstream effects remain unpredictable. The mechanism of action has been studied across multiple macrophage cell models [**Figure 4.**, A: (Lu et al., 2015), B:(Choi et al., 2014), C:(Shin et al., 2009a), D:(Kim et al., 2006), E:(Imamura et al., 2015), F:(Yang et al., 2017), G:(Shin et al., 2009b), H:(Qing et al., 2017), I:(Liu et al., 2025), J:(Jeong et al., 2010), K:(Peng et al., 2015), L:(Dou et al., 2016)]

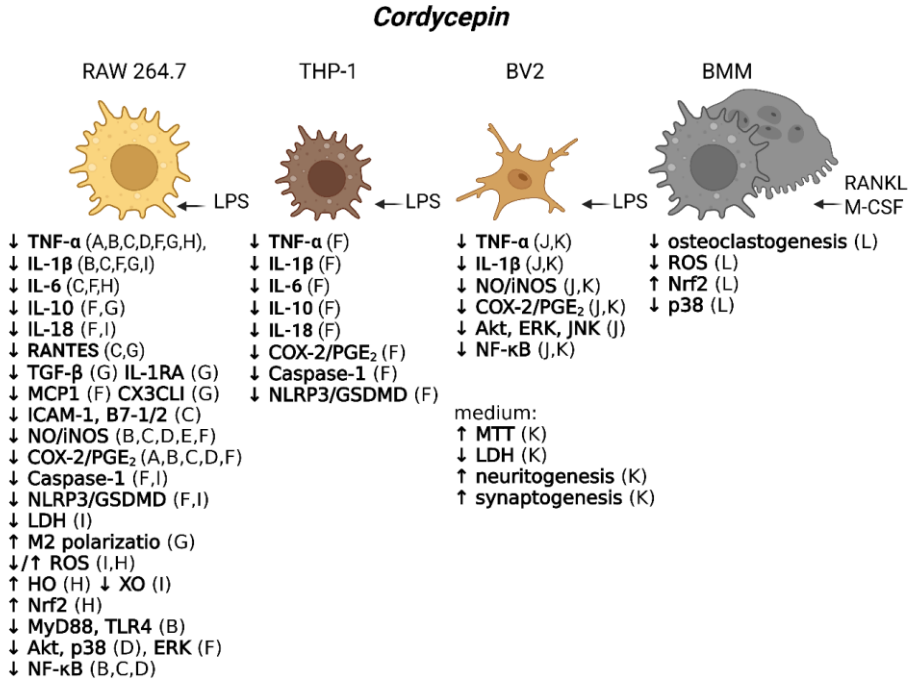

Figure 4. Effects of cordycepin on different macrophage cultures (literature data). Own figure, created by BioRender.com. Literature data indicated by letters A-L according to the citations of the related text above. BV2= murine microglial cell line, RAW 264.7=murine macrophage-like cell line, THP-1= human leukemia monocytic cell line, BMM=bone marrow-derived primary macrophages, RANKL= receptor activator of nuclear factor kappa-B ligand (osteoclast precursor), M-CSF=macrophage colony-stimulating factor IL-1RA= interleukin-1 receptor antagonist, MCP1= monocyte chemoattractant protein-1, CX3CLI= C-X3-C motif chemokine ligand 1, B7-(1/2)=cluster of differentiation (80/86), GSDMD=gasdermin D. The explanations of the rest of the abbreviations can be found in the Introduction and literature overview

3.9 Cell cultures and *ex vivo* models

Cell culturing began in the late 19th and early 20th centuries, with foundational work by scientists such as Wilhelm Roux, who maintained living cells outside the body in saline in 1885, and Ross Granville Harrison, who developed the first *in vitro* techniques using frog embryonic tissue in 1907 (Moro et al., 2024). The introduction of antibiotics in the 1940s revolutionized the field by reducing contamination, and the establishment of immortalized cell

lines like HeLa in the 1950s enabled more consistent and long-term studies (Duval et al., 2017). Primary cell cultures are created by isolating cells directly from living tissues. These cultures closely mimic the natural physiological state and cellular diversity of their tissue of origin, making them essential tools for studying normal cell function and disease mechanisms *in vitro*. Unlike immortalized cell lines, which can proliferate indefinitely but often lose key physiological traits, primary cultures retain the native characteristics and behavior of the original tissue, though they have a limited lifespan (Moro et al., 2024).

The concept significantly precedes the establishment of cell lines as the experiments of Ross Granville Harrison were carried out with frog embryonic nerve cells cultured according to the principles of primary cell cultures. Around the same time, Alexis Carrel developed techniques to culture chicken embryonic heart tissue. Even earlier, in 1885, Wilhelm Roux maintained chicken embryo cells in a saline solution, laying foundational groundwork for modern cell culturing (Moro et al., 2024). Two-dimensional (2D) cell cultures, in case of both cell lines and primary cultures, quickly became the standard due to their simplicity, cost-effectiveness, and ease of analysis. In case of these, cells grow simply as a monolayer on flat surfaces. These 2D cultures have been instrumental in advancing our understanding of cell biology, disease mechanisms, drug screening, and protein production. However, while 2D cell cultures remain widely used, their inability to fully mimic the three-dimensional environment and cell interactions of living tissues has led to the development of more physiologically relevant 3D models in recent years (Duval et al., 2017). In contrast, 3D cell culture models better reproduce the *in vivo* cellular environment with realistic cellular responses and interactions and more complex morphology. Modern 3D techniques—such as hydrogels, polymeric scaffolds, spheroids, and organoids—enable advanced disease modeling, and drug testing. These systems offer versatile applications and improve experimental outcomes compared to traditional 2D culture (Jensen and Teng, 2020). Spheroid cultures created by scaffold-free techniques like the hanging drop method mimic tissue architecture by suspending cells in small droplets, where gravity or other forces (e.g., magnetic force) induce aggregation to form compact 3D cell clusters typically ranging from 100 to 600 μm in diameter; however, some studies have reported spheroids exceeding 1,000 μm in diameter, demonstrating the capability to model thick tissue structures with multiple cellular layers and complex microenvironments (Brophy et al., 2009; Shi et al., 2018; Zanoni et al., 2016). Polymeric scaffolds are synthetic or natural biomaterials offering tunable mechanics and architecture that guide cell adhesion, migration, and differentiation in 3D, useful for tissue engineering (Jensen and Teng, 2020). Matrigel cultures use a protein-rich, extracellular matrix-like hydrogel as

natural scaffold that supports cells growing in three dimensions supplying biochemical cues and structural support. The Matrigel scaffold is capable of promoting the formation of organoids with the highest structural complexity in cell culturing (Hughes et al., 2010) . With complex tissue-like architecture these so-called organotypic cultures are complex 3D structures grown from patient-derived stem cells or tissue sample, recapitulating organ-specific architecture and function for personalized disease modeling and therapy (Hu et al., 2018). Primary tissue fragments, retaining the extracellular matrix from the donor tissue, cultured *in vitro* with special growth media, fall under the category of organoids (Hu et al., 2018). Explant models were developed as an intermediate solution between animal and cell culture systems, thus enabling the study of the physiological function and pathological states of target organs and tissues (Randall et al., 2011). The history of the first explants, or ex vivo models, dates back to the 1950s, when the mechanisms of nutrient absorption were studied using excised segments of the intestine. Initially, tissue and organ cultures were entirely restricted to embryonic tissues, until 1959, when Trowell first cultured mature tissues in synthetic media (Trowell, 1959; Wilson and Wiseman, 1954). A decade later, Browning and Trier successfully established an ex vivo culture of intestinal mucosa based on Trowell's method (Browning and Trier, 1969). Explant culturing necessitates a liquid-tissue-gas interface and an oxygen-rich environment since anoxia is the main cause of cell necrosis in explant cultures. Therefore, Browning and Trier introduced the intestinal explant technique at a relatively late point in the scale of explant studies. The breakthrough happened thanks to providing 95% O_2. Today, both human and animal research extensively utilize intestinal explants. Their layered and polarized structure, which includes essential cell-cell interactions, is its primary benefit over gut epithelial cell cultures. Intestinal explants could replicate the gut-specific cytokine production. Thus the model allows for the investigation of the effects of various substances. For instance, the role of gliadin in the pathogenesis of human coeliac disease has been described using explant cultures, and the model has significantly advanced the understanding of the inflammatory responses occurring in the gut in relation to other diseases, such as inflammatory bowel disease (Randall et al., 2011).

4 Significance and aims of the study

With the emergence of microbes resistant to certain antibiotics, the WHO forecast warns us that antimicrobial resistance will imply a significant threat: the number of deaths caused by resistant bacterial infections worldwide is predicted to increase 15-fold by 2050 (Manyi-Loh et al., 2018; Robinson et al., 2016). Notwithstanding that the application of antimicrobials as growth promoters was banned in the EU in 2006, the still abundant, often prophylactic or metaphylactic overuse of these agents in the livestock sector can strongly increase the risk of resistance development (Fair and Tor, 2014; Manyi-Loh et al., 2018). In poultry farming, viral infections constitute a major predisposing factor—alongside suboptimal husbandry conditions—by compromising the integrity of the intestinal barrier and impairing immune function, thereby facilitating the pathogenic activity of facultative bacteria.

Broiler farming faces profound economic loss because of virus-induced immunosuppression during the first few weeks of production. According to our present knowledge, infectious bursal disease virus and chicken anemia virus are presumed to be able to break through immunity in the first few weeks of production and can deplete the primary lymphoid organs via cytokine overproduction, cytotoxic T cell response, and apoptosis of the lymphocytes (Hoerr, 2010; Santen et al., 2004; Sharma et al., 2000). Avian reoviruses could harm the liver and the intestines as well, leading to malabsorption syndrome together with other non-enveloped ubiquitous viruses of RSS (Lin et al., 2007; Rehman et al., 2018). The detachment of the epithelial lining facilitates *Salmonella* septicemia and enterotoxemia. Incidental fowl adenovirus causes liver damage in immunosuppressed individuals and manifests a potential threat during the first four weeks of production (Choi et al., 2012; Mariani et al., 2001; Niu et al., 2018).

These data demonstrate that virus-induced diseases in chickens play an outstanding role. The application of novel natural feed additives could mitigate the damage and, therefore, assist the industry in curtailing unnecessary antibiotic application for growth promotion and against secondary pathogens. In the poultry industry, there is growing evidence that the enhanced performance previously obtained with the subtherapeutic application of antibiotics is attainable with natural feed additives of plant origin (Aljumaah et al., 2020; Ayalew et al., 2022; Buchanan et al., 2008; Demir et al., 2003). The beneficial effect of these substances on animal health and productivity could be mediated by their anti-inflammatory, cytoprotective, and antioxidant

properties (Alagawany et al., 2021; Rathee et al., 2009), which should be extensively investigated.

The scientific literature on specific plant- and fungus-derived metabolites is detailed and comprehensive. However, studies using mammalian models dominate existing academic sources. The broiler industry operates on tight profit margins, making it highly sensitive to even minor increases in production costs. As the broiler industry is one of the most intensive sectors in food production, I believe any approach aimed at enhancing the quantity and quality of the final product should be welcomed, given its particular significance from both economic and public health perspectives.

In vitro studies are inevitable to obtain beneficial feed additives and paramedical products. By screening potential pro-inflammatory substances (flagellin, poly I:C and LPS) on chicken primary hepatocyte – non-parenchymal cell co-cultures, it was aimed to set and characterize an inflammatory chicken hepatic cell model suitable for challenging substances with antioxidant and immunomodulatory effects. However, according to the body of literature, the cell-cell interactions within the gut are even more complex to replicate. Explant systems comply with the 3R principles of laboratory animal experiments since they enable the preparation of multiple cultures from a single animal while providing a tissue-specific response within a regulated environment (Randall et al., 2011; Russo et al., 2016). Nevertheless, preventing anoxia-induced damage to the explants is challenging (Randall et al., 2011). Moreover, the use of uniform and size-standardized explant cultures remains limited. Therefore, the author aimed to follow the histology and viability parameters of 1 and 2 mm diameter-sized explants obtained with biopsy punch to describe possible size-dependent alterations and intended to describe the effects of PAMPs on the explant model of the ideal size after 12 h culturing. This work leads to the development and characterization of a potential *ex vivo* model of the chicken ileum.

<u>The objectives of the PhD thesis are presented below:</u>

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

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

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

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

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

5 Materials and methods

5.1 Ethic statement

The cells for the chicken primary hepatocyte – non-parenchymal cell co-culture s and the ileal explants were isolated from a 3-week-old male Ross-308 broiler chicken. The animals were provided by Gallus Ltd. (Devecser, Hungary) and were raised and fed following the guidelines of the breeder (Aviagen Ross Broiler Management Handbook 2018, 2018). The experiments were executed in conformity with institutional policies, approved by the Local Animal Welfare Committee of the University of Veterinary Medicine Budapest and by the Government Office of Zala County, Food Chain Safety, Plant Protection, and Soil Conservation Directorate, Zalaegerszeg, Hungary (number of permission: GK-419/2020; approval date: May 11, 2020).

5.2 Chemicals and reagents

All chemicals applied for cell and tissue isolation, establishment and treatment of cell cultures and for the measurements were obtained from Merck KGaA (Darmstadt, Germany), except where the source of the chemical was specifically indicated.

5.3 Study design

The present thesis aimed to investigate the anti-inflammatory and cell protective effects of two plant derived secondary metabolites, CA and luteolin and further *Cordyceps militaris* extract (CME) rich in fungal active metabolites (e.g. cordycepin) on chicken *in vitro* and *ex vivo* experimental models. First and foremost, reliable inflammatory models were necessary to carry out the exact experiments. Therefore, in Preliminary Study I. the proper PAMPs were selected to induce elevated cytokine production on the chicken primary hepatocyte – non-parenchymal cell co-culture, previously established and characterized by our research group. Firstly, it enabled us to carry out Study I.A and Study I.B As flagellin and poly I:C both contributed to proper inflammatory models, these PAMPs were further applied (Study I.A: poly I:C, Study I.B: flagellin), meanwhile LPS was excluded from the subsequent experiments. Afterwards a chicken gastrointestinal model was established and characterized, which could be used for higher-throughput microplate format experiments. This model provides uniform and

size-standardized chicken gut explant cultures. The inflammatory response of these explants was tested under exposure to the previously favoured PAMPs and in addition LTA. The miniature chicken ileal explant model was successfully applied with all the PAMPs, therefore poly I:C as a robust interferon inducer mimicking viral RNA induced inflammation was selected to carry out Study II. Moreover, as the importance of cordycepin and *Cordyceps militaris extracts* are investigated for the supplementary treatment of viral infectious diseases, it was the selected protective extract for the last experiment (Study II.) of the present PhD thesis.

In Preliminary Study I, a chicken primary hepatocyte – non-parenchymal cell co-culture model was applied to establish a reliable and reproducible inflammatory platform. In the present study, the impact LPS, flagellin, as well as poly I:C were investigated. Cell viability was evaluated by quantifying metabolic activity and extracellular LDH activity, whereas the concentrations of interleukin-6 (IL-6) and interleukin-8 (IL-8) were determined as inflammatory parameters. Redox parameters were not evaluated in this preliminary study.

Study I.A employed the same chicken primary hepatocyte – non-parenchymal cell co-culture model to examine the immunomodulatory effects of poly I:C and CA compared to N-acetylcysteine (NAC) as a reference treatment. Cell viability was assessed via metabolic activity, extracellular LDH activity, and caspase-3 levels. The inflammatory response was characterized through quantification of the concentrations of IL-6, IL-8, IL-10, interferon-alpha (IFN-α), interferon-gamma (IFN-γ), and macrophage colony-stimulating factor (M-CSF), whereas MDAlevels were quantified to assess redox status.

In Study I.B, the chicken primary hepatocyte – non-parenchymal cell co-culture model was further employed, this time focusing on the impact of flagellin and luteolin. Cell viability was evaluated by metabolic activity and extracellular LDH activity, and the inflammatory response was evaluated by quantifying IL-6, IL-8, IL-10, IFN-α, and IFN-γ concentrations. Redox parameters comprised quantification of H_2O_2 and MDA levels.

Preliminary Study II. was performed employing a chicken ileal explant model to extend the scope of the investigation. In this experiment LTA, flagellin, and poly I:C were tested. Cell viability was determined by metabolic activity and extracellular LDH activity, whereas the inflammatory response was assessed through quantification of the concentrations of IL-2, IL-6, IL-8, IL-10, IFN-α, IFN-γ, and regulated on activation, normal T cell expressed and secreted (RANTES). Redox parameters were not incorporated in the present study.

Study II. utilized the chicken ileal explant model to evaluate the impact of poly I:C and Cordyceps militaris extract. Cell viability was evaluated by quantifying metabolic activity, extracellular LDH activity. The inflammatory response was characterized by determining the

concentrations of IL-1β, IL-6, IFN-γ, and RANTES, whereas redox status was evaluated by quantifying H_2O_2 and MDA levels.

The detailed study design can be seen in **Table 3**.

Table 3. Study design.

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

5.4 Preliminary study I.: Establishment of a hepatic inflammatory model

5.4.1 Cell isolation and culturing of chicken hepatic co-cultures

A single chicken was anesthetized with carbon dioxide and then slaughtered via decapitation. Then access to the liver through the gastropancreaticoduodenal vein of the hepatic portal system was provided (**Figure 5.**). The buffers for the perfusion of the liver were all incubated at 40°C and pre-oxygenated with Carbogen (95% O_2 and 5% CO_2). A perfusion rate of about 30 mL/min was used. First, 150 mL Hanks' Balanced Salt Solution (HBSS) buffer containing 0.5 M ethylene glycol tetra-acetic acid (EGTA) was applied, followed by 150 mL EGTA-free HBSS. Thereafter the liver was perfused with 100 mL HBSS buffer freshly supplemented with 100 mg collagenase type IV (Nordmark, Uetersen, Germany), 7 mM $CaCl_2$ and 7 mM $MgCl_2$, to disintegrate the hepatic interstitium.

Figure 5. Perfusion of the chicken liver through the gastropancreaticoduodenal vein.

Following the excision of the liver, the Glisson's capsule was disrupted, and the freshly gained cells were suspended in 50 mL ice-cold HBSS which contained bovine serum albumin (2.5%) to avoid cluster formation. The cell suspension was filtered through three layers of sterile gauze to remove any leftover cell aggregates and the undigested interstitium, then it was incubated on ice for 50 min. Afterward, the hepatocyte and non-parenchymal cell containing fractions were separated using multistep differential centrifugation. The cell suspension was centrifuged three times at 100x g for 3 min in Williams' Medium E, which was 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). After each step, the supernatants containing the non-parenchymal cells were collected separately and hepatocyte containing pellets were resuspended in the cell culture medium. Finally, a purified hepatocyte fraction was received.

The supernatants collected were centrifuged at 350x g for 10 min to separate the non-parenchymal cell fraction via the sedimentation of the remaining hepatocytes and red blood cells. Thereafter, the gained supernatant was again centrifuged at 800x g for 10 min. After resuspending, the final sediment contained the non-parenchymal cell enriched fraction. Then the viability of both the hepatocytes and non-parenchymal cells was examined separately by trypan blue exclusion test: the number of living cells should be over 90%, and the cell load was determined by cell counting in Bürker's chamber to adjust the appropriate cell concentrations (10^6 cells/mL). Previously, both hepatocyte and non-parenchymal cell enriched fractions have been characterized by flow cytometry and immunofluorescent detection using anti-albumin and chicken macrophage specific antibodies (Mackei et al., 2020a, 2020c; Sebők et al., 2021).

The cell suspensions were mixed in the ratio of 6:1 (hepatocytes to non-parenchymal cells) and then seeded onto 24-well and 96-well plates (Greiner Bio-One Hungary Kft., Mosonmagyaróvár, Hungary) which were previously coated with collagen type I (10 µg/cm²). The seeding volume was 400 µL/well for the 24-well plates and 100 µL/well for the 96-well plates. They were incubated at 37°C in a humid atmosphere with 5% CO_2. After 4 h the culture media were changed and the confluent co-cultures were attained after 24 h. The medium during the experiments was supplemented correspondingly to the Williams' Medium E (WM) used for the seeding. FBS was only applied for the first 24 h of culturing. After 24 h incubation and Giemsa staining of some wells of the 24-well plates, the confluent structure of the cultures was visible (**Figure 6.**).

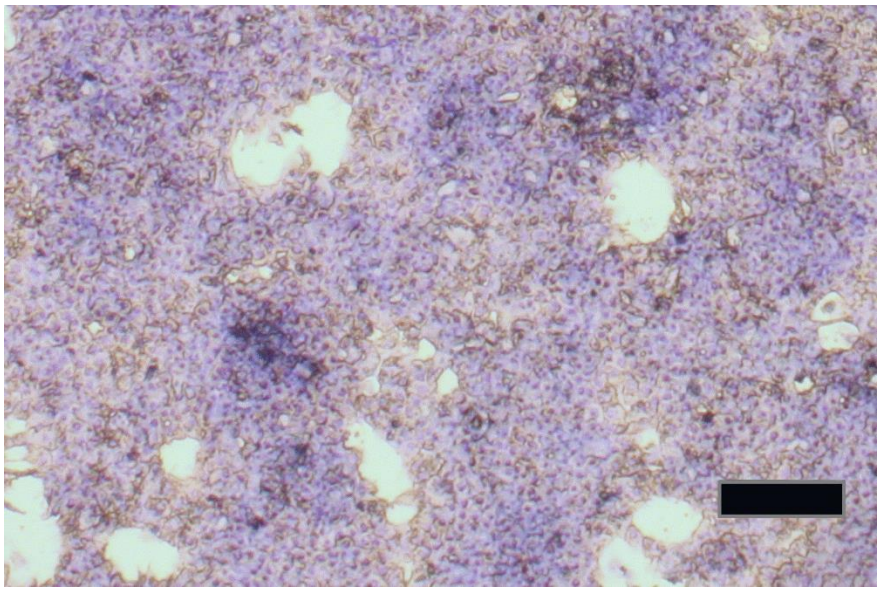

Figure 6. Giemsa staining of hepatocyte – non-parenchymal cell co-cultures after 24 h culturing (bar = 200 µm).

5.4.2 Treatment of the cultures

The medium of the cultures was supplemented with 0 (control), 10 or 50 µg/mL LPS from *Escherichia coli* (O55:B5) for 24 h (Farnell et al., 2003; Kalaiyarasu et al., 2016; Kogut et al., 2005; Zhang et al., 2017), and in a separate plate cell cultures were exposed to culture media supplemented with 0 (control), 100 or 250 ng/mL *Salmonella* Typhimurium derived flagellin (Abdel-Mageed et al., 2014; Kamimura et al., 2017; Kogut et al., 2008), further with 50 or 100 µg/mL poly I:C (Abdel-Mageed et al., 2014; Kamimura et al., 2017) for 24 h. To achieve the re-annealing the poly I:C was heated at 50 °C for 3 min then cooled down before added to cell culture media.

5.4.3 Measured parameters

Metabolic activity was measured by a CCK-8, while extracellular LDH activity by a colorimetric assay kit (Diagnosticum Ltd, Budapest, Hungary). The concentration of IL-6 and IL-8 were determined by a chicken-specific sandwich ELISA test (MyBioSource, San Diego, CA, USA).

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

5.5.1 Cell isolation and culturing

The cells were isolated and cultured according to the protocol of 5.4.1.

5.5.2 Treatment of the cultures

After 24 h, the cultured cells were treated with WM which was 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 100 or 200 µg/mL NAC and 10 or 100 µg/mL CA. The remaining treatments consisted of combinations of 50 µg/mL poly I:C and 100 or 200 µg/mL NAC or 50 µg/mL poly I:C together with 10 or 100 µg/mL CA. The treatment groups are listed in **Table 4.** (n=6/group).

Table 4. Overview of treatment groups in Study I.A. Poly I:C = polyinosinic-polycytidylic acid.

Group	Poly I:C	Chicoric acid	N-acetylcysteine
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**

The treated cells were incubated for 24 h. Then cell culture media were collected from the 24-well plates in aliquots, and 50 µl/well mammalian protein extraction reagent (M-PER) lysis buffer was added to the wells. The removal of the cells from the plate was facilitated with a cell scraper and by repeated up and down pipetting to gain cell lysates. Cell culture medium and cell lysate samples were stored at -80°C until the measurement of certain parameters.

5.5.3 Measured parameters

Metabolic activity was measured by a CCK-8, while extracellular LDH activity by a colorimetric assay kit (Diagnosticum Ltd, Budapest, Hungary). The concentration of IL-6 and IL-8 were determined by a chicken-specific sandwich ELISA test (MyBioSource, San Diego, CA, USA). Meanwhile, IL-10, IFN-α, IFN-γ, M-CSF concentrations were quantified by a Luminex Milliplex Chicken Cytokine/Chemokine Panel. MDA level was determined by a colorimetric assay.

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

5.6.1 Cell isolation and culturing

The cells were isolated and cultured according to the protocol of 5.4.1.

5.6.2 Treatment of the cultures

After 24 h of incubation, cells were cultured in WM supplemented as detailed in chapter 2.1 but without FBS and further supplemented with flagellin derived from *S.* Typhimurium at the concentrations of 0 (control) and 250 ng/mL, with 0, 4 or 16 µg/mL luteolin, or with the combination of flagellin (250 ng/mL) and luteolin (4 or 16 µg/mL, Cat. L9283, Merck KGaA) for 24 h (**Table 5.**). Flagellin and luteolin stock solutions were freshly prepared with pure WM.

Table 5. Overview of treatment groups in Study I.B.

Group	Flagellin	Luteolin
Control	**-**	**-**
L1	**-**	**4 µg/mL**
L2	**-**	**16 µg/mL**
F	**250 ng/mL**	**-**
FL1	**250 ng/mL**	**4 µg/mL**
FL2	**250 ng/mL**	**16 µg/mL**

The 400 µL/well culture medium of cells in 24-well plates was then collected, and they were lysed in 100 µL/well M-PER lysis buffer. Samples of cell lysate and culture medium were stored at -80 °C until the measurements.

5.6.3 Measured parameters

Metabolic activity was measured by a CCK-8, while extracellular LDH activity by a colorimetric assay kit (Diagnosticum Ltd, Budapest, Hungary). The concentration of IL-6 and IL-8 were determined by a chicken-specific sandwich ELISA test (MyBioSource, San Diego, CA, USA). Meanwhile, IL-10, IFN-α, IFN-γ, concentrations were quantified by a Luminex Milliplex Chicken Cytokine/Chemokine Panel. H_2O_2 and MDA level was determined by a colorimetric assay.

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

5.7.1 Tissue sample isolation and excision of the explants

The used Penicillin-Streptomycin (Pen-Strep) is a product of Gibco (Waltham, MA, USA), and HCMTM SingleQuotsTM Kit (Catalog No. CC-4182) is a product of Lonza-Biocenter (Szeged, Hungary). The latter contains ascorbic acid, bovine serum albumine, hydrocortisone, transferrin, human epidermal growth factor, insulin, gentamicin and amphotericin-B. Reusable biopsy punches with plungers were purchased from MDE GmbH (Heidelberg, Germany).

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 10cm 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 according to Udden et al (Udden et al., 2017). The intestinal section was cut longitudinally on the mesenteric side and washed three consecutive times with PBS + Pen-Strep solution. This step might be repeated until no physical contaminants are visible. After that, 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., Mosonmagyaróvár, Hungary) coated with collagen type I

(10g/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 (DMEM-F12) supplemented with FBS 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 (**Figure 7.**):

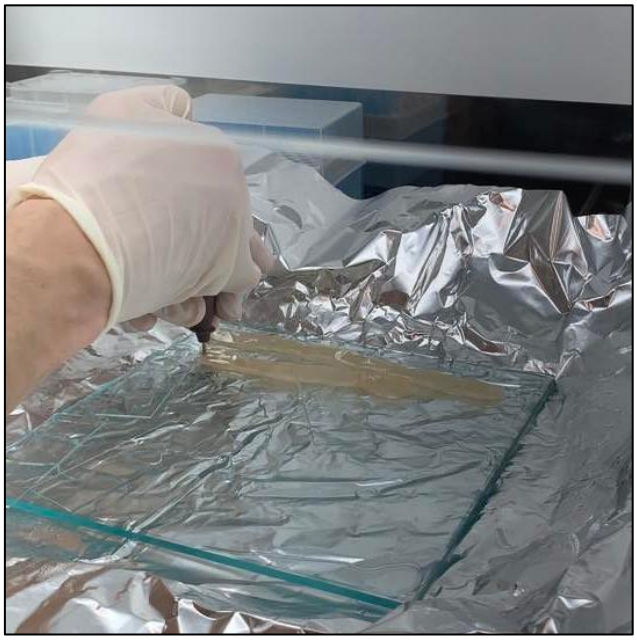

Figure 7. Excision of the intestinal explants.

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

5.7.2 Culturing and treatment of the explants

5.7.2.1 Preliminary Study II.1

The viability, cell membrane damage and histology of 1 and 2 mm diameter explant cultures were assessed. The cultures were obtained according to section 5.7.1. Six 1 and 2 mm diameter explants were fixed instantly with 10% neutral buffered formalin on biopsy sponges at room temperature for further evaluation (H&E stain). The remaining explants were directly deposited into the cell culture plate wells. 1 mm explants were cultured on 96-well plates in 100 µl, and the 2 mm explants were placed in the wells of 24-well plates in 400 µl media proportionately to the volume of the explant to make the biological response of 1 and 2 mm explants comparable. The shape of the 1 and 2 mm explants was 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 LDH measurements (H&E stain and pan-cytokeratin immunohistochemistry) from both the 1 and the 2 mm diameter explants. The media of the explants was changed every 12 h, and the removed medium samples were stored for LDH measurement in the case of separate LDH groups of explants. A CCK-8 assay was performed with concomitant medium changes before and after a measurement on the separate CCK-8 groups. In case of the 24 h histology groups a simple change of the medium has been carried out. Most of the 2 mm explants for the CCK-8 and LDH tests were macroscopically disrupted after approximately 48 h of culturing, therefore the measurements were terminated after 36 h. 1 mm samples were maintained longer with regular medium changes every other day. After 9 days, the study was concluded, and the 1 mm explants were fixed on the plate wells and stained according to Giemsa.

5.7.2.2 Preliminary Study II.2

Based on the results of Preliminary Study II.1 and for practical reasons (The repetitive change of media can be technically difficult in case of 1 mm explants.), the smallest obtainable and manageable, 1.5 mm diameter explants were excised. Five explants were incubated on 96 well plates in separate wells filled with 200 µl of medium. The medium was changed 1 h after seeding. The explants were left untreated for 12 h, fixed with formalin on biopsy sponges, and stored for H&E staining and pan-cytokeratin immunohistochemistry.

5.7.2.3 Preliminary Study II.3

Based on the results of Preliminary Study II.1 and II.2, 1.5 mm diameter explants were obtained. The samples (n=5/group) were placed in 200 µl of medium on 96-well plates. The medium was changed 1 h after seeding for the treatment media supplemented with the following chemicals in six separate groups: 10 or 50 µg/mL LTA from *Staphylococcus aureus* or 100 and 250 ng/mL *Salmonella* Typhimurium-derived flagellin, or with 50 and 100 µg/mL poly I:C for 12 h, respectively. The concentrations were selected based on preliminary results and previous studies on chicken cell cultures (Sebők et al., 2021). Poly I:C was heated at 50°C for 3 min and then cooled down before adding it to cell culture media to achieve the re-annealing. 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.

5.7.3 Measured parameters and histology

Metabolic activity of explants was measured by CCK-8 assay after 1, 12, 24 and 36 h for Preliminary Study II.1 and after 12 h for Preliminary Study II.3. The extracellular LDH enzyme activity indicating membrane damage was determined by a kinetic photometric assay from samples of culture medium obtained after 1, 12, 24 and 36 h for Preliminary Study II.1 and after 12 h for Preliminary Study II.3. The concentration of IL-8 was determined by a chicken-specific sandwich ELISA test (MyBioSource, San Diego, CA, USA). Meanwhile, IFN-α, IFN-γ, IL-2, IL-6, IL-10 and RANTES concentrations were quantified by a Luminex Milliplex Chicken Cytokine/Chemokine Panel in case of Preliminary Study II.3.

The explants from Preliminary Study II.1 directly after the sampling and after 24 h of culturing and in case of Preliminary Study II.2 after 12 h of culturing were fixed on foam biopsy pads. After 24 h of fixation at room temperature in 10% neutral buffered formalin, 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 with the orientation to allow the largest possible surface area to be cut. Thus 3-4 µm thin sections were made 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 using an automatic staining instrument. The H&E slides were examined to evaluate and compare the explants focusing on three criteria of Kolf-Clauw et al. investigated on a porcine jejunal explant culture, precisely the lesion of the tissue, the number of crypts and the enterocyte morphology (Kolf-Clauw et al., 2009).

To compare 1 and 2 mm samples from Preliminary Study II.1 after 24 h of culturing and to assess the morphology of the samples from Preliminary Study II.2 after 12 h of culturing, pan-cytokeratin immunohistochemistry was applied to evaluate the integrity of intestinal epithelium via the assessment of cytokeratin intermediate filaments. Sections were deparaffinized in xylene (2×15 min) and rehydrated in 96% and absolute ethanol for 5-5 mins. It was followed by a 2x rinse in distilled water for a few mins. Antigen retrieval was performed in EnVision FLEX target retrieval solution, High pH 9 (50x) in a microwave (800 Watts for 5 mins, then 180 Watts for 10 mins), then the sections were flushed with PBS 2 times. EnVision FLEX Peroxidase-Blocking reagent was added for 5 mins, 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 mins at room temperature. The slides were rinsed 2x in PBS before and after the 20-min incubation of the secondary antibody (Dako, EnVision Flex HRP). The staining was displayed with DAB Chromogen (Dako Envision Flex DAB + Chromogen 1 drop + 1 mL Envision Flex Substrate Buffer) for 1-3 mins 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 mins. The slides were covered with coverslips after dehydration in ethanol and xylene (3-3 min in 96% and absolute ethanol, and 2×3 min in xylene).

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

5.8.1 Tissue sample isolation and excision of the explants

The explants were obtained according to the protocol of 5.7.1 Tissue sample isolation and excision of the explants.

5.8.2 Culturing and treatment of the explants

The 1.5 mm diameter-sized explants were cultured and treated according to 5.7.2.3. Preliminary Study II.3 for 12 h. The treatment groups were established as follows. The experimental groups received either 0.12% (m/m) or 0.6% (m/m) CME formulated with dextrose (COR1 and COR2). According to the analytical examination of the CME, which was performed using liquid chromatography–tandem mass spectrometry (LC-MS/MS) at the

Institute of Bioanalytics and Agro-Metabolomics, Department of Agrobiotechnology, University of Natural Resources and Life Sciences, Vienna (BOKU), the powder contains 5264 ng/g cordycepin, 4592 beauveriolide I and 9912 ng/g beauveriolide III, and approximately 1‰ β-glucan. The components and the mycotoxin profile of the CME is detailed in the Supplementary Material. Respectively, the control groups were treated with 0.12% (m/m) dextrose (C1) and 0.6% (m/m) dextrose (C2). To induce inflammation, explants were treated with 50 µg/mL poly I:C in combination with either 0.12% (m/m) or 0.6% (m/m) dextrose (PC1 and PC2). Furthermore, combined treatment groups were established, in which explants received 0.12% (m/m) or 0.6% (m/m) CME formulated with dextrose and 50 µg/mL poly I:C (PCOR1 and PCOR2) (**Table 6.**).

Table 6. Overview of treatment groups in Study II. Poly I:C = polyinosinic-polycytidylic acid.

Group	Poly I:C	Dextrose	Cordyceps militaris extract
C1 (Control)	**-**	**0.12% (m/m)**	**-**
C2 (Control)	**-**	**0.6% (m/m)**	**-**
COR1	**-**	**-**	**0.12% (m/m)**
COR2	**-**	**-**	**0.6% (m/m)**
PC1	**50 µg/mL**	**0.12% (m/m)**	**-**
PC2	**50 µg/mL**	**0.6% (m/m)**	**-**
PCOR1	**50 µg/mL**	**-**	**0.12% (m/m)**
PCOR2	**50 µg/mL**	**-**	**0.6% (m/m)**

After 12 h of incubation, the culture supernatants were collected and stored at -80°C for subsequent analysis.

5.8.3 Measured parameters

Metabolic activity was measured by a CCK-8, while extracellular LDH activity by a Lactate Dehydrogenase Activity Assay Kit. The concentration of IL-1β was determined by a chicken-specific sandwich ELISA test (MyBioSource, San Diego, CA, USA). Meanwhile, IL-

6, IFN-γ, and RANTES concentrations were quantified by a Luminex Milliplex Chicken Cytokine/Chemokine Panel. H_2O_2 and MDA level was determined by a colorimetric assay.

5.9 Assays of the measured parameters

Metabolic activity of explants and the cultured cells was measured by CCK-8 assay (Cell counting Kit-8, Dojindo Molecular Technologies, Rockville, MD, USA). CCK-8 reagent working solution was diluted in a ratio of 1:10 with fresh indicator free medium (in case of the chicken primary hepatocyte – non-parenchymal cell co-cultures WM, in case of the chicken ileal explant DMEM-F12) and it was pipetted on the cultures. After 2 h of incubation under culturing conditions, 100µl of culture media sample was transferred to an empty 96-well plate to measure the absorbance according to the manufacturer's instructions at 340nm with a Multiskan GO 3.2 reader (Thermo Fisher Scientific, Waltham, MA, USA). By the assay, the amount of reduced nicotinamide adenine dinucleotide phosphate [NAD(P)H+H^+] produced in the cellular catabolic reactions was monitored.

The LDH activity is commonly applied as the tissue viability/breakdown parameter of *ex vivo* cultures and used as a common indicator of plasma membrane damage in case of monolayer cultures (Lim et al., 2012; Peñaranda et al., 2020; Zhang et al., 2017). The high LDH activity in the medium indicates that the cell membranes are damaged, and the intracellular enzyme is released into the medium. The extracellular LDH enzyme activity indicating membrane damage was determined by a kinetic photometric assay from samples of culture medium.

- An enzyme kinetic photometric assay (Diagnosticum Ltd., Budapest, Hungary) was applied in the first three experiments. Briefly, 200 µL of the working reagent [containing 56 mM phosphate buffer, pH 7.5; 1.6 mM pyruvate and 240 µM red reduced nicotinamide adenine dinucleotide (NADH+H^+)] was mixed with 10 µL cell culture medium and then the absorbance was measured at six consecutive time points with 1 min delay in a Multiskan GO 3.2 reader at 340 nm.
- In the explant experiments, a lactate dehydrogenase activity assay kit (Merck KGaA, Darmstadt, Germany) was utilized to measure the parameter. The enzyme reduces NAD^+ to NADH+H^+, which is detectable at 450 nm with a photometric reader. 50 µL of culture medium samples diluted with LDH Assay Buffer were added to 96-well microplates and mixed with 50 µL of freshly prepared Master Reaction Mix. After two mins of incubation at 37°C, the

absorbance was measured for the first time at 450 nm using a Multiscan GO 3.2 reader (Thermo Fisher Scientific Inc., Waltham, MA, United States). Every five mins, measurements were taken until the absorbance of the most active sample exceeded the value of the highest standard.

The IL-1β, IL-8 and in Preliminary study I and Study I.A, I.B, the IL-6 concentration was determined using a chicken-specific sandwich ELISA kit (MyBioSource, San Diego, CA, USA) according to the manufacturer's instructions. Following the principles of the ELISA assay, the target interleukin bound to the immobilized antibody on the plate surface. To ensure reaction specificity, a biotin-conjugated secondary antibody was applied to the plate, followed by the addition of an avidin-linked conjugate. Subsequently, the added color reagent produced a colored product, the intensity of which was measured photometrically at 450 nm using a Multiskan GO 3.2 instrument.

Luminex xMAP method was used to measure the protein concentrations of IL-2, IL-6, IL-10, IFN-α, IFN-γ, M-CSF and RANTES in the medium performing Milliplex Chicken Cytokine/Chemokine Panel (Cat.Nr.: GCYT1-16K, Merck KGaA, Darmstadt, Germany) following the instructions of the company. Concisely, all samples were thawed and tested in blind fashion. Two technical replicates were included. 25 µl of each sample, standard, control, and reaction buffer was added to a 96-well plate. An additional 25 µl of four, distinctly colored, primary antibody-coated bead sets was added to each well. Biotinylated detection antibody mixture and phycoerythrin-conjugated streptavidin were added to the plate following steps of washing after overnight incubation. After the last step, 150 mL drive fluid was added to the wells, the beads were resuspended for an additional 5 min on a plate shaker and read of the Luminex MAGPIX® instrument (Luminex Corporation, Austin, TX, USA). Luminex xPonent 4.2 was used for data acquisition. Five parameters logistic regression curves were generated as standard curves for all analytes by the Milliplex Analyst 5.1 (Merck Millipore, Darmstadt, Germany) or the Millipore Belysa 1.1 (Merck Millipore, Darmstadt, Germany) software calculating with bead median fluorescence intensity values.

The measurement of extracellular H_2O_2 level from the culture medium samples was performed using the Amplex Red assay (Thermo Fisher Scientific, Waltham, MA, USA). After incubation of 50 µL of Amplex Red (100 µM), horseradish peroxidase (0.2 U/mL) and 50 µL of culture medium at 21°C for 30 min, the fluorescence of the samples was measured at 531 nm with an excitation set at 590 nm using a Victor X2 2030 fluorimeter (Perkin Elmer, Waltham, MA, USA).

The concentration of intracellular malondialdehyde (MDA) was determined from cell or tissue lysates. This product is formed during lipid peroxidation and can be measured using a Lipid peroxidation (MDA) assay kit detecting thiobarbituric acid reactive substances. To 100 µL of the lysate samples, 300 µL of thiobarbituric acid was added, and the mixture was then incubated at 95°C for 1 h according to the manufacturer's instructions. This was followed by a 10-min cooling on ice, after which the absorbance of the sample was measured at 532 nm using a Multiskan GO 3.2 reader.

To investigate the rate of apoptosis, caspase-3 level was evaluated from cell lysate samples with chicken specific caspase-3 ELISA Kit (MyBioSource, San Diego, CA, USA) according to the instructions of the manufacturer. The absorbance was assessed at 450 nm with a Multiskan GO 3.2 reader.

The yielded concentrations of caspase-3 and MDA and each cytokine of the explant studies were corrected to the total protein content of the cell/explant suspension samples measured with BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

5.10 Statistical analysis

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

5.10.1 Preliminary study I.

For visualization, relative intensity (for the CCK results), relative concentration values (for interleukin measures), and relative change in absorbance (for the LDH activity) were calculated from the data by dividing each value by the average of the corresponding control group. Statistical significance was evaluated for each treatment to the corresponding control group using Wilcoxon signed rank test.

5.10.2 Study I.A

A correlogram was generated using the corrplot and the RColorBrewer package and the correlation coefficients (R2) were interpreted according to Mukaka (44). Results were assessed as the mean and the standard error of mean (SEM). Statistical significance was evaluated for each treatment to the corresponding absolute or poly I:C exposed control group using Wilcoxon signed-rank test. Specifically, the groups C1, C2, N1, N2 and P were compared to the absolute control, while groups with combined poly I:C + CA/NAC treatments (PC1, PC2, PN1 and PN2) were compared to group P.

5.10.3 Study I.B

Results were plotted as the mean and standard deviation (SD) on bar graphs using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). The measured concentrations of MDA, IL-6, IL-8, IFN-α, IFN-γ and IL-10 were standardized to the total protein concentration of the appropriate cell lysate as assessed with the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The Wilcoxon signed-rank test was applied to evaluate the significance of the differences between the absolute control (without flagellin and luteolin) and the single treatment (flagellin or luteolin) groups or between the flagellin exposed and the combined treatment (flagellin+luteolin) groups.

5.10.4 Preliminary study II.

Results were plotted as mean and SEM on bar graphs using GraphPad Prism software. In Preliminary Study II.1 paired T-test was applied to compare the metabolic activity of the same explant group at different time points. An unpaired T-test was used to compare the metabolic activity and the LDH activity of 1 and 2 mm explants. In Preliminary Study II.3 group means were compared with those of the untreated control group, using Wilcoxon signed-rank test. Cytokine values were normalized in GraphPad Prism and displayed as relative percentages. The smallest mean in each data set was defined as 0% and the largest as 100%. The difference between means was considered significant if the p-value was less than 0.05.

5.10.5 Study II.

The results were presented in bar charts using the GraphPad Prism software, expressed as mean values ± SEM. Groups PC1 and PC2 were compared to C1 and C2, while groups PCOR1 and PCOR2 were compared to groups PC1 and PC2, respectively. To compare group means, the Wilcoxon signed-rank test was used.

6 Results

6.1 Preliminary study I.: Establishment of a hepatic inflammatory model

No alteration was found in metabolic activity after LPS exposure, although there was a significant decrease after applying 50 and 100 µg/mL poly I:C ($p<0.001$, $p=0.004$, respectively) in cultured cells (**Figure 8.**). Concerning the extracellular LDH activity significant elevation was detected after 50 ng/mL poly I:C treatment ($p=0.032$, **Figure 8.**).

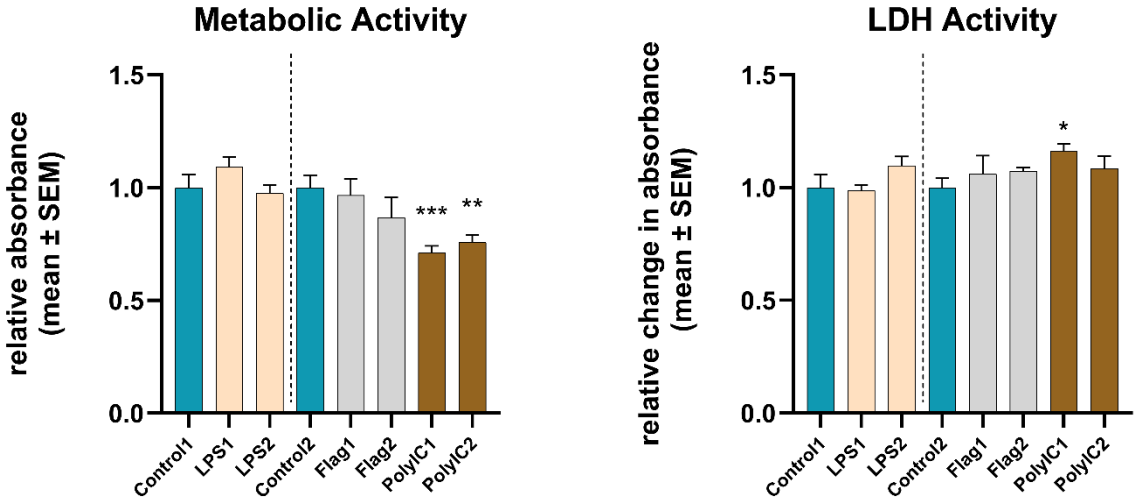

Figure 8. Metabolic activity ($n_{Control1,\ LPS}=6$/group, $n_{Control2,\ Flag,\ PolyIC}=10$/group) measured by CCK-8 assay and extracellular lactate dehydrogenase (LDH) activity ($n_{Control1,\ LPS}=6$/group, $n_{Control2,\ Flag,\ PolyIC}=5$/group) measured by an enzyme kinetic photometric assay. PolyIC1=50 µg/ml polyinosinic-polycytidylic acid, PolyIC2=100 µg/ml polyinosinic-polycytidylic acid, LPS1=10 µg/ml lipopolysaccharide (LPS) from *Escherichia coli*, LPS2=50 µg/ml lipopolysaccharide (LPS) from *Escherichia coli*, Flag1=100 ng/ml *Salmonella* Typhimurium flagellin, Flag2=250 ng/ml *Salmonella* Typhimurium flagellin. Mean ± SEM, *p≤0.05, **p<0.01, ***p<0.001. Group LPS1 and LPS2 were compared to Control1, while group Flag1, Flag2, PolyIC1 and PolyIC2 were compared to group Control2.

The IL-6 concentration was significantly elevated in the culture medium after 250 ng/mL flagellin (p=0.020) and 50 ng/mL poly I:C treatments (p=0.020, **Figure 9.**). 50 µg/mL poly I:C challenges significantly increased the concentration of IL-8 (p=0.036, **Figure 9.**).

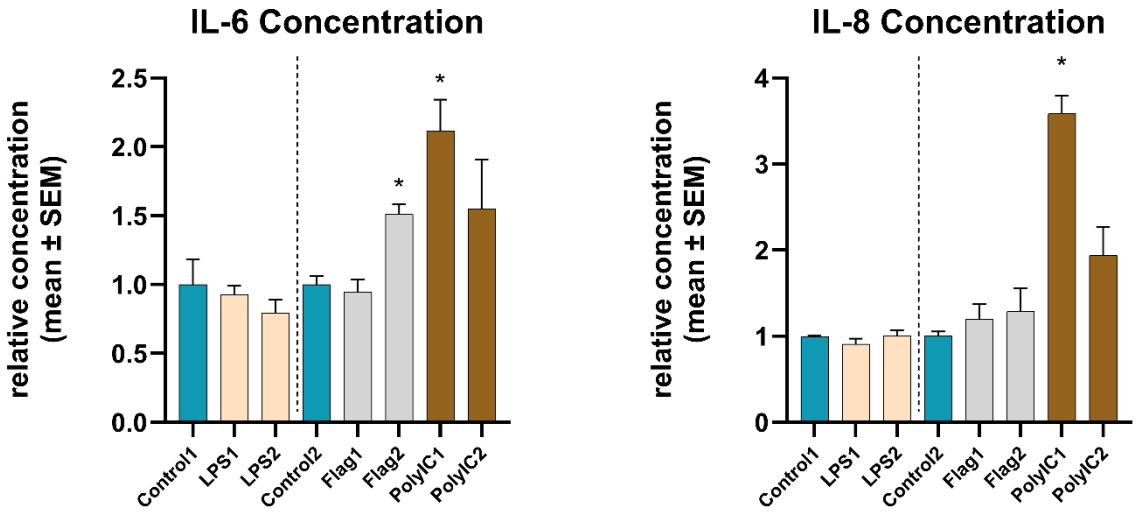

Figure 9. IL-6 ($n_{Control1, LPS}$=6/group, $n_{Control2, Flag, PolyIC}$ =5/group) and IL-8 concentration ($n_{Control1, LPS}$=6/group, $n_{Control2, Flag, PolyIC}$=5/group) of the cell culture medium measured by chicken specific ELISA. PolyIC1=50 µg/ml polyinosinic-polycytidylic acid, PolyIC2=100 µg/ml polyinosinic-polycytidylic acid, LPS1=10 µg/ml lipopolysaccharide (LPS) from *Escherichia coli*, LPS2=50 µg/ml lipopolysaccharide (LPS) from *Escherichia coli*, Flag1=100 ng/ml *Salmonella* Typhimurium flagellin, Flag2=250 ng/ml *Salmonella* Typhimurium flagellin. Mean ± SEM, *p≤0.05. Group LPS1 and LPS2 were compared to Control1, while group Flag1, Flag2, PolyIC1 and PolyIC2 were compared to group Control2.

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

The results show that NAC diminished the metabolic activity significantly (N1: p=0.002, N2: p=0.004). Moreover, a significant decrease in metabolic activity was observed in the group treated with poly I:C (p=0.009; **Figure 10.**) 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 suppressed by poly I:C.

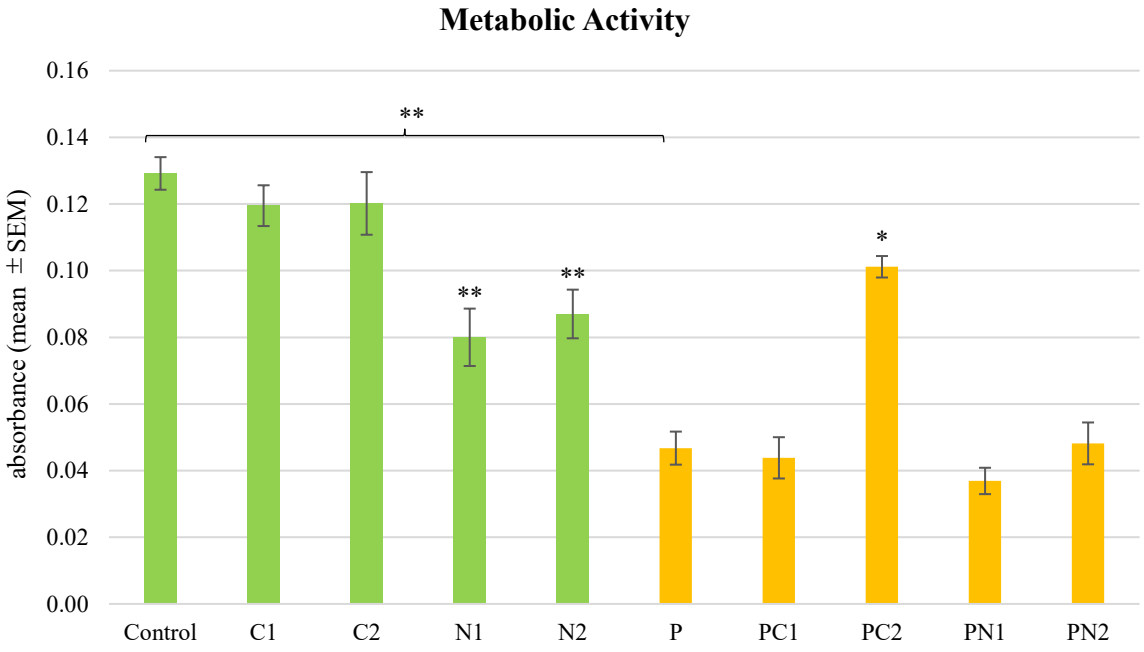

Figure 10. Metabolic activity measured by CCK-8 assay. C1=chicoric acid (10 µg/mL), C2=chicoric acid (100 µg/mL), N1=N-acetylcysteine (100 µg/mL), N2=N-acetylcysteine (200 µg/mL), P=poly I:C (50 µg/mL), PC1=poly I:C (50 µg/mL) and chicoric acid (10 µg/mL), PC2=poly I:C (50 µg/mL) and chicoric acid (100 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 (n=6/group) ± 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.

The results of the extracellular LDH activity indicating the increase of membrane permeability, reveal that the treatment with poly I:C induced the damage of the cultured cells (p=0.002; **Figure 11.**), while the combined exposure to poly I:C and CA resulted in a significant reduction in LDH activity (PC1, PC2: p=0.002). Correspondingly compared to the 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 elevated LDH level (p=0.041).

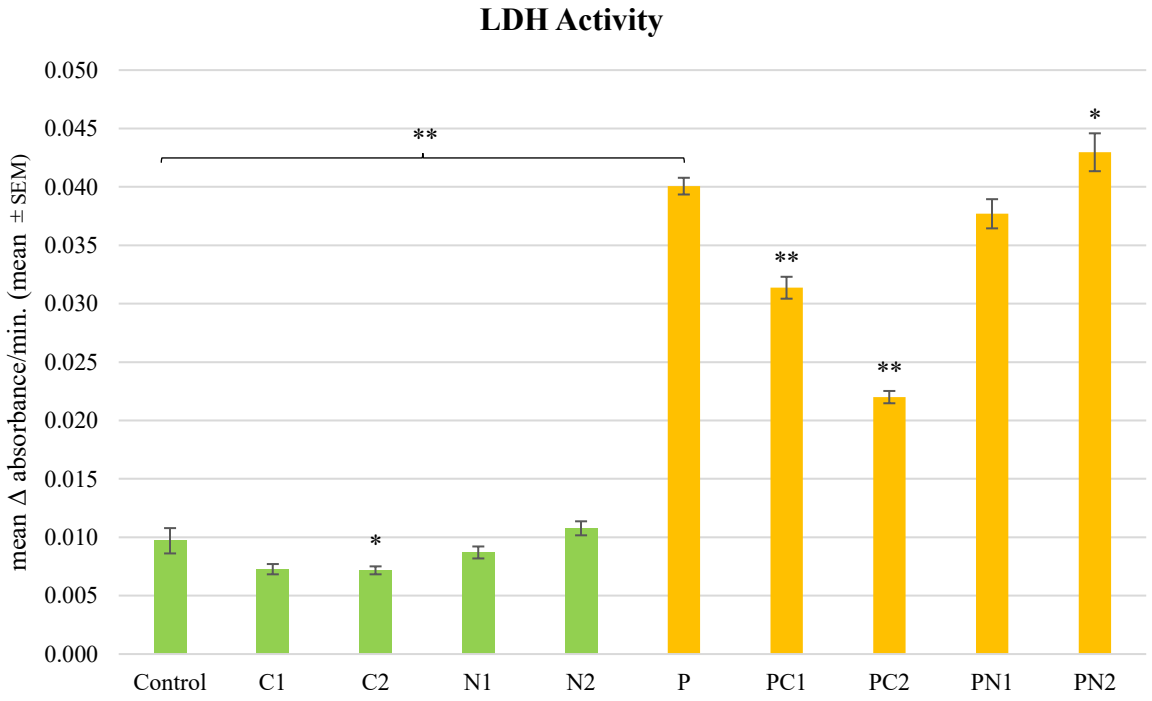

Figure 11. Extracellular lactate dehydrogenase activity measured by enzyme kinetic photometric assay. C1=chicoric acid (10 µg/mL), C2=chicoric acid (100 µg/mL), N1=N-acetylcysteine (100 µg/mL), N2=N-acetylcysteine (200 µg/mL), P=poly I:C (50 µg/mL), PC1=poly I:C (50 µg/mL) and chicoric acid (10 µg/mL), 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 (n=6/group) ± 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 assess the extent of lipid peroxidation and the damage of membrane components, MDA concentration was measured in the cell lysate. The MDA concentration was not affected by most of the treatments, which means that no significant lipid peroxidation was detected following the applied treatments compared to the control. Only the treatment with 10 µg/mL CA and poly I:C showed a significant decrease in MDA concentration (p=0.038; **Figure 12.**) compared to the treatment with poly I:C alone.

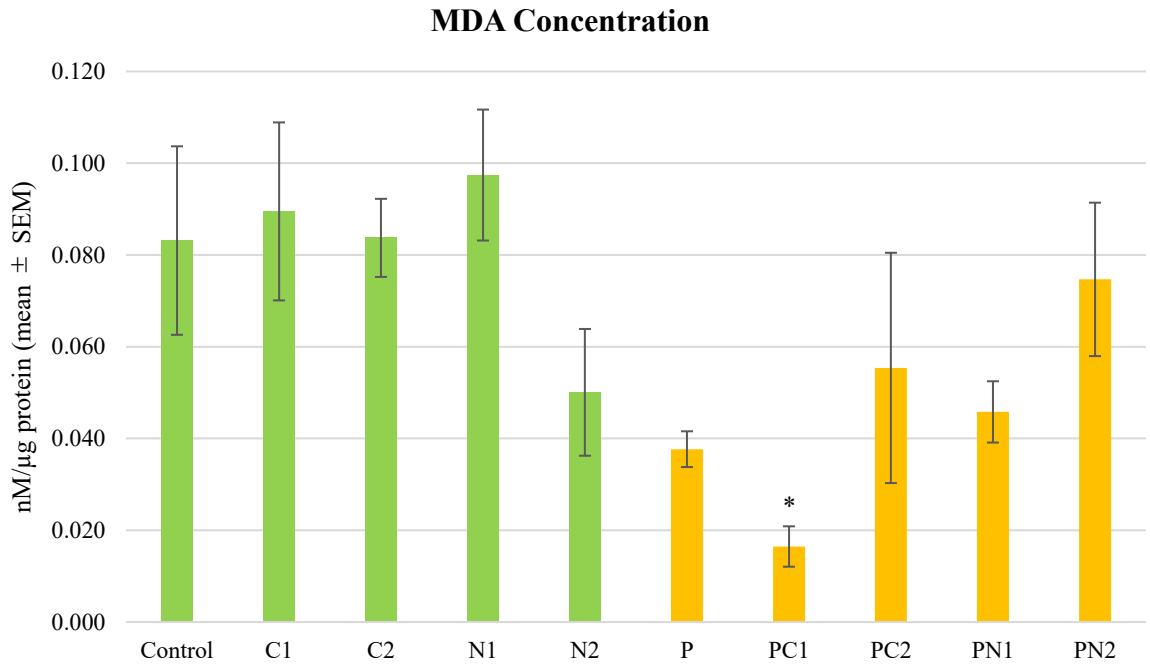

Figure 12. Malondialdehyde (MDA) concentration measured by colorimetric test. C1=chicoric acid (10 µg/mL), C2=chicoric acid (100 µg/mL), N1=N-acetylcysteine (100 µg/mL), N2=N-acetylcysteine (200 µg/mL), P=poly I:C (50 µg/mL), PC1=poly I:C (50 µg/mL) and chicoric acid (10 µg/mL), PC2=poly I:C (50 µg/mL) 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 (n=6/group) ± 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 application of CA induced a significant decrease in IL-6 (C1, C2: p=0.004; **Figure 13.**) and IL-8 concentration (C1: p=0.008, C2: p=0.004; **Figure 14.**) compared to control. Furthermore, the concentrations of IL-6 and IL-8 as pro-inflammatory 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 diminished the IL-6 (p=0.041) and IL-8 (p=0.002) concentrations significantly 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) in the combined treatment group compared to the level of the poly I:C treatment group.

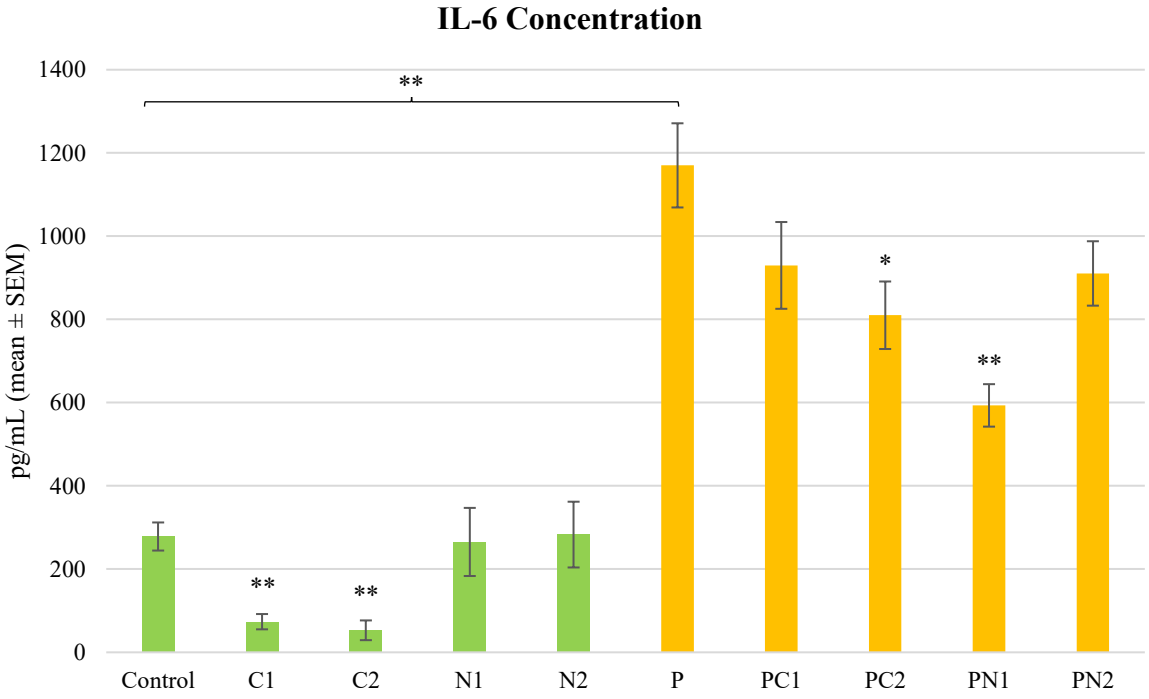

Figure 13. Measurements of IL-6 concentration using chicken specific ELISA. C1=chicoric acid (10 µg/mL), C2=chicoric acid (100 µg/mL), N1=N-acetylcysteine (100 µg/mL), N2=N-acetylcysteine (200 µg/mL), P=poly I:C (50 µg/mL), PC1=poly I:C (50 µg/mL) and chicoric acid (10 µg/mL), PC2=poly I:C (50 µg/mL) and chicoric acid (100 (50 µg/mL) and N-acetylcysteine (200 µg/mL). Mean (n=6/group) ± 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.

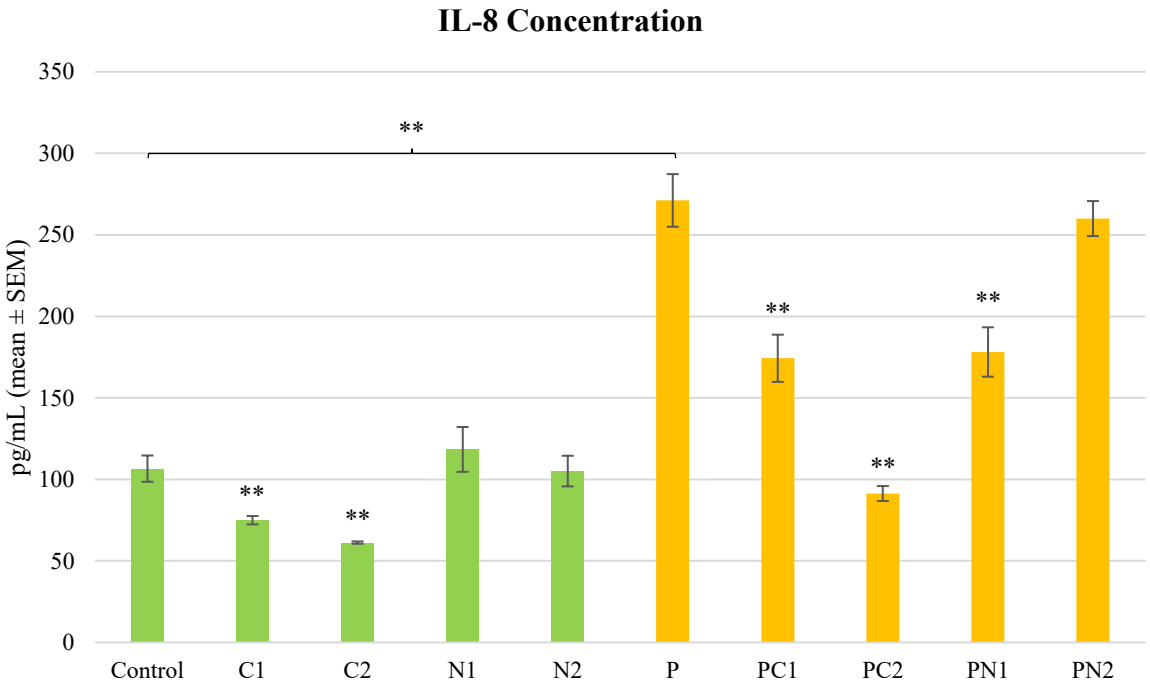

Figure 14. Measurements of IL-8 concentration using chicken specific ELISA. C1=chicoric acid (10 µg/mL), C2=chicoric acid (100 µg/mL), N1=N-acetylcysteine (100 µg/mL), N2=N-acetylcysteine (200 µg/mL), P=poly I:C (50 µg/mL), PC1=poly I:C (50 µg/mL) and chicoric acid (10 µg/mL), PC2=poly I:C (50 µg/mL) 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 (n=6/group) ± 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 a diminished level following 200 µg/mL NAC administration (p=0.036; **Figure 15.**) compared to the control group. Combined supplementation of poly I:C with lower concentration of NAC induced a significant decrease in the IL-10 concentration (p=0.030) set against the poly I:C exposed group. Meanwhile, the application of CA had no significant effect on IL-10 concentration.

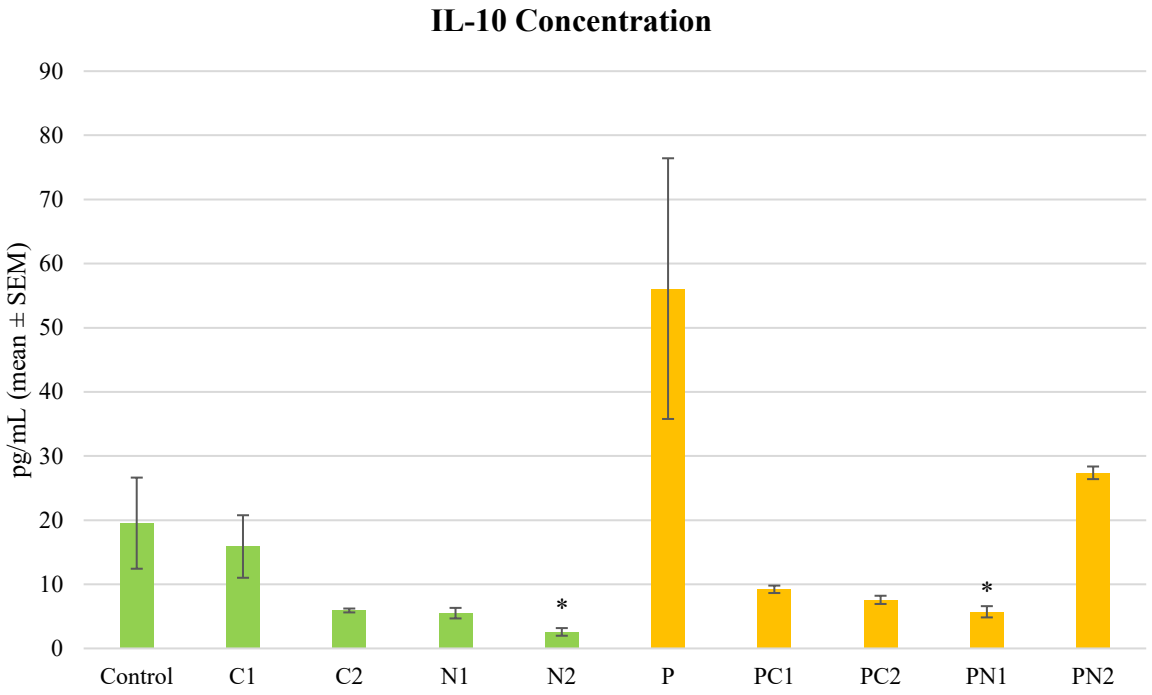

Figure 15. Measurements of IL-10 concentration using Luminex-based Milliplex immunoassay method. C1=chicoric acid (10 µg/mL), C2=chicoric acid (100 µg/mL), N1=N-acetylcysteine (100 µg/mL), N2=N-acetylcysteine (200 µg/mL), P=poly I:C (50 µg/mL), PC1=poly I:C (50 µg/mL) and chicoric acid (10 µg/mL), PC2=poly I:C (50 µg/mL) and chicoric acid (100 (50 µg/mL) and N-acetylcysteine (200 µg/mL). Mean (n=6/group) ± 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 M-CSF measurement revealed a significant decrease in the concentration of this chemokine in cells exposed to NAC (N1: p=0.002, N2: p=0.009; **Figure 16.**) 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).

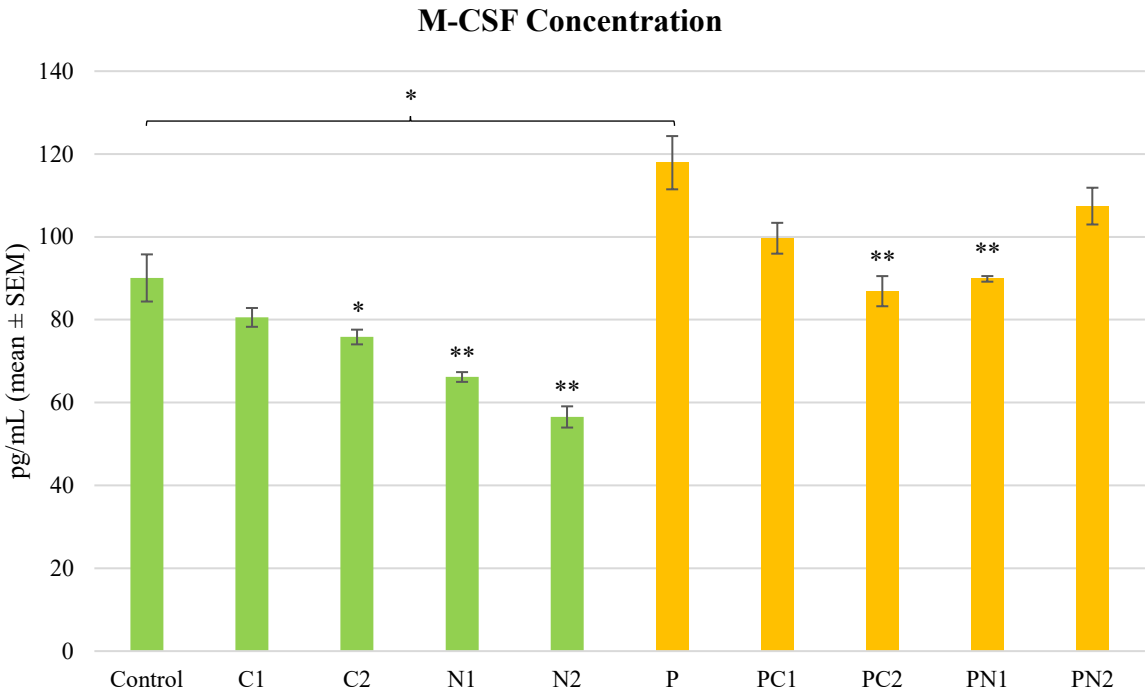

Figure 16. Measurements of M-CSF concentration using Luminex-based Milliplex immunoassay method. C1=chicoric acid (10 µg/mL), C2=chicoric acid (100 µg/mL), N1=N-acetylcysteine (100 µg/mL), N2=N-acetylcysteine (200 µg/mL), P=poly I:C (50 µg/mL), PC1=poly I:C (50 µg/mL) and chicoric acid (10 µg/mL), PC2=poly I:C (50 µg/mL) and chicoric acid (100 (50 µg/mL) and N-acetylcysteine (200 µg/mL). Mean (n=6/group) ± 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 17.**) and IFN-γ ($p=0.050$; **Figure 18.**) 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 ($p=0.044$). Neither CA, nor NAC application resulted in significant change compared to the solely poly I:C exposed group in the case of IFN-γ. Nevertheless significant difference between poly I:C exposed and control group was observed, but comparing the level of IFN-γ of PC1, PC2, PN1 group to the absolute controls, no significant difference could be noted, 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: $p=0.314$).

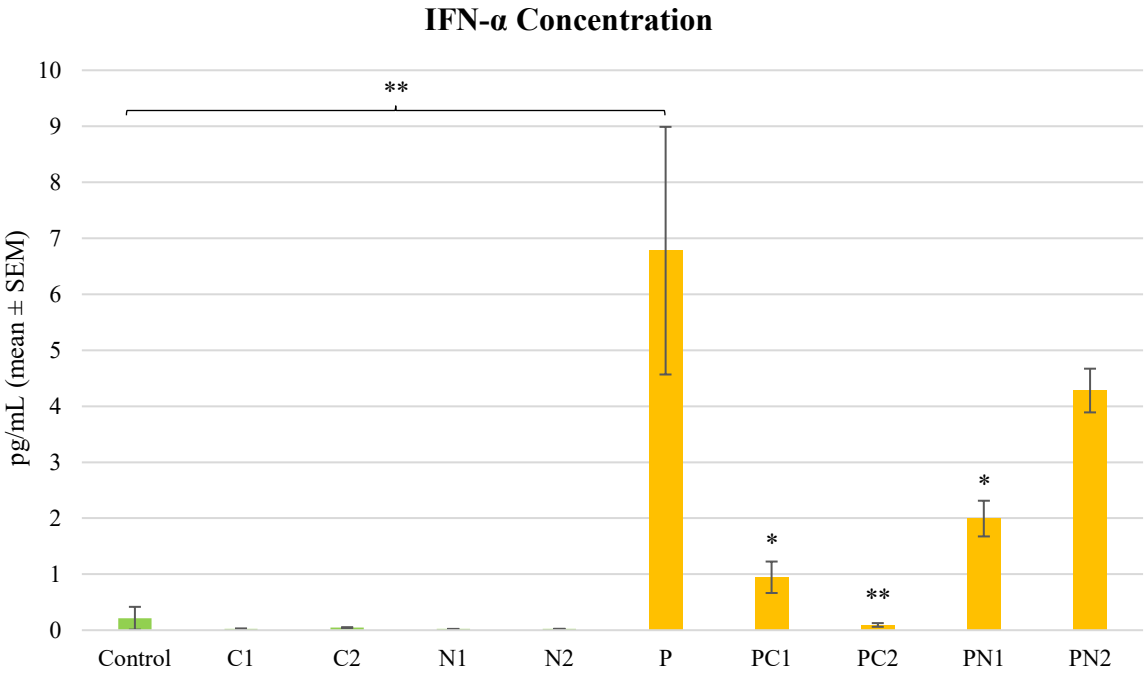

Figure 17. Measurements of IFN-α concentration using Luminex-based Milliplex immunoassay method. C1=chicoric acid (10 µg/mL), C2=chicoric acid (100 µg/mL), N1=N-acetylcysteine (100 µg/mL), N2=N-acetylcysteine (200 µg/mL), P=poly I:C (50 µg/mL), PC1=poly I:C (50 µg/mL) and chicoric acid (10 µg/mL), PC2=poly I:C (50 µg/mL) and chicoric acid (100 (50 µg/mL) and N-acetylcysteine (200 µg/mL). Mean (n=6/group) ± 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.

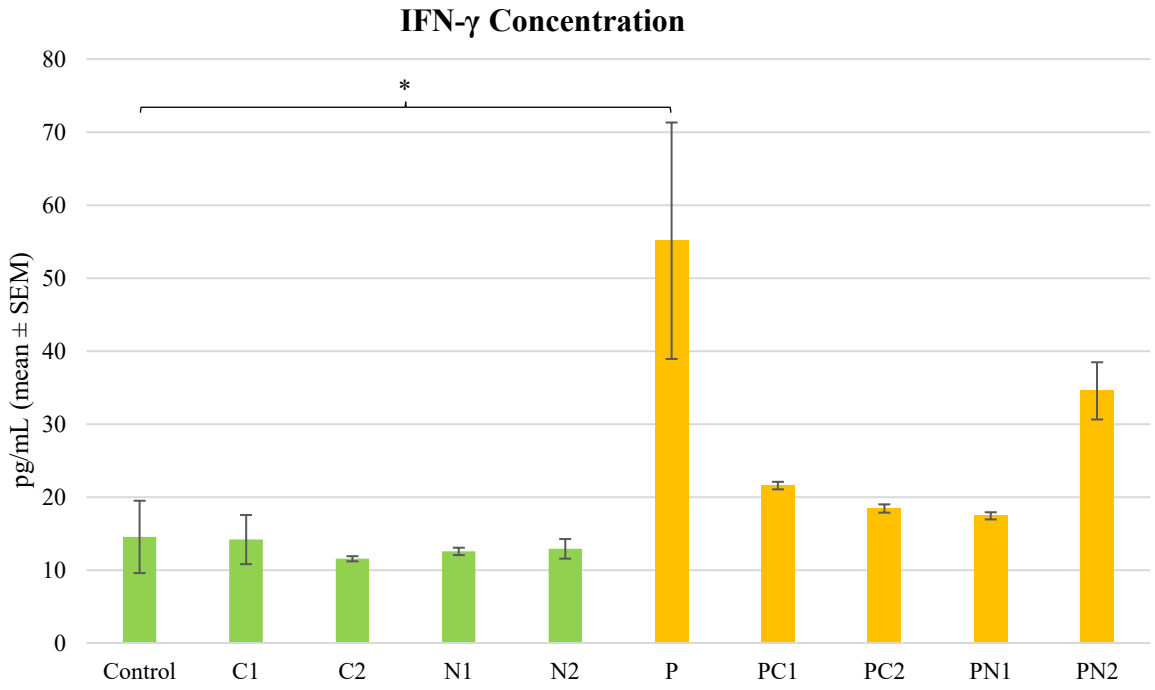

Figure 18. Measurements of IFN-γ concentration using Luminex-based Milliplex immunoassay method. C1=chicoric acid (10 µg/mL), C2=chicoric acid (100 µg/mL), N1=N-acetylcysteine (100 µg/mL), N2=N-acetylcysteine (200 µg/mL), P=poly I:C (50 µg/mL), PC1=poly I:C (50 µg/mL) and chicoric acid (10 µg/mL), PC2=poly I:C (50 µg/mL) and chicoric acid (100 (50 µg/mL) and N-acetylcysteine (200 µg/mL). Mean (n=6/group) ± 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.

Caspase-3 concentration was measured from the cell lysate to determine the rate of apoptosis. The results indicate that none of the treatments had a significant effect on this parameter (**Figure 19.**). Nevertheless, poly I:C induced a notable decrease in caspase-3 level (p=0.093) compared to the control. Furthermore, PC2 treatment group showed remarkable elevation in caspase-3 concentration (p=0.066) compared to the decreased value of group P.

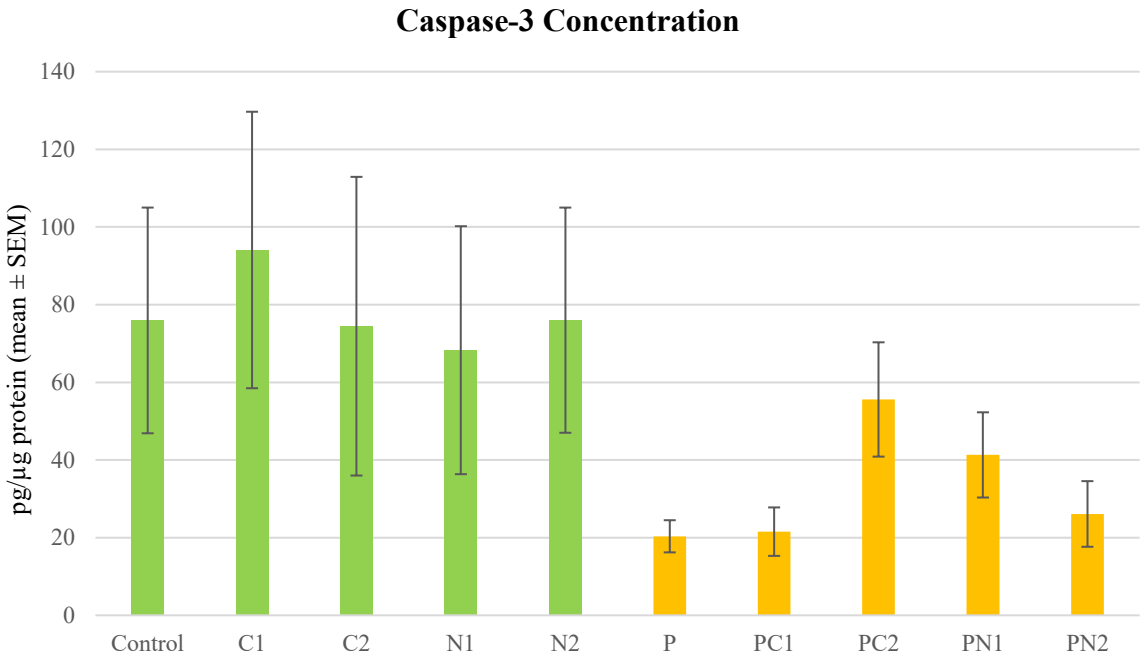

Figure 19. Measurements of Caspase-3 concentration using chicken specific ELISA. C1=chicoric acid (10 µg/mL), C2=chicoric acid (100 µg/mL), N1=N-acetylcysteine (100 µg/mL), N2=N-acetylcysteine (200 µg/mL), P=poly I:C (50 µg/mL), PC1=poly I:C (50 µg/mL) and chicoric acid (10 µg/mL), PC2=poly I:C (50 µg/mL) 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 (n=6/group) ± SEM. 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)

High ($90>R^2>70$) or moderate ($R^2>50$) positive correlation was found between each of the measured cytokines and between all cytokines and LDH apart from IL-6 and IL-10 plus LDH and IL-10 (**Figure 20.**). A high or moderate negative correlation was found between the metabolic activity and the concentration of cytokines: IL-6, IL-8, and IFN-α. Additionally, the highest negative correlation was detected between the LDH level and metabolic activity.

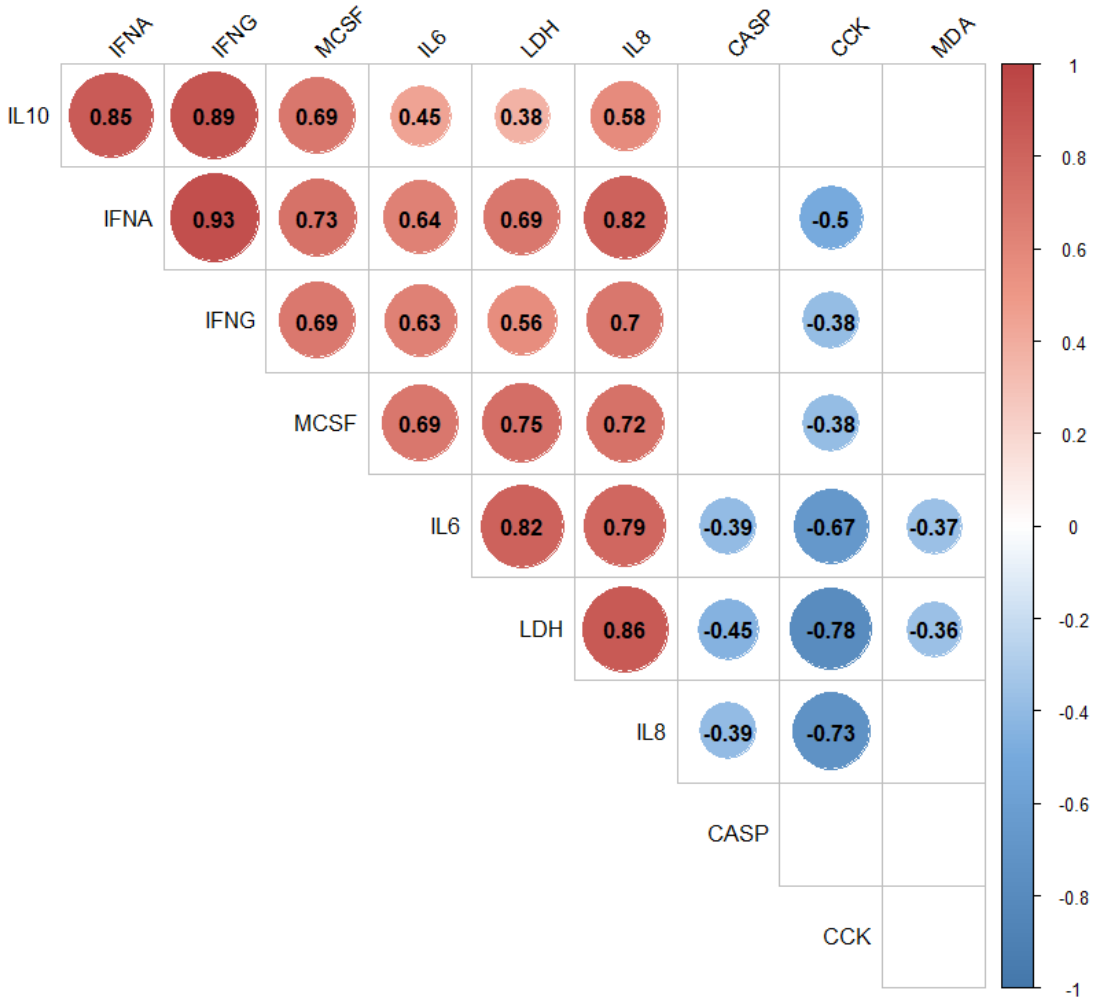

Figure 20. The Correlogram presents the significant positive (red) and negative (blue) correlations with the respective R2 values indicated between the measured parameters. IFNA=interferon-α, IFNG=interferon-γ, MCSF=macrophage colony-stimulating factor, IL6=interleukin-6, IL8=interleukin-8, IL10=interleukin-10, LDH=lactate dehydrogenase, CASP=caspase-3,

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

The higher applied dose of luteolin (L2, 16 µg/mL) significantly (p=0.002) reduced the metabolic activity of the cells (**Figure 21.**). 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**

22.). 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.

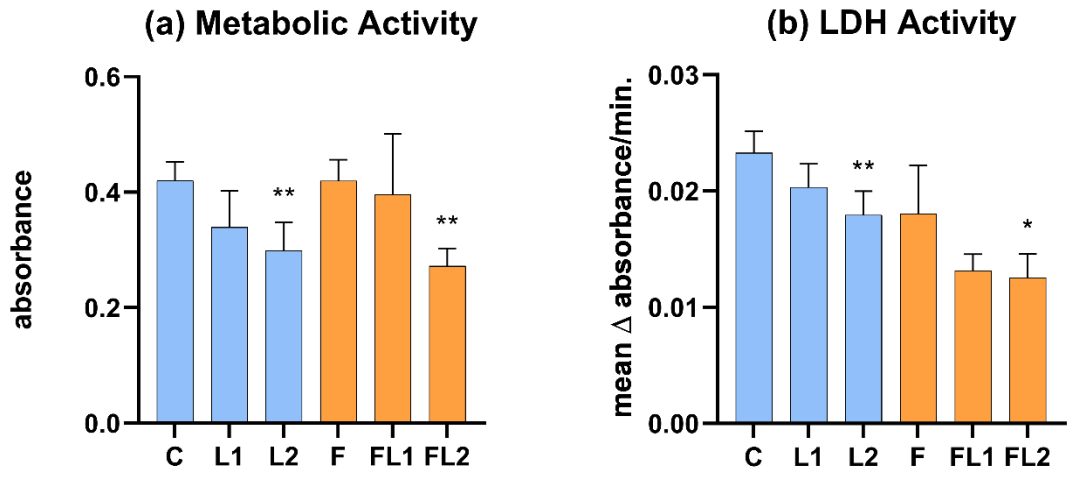

Figure 21. Metabolic activity measured by CCK-8 assay (a) and extracellular lactate dehydrogenase activity measured by an enzyme kinetic photometric assay (b). C=control, L1=luteolin (4 µg/mL), L2=luteolin (16 µg/mL), F=flagellin (250 ng/mL), FL1=flagellin (250 ng/mL) and luteolin (4 µg/mL), FL2=flagellin (250 ng/mL) and luteolin (16 µg/mL). Mean (n = 6/group) ± SD, *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 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 22.**). No significant change has been observed in the level of IFN-γ (**Figure 22.**). 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 22.**). 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 22.**).

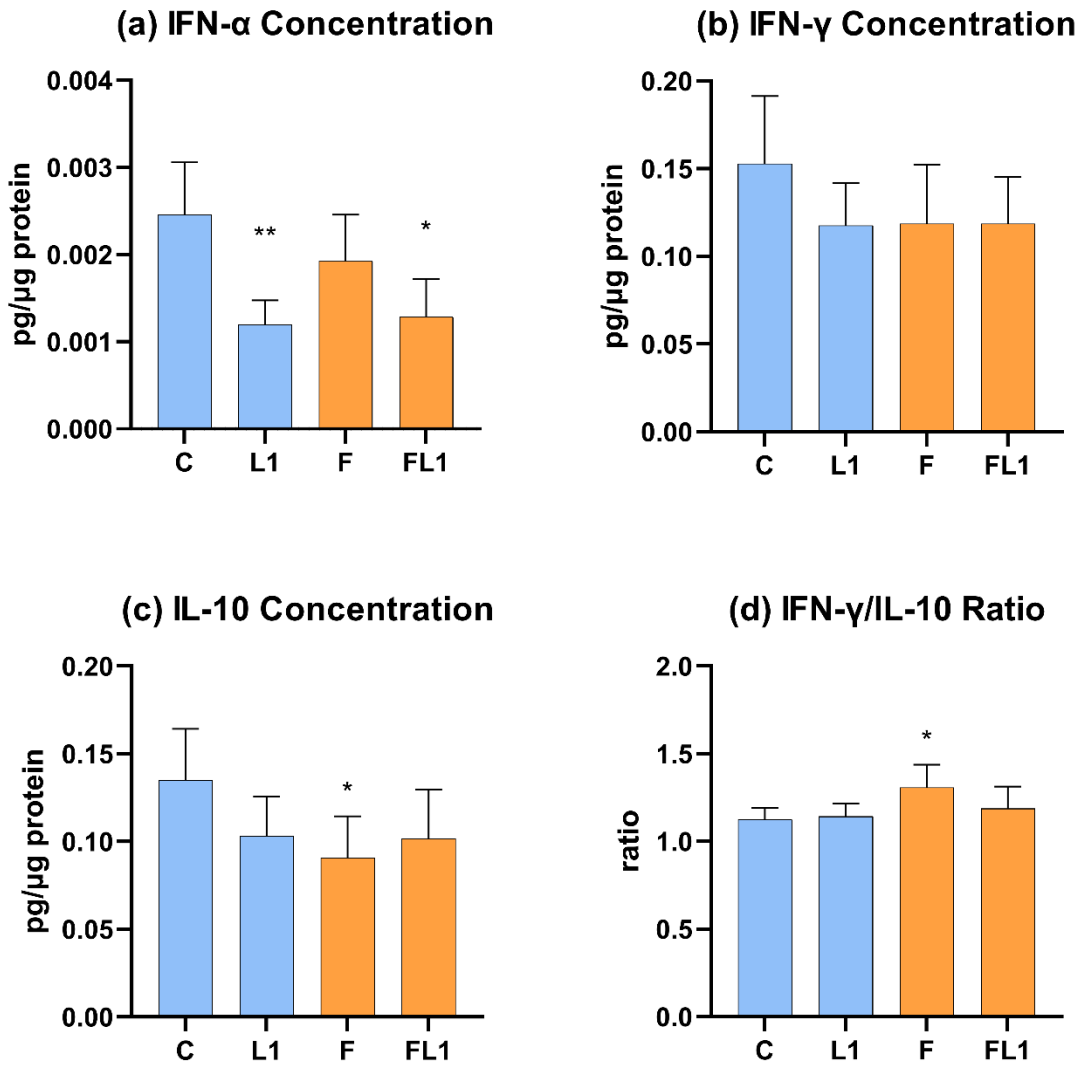

Figure 22. IFN-α (a), IFN-γ (b), IL-10 (c) concentration of the cell culture medium measured using Luminex-based Milliplex immunoassay method and the calculated IFN-γ/ IL-10 ratio (d). C=control, L1=luteolin (4 µg/mL), F=flagellin (250 ng/mL), FL1=flagellin (250 ng/mL) and luteolin (4 µg/mL). Mean (n = 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.

No significant difference in IL-6 concentration was observed between any of the investigated treatment groups (**Figure 23.**). IL-8 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 23.**).

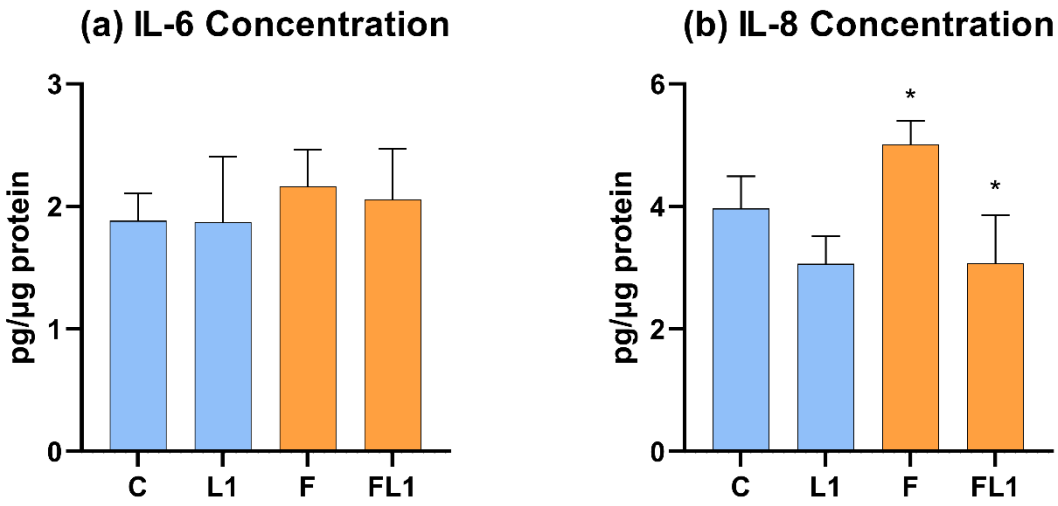

Figure 23. IL-6 (a) and IL-8 (b) concentration of the cell culture medium measured by chicken specific ELISA. C=control, L1=luteolin (4 µg/mL), F=flagellin (250 ng/mL), FL1=flagellin (250 ng/mL) and luteolin (4 µg/mL). Mean (n = 6/group) ± SD, *p≤0.05. Group L1 and F were compared to C, while group FL1 was compared to group F.

The decrease in extracellular H_2O_2 levels 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 24.**). 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 24.**).

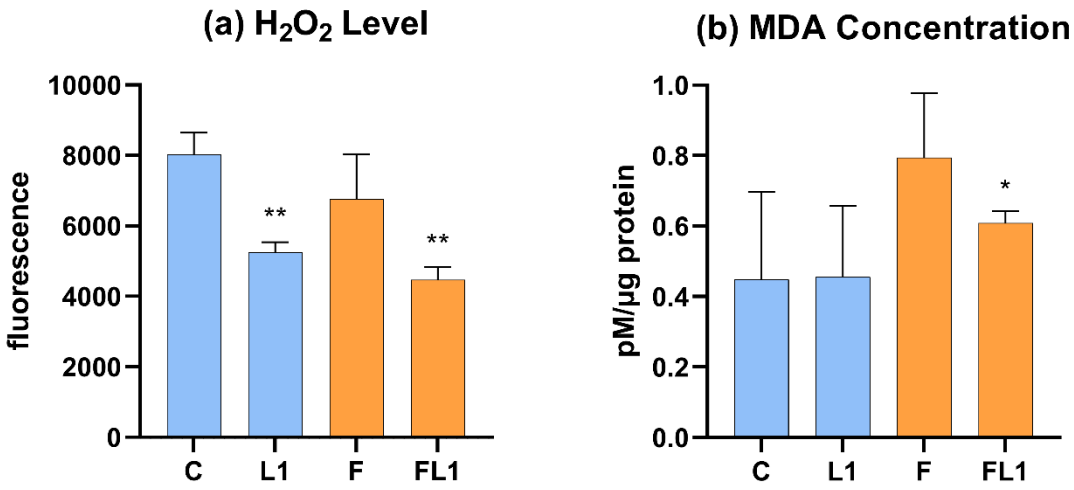

Figure 24. H_2O_2 level of the cell culture medium measured by Amplex Red fluorimetric assay (a) and MDA concentration of the cell lysate measured by a colorimetric assay (b). C=control, L1=luteolin (4 µg/mL), F=flagellin (250 ng/mL), FL1=flagellin (250 ng/mL) and luteolin (4 µg/mL). Mean (n = 6/group) ± SD, *p≤0.05, **p<0.01. Group L1 and F were compared to C, while group FL1 was compared to group F.

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

6.4.1 Preliminary Study II.1

The cellular metabolic and total LDH activities of the explants were measured in the first 36 h 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 25.**). 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 25.**).

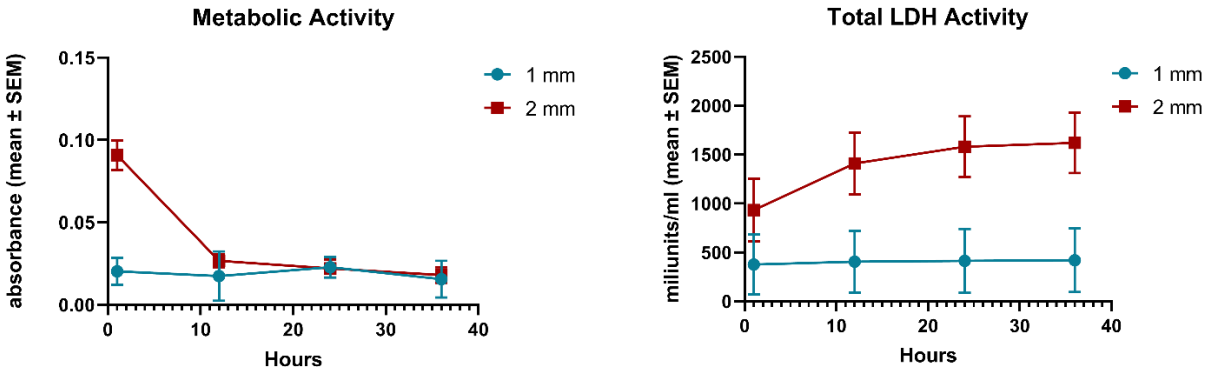

Figure 25. Cellular metabolic activity measured by CCK-8 assay and total lactate dehydrogenase (LDH) activity measured by an enzyme kinetic photometric assay of 1 and 2 mm explants. Mean (n=5/group) ± SEM.

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 26.**).

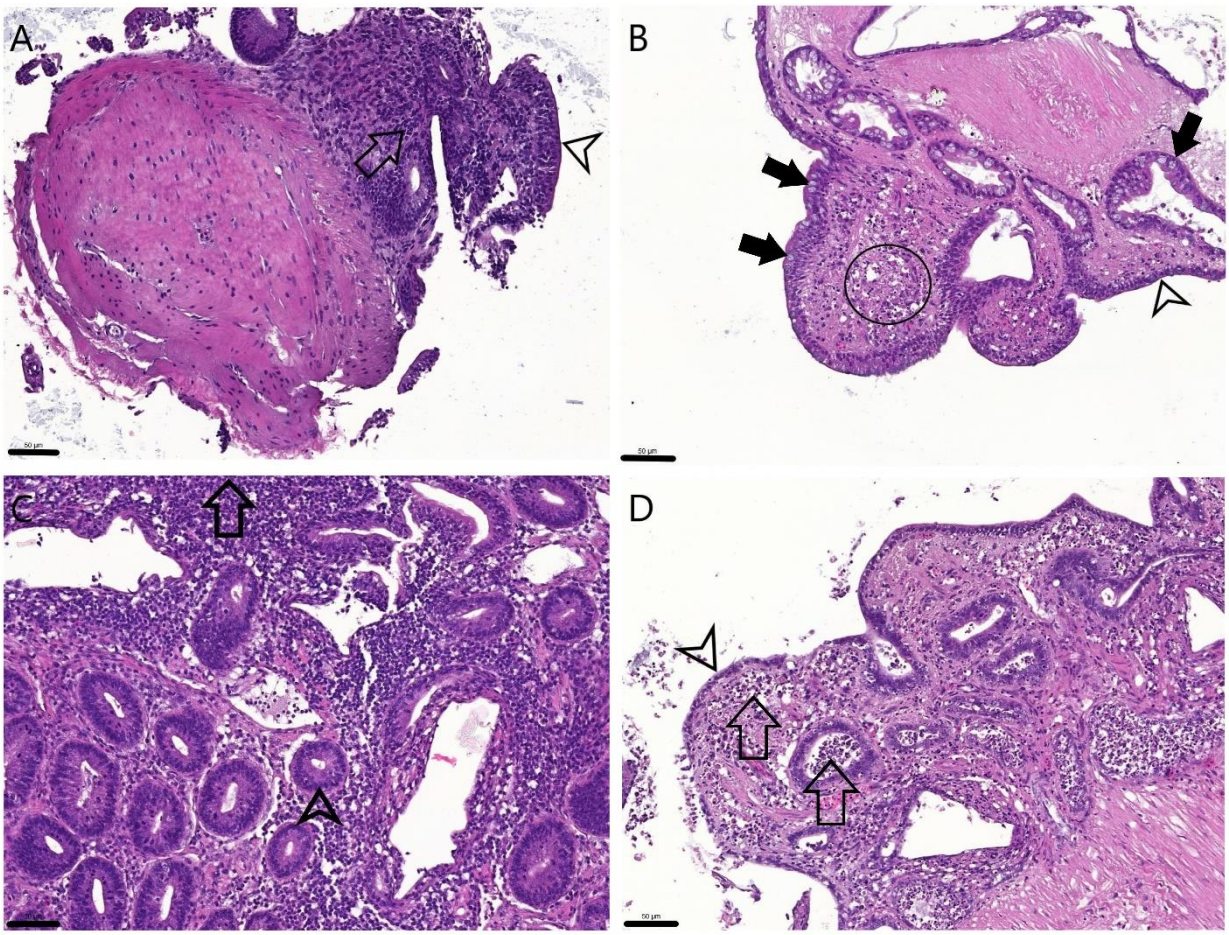

Figure 26. H&E stained sections of the explants. 0h 1 mm (A) and 2 mm (C): The physiologic columnar epithelial morphology (arrowheads) is visible on the surface and within the crypts, and the proprial mononuclear infiltrate is observable (arrows). 24 h 1 mm (B): The cuboidal surface enterocytes (arrowhead) and Goblet cells (full arrows) are visible both inside the crypts and on the surface while in the *lamina propria* apoptotic cell debris appears (circle). 24 h 2 mm (D): The surface enterocytes are flattened or detached (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 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 27.**)

The disruption of the 2 mm explants was evident after 48 h of culturing as they scattered into smaller pieces and cell debris, making their further manipulation and culturing impossible.

After 9 days of culturing 1 mm, explants were macroscopically intact with epithelial cells budding out from their surface visible after fixation and Giemsa staining with inverse microscopy (**Figure 27.**).

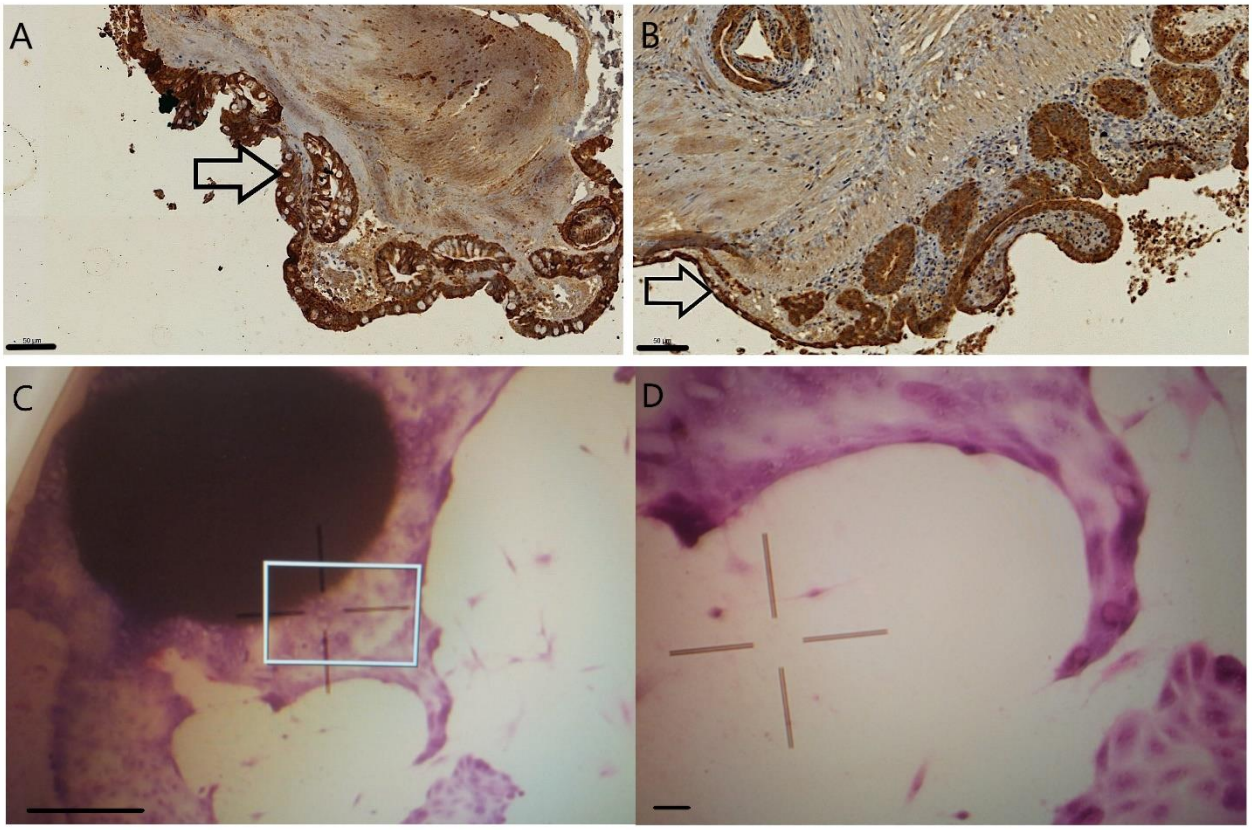

Figure 27. Pan-cytokeratin stained 24 h sections of 1 mm (A) and 2 mm (B) explants. The epithelial cells are marked in dark brown, and Goblet cells, with their unstained intracytoplasmic vacuoles, are primarily seen in 1 mm explants (arrows). Giemsa staining of the 1 mm explants after 9 days of culturing visualized with inverse microscope (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).

6.4.2 Preliminary Study II.2

The 1.5 mm explants 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 28.**).

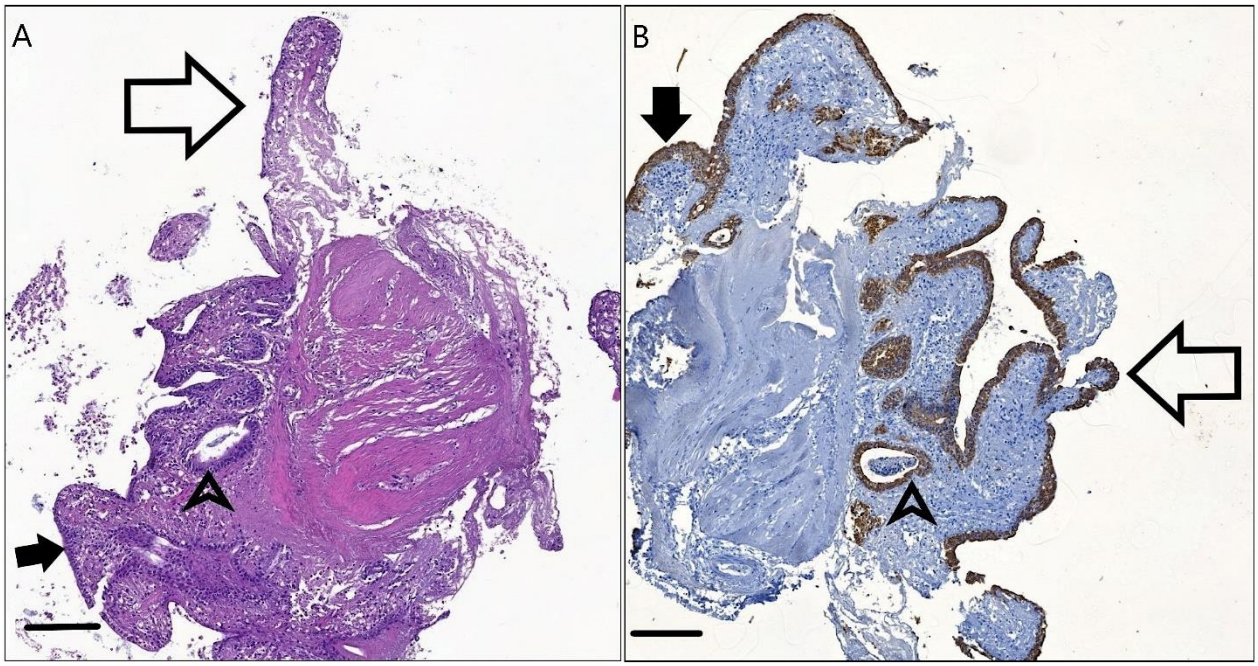

Figure 28. H&E stained (A) and Pan-cytokeratin stained (B) sections of the 1.5 mm explants after 12 h incubation. The physiologic columnar or cuboidal epithelial morphology (arrowheads) is visible on the surface (full arrows) and within the crypts (arrowheads). Remnants of the intestinal villi are present (empty arrows.) Bar line=100 μm.

6.4.3 Preliminary Study II.3

The CCK-8 assay carried out 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 29.**). No significant change was observed between PAMP-exposed and control explants in case of the lactate dehydrogenase activity (**Figure 29.**)

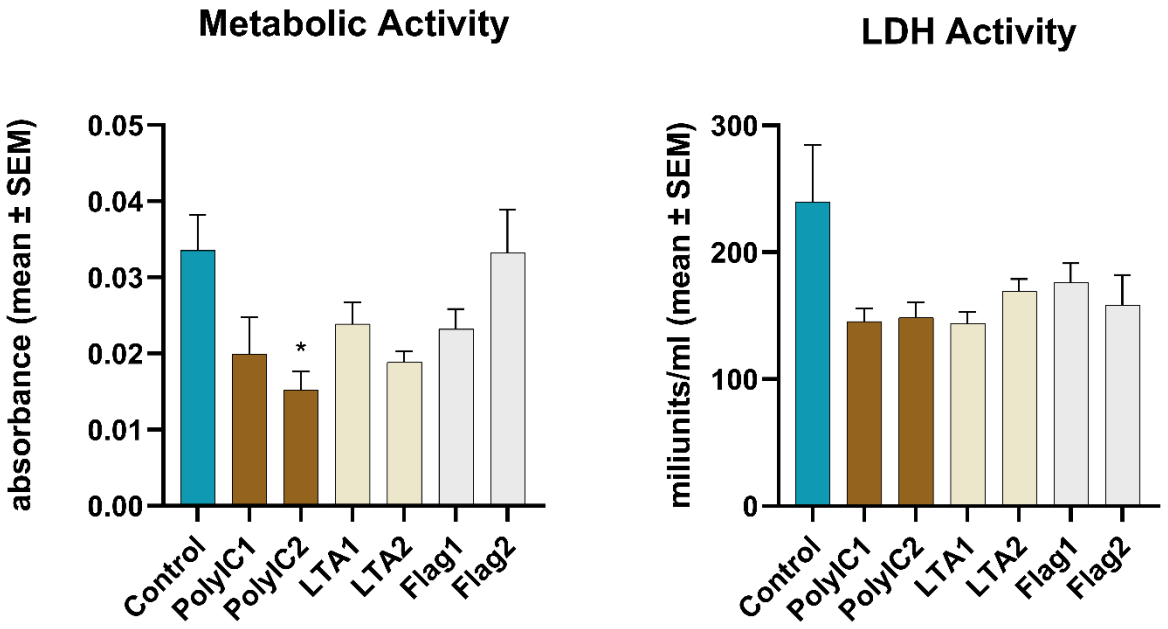

Figure 29. Metabolic activity measured by CCK-8 assay and extracellular lactate dehydrogenase (LDH) activity measured by an enzyme kinetic photometric assay of the 1.5 mm explants. PolyIC1=50 µg/mL polyinosinic-polycytidylic acid, PolyIC2=100 µg/mL polyinosinic-polycytidylic acid, LTA1=10 µg/mL *Staphylococcus aureus* lipoteichoic acid, LTA2=50 µg/mL *Staphylococcus aureus* lipoteichoic acid, Flag1=100 ng/mL *Salmonella* Typhimurium flagellin, Flag2=250 ng/mL *Salmonella* Typhimurium flagellin. Mean (n=5/group) ± SEM, *p<0.05.

IFN-α concentration was significantly increased by 50 µg/mL poly I:C treatment. LTA and poly I:C elevated IFN-γ 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 level of RANTES (**Figure 30.**, **Table 7.**). 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 31.**, **Table 7.**) No significant change in the IL-8 concentration was induced by either of the applied treatments (**Figure 30.**, **Table 7.**).

Table 7. Significant differences 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		

Poly I:C, polyinosinic:polycytidylic acid.

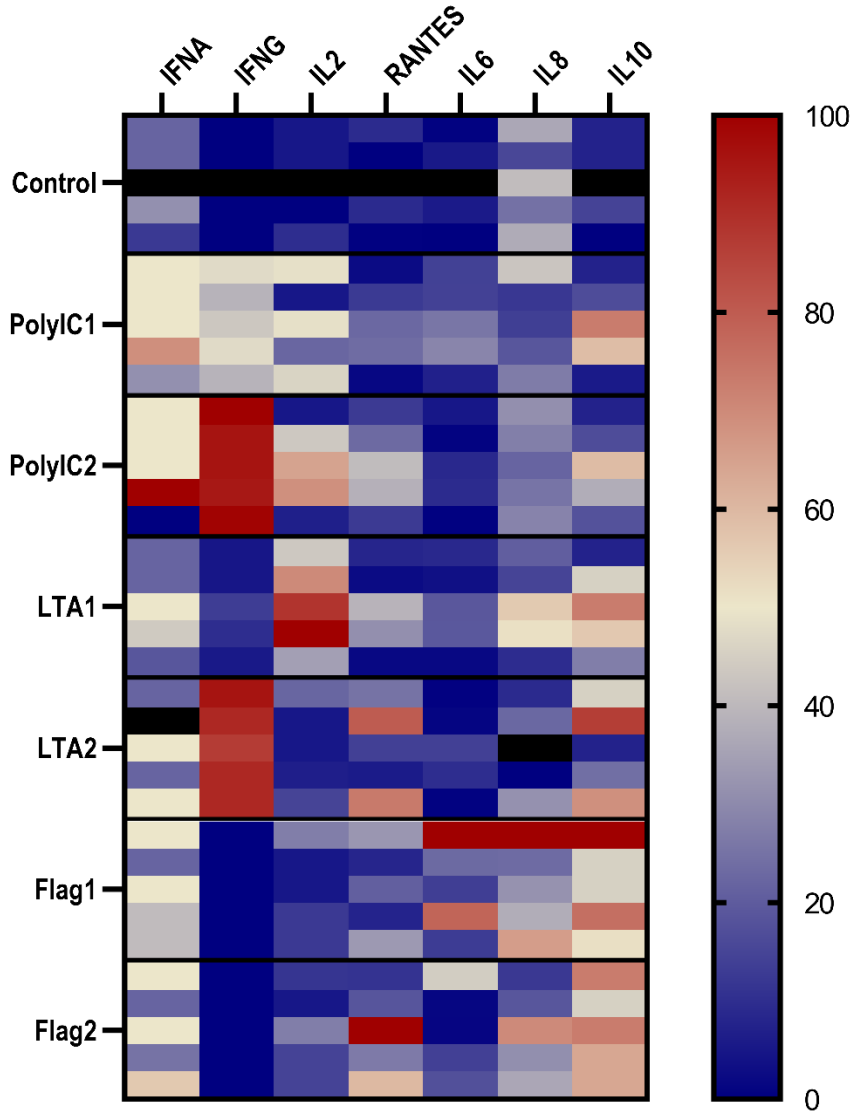

Figure 30. The heatmap represents the normalized relative percentages of the investigated cytokines. IFNA=interferon-α, IFNG=interferon-γ, RANTES=regulated on activation, normal T cell expressed and secreted, IL2=interleukin-2, IL6= interleukin-6, IL8=interleukin-8, IL10=interleukin-10, IL2=interleukin-2, PolyIC1=50 µg/mL polyinosinic-polycytidylic acid, PolyIC2=100 µg/mL polyinosinic-polycytidylic acid, LTA1=10 µg/ml *Staphylococcus aureus* lipoteichoic acid, LTA2=50 µg/mL *Staphylococcus aureus* lipoteichoic acid, Flag1=100 ng/mL *Salmonella* Typhimurium flagellin, Flag2=250 ng/mL *Salmonella* Typhimurium flagellin.

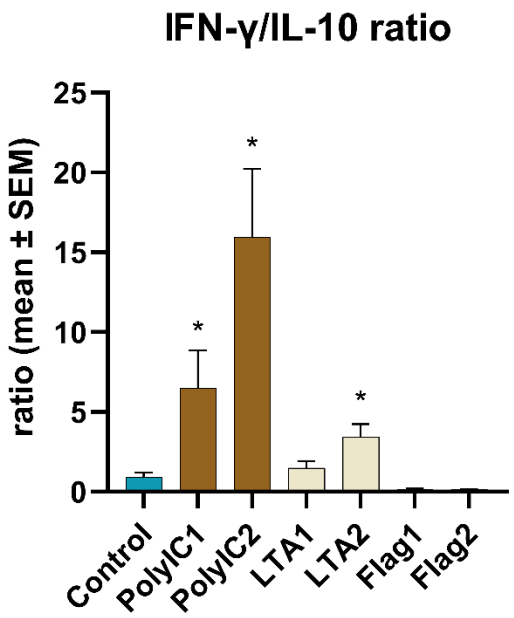

Figure 31. IFN-γ/IL-10 ratio. PolyIC1=50 µg/mL polyinosinic-polycytidylic acid, PolyIC 2=100 µg/mL polyinosinic-polycytidylic acid, LTA1=10 µg/mL *Staphylococcus aureus* lipoteichoic acid, LTA2=50 µg/mL *Staphylococcus aureus* lipoteichoic acid, Flag1=100 ng/mL *Salmonella* Typhimurium flagellin, Flag2=250 ng/mL *Salmonella* Typhimurium flagellin. Mean (n=5/group) ± SEM, *p<0.05.

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

The metabolic activity and LDH activity of the explants was not influenced by the CME treatment (**Figure 32.**).

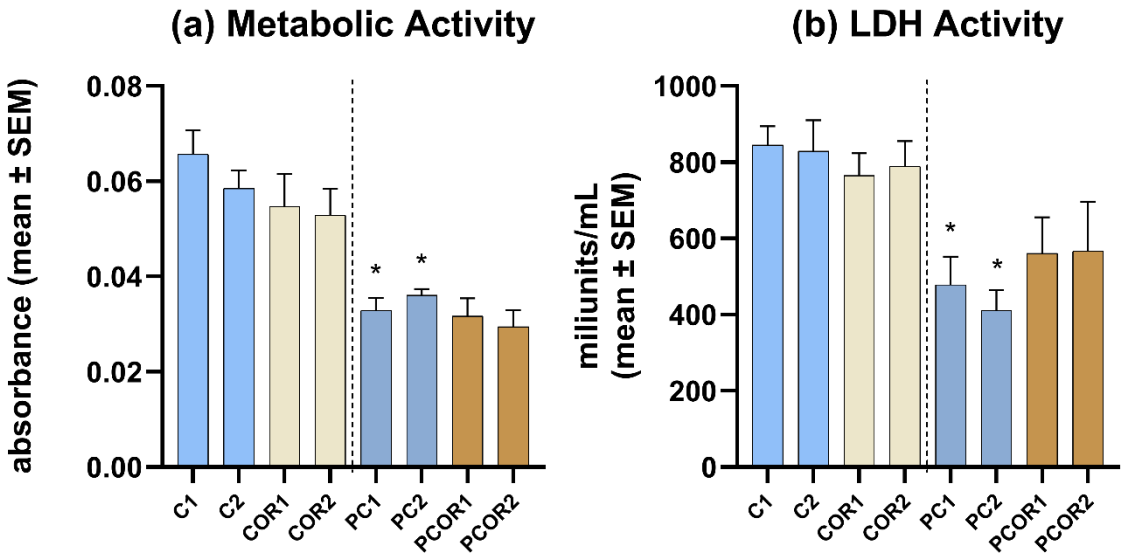

Figure 32. Metabolic activity measured by the CCK-8 assay (a) and extracellular lactate dehydrogenase activity measured by an enzyme kinetic photometric assay (b). C1=control 0.12% dextrose, C2=control 0.6% dextrose, COR1=*Cordyceps militaris* extract 0.12%, COR2=*Cordyceps militaris* extract 0.6%, PC1=poly I:C (50 µg/mL) and dextrose 0.12%, PC2=poly I:C (50 µg/mL) and dextrose 0.6%, PCOR1=poly I:C (50 µg/mL) and *Cordyceps militaris* extract 0.12%, PCOR2=poly I:C (50 µg/mL) and *Cordyceps militaris* extract 0.6%. Mean ± standard error of the mean (SEM), *p<0.05. Groups PC1 and PC2 were compared to C1 and C2, while groups PCOR1 and PCOR2 were compared to groups PC1 and PC2, respectively.

Without PAMP exposure, the 0.6% CME treatment significantly reduced IFN-γ cytokine levels compared to the untreated control (p=0.016; **Figure 33.**). Compared to the inflammatory control, both 0.6% (p=0.047) and 0.12% (p=0.016) CME treatments reduced IFN-γ levels (**Figure 33.**). For RANTES, the 0.12% dextrose-formulated CME treatment was effective (p=0.032, **Figure 33.**), while 0.6% CME reduced IL-1β levels in the presence of poly I:C (p=0.016, **Figure 33.**).

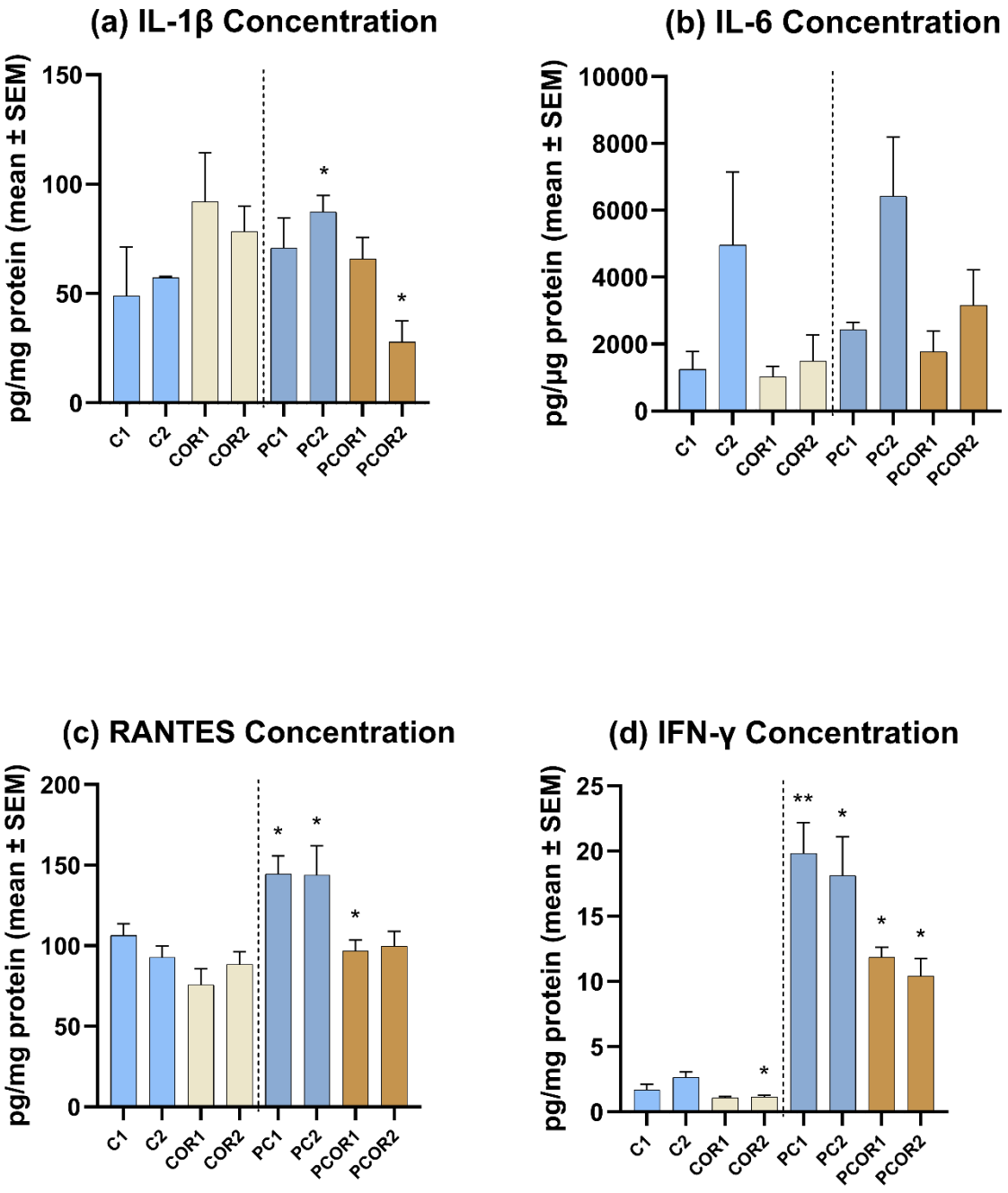

Figure 33. IL-1β concentration measured by chicken specific ELISA (a) and IL-6 (b), RANTES (c) and IFN-γ (d) concentration measured by chicken specific Luminex MAGPIX Panel from the cell culture medium. C1=control 0.12% dextrose, C2=control 0.6% dextrose, COR1=*Cordyceps militaris* extract 0.12%, COR2=*Cordyceps militaris* extract 0.6%, PC1=poly I:C (50 µg/ml) and dextrose 0.12%, PC2=poly I:C (50 µg/ml) and dextrose 0.6%, PCOR1=poly I:C (50 µg/ml) and *Cordyceps militaris* extract 0.12%, PCOR2=poly I:C (50 µg/ml) and *Cordyceps militaris* extract 0.6%. Mean ± standard error of the mean (SEM), *p<0.05, **p<0.01. Groups PC1 and PC2 were compared to C1 and C2, while groups PCOR1 and PCOR2 were compared to groups PC1 and PC2, respectively.

H_2O_2 concentration was not affected by CME treatment; meanwhile, applied at 0,6%, it increased the MDA level (p=0.029, **Figure 34.**), indicating lipid peroxidation.

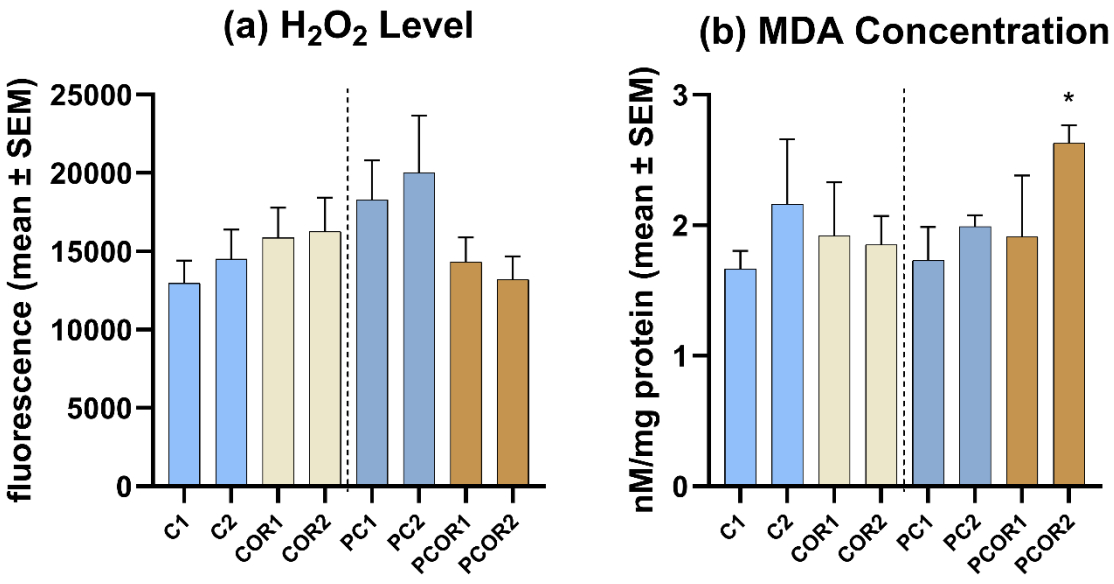

Figure 34. H_2O_2 level of the cell culture medium measured by Amplex Red fluorimetric assay (a) and MDA concentration of the cell lysate measured by a colorimetric assay (b). C1=control 0.12% dextrose, C2=control 0.6% dextrose, COR1=*Cordyceps militaris* extract 0.12%, COR2=*Cordyceps militaris* extract 0.6%, PC1=poly I:C (50 µg/ml) and dextrose 0.12%, PC2=poly I:C (50 µg/ml) and dextrose 0.6%, PCOR1=poly I:C (50 µg/ml) and *Cordyceps militaris* extract 0.12%, PCOR2=poly I:C (50 µg/ml) and *Cordyceps militaris* extract 0.6%. Mean ± standard error of the mean (SEM), *p<0.05. Groups PC1 and PC2 were compared to C1 and C2, while groups PCOR1 and PCOR2 were compared to groups PC1 and PC2, respectively.

7 Discussion

7.1 Preliminary study I.: Establishment of a hepatic inflammatory model

The applied hepatocyte and non-parenchymal cell fractions have been previously characterized by flow cytometry and immunofluorescent detection of specific markers for hepatocytes and non-parenchymal macrophages (Mackei et al., 2020a).

Our research aimed to create an inflammatory model on hepatic cell cultures of chicken origin. Only limited data on this issue is available; however, it would be an essential basis for future studies concerning the *in vitro* testing of anti-inflammatory agents. The applied chicken primary hepatocyte – non-parenchymal cell co-cultures have already been utilized by our research group previously to investigate the cellular effects of acute heat stress and T-2 toxin (Mackei et al., 2020c, 2020b). Furthermore, even inflammatory models were designed using similar cultures of porcine origin (Mátis et al., 2017). The inclusion of the non-parenchymal cell fraction in co-cultures at cell ratio 6:1 (hepatocytes to non-parenchymal cells) refers to a mild hepatic inflammatory state with moderate intrahepatic macrophage migration (Mackei et al., 2020b), enabling the investigation of the link between the inflammatory and stress response. The authors intended to examine the effects of the traditional Gram-negative endotoxin, LPS, against flagellin from *Salmonella* Typhimurium and poly I:C.

At first, the aim was to monitor how the applied pro-inflammatory agents affected the metabolic activity and membrane damage of the cultured cells. Poly I:C treatment decreased the metabolic activity of the 2D cell cultures, demonstrating a metabolically depressed state because of the harmful effect of these compounds. However, cell membrane damage was only detectable after the poly I:C treatment based on the extracellular LDH measurements.

The pro-inflammatory effects of our candidate molecules were screened by measuring the concentrations of IL-6 and IL-8 in culture media. The lower-dose, 50 μg/mL poly I:C supplementation turned out to be the most potent and versatile pro-inflammatory treatment as it has triggered the increase of both cytokines. This novel molecule showed a potent effect in splenocyte-derived leukocyte and oviduct-originated primary chicken cell cultures inducing high IL-1β, IL-6, IFN-α, and β mRNA level elevation (Abdel-Mageed et al., 2014; Kamimura et al., 2017; Villanueva et al., 2011b).

It can be stated that LPS had no pro-inflammatory effect on the applied chicken hepatic model. The inadequate IL response caused by *Escherichia coli* LPS is a possible result of the deficient signal transduction of chicken TLR4.

TLRs specified for extracellular pathogen recognition exclusively activate MyD88 dependent pathway which is needed for the prompt activation of Nf-κB. In human, TLR-3 and -4 agonists (double-stranded RNA and LPS) can set off interferon production and a so-called delayed or late-phase activation of Nf-κB via a MyD88 independent route which results in IL and TNF-α production. But the TLR4 related branch of this alternative pathway is assumed to be nonexistent in chicken. Thus solely double-stranded RNA via TLR3 initiated signal downstream leads to both late-phase activation of NF-κB and interferon response factors in chicken. The inadequate IL response in case of *Escherichia coli* LPS challenge is possibly related to the deficient TLR4 signal transduction in chicken and the lack of this additional route(Gao et al., 2008; Kannaki et al., 2010; Seki and Brenner, 2008a). Keestra and van Putten investigated this deficiency. They corroborated this hypothesis by the lack of IFN production in response to different *Salmonella* Enteritidis and Gallinarum as well as *Pasteurella multocida* strains and secondly by the absence of certain TLR4 signal mammalian gene orthologs in chicken (Gao et al., 2008; Kannaki et al., 2010; Keestra and Putten, 2008; Kiziltas, 2016; Seki and Brenner, 2008b). This concept is still controversial as two different research groups managed to trigger INF response with *E. coli* and *Salmonella* Typhimurium in cell cultures of chicken origin (Esnault et al., 2011; L. Lian et al., 2012); however, only on the level of gene expression. It can be hereby stated that IL-6 and IL-8 protein level elevation could not be induced in cell culture media with LPS from the chicken pathogenic O55:B5 serotype of *E. coli* in accordance with the relatively high tolerance to LPS of avian species (Keestra and Putten, 2008). Nonetheless, there is limited data on the effect of LPS on the secreted IL-6 and IL-8 concentrations in chicken. The level of the interleukin proteins showed only a slight or no increase as opposed to the remarkable elevation of the expression of the respective genes (Abdel-Mageed et al., 2014; Cheng et al., 2017; Kamimura et al., 2017; Rajput et al., 2014). As both interleukins were measured directly from cell culture media, the differences between the present results and those of studies assessing mRNA levels might arise from post-transcriptional, translational and post-translational regulatory mechanisms. These processes, for example interleukin mRNA accumulation or degradation by ribonuclease enzymes and protein kinase C dependent translational regulation of IL-6 level are included in the determination of the final inflammatory cytokine production of the cell (Kim et al., 2011; Rola-Pleszczynski and Stankova, 1992; Seno et al., 2018).

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

According to our present knowledge, infectious bursal disease virus and chicken anemia virus are presumed to be able to break through immunity and can deplete the primary lymphoid organs via cytokine overproduction, cytotoxic T-cell response, and apoptosis of the lymphocytes (Hoerr, 2010; Santen et al., 2004; Sharma et al., 2000). Avian reoviruses similarly induce apoptosis, lipid peroxidation and DNA damage of the cells in the bursa and the thymus, but they might harm the liver and the intestines as well, leading to malabsorption syndrome together with other non-enveloped ubiquitous viruses (Lin et al., 2007; Rehman et al., 2018). These data demonstrate that virus-induced diseases in chickens play an outstanding role. The application of novel natural feed additives could mitigate the damage, and therefore, they could assist the industry in curbing unnecessary antibiotic application for secondary pathogens and growth promotion.

The primary goal of this study was to investigate the immunomodulatory effects of CA and NAC on a chicken hepatic cell culture model, mimicking the viral infection by poly I:C exposure. Therefore, chicken primary hepatocyte – non-parenchymal cell co-cultures were used, because the inclusion of non-parenchymal cells at the ratio 6:1 mimics a mild hepatic inflammatory state with moderate macrophage migration and is suitable to study inflammatory and stress responses as corroborated in the previous study and prior investigations of our research group (Mackei et al., 2020b).

CA concentrations of 10 µg/mL and 100 µg/mL were used according to literature data reporting that no significant toxicity against human normal (HL-7702) liver cell line occurred with these concentrations. According to further studies, under cell damage induced by D-galactosamine, CA had the utmost effect at 100 µg/mL on hepatocytes and showed a comparable protective effect to silybin (Zhang et al., 2014). Concentrations of 100 and 200 µg/mL of NAC were chosen based on available literature. Zhang et al. (2020) revealed that 500 µmol/L (81,5 µg/mL) NAC could alleviate cadmium-induced toxicity in chicken peritoneal macrophages (Zhang et al., 2020). Furthermore, according to Preliminary Study I, poly I:C applied at 50 µg/mL rate was a potent pro-inflammatory treatment. Hence poly I:C was applied in this concentration in Study I to mimic inflammatory response triggered by viral infection.

The cellular viability was monitored with the CCK-8 metabolic activity assay, which gives information about the aerobic catabolic activity via the measurement of $NAD(P)H+H^+$. A

significant decrease of the parameter was observed in case of the cells treated with poly I:C, consistent with the results of Preliminary Study I and literature data, indicating that the cells are metabolically depressed due to the harmful effect (Chen et al., 2019). But with the addition of CA in high concentration (100 µg/mL), the magnitude of the cellular catabolism could be maintained, meaning CA was able to provide sufficient protective effects on cell viability. Intriguingly, NAC treatment resulted in a suppressed metabolic state and failed to restore the poly I:C evoked metabolic depression, which is contradictory to the study performed by He et al. (2012), indicating that NAC was able to sustain the viability of T-2 toxin treated chicken tibial growth plate chondrocytes (He et al., 2012). However, it is possible that NAC at lower concentrations or with shorter exposure times could have a cytoprotective effect, which may not have been detected due to the limitations of the present study.

Contrasting it to a broad scale of phytochemicals, CA proved to maintain cell viability as the second most effective agent from 85 tested molecules on microglial cells (Mu et al., 2019). Besides, it was proved to compensate D-galactosamine induced metabolic depression identically to silybin on a human hepatic cell line, HL-7702 and alleviated the damage caused by doxorubicin (Jabłońska-Trypuć et al., 2018; Zhang et al., 2014). Based on the observations of the present study and the data of the relevant literature, CA should be considered as a biomolecule of which main therapeutic effect resides in its cell protectant nature. It has maintained the metabolic activity of a large spectrum of cell lines under various stress stimuli just as in the case of our primary hepatic co-culture.

LDH leakage was measured from the cell culture medium to assess the membrane damage and cytotoxicity. In our study, the cells treated with poly I:C only showed higher extracellular LDH activity, but the induced elevation was significantly reduced when combined treatment with CA was applied, indicating that CA could attenuate membrane damage. Primarily, the inflammatory nature of this impairment should be accentuated as a high positive correlation between LDH and the level of pro-inflammatory cytokines (IL-6, IL-8, M-CSF) was observed, which would imply a cellular mechanism different from necrosis. Beforehand, it was indicated that poly I:C can induce pyroptosis (L.-H. Lian et al., 2012). The mechanism of action is not elucidated, but hypothetically, pyroptosis is a caspase-3 independent process resulting in extracellular LDH and cytokine level elevation. In avian species bibliographic data is limited, but in other respects, pyroptosis was proved to occur in chicken primary hepatocyte cell cultures under copper induced oxidative stress (Liao et al., 2019; Shi et al., 2017). The pyroptosis-related cell damage in the present study could be supported by the notable but not significant caspase-3 level decrease under poly I:C exposure recovered in the CA treatment group. This

phenomenon is in line with the observation of Ding et al. (2019) on an LPS treated acute lung injury mouse model. CA suppressed NLRP3 activation, IL-6, IL-1β, and caspase-1 production with a parallel reduction in reactive oxygen species (ROS) generation and lipid peroxidation (Ding et al., 2019). However, it was demonstrated beforehand in a mouse connective tissue cell line that poly I:C could induce cytotoxic effect independently from interferon production, which statement might be described by a cellular response induced by a pattern recognition receptor different from TLR3. Interestingly suppression of NLRP3 is a potential mechanism of action of NAC under LPS-induced inflammation (X. Wang et al., 2020). Additionally, as negative correlation between LDH level and cellular metabolic activity was observed, these two parameters are suspected to change in conjunction in response to the inflammatory stimulus.

Furthermore, MDA concentration was measured to examine lipid peroxidation, revealing the extent of oxidative stress and the status of the cell membranes. Interestingly, according to the literature CA may provoke ROS generation in melanoma cells and preadipocytes, but in fibroblast cells CA pretreatment significantly diminished the concentration of MDA and intracellular ROS, concomitantly elevating the level of antioxidant parameters (e.g. reduced glutathione, protein thiol group content) under stress conditions induced by doxorubicin (Jabłońska-Trypuć et al., 2020, 2018; Xiao et al., 2013). Bibliographic data are partly supported by the present results considering that cells treated with poly I:C showed decreased MDA concentration after CA supplementation. On the other hand, NAC did not affect the MDA levels significantly, which is barely corresponding to other studies where NAC was able to reduce lipid peroxidation in cells exposed to noxious substances (Faghfouri et al., 2020; He et al., 2012; J. Wang et al., 2020). CA possibly regulates the expression of key enzymes in antioxidant protection like HO-1 and glutamate-cysteine ligase in glutathione synthesis via NRF2. Thus, it has elevated glutathione level, catalase and SOD activity both in the brain of mice and microglial cell lines (Q. Liu et al., 2017, p. 2; Steele et al., 2013, p. 2).

A healthy liver has a relative immune tolerance against PAMPs, nevertheless upregulation of TLR gene transcription and the overactivation of PAMP triggered signal transduction implicate the breakdown of the tolerance, therefore, leads to overexpression of pro-inflammatory cytokines (Gao et al., 2008; Kiziltas, 2016; Seki and Brenner, 2008a). IL-8 is a chemokine responsible for neutrophil migration and activation. It is released excessively at the site of inflammation related tissue damage in conjunction with a multi-faceted pro-inflammatory interleukin, IL-6 (Guo et al., 2018; Harada et al., 1994). IL-6 and IL-8 as bioindicators were used to investigate the pro-inflammatory effects of poly I:C and the inhibitory effects of CA on inflammatory cytokine production. Poly I:C alone was able to

increase the secretion of these cytokines, which is in line with our previous results and a study conducted by Chen et al (Chen et al., 2019). CA had a robust inhibitory effect on IL-8 release, even when cells were treated with the applied relatively high poly I:C concentration. Accordant to another experiment, 100 μg/mL CA was also able to limit the IL-6 production through the inhibition of the NF-κB pathway in palmitate-induced peripheral blood mononuclear cells from diabetic patients (Sadeghabadi et al., 2019). Our results were also compatible with the study of Oláh et al., reporting that Echinacea extract was able to reduce mRNA expression of IL-6 and IL-8 in a poly I:C treated HaCaT human keratinocyte cell line (Oláh et al., 2017). Similarly to CA, NAC decreased the pro-inflammatory cytokine secretion of cells exposed to the virus nucleic acid analog; however, in line with the bibliographic data on chicken peritoneal macrophages (Zhang et al., 2020), only the lower dosage elicited a significant decrease in the cellular IL-6 release.

In the present study, the treatment of cells with poly I:C did not affect the caspase-3 concentration significantly. This could be because poly I:C, as a TLR3 agonist, activates the inflammatory pathway through TRIF instead of MyD88, which further activates the NF-κB without any influence on apoptosis-related pathways (Chen et al., 2012; Stowell et al., 2009). However, according to literature data, poly I:C induced apoptosis via caspase-3 and -8 activation in intestinal epithelial cells of mice (McAllister et al., 2013). Furthermore, Xiao et al. (2013) observed increased caspase concentration after CA treatment in mouse preadipocytes. They reported that CA was able to induce apoptosis via the mitochondria-dependent pathway resulting in mitochondrial membrane potential loss, cytochrome c release, and caspase-3 activation (Xiao et al., 2013). In the present study, a remarkable but insignificant increasing effect of CA on the apoptosis rate was found in the poly I:C cotreatment group.

There is a lack of information on the beneficial effects of CA concerning virus-induced inflammation. Type I interferons, like IFN-α, are crucial first-wave cytokines both for infected cells and extracellular responses against invader viruses. Their role in the cell is primarily to induce IFN-stimulated genes, which will hinder viral replication by the expression of RNase enzymes, inhibition of the cellular transcription, translation, and the prevention of virus entry and virion release. Nearby in the microenvironment of the inflammation, type I IFNs facilitate monocyte and natural killer cell migration and the production of IL-15 in dendritic cells and IL-18 in monocytes, finally inducing IFN-γ production in the target lymphocytes, NK-cells or monocytes (Lee and Ashkar, 2018). This cytokine as a final signal terminates the antiviral response via the stimulation of antigen presentation, the induction of Th1 cytotoxic response, and the induction of M1 macrophage polarization, phagocytosis, and production of NO and

ROS. Therefore, type I interferons are vital for effective antiviral immunity. Meanwhile, their long-term production suppresses the function of macrophages and NK cells. Furthermore, excessive production of IFN-γ could generate a detrimental cytotoxic response, therefore contributing to the pathogenesis of chicken diseases, such as chicken anemia and IBD (Hoerr, 2010; Lee and Ashkar, 2018; Majde, 2000). CA remarkably yet not significantly reduced IFN-γ gene expression level in the brain of chicken embryos exposed to thiacloprid insecticide induced inflammation and oxidative stress (Farag et al., 2021). Meanwhile, long-term CA treatment might augment Th1 mediated immune response indicated by the elevated level of IL-2, IL-12, and IFN-γ, and decreased corticosterone concentration in chronically stressed, therefore immunosuppressed mice (Kour and Bani, 2011). The present study demonstrates that CA under virus analog induced stress could alleviate both IFN-α and IFN-γ production in a dose-dependent manner just as 100 μg/mL NAC treatment.

M-CSF is one of the three substantial cytokines responsible for myeloid cell maturation, migration and differentiation. On contrary to the rest, the M-CSF polarized macrophages have a fundamental role in pro-inflammatory and regulatory response and the clearance of early apoptotic cells, therefore they maintain a steady state at the site of inflammation (Hamilton and Achuthan, 2013; Wiktor-Jedrzejczak and Gordon, 1996; Xu et al., 2007). The level of this chemokine measured from the cell culture media according to the present study would serve as a reliable indicator of the inflammatory response as a very low standard deviation in its level has been registered, meanwhile, M-CSF shows a moderate to high correlation with all the measured cytokines and LDH. IL-10 production after TLR3 stimuli was exclusively described in macrophages from the whole set of inflammatory cells. The fact that the IL-10 production of bone marrow derived macrophages after LPS stimulus relies on TRIF dependent signaling and type-I IFNs further intrigues the crosstalk of these cytokines, moreover IFN-γ could intrinsically inhibit IL-10 production (Chang et al., 2007; Saraiva and O'Garra, 2010, p. 10). In line with literature data, the induction and production of IL-10 as an overcompensation happened parallelly with pro-inflammatory cytokines in the present model. The anti-inflammatory role of IL-10 in chicken has been described; however, it is not yet known whether hepatocytes could produce this cytokine (Wu et al., 2016). It is hypothesized that similarly to IL-10, M-CSF serves as a compensatory anti-inflammatory cytokine in the present cellular crosstalk.

Nonetheless poly I:C solely mimics viral double-stranded RNA to activate TLR3-mediated antiviral responses but cannot induce certain virus-specific cellular effects such as activation of alternative viral RNA sensing pathways (e.g., RLRs), virus-mediated modulation

of intracellular signaling cascades, or the full spectrum of cytokine and chemokine profiles triggered by live viral replication. Although this limitation exists, poly I:C induced significant changes in most of the measured pro-inflammatory cytokines, and the elevation of certain cytokine levels showed a strong positive correlation with LDH release, suggesting inflammatory cell damage or cell death. This highlights the overall value of the research despite the noted limitation.

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

In the poultry industry, increasing evidence suggests that natural plant-based feed additives can achieve the same improvements in performance that were once obtained through the subtherapeutic use of antibiotics (Aljumaah et al., 2020; Buchanan et al., 2008; Demir et al., 2003). The beneficial effect of these substances on animal health and productivity could be mediated by their anti-inflammatory, cytoprotective and antioxidant properties (Alagawany et al., 2021; Rathee et al., 2009), which should be extensively investigated.

In the present study, a molecular pattern recognized by TLR5, flagellin of *S.* Typhimurium was used to induce the hepatic inflammatory response. Following flagellin stimulation of chicken heterophilic granulocytes, Kogut et al. reported NFκ-B activation, an increase in IL-1β, IL-6 and IL-8 mRNA levels, and described degranulation and free radical generation (Kogut et al., 2008, 2005). Furthermore, recombinant *S.* Typhimurium flagellin stimulated IL-4, IL-6 and IL-12 (Gupta et al., 2013), while *S.* Enteritidis infection *in vitro* elevated the IL-1β, IL-6 and IL-8 gene expression in chicken peripheral blood mononuclear cells (Pan et al., 2012). These data accentuate the pro-inflammatory response of chicken inflammatory cell monocultures after flagellin exposure, albeit the liver and its resident cells are postulated primary targets of flagellated bacteria, and the TLR receptor toolkit of the organ might be crucial in the elimination of the bacterial burden from the portal circulation. A study on bioluminescent imaging of NF-κB-dependent luciferase expression in TLR5 agonist treated mice unveiled that the liver is the major organ to respond to flagellin-like products in contrast to bacterial LPS, a surge of luminescence was detected in hepatocytes early after the TLR5 agonist administration (Burdelya et al., 2013).

The liver is one designated place of paratyphoid *S. enterica* multiplication in chicken, therefore it could damage the organ in case of early infection of hatchlings (Barbosa et al., 2017; Barrow, 2000). Hence, the primary aim of our work was the *in vitro* modeling of inflammation and oxidative stress induced by ciliated bacteria in chicken primary hepatocyte – non-

parenchymal cell co-cultures of chicken origin to recapitulate the response of the avian liver. Preliminary Study I confirmed that flagellin at concentrations of 100 and 250 ng/mL did not induce cellular damage in terms of increased LDH activity or decreased metabolic activity of the cells. However, flagellin significantly increased the production of pro-inflammatory IL-6 at 250 ng/mL. In Study II a significant elevation of the expression of IL-8 protein in chicken primary hepatocyte – non-parenchymal cell co-cultures was observed, which is in agreement with the data published on chicken heterophilic granulocytes and peripheral blood mononuclear cells.

The effect of luteolin on cell cultures of chicken origin was investigated for the first time to the best of our knowledge; however, luteolin has already been applied to many mammalian cell lines. The protective effect of the molecule on cell viability and membrane integrity was confirmed on NRK-52E rat kidney cells when applied at as high as 50-200 μM concentration (Liu et al., 2020). Wang et al. (2021) examined the cellular metabolic activity of mouse primary hepatocyte culture following LPS exposure. The authors have reported that in relatively low, 10 and 20 μM concentrations it has restored the viability of the cells (Wang et al., 2021). Luteolin also served as a cytoprotective agent under LPS and ATP-induced inflammatory cell death in 50 μM in human THP-1 monocyte cell line indicated by decreased LDH leakage and restored cell metabolic activity. This concentration proved to be the highest tolerable to the monocyte culture as 100 μM luteolin significantly decreased cellular viability (Zou et al., 2021). In our chicken primary hepatic cell model, 16 μg/mL concentration, a dose similar to 50 μM (equal to 14.132 μg/mL) of luteolin, proved cytotoxic. In contrast, exposure to low concentrations of luteolin (5 μM, referring to 1.413 μg/mL) in RAW 264.7 macrophages effectively modified the inflammatory response of cells without any effect on LDH-indicated cytotoxicity, in consonance with the 4 μg/mL dosage applied in the present study (SungJeehye and LeeJunsoo, 2015).

IFN-γ plays a vital role in eliminating intracellular pathogens, including bacteria. It is hypothesized to be a vital cytokine to terminate the spread of *S. enterica* as it contributes to the processing of pathogens in phagocytes (Ingram et al., 2017). Both human and murine model studies confirmed that high IFN-γ and low IL-10 levels were connected to the successful activation of the immune system and the elimination of enteric Salmonellosis in the early phase of the infection (Ingram et al., 2017; Pie et al., 1996; Stoycheva and Murdjeva, 2005). The cytokine level has not been proven to be associated with the pace of clearance in chickens. However, the IFN-γ level surge in the spleen and cecal tonsils is vital to eliminating *S. enterica* from the gastrointestinal tract (Beal et al., 2005). The present study, therefore, aimed to examine

the changes in the level of IFN-γ and IL-10, two crucial cytokines in paratyphoid *S. enterica* induced response and their ratio consequently. The decrease observed in IL-10 concentration and the increase in IFN-γ/IL-10 ratio in case of flagellin exposure corroborate with a pro-inflammatory response similar to that observed in mammals and therefore, validate the applied *in vitro* model (Ingram et al., 2017; Pie et al., 1996; Stoycheva and Murdjeva, 2005). Type I IFNs as discussed earlier (Study I) are essential for efficient immunity, while their sustained synthesis inhibits macrophage and NK cell activity and leads to the production of ROS. In the present study, luteolin effectively diminished the hepatic IFN-α release both in sole and combined application with flagellin, indicating the suggested anti-inflammatory role of the flavonoid in maintaining the physiological inflammatory homeostasis.

Luteolin exposure at 4 μg/mL decreased the IL-8, but not the IL-6 production of chicken hepatic co-cultures. Chicken IL-8 attracts monocytes, macrophages and lymphocytes to the site of inflammation, but in higher concentrations, it also stimulates angiogenesis (Poh et al., 2008). IL-8 production of human HT29 colon adenocarcinoma cells following the TNF-α challenge was diminished by luteolin (Kim et al., 2005). In another study, luteolin reduced the accumulation of the pro-inflammatory cytokines IL-6 and TNF-α induced by LPS exposure in mouse primary hepatic cell cultures (Wang et al., 2021). These results could be further supported by the observation of Wang et al. In their study, RAW 264.7 macrophages were treated with LPS and IFN-γ to generate cultures of pro-inflammatory branching M1 phenotype macrophages. Subsequently, luteolin treatment resulted in a concentration-dependent alteration in cell morphology towards the appearance of mainly anti-inflammatory M2 macrophages and a significant decrease in gene expression of the cytokines IL-1β and IL-6 (S. Wang et al., 2020). The present study demonstrates that luteolin could decrease the protein level of IL-8 in a chicken primary co-culture recapitulating the cell ratio of the avian liver. The mechanism of action in mammalian cells is presumed to be connected to inhibitors of NF-κB (IκBs), which prevent NF-κB from binding to its binding site on the DNA (Aziz et al., 2018; Kim et al., 2005; Li et al., 2019; SungJeehye and LeeJunsoo, 2015). The blockage of the NF-κB signal might be a putative mechanism also in chicken as no remarkable difference between the TLR5 signal pathway in avian and mammalian cells has been described. The flagellin-induced alarm of the immune system is highly conserved (Keestra et al., 2013).

Excessive stress and disturbance of the redox signaling could lead to the overproduction of reactive species (Surai et al., 2019). This oxidative stress could be compensated by nutritional antioxidants or by stimulating the production of antioxidant enzymes (e.g. SOD, glutathione peroxidase, heme oxygenase) regulated by Nrf2 (Ma, 2013; Surai et al., 2019). The activation

of the Nrf2 signaling pathway may be one of the potential mechanisms by which luteolin diminishes cellular oxidative stress (Li et al., 2019). Flavonoids are able to scavenge reactive oxygen species structure specifically and reduce the rate of lipid peroxidation (Cai et al., 1997). The results of the present study confirm that luteolin could alleviate lipid peroxidation and decrease the extracellular H_2O_2 level. In line with the observation of the authors, a decrease in the intracellular reactive oxygen species level was reported in cardiomyocytes and IPEC-J2 intestinal porcine enterocyte cultures after luteolin supplementation (Kovács et al., 2020; Li et al., 2019).

The present *in vitro* model study has provided some initial evidence concerning the anti-inflammatory and antioxidant role of luteolin in the chicken liver, suggesting that it might be a potential and safe candidate in poultry nutrition to ameliorate the deteriorative effects of enteric bacterial infections. However, it has to be stressed that further studies are required to assess the effects of this plant-derived metabolite, even under *in vivo* conditions, when applied as a feed additive.

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

Explant systems comply with the R principles of laboratory animal experiments since they enable the preparation of multiple cultures from a single animal while providing a tissue-specific response within a regulated environment (Randall et al., 2011; Russo et al., 2016). Therefore, animal gut explant studies were done on a large scale to investigate the effects of mycotoxins and pathogens and to assess the impact of specific dietary components (Costa et al., 2016; Peñaranda et al., 2020; Zhang et al., 2017). The merit of the immersion technique is that the liquid phase, the intestinal fluid of the intestine is more accurately modeled in contrast to air-liquid interface cultures. Nevertheless, preventing anoxia-induced damage to the explants is challenging (Randall et al., 2011). Therefore, the authors aimed to follow the histology and viability parameters of 1 and 2 mm diameter sized smaller and bigger explants to describe possible size-dependent alterations. In view of the fact that 1 mm, smaller sized explants are hard to obtain and visualize during the change of the media the authors opted for the smallest possible 1.5 mm diameter explants in Preliminary Study II.2. In that study the morphology of 1.5 mm sized explants was examined after 12 h incubation. In Preliminary Study II.3 according to the results of the preceding studies the authors aimed to describe the effects of PAMPs on the 1.5 mm sized explant model after 12 h culturing.

In Preliminary Study II.1, routine histology showed altered epithelial morphology and damaged *lamina propria* layer after 24 h of culturing in case of both 1 and 2 mm explants. Different-sized explants showed different extent of alteration in favor of 1 mm explants. The changes in epithelial shapes are difficult to interpret. However, it is conceivable that the cells took on a flatter morphology, particularly in case of the 2 mm explants to cover the basement membrane due to the absence of the detached adjacent cells, or that they became flatter through another mechanism. The cells had supposedly undergone apoptosis and necrosis in the *lamina propria* of the specimens for both explants as inferred mainly from the nuclear alterations. As the degradation is much more visible deeper in the propria, the lack of nutrients or anoxia is suspected to be the reason. Although appreciable villus is absent either in the 24 h or the 0 h control slides, the 1 and 2 mm explants maintained their epithelial layer. Crypt cellular debris and the lack of differentiated Goblet cells are presumably associated with epithelial injury in the 2 mm samples. Randall et al. described that explants generally smaller than 3 mm are easy to lose during processing and challenging to be embedded in an appropriate orientation, which along with the possible damage caused by the punch could describe the loss of the villi in the present study (Randall et al., 2011).

Moreover, the Giemsa stained 1 mm cultures proved further the viability of the epithelium and the superficial layers after multiple days of maintenance. After 2-3 days of culturing the 2 mm explants were fragmented, which is in line with the prompt decrease in the metabolic activity and increase in the extracellular LDH activity. Although the original metabolic activity of the cells of 2 mm explant was higher, these changes suggest that the smaller 1 mm ileal explants are more resilient under extended (12 h or above) culturing. Nevertheless, prolonged incubation is controversial because of the histological alterations observed after 24 h in both explants. Under hypoxic conditions, the pro-inflammatory response related genes of human mature dendritic cells are over-expressed (Blengio et al., 2013). The hypoxia inducible factor signaling, and NF-κB pathway are closely connected. Under anoxia the pro-inflammatory cytokine gene expression is increased, which might assist tissue recovery under physiologic conditions (Pham et al., 2021). Therefore, to prevent the influence of excessive anoxia on inflammatory response, the authors suggest a shorter incubation time and thus applied 12 h incubation in Preliminary Study II.2. Following this duration, the peak translation of cytokine proteins could be assessed according to studies on different cell lines (Israelsson et al., 2020). Practically, the authors found the 1.5 mm and higher diameter biopsy punch more convenient to use as 1 mm explants might be stuck to the plunger or lost during the change of media. To shorten the time spent on the excision and to further decrease the difference

between explants when a high amount of treatment groups and replicates are required, 1.5 mm explants could be a choice with minimal compromise. Therefore, in Studies 2 and 3, the smallest feasible 1.5 mm explants were examined. The length of the incubation was also limited to 12 h to assess the morphological status of untreated 1.5 mm explants and further in Preliminary Study II.3 to determine their acute inflammatory response under PAMP exposure.

Preliminary Study II.3 shows that TLR agonists unequivocally induce specific cytokine responses. A previous chicken ileal explant culture study of Zhang et al. demonstrates that explants remain viable for 2 h under ambient gas pressure conditions. Furthermore, according to the cited article, the culture served as a proper model to evaluate inflammatory response induced by LPS stimuli as they observed that 20 μg/mL LPS could elevate the gene expression of IL-1β and IL-8. In contrast, 50μg/mL could significantly increase the extracellular LDH activity, indicating cell injury (Zhang et al., 2017). Most of the described TLRs have been characterized in heterophils, macrophages and in the intestinal samples of chicken (Kannaki et al., 2010). Therefore, the authors aimed to assess the inflammatory response of ileal explants exposed to TLR agonist PAMPs for 12 h of culturing: poly I:C, LTA of *Staphylococcus aureus* and *Salmonella* Typhimurium derived flagellin. A human intestinal explant study underpinned that PAMPs have an essential role in gut inflammatory homeostasis which could be described accurately by IFN-γ/IL-10 ratio (Jarry et al., 2008). Lymphocytes are the prevailing immune cells in the intestine. T cells are a type of lymphocyte essential for recognizing and responding to pathogens and thus orchestrating adaptive immune response. The Th1 subset of this group promotes cytotoxic T cell responses against intracellular pathogens during which inflammatory cytokines such as IFN-γ are released. In contrast, regulatory T cells (Tregs) are specialized to suppress immune responses and maintain tolerance, preventing excessive inflammation and damage by producing anti-inflammatory cytokines. Amongst these cytokines, IL-10 is of utmost importance. Therefore, the balance between Th1 and Treg responses, often represented by the IFN-γ/IL-10 ratio, is critical in organs such as the gut and liver, where controlled immune activity is essential to protect against pathogens while avoiding chronic inflammation or tissue injury (Jarry et al., 2008; Ma et al., 2021; O'Shea and Paul, 2010). This ratio is of high importance and indicative of LPS-dependent decrease in the constitutively expressed mucosal IL-10. This IL-10 decrease according to the authors could lead to IFN-γ elevation and concomitant damage and apoptosis of the cells of human colon explants (Jarry et al., 2008). The ratio similarly indicates inflammatory status in colitis models and in an LPS-stimulated macrophage culture (Ma et al., 2021). In the present study flagellin induced significant elevation of IL-6, IL-10 and RANTES levels, although the IFN-γ/IL-10 was not altered. This observation

might be described by the fact that TLR5, the Toll-like receptor of flagellin is confined to the basolateral side of enterocytes in human (Hug et al., 2018). The localization of this receptor has not been investigated, and this similarity has not been disproved in chickens so far. Therefore, flagellin might act on a more permeable epithelial barrier and stimulates a niche of cells deeper in the mucosa. Meanwhile, LTA and Poly I:C possibly induce TLR signals in enterocytes irrespective of the polarity. The capability of Poly I:C to induce inflammation on primary cell cultures of chicken origin was confirmed; however, its effect in mammalian intestinal *ex vivo* studies is controversial as it caused no cytokine production but increased the permeability of mucosal explants (Brand et al., 2018; Moyano-Porcile et al., 2015). LTA exposure has not been investigated before either on mammalian or on avian gut explant cultures, therefore the effect of LTA to increase the secreted IFN-γ and IL-2 concentrations and to elevate the IFN-γ/IL-10 ratio was first described in the present study. The elevation of the latter parameter might render LTA and also poly I:C challenge suitable for screening of anti-inflammatory candidates in line with the study on mouse model systems of Ma et al. In the experiment of the cited authors, IFN-γ/IL-10 ratio was used to quantify LPS or dysbiosis-induced colonic and systemic pro-inflammatory response and concomitant neuroinflammation which were counteracted by probiotic bacterial strains (Ma et al., 2021).

The results of Preliminary Study I and Preliminary Study II should be contrasted as follows. On chicken primary hepatocyte – non-parenchymal cell co-cultures, only the higher LTA concentration was able to cause an increase in the IL-8 secretion. However, the cultures responded under flagellin and poly I:C exposure with the elevation of IL-6 concentration. Comparison between monolayer primary cell cultures and explants further highlights that the polarity of defined cell types in a model highly determines the inflammatory response. The gut explants secrete cytokines and growth factors that are specific to the mucosal membrane and not to a particular cell type. This is an advantage of explant models as several pro-inflammatory interleukins are not or not constitutively secreted by single cell types e.g. human enterocytes or macrophages (Daig et al., 2000; Noel et al., 2017).

IFN-γ and IL-6 are secreted in small concentrations by the intestinal epithelial cells (Noel et al., 2017). Therefore, the effect of different agents and anti-inflammatory drugs is commonly tested also on intestinal *ex vivo* cultures to gain more representative data about the response of the complete gut mucosa (Russo et al., 2016). In the present investigation a marked significant increase in IFN-γ level was induced by LTA and poly I:C at both applied doses, while IFN-α concentration was considerably increased by 50 µg/mL poly I:C treatment. Concerning IL-2 concentration, 10 µg/mL LTA had an increasing effect, whereas 100 ng/ml

flagellin and 50 μg/mL poly I:C elevated the level of IL-6. When flagellin was administered, IL-10 concentration was elevated at both doses, and RANTES levels rose in response to the higher poly I:C and flagellin concentrations. Elevated LTA and poly I:C concentrations considerably raised the IFN-γ/IL-10 ratio compared to the control. Therefore, the application of the examined agents, LTA, poly I:C and flagellin could induce a satisfactory pro-inflammatory response on chicken ileal explant cultures based on the cytokines of interest.

The applied 1 and 2 mm explants showed a mild alteration of the epithelium and notable damage of the *lamina propria* after 24 h of culturing, meanwhile after 12 h culturing in Preliminary Study II.2 the 1.5 mm explants possessed a mostly columnal epithelial lining with moderate impairment in the propria. Mucosal damage is a characteristic of most of the gut leakage models as it captures the two-hit nature of gut inflammation. This aspect of inflammatory bowel disease is exploited and emphasized in case of the mouse model of dextran sodium sulfate (DSS) induced ulcerative colitis. DSS primarily induces damage of the epithelial barrier and therefore facilitates the intestinal pathogens and their antigens to reach the deeper layers of the mucosa (Chassaing et al., 2014). At the site of the inflammation, DAMPs are released from the destructed cells. They conjointly with PAMPs, are triggers in regulating inflammation and thus play a substantial role in gastrointestinal diseases, e.g., inflammatory bowel disease. The faulty healing of the gut barrier plays an unequivocal role in maintaining inflammatory bowel disease (Boyapati et al., 2016). This tissue damage is indeed a characteristic of the present *in vitro* model, which is also a limitation of it. However, the involvement of DAMPs and impaired tissue integrity related factors could make the explant model even more reasonable if mild damage of the propria and the epithelial layer are not objections for the research setting. Particularly if the study aims to investigate the anti-inflammatory potential of drugs and drug metabolites that could not penetrate through the intact mucosal barrier (e.g. host defense peptides) (Mátis et al., 2024).

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

The present study investigated the immunomodulatory effect of a CME extract. The powder contains 5264 ng/g cordycepin, 4592 beauveriolide I and 9912 ng/g beauveriolide III, and approximately 1‰ β-glucan. The components and the mycotoxin profile of the CME is detailed in Supplementary Material.

Investigating the mechanism of action of cordycepin and CME in poultry is advisable and particularly timely, as a few *in vitro* studies reported general health benefits and effects on the performance of production animals. Han et al. (2015) demonstrated the osteometabolic benefits of the supernatant derived from *Cordyceps militaris* fermentation broth in broiler chickens. The fermentation broth was incorporated into the diet at concentrations of 1, 2, and 4 g/kg. Administration of 1 g/kg significantly enhanced body weight gain, whereas the 4 g/kg dosage markedly increased feed intake of Ross-308 hybrid broilers. Notably, the 2 g/kg supplementation elevated tibial calcium content, indicating enhanced bone mineralization (Han et al., 2015). In a separate 12-week study on Hendrix laying hens, a *Cordyceps militaris* intervention significantly improved feed conversion ratio by reducing it by 10–15% during weeks 9 to 12. Concurrently, there was a statistically significant increase in egg white mass and total egg mass during weeks 5 to 12 and cholesterol content in eggs was significantly reduced by 12–18% in groups receiving 10 and 20 g/kg supplementation by the end of the 12-week period (Wang et al., 2015). The results observed are assumed to be in connection with the general inflammatory homeostasis of the animals. Moreover, the effect on bone mineralization could be understood in light of *in vitro* data from primary bone marrow macrophages. The authors of the respective study concluded that cordycepin could ameliorate osteoclastogenesis and bone resorption while inhibiting ROS generation via the activation of Nrf2 signaling pathway (Dou et al., 2016).

The *in vivo* general health effects of the extract observed *in vivo* could be further supported by the positive effect of the extract on cell viability decreasing LDH activity and inhibiting macrophage pyroptosis and caspase-1 activity (Liu et al., 2025; Peng et al., 2015; Yang et al., 2017). Nonetheless, CME in the present study had no effect on the metabolic activity and LDH release of the cells.

Cordycepin, the primary bioactive compound in CME, alleviated colitis in mice by balancing Th1/Th2 and Th17/Treg immune responses, while in LPS-treated piglets, the full extract (but not cordycepin alone) reduced colon IL-6 and IL-8 levels, though both treatment, pure cordycepin and CME, similarly ameliorated histopathological alterations (Liu et al., 2024; Xiong et al., 2025). Further studies on DSS-induced mice colitis models corroborate a decrease in cytokine levels (e.g. IL-1β, TNF-α) in the blood plasma ascribed to feeding CME (Park and Park, 2013; Wu et al., 2023). These findings underscore the therapeutic potential of cordycepin and CME in colitis, targeting immune dysregulation. Cordycepin exerts its anti-inflammatory effects primarily by inhibiting NF-κB signal pathway, but additional anti-inflammatory effect elicited on MAPK signaling pathways or $A2_A$/A3 adenosine receptors cannot be excluded (Du

et al., 2021; Tan et al., 2021). Systematic reviews highlight its dual regulation of metabolic pathways: activating AMPK (enhancing catabolic energy balance) and inhibiting the PI3K/AKT axis (reducing anabolic processes and cell proliferation). While cordycepin clearly suppresses the ERK pathway, imparting antiproliferative effects, its impact on other MAPK pathways (e.g., JNK, p38), which regulate apoptosis and stress responses, remains ambiguous. Together, these mechanisms position cordycepin as a multifaceted modulator of inflammation, balancing cytokine suppression, metabolic regulation, and targeted pathway inhibition (Radhi et al., 2021).

The present study genuinely investigated the anti-inflammatory effect of CME on a gut explant model to understand the outcome of this complex regulation of cellular pathways by means of the level of produced cytokines (IL-1β, IL-6, IFN-γ, RANTES). Compared to the inflammatory control group, both 0.6% and 0.12% CME treatments elicited suppression of IFN-γ concentration. The IFN-inducing properties of cordycepin have also been documented. Type I IFNs are critically involved in restricting viral replication *in vivo*; thus, the ability of cordycepin to enhance their production is notable. It might be initiated by activating the intracellular pattern recognition receptor RIG-I and its associated signaling pathway (Chanda and Banerjee, 2015). Moreover, two *in vitro* studies report the IFN-γ level increasing effect of CME in mouse splenocytes and RAW 264.7 macrophage cell line (Ha et al., 2006; Kim et al., 2008). This effect is also triggered by cordycepin on a natural killer cell line (Chaicharoenaudomrung et al., 2023). However, neither of the listed articles included PAMP stimulation. Additionally, *Cordyceps militaris* hot water extract treatment attenuated LPS-induced IFN-*γ* expression in the spleen and bursa Fabricii of Ross 308 broiler chicken (Cheng et al., 2019). Based on literature data and our findings, robust IFN-γ production induced by poly I:C or other PAMPs could theoretically be attenuated, whereas CME generally enhances baseline IFN-γ expression. However, in the present study, the higher concentration of CME exposure unless poly I:C treatment also decreased the concentration of IFN-γ. This result highlights the dual regulatory role of CME (and potentially cordycepin) in modulating antiviral immune responses during viral infections. Balancing pro-/anti-inflammatory signals could optimize viral and bacterial clearance while minimizing tissue damage.

In contrast, multiple studies on macrophages have described the unambiguous anti-inflammatory effect of cordycepin by curbing LPS-induced elevated TNF-α and IL production. Peng et al. observed a decrease of IL-1β and TNF-α in a BV2 microglia culture (Peng et al., 2015). Furthermore, in case of RAW 264.7 macrophage cultures exposed to LPS cordycepin decreased the level of TNF-α (Choi et al., 2014; Kim et al., 2006; Lu et al., 2015; Qing et al.,

2017; Shin et al., 2009b, 2009a; Yang et al., 2017), IL-1β (Choi et al., 2014; Liu et al., 2025; Shin et al., 2009b, 2009a; Yang et al., 2017) and IL-6 (Qing et al., 2017; Shin et al., 2009a; Yang et al., 2017). Corroborating these findings, CME treatment significantly reduced the levels of RANTES and IL-1β cytokines produced by explants exposed to poly I:C according to our observations. A similar reduction in RANTES was reported in RAW 264.7 cell line experiments. In the first study, this decrease was associated with lowered nitric oxide (NO) and COX-2 levels and inhibition of the NFκB signaling pathway. In the latter, the reduction in RANTES was linked to a shift in the cell morphology toward an M2 anti-inflammatory phenotype (Shin et al., 2009a, 2009b).

The antioxidant properties of CME are primarily attributed to its polysaccharides, phenolic compounds, and carotenoids, although its efficacy may also be influenced by the presence of cordycepin, ergothioneine, and selenium (Jędrejko et al., 2021). Several studies have highlighted the significant role of cordycepin in mediating the redox effects of CME, with *in vitro* findings indicating that cordycepin can enhance the activity of the Nrf2 transcription factor and the associated antioxidant pathways (Dou et al., 2016; Qing et al., 2017). Notably, Qing et al. reported that cordycepin initially increases reactive oxygen species (ROS) levels, which subsequently leads to the activation of Nrf2 signaling. This observation is consistent with the present study, where a pro-oxidant effect of CME was observed at higher dosages. Importantly, no studies in the reviewed literature have reported an increase in MDA level following treatment with CME. On the contrary, most *in vivo* studies have demonstrated reduced lipid peroxidation, as evidenced by decreased MDA levels. For example, treatment with polysaccharides from the fruiting body of *Cordyceps militaris* significantly reduced MDA levels in RAW 264.7 cells subjected to H_2O_2-induced oxidative stress, whereas mycelial intracellular and extracellular polysaccharides with higher reported antioxidant capacity did not produce the same effect (Guo et al., 2024). However, certain compounds, such as flavonoids, can exert dual effects on redox homeostasis, with these actions being hypothetically dependent on their concentration and molecular structure (Vörösházi et al., 2025). This effect of CME, observed exclusively in the higher concentration treatment group, may be attributable to any of its various bioactive constituents.

7.6 Conclusion

The present thesis has successfully characterized and refined *in vitro* and *ex vivo* inflammatory models employing chicken hepatic co-cultures and ileal explants, thereby

providing a platform for the evaluation of presumed immunomodulatory agents. These models served as a reliable way to test how different substances affect inflammation and inflammation related cell and tissue damage in chicken. The chicken primary hepatocyte – non-parenchymal cell co-culture model, responded clearly to inflammatory triggers like flagellin and poly I:C. This allowed for careful measurement of changes in cell metabolism, membrane integrity, and cytokine production. Similarly, the gut explant system, optimized for explant size and incubation time, proved sensitive to PAMP-induced inflammatory responses, reflecting the complexity of mucosal immunity.

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

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

8 New scientific results

Ad 1,

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

Ad 2,

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

Ad 3,

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

Ad 4,

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

Ad 5,

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

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10 Own scientific publications

Publications related to the topic of the present thesis

Full text papers in peer-reviewed journals:

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Supervision of DVM theses:

Herrmann, Eva Madeleine: **The effects of chicoric acid on chicken primary hepatic cell co-cultures under viral RNA analog induced inflammation.** TDK thesis. Supervisors: Patrik Tráj and Gábor Mátis, Budapest, 2021.

Kákonyi Ákos Richárd: **The anti-inflammatory effect of luteolin on flagellin treated chicken hepatic cell culture**. TDK thesis. Supervisors: Patrik Tráj and Gábor Mátis, Budapest, 2022.

Dudás Réka: **Effects of Bacillus extracts on ileal explant cultures of chicken origin**. TDK thesis. Supervisor: Patrik Tráj, Gábor Mátis Budapest, 2023.

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12 Supplementary material

Report on the results of the analysis of mycotoxins in fermentation samples

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Samples: CME (2023.01.20)

Analytical task:

Quantitative determination of mycotoxins and secondary metabolites.

Instrumental parameters

Detection and quantification was performed with a QTrap 5500 MS/MS system (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray electrospray ionization (ESI) source and a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was performed at 25 °C on a Gemini® C18-column, 150×4.6 mm i.d., 5 µm particle size, equipped with a C18 security guard cartridge, 4×3 mm i.d. (all from Phenomenex, Torrance, CA, US. ESI-MS/MS was performed in the scheduled multiple reaction monitoring (sMRM) mode both in positive and negative polarities in two separate chromatographic runs. The sMRM detection window of each analyte was set to the respective retention time ±27 s and ±42 s in positive and in negative mode, respectively. The target scan time was set to 1 s. The settings of the ESI-source were as follows: source temperature 550°C, curtain gas 30 psi (206.8 kPa of max. 99.5% nitrogen), ion source gas 1 (sheath gas) 80 psi (551.6 kPa of nitrogen), ion source gas 2 (drying gas) 80 psi (551.6 kPa of nitrogen), ion-spray voltage −4,500 V and +5,500 V, respectively, collision gas (nitrogen) medium.

Description of the LC-MS/MS protocol and validation data for seven matrices is available free of charge at Analytical and Bioanalytical Chemistry https://link.springer.com/article/10.1007/s00216-020-02489-9

Identification and quantification

Confirmation of positive analyte identification is obtained by the acquisition of two sMRMs per analyte (with the exception of moniliformin and 3-nitropropionic acid, that each exhibit only one fragment ion), which yields 4.0 identification points according to commission decision 2002/657/EC. In addition, the retention time and the intensity ratio have to agree within 0.03 min and 30% rel., respectively, with an authentic standard.

Quantification is based on serial dilution of a multianalyte stock solution. The accuracy of the method is verified on a routine basis by participation in interlaboratory testing schemes including a broad variation of matrices of grains, nuts, dried fruits and spices. Satisfactory z-scores between -2 and 2 have been obtained for > 95% of the > 1800 results submitted so far. The expanded measurement uncertainty has been determined to be 50 % (coverage factor 2).

Limits of detection and quantification were calculated according to the EURACHEM guide.

Extraction

0.5 g powdered sample were extracted for 90 min with 10 mL of acetonitrile/water/acetic acid 79/20/1. The raw extract was diluted 1:1 with acetonitrile/water/acetic acid 20/79/1 and 5 µL of the diluted extract was injected into the LC-MS/MS system without further pre-treatment.

Results

Table 8. Concentrations (expressed as ng/g, respectively) of positively identified analytes in the investigated sample.

Analyte class	Analyte	CMAD-0117
Fusarium metabolites	Enniatin B	1,00
Penicillium metabolites	1-Deoxypebrolide	0,26
	Andrastin A	0,26
	Citreohybridinol	1,86
	Dechlorogriseofulvin	0,51
	Griseofulvin	7,83
	Mycophenolic acid	4,55
	Pyrenocin A	3,44
	Quinolactacin A	0,13
Alternaria metabolites	Tenuazonic acid	42,1
	Infectopyron	33,5
	Radicinin	0,66
Metabolites from other fungal genera	Abscisic acid	56,2
	Beauveriolide I	4592
	Beauveriolide III	9912
	Calphostin C	379
	Cordycepin*	5264
	Dichlordiaportin	13,7
Plant metabolites	Daidzein	< LOD
	Daidzin	< LOD
	Genistein	< LOD
	Genistin	< LOD
	Glycitein	< LOD
	Glycitin	< LOD
Unspecific metabolites	Brevianamid F	145
	cyclo(L-Pro-L-Tyr)	881

	cyclo(L-Pro-L-Val)	846
	Fellutanine A	41,8
	N-Benzoyl-Phenylalanine	1,08
	Rugulusovin	70,3
	Tryptophol	< LOD

*The peak of cordycepin in the samples exhibited a small, but yet significant shift in LC retention time compared to the standards (4.83 min vs. 4.87 min), which is just outside internal criteria of ± 0.03 min.

All other investigated analytes were below their respective limits of detection in the investigated samples (see also Appendix).

Tulln, January 26th, 2023 Michael Sulyok