University of Veterinary Medicine
Department of Pharmacology & Toxicology

Thesis

Luteolin and chrysin could prevent lipopolysaccharide-ochratoxin combination-caused inflammation and oxidative stress *in vitro*

Annelie Wohlert

Supervisor:
Dr. Pásztiné dr. Gere Erzsébet, associate researcher
Department of Pharmacology & Toxicology

2024

Table of content

1. List of abbreviations 2
2. Introduction 4
3. Literature overview 5
3.1 Characterization of IPEC-J2 cell line and minipig hepatocytes 5
3.2 Reactive oxygen species and oxidative stress 7
3.3 Production of inflammatory cytokines IL-6 and IL-8 8
3.4 The effects of OTA in the intestine 8
3.5 The impact of bacterial lipopolysaccharide 11
3.6 Characterization of chrysin and luteolin 11
4. Aim of study 13
5. Material and methods 14
5.1 Reagents 14
5.2 Cells and culturing conditions 14
5.3 Treatments of IPEC-J2 cells with OTA, LPS and flavonoids LUT and CHR 16
5.4 Cell viability evaluations 16
5.5 Detection of changes in redox status of IPEC-J2 cells and minipig hepatocytes 17
5.6 Determination of proinflammatory cytokine IL-6 and IL-8 expression 18
5.7 Statistic analysis 19
6. Results 19
6.1 Cell viability assay on the IPEC-J2 cell line 19
6.2 Detection of extracellular hydrogen-peroxide production 20
6.3 IC ROS determination 21
6.4 The changes in IL-6 and IL-8 levels after exposure to OTA, LPS and the selected flavonoids in IPEC-J2 cells 22
6.5 Cell viability of minipig hepatocytes 24
6.6 Extracellular hydrogen-peroxide evaluation in minipig hepatocytes 25
7. Discussion and conclusion 26
8. Summary 31
9. Összefoglaló 32
10. References 33
11. Acknowledgements 39
12. HuVetA statement 40
13. Thesis Progress Report 42
14. Supervisor counter signature 44
15. TDK equivalence statement 45

1. List of abbreviations

AFB1: Aflatoxin B1

ANOVA: Analysis of variance

AP-1: Activator protein 1

BLAST: Basic local alignment search tool

C7G: Chrysin-7-glucuronide

C7S: Chrysin-7-sulfate

CaCo-2: Human colorectal adenocarcinoma cells

CAT: Catalase

CCK-8: Cell counting kit 8

CHR: Chrysin

COX-2: Cyclooxygenase-2

DCF: Dichloro-fluorescein

DCFH-DA: 2'-7'dichlorodihydrofluorescein diacetate

DMEM: Dulbecco's modified Eagle's medium

DNA: Deoxyribonucleic acid

EC: Extracellular

EGF: Epidermal growth factor

ELISA: Enzyme-linked immunosorbent assay

E. coli: *Escherichia coli*

FBS: Fetal bovine serum

FFA: Free fatty acids

FHs74: Human fetal small intestinal epithelial

GSH: Glutathione

GSH-Px: Glutathione-peroxidase

HIF-1α: Hypoxia-inducible factor 1-alpha

HT29-MTX: Human colorectal adenocarcinoma cells (differentiated using methotrexate)

H_2O_2: Hydrogen-peroxide

IC: Intracellular

IgA: Immunoglobulin A

IL-1β: Interleukin-1 beta

IL-6: Interleukin-6

IL-8: Interleukin-8

IFN-γ: Interferon gamma

iNOS: Inducible nitric oxide synthase

IPEC-J2: Porcine intestinal jejunal epithelial cells

JAK-STAT: Janus kinases -signal transducer and activator of transcription proteins

LDH: Lactate dehydrogenase

LPS: Lipopolysaccharide

LUT: Luteolin

MAPK: Mitogen-activated protein kinase

MDA: Malondialdehyde

MLCK: Myosin light chain kinase

MPO: Myeloperoxidase

mRNA: Messenger ribonucleic acid

MTT/MTS: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NOX: NADPH oxidase

Nrf 2: Nuclear factor erythroid 2-related factor 2

NRK-52E: Rat renal proximal tubular cell line

OTA: Ochratoxin A

PLA2: Phospholipase A2

PTEN: Phosphatase and tensin homolog

PUFA: Polyunsaturated fatty acids

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

RT-PCR: Real-time polymerase chain reaction

SEM: Standard error of mean

SOD: Superoxide dismutase

TJ: Tight junctions

TLR4: Toll like receptor 4

TNF-α: Tumor necrosis factor-alpha

2. Introduction

One of the recent major worldwide issues has been the extensive global climate change, which has a significant impact on the production of good-quality agricultural feeds and thus on maintenance of appropriate conditions for the livestock. Mycotoxins, as secondary metabolites of fungi, can contaminate crops and cereals during the vegetative period or during storage. These plant parts can be present in the cereal-based foods for human consumption and in feeds for farm animals. Fungal infestation can lead to deterioration of the crops and mycotoxins such as ochratoxin A (OTA) can confer acute and chronic safety risk upon oral intake [1,2]. Grain-based products consumed by the population of poorer countries are mostly contaminated with mycotoxins [3]. Poisoning with mycotoxins usually occurs through the gastrointestinal system. The extent of this adverse mycotoxin effect depends on the frequency and the dose as well as on the type of mycotoxin and on the species. The synergistic effect of other mycotoxins and other chemicals cannot be neglected either [4]. Among monogastric animals, poultry and pigs are particularly sensitive to contamination caused by certain mycotoxins. They can cause an unpleasant taste, thereby reduce feed intake and growth, and consequently the performance of farm animals [5].

The consumption of animal-deriving products raises yearly. This can lead to extensive spread of bacterial infections within the livestock. Small farm numbers are getting less every year while large scale farms keep growing leading to more stress and population density and to potential danger of spreading diseases faster. Gram negative bacteria containing lipopolysaccharides (LPS) can result in damage and dysfunction of enterocytes. The intestinal system has a critical role in the uptake of LPS thus needs to be protected [6]. One of the most common bacteria within a pig livestock is *Escherichia coli* (*E.coli)* which in case of infection commonly leads to damage to the enterocytes. The induced intestinal epithelium barrier integrity impairment can cause secondary infections and as well lowers performance of the animals overall such as growth rate [7]. Antibiotics can be used to regulate *E.coli*-caused infection, but antibiotic resistances within bacteria are developing even faster thus regulations considering safety of food production are getting stricter every year. Multi-resistant bacteria in human medicine often need treatment with antibiotics belonging to the restrictive class (AMEG A) of antibiotics, which are preserved for mainly human use and companion animals in special circumstances. The EU government decided on new regulations (Regulation (EU) 2019/4 and EU Regulation (EU) 2019/6) to act against resistant bacteria from the 28th of January 2022 [8–10]. Alternatives to antibiotics such as

pre- and probiotics, feed supplements and additives, vitamins and flavonoids have recently been investigated more intensely to maintain animal health and to reduce application of antibiotics. Moreover, the utilization of these bioactive compounds can lead to less economic loss, less or no withdrawal period, safer and stress-free application and better overall herd health.

Plant-derived extracts containing polyphenols have been shown to have beneficial effects on the intestinal epithelium *in vitro* and in animal experiments exposed to various xenobiotics [11–15]. Among the flavonoids, luteolin (LUT) and chrysin (CHR) can be found in honey, nuts, grains, propolis, blue passionflower and in various fruits such as bitter lemon [12,16,17]. Polyphenols are rarely found in the form of aglycones, mostly they are conjugated with sugar components in plants and can gain their active forms only in the digestive tract after enzymatic degradation. *In vitro* research on plant antioxidants might contribute to prevention of intestinal diseases caused by harmful xenobiotics such as OTA and bacterial LPS.

The aim of our work was to create a porcine intestinal jejunal epithelial (IPEC-J2) cell model and to work on minipig hepatocytes, on which we can study the effects of OTA and LPS on the viability of intestinal epithelial cells, redox and inflammatory processes, and study the effects of LUT and CHR in preventing the harmful effects caused by harmful xenobiotics. During our research, we sought an answer to whether LUT or CHR can mitigate the negative effects of OTA and LPS or their combinations, and based on the data of *in vitro* tests, whether they are suitable for being an alternative feed supplement to prevent the adverse effects of mycotoxicosis and LPS exposure.

3. Literature overview

3.1 Characterization of IPEC-J2 cell line and minipig hepatocytes

The IPEC-J2 cell line is a non-tumorigenic cell line of neonatal porcine small intestinal epithelial origin, which was isolated from the jejunum of newborn pigs, even before colostral milk intake. The cells are characterized by a rapid proliferation and colonization ability [18,19] which makes them appropriate model for *in vitro* investigation of crosstalks between xenobiotics such as LPS [6,7,20–24] and mycotoxins such as OTA [25–28] and swine enterocytes. During differentiation, tight junction (TJ) is formed between the cells with the help of the cell junction proteins claudin-1, -3, -4, -5, -7, -8, -12, tricellulin,

occludin, E-cadherin and zonula occludens-1 [19,29], as well as microvilli [30]. Their mucosal production *in vitro* depends on the serum added to the medium [30,31]. Messenger ribonucleic acids (mRNAs) of several interleukins such as IL-1, -1, -6, -7, -8, -10, -12A, -12B, -15, -18 were detected [32] and the production of IL-6, -8 by IPEC-J2 cells was also demonstrated at the protein level [33,34]. IPEC-J2 cells are widely used *in vitro* studies because they can survive and differentiate under suitable culturing conditions. In addition, they can mimic the human gut physiology due to the fact that pig intestine is the closest to the human one [35–37]. The interactions between the intestinal epithelium and bacteria [38,39], viruses [40], mycotoxins [41,42] or other xenobiotics such as flavonoids [12,13,24,43,44] are shown in several studies (Figure 1).

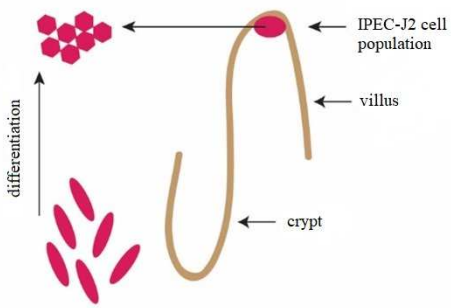

Figure 1: Schematic representation of the IPEC-J2 cell population by *[45]* modified by Annelie Wohlert

The Göttingen minipig is a crossbred of three breeds: the Minnesota minipig, the German landrace pig and the Vietnamese potbelly pig. This breed is raised under pathogen-free conditions for investigative purposes [46]. The liver of minipigs morphologically differ from that of human, as there are more prominent lobes and fibrinous connective tissue between portal triads [47]. CYP450 may be the most important enzyme within the hepatocytes and it is found that minipig hepatocytes are very much alike the human ones [48,49]. In particular the enzymes CYP1A2, CAP2A6, CYP2E1, CYP2C9, CYP3A4 have very minor differences in activity compared to human and only medium differences where detected in CYP2C19, CYP2D6 and CYP3A4 activity [50]. Immunoblotting showed that minipig CYP3A29 is 60% similar to human CYP3A4, while minipig CYP2A showed even 70% similarity to human CYP2A6 [51].

Studies showed that investigations with basic local alignment search tool (BLAST) lead to recognition that minipig hepatocyte transporters such as ABC-efflux-transporter and SLC influx-transporter systems are very homologous to human hepatocytes in a range of 72-90% [50].

3.2 Reactive oxygen species and oxidative stress

In animals and plants, as well as in fungi, the oxidative (or redox) state plays a central role in biological processes. Redox homeostasis in animal cells is highly conserved, but there may be significant differences between taxa and in the amount of prooxidant and antioxidant compounds [52]. The redox reactions take part in important signaling mechanisms in cellular metabolism. Free radicals have one or more unpaired electrons in the outermost electron shell, and they can be divided into two main groups: reactive oxygen species (ROS) and reactive nitrogen species (RNS). They have been identified in the literature as key players in the cellular and oxidative stress in initiating, mediating and regulating biochemical processes [53].

The group of ROS molecules includes the superoxide anion ($O_2^{\cdot -}$) and the hydroxyl radical (OH^-), which have unpaired electrons and are therefore highly reactive and they can react with the proteins, lipids and deoxyribonucleic acid (DNA) of the cell. Hydrogen peroxide (H_2O_2) is a non-radical two-electron reduction product of oxygen, which can function as a signaling molecule [54]. ROS play a role in the modification of several signaling pathways [55,56]. They can modulate division and are linked to viability pathways, such as mitogen-activated protein kinase (MAPK) [57] and phosphatase and tensin homolog (PTEN) routes.

ROS species are produced in mitochondria under physiological conditions [58], and through the NADPH oxidase (NOX) enzyme family [59]. However, under the influence of cell-damaging agents, intracellular (IC) production of free radicals can increase rapidly and cause oxidative stress [60].

Oxidative stress occurs when the concentration of ROS exceeds cellular antioxidant capacity. Among the free radicals, hydroxyl radicals can react with polyunsaturated fatty acids (PUFA) present in high concentrations in cell membranes as part of the lipid peroxidation. As a result of lipid peroxidation membrane proteins adopt a cross-linked structure, based on the decrease in rotational and lateral mobility [61]. Amount of malondialdehyde (MDA) also increases thus ultimately the integrity of the membrane is damaged.

3.3 Production of inflammatory cytokines IL-6 and IL-8

Cytokines, such as IL-6 and IL-8, mainly give responses in case of infection or inflammation. But IL-6 additionally regulates regenerative, neural and metabolic processes [62,63]. The most important function of IL-6 is the accumulation of neutrophiles 24-48 hrs after infection. Although it is mainly considered as pro-inflammatory cytokine, IL-6 also has anti-inflammatory activity thus also helps regeneration [62]. IL-6 supports liver regeneration as well as stimulates the hepatic acute phase proteins [63], thus mice missing IL-6 showed liver impairment after a 2/3 hepatectomy [62]. IL-8 can be found in different tissue types as well as in blood cells. It is very specifically targeting neutrophiles thus IL-8 is a major cytokine in pro- and inflammatory processes [64,65]. The prominent role of IL-8 is to attract neutrophiles into misfunctioning tissues. IL-8 gets activated by LPS, living bacteria, tumor necrosis factor alpha (TNF-α) and IL-1 [66,67] within 24 hrs [65].

3.4 The effects of OTA in the intestine

Ochratoxin A (OTA) is a toxin derived from *Aspergillus ochraceus* and *Penicillium verrucosum* fungi and they are found mostly in the food chain and the feedstuff of animals [68,69]. Chemically OTA is L-phenylalanine-coupled chlorophenolic group containing a dihydroisocoumarin moiety [70] (Figure 2). OTA is proven to be nephrotoxic, teratogenic, neurotoxic as well as immunotoxic and possibly carcinogenic to humans [69,70].

The most important port of entry for OTA is the intestinal cell barrier and this toxin will destroy the intestinal barrier function and thus can have access to the circulation [28]. Upon entering the body circulation, it will bind to the blood serum and start accumulating different organ systems with different affinities. The highest affinity to OTA is known to have the liver, kidney and the blood system [71]. OTA levels were found to be significantly

elevated in the blood serum and kidneys of porcine [72]. Consumption of contaminated pork meat-related products may lead to chronic toxicosis with OTA in humans. In addition, OTA is proven to have a much longer half-life in the blood of the humans and pigs in comparison to any other animal species, which indicates a higher sensitivity to OTA of these species [69]. OTA has shown to affect the cell proliferation of the gut *in vitro* models and moreover influences the cell variability on human colorectal adenocarcinoma (Caco-2) cells as well as on human colorectal adenocarcinoma (differentiated using methotrexate) (HT29-MTX) cell lines which are tumor cell lines [28]. It can cause ROS on IPEC-J2 cells through apoptosis of these and inhibition of mitochondrial respiration chain [68]. OTA is known to elevate ROS by increasing the IC calcium-level as well as the myosin light chain kinase (MLCK) activity [27]. Furthermore, it elevated ROS production in concentration-dependent manner detected by MitoSOX Red staining via increasing the IC calcium-level thus the significant MLCK activity [27].

The first line of defense within the body is known to be the intestinal epithelial barrier. Moreover, it is apoptotic to more organs in addition to the intestinal system including the kidney, the liver, the immune system as well as even lymphocytes and single-stranded DNA [73]. OTA has shown to elevate lipid peroxidation and producing free radicals [74]. Another important factor in decreasing oxidative stress in the body is the glutathione system [74]. A significant elevation of ROS-levels and lowering of antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione-peroxidase (GSH-Px) by an OTA+ aflatoxin B1 (AFB1) combination was found in the serum, in the kidney and in the liver of chicken after 42-days of feeding the mycotoxins in a concentration of 101.41 µg/kg OTA and 20.10 µg/kg AFB1 in the diet [75]. Furthermore, OTA is proven to show significant ROS elevation in human fetal small intestinal epithelial (FHs74) cells *in vitro* in a concentration of 2 µM after 48 hrs of incubation and measurement with 2'-7'dichlorodihydrofluorescein diacetate (DCFH-DA) method [76].

The inflammatory response in the form of cytokines as well as interleukins and immunoglobulin A (IgA) is one of the first reactions of the body to get rid of the mycotoxins. The intestinal epithelium produces mucin containing antimicrobial peptides in order to protect the epithelial layer [28]. These toxins have shown to be capable of elevating the pro-inflammatory cytokines, such as IL-6, IL-8, interleukin-1 beta (IL-1β), cyclooxygenase-2 (COX-2) as well as TNF-α in IPEC-J2 cell lines [28,73]. It was also evaluated by enzyme-linked immunosorbent assay (ELISA) that OTA+ AFB1 in the diet can cause elevation in IL-6 and IL-8 levels as well as a reduction of IL-10 *in vivo* in chicken serum, liver and kidney

when the treatment lasted 42 days [75]. It was also found that OTA did not increase pro-inflammatory IL-8 secretion in Caco-2 cells directly, however, it can facilitate the impact of IL-1β -induced cytokine production in lower concentrations and it enhances transepithelial passage of non-invasive commensal *E. coli*. In this case IL-8 secretion was measured indirectly through IL-1β and evaluated by real-time polymerase chain reaction (RT-PCR) showing significant elevation of IL-8 secretion after treatment of 12 hrs in concentrations 1 µM, 10 µM and 100 µM OTA in Caco-2 cells after seeded basolaterally [77]. IL-6 values were significantly increased after 24 hrs in FHs74 cells exposed to 4 µM OTA [76].

Mycotoxins showed to be cytotoxic mainly on the intestinal cells leading to apoptosis and destroying the intestinal barrier so OTA can possibly cause a disbalance of the microbiome as well as leading to a secondary infection [27]. Cell viability investigated by cell counting kit-8 (CCK-8) was found to be significantly decreased in FHs74 cells by aflatoxin M1 or OTA (0.05 µM-4 µM) or with their combination after 48 hrs of treatment [76]. OTA is reported to affect IPEC-J2 cell viability using both 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and Lactate dehydrogenase (LDH) method, and it also caused barrier dysfunction such as damage to the integrity of IPEC-J2 monolayers [27]. Cell viability assay, MTT showed significant cytotoxic effects of OTA (in a range of 1 µM to 180 µM) in Caco-2 (from 80.6 to 53.8%) /HepG2 (from 87.8 to 66.3%) cell lines after 24 hrs [78].

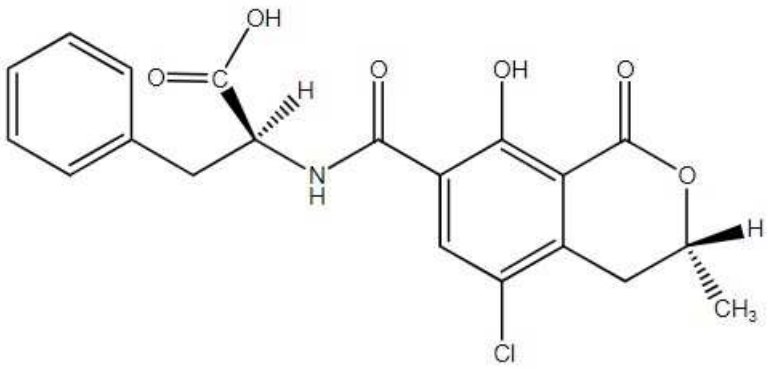

Figure 2: OTA chemical structure

3.5 The impact of bacterial lipopolysaccharide

LPS is an endotoxin deriving from Gram-negative bacteria such as *E.coli*. LPS is characterized by a hydrophobic part called lipid A [79,80]. This endotoxin can cause oxidative stress by alteration in the metabolism and as well leading to inflammatory processes and morphological damage within the body [12] as well as septic shock [79].

IC ROS showed a significant increase based on DCFH-DA after 24 hrs treatment with LPS deriving from *E.coli* in addition to endotoxin-induced cytotoxicity examined by Neutral red assay [11,14]. Extracellular (EC) H_2O_2 levels measured by Amplex red method in IPEC-J2 cell line did not seem to be elevated significantly upon the administration of 10 μg/ml LPS for 24 hrs [14]. Other studies showed a significant enhancement in IC ROS in DCFH-DA evaluation after 24 hrs treatment of IPEC-J2 cells with LPS of *E.coli* (strain O127:B8 or O111:B4) [12,14].

LPS as a component of Gram-negative bacteria cell wall can release pro-inflammatory cytokines mostly in macrophages, such as TNF-α, IL-6, IL-8 [7,12,79], which can lead to a septic shock. IL-6 [7,81] and IL-8 [81] were elevated significantly in IPEC-J2 cells after treatment with 10 μg/mL LPS for 12 hours and measured with ELISA assay. A different study showed the same significant elevation of IL-6 and IL-8 in IPEC-J2 cells after 1 hr incubation with LPS deriving from *E.coli* [11].

3.6 Characterization of chrysin and luteolin

Chrysin (also called 5,7-dihydroxy-2-phenyl-4H-chromen-4-on) is a flavonoid of the class of flavones which is mainly plant- and honey-derived and occurs naturally in fruits and vegetables (Figure 3). CHR contains two benzene rings and a heterocyclic ring containing oxygen and has 2 or 3 double bond carbon and a carbonyl group linked to the fourth carbon [82]. Studies showed that chrysin has many positive effects on different diseases and includes to be anti-inflammatory, antiviral, antibacterial, anticancer and antioxidant [16,17,83].

CHR can improve the lipid peroxidation thus lower the oxidative stress [82] and shows to be neuro protective. CHR is hepatoprotective via tumor necrosis factor-alpha (TNF-α) and lowers free fatty acids (FFA), cholesterol and triglyceride [16,17,82]. It has anti-inflammatory properties due to inhibition of phospholipase A2 (PLA2) and more specifically the COX-2 binding site as well as blocker of the inducible nitric oxide synthase

(iNOS) and myeloperoxidase (MPO). CHR can also decrease inflammatory cytokines such as interferon gamma (IFN-γ), TNF-α, IL-1β, IL-6 and IL-12 and inhibit prostaglandin production. IL-6 was successfully quenched by CHR after 72 hrs of incubation in rats after 16 days of treatment [84]. *In vivo* investigation showed a reducing in oxidative stress by CHR in mice by elevating SOD and CAT as well as GSH-Px and intervening Na-K-ATP-ase [82].

However, it has a relatively poor oral bioavailability and needs suitable carriers to reach the effective dose [16]. Through the metabolism CHR is mainly broken down to the active metabolites chrysin-7-sulfate (C7S) and chrysin-7-glucuronide (C7G) within humans and mice [17].

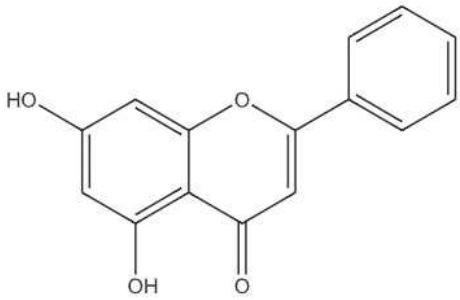

Figure 3: Chemical structure of CHR

Luteolin is a 3´,4´,5,7-tetrahydroxy flavone and polyphenolic compound (Figure 4), and it can be found in plants and fruits such as pepper, carrots, rooibos tea or broccoli. The properties of LUT include neuroprotective, anti-inflammatory, antioxidant, cardioprotective and anticancer effects [12,85] as well as antimicrobial, chemo preventive and antidiabetic effects [15].

Although the oral bioavailability is thought to be very low in flavonoids overall, LUT seems to be more absorbed by specific parts of the intestines such as colon and ileum [85] and is non-toxic [15]. The anti-inflammatory properties are exerted via inhibition of inflammatory cytokines such as IL-1β, IL-2, IL-6, IL-8, IL-12, IL-17 and TNF-α [14,15] as well as by regulating nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), Activator protein 1 (AP-1) and Janus kinases -signal transducer, activator of transcription proteins (JAK-STAT) and through increasing anti-inflammatory cytokine IL-10. LUT is also able to modulate the MAPK [15] pathway, which produces ROS. Moreover, LUT has proven effects against various pathogens [14]. In injured cells, damaged by TNF-α, LUT can elevate the GSH level, which is an effective system against ROS [15]. *In vivo*

LUT can inhibit LPS-induced TNF-α production in rats and IL-6 and TNF-α expression in kidney of mice and is proven to show positive effects on inflammatory diseases in both *in vivo* and *in vitro* models [15].

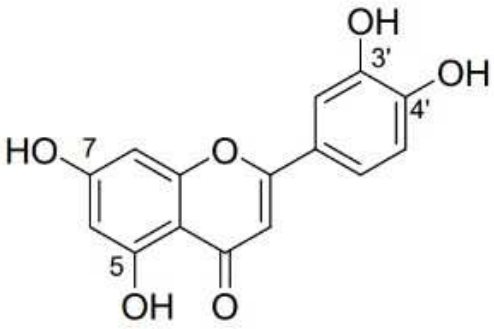

Figure 4: LUT chemical structure

4. Aim of study

OTA and LPS affect the physiological function of the intestine and the liver negatively thus *in vitro* investigation of damaging effects of these toxins applied either individually or in combination in porcine IPEC-J2 cells and minipig hepatocytes is of crucial importance from the point of developing strategies in the treatment of enterohepatic disorders. Our main goal was to investigate if naturally occurring, plant-derived flavonoids such as LUT and CHR can exert positive anti-inflammatory and antioxidant effects in porcine intestinal cells and hepatocytes exposed to 24 hrs-lasting OTA and LPS administration. In our study we planned to carry out the following experiments:

- In our study cytotoxicity of individually applied and co- administered OTA and *E. coli* LPS and the potential beneficial effects of LUT or CHR were assessed measuring cell viability rates in IPEC-J2 cells and in minipig hepatocytes.
- In addition, markers of oxidative stress such as EC hydrogen-peroxide production and IC ROS levels were monitored after 24 hrs exposure of IPEC-J2 cells to OTA or LPS alone or to their combinations either in the presence or in the absence of LUT and CHR.

- In minipig hepatocytes the impact of OTA exposure on EC peroxide secretion was monitored and the effects of CHR on OTA-mediated oxidative stress was evaluated in three different concentrations (100 nM, 500 nM, 1000 nM).
- In addition, changes in secretion of proinflammatory cytokines such as IL-6 and IL-8 were followed upon LPS/OTA treatment at protein level. Furthermore, potential beneficial effects of LUT and CHR were also elucidated in the restoration of upregulated IL-6 and IL-8 production.

There have been several studies about the efficiency of flavonoids in bacterial and viral infections, but little is known about the potential of flavonoids to alleviate adverse effects of co-occurred bacterial LPS and OTA. This *in vitro* study was designed to predict properties of flavonoids to counteract intestinal and hepatic damage produced by the mycotoxin OTA and *E. coli* LPS.

5. Material and methods

5.1 Reagents

Chemicals used in this study such as LUT, LPS solution originated from *Escherichia coli* O111:B4, fluorimetric hydrogen peroxide assay, and porcine IL-6 and IL-8 ELISA kits were obtained from Merck (Darmstadt, Germany). OTA and the flavonoid CHR were kindly provided by Dr. Miklós Poór, (University of Pécs, Pécs, Hungary). Amplex red hydrogen peroxide assay kit was purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA) and CellTiter96 Aqueous One Solution reagent was from Promega (Bioscience, Budapest, Hungary).

5.2 Cells and culturing conditions

The cell line used for the experiments was the IPEC-J2 from the jejunum of porcines. The IPEC-J2 epithelial cell line was a kind gift from Dr. Jody Gookin (Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, USA). The cell monolayers were maintained in 75 cm^2 cell culture flasks with filtered caps (Orange Scientific, Braine-l'Alleud, Belgium) at 37°C in a humidified atmosphere of 5% CO^2. The culture medium contained 50% Dulbecco's modified Eagle's

medium (DMEM) and 50% Ham's F12 Nutrient Mixture (Merck, Darmstadt, Germany) supplemented with 1.5 mmol/L HEPES, 5% fetal bovine serum (FBS) (Biocenter, Budapest, Hungary), 1% insulin/transferrin/sodium selenite medium supplement, 5 ng/mL epidermal growth factor (EGF), and 1% penicillin/streptomycin (all purchased from Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Cells were used between passages 42 and 45 (Figure 5). The media were changed every second day.

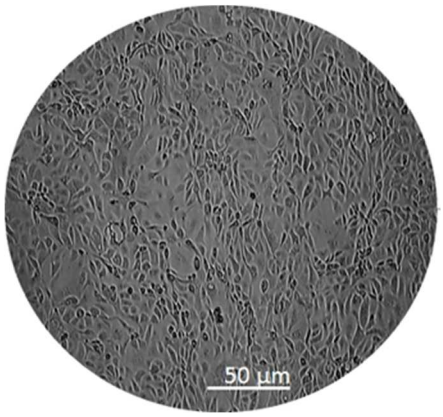

Figure 5: Microscopic picture of IPEC-J2 cells (100x magnitude). The scale bar indicates 50 µM.

Hepatocytes originating from minipigs were used as an additional cell line. Cryopreserved Göttingen minipig hepatocytes (Figure 6) were purchased from Primacyt Cell Culture Technology GmbH (Schwerin, Germany). Hepatocytes were thawed using minipigs hepatocyte recovery medium (Lonza, Biocenter Ltd., Szeged, Hungary) and cells were centrifuged (10 min, 100 g, 20 °C) to remove supernatants. Cells were incubated with 5% FBS-containing plating medium for 6 hrs, then with FBS-free medium (both media were obtained from Lonza, Biocenter Ltd., Szeged, Hungary) on collagen-coated membrane inserts (Costar Transwell-COL PTFE permeable supports, 6.5 mm inserts, 0.4 µm pore size, 24-well plate, Corning Incorpo-rated, Kennebunk ME, US) for CCK-8 investigations. Maintenance medium without FBS was replaced every 24 hrs and the cells were incubated at 37 °C with 5% CO_2.

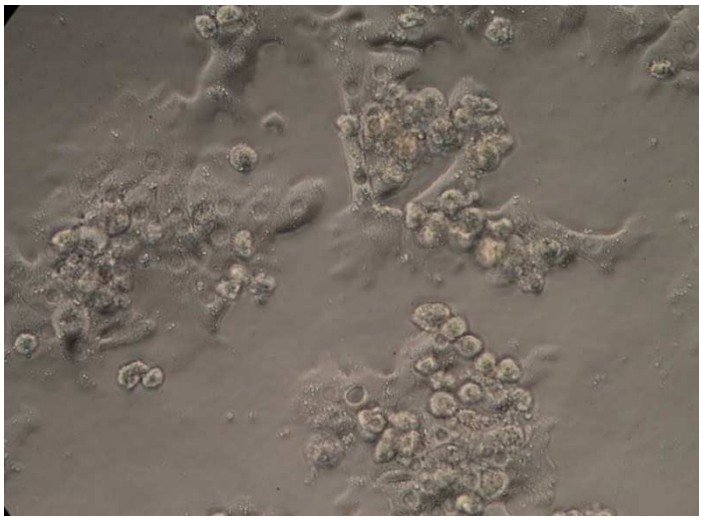

Figure 6: Göttingen minipig hepatocytes (40x magnitude)

5.3 Treatments of IPEC-J2 cells with OTA, LPS and flavonoids LUT and CHR

The stock solutions were freshly made with phenol red-free DMEM/F12 (Merck, Darmstadt, Germany). OTA were used at concentrations of 1 µM, 5 µM, 20 µM. CHR was applied at 1 µM, LUT was at 2.5 µg/ml and LPS was used at 10 µg/ml. Cell cultures were exposed to the treatments for an incubation time of 24 hrs. After the treatment cell-free supernatants were collected for subsequent procedures such as IL-6/IL-8 and EC H_2O_2 determination. DCFH-DA and MTS assays were completed with cells seeded on 24-well and 96-well plates, respectively.

5.4 Cell viability evaluations

Cytotoxicity was examined with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTS) reagent (CellTiter96 Aqueous One Solution, Promega, Bioscience, Budapest, Hungary) in IPEC-2 cells. This test measures the rate of viable cells by determining the soluble tetrazolium salt conversion in the metabolically active cells to a colored formazan product with the advantage over MTT that the solubilization step is not required for avoiding formazan precipitation in the aqueous medium.

IPEC-J2 cells were seeded on 96-well culture plates and allowed 24 hrs to reach confluence. OTA in the range of 1 µM to 20 µM, 10 µg/ml LPS, 2.5 µg/ml LUT, 1 µM CHR solutions as well as their combinations were added to the cells using a multichannel pipette and were incubated for 24 hrs at 37°C, 5% CO_2. After the incubation time, the solutions were removed, and each well received 100 µl of fresh phenol red-free medium containing 20 µl of MTS solution. After an incubation time of 2 hrs at 37°C, the absorbance values were measured at 490 nm using the SpectraMax iD3 (Figure 7).

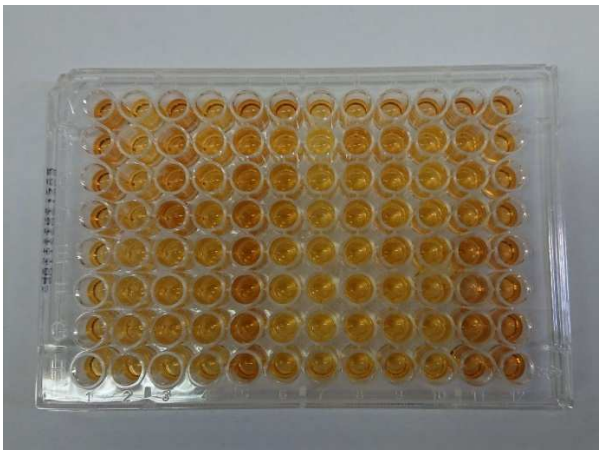

Figure 7: MTS on a 96-well plate after 2 hrs of incubation time with the dye

For the measurement of cell viability in the hepatocytes on collagen-coated membrane inserts CCK-8 assay was used (Dojindo Molecular Technologies, Rockville, MD, USA) to detect the amount of $NADH+H^+$ gained in the catabolic pathways. The test was performed according to the manufacturer's protocol. First, 10 µl CCK-8 reagent and 100 µl fresh Williams' Medium E were added to the cultured cells, and after a 2 hrs incubation, the absorbance was then measured at 450 nm using the SpectraMax iD3.

5.5 Detection of changes in redox status of IPEC-J2 cells and minipig hepatocytes

Effects of *E. coli* LPS (10 µg/ml), OTA (in the concentration range of 1 µM and 20 µM), the flavonoids (1 µM CHR and 2.5 µg/ml LUT) alone or in combinations were tested on redox status of IPEC-J2 cells. In minipig hepatocytes OTA (20 µM) and CHR (in the range of 100 nM to 1000 nM) were administered alone or in combinations. All chemicals were dissolved in plain medium and were incubated with the cells for 24 hrs on 24-well

plates. EC H_2O_2 production was monitored in IPEC-J2 cells by using the Amplex red hydrogen peroxide assay kit. The Amplex red reagent reacts with H_2O_2 (in 1: 1 stoichiometry) to produce a red fluorescent product called resorufin in the presence of horseradish peroxidase. After 24 hrs incubation time, 50 µl of the cell-free supernatants was collected and was mixed with the Amplex red working solution according to the manufacturer's instructions. The fluorescence intensity was measured with Victor X2 2030 fluorometer (excitation and emission wavelengths were 530 nm and 590 nm, respectively) (Figure 8). Cells treated only with plain medium served as control.

To detect the amount of IC ROS, 10 µM DCFH-DA dye (Merck, Budapest, Hungary) was used. IC ROS can oxidize DCFH-DA to a detectable fluorescent product, dichloro-fluorescein (DCF); therefore, elevated fluorescence values show an increased amount of IC ROS. The dye was added to the cells for 60 min, followed by rinsing with medium, scraping and centrifugation for 10 min (at 3000×g). A Victor X2 2030 fluorometer was used to determine fluorescence of the samples (excitation wavelength: 480 nm, emission wavelength: 530 nm).

Figure 8: Amplex red assay on 96-well-plate after 30 min of incubation with dye

5.6 Determination of proinflammatory cytokine IL-6 and IL-8 expression

The cell free supernatants of IPEC-J2 were collected after 24 hrs incubation time. Porcine sandwich ELISA kits were used to monitor the changes in IL-6 and IL-8 levels in IPEC-J2 cells exposed to LPS, OTA, different flavonoids and their combinations. The supernatants were treated according to the instructions of the manufacturer and measured by SpectraMax iD3 at 450 nm.

5.7 Statistic analysis

The statistical analysis of the results was performed by using R Core Team (version of 2016). Differences between groups were analyzed by one-way ANOVA coupled with the post hoc Tukey test for multiple comparisons. $p<0.05$, $p<0.01$ and $p<0.001$ were considered to be statistically significant.

6. Results

6.1 Cell viability assay on the IPEC-J2 cell line

After 24 hrs of incubation, viability of IPEC-J2 cells was measured with MTS (Figure 9) on the IPEC-J2 cell line. CHR (1 µM) and LUT (2.5 µg/ml) alone did not show any negative effect on the viability of cells. LPS (10 µg/ml) alone could reduce cell viability ($p=0.0486$), and in combination with OTA (1 µM, 5 µM, 20 µM) a significant worsening of the viability was seen ($p<0.001$). OTA alone had shown to be capable of inducing cell death (1 µM, 5 µM, 20 µM $p<0.001$ in each case). As seen in Figure 9 a significant reduction of cell viability had been detected in case of all the combinations of LPS+OTA+CHR (10 µg/ml+ 1 µM / 5 µM / 20 µM + 1 µM) as well as LPS+OTA+CHR+LUT (10 µg/ml+ 1 µM / 5 µM / 20 µM + 1 µM+ 2.5 µg/ml) and LPS+OTA+LUT (10 µg/ml+ 1 µM / 5 µM / 20 µM + 2.5 µg/ml) ($p<0.001$). After our investigation it can be stated that significant cytotoxic effects of OTA and LPS+OTA could not be quenched by the application of flavonoids LUT and CHR.

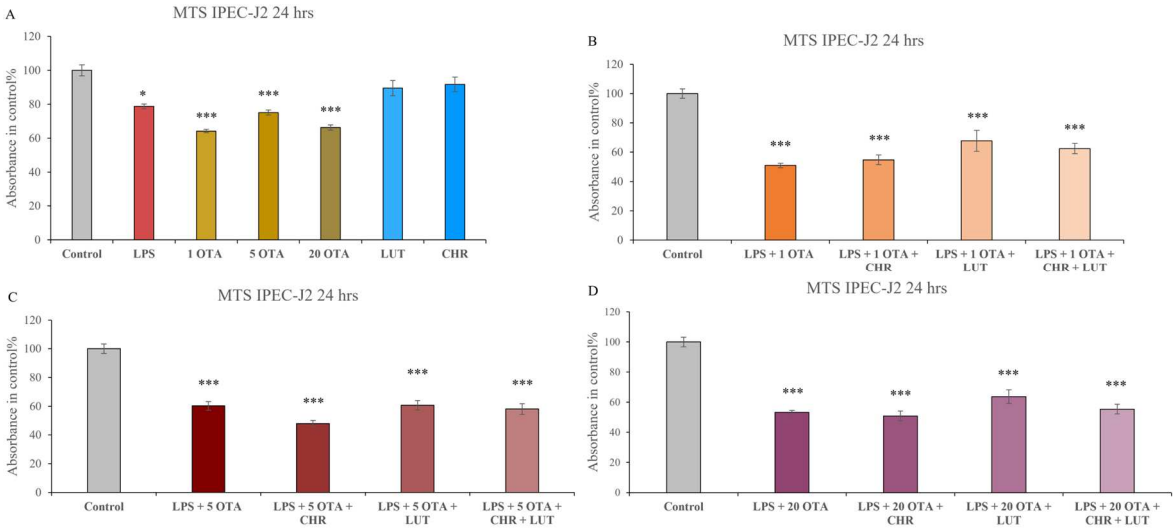

Figure 9: Cell viability determination. Changes in IPEC-J2 cell viabilities were detected upon 24 hrs administration of 10 µg/ml LPS, 1 µM OTA, 5 µM OTA, 20 µM OTA and their combinations even in the presence of 1 µM CHR and 2.5 µg/ml LUT. Data are shown as means of absorbances expressed in control % with standard errors of mean (SEM); n= 3-4 samples per group; * indicates $p<0.05$, *** indicates $p<0.001$ compared to controls

6.2 Detection of extracellular hydrogen-peroxide production

No significant elevation in hydrogen-peroxide secretion could be seen in IPEC-J2 cells exposed to LPS (10 µg/ml) or to OTA (1 µM, 5 µM, 20 µM) or to their combination ($p>0.05$). CHR at 1 µM did not cause significantly lower H_2O_2 production, however, LUT (2.5 µg/ml) could decrease significantly the peroxide levels ($p<0.001$). A combination of LPS (10 µg/ml) + OTA (1 µM, 5 µM, 20 µM) with LUT (2.5 µg/ml) or LUT+CHR (2.5 µg/ml + 1 µM) has shown to be capable of lowering the EC H_2O_2 ($p<0.001$). CHR alone however as an additive to LPS+ OTA (1 µM, 5 µM, 20 µM) was not capable of reducing the H_2O_2 level. Based on our results after 24 hrs of incubation LPS and OTA cannot significantly elevate the EC H_2O_2 level, but LUT alone could reduce the hydrogen-peroxide level effectively, while CHR cannot enhance the antioxidant effect of LUT. CHR alone was not able to affect the hydrogen-peroxide level (Figure 10).

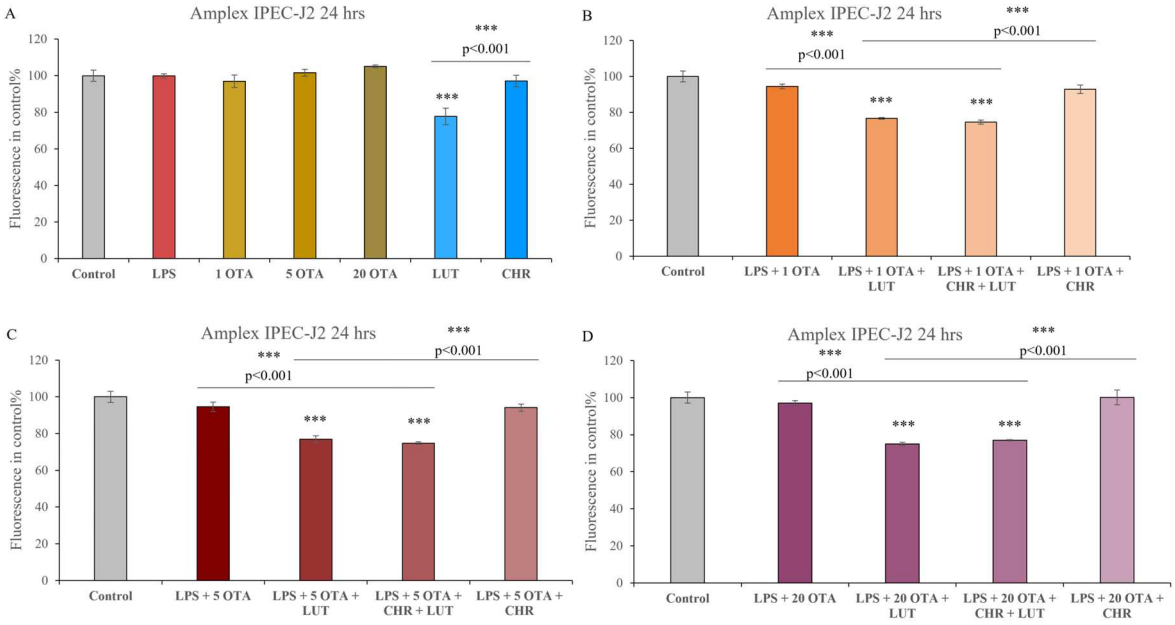

Figure 10: Relative fluorescence intensity in control% using Amplex red method. Changes in fluorescence intensities can be seen after 24 hrs of treatment with 1 µM OTA, 5 µM OTA, 20 µM OTA, 1 µM CHR, 2.5 µg/ml LUT, 10 µg/ml LPS and their combinations; data are shown as means of relative fluorescence intensities with SEM; n= 4 samples per group; *** indicates $p<0.001$; λexc=530 nm, λem=590 nm. Asterisks alone indicate significant differences between control and treated groups, asterisks with line shows significant changes between the designated groups.

6.3 IC ROS determination

OTA alone (1 µM, 5 µM, 20 µM) did not cause significantly higher IC ROS production in the IPEC-J2 cells (Figure 11). CHR (1 µM) alone did not seem to influence the IC redox status. Cells exposed to LPS (10 µg/ml) alone had a significantly higher level of ROS ($p=0.0443$) similarly to those in IPEC-J2 cells challenged with coadministration of LPS+ OTA (10 µg/ml+ 1 µM) ($p=0.0109$). LUT (2.5 µg/ml) alone as well as CHR (1 µM) alone and their combination LUT+CHR (2.5 µg/ml + 1 µM) have shown to be capable of lowering IC ROS induced by LPS+ OTA (10 µg/ml+ 1 µM) (all $p<0.001$). LUT (2.5 µg/ml) could significantly decrease the oxidative stress within the cells ($p<0.001$) and seems to be significantly more effective than CHR (1 µM) alone ($p<0.01$). Based on our 24 hrs findings it can be stated that LPS alone as well as LPS+ OTA resulted in a higher level of ROS in the cells which has been successfully prevented by LUT and CHR alone as well as by their combination (Figure 11).

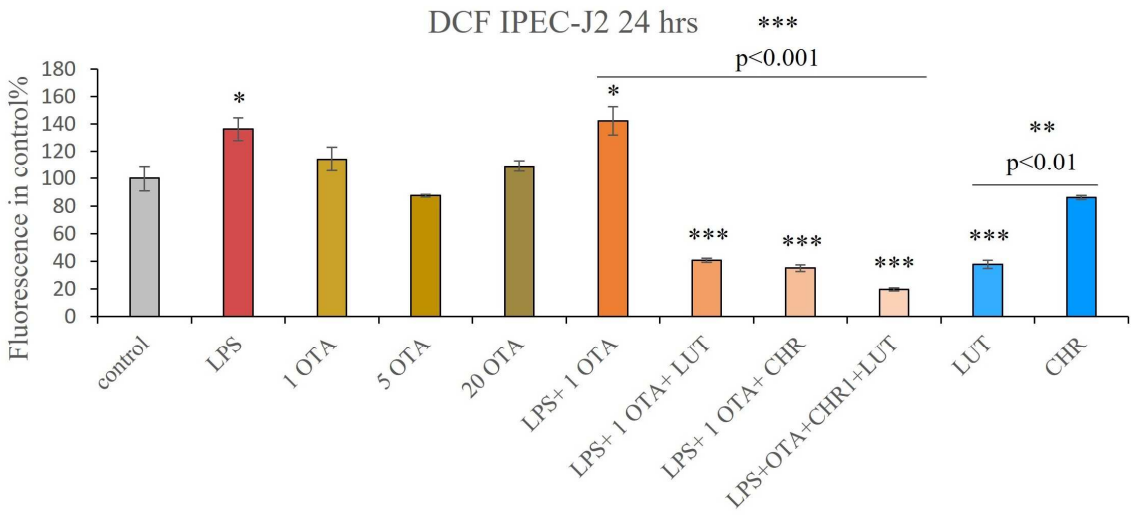

Figure 11: Relative fluorescence intensity in control% using DCFH-DA method. Measurement of IC ROS levels after 24 hrs treatment of IPEC-J2 cells with 1 µM OTA, 5 µM OTA, 20 µM OTA, 1 µM CHR, 2.5 µg/ml LUT, 10 µg/ml LPS and their combinations; data are shown as means of relative fluorescence intensities with SEM; n= 3 samples per group, * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$; λexc=480 nm, λem=530 nm. Asterisks alone indicate significant differences between control and treated groups, asterisks with line shows significant changes between the designated groups.

6.4 The changes in IL-6 and IL-8 levels after exposure to OTA, LPS and the selected flavonoids in IPEC-J2 cells

Different concentrations of OTA (1 µM, 5 µM and 20 µM) were used in cells for IL-6/IL-8 examinations. 10 µg/ml LPS alone, LPS+OTA combination (10 µg/ml+ 1 µM), flavonoids 1 µM CHR, 2.5 µg/ml LUT alone and these in combinations with LPS+OTA were also used to see their inflammatory effects (Figure 12). Based on our 24 hrs findings IL- 6 production was not increased in IPEC-J2 cells exposed to OTA 1 µM, 5 µM and 20 µM compared to untreated controls. LUT and CHR did not influence the basal IL-6 secretion of the cells. However, when LPS (10 µg/ml) or LPS+OTA combination was used (10 µg/ml LPS+ 1 µM OTA) significant increase in IL-6 levels was detected ($p=0.04431$ and $p<0.001$, respectively). LUT (2.5 µg/ml) and LUT+CHR (2.5 µg/ml+1 µM CHR) could efficiently reduce IL-6 production to the control level when these flavonoids were administered simultaneously with LPS+OTA for 24 hrs (both $p<0.001$). It was also found that there were no significant differences between LUT and LUT+CHR promoted IL-6 reduction in cells exposed to OTA and LPS (1 µM + 10 µg/ml) together (Figure 12).

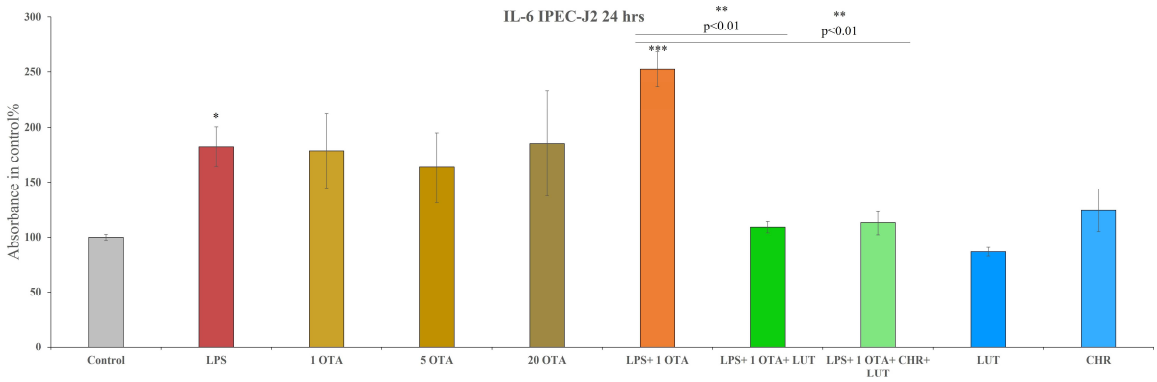

Figure 12: Measurements of IL-6 levels after 24 hrs treatment of IPEC-J2 cells with 1 µM OTA, 5 µM OTA, 20 µM OTA, 1 µM CHR, 2.5 µg/ml LUT, 10 µg/ml LPS and their combinations; data are shown as means of absorbance values with SEM; n= 3 samples per group; * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001; Asterisks alone indicate significant differences between control and treated groups, asterisks with line shows significant changes between the designated groups.

LPS at 10 µg/ml could elevate proinflammatory IL-8 levels significantly (p=0.0489) after 24 hrs treatment. OTA alone at very high concentration (at 20 µM) could not promote IL-8-mediated inflammation (p>0.05). Beneficial effects could be seen with application of LUT and CHR at 2.5 µg/ml and 1 µM when they were administered simultaneously, however, LUT alone could suppress LPS+OTA-caused IL-8 production in very similar manner as it could be observed in case of administration for LUT+CHR combination (both p<0.001). No significant changes in IL-8 secretion were found between LPS+OTA+LUT and LPS+OTA+CHR+LUT thus CHR alone did not exert beneficial effect on decreasing upregulated IL-8 levels (Figure 13).

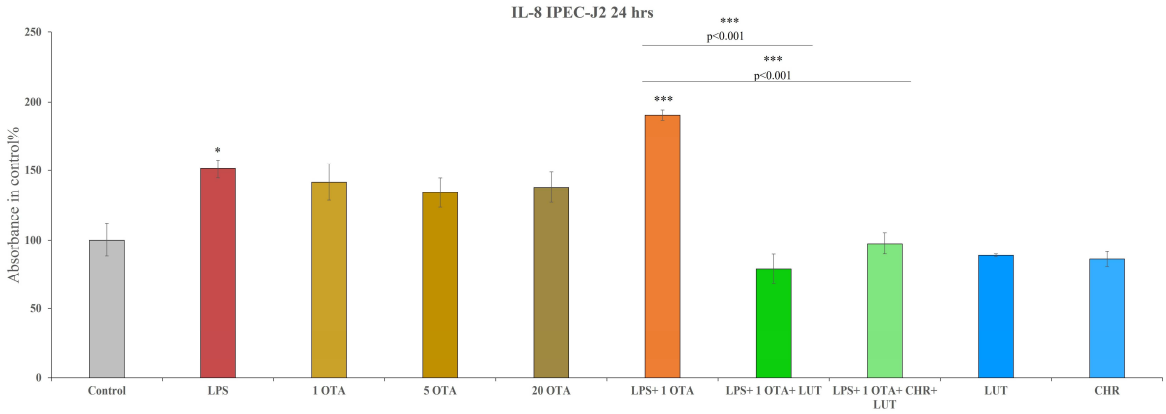

Figure 13: Measurements of IL-8 levels after 24 hrs treatment of IPEC-J2 cells with 1 µM OTA, 5 µM OTA, 20 µM OTA, 1 µM CHR, 2.5 µg/ml LUT, 10 µg/ml LPS and their combinations; data are shown as means with SEM; n= 3 samples per group, * indicates p<0.05, *** indicates p<0.001; Asterisks alone indicate significant differences between control and treated groups, asterisks with line shows significant changes between the designated groups.

In accordance with our 24 hrs results treatment of IPEC-J2 cells with OTA and LPS lead to significant elevation of proinflammatory IL-6 and IL-8 cytokine productions which were successfully alleviated by LUT alone. CHR addition did not improve the efficacy of LUT in restoring LPS+OTA -facilitated inflammatory processes.

6.5 Cell viability of minipig hepatocytes

20 µM OTA and CHR (100 nM, 500 nM, 1000 nM) were used either alone or in combination. CHR (100 nM, 500 nM, 1000 nM) did not exert significant effects on the viability of the cells ($p>0.05$). In contrast, OTA (20 µM) promoted cell death to great extent ($p<0.001$). The flavonoid CHR (100 nM, 500 nM) in the combination with OTA (20 µM) did not completely restore cell viability to control level (100 nM $p=0.00474$; 500 nM $p=0.00939$). OTA (20 µM) in combination with a higher concentration of CHR (1000 nM) showed a lower negative, but still significant effect on cell viability ($p=0.04682$). Cytotoxic effects of OTA (at 20 µM) could not be quenched by combining OTA with CHR (100 nM, 500 nM, 1000 nM) as there were no significant differences between OTA and OTA+CHR combinations in all three cases ($p>0.05$). Our results had shown that the treatment of hepatocytes with OTA at 20 µM for 24 hrs significantly led to cell death which could not be completely alleviated by CHR ($p>0.05$) (Figure 14).

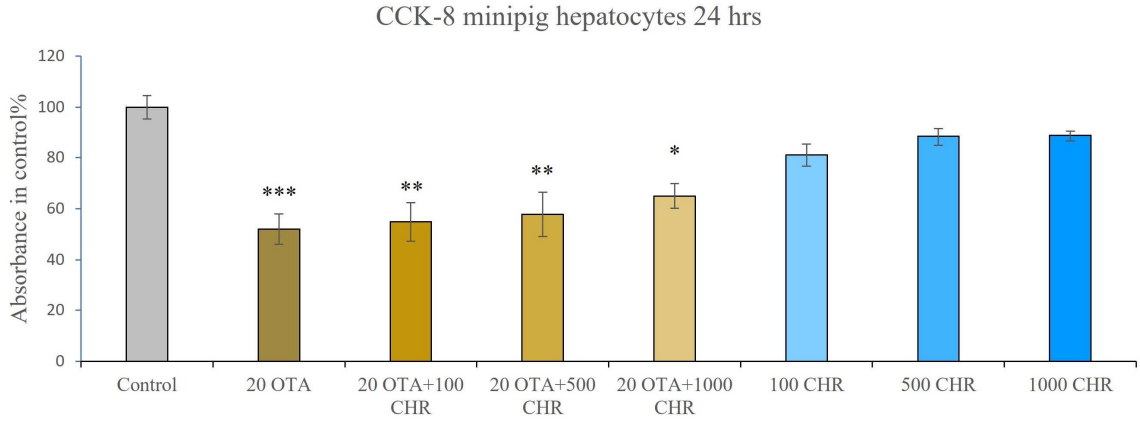

Figure 14: CCK-8 measurement in minipig hepatocytes after 24 hrs of treatment with 20 µM OTA, 100 nM CHR, 500 nM CHR, 1000 nM CHR and their combinations; data are shown as means of absorbances in control % with SEM; n= 3 samples per group, * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

The effects of OTA (20 µM), OTA+ CHR (20 µM + 1 µM) and CHR (1 µM) could also be seen morphologically on microscopic pictures of minipig hepatocytes in direct comparison to control (Figure 15). OTA treatment induced cell floating due to cell death, which can be observed based on the excessive presence of rounded cells and cell islands.

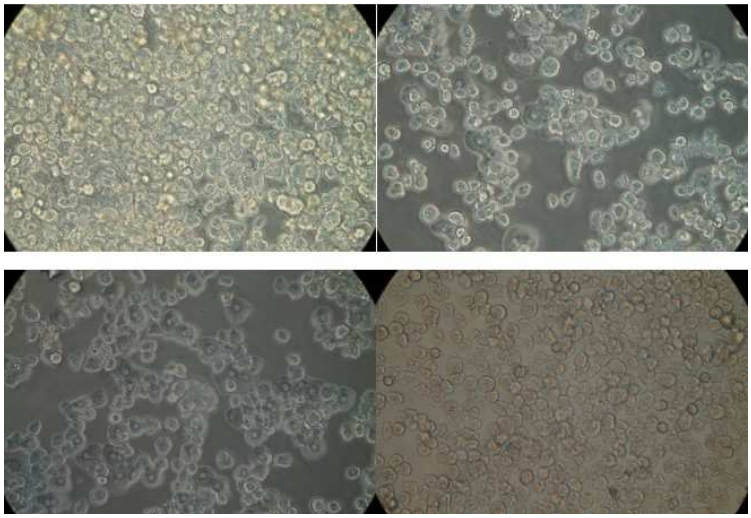

Figure 15: Microscopic pictures of the minipig hepatocytes on collagen-coated membrane inserts; top left: control group; top right: after treatment with 20 µM OTA; bottom left: 20 µM OTA+ 1 µM CHR; bottom right: 1 µM CHR. 40x magnification. Duration of the treatment was 24 hrs.

6.6 Extracellular hydrogen-peroxide evaluation in minipig hepatocytes

CHR (100 nM, 500 nM, 1000 nM) did not exert significant effects on the EC hydrogen-peroxide production ($p>0.05$). OTA (20 µM) could elevate H_2O_2 production ($p=0.00411$). CHR (100 nM, 500 nM, 1000 nM) in combination with OTA (20 µM) could successfully decrease hydrogen-peroxide levels (100 nM $p=0.00132$; 500 nM $p=0.00279$; 1000 nM $p=0.00506$). Based on our results after 24 hrs treatment OTA could elevate hydrogen-peroxide levels significantly which was successfully counteracted by CHR (100 nM, 500 nM, 1000 nM) (Figure 16).

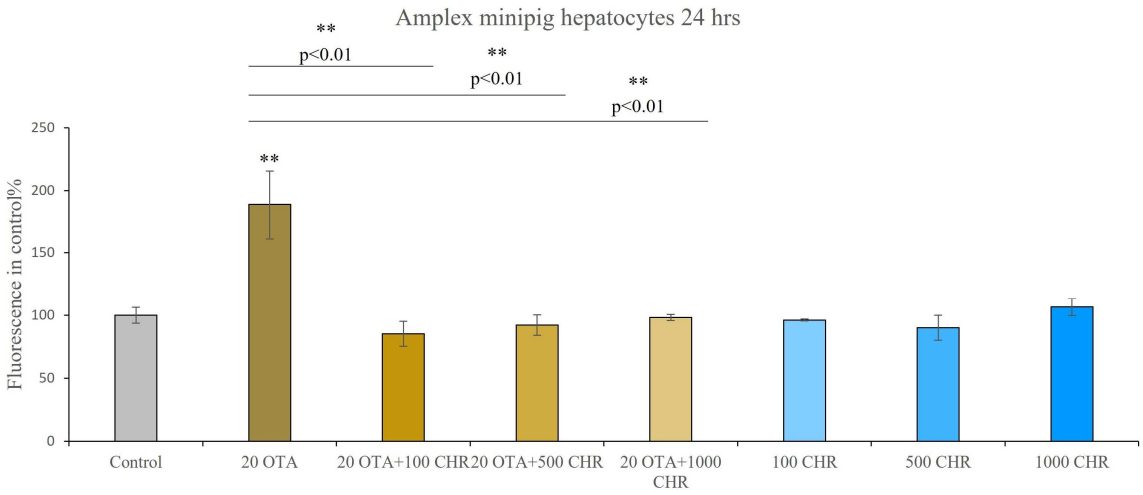

Figure 16: Fluorescence intensities expressed in control% using Amplex red method. Treatment of minipig hepatocytes were completed with 20 µM OTA, 100 nM CHR, 500 nM CHR, 1000 nM CHR and their combinations; data are shown as means of absorbances in control % with SEM; n= 3 samples per group, ** indicates p<0.01; λexc=530 nm, λem=590 nm. Asterisks alone indicate significant differences between control and treated groups, asterisks with line shows significant changes between the designated groups.

7. Discussion and conclusion

Antibiotic resistances are one of the most recent dangers and challenges nowadays and EU guidelines concerning the prudent antibiotic use in veterinary medicine were formed to tackle these (2019/6 EU) [10]. Farmers of large-scale facilities try to lower environmental stress, improve husbandry and feed as well as using alternatives such as natural bioactive substances to improve herd health. A positive side effect of alternative products is the lack of a withdrawal period because of the missing maximum residue limit thus they are mostly defined safe. Flavonoids are present in a lot of food sources, such as fruits, and are known to have beneficial effects, but in animals the ways of action and effects are still not well investigated thus need to be studied further [86].

Balance of the microbiome is a key role in resisting environmental stressors, maintaining balance and preventing dysbiosis [87]. Oxidative stress, inflammation and reduced intestinal barrier function thus can lead to the increased entry of toxins and antigens. These negative phenomena can be caused by bacterial overgrowth [88]. Cell viability and division is critically influenced by an insufficient redox status which is leading to harmful changes in the DNA, structural proteins and lipids and ultimately impairing organ function. These effects can get potentiated by environmental stressors.

Mycotoxins such as OTA and LPS deriving from Gram negative bacteria are capable of reducing viability of enterocytes. To administer flavonoids, which may have a beneficial function, duration of treatment and concentration have to be investigated further to exclude any potential unsafe effect. Studies showed that luteolin (100 µM) did not decrease cell viability in IPEC-J2 cells after 24 hrs of treatment [12]. Similar result can be seen in our study, as LUT did not exert any cytotoxic effect. Effects of CHR on cell toxicity was not investigated in IPEC-J2 cells and minipig hepatocytes to our best knowledge. We observed that CHR (in a range of 100 nM-1000 nM) did not exert any negative effect on minipig hepatocytes viability after 24 hrs treatment. In our study we have shown that after 24 hrs of treatment CHR (1 µM) had no negative effect on the viability in IPEC-J2 cells and therefore can be considered safe. Cell death was reported to happen after 48 hrs of treatment in case of OTA in combination with aflatoxin M1 treatment (0.05 µM-4 µM) in FHs74 cells measured with CCK-8 method [76]. Other studies showed significant cytotoxic effects of OTA (in a range of 1 µM to 180 µM) in Caco-2 cell lines after 24 hrs treatment [78]. Our study confirmed that 24 hrs of treatment with OTA (in the range of 1 µM to 20 µM) in IPEC-J2 cells lead to a significant cytotoxic effect, as well as the combination of LPS with OTA (10 µg/ml+ 1 µM / 5 µM / 20 µM). In primary rat hepatocytes cell death was described after treatment with OTA (12.5 µM) [89]. This agreed with our results that treatment with OTA (20 µM) in minipig hepatocytes for 24 hrs could cause decreased cell viability. Wang et al. investigated the cytotoxicity after 12 hrs in IPEC-J2 cells treated with LPS from *E.coli* [90]. In our studies we came to the same conclusion considering the toxic effect of 24 hrs LPS treatment in IPEC-J2 cells. In IPEC-J2 cells LUT and CHR were not able to prevent cell death, this is consistent with the fact that CHR was not able to elevate cell viability in minipig hepatocytes.

Flavonoids such as CHR and LUT have potent effect on oxidative stress because of their chemical structure having hydroxyl groups on the rings as well as a double bound on 2-3 positions at the C ring [91]. The catechol group on the B ring even accelerates the antioxidant effect and LUT showed to be effective in lowering ROS in rat serum and human leukocytes leading to the prevention of lipid peroxidation [92]. The antioxidant properties of LUT (25-100 µM) were able to counteract the ROS induction of *Salmonella enterica,* and

E. coli endotoxins in IPEC-J2 cells without concentration dependency [12]. Our results confirmed that LUT (even at 2.5 µg/ml) was able to successfully quench the LPS-induced oxidative stress. We showed here first, that CHR has similar antioxidant properties as it positively influenced the oxidative stress induced by OTA and LPS in IPEC-J2 cells and minipig hepatocytes as well.

Oxidative stress-inducing effects of OTA have been investigated in *in vitro* studies. In FHs74 cells it was found that OTA in a concentration of 2 µM after 48 hrs of treatment lead to an increase in ROS investigated by DCFH-DA method [76]. In another study OTA (10 µM) treatment of Vero cells for different time intervals resulted in increased IC ROS levels [93]. *In vitro* LUT (100 µM) was able to elevate OTA-induced (50 µM) oxidative stress after 24 hrs treatment in rat renal proximal tubular cell line (NRK-52E) by regulating nuclear factor erythroid 2-related factor 2 (Nrf2) and hypoxia-inducible factor 1-alpha (HIF-1α) [94]. Wang et al. investigated the oxidative stress induced by OTA (in a range of 2 µM-8 µM) with DCFH-DA after 12 hrs treatment and had shown a concentration-dependent effect in IPEC-J2 cells [25]. We did not see this phenomenon in our study as OTA did not increase IC ROS levels after 24 hrs which is maybe to the fact that mycotoxin resulted in a viable cell number reduction as a compensatory effect. In case of LPS deriving from *E.coli* studies showed that this endotoxin was able to exaggerate IC ROS levels using DCFH-DA method in a concentration of 10 µg/ml in IPEC-J2 cells after 24 hrs of treatment [12,14] which was also proven by our experiments by using the same concentration, method, time and cell model. EC H_2O_2 by Amplex red method showed no significant elevation in response to 24 hrs 10 µg/ml LPS exposure in IPEC-J2 cells [14] in agreement to our studies. LPS of different origins could not elevate EC H_2O_2 [95]. Mycotoxins like T-2 and DON alone and in combination could increase EC H_2O_2 in IPEC-J2 cells [96]. To our best knowledge we were the first ones to investigate EC H_2O_2 levels in minipig hepatocytes. We have seen that OTA (20 µM) increased EC H_2O_2 level after 24 hrs which was successfully counteracted by CHR (100 nM, 500 nM, 1000 nM).

For Gram-negative bacteria the activation of the innate immune system by binding to toll like receptor 4 (TLR4) is an important factor with which the bacteria can bind to the host cell and activate the immune response [97]. ROS production induced by LPS leads to apoptosis [98] in those cells thus ultimately higher vulnerability of the cell barrier [99] and elevated secretion of cytokines in the blood and intestinal system [100]. Epithelial cells play a key role in the regulation of cytokine overproduction against adverse effects [101,102]. As

far as we know there are very few data concerning interleukin production in IPEC-J2 cells. After treatment of chicken with OTA at 101.41 µg/kg in combination with 20.10 µg/kg AFB1 for 42 days, it was found that IL-6 and IL-8 levels were increased in their serum, liver and kidney measured by ELISA [75]. In another study IL-6 levels were elevated by OTA (4 µM) after 24 hrs treatment in FHs74 cells measured by ELISA [76]. According to Maresca et al. IL-8 level were indirectly elevated by OTA (1 µM, 10 µM, 100 µM) after 12 hrs treatment in Caco-2 cells evaluated by RT-PCR [77]. According to literature data, after a 12-hour treatment period, the LPS used at a concentration of 10 µg/ml increased the IL-6 and IL-8 levels in IPEC-J2 cell line in ELISA test [7,81]. Studies showed that chrysin (50 mg/kg body weight/day) given to Lewis rats for 16 days lowered IL-6 levels significantly [84]. We have proven that LPS (10 µg/ml) could elevate the IL-8 levels after 24 hrs of treatment in IPEC-J2 cells measured with ELISA. In our experiments LPS (10 µg/ml) alone and in combination with OTA (1 µM) elevated IL-6 and IL-8 levels in IPEC-J2 cells after 24 hrs incubation. The secretion of these inflammatory cytokines was successfully quenched by LUT.

In conclusion, we showed that LUT and CHR could counteract some of the negative effects of the combined effects of LPS and OTA, but further *in vivo* tests are needed. The co-administration of OTA and LPS in IPEC-J2 cells models the complexity of exposure of enterocytes to environmental stressors. As the intestinal system is one of the first barriers exposed to potentially harmful substances and the liver is the main organ of detoxification, it is very important to investigate possibilities to protect these organs (Figure 16-17). The bioactive substances such as flavonoids can counteract some of the negative effects exerted by harmful substances and therefore possibly can reduce the usage of antibiotics in the future. This investigation of combined effects of LPS and OTA are resembling the multifactorial origin of infections and their effects on oxidative and inflammatory processes which have not been examined *in vitro* yet.

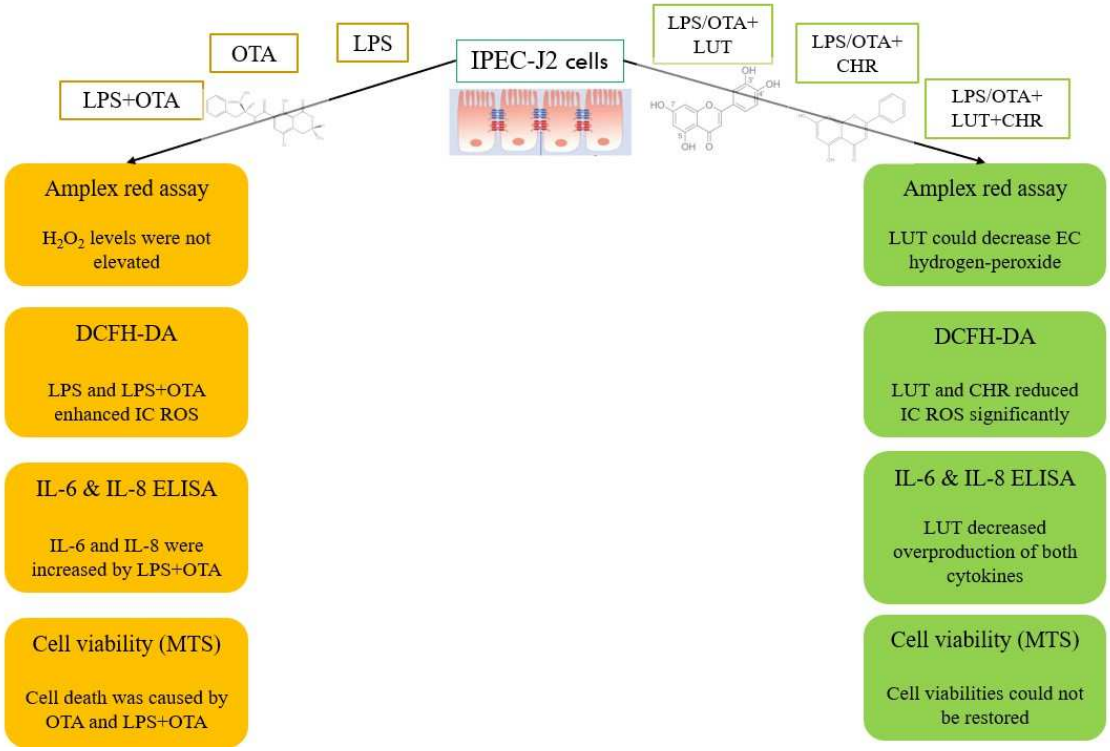

Figure 16: Schematic diagram of beneficial effects of LUT and CHR on intestinal epithelial damage caused by LPS, OTA and their combinatorial treatment. LUT reduced the expression of the investigated inflammatory cytokines. LUT and CHR decreased the IC ROS level significantly. EC stands for extracellular.

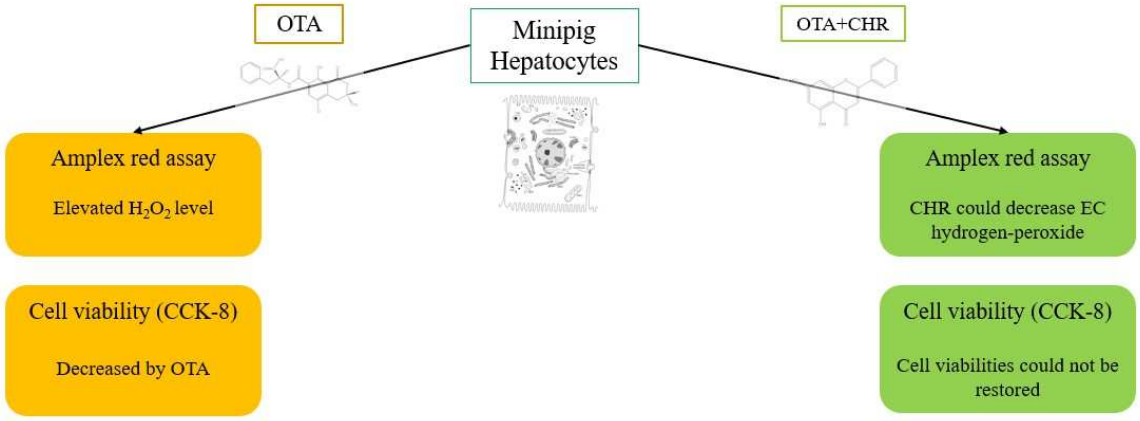

Figure 17: Schematic diagram of beneficial effects of CHR on minipig hepatocyte damage caused by OTA treatment. CHR successfully reduced the EC H_2O_2 overproduction of IPEC-J2 cells. EC means extracellular.

8. Summary

Ochratoxin A (OTA) produced by *Aspergillus* and *Penicillium* spp. can contaminate cereal-based foods and feedstuffs and their oral consumption can cause severe gastrointestinal, kidney and liver disorders both in humans and in the livestock. Gram-negative bacterial lipopolysaccharide (LPS) is also reported to cause chronic inflammation of the gut and is capable of stimulating liver injury. However, plant-derived flavonoids such as chrysin (CHR) and luteolin (LUT) display many positive aspects, such as anti-inflammatory and antioxidant properties.

In this study, not cancerous IPEC-J2 cells from neonatal piglets as well as minipig hepatocytes were used to study on the 24 hrs lasting impact of OTA/LPS and for screening beneficial effects of the applied flavonoids. The cells were treated with OTA (1 µM, 5 µM and 20 µM), LPS (10 µg/ml), CHR (1 µM) and LUT (2.5 µg/ml) alone and in combinations in IPEC-J2. The minipig hepatocytes were exposed to OTA (20 µM) in the absence or in the presence of CHR (100 nM, 500 nM, 1000 nM). To determine the cell viabilities MTS assay (in IPEC-J2 cells) and CCK-8 test (minipig hepatocytes) were performed and to evaluate extracellular (EC) hydrogen-peroxide (H_2O_2) Amplex red assay was applied. Intracellular (IC) reactive oxygen species (ROS) were assessed using 2'-7'dichlorodihydrofluorescein diacetate (DCFH-DA) method. ELISA kit assay was used to assess IL-6 and IL-8 secretion in IPEC-J2 cells.

It was found that OTA decreased cell viabilities both in IPEC-J2 cells and in minipig hepatocytes ($p<0.001$), while LPS reduced viability in IPEC-J2 cells ($p<0.01$). This effect could not be alleviated by LUT or CHR ($p>0.05$), however EC H_2O_2 was successfully suppressed by LUT in IPEC-J2 cells ($p<0.001$) and CHR in minipig hepatocytes ($p<0.01$). OTA in combination with LPS would significantly elevate the IC ROS which was successfully counteracted by CHR and LUT ($p<0.001$). Inflammatory cytokines IL-6 and IL-8 secretion was elevated by LPS ($p<0.01$) and LPS+ OTA ($p<0.001$) and could be successfully quenched by LUT (IL-6 $p<0.01$; IL-8 $p<0.001$).

Based on our results flavonoids (CHR and LUT) exert beneficial effects on IC ROS levels as well as on cytokine secretion (LUT) *in vitro*. These flavonoids might be used as dietary and feed supplements in the future to avoid OTA and LPS- related health risks in humans and in farm animals after further *in vivo* safety studies.

9. Összefoglaló

Az *Aspergillus* és *Penicillium* spp. által termelt ochratoxin A (OTA) a gabonaalapú élelmiszereket és takarmányokat szennyezheti, szájon át történő fogyasztása súlyos gyomor-bélrendszeri, vese- és májbetegségeket okozhat mind az emberekben, mind a haszonállatokban. A Gram-negatív baktériumokban található lipopoliszacharidok (LPS-ok) szintén krónikus bélgyulladást és májkárosodást képesek előidézni. A növényi eredetű flavonoidok, mint a krizin (CHR) és a luteolin (LUT) azonban gyulladáscsökkentő és antioxidáns hatásúak.

Ebben a tanulmányban újszülött malacokból származó IPEC-J2 sejteket, valamint minisertés hepatocitákat használtunk az OTA/LPS 24 órás hatásának vizsgálatára és az alkalmazott flavonoidok jótékony hatásainak szűrésére. Az IPEC-J2 sejteket OTA-val (1 µM, 5 µM és 20 µM), LPS-sel (10 µg/ml), CHR-nel (1 µM) vagy LUT-nal (2,5 µg/ml) kezeltük önmagukban vagy kombinációkban. A minisertés májsejtekhez OTA-t (20 µM) adtunk CHR (100 nM, 500 nM, 1000 nM) hiányában és annak jelenlétében is. A sejtek életképességének meghatározására MTS tesztet (IPEC-J2 sejtekben) és CCK-8 tesztet (minisertés májsejtekkel) végeztünk, az extracelluláris (EC) hidrogén-peroxid (H_2O_2) szint változásának nyomonkövetésére Amplex red technikát alkalmaztunk. Az intracelluláris (IC) reaktív oxigénfajtákat (ROS) a 2'-7'-dikloro-dihidrofluoreszcein-diacetát (DCFH-DA) módszerrel vizsgáltuk. Az IPEC-J2 sejtek IL-6 és IL-8 termelésének értékelésére ELISA kitet használtunk.

Kísérleti eredményeink szerint megállapítható, hogy az OTA csökkentette a sejtek életképességét mind az IPEC-J2 sejtekben, mind a minisertés hepatocitákban ($p<0.001$), amit nem tudott kompenzálni sem a LUT, sem a CHR ($p>0.05$), azonban az EC H_2O_2 termelést hatékonyan mérsékelte a LUT az IPEC-J2 sejtekben ($p<0.001$) és a CHR a minisertés májsejtekben ($p<0.01$). Az OTA LPS-sel kombinálva jelentősen megemelte az IC ROS-t, amit a CHR és az LUT sikeresen ellensúlyozott ($p<0.001$). Az IL-6 és IL-8 gyulladásos citokinek szekrécióját az LPS+ OTA megemelte ($p<0.001$), amit a LUT sikeresen csökkentett (IL-6 $p<0.01$; IL-8 $p<0.001$).

Kutatási adataink alapján a CHR és a LUT jótékony hatást gyakoroltak az IC ROS szintjére, valamint a vizsgált citokinek termelődésére (LUT) *in vitro*. Ezeket a flavonoidokat a jövőben étrend- és takarmánykiegészítőként lehetne használni az OTA és az LPS által okozott egészségügyi károsodások elkerülése érdekében az emberekben és a haszonállatokban egyaránt további *in vivo* biztonsági vizsgálatok elvégzését követően.

10. References

1. van Egmond, H. P.; Schothorst, R. C.; Jonker, M. A. Regulations relating to mycotoxins in food. *Analytical and Bioanalytical Chemistry* **2007**, *389* (1), 147–157. https://doi.org/10.1007/S00216-007-1317-9.
2. Alshannaq, A.; Yu, J. H. Occurrence, toxicity, and analysis of major mycotoxins in food. *International Journal of Environmental Research and Public Health* **2017**, *14* (6), 632. https://doi.org/10.3390/IJERPH14060632.
3. Wild, C. P.; Gong, Y. Y. Mycotoxins and human disease: a largely ignored global health issue. *Carcinogenesis* **2010**, *31* (1), 71–82. https://doi.org/10.1093/CARCIN/BGP264.
4. Gajecka, M.; Stopa, E.; Tarasiuk, M.; Zielonka, L.; Gajecki, M. The expression of type-1 and type-2 nitric oxide synthase in selected tissues of the gastrointestinal tract during mixed mycotoxicosis. *Toxins* **2013**, *5* (11), 2281–2292. https://doi.org/10.3390/TOXINS5112281.
5. Iheshiulor, O. O. M.; Esonu, B. O.; Chuwuka, O. K.; Omede, A. A.; Okoli, I. C.; Ogbuewu, I. P. Effects of mycotoxins in animal nutrition: a review. *Asian Journal of Animal Sciences* **2011**, *5* (1), 19–33. https://doi.org/10.3923/ajas.2011.19.33.
6. Wang, X.; Liu, Y.; Li, S.; Pi, D.; Zhu, H.; Hou, Y.; Shi, H.; Leng, W. Asparagine attenuates intestinal injury, improves energy status and inhibits AMP-activated protein kinase signalling pathways in weaned piglets challenged with Escherichia coli lipopolysaccharide. *British Journal of Nutrition* **2015**, *114* (4), 553–565. https://doi.org/10.1017/S0007114515001877.
7. Wan, J.; Zhang, J.; Wu, G.; Chen, D.; Yu, B.; Huang, Z.; Luo, Y.; Zheng, P.; Luo, J.; Mao, X.; Yu, J.; He, J. Amelioration of enterotoxigenic Escherichia coli-induced intestinal barrier disruption by low-molecular-weight chitosan in weaned pigs is related to suppressed intestinal inflammation and apoptosis. *International Journal of Molecular Sciences 2019, Vol. 20, Page 3485* **2019**, *20* (14), 3485. https://doi.org/10.3390/IJMS20143485.
8. Aslam, B.; Wang, W.; Arshad, M. I.; Khurshid, M.; Muzammil, S.; Rasool, M. H.; Nisar, M. A.; Alvi, R. F.; Aslam, M. A.; Qamar, M. U.; Salamat, M. K. F.; Baloch, Z. Antibiotic resistance: a rundown of a global crisis. *Infection and Drug Resistance* **2018**, *11*, 1645. https://doi.org/10.2147/IDR.S173867.
9. Livermore, D. M. Minimising antibiotic resistance. *The Lancet Infectious Diseases* **2005**, *5* (7), 450–459. https://doi.org/10.1016/S1473-3099(05)70166-3.
10. Iatridou, D. The impact of the new VMR on medicines availability, antimicrobials use and antimicrobial resistance. *Regulatory Rapporteur* **2021**, *18* (5), 26–29.
11. Kovács, D.; Pézsa, N. P.; Jerzsele, Á.; Süth, M.; Farkas, O. Protective effects of grape seed oligomeric proanthocyanidins in IPEC-J2-Escherichia coli/Salmonella typhimurium co-culture. *Antibiotics* **2022**, *11* (1), 110. https://doi.org/10.3390/ANTIBIOTICS11010110.
12. Kovács, D.; Karancsi, Z.; Farkas, O.; Jerzsele, Á. Antioxidant activity of flavonoids in LPS-treated IPEC-J2 porcine intestinal epithelial cells and their antibacterial effect against bacteria of swine origin. *Antioxidants* **2020**, *9* (11), 1267. https://doi.org/10.3390/antiox9121267.
13. Karancsi, Z.; Móritz, A. v; Lewin, N.; Veres, A. M.; Jerzsele, Á.; Farkas, O. Beneficial effect of a fermented wheat germ extract in intestinal epithelial cells in case of lipopolysaccharide-evoked inflammation. *Oxidative medicine and cellular longevity* **2020**, *2020*. https://doi.org/10.1155/2020/1482482.
14. Kovács, D. In vitro investigation of the effects of flavonoids in porcine gastrointestinal infection models. PhD Thesis, University of Veterinary Medicine, Doctoral School, Budapest, Hungary, **2022**.
15. Aziz, N.; Kim, M. Y.; Cho, J. Y. Anti-inflammatory effects of luteolin: A review of in vitro, in vivo, and in silico studies. *Journal of Ethnopharmacology* **2018**, *225*, 342–358. https://doi.org/10.1016/J.JEP.2018.05.019.
16. Mani, R.; Natesan, V. Chrysin: Sources, beneficial pharmacological activities, and molecular mechanism of action. *Phytochemistry* **2018**, *145*, 187–196. https://doi.org/10.1016/J.PHYTOCHEM.2017.09.016.
17. Stompor-gorący, M.; Bajek-bil, A.; Machaczka, M. Chrysin: Perspectives on contemporary status and future possibilities as pro-health agent. *Nutrients 2021, Vol. 13, Page 2038* **2021**, *13* (6), 2038. https://doi.org/10.3390/NU13062038.
18. Geens, M. M.; Niewold, T. A. Optimizing culture conditions of a porcine epithelial cell line IPEC-J2 through a histological and physiological characterization. *Cytotechnology* **2011**, 415–432. https://doi.org/10.1007/s10616-011-9362-9.
19. Vergauwen, H. The impact of food bioactives on health In vitro and ex vivo models: Chapter 12 the IPEC-J2 cell line. *The impact of food bio-actives on gut health: In vitro and ex vivo models* **2015**, 125–134. https://doi.org/10.1007/978-3-319-16104-4_12.

20. Sundaram, T. S.; Giromini, C.; Rebucci, R.; Baldi, A. Omega-3 polyunsaturated fatty acids counteract inflammatory and oxidative damage of non-transformed porcine enterocytes. *Animals* **2020**, *10* (6), 956. https://doi.org/10.3390/ANI10060956.
21. Tang, Z.; Liu, J.; Sun, Z.; Li, J.; Sun, W.; Mao, J.; Wang, Y. Protective effects of taurine on growth performance and intestinal epithelial barrier function in weaned piglets challenged without or with lipopolysaccharide. *Animal Production Science* **2017**, *58* (11), 2011–2020. https://doi.org/10.1071/AN16249.
22. Pi, D.; Liu, Y.; Shi, H.; Li, S.; Odle, J.; Lin, X.; Zhu, H.; Chen, F.; Hou, Y.; Leng, W. Dietary supplementation of aspartate enhances intestinal integrity and energy status in weanling piglets after lipopolysaccharide challenge. *The Journal of Nutritional Biochemistry* **2014**, *25* (4), 456–462. https://doi.org/10.1016/J.JNUTBIO.2013.12.006.
23. Farkas, O.; Pászti-Gere, E.; Palócz, O.; Csikó, G.; Gálfi, P.; Mátis, G.; Kulcsár, A.; Petrilla, J.; Neogrády, Z. Effects of Lactobacillus plantarum 2142 and sodium n-butyrate in lipopolysaccharide-triggered inflammation: Comparison of a porcine intestinal epithelial cell line and primary hepatocyte monocultures with a porcine enterohepatic co-culture system. *Journal of Animal Science* **2014**, *92* (9), 3835–3845. https://doi.org/10.2527/JAS.2013-7453.
24. Palócz, O.; Pászti-Gere, E.; Gálfi, P.; Farkas, O. Chlorogenic acid combined with Lactobacillus plantarum 2142 reduced LPS-induced intestinal inflammation and oxidative stress in IPEC-J2 cells. *PLOS ONE* **2016**, *11* (11), e0166642. https://doi.org/10.1371/JOURNAL.PONE.0166642.
25. Wang, H.; Chen, Y.; Zhai, N.; Chen, X.; Gan, F.; Li, H.; Huang, K. Correction to ochratoxin A-induced apoptosis of IPEC-J2 cells through ROS-mediated mitochondrial permeability transition pore opening pathway. *Journal of Agricultural and Food Chemistry* **2018**, *66* (33), 8885. https://doi.org/10.1021/ACS.JAFC.8B04009.
26. Frangiamone, M.; Cimbalo, A.; Alonso-Garrido, M.; Vila-Donat, P.; Manyes, L. In vitro and in vivo evaluation of AFB1 and OTA-toxicity through immunofluorescence and flow cytometry techniques: A systematic review. *Food and Chemical Toxicology* **2022**, *160*, 112798. https://doi.org/10.1016/J.FCT.2021.112798.
27. Wang, H.; Zhai, N.; Chen, Y.; Fu, C.; Huang, K. OTA induces intestinal epithelial barrier dysfunction and tight junction disruption in IPEC-J2 cells through ROS/Ca2+-mediated MLCK activation. *Environmental Pollution* **2018**, *242*, 106–112. https://doi.org/10.1016/J.ENVPOL.2018.06.062.
28. Gao, Y.; Meng, L.; Liu, H.; Wang, J.; Zheng, N. The compromised intestinal barrier induced by mycotoxins. *toxins* **2020**. https://doi.org/10.3390/toxins12100619.
29. Brosnahan, A. J.; Brown, D. R. Porcine IPEC-J2 intestinal epithelial cells in microbiological investigations. *Veterinary Microbiology* **2012**, *156* (3–4), 229–237. https://doi.org/10.1016/J.VETMIC.2011.10.017.
30. Schierack, P.; Marcel, A. E.; Ae, N.; Pollmann, M.; Dietrich, K.; Ae, W.; Amasheh, S.; Lodemann, U.; Jo¨rg, J.; Ae, J.; Tachu, B.; Kleta, S.; Anthony, A. E.; Ae, B.; Tedin, K.; Wieler, L. H. Characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine. *Histochemistry and cell biology* **2005**, *125*, 293–305. https://doi.org/10.1007/s00418-005-0067-z.
31. Zakrzewski, S. S.; Richter, J. F.; Krug, S. M.; Jebautzke, B.; Lee, I.-F. M. Improved cell line IPEC-J2, characterized as a model for porcine jejunal epithelium. *PLoS ONE* **2013**, *8* (11), 79643. https://doi.org/10.1371/journal.pone.0079643.
32. Mariani, V.; Palermo, S.; Fiorentini, S.; Lanubile, A.; Giuffra, E. Gene expression study of two widely used pig intestinal epithelial cell lines: IPEC-J2 and IPI-2I. *Veterinary Immunology and Immunopathology* **2009**, *131* (3–4), 278–284. https://doi.org/10.1016/J.VETIMM.2009.04.006.
33. Arce, C.; Ramírez-Boo, M.; Lucena, C.; Garrido, J. J. Innate immune activation of swine intestinal epithelial cell lines (IPEC-J2 and IPI-2I) in response to LPS from Salmonella typhimurium. *Comparative Immunology, Microbiology and Infectious Diseases* **2010**, *33* (2), 161–174. https://doi.org/10.1016/J.CIMID.2008.08.003.
34. Razzuoli, E.; Villa, R.; Amadori, M. IPEC-J2 cells as reporter system of the anti-inflammatory control actions of interferon-alpha. *Journal of Interferon & Cytokine Research* **2013**, *33* (10), 597–605. https://doi.org/10.1089/JIR.2012.0127.
35. Zhang, Q.; Widmer, G.; Tzipori, S. Gut microbes a pig model of the human gastrointestinal tract. *Landes Bioscience* **2013**, *4*, 193–200. https://doi.org/10.4161/gmic.23867.
36. Gonzalez, L. M.; Moeser, A. J.; Blikslager, A. T. Porcine models of digestive disease: The future of large animal translational research. *Translational Research* **2015**, *166* (1), 12–27. https://doi.org/10.1016/J.TRSL.2015.01.004.

37. Nossol, C.; Barta-Böszörményi, A.; Kahlert, S.; Zuschratter, W.; Faber-Zuschratter, H.; Reinhardt, N.; Ponsuksili, S.; Wimmers, K.; Diesing, A.-K.; Rothkötter, H.-J. Comparing two intestinal porcine epithelial cell lines (IPECs): Morphological differentiation, function and metabolism. *PLoS ONE* **2015**, *10 (7)*, e0132323. https://doi.org/10.1371/journal.pone.0132323.
38. Liu, F.; Li, G.; Wen, K.; Bui, T.; Cao, D.; Zhang, Y.; Yuan, L. Porcine small intestinal epithelial cell line (IPEC-J2) of rotavirus infection as a new model for the study of innate immune responses to rotaviruses and probiotics. *Viral Immunology* **2010**, *23* (2), 135–149. https://doi.org/10.1089/VIM.2009.0088.
39. Spitzer Speiser W Vahjen J Zentek, F. S. Effect of different feed ingredients and additives on IPEC-J2 cells challenged with an enterotoxigenic Escherichia coli strain. *Cytotechnology* **2015**, *68*, 1463–1471. https://doi.org/10.1007/s10616-015-9905-6.
40. Jung, K.; Miyazaki, A.; Hu, H.; Saif, L. J. Susceptibility of porcine IPEC-J2 intestinal epithelial cells to infection with porcine deltacoronavirus (PDCoV) and serum cytokine responses of gnotobiotic pigs to acute infection with IPEC-J2 cell culture-passaged PDCoV. *Veterinary Microbiology* **2018**, *221*, 49–58. https://doi.org/10.1016/J.VETMIC.2018.05.019.
41. Pomothy, J. M.; Szabó, O.; Czimmermann, Á. E.; Babiczky, Á.; Jerzsele, Á.; Pászti-Gere, E. Investigation of the inflammatory and oxidative stress-inducing effects of deoxynivalenol and T-2 toxin exposure in non-tumorigenic human intestinal cell model. *Toxicon* **2021**, *200*, 78–86. https://doi.org/10.1016/J.TOXICON.2021.07.002.
42. Pomothy, J. M.; Pászti-Gere, E.; Barna, R. F.; Prokoly, D.; Jerzsele, Á. The impact of fermented wheat germ extract on porcine epithelial cell line exposed to deoxynivalenol and T-2 mycotoxins. *Oxidative Medicine and Cellular Longevity* **2020**, *2020*. https://doi.org/10.1155/2020/3854247.
43. Farkas, O.; Palócz, O.; Pászti-Gere, E.; Gálfi, P. Polymethoxyflavone apigenin-trimethylether suppresses LPS-induced inflammatory response in nontransformed porcine intestinal cell line IPEC-J2. *Oxidative medicine and cellular longevity* **2015**, *2015*. https://doi.org/10.1155/2015/673847.
44. Pászti-Gere, E.; Czimmermann, E.; Ujhelyi, G.; Balla, P.; Maiwald, A.; Steinmetzer, T. In vitro characterization of TMPRSS2 inhibition in IPEC-J2 cells. *Journal of Enzyme Inhibition and Medicinal Chemistry* **2016**, *31* (S2), 123–129. https://doi.org/10.1080/14756366.2016.1193732.
45. Bús-Pomothy Judit Mercédesz. Potenciálisan védő hatású anyagok alkalmazhatósága mikotoxinok által károsított bélbarrier épségének fenntartására in vitro modellben. PhD Thesis, University of Veterinary Medicine, Budapest, **2022**.
46. Lignet, F.; Sherbetjian, E.; Kratochwil, N.; Jones, R.; Suenderhauf, C.; Otteneder, M. B.; Singer, T.; Parrott, N. Characterization of pharmacokinetics in the Göttingen Minipig with reference human drugs: An in vitro and in vivo approach. *Pharmaceutical Research* **2016**, *33* (10), 2565–2579. https://doi.org/10.1007/s11095-016-1982-5.
47. Bode, G.; Clausing, P.; Gervais, F.; Loegsted, J.; Luft, J.; Nogues, V.; Sims, J. The utility of the minipig as an animal model in regulatory toxicology. *Journal of Pharmacological and Toxicological Methods* **2010**, *62* (3), 196–220. https://doi.org/10.1016/J.VASCN.2010.05.009.
48. Skaanild, M. T.; Friis, C. Characterization of the P450 system in Göttingen minipigs. *Pharmacology & Toxicology* **1997**, *80* (SUPPL. 2), 28–33. https://doi.org/10.1111/J.1600-0773.1997.TB01986.X.
49. van Peer, E.; Verbueken, E.; Saad, M.; Casteleyn, C.; van Ginneken, C.; van Cruchten, S. Ontogeny of CYP3A and P-Glycoprotein in the liver and the small intestine of the Göttingen minipig: An immunohistochemical evaluation. *Basic & Clinical Pharmacology & Toxicology* **2014**, *114* (5), 387–394. https://doi.org/10.1111/BCPT.12173.
50. Dalgaard, L. Comparison of minipig, dog, monkey and human drug metabolism and disposition. *Journal of Pharmacological and Toxicological Methods* **2015**, *74*, 80–92. https://doi.org/10.1016/J.VASCN.2014.12.005.
51. Soucek, P.; Zuber, R.; Anzenbacherová, E.; Anzenbacher, P.; Guengerich, F. P. Minipig cytochrome P450 3A, 2A and 2C enzymes have similar properties to human analogs. *BMC Pharmacology* **2001**, *1* (1), 1–5. https://doi.org/10.1186/1471-2210-1-11.
52. Costantini, D. Understanding diversity in oxidative status and oxidative stress: The opportunities and challenges ahead. *Journal of Experimental Biology* **2019**, *222* (13). https://doi.org/10.1242/jeb.194688.
53. Zarkovic, N. Editorial roles and functions of ROS and RNS in cellular physiology and pathology. *cells* **2020**, *9*, 767. https://doi.org/10.3390/cells9030767.
54. Stone, J. R.; Yang, S. Hydrogen peroxide: A signaling messenger. *Antioxidants & Redox Signaling* **2006**, *8* (3–4), 243–270. https://doi.org/10.1089/ARS.2006.8.243.
55. Ray, P. D.; Huang, B. W.; Tsuji, Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cellular Signalling* **2012**, *24* (5), 981–990. https://doi.org/10.1016/J.CELLSIG.2012.01.008.

56. Schieber, M.; Chandel, N. S. ROS function in redox signaling and oxidative stress. *Current Biology* **2014**, *24* (10). https://doi.org/10.1016/J.CUB.2014.03.034.
57. Son, Y.; Kim, S.; Chung, H. T.; Pae, H. O. Reactive oxygen species in the activation of MAP kinases. *Methods in Enzymology* **2013**, *528*, 27–48. https://doi.org/10.1016/B978-0-12-405881-1.00002-1.
58. Murphy, M. P. How mitochondria produce reactive oxygen species. *Biochemical Journal* **2009**, *417* (1), 1–13. https://doi.org/10.1042/BJ20081386.
59. Lassègue, B.; Martín, A. S.; Griendling, K. K. Review biochemistry, physiology, and pathophysiology of NADPH oxidases in the cardiovascular system. *Circulation research* **2012**, *110*, 1364–1390. https://doi.org/10.1161/CIRCRESAHA.111.243972.
60. Winterbourn, C. C. Reconciling the chemistry and biology of reactive oxygen species. *Nature Chemical Biology 2008 4:5* **2008**, *4* (5), 278–286. https://doi.org/10.1038/nchembio.85.
61. Richter, C. Biophysical consequences of lipid peroxidation in membranes. *Chemistry and Physics of Lipids* **1987**, *44* (2–4), 175–189. https://doi.org/10.1016/0009-3084(87)90049-1.
62. Scheller, J.; Chalaris, A.; Schmidt-Arras, D.; Rose-John, S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **2011**, *1813* (5), 878–888. https://doi.org/10.1016/J.BBAMCR.2011.01.034.
63. Rose-John, S.; Leonard, W. J.; Schreiber, R. D. Interleukin-6 family cytokines. *Cold Spring Harbor Perspectives in Biology* **2018**, *10* (2), a028415. https://doi.org/10.1101/CSHPERSPECT.A028415.
64. M, B. The role of interleukin-8 in inflammation and mechanisms of regulation. *Journal of Periodontology* **1993**, *64* (5 Suppl), 456–460.
65. Altstaedt, J.; Kirchner, H.; Rink, L. Cytokine production of neutrophils is limited to interleukin-8. *Immunology* **1996**, *89* (4), 563–568. https://doi.org/10.1046/J.1365-2567.1996.D01-784.X.
66. Remick, D. G. Interleukin-8. *Critical Care Medicine* **2005**, *33* (12 SUPPL.). https://doi.org/10.1097/01.CCM.0000186783.34908.18.
67. Hoch, R. C.; Schraufstätter, I. U.; Cochrane, C. G. In vivo, in vitro, and molecular aspects of interleukin-8 and the interleukin-8 receptors. *Journal of Laboratory and Clinical Medicine* **1996**, *128* (2), 134–145. https://doi.org/10.1016/S0022-2143(96)90005-0.
68. Wang, H.; Chen, Y.; Zhai, N.; Chen, X.; Gan, F.; Li, H.; Huang, K. Ochratoxin A-induced apoptosis of IPEC-J2 cells through ROS-mediated mitochondrial permeability transition pore opening pathway. *Journal of Agricultural and Food Chemistry* **2017**, *65* (48), 10630–10637. https://doi.org/10.1021/ACS.JAFC.7B04434.
69. Petzinger, E.; Ziegler, K. Ochratoxin A from a toxicological perspective. *Journal of Veterinary Pharmacology and Therapeutics* **2000**, *23* (2), 91–98. https://doi.org/10.1046/J.1365-2885.2000.00244.X.
70. el Khoury, A. E.; Atoui, A. Ochratoxin A: General overview and actual molecular status. *Toxins 2010, Vol. 2, Pages 461-493* **2010**, *2* (4), 461–493. https://doi.org/10.3390/TOXINS2040461.
71. Ringot, D.; Chango, A.; Schneider, Y. J.; Larondelle, Y. Toxicokinetics and toxicodynamics of ochratoxin A, an update. *Chemico-Biological Interactions* **2006**, *159* (1), 18–46. https://doi.org/10.1016/J.CBI.2005.10.106.
72. Vlachou, M.; Pexara, A.; Solomakos, N.; Govaris, A. Ochratoxin A in slaughtered pigs and pork products. *Toxins* **2022**, *14* (2), 67. https://doi.org/10.3390/TOXINS14020067.
73. Al-Anati, L.; Petzinger, E. Immunotoxic activity of ochratoxin A. *Journal of Veterinary Pharmacology and Therapeutics* **2006**, *29* (2), 79–90. https://doi.org/10.1111/J.1365-2885.2006.00718.X.
74. Guilford, F. T.; Hope, J. Deficient glutathione in the pathophysiology of mycotoxin-related illness. *Toxins* **2014**, *6*, 608–623. https://doi.org/10.3390/toxins6020608.
75. Qing, H.; Huang, S.; Zhan, K.; Zhao, L.; Zhang, J.; Ji, C.; Ma, Q. Combined toxicity evaluation of ochratoxin A and aflatoxin B1 on kidney and liver injury, immune inflammation, and gut microbiota alteration through pair-feeding pullet model. *Frontiers in Immunology* **2022**, *0*, 3539. https://doi.org/10.3389/FIMMU.2022.920147.
76. Wang, Z. W.; Gao, Y. N.; Huang, S. N.; Wang, J. Q.; Zheng, N. Ex vivo and in vitro studies revealed underlying mechanisms of immature intestinal inflammatory responses caused by aflatoxin M1 together with ochratoxin A. *Toxins* **2022**, *14* (3), 173. https://doi.org/10.3390/TOXINS14030173.
77. Maresca, M.; Yahi, N.; Younès-Sakr, L.; Boyron, M.; Caporiccio, B.; Fantini, J. Both direct and indirect effects account for the pro-inflammatory activity of enteropathogenic mycotoxins on the human intestinal epithelium: Stimulation of interleukin-8 secretion, potentiation of interleukin-1β effect and increase in the transepithelial passage of commensal bacteria. *Toxicology and Applied Pharmacology* **2008**, *228* (1), 84–92. https://doi.org/10.1016/J.TAAP.2007.11.013.

78. González-Arias, C. A.; Crespo-Sempere, A.; Marín, S.; Sanchis, V.; Ramos, A. J. Modulation of the xenobiotic transformation system and inflammatory response by ochratoxin A exposure using a co-culture system of Caco-2 and HepG2 cells. *Food and Chemical Toxicology* **2015**, *86*, 245–252. https://doi.org/10.1016/J.FCT.2015.10.007.
79. Raetz, C. R. H.; Whitfield, C. Lipopolysaccharide endotoxins. *Annual review of biochemistry* **2002**, *71*, 635. https://doi.org/10.1146/ANNUREV.BIOCHEM.71.110601.135414.
80. Ziegler-Heitbrock, H. W. l. Molecular mechanism in tolerance to lipolysaccharide. *Journal of inflammation* **1995**, *45.1*, 13–26.
81. Dong, N.; Xu, X.; Xue, C.; Wang, C.; Li, X.; Bi, C.; Shan, A. Ethyl pyruvate inhibits LPS induced IPEC-J2 inflammation and apoptosis through p38 and ERK1/2 pathways. *Cell cycle* **2019**, *18* (20), 2614–2628. https://doi.org/10.1080/15384101.2019.1653106.
82. Naz, S.; Imran, M.; Rauf, A.; Orhan, I. E.; Shariati, M. A.; Iahtisham-Ul-Haq; IqraYasmin; Shahbaz, M.; Qaisrani, T. B.; Shah, Z. A.; Plygun, S.; Heydari, M. Chrysin: Pharmacological and therapeutic properties. *Life Sciences* **2019**, *235*, 116797. https://doi.org/10.1016/J.LFS.2019.116797.
83. Khoo, B. Y.; Chua, S. L.; Balaram, P. Apoptotic effects of chrysin in human cancer cell lines. *International Journal of Molecular Sciences* **2010**, *11* (5), 2188–2199. https://doi.org/10.3390/IJMS11052188.
84. Xiao, J.; Zhai, H.; Yao, Y.; Wang, C.; Jiang, W.; Zhang, C.; Simard, A. R.; Zhang, R.; Hao, J. Chrysin attenuates experimental autoimmune neuritis by suppressing immuno-inflammatory responses. *Neuroscience* **2014**, *262*, 156–164. https://doi.org/10.1016/J.NEUROSCIENCE.2014.01.004.
85. Nabavi, S. F.; Braidy, N.; Gortzi, O.; Sobarzo-Sanchez, E.; Daglia, M.; Skalicka-Woźniak, K.; Nabavi, S. M. Luteolin as an anti-inflammatory and neuroprotective agent: A brief review. *Brain Research Bulletin* **2015**, *119*, 1–11. https://doi.org/10.1016/J.BRAINRESBULL.2015.09.002.
86. Panche, A. N.; Diwan, A. D.; Chandra, S. R. Flavonoids: An overview. *Journal of nutritional science* **2016**, *5*. https://doi.org/10.1017/JNS.2016.41.
87. Tomasello, G.; Mazzola, M.; Leone, A.; Sinagra, E.; Zummo, G.; Farina, F.; Damiani, P.; Cappello, F.; Gerges Geagea, A.; Jurjus, A.; Assi, T. B.; Messina, M.; Carini, F. Nutrition, oxidative stress and intestinal dysbiosis: Influence of diet on gut microbiota in inflammatory bowel diseases. **2016**, *160* (4), 461–466. https://doi.org/10.5507/bp.2016.052.
88. Iizuka, M.; Sasaki, K.; Hirai, Y.; Shindo, K.; Konno, S.; Itou, H.; Ohshima, S.; Horie, Y.; Watanabe, S. Morphogenic protein epimorphin protects intestinal epithelial cells from oxidative stress by the activation of EGF receptor and MEK/ERK, PI3 kinase/Akt signals. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **2007**, *292* (1). https://doi.org/10.1152/AJPGI.00181.2006.
89. Essid, E.; Dernawi, Y.; Petzinger, E. Apoptosis induction by OTA and TNF-α in cultured primary rat hepatocytes and prevention by silibinin. *Toxins* **2012**, *4* (11), 1139–1156. https://doi.org/10.3390/TOXINS4111139.
90. Wang, J.; Zeng, Y.; Wang, S.; Liu, H.; Zhang, D.; Zhang, W.; Wang, Y.; Ji, H. Swine-derived probiotic Lactobacillus plantarum inhibits growth and adhesion of enterotoxigenic Escherichia coli and mediates host defense. *Frontiers in Microbiology* **2018**, *9* (JUN), 1364. https://doi.org/10.3389/FMICB.2018.01364.
91. Harris, G. K.; Qian, Y.; Leonard, S. S.; Sbarra, D. C.; Shi, X. Luteolin and chrysin differentially inhibit cyclooxygenase-2 expression and scavenge reactive oxygen species but similarly inhibit prostaglandin-E2 formation in RAW 264.7 cells. *The Journal of nutrition* **2006**, *136* (6), 1517–1521. https://doi.org/10.1093/JN/136.6.1517.
92. Bustos, P. S.; Deza-Ponzio, R.; Páez, P. L.; Cabrera, J. L.; Virgolini, M. B.; Ortega, M. G. Flavonoids as protective agents against oxidative stress induced by gentamicin in systemic circulation. Potent protective activity and microbial synergism of luteolin. *Food and Chemical Toxicology* **2018**, *118*, 294–302. https://doi.org/10.1016/J.FCT.2018.05.030.
93. Ramyaa, P.; Padma, V. V. Ochratoxin-induced toxicity, oxidative stress and apoptosis ameliorated by quercetin – Modulation by Nrf2. *Food and Chemical Toxicology* **2013**, *62*, 205–216. https://doi.org/10.1016/J.FCT.2013.08.048.
94. Liu, M.; Cheng, C.; Li, X.; Zhou, S.; Hua, J.; Huang, J.; Li, Y.; Yang, K.; Zhang, P.; Zhang, Y.; Tian, J. Luteolin alleviates ochratoxin A induced oxidative stress by regulating Nrf2 and HIF-1α pathways in NRK-52E rat kidney cells. *Food and Chemical Toxicology* **2020**, *141*. https://doi.org/10.1016/J.FCT.2020.111436.
95. Allegra, M.; Tesoriere, L.; Karancsi, Z.; Kovács, D.; Pézsa, P.; Gálfi, P.; Jerzsele, Á.; Farkas, O. The Impact of quercetin and its methylated derivatives 3-o-methylquercetin and rhamnazin in lipopolysaccharide-induced inflammation in porcine intestinal cells. *Antioxidants 2022, Vol. 11, Page 1265* **2022**, *11* (7), 1265. https://doi.org/10.3390/ANTIOX11071265.

96. Pomothy, J. M.; Barna, R. F.; Pászti, E. A.; Babiczky, Á.; Szóládi, Á.; Jerzsele, Á.; Gere, E. P. Beneficial effects of rosmarinic acid on IPEC-J2 cells exposed to the combination of deoxynivalenol and T-2 toxin. *Mediators of Inflammation* **2020**, *2020*. https://doi.org/10.1155/2020/8880651.
97. Akira, S. TLR signaling. *Current Topics in Microbiology and Immunology* **2006**, *311*, 1–16. https://doi.org/10.1007/3-540-32636-7_1.
98. Chen, Z.; Yuan, Q.; Xu, G.; Chen, H.; Lei, H.; Su, J. Effects of quercetin on proliferation and H_2O_2-induced apoptosis of intestinal porcine enterocyte cells. *Molecules 2018, Vol. 23, Page 2012* **2018**, *23* (8), 2012. https://doi.org/10.3390/MOLECULES23082012.
99. König, J.; Wells, J.; Cani, P. D.; García-Ródenas, C. L.; MacDonald, T.; Mercenier, A.; Whyte, J.; Troost, F.; Brummer, R. J. Human intestinal barrier function in health and disease. *Clinical and Translational Gastroenterology* **2016**, *7* (10). https://doi.org/10.1038/CTG.2016.54.
100. Hou, Y.; Wang, L.; Zhang, W.; Yang, Z.; Ding, B.; Zhu, H.; Liu, Y.; Qiu, Y.; Yin, Y.; Wu, G. Protective effects of N-acetylcysteine on intestinal functions of piglets challenged with lipopolysaccharide. *Amino Acids* **2012**, *43* (3), 1233–1242. https://doi.org/10.1007/S00726-011-1191-9.
101. Kishimoto, T. Interleukin-6 and its receptor; from cloning to clinic. *International Archives of Allergy and Immunology* **1992**, *99* (2–4), 172–177. https://doi.org/10.1159/000236243.
102. Sallusto, F.; Baggiolini, M. Chemokines and leukocyte traffic. *Nature Immunology* **2008**, *9* (9), 949–952. https://doi.org/10.1038/ni.f.214.

11. Acknowledgements

I would like to express my gratitude towards Dr. Ákos Jerzsele, the Head of the Department of Pharmacology and Toxicology, who provides the facilities for my studies.

I am so grateful to have such an amazing supervisor, Dr. Erzsébet Pásztiné Gere, who never lost faith in me and whose support was outstanding. Her work setting, kindness and motivation truly inspired me to give the best I can and to extend my knowledge. I would also like to express my thanks to Dr. Nikolett Palkovicsné Pézsa, Dr. Orsolya Farkas and Dr. Alma Virág Móritz who supported my work tremendously with their knowledge, kindness and advice.

Special thanks to my love Elias, who always supported me and whose patience, advice and company during this time pushed me through the hardest times.

Thank you to my parents and my brother who enable me to study veterinary medicine here. Your love, advice and support make my dream to become a vet and the best version of myself come true each and every day.

Thank you to all my friends at this university, who became an important part of my life and always support me no matter what.

Thank you to my former work colleagues at the “Pferdeklinik Seester” who always believed in me becoming a vet.

This research was funded by project no. TKP2020-NKA-01 and has been implemented with the support provided by the National Research, Development and Innovation Fund of Hungary, financed under the Tématerületi Kiválósági Program 2020 (2020-4.1.1-TKP2020) funding scheme. This work was supported by the Hungarian National Research, Development and Innovation Office under grant number 124522.

12. HuVetA statement

HuVetA

ELECTRONIC LICENSE AGREEMENT AND COPYRIGHT DECLARATION*

Name: Wohlert, Annelie

Contact information (e-mail): annelie@wohlert.io

Title of document (to be uploaded): Luteolin and chrysin could prevent lipopolysaccharide-ochratoxin combination-caused inflammation and oxidative stress *in vitro*

Publication data of document: TDK

Number of files submitted: 1

By accepting the present agreement the author or copyright owner grants non-exclusive license to HuVetA over the above mentioned document (including its abstract) to be converted to copy protected PDF format without changing its content, in order to archive, reproduce, and make accessible under the conditions specified below.

The author agrees that HuVetA may store more than one copy (accessible only to HuVetA administrators) of the licensed document exclusively for purposes of secure storage and backup, if necessary.

You state that the submission is your original work, and that you have the right to grant the rights contained in this license. You also state that your submission does not, to the best of your knowledge, infringe upon anyone's copyright. If the document has parts which you are not the copyright owner of, you have to indicate that you have obtained unrestricted permission from the copyright owner to grant the rights required by this Agreement, and that any such third-party owned material is clearly identified and acknowledged within the text of the licensed document.

The copyright owner defines the scope of access to the document stored in HuVetA as follows (**mark the appropriate box with an X**):

☐	I grant unlimited online access,
X	I grant access only through the intranet (IP range) of the University of Veterinary Medicine,
☐	I grant access only on one dedicated computer at the Ferenc Hutÿra Library,
☐	I grant unlimited online access only to the bibliographic data and abstract of the document.

Please, define the **in-house accessibility of the document** by marking the below box with an **X**:

X	I grant in-house access (namely, reading the hard copy version of the document) at the Library.

If the preparation of the document to be uploaded was supported or sponsored by a firm or an organization, you also declare that you are entitled to sign the present Agreement concerning the document.

The operators of HuVetA do not assume any legal liability or responsibility towards the author/copyright holder/organizations in case somebody uses the material legally uploaded to HuVetA in a way that is unlawful.

Date: Budapest, 17.09.2022

A. Wohlert

Author/copyright owner signature

HuVetA Magyar Állatorvos-tudományi Archívum – Hungarian Veterinary Archive *is an online veterinary repository operated by the Ferenc Hutÿra Library, Archives and Museum. It is an electronic knowledge base which aims to collect, organize, store documents regarding Hungarian veterinary science and history, and make them searchable and accessible in line with current legal requirements and regulations.*

HuVetA relies on the latest technology in order to provide easy searchability (by search engines, as well) and access to the full text document, whenever possible.
Based on the above, HuVetA aims to:

- *increase awareness of Hungarian veterinary science not only in Hungary, but also internationally;*
- *increase citation numbers of publications authored by Hungarian veterinarians, thus improve the impact factor of Hungarian veterinary journals;*
- *present the knowledge base of the University of Veterinary Medicine Budapest and its partners in a focussed way in order to improve the prestige of the Hungarian veterinary profession, and the competitiveness of the organizations in question;*
- *facilitate professional relations and collaboration;*
- *support open access.*

13. Thesis Progress Report

UNIVERSITY OF VETERINARY MEDICINE, BUDAPEST
founded in 1787, EU-accredited since 1995

INTERNATIONAL STUDY PROGRAMS
secretary, student@univet.hu

Thesis progress report for veterinary students

Name of student: Annelie Wohlert

Neptun code of the student: FBMJRT

Name and title of the supervisor: Pásztiné Dr. Gere Erzsébet, associate researcher

Department: Department of Pharmacology and Toxicology

Thesis title: Luteolin and chrysin could prevent lipopolysaccharide-ochratoxin combination-caused inflammation and oxidative stress *in vitro*

Consultation – 1st semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2022	02	15	Method application and development	
2.	2022	03	04	Time plan, protocol writing	
3.	2022	04	07	Scientific literature overview, summary of results, research work	
4.	2022	05	05	Evaluation of the research data, experimental work	
5.	2022	06	09	TDK thesis writing, milestones	

Grade achieved at the end of the first semester: jeles (5)

Consultation – 2nd semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2022	09	09	Statistical assessment of the results, experimental works	
2.	2022	09	12	Statistical assessment of the results, experimental works	
3.	2022	09	14	TDK writing and revision	

1

4.	2022	10	03	TDK writing and revision	
5.	2022	10	05	TDK writing and revision, preparation for TDK conference	

Grade achieved at the end of the second semester: jeles (5)

The thesis meets the requirements of the Study and Examination Rules of the University and the Guide to Thesis Writing.

I accept the thesis and found suitable to defence.

.....
signature of the supervisor

Signature of the student: A. Wohlert

Signature of the secretary of the department: .....

Date of handing the thesis in 13.11.2024

14. Supervisor counter signature

I hereby confirm that I am familiar with the content of the thesis entitled “Luteolin and chrysin could prevent lipopolysaccharide-ochratoxin combination-caused inflammation and oxidative stress *in vitro*” written by Annelie Wohlert (student name) which I deem suitable for submission and defence.

Date: Budapest, 08. (day) November (month) 2024 (year)

Pásztiné Dr. Gere Erzsébet

.....

Supervisor name and signature

Department of Pharmacology and Toxicology

15. TDK equivalence statement

DECLARATION

I hereby declare that the thesis entitled "Luteolin and chrysin could prevent lipopolysaccharide-ochratoxin combination-caused inflammation and oxidative stress *in vitro*" is identical in terms of content and formal requirements to the TDK research paper submitted in 2022 (year).

Date: Budapest, 05. (day) November (month) 2024 (year)

Annelie Wohlert, A. Wohlert

……..

Student name and signature