THE EFFECTS OF PROBIOTICS AND ANTIOXIDANTS ON DAMAGE TO PORCINE EPITHELIAL CELLS CAUSED BY VETERINARY PHARMACEUTICAL, TOXIC AND INFECTIOUS AGENTS IN VITRO

FINAL REPORT

1. Main tasks of the project

Our goals in this research project were to establish functional *in vitro* models of the intestine to mimic oxidative stress and inflammation upon administration of xenobiotics such as deoxynivalenol (DON), T-2, ochratoxin A (OTA), lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 and *Lawsonia (L.) intracellularis*. Polyphenolic rosmarinic acid (RA), luteolin (LUT), chrysin (CHR), quercetin (Que) and fermented wheat germ extract (FWGE) were screened to evaluate their potential beneficial effects in case of mycotoxin-induced redox imbalance and inflammation. In addition, the efficacy of sodium- butyrate (SB) and spent culture supernatants (SCS) of probiotic *Bacillus licheniformis* was also assessed in IPEC-J2 cells exposed to *Lawsonia (L.) intracellularis*.

Furthermore, safety profiles of pharmaceutical agents such as matriptase/transmembrane serine-protease 2 (TMPRSS2) inhibitors were planned to be evaluated in colon-originated spheroids, in hydrogels with liver epithelial cells/Kupffer co-cultures derived from male pigs of the Hungarian Large White breed and in primary hepatocytes of rat, beagle, cynomolgus monkey and human species.

2. **Main results of the project**

2.1 *In vitro* effects of DON, T-2 and their combinations with antioxidant compounds

Mycotoxin contamination in feedstuffs is a worldwide problem that causes serious health issues in swine. DON and T-2 are known to challenge mainly intestinal barrier functions and may contribute to the formation of the secondary infections. Intestinal infections in pigs are often treated with antibiotics, but the inappropriate use of these agents leads to the development of resistant bacterial strains, which renders therapy ineffective and may also pose a potential risk to humans consuming pork. Thereby there is a growing demand to find alternative

substances for substitution or supplementation of antibiotic therapy. Such, potentially beneficial compounds are plant-derived flavonoids.

In our study the impact of Que, a plant- derived flavonol and DON was evaluated on cell cytotoxicity, TER of cell monolayers and extracellular (EC)/IC redox status. It was found that DON decreased significantly TERs and triggered oxidative stress, while Que pretreatments were beneficial in maintaining the integrity of the monolayers. However, co-treatment with Que was unable to preserve the integrity and redox balance of the cells exposed to DON. These results indicated that only pre-incubation of cells with Que at 20 μmol/L for 24 h was beneficial in compensating the DON-disrupted extracellular oxidative status.

The effect of FWGE containing apigenin was evaluated with co-treatments with DON and T-2. The effects of FWGE on IPEC-J2 contaminated with DON and T-2 have not been studied until now. The cells were treated for 24 h with the selected mycotoxin solutions, then the IPEC-J2 cells were allowed to regenerate in culture medium for an additional 24 h. Our study found that 8 μmol/L DON and 5 nmol/L T-2 significantly reduced the TER values during and after the treatments, while 1% and 2% FWGE significantly increased them *in vitro*. To our knowledge, these are the first findings of determination for the impact of FWGE on the barrier integrity of the IPEC-J2 cell monolayer exposed to mycotoxins. FWGE used in 1% and 2% concentrations appeared to be beneficial to IPEC-J2 exposed to fusariotoxins since the aqueous extract significantly decreased the ROS levels. In conclusion, the results demonstrate that FWGE is effective in barrier integrity reinforcement and redox homeostasis maintenance when cells were treated with the extract during contamination with mycotoxins.

RA was also used in our experiments based on IPEC-J2 cells. Significant cell death was observed upon exposure of cells to DON at 50 μmol/L after 48 and 72 h incubation. After 48 and 72 h treatments at 20 nmol/L and at higher concentrations T-2 showed cytotoxic effects. Our results demonstrated that moderate to low concentrations of RA improved cell viability significantly using 24 h exposure time. However, high concentrations of RA (above 200 μM) for 24 h caused cell death to great extent. This study demonstrated that binary mixture of DT2 could deteriorate barrier integrity of IPEC-J2 cells and it could elevate levels of inflammatory cytokines. RA appeared to have anti-inflammatory, antioxidant and barrier-reinforcing potential in the prevention of DT-2-caused detrimental intestinal effects *in vitro*. Further *in vivo* studies are needed, however to confirm *in vitro* observed beneficial effects of RA and to establish daily application schedule of RA as porcine feed additive in the future.

2.2 The impact of LUT and CHR in porcine IPEC-J2 cells exposed to *E. coli* LPS and OTA combination

OTA is found mostly in the food chain and the feedstuff of animals. 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-lives 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. Gram-negative bacteria in contact with intestinal epithelial cells can lead to apoptosis of infected cells.

The study of the combined effect of OTA and LPS, modelling the complexity of environmental factors and their effects on immunological parameters, has not been done before. Non- cancerous porcine IPEC-J2 cells were treated with OTA (1 μmol/L, 5 μmol/L and 20 μmol/L), *E. coli* LPS (10 μg/ml), CHR (1 μmol/L) and LUT (2.5 μg/ml) alone and in their combinations. OTA decreased cell viabilities, which could not be alleviated by LUT or CHR, however, EC H_2O_2 production was successfully suppressed by LUT in IPEC-J2 cells. OTA with LPS elevated the IC ROS which was counteracted by CHR and LUT. In our study, applied LPS induced changes in IL-6 and IL-8 levels after 24 h. OTA treatments were not associated with increases in these cytokine levels. It is noteworthy, however, that co-administration of OTA and LPS induced significant increases in both cytokines tested, thus modelling multiple exposures to environmental stress. Moreover, the application of both CHR and LUT decreased the levels of IL-6 and IL-8 in IPEC-J2 cell culture. In conclusion, LUT and CHR could be able to counteract the combined damaging effects of *E. coli* LPS and OTA, but further *in vivo* tests are needed to understand the background of the complex effects. The presence of natural substances such as flavonoids in feed can counteract the effects of these environmental stressors and contribute to reducing the use of antibiotics, thereby reducing the spread of antimicrobial resistance, and reducing the likelihood of harmful factors entering the food chain.

2.3 Study on the combined effects of DON and T-2 on non- tumorigenic HIEC cells

DON and T-2 toxin can co-occur in infected plants, so mapping their impact on other intestinal epithelial cell models is of key importance. In our study, DON and T-2 toxin alone and in combination were administered to non-cancerous human intestinal epithelial cell line, HIEC-6. 24 h treatment of HIEC-6 cells with 1 μmol/L DON, 5 nmol/L T-2 and 1 μmol/L DON + 5 nmol/L T-2 combination significantly increased EC H_2O_2 production and IC ROS compared

to control values. Each of the treatments elevated IL-6 and IL-8 levels compared to basal cellular IL-6 and IL-8 production. Mycotoxin exposure decreased the membranous localization of claudin-1 protein, while the expression of occludin remained unchanged. Our study ist the first in which the effects of mycotoxins DON and T-2 were evaluated on the viability of HIEC-6 cells. Based on our findings, the applied mycotoxins administered non-cytotoxic concentrations could raise both EC/IC oxidative stress and the production of inflammatory cytokines such as IL-6 and IL-8 could change distribution pattern of TJ protein claudin-1, but not the expression of occludin.

2.4 The effects of feed-additive sodium- butyrate and *Bacillus licheniformis* SCS in *Lawsonia intracellularis* -caused infection

The project work involved *in vitro* investigation of *L. intracellularis*- caused enteropathy and selection of beneficial antibiotic alternatives in IPEC-cells Proliferative enteropathy of weaning pigs has significant economical importance in the swine industry. Administration of antibiotics against *L. intracellularis* results in drug residues in the meat, resistance formation among pathogenic bacteria and cause further economic losses. To avoid the usage of antibiotics in proliferative enteropathy caused by intracellularly acting enteropathogen, *L. intracellularis*, SCS of *Bacillus licheniformis* (*B. licheniformis*) CECT 4536 and SB were used to test their potential beneficial effects *in vitro*. Porcine IPEC-J2 cell line was infected with $5x10^5$ CFU *L. intracellularis* (strain PHE/MN1-00) two times. Our findings suggest that SCS of *B. licheniformis* (0.5%) could improve the barrier integrity and was capable of exerting anti-inflammatory effect in IPEC-J2 cell model of *L. intracellularis*-caused porcine proliferative enteropathy.

2.5 Applicability of colon adenocarcinoma- and liver epithelial cells-based 3D cultures

In 3D or multilayer *in vitro* cell cultures such as spheroids from cancerous cells and hepatocyte mono- and the hepatocyte- Kupffer cell co-cultures embedded in a hydrogel better resemble the *in vivo* conditions than the widely used monolayer cell culture systems. It was found that matriptase/TMPRSS2 inhibitors such as MI-432, MI-453, MI-460 and MI-463 at 50 µmol/L did not cause murine C-26 and a human HT-29 cell deaths to a significant degree in spheroids with diameters of approximately 200 µm for C-26 and 250-300 µm for HT-29 after a growth period of 3 days. Matriptase/TMPRSS2 inhibitors at 50 µmol/L did not alter the HT-

29 spheroid growth rate within 7 days. Only MI-432 caused a remarkable decrease in C-26 spheroid growth thus the altered matriptase/TMPRSS2 activity did not affect cancer cells' growth tendency (except MI-432) during formation of C-26/HT-29 spheroids.

The matriptase-2 (MT-2) on the surface of the hepatocytes regulates the production of the hepcidin responsible for the iron homeostasis. In our experiments, the effects of MI 441 and MI-461 were investigated in hepatocyte mono- and hepatocyte- Kupffer cell co-cultures (with cell proportion 6:1). According to our results, EC H_2O_2 levels were not elevated significantly. Matriptase inhibitors did not cause increases in IL-6 and IL-8 levels. However, in the case of the inflammatory 3D co-culture model, MI-441 and MI-461 caused significant decrease in IL-8 levels. In summary it can be concluded that different results between 2D and 3D measurements necessitate further studies to investigate MI-441 and MI-461-caused pharmacological effects in-depth.

2.6 *In vitro* safety evaluation of modulators of TMPRSS2/furin as enzymatic targets for antiviral therapy using liver epithelial cells from different species

The One Health approach, which includes collaborative application of human and veterinary pharmacological and clinical knowledge, can provide strategies against zoonotic coronaviruses. Due to worlwide presence of COVID-19 (607083820 confirmed human cases including 6496721 deaths until October 2022, https://covid19.who.int/) during the project period, *in vitro* characterization of potental drug candidates with inhibitory effects of TMPRSS2 and furin was of prioritized importance. Recently, it has been discovered that several mammals including dogs, domestic cats, tigers, lions, minks, ferrets can be infected with the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) showing vulnerability mainly to human-to-animal transmission.

It was previously reported that SARS-CoV-2 titers decreased in a dose-dependent manner after treatment with MI-432 and MI-1900 in Calu-3 cells most likely via suppression of TMPRSS2. Based on our data, MI-1900 and MI-1907 did not cause cell death significantly up to 50 μmol/L and did not affect the levels of IL-6 and IL-8 in HIEC-6 cells and primary human hepatocytes (PHHs). Relatively low IC_{50} values (<1 μmol/L) indicate that cytochrome P 450 (CYP) 3A4 was affected in human *in vitro* models. It was also observed that the primate cynomolgus monkey microsomal CYP1A2 was modulated by administration of MI-432, MI-463, MI-482 and MI-1900 compounds at 50 μmol/L and IC_{50} values were determined to be lower than 5 μmol/L in each case. It was also reported that the priming of the SARS-CoV-2 S

protein could be also inhibited by furin inhibitor MI-1851. In our study it was confirmed that MI-1851 can be safely used at 50 μmol/L in porcine IPEC-J2 cells and PHHs. In addition, the lack of oxidative stress-inducing properties of inhibitor MI-1851 was also demonstrated. Compared to the *in vitro* impact of potential antiviral TMPRSS2 inhibitors on CYP3A4, MI-1851 showed only a minimal safety risk from the perspective of drug-drug interaction.

3. Other relevant information

In 2017, I gave birth to my third child. Due to this circumstance 2 years prolongation of the project was permitted.

SUPPLEMENTARY MATERIAL

PHD THESIS WORK:

Bús-Pomothy Judit: Applicability of potential protective agents in the restoration of healthy intestinal barrier damaged by mycotoxins in an *in vitro* model 2022

TDK AND THESIS WORKS:

Annelie Wohlert: Luteolin and chrysin could prevent lipopolysaccharide-ochratoxin combination-caused inflammation and oxidative stress *in vitro* 2022
Szentkirályi-Tóth Anna: Optimisation of microsomal studies to test pharmaceutical candidate compounds 2021
Fedor Zsófia: *In vitro* comparison of biotransformation of 3-amidinophenylalanine-type compounds in different animal species 2021
Szabó Orsolya: The effects of deoxynivalenol, T-2 toxin and their combination on HIEC-6 cells 2020.
Szóládi Áron: Beneficial effects of rosmarinic acid on IPEC-J2 cells exposed to combination of deoxynivalenol and T-2 toxin 2020.
Gatt Katrina: *In vitro* evaluation of DON-induced intestinal barrier damage 2019.
Prokoly Dorottya: Study on the effect of fermented wheat germ extract on intestinal epithelial cells exposed to fusariotoxins 2019.
Kiss Zsófia: Modelling of intestinal damage caused by zearalenone using IPEC-J2 cells 2017.

Szombath Gergely: 3D physiological and inflammatory hepatic *in vitro* models for screening drug candidates National TDK Conference 1st place 2017.

Czimmermann Ágnes Eszter: *In vitro* pharmacological characterization of selective TMPRSS2 inhibitor National TDK Conference 3rd place 2017.