Theses of doctoral (PhD) dissertation

IN VITRO INVESTIGATION OF THE EFFECTS OF FLAVONOIDS IN PORCINE GASTROINTESTINAL INFECTION MODELS

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1. Introduction and aims of research

Antimicrobial resistance (AMR) is a leading health threat of the 21st century that impacts humans, animals, and the environment inextricably. Even though the term AMR can refer to insusceptibility of any microorganisms to the drugs developed against them, it is most commonly used to describe antibiotic resistance in bacteria. The widespread presence of resistant bacteria, and especially the occurrence of multidrug-resistant (MDR) strains can remarkably reduce available treatment options against these infections, and consequently increase their mortality. Bacterial AMR has developed as the result of improper usage of antibiotics on both the human and veterinary fields, and must also be tackled with coordinated, interdisciplinary measures from these sectors, under the so-called "One Health" approach. From this point of view, antibiotic usage in food-producing animals requires special attention, as it has been linked to the spread of resistance in humans mainly via the foodborne route. In pigs, gastrointestinal infections caused by *Escherichia coli* and *Salmonella* spp. are of high importance, as both pathogens are widespread, potentially zoonotic, and highly prone to develop resistance. Therefore, these infections are difficult to treat, and have public health importance due to the possible foodborne transmission, which might include the spread of resistance conferring genes as well.

Among the strategies developed and supported by researchers, international organizations, and authorities to overcome AMR, the usage of antibiotic alternatives has great importance. These substances have the potential to be effective in the prevention and treatment of bacterial infections alone, or in combination with antibiotics. Among many other conceivable alternatives, flavonoids – bioactive compounds of plant origin – represent a large, promising group with several beneficial health promoting activities. In this study, our aim was to test the efficacy of flavonoids, grape seed oligomeric proanthocyanidins (GSOP) and luteolin (LUT) *in vitro*, in porcine gastrointestinal infection models caused by potentially zoonotic bacteria (*E. coli* and *S. enterica* ser. Typhimurium) to evaluate their usage as potential antibiotic alternatives for the prevention and treatment of swine intestinal bacterial infections.

Firstly, we investigated the impact of these flavonoids on cell viability to determine their concentrations that can be used safely on IPEC-J2 porcine intestinal epithelial cells (Neutral Red method). Then we have treated IPEC-J2 cells with bacterial endotoxin (lipopolysaccharide, LPS) of *E. coli* and *S.* Typhimurium origin and investigated the antioxidant effect of GSOP and LUT against oxidative stress caused by LPS (dichlorodihydrofluorescein diacetate [DCFH-DA]

assay, Amplex Red method). In case of Gram-negative infections, LPS plays an important role in worsening the symptoms, especially when it is released in high amount during antibiotic therapy. Both GSOP and LUT were applied in different concentrations in combination with LPS to evaluate potential correlation between their concentration and the observed activity.

Afterwards, we determined minimum inhibitory concentration (MIC) values of GSOP and LUT against *E. coli* and *S.* Typhimurium field isolates of porcine origin to obtain information on their potential bacteriostatic activity (broth microdilution method). Furthermore, we have tested the flavonoids' interaction with three antibiotics used frequently in pigs (amoxicillin, gentamicin, enrofloxacin) to evaluate whether they improve, decrease, or do not influence the activity of these substances (checkerboard assay). These antibiotics are generally active against *E. coli* and *S.* Typhimurium, and are used in both human and veterinary medicine, but resistance is common against them.

In the third phase of the study, we have established a co-culture model, in which IPEC-J2 cells were infected with *E. coli* and *S.* Typhimurium of porcine origin. In this system, beneficial effects of GSOP and LUT were tested in different concentrations and experimental settings, including pre-, parallel, and post-treatment, based on the time of flavonoid addition compared to the time of bacterial infection. Our goal was to test dose-dependence of the flavonoids' effect, as well as to model and compare their potential usage as prevention or treatment options in swine enteric bacterial infections. In this model, the effect of GSOP and LUT were examined on reactive oxygen species (DCFH-DA assay, Amplex Red method) and interleukin-6, -8 (IL-6, -8) levels (enzyme-linked immunosorbent assay [ELISA]) in IPEC-J2 cells that were elevated due to bacterial infection. Furthermore, flavonoids were tested if they can alleviate barrier integrity damage in IPEC-J2 cells caused by bacteria (fluorescein isothiocyanate–dextran [FD4] assay), and if they can inhibit the adhesion of *E. coli* and *S.* Typhimurium to the cells (colony forming unit [CFU] counting).

The obtained results are not only important on the field of veterinary medicine, but might be extrapolated to public health, due to zoonotic potential of the investigated pathogens.

2. Overview of new scientific results

For the first time, we have investigated protective effects of grape seed oligomeric proanthocyanidins (GSOP) and luteolin (LUT) in IPEC-J2 porcine intestinal epithelial cells treated with bacterial endotoxin (lipopolysaccharide, LPS) and in IPEC-J2 – bacterium co-culture (i.e. IPEC-J2 cells infected with *Escherichia coli* and *Salmonella enterica* ser. Typhimurium). Furthermore, we have tested antibacterial activity of these flavonoids and their potential interaction with antibiotics against several *E. coli* and *S.* Typhimurium field isolates of porcine origin.

Main findings of the study are as follows:

1. GSOP (up to 200 μg/ml) and LUT (up to 100 μg/ml) did not show cytotoxic effect on IPEC-J2 cells when applied for 1, 12 and 24 hours.
2. GSOP and LUT were able to decrease oxidative stress (intracellular reactive oxygen species levels) in IPEC-J2 cells triggered by LPS of *E. coli* (O111:B4, O127:B8) and *S.* Typhimurium origin, as well as oxidative stress inflicted by *E. coli* and *S.* Typhimurium *in vitro* infections.
3. In certain treatment types, GSOP and LUT showed anti-inflammatory effect (decreased interleukin-6 and interleukin-8 levels) and barrier protective activity in IPEC-J2 cells in case of inflammation and barrier integrity damage caused by *E. coli* and *S.* Typhimurium *in vitro* infections.
4. GSOP could inhibit the adhesion of *E. coli* and *S.* Typhimurium to IPEC-J2 cells, while LUT did not show anti-adhesive activity against any of the tested bacteria.
5. GSOP inhibited the growth of *E. coli* and *S.* Typhimurium field isolates of porcine origin with minimum inhibitory concentration (MIC) values of 2048 μg/ml, while LUT showed bacteriostatic effect against the same bacteria at 256 μg/ml concentrations. The addition of GSOP and LUT did not influence activity of conventionally used antibiotics (amoxicillin, gentamicin, enrofloxacin) against the same strains (average FIC indexes between 1.0000 and 1.4375).

Based on our findings, GSOP and LUT are promising candidates to be used in the future as feed additives for the prevention and/or treatment of gastrointestinal infections in swine caused by *E. coli* and *S.* Typhimurium.

3. Own scientific publications related to the topic of the dissertation

Full text papers in peer-reviewed journals

Kovács D., Palkovicsné Pézsa N., Jerzsele Á., Süth M., Farkas O.: **Protective Effects of Grape Seed Oligomeric Proanthocyanidins in IPEC-J2-*Escherichia coli/Salmonella* Typhimurium Co-Culture**, Antibiotics (Basel), 11(1). 110, 2022.

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, 9(12). 1267, 2020.

Kovács D., Palkovicsné Pézsa N., Farkas O., Jerzsele Á.: **Antibiotikum-alternatívák a sertéstartásban**, Magyar Állatorvosok Lapja, 143. 281-292, 2021.

Kovács D., Karancsi Z., Palkovicsné Pézsa N., Jerzsele Á., Farkas O.: **Polifenolok bélhámra gyakorolt antioxidáns és gyulladáscsökkentő hatásának modellezése**, Új Diéta: A Magyar Dietetikusok Lapja, 30. 10–13, 2021.

Kovács D., Karancsi Z., Palkovicsné Pézsa N., Farkas O.: **Bélhámsejt-modell gyulladáscsökkentő és antioxidáns hatású anyagok vizsgálatára**. In: Poór P., Mézes M., Blázovics A.: Oxidatív stressz és antioxidáns védekezés a növényvilágtól a klinikumig. Budapest, Magyarország, Magyar Szabadgyök-Kutató Társaság, 218. 136-145, 2020.

Conference presentations

Kovács D., Palkovicsné Pézsa N., Jerzsele Á., Farkas O.: **Szőlőmag proantocianidinek hatásai sertés bélhámsejt – baktérium kokultúrában.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2022.

Kovács D., Karancsi Z., Palkovicsné Pézsa N., Farkas O., Jerzsele Á.: **Baktérium-bélhámsejt ko-kultúra létrehozása a bakteriális eredetű bélhámkárosodás, valamint potenciális antibiotikum alternatívák tanulmányozására**. MTA Akadémiai Beszámolók, Budapest, Hungary, 2021.

Kovács D., Karancsi Z., Farkas O., Jerzsele Á.: **Proantocianidinek használata sertések bakteriális bélfertőzéseiben**. MTA Akadémiai Beszámolók, Budapest, Hungary, 2020.