Theses of doctoral (PhD) dissertation

EVALUATION OF PROBIOTICS ON PORCINE INTESTINAL EPITHELIAL CELLS

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1. Introduction and aims

The growth of human population increasingly demands food of animal origin, including pork meat. Intestinal diseases caused by *Salmonella* spp. and *Escherichia coli* (*E. coli*) may lead to significant economic loss in pigs and often require antibiotic therapy. In the past, swine industry has largely relied on prophylactic and metaphylactic use of antibiotics to control gastrointestinal diseases. However, the misuse of antibiotics led to the emergence of antibiotic resistance and residues in the human food chain may appear, thus threatening human health. Consequently, it has become pivotal for the swine industry to seek for feed additives that can contribute to the health of the gastrointestinal tract. Probiotics are promising candidates for this purpose. Probiotic action is complex, the exact mechanism has been widely studied, but still needs to be elucidated. Among the beneficial effects exerted by probiotic bacteria are inhibition of pathogen adhesion, stimulation of heat shock proteins, alteration of cytokine production, antioxidant properties and enhancement of barrier function. Therefore, this study aims to examine the effect of multiple probiotic candidates (*Enterococcus faecium, Lactobacillus rhamnosus, Bacillus licheniformis and Bacillus subtilis*) in porcine gastrointestinal infection models, *in vitro*. Two economically important swine pathogens *E. coli* and *S. enterica* serovar Typhimurium (*S.* Typhimurium) or lipopolysaccharide (LPS) of *S.* Typhimurium or *E. coli* origin were used to model gastrointestinal infections.

First, we aimed to determine the optimal treatment conditions for our experiments. The impact of spent culture supernatants and bacterial cells on the viability of intestinal porcine epithelial cells **(**IPEC-J2) cells was tested (Neutral Red method).

Second, our goal was to examine whether cell-free bacterial spent culture supernatants (SCSs) of *Enterococcus faecium (E. faecium)*, *Lactobacillus rhamnosus* (*L. rhamnosus)*, *Bacillus licheniformis (B. licheniformis)* and *Bacillus subtilis (B. subtilis)* can achieve beneficial effects. Therefore IPEC-J2 cells were challenged with three different types of LPS, namely *S.* Typhimurium LPS, *E. coli* O111:B4 LPS and *E. coli* O127:B8 LPS and treated with the SCSs of *E. faecium, L. rhamnosus, B. licheniformis* and *B. subtilis.* The effect on reactive oxygen species (ROS) production was measured using dichlorodihydrofluorescein

diacetate (DCFH-DA) method and we determined the antioxidant capacity of the SCSs. Moreover, the potential antibacterial effect of the SCSs were tested against *E. coli* and *S.* Typhimurium field isolates of porcine origin (microdilution method).

The third objective was to evaluate the *in vitro* probiotic potential of four probiotic candidates *E. faecium*, *L. rhamnosus*, *B. licheniformis* and *B. subtilis* against pathogen-induced damages using bacteria. Therefore, we established a co-culture model, in which gastrointestinal infection was modelled using *E. coli* and *S.* Typhimurium of porcine origin. Different treatment conditions were applied, pre-treatment, co-treatment and post-treatment. Pre-treatment meant that the probiotic bacterium was added one hour before the addition of the pathogenic bacterium to IPEC-J2 cells, during co-treatment probiotic and pathogenic bacteria were added at the same time and in the post-treatment assay, pathogenic bacteria were added prior to the addition of the probiotic bacteria. These different treatment regimens were included in the study in order to evaluate the probiotics' action as preventive or therapeutic agents. The effects on paracellular permeability (fluorescein isothiocyanate-dextran [FD4] method), inflammatory response (ELISA method), ROS production (DCFH-DA method), and adhesion inhibition of pathogens (colony forming unit [CFU] counting) were investigated using IPEC-J2 cell line.

Beneficial effects of probiotics have been extensively studied, however they are strain/species-specific, to put it another way promising effects must be determined for every single probiotic strain/species. The present work significantly contributes to the characterization of probiotic-specific potentially beneficial effects. Results can serve as a basis for further *in vivo* studies carried out in pigs. Moreover, given the similarities between human and swine gastrointestinal tract, the results may also contribute to the application of probiotics in human health.

2. Overview of scientific results

Our study was the first to comprehensively test protective effects of *E. faecium*, *L. rhamnosus*, *B. licheniformis* and *B. subtilis* on IPEC-J2 cells. Antioxidant capacity of bacterial SCSs was evaluated under LPS induced oxidative damage and antimicrobial activity of SCSs against several swine-derived *E. coli* and *S.* Typhimurium field isolates was investigated. Furthermore, the beneficial effects on intracellular ROS production,

inflammatory cytokine response, paracellular permeability and adhesion inhibition of pathogens were tested using IPEC-J2 – bacterium co-culture model.

Main findings of the study are as follows:

1. *E. faecium, L. rhamnosus, B. licheniformis* and *B. subtilis* affect the viability of IPEC-J2 cells in a species-specific manner. Spent culture supernatants of *E. faecium, L. rhamnosus, B. licheniformis* and *B. subtilis* (6% concentration for 1 hour) and bacterial suspensions of *E. faecium, L. rhamnosus, B. licheniformis* and *B. subtilis* (10^8 CFU/ml for 1 hour) did not show cytotoxic effects on IPEC-J2 cells.
2. Intracellular ROS reducing ability of SCSs of *B. licheniformis* and *B. subtilis* is independent of the type of LPS used to induce oxidative stress. Intracellular ROS reducing effects of SCSs of *E. faecium, L. rhamnosus* depend on the applied type of LPS used to evoke oxidative stress.
3. The effect of probiotic bacterial suspensions on barrier integrity of IPEC-J2 cells is species-specific; *L. rhamnosus* enhances, *B. subtilis* reduces, while *E. faecium and B. licheniformis* do not significantly affect barrier integrity. *E. faecium* and *L. rhamnosus* can counteract barrier damage in IPEC-J2, independently of the barrier disruptions' causative agent (*E. coli* or *S.* Typhimurium) and of the time of addition (pre-, co-, post-treatment). *B. licheniformis* and *B. subtilis* do not exert beneficial effects against barrier impairment of IPEC-J2 cells caused by *E. coli* or *S.* Typhimurium.
4. In certain treatment types, *E. faecium, L. rhamnosus, B. licheniformis* and *B. subtilis* showed anti-inflammatory effect (reduced IL-6 and IL-8 levels) in IPEC-J2 cells challenged with *S.* Typhimurium. The effect of probiotics on proinflammatory response of IPEC-J2 cells is species-specific and also depends on the type of proinflammatory cytokine examined and on the causative agent (*E. coli* or *S.* Typhimurium) used to evoke inflammation. The time of addition of probiotics also influences the inflammation-reducing effect.
5. *E. faecium, L. rhamnosus, B. licheniformis* and *B. subtilis* have a general intracellular ROS reducing effect in IPEC-J2 cells, moreover, this effect is not species-specific

and is independent of the causative agent (*E. coli* or *S.* Typhimurium) of oxidative stress.

6. The adhesion of both *E. coli* and *S.* Typhimurium to IPEC-J2 cells can be significantly inhibited by *E. faecium, L. rhamnosus and B. licheniformis* regardless of the time of addition (pre-, co- or post-treatment). Pathogen adhesion inhibiting properties of *B. subtilis* depend on the type of applied pathogenic bacteria.

Based on our results, *E. faecium, L. rhamnosus, B. licheniformis* and *B. subtilis* are attractive candidates as feed additives that can contribute to the prevention and treatment of *E.coli* or *S.* Typhimurium induced gastrointestinal diseases.

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

Full text papers in peer-reviewed journals

Palkovicsné Pézsa N., Kovács D., Rácz B., Farkas O**.: Effects of *Bacillus licheniformis* and *Bacillus subtilis* on gut barrier function, proinflammatory response, ROS production and pathogen inhibition properties in IPEC-J2 — *Escherichia coli/Salmonella* Typhimurium co-culture**, Microorganisms, 10. 936, 2022.

Palkovicsné Pézsa N., Kovács D., Gálfi P., Rácz B., Farkas O**.: Effect of *Enterococcus faecium* NCIMB 10415 on gut barrier function, internal redox state, proinflammatory response and pathogen inhibition properties in porcine intestinal epithelial cells**, Nutrients, 14. 1486, 2022.

Palkovicsné Pézsa N., Kovács D., Somogyi Z., Rácz B., Farkas O., **Probiotikumok hatásának vizsgálata sertésekben**, Magyar Állatorvosok Lapja, 144. 613-622., 2022

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., 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

Palkovicsné Pézsa N., Karancsi Z., Farkas O., Rácz B.: ***Lactobacillus plantarum* 2142 hatása bélhámsejtek morfológiájára fény- és elektronmikroszkópos vizsgálatokban.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2018

Palkovicsné Pézsa N., Karancsi Z., Bowles H., Rácz B., Farkas O.: **Probiotikumokkal történő kezelés hatásának nyomonkövetése IPEC-J2 sertés bélhám sejteken.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2019

Palkovicsné Pézsa N., Karancsi Z., Farkas O., Rácz B. **Probiotikumok hatása IPEC-J2 bélhám sejtekre.** Magyar Szabadgyök-Kutató Társaság X. Kongresszusa, Szeged, Hungary, 2019.

Palkovicsné Pézsa N., Karancsi Z., Rácz B., Farkas O.: **Enterococcus faecium felülszóval történő kezelés hatásának nyomonkövetése IPEC-J2 sejtkultúrán.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2020

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.

Palkovicsné Pézsa N., Kovács D., Farkas O., Rácz B.:***Enterococcus faeciummal* történő kezelés hatásának nyomonkövetése IPEC-J2 sejtkultúrán.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2022

Farkas O., Palkovicsné Pézsa N., Kovács D., Pászti-Gere E., Rácz B.: ***In Vitro* Porcine Intestinal Co-Culture Model to Study the Effect of *Enterococcus faecium* in *Escherichia coli* and *Salmonella Typhimurium* Infection** IPC 2022 - International Conference of Probiotics and Prebiotics, Bratislava, Slovakia, 2022

Palkovicsné Pézsa N., Kovács D., Farkas O., Rácz B.*: **Lactobacillus rhamnosusszal* történő kezelés hatásának vizsgálata IPEC-J2— baktérium ko-kultúrán.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2023