

SZENT ISTVÁN UNIVERSITY
Doctoral School of Veterinary Science

**Strategies for protecting enterocytes from oxidative stress-
induced inflammation**

PhD thesis

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1. INTRODUCTION

The oxidative stress via causing cell and tissue damage can lead to formation of acute and chronic inflammation. Intestinal epithelium acts as a strong physical and chemical barrier against invading bacteria, toxins, oxidative stress and various chemical agents. Malfunction of the epithelial defense mechanisms as a result of damaged gut mucosa and altered intestinal microbial homeostasis can easily lead to leaky gut syndrome which can influence the general health condition of animals even on optimal nutritional regimens.

The first aim of this experimental work was the development of an *in vitro* system mimicking intestinal epithelium, where oxidative stimuli can be introduced by peroxide treatment and the regulatory effect of acute oxidative stress can be monitored continuously. The prerequisite for finding the optimal dose and treatment time of peroxide administration was the maintenance of cell viability whereas the changes in relative gene expression level of proinflammatory cytokines could indicate the acute phase of inflammatory processes. IPEC-J2 cells isolated from the jejunum of a neonatal piglet were selected for *in vitro* study as their glycosylation pattern, proliferation rate and colonisation ability can characterize better the *in vivo* conditions in the gut ecosystem. The high transepithelial electrical resistance value of IPEC-J2 monolayers grown on collagen-coated polyester membrane inserts demonstrated the functional integrity of the continuous cell association, acting as a single-layer tight physical barrier separating apical and basolateral compartments in the 3D model system .

Recently, the administration of low-dose dietary antibiotics has been banned in EU for growth promotion of livestock to avoid the development of antibiotic resistance of some pathogenic bacteria. For this reason nowadays there is a growing interest to replace various antibiotics with safely applicable growth promoters. Probiotics are defined as live microbial food/feed ingredients that have beneficial effects on the host health and well-being. They are normal inhabitants of the healthy gut microbiota and present in several fermented foods. Currently, pre- and probiotics have been representing one of the most promising alternatives to antibiotics to protect animal health and increase the efficiency of nutrient utilization. It has already been reported that they can improve the intestinal microbial balance, confer protection against enteropathogenic bacteria, and prevent or cure intestinal diseases, however, the underlying mechanisms remained to be elucidated in detail. These effects are partially mediated via the production of antimicrobial metabolites such as various salts of short chain

organic acids (lactate, acetate and butyrate), hydrogen peroxide or bacteriocins, and competition with harmful bacteria for nutrients or adhesion sites. Organic acids are widely used to reduce mucosal damage caused by infection or oxidative stress in swine. One of the most important organic acids produced by intestinal microbiota (mostly probiotic bacteria) is butyric acid, which can play important role in the physiology and metabolism of the rumen, the intestine and the ruminal and intestinal mucosa.

Further aim of this dissertation was to reveal the influence of spent culture supernatant (SCS) of probiotics (*Lactobacillus plantarum* 2142, *Lactobacillus casei* Shirota, *Bifidobacterium animalis* subsp. *lactis* Bb-12, *Bacillus amyloliquefaciens* CECT 5940, *Enterococcus faecium* CECT 4515) on the response of enterocytes to oxidative stress, and the supernatant's ability to protect them from oxidative injury and to find out which active components can play key role in this beneficial effect. To determine the impact of probiotics on acute oxidative stress-induced inflammation, experiments were performed on IPEC-J2 intestinal epithelial cell line cultured on membrane inserts. As fast and reliable screening method for the antiinflammatory properties of selected probiotics or their metabolites, the relative gene expression of two proinflammatory cytokines (interleukin-8, IL-8 and tumor necrosis factor- α , TNF- α) and that of cytoprotective 70 kDa heat shock protein (Hsp70) were assessed in peroxide-treated IPEC-J2 cells using a qRT-PCR after addition of the supernatant or short chain organic acids.

The practical aspect of this dissertation is to provide important scientific data for the swine industry about the beneficial effect of probiotics and their metabolites in the prevention or treatment of oxidative stress-induced inflammatory processes. The in-depth characterization of potential antiinflammatory properties of protective agents such as short chain organic acids in the porcine intestinal epithelial cell line, IPEC-J2-based 3D model and in swine exposed to reactive oxygen species included the following steps:

1. The investigation of subcellular effect of acute oxidative stress on optimized *in vitro* 3D intestinal model systems
 - Development of H₂O₂ treatment regimen with optimized incubation time and H₂O₂ dose for achieving the peak level of investigated proinflammatory cytokines without detectable cell death.
 - Quantitative determination of IL-8 in apical and basolateral compartments using ELISA technique.
2. Evaluation of peroxide-triggered cell response profile

- Via indicators of cell monolayer integrity such as changes in TER, lipid peroxidation and distribution of tight junctional (TJ) proteins.
 - Analysis of differentiated protein kinase C (PKC) isoenzymes to reveal which one if elevated can be responsible for oxidative-stress induced changes at signal transduction level during recovery period after H₂O₂ administration
3. Screening of immunomodulatory effects of spent culture supernatants from selected bacterial strains
- Pharmaceutically and therapeutically important target: Probiotic supernatant capable of alleviating effectively inflammatory processes via restoration of upregulated IL-8 and TNF- α relative gene expression and via elevation of cytoprotective Hsp 70 level
4. Study on effects of microbial organic acids
- Elucidation of potential beneficial effects of lactic acid and acetic acid in prevention of intestinal epithelial cells from cell injury when they were exposed to acute oxidative stress.
 - Study on how butyrate can act on oxidative stress-affected gastrointestinal epithelium and how it can influence enterocyte proliferation in swine intestine.

2. MATERIALS AND METHODS

2.1 Cell culture

- Two cell lines were used for *in vitro* experiments:
 - Non-transformed IPEC-J2 cell line derived from jejunal epithelia isolated from a neonatal piglet cultured on polycarbonate or on collagen-coated polyester membrane inserts
 - Human colon adenocarcinoma Caco-2 cell lines
- TER measurement of monolayers was performed on alternate days after seeding from days 5 to 21 of culture using EVOM Epithelial Tissue Volt/Ohmmeter.
- Cells were given different concentrations of H₂O₂ (0-10 mM) in plain medium apically for different time intervals.

2.2 Cytotoxicity evaluation

- 1 µg/ml DAPI in PBS was added for 10 min. Cells were then washed and fixed with 4 v/v% formaldehyde/PBS solution.

2.3 Immunohistochemistry

- Immunohistochemical measurements for evaluation of the number of dividing cells in ileal samples were performed after dehydration, embedding in paraffin and section cutting using Ki67 proliferation marker.
- For immunohistochemistry 3-4 µm thick sections were cut and stained with antibodies to E-cadherin, claudin-1 and claudin -4. The sections were dewaxed in xylene and graded ethanol. After treatment with appropriate antigen retrieval solution the sections were incubated with primary antibodies against anti-claudin-1, anti-claudin-4 and anti-E-cadherin. Antigen-bound primary antibody was detected using standard avidin-biotin immunoperoxidase complex (DAKO, LSAB2 Kit). The chromogen substrate was 3, 3-diaminobenzidine in each case. Sections were counterstained with Mayer's haematoxylin.

2.4 Investigation of PKC isoenzymes using Western blot

- Cells were lysed and equal amounts of proteins were loaded onto 8-16% HEPES-SDS-polyacrylamide gels, which were then blotted to Immobilon-P membranes. For immunodetection, membranes were first blocked with 5% (w/v) non-fat dry milk and 0.1% (v/v) Tween 20 in Tris-buffered saline and then treated with the antibodies. The following antibodies were used: rabbit polyclonal antibodies against PKC α , PKC γ , PKC δ , PKC ϵ , PKC ζ , PKC ζ -Thr^{410/403} and mouse monoclonal antibody against GAPDH. Secondary antibodies conjugated with horseradish peroxidase were: goat anti-rabbit IgG, goat anti-mouse IgG and donkey anti-goat IgG. Visualization of blots was performed with enhanced chemiluminescence.

2.5 Lipid peroxidation in IPEC-J2 cells exposed to peroxide treatment

- The rate of lipid peroxidation was determined by quantitative measurement of conjugated dienes (CDs) at 234 nm, conjugated trienes (CTs) 268 nm and malondialdehyde (MDA) with TBA assay at 535 nm detection wavelength in cell supernatants.

2.6 Quantitative Real Time PCR

- After the H₂O₂ treatment 1 ml of ice-cold TRIzol reagent was added to the cell monolayers.
- Isolated RNA was treated with the AMP-D1 Dnase I.
- Synthesis of the first strand of cDNA was achieved using RevertAid H Minus First Strand cDNA Synthesis kit.
- qRT-PCR was performed using the iQ SYBR Green Supermix kit. Tested genes of interests were IL-6, IL-8, TNF- α and Hsp 70. Reference genes were hypoxanthine phosphoribosyl-transferase (HPRT) and cyclophilin-A (CycA).

2.7 IL-8 ELISA

- The apical and basolateral culture media were collected and the level of IL-8 secretion was determined by a porcine-specific IL-8 ELISA Kit.

2.8 Exposure of epithelial cells to SCS treatment

- *L. plantarum* 2142, *L. casei* Shirota, *Enterococcus faecium* CECT 4515 were grown in DeMan, Rogosa, Sharpe, *Bacillus amyloliquefaciens* CECT 5940 in tryptone soya broth *Bifidobacterium animalis* subsp. *lactis* Bb 12 in tryptone phytone yeast medium. SCSs were prepared by centrifugation of the bacterial suspension (with final bacterial concentrations of 10^9 CFU/ ml) at 3000 g at 5°C for 10 min. Centrifuged supernatant was then passed through a sterile 0.22 µm pore size filter unit.

2.9 Quantification of peroxide content after peroxide-supernatant interaction

- Concentration of H₂O₂ in aqueous solutions was determined by a colorimetric method according to Nowak (Nowak D., Biomed. Biochim. Acta 49, 353-362, 1990).

2.10 Determination of D- and L-lactic acid produced by lactobacilli

- Enzymatic analysis of D-and L- lactic acid (LA) was performed to assess the concentration of the lactic acid present in SCS of *lactobacilli* with a lactate dehydrogenase-based UV detection kit.

2.11 Separation of *lactobacilli* –specific bioactive peptides

- Capillary zone electrophoresis was applied for separation of underivatized peptides with UV detection at 200 nm wavelength using 0.1 M phosphate buffer.
- Gel electrophoresis was performed using 15% acrylamide gel. 10 mg freeze-dried sample was dissolved in 300 µl buffer (3% sodium dodecyl sulfate, 62 mM tris (hydroxymethyl) aminomethane, 8.7% glycerol, 10% β-mercaptoethanol pH=6.8). Components of running buffer were 25 mM Tris-HCl, 200 mM glycine and 0.1 w/v% SDS. Gel staining was performed with the application of Coomassie R-250 solution combined with silver staining.

2.12 Swine experiments

- For *in vivo* research a total of 40 (mixed male and female) commercial-cross pigs (Hungarian Large White x Hungarian Landrace /HLW x HL/) were used. The treatment, housing, husbandry and slaughter conditions were set to meet the

requirements of European Union Guidelines. The only difference was that diet supplemented with sodium-n-butyrate (0.15%, 13.6 mM) was administered to treated animals, while butyrate-free feed was added to controls. The animals were slaughtered at the age of 115 days under permission in the Research Institute for Animal Breeding and Nutrition (Herceghalom).

- The count of *lactobacilli* in swine ileum was determined on MRS broth based on visual colony inspection (20-150 colonies per Petri plate). The incubation lasted at 37°C for 72 hours.

2.13 Statistical analysis

- Relative gene expression levels of the genes of interest were calculated by the Relative Expression Software Tool (REST) 2009 Software.
- For statistical evaluation R 2.11.1 software package (2010) was applied. Differences between means were evaluated by one-way ANOVA with post-hoc Tukey test, where data were of normal distribution and homogeneity of variances was confirmed or Kruskal-Wallis nonparametric test.
- ANCOVA was used for interpretation of change in TER versus culturing time in case of different membrane inserts. A p value of < 0.05 was accepted to indicate statistical significance.

3. RESULTS AND DISCUSSION

3.1 Acute oxidative stress-induced changes in IPEC-J2 and Caco-2 cells

IPEC-J2 and Caco-2 cell lines were used as an *in vitro* model for studying on interaction of oxidative stress on normal intestinal epithelium. To mimic *in vivo* conditions they were cultured on Transwell membrane inserts which allowed them to reconstruct the polarized functional epithelia and form tight junctions (TJs) *in vitro*.

Remarkable differences in reactivity towards oxidative stress could be found comparing these two cell lines: Differentiated IPEC-J2 cell monolayer integrity can be partially disrupted by 1 h 2 mM hydrogen peroxide administration in contrast to filter-grown Caco-2 P cells, where 4 mM H₂O₂ treatment did not exert impact on cells, and more than 10 mM H₂O₂ for 1 h could only decrease TER significantly. Viability of the cells is decreased in concentration-dependent manner due to the cell-damaging properties of the ROS. Studying early effects of H₂O₂ on filter-grown Caco-2 P cells, it was found that 1 to 4 mM H₂O₂ had no impact on cell viability, while 20% of nuclei were stained with DAPI after 10 mM H₂O₂ exposure. However, IPEC-J2 cells seem to be more sensitive since 10% and 15% of the differentiated IPEC-J2 cells lost their viability after 2 mM and 4 mM H₂O₂ treatment, respectively.

For optimization of IPEC-J2 cell culturing conditions, PC and cc-PE membrane inserts were tested and compared in 3D functional cell model. No significant difference was found between the rates of IPEC-J2 cell differentiation on PC and cc-PE membrane inserts till the 8th day of cell culture. Between 9th and 19th days of cell culture increase in TER of IPEC-J2 cells grown on cc-PE membrane inserts was significantly higher (slope=802.7±24.2 Ohm*cm²/day, R=0.9608) than that on PC membrane inserts (slope= 663.0±37.5 Ohm*cm²/day, R=0.8789). On the 21st day of cell culture the final TER values were significantly higher on cc-PE (8702.8±45.9 Ohm*cm²) than those on PC (6134.8±154.3 Ohm*cm²) membrane inserts.

It was supposed that one of the most characteristic consequences of oxidative stress-triggered damage would be the elevated rate of lipid peroxidation depending on the applied peroxide concentration and the gradual decrease in capacity of cellular antioxidant defence mechanisms. In contrast, no significant change occurred in the level of any investigated parameters, neither in concentration of early stage lipid peroxidation indicators, such as CDs,

CTs nor in amount of the late phase marker, MDA by the effect of oxidative intervention, independently of applied peroxide concentration up to 4 mM.

IPEC-J2 cells showed membranous claudin-1, claudin-4 and E-cadherin positivity confirming the development of differentiated phenotype. After a 1 hour treatment with H₂O₂, up to the concentration of 4 mM, the cellular distribution and staining expression of these proteins remained unaltered. The signalling pathways behind acute oxidative stress-induced cellular changes were also investigated with partial mapping of PKC isoenzymes activity. In differentiated IPEC-J2 cells, PKC α , δ , ϵ , ζ , és η isoenzymes could be detected. When IPEC-J2 cells were treated with 1 mM peroxide for 1 h, level of PKC ζ was increased significantly 6-24 h after peroxide administration during recovery period.

In this work dose-and time-course response of cytokine expression was determined in filter-grown IPEC-J2 cells after H₂O₂ treatment. H₂O₂ administration over 0.5-4 mM concentration range did not alter relative gene expression level of IL-6 in IPEC-J2 cells. However, the gene expression of IL-8 and TNF- α was significantly elevated after 0.5, 1 and 2 mM H₂O₂ addition compared to controls, the peak level was reached using 1 mM H₂O₂ for 1 h. IL-8 concentration was significantly elevated at 4 h after peroxide administration and it increased continuously until the end of recovery time. After 24 h of recovery 101 pg/ml IL-8 was detected in the apical medium of IPEC-J2 cells.

3.2 Preventive effect of probiotics and metabolites against oxidative stress

One of the prerequisites necessary for elucidation of the precise role of probiotic strains and their metabolites in acute inflammatory mechanisms was that even partial agonistic and antagonistic effect of probiotics could be traced on basal and elevated cytokine regulation. IPEC-J2 cell system enables the screening for potential protective immunomodulatory substances operating on preventing intestinal epithelium from harmful oxidative stimuli or on restoration of physiological microbial communities disturbed by exogenous or endogenous agent-provoked inflammatory processes.

It was found that SCS of *L. plantarum* 2142 could downregulate the effect of acute oxidative stress evoked by 1 h 1 mM H₂O₂ treatment on proinflammatory cytokine, IL-8 and TNF- α relative gene expression level in IPEC-J2 cells. 1.8 fold decrease in IL-8 and 2.6 fold reduction in TNF- α level were detected when *L. plantarum* 2142 was used in 13.3 v/v% concentration. In accordance, the level of protective Hsp70 increased significantly in the presence of *L. plantarum* 2142 supernatant by 2.7 fold compared to samples only treated with

1 mM hydrogen-peroxide. Antiinflammatory effect of SCS was not based on the ability of peroxide decomposition.

This research work confirmed that neither acetic acid nor racemic, L- and D- LA exerted beneficial effect against excessive oxidative stimuli. Among metabolites secreted by *L. plantarum* 2142, molecular masses (21 and 31 kDa) of two *L. plantarum* 2142-specific proteins were analysed with SDS-PAGE method. CZE method tailored to detect low molecular weight peptides also managed to establish the presence of another specific peptide present in SCS of *L. plantarum* 2142.

Incubation of IPEC-J2 cells with sodium butyrate at 2 mM could increase TERs in the presence of peroxide which elevation was significant even after 24 h recovery period. Butyrate feeding increased the amount of *lactobacilli* and consequently the production of L-LA significantly with concomitant reduction in pH. Significantly higher amount of Ki 67 positive crypts in ileum was found in butyrate-fed pigs. The number of microvilli on ileal mucosa was significantly higher when pigs were fed with diet containing 0.15% sodium butyrate. In butyrate-treated animals the number of microvilli was 27 ± 3 per plica versus 19 ± 2 per plica in controls ($p < 0.05$).

4. NEW SCIENTIFIC RESULTS

1. It was shown that IPEC-J2 cell line cultured on collagen-coated polyester membrane inserts provides a reliable model for investigation of porcine-specific oxidative stress-induced inflammatory processes involving regulatory changes in the cytokine network.
2. Spent culture supernatant of *L. plantarum* 2142 significantly reduced the peroxide-induced level of IL-8 and TNF- α mRNS, while elevated the expression level of Hsp70 mRNS with respect to control data. The protective properties of *L. plantarum* 2142 SCS can not be attributed to metabolite-triggered chemical decomposition of H₂O₂.
3. *Bacillus amyloliquefaciens* CECT 5940 manifested in upregulated IL-8 and TNF- α relative gene expression level in IPEC-J2 cells indicating a change in the pattern of the inflammatory cytokine network.
4. D-lactic acid, L-lactic acid, their racemic mixture in the concentration present in *L. plantarum* 2142 SCS and acetic acid did not reduce significantly peroxide-induced increase in IL-8 and TNF- α relative gene expression pointing out that the major downregulators of oxidative stress are not short chain organic acids in the probiotic supernatant.
5. Butyric acid at millimolar concentration contributed to enhanced enterocyte proliferation and stimulated formation of microvilli on plicae *in vivo*, and it was capable of suppression *E. coli* 30037 growth and at the same time it increased *lactobacilli* colony forming *in vitro*.

5. LIST OF PUBLICATION

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