Theses of doctoral (PhD) dissertation

DEVELOPMENT OF *IN VITRO* MODELS TO INVESTIGATE CHRONIC INFLAMMATORY ENTEROPATHIES IN DOGS

dr. Alma Virág Móritz

Supervisors: Dr. Orsolya Farkas

Dr. Roland Psáder

UNIVERSITY OF VETERINARY MEDICINE BUDAPEST

Doctoral School of Veterinary Sciences

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Supervisors and Consultants

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Dr. Orsolya Farkas

Senior Research Fellow

Department of Pharmacology and Toxicology, University of Veterinary Medicine Budapest

Supervisor

…….

Dr. Roland Psáder

Associate Professor

Department of Internal Medicine, University of Veterinary Medicine Budapest

Supervisor

Dr. Mátis Gábor

Associate Professor

Department of Physiology and Biochemistry, University of Veterinary Medicine Budapest

Consultant

Dr. Jerzsele Ákos

Associate Professor

Department of Pharmacology and Toxicology, University of Veterinary Medicine Budapest

Consultant

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dr. Alma Virág Móritz

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1. Introduction and aims

Canine chronic inflammatory enteropathy (CIE) is a common and complex disorder characterized by persistent gastrointestinal signs, including vomiting, diarrhea, anorexia, and weight loss, which substantially impair the quality of life of affected animals. The disease typically follows a relapsing–remitting course, and its pathogenesis is multifactorial, involving dysregulation of the intestinal immune system, disruption of intestinal epithelial barrier function, and alterations in gut microbiota composition. Impairment of the intestinal barrier allows luminal microorganisms and their components—particularly lipopolysaccharides (LPS) derived from Gram-negative bacteria—to translocate into the circulation, thereby contributing to the development of a low-grade but persistent systemic inflammatory condition referred to as metabolic endotoxemia. LPS-induced immune activation is associated with increased production of reactive oxygen and nitrogen species, leading to oxidative stress. This process further damages the intestinal epithelium, compromises barrier integrity, and promotes the translocation of additional bacterial components, thereby establishing a self-perpetuating pathological cycle. These mechanisms may explain why sustained clinical improvement is difficult to achieve in certain cases of CIE using conventional therapeutic approaches alone. Current treatment strategies primarily rely on dietary intervention, modulation of the intestinal microbiota, and administration of immunosuppressive agents. Although these therapies are often effective, their long-term use may be associated with significant adverse effects. Consequently, there is increasing interest in complementary or alternative therapeutic approaches capable of reducing inflammation and oxidative stress while supporting intestinal barrier function.

In recent years, naturally occurring bioactive compounds, particularly flavonoids, have gained increasing attention in biomedical research. These plant-derived secondary metabolites are widely distributed in food sources and possess a broad range of beneficial biological activities, including antioxidant, anti-inflammatory, and immunomodulatory properties. Flavonoids such as quercetin, luteolin, and grape seed oligomeric proanthocyanidins (GSOP) are capable of scavenging reactive oxygen species, modulating inflammatory signaling pathways, and reducing the production of pro-inflammatory mediators. In addition, accumulating evidence suggests that these compounds may positively

influence gut microbiota composition and intestinal epithelial integrity. Based on these findings, it can be hypothesized that flavonoids may attenuate LPS-induced inflammatory and oxidative processes, thereby reducing systemic endotoxin burden and contributing to the alleviation of pathological mechanisms associated with CIE. Investigation of these effects, however, requires reliable and reproducible experimental models suitable for studying both local and systemic inflammatory responses.

Therefore, the overall objective of my doctoral research was to establish and apply *in vitro* model systems related to canine chronic intestinal inflammation in order to investigate the mechanisms of oxidative stress and inflammation, as well as the effects of naturally derived flavonoids. Particular emphasis was placed on evaluating the antioxidant, anti-inflammatory, and potential antibacterial properties of quercetin, luteolin, and GSOP. One major focus of the research was the modeling of systemic endotoxin exposure. For this purpose, canine peripheral blood mononuclear cells (PBMCs) and polymorphonuclear cells (PMNs) were utilized. Inflammatory and oxidative responses were induced using LPS derived from different sources, including *Escherichia coli* O111:B4, *Salmonella* Enteritidis, and canine-origin *E. coli* strains isolated from septicemic cases. During model optimization, the applicability of PBMC- and PMN-based systems was compared, revealing that PBMC cultures were more suitable for representing the investigated processes. In the standardized model, the effects of flavonoids were evaluated at concentrations of 25 and 50 μg/mL, either alone or in combination with LPS or bacterial stimulation. Cellular metabolic activity was assessed using the CCK-8 assay, while cytotoxicity was determined by measuring extracellular lactate dehydrogenase (LDH) activity. Oxidative stress was characterized by intracellular reactive oxygen species (ROS) levels, as well as extracellular hydrogen peroxide (H_2O_2) and reactive nitrogen species (RNS) concentrations. Inflammatory responses were primarily evaluated by monitoring changes in TNF-α production.

The investigations also included analysis of the antibacterial activity of flavonoids. Using canine-origin *E. coli* strains, the minimum inhibitory concentration (MIC) and mutant prevention concentration (MPC) were determined by broth microdilution methods. Furthermore, the combined effects of flavonoids and enrofloxacin were assessed by calculating the fractional inhibitory concentration (FIC) index.

Another major component of the doctoral work involved the establishment of a duodenal explant-based *in vitro* model, enabling the investigation of local inflammatory and oxidative processes. Duodenal explants obtained from healthy dogs using a punch biopsy technique were incubated for 12 hours and treated with LPS (*E. coli* O111:B4 and *S. Enteritidis*), flavonoids (25 and 50 μg/mL), or their combinations. Within this system, cell viability (CCK-8), cytotoxicity (LDH), markers of oxidative and nitrosative stress (intracellular ROS, extracellular H_2O_2, and RNS), as well as the production of inflammatory cytokines (TNF-α and IL-6) were investigated.

Overall, the aim of my research was to elucidate the extent to which the investigated flavonoids are capable of modulating LPS-induced inflammatory and oxidative processes in both systemic and local experimental models. My findings may contribute to a better understanding of the mechanisms through which these natural bioactive compounds can alleviate pathological processes associated with CIE and may provide a basis for the development of complementary therapeutic strategies capable of reducing the long-term reliance on antibiotics and immunosuppressive agents.

2. Overview of scientific results

The present study demonstrated for the first time in canine peripheral blood immune cells that lipopolysaccharides (LPS) derived from *Escherichia coli* and *Salmonella Enteritidis*, as well as canine-origin *Escherichia coli* isolates, induce pronounced oxidative and inflammatory responses characterized by increased intracellular ROS and TNF-α production. Furthermore, significant attenuation of these responses by quercetin, luteolin, and GSOP was observed without cytotoxic effects, while only limited direct antibacterial activity and no antagonistic interaction with enrofloxacin were detected. In parallel, a canine duodenal explant model was established, in which the investigated flavonoids effectively reduced LPS-induced oxidative stress and inflammatory cytokine production (TNF-α and IL-6), thereby supporting both their immunomodulatory potential and the applicability of the model for investigating intestinal inflammatory processes.

The main findings of the research are summarized as follows:

1. Treatment with 1 μg/mL *E. coli*- and *S. Enteritidis*-derived LPS was shown to induce a pronounced oxidative and inflammatory response in PBMCs, characterized by increased ROS and TNF-α production, while cellular metabolic activity remained largely unaffected.

2. Quercetin and luteolin at concentrations of 25 and 50 μg/mL were shown to significantly reduce *E. coli*- and *S. Enteritidis* LPS-induced intracellular ROS levels in both PBMCs and PMNs without exerting cytotoxic effects. In addition, treatment with quercetin at 50 μg/mL and luteolin at 25 and 50 μg/mL effectively attenuated LPS-induced TNF-α production in PBMCs.

3. Canine-origin *E. coli* isolates were demonstrated to induce oxidative stress and pro-inflammatory responses in PBMCs in a strain- and treatment-dependent manner. Furthermore, quercetin, luteolin, and GSOP at concentrations of 25 and 50 μg/mL significantly reduced bacteria-induced ROS and TNF-α production in *E. coli*-stimulated PBMCs.

4. Only limited direct antibacterial activity was observed for the investigated flavonoids, and no antagonistic interaction with enrofloxacin was detected. These findings suggest

that co-administration with enrofloxacin may be considered safe, while the observed effects are primarily associated with immunomodulatory mechanisms.

5. A canine duodenal explant model suitable for the *in vitro* investigation of oxidative stress and inflammation was successfully established. Within this model, quercetin, luteolin, and GSOP were shown to reduce *E. coli*- and *S. Enteritidis* LPS-induced oxidative stress and attenuate TNF-α release without causing cellular damage. Moreover, treatment with quercetin and luteolin at 25 μg/mL reduced IL-6 concentrations compared to the untreated control.

The obtained results demonstrate that LPS and canine-origin *E. coli* strains induce significant oxidative and inflammatory responses in canine immune cells, whereas quercetin, luteolin, and GSOP effectively attenuate these processes without inducing cellular damage, primarily through immunomodulatory mechanisms. In addition, the established duodenal explant model proved suitable for the *in vitro* investigation of these pathological processes. Collectively, the findings indicate that the investigated flavonoids may represent promising complementary therapeutic agents for canine chronic inflammatory enteropathies.

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

Full text papers in peer-reviewed journals

Móritz A.V.; Jerzsele Á.; Psáder R.; Farkas O. **Kutya bélrendszeri megbetegedéseinek *in vitro* modellezési lehetőségei - Irodalmi összefoglaló.** *Magyar Állatorvosok Lapja* 2023, 145 (8) 477-486. ISSN 0025-004X.

Móritz, A.V.; Kovács, H.; Jerzsele, Á.; Psáder, R.; Farkas, O. **Flavonoids in Mitigating the Adverse Effects of Canine Endotoxemia.** *Frontiers in Veterinary Science* 2024, 11, doi:10.3389/fvets.2024.1396870. (D1)

Móritz, A.V.; Fónagy, V.; Psáder, R.; Jerzsele, Á.; Farkas, O. **Anti-Inflammatory and Antioxidant Effects of Quercetin, Luteolin, and Proanthocyanidins in Canine PBMCs Stimulated with *Escherichia Coli.*** *Animals* 2025, 15, doi:10.3390/ani15243622. (VetQ1)

Móritz, A.V.; Horváth N.L., Márton, RA.; Szilasi A.; Jerzsele, Á.; Psáder, R.; Farkas, O. **Anti-Inflammatory Effects of Flavonoids in an LPS-Induced *In Vitro* Model of Canine Chronic Enteropathy.** *Animals* 2026, 16, doi: 10.3390/ani16030450. (VetQ1)

Conference presentations

Móritz AV, Kovács H, Psáder R, Farkas O, **Flavonoidok szerepe kutya endotoxémia káros hatásainak mérséklésében.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2024.

Móritz AV, Jerzsele Á, Psáder R, Farkas O, **Flavonoidok immunmoduláns hatása kutya PBMC modellben.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2026.