Department of Pharmacology and Toxicology
University of Veterinary Medicine Budapest

Antioxidant and anti-inflammatory effects of proanthocyanidins in porcine intestinal epithelial cell – bacterium co-culture

By
Bella Gál

Supervisor:
Dóra Kovács DVM

Budapest, Hungary
2021

TABLE OF CONTENTS

ABBREVIATIONS

AMEG - Antimicrobial Advice ad hoc Expert Group

AMR – Antimicrobial resistance

CFU – Colony forming unit

CIPARS – Canadian Integrated Program for Antimicrobial Resistance Surveillance

DCF – Dichloro-dihydro-fluorescein

DCFH-DA - 2',7' dichloro-dihydro-fluorescein diacetate

DMEM/F12 – Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient (1:1)

ECDC – European Centre for Disease Prevention and Control

EFSA – European Food Safety Authority

ELISA – Enzyme-linked immunosorbent assay

EMA – European Medicines Agency

ESVAC – European Surveillance of Veterinary Antimicrobial Consumption

EU – European Union

GI – Gastrointestinal

HP-CIA – Highest priority critically important antibiotics

IC ROS – Intracellular reactive oxygen species

IL-6 – Interleukin-6

IL-8 – Interleukin-8

LPS – Lipopolysaccharides

MDR – Multi-drug resistance

MRSA – Methicillin-resistant *Staphylococcus aureus*

PACs – Proanthocyanidins

PBPs – Penicillin-binding proteins

PBS – Phosphate buffered saline

PCU – Population correction unit

PDR – Pandrug-resistance

TSA – Tryptone soy agar

UK-VARSS – United Kingdom Veterinary Antimicrobial Resistance and Sales Surveillance

WHO – World Health Organization

XDR – Extensive drug-resistance

1. INTRODUCTION

Antibiotics have been an essential part of both human and animal medicine for the past 100 years since the discovery of penicillin by Alexander Fleming in 1928. In recent years, however, as a result of excessive and improper use in both fields, antibiotic resistance has come to light whereby, the efficacy of antibiotics has decreased against many bacteria due to the increasing prevalence of antibiotic resistant microorganisms. Infections caused by resistant bacteria are difficult to treat, can relapse and might even lead to treatment failure in both human and veterinary medicine. If doctors and veterinarians continue this cycle of over-prescribing antibiotics, they may no longer be an option for treatment in the future. As a result of the above, it is essential to promote prudent use of these agents and to reduce the overall consumption, which can be achieved with the usage of alternative therapies for preventing and treating bacterial infections.

The phenomenon of antibiotic resistance can only be overcome with the 'One Health' concept, meaning the joint effort of human and veterinary healthcare professionals. It has already been demonstrated that resistant bacteria and resistance genes developed in food producing animals can spread to consumers via the food chain. This is of the highest importance in case of zoonotic and frequently multi-resistant bacteria such as *Escherichia coli*, *Salmonella* spp. and *Campylobacter* spp. Therefore, decreasing the usage of antibiotics in food producing animals has significant public health implications as well.

Flavonoids, a group of polyphenolic plant and fungus secondary metabolites are among the alternative therapeutic options that might be used for the prevention and treatment of bacterial infections caused by pathogens such as *E. coli* and *Salmonella* spp. Flavonoids can have several beneficial effects, including antioxidant, anti-inflammatory, and antibacterial activities, which make them eligible as potential antibiotic alternatives. Proanthocyanidins (PACs) are among these promising candidates that might serve as feed additives in food producing species leading to a decreased usage of antibiotics in the food chain.

Aims of this study were to test the protective effects of grape seed PACs in a porcine intestinal epithelial cell – bacterium (*E. coli*, *S.* Typhimurium) co-culture, in order to model their potential usage against intestinal bacterial infections of swine.

2. LITERATURE REVIEW

2.1. Antimicrobial resistance

2.1.1. Definition

It is the change in the response of microorganisms including bacteria, fungi, viruses and parasites, to the use of medicines such as antibiotics, antifungals, antivirals and antiparasiticides, which describes the term antimicrobial resistance (AMR). This phenomenon represents one of the most important health threats nowadays in both human and veterinary medicine [1], especially in case of bacteria resistant to antibiotics. Infections caused by antibiotic resistant bacteria can lead to significant morbidity and mortality and are predicted to be responsible for 10 million human deaths per year by 2050. In both the human and veterinary fields of medicine, AMR is associated with the widespread use and misuse of antibiotics and should be addressed with the 'One Health' approach since the health of humans, animals and the ecosystem is strongly connected [2].

Antibiotic resistance can be categorised into three main types: intrinsic, acquired and adaptive. Intrinsic, also known as *ab ovo* or primary resistance is the consequence of inherent properties in bacteria [2], meaning that the bacteria have never been and will never be susceptible to the concerned drug. Intrinsic resistance is common for example, against penicillins and refers to the absence of cell wall in some bacteria, while anaerobic bacteria show *ab ovo* resistance to aminoglycosides since these drugs require oxygen for their activity [3]. Acquired resistance is the evolutionary adaptation of microorganisms resulting in a previously susceptible bacterium becoming resistant to an antibiotic due to acquiring resistance mechanisms either by mutation or horizontal gene transfer [2, 4]. Horizontal gene transfer, which can occur via transformations, transduction, and conjugation [2], is an important route in the spread of resistance. With these mechanisms, resistance can not only be transferred between pathogenic bacteria, but also to commensal strains [5]. Adaptive resistance is the transient result of gene expression modulations induced by environmental factors (e.g. pH, ion concentrations, nutrient conditions), which – in contrast to the previous types – disappears whenever the inducing signal is removed [2].

Resistance appears in several forms such as multi-drug resistance (MDR), extensive drug-resistance (XDR) and pandrug-resistance (PDR). MDR is defined as "acquired non-

susceptibility to at least one agent in three or more antimicrobial categories". XDR is the non-susceptibility to at least one agent in all but two or fewer antimicrobial categories. PDR is the non-susceptibility to all agents in all antimicrobial categories [6]. A group of pathogens with a high rate of antimicrobial resistance has been classified as the ESKAPE organisms, which include *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species [7]. These bacteria have also been listed by the World Health Organization (WHO) as critical or high priority pathogens based on the need for developing new and clinically effective antibiotics against them [8].

Antibiotic resistance can be the result of different mechanisms, such as destruction or modification of the antibiotic (e.g. through beta-lactamase production); protection, overproduction or modulation of the antibiotic targets (e.g. gene mutations in penicillin-binding proteins [PBPs] in methicillin-resistant *S. aureus* [MRSA]) and reduced accumulation of the antibiotic (e.g. through the expression of efflux pumps) [2, 3].

2.1.2. Prevalence and spread of resistance

Resistance to antibiotics in the world has now become so extreme that over 70% of bacteria are resistant to at least one of the antibiotics used worldwide [9]. According to the WHO, in 2016, almost 500,000 people developed multi-drug resistant tuberculosis globally, and drug resistance is also having further impacts on the treatment struggle of other diseases such as infections caused by *E. coli* [10].

Worldwide, the dairy and meat industry has contributed greatly to the spread of multi-resistant bacterial strains due to the increasing demand for food of animal origin. In order to increase productivity, antibiotics have been widely used for growth promotion in food producing species via mixing them in the feed of animals in subtherapeutic dosage. This practice could help in preventing infections and promoting weight gain of animals, however, it has also led to an increased number of resistant bacteria in the food chain [11, 12]. Furthermore, as most farm animals are kept in large groups or herds, individual treatment of diseases is rare, most commonly they receive mass or herd treatment. Individual treatment parentally is generally considered less on large-scale farms as it has higher costs and labour demand [13]. The ease of antibiotic administration mixed in feed and drinking water further contributes to the increased risk of resistance [14], due to

frequently unprecise dosing in case of these administration routes. Metaphylaxis is also used on large-scale farms where, all animals, both sick and healthy are being given the same drug, e.g. antibiotic, which can increase the risk of resistance development compared to individual antibiotic application to the sick animals only [15].

Even though the use of antibiotics for growth promotion has already been banned in several countries (including the European Union (EU), in 2006) [11], and further restrictions are being placed on their in-feed use for any purpose [16] and on their prophylactic and metaphylactic usage [17], they are still being used worldwide in intensive farming for growth promotion, and for the prevention and treatment of diseases [11, 18]. As an example, pigs are highly susceptible to respiratory and intestinal disorders, and as a result, are most frequently treated with the appropriate antibiotics such as: beta-lactams, tetracyclines, polymyxins and macrolides [14]. In line with their frequent usage, research has shown that in swine, resistance to ampicillin, tetracycline and azithromycin in *E. coli* has been common [5]. Resistance against macrolides in bovine pathogens is also frequent [15].

Resistance development in food producing animals is not only a threat to veterinary medicine, but it also has public health implications. Resistant bacteria evolved in animals can be transmitted to humans through foods of animal origin, contributing to the spread of multi-drug resistant bacteria [19]. The European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) regularly publish a summary report about antibiotic resistance in zoonotic bacteria from humans, animals, and food, including *Salmonella* spp., *Campylobacter* spp., *E. coli* and MRSA, based on data collected from EU member states. The report shows that resistance is still very common in these pathogens, and that their resistance patterns are usually similar regardless of their origin, which also supports the role of food chain in the spread of resistance. For example, in the period of 2018-2019, high levels of ampicillin, tetracycline and sulphonamide resistance could be observed in *Salmonella* isolates obtained from human, animal and food samples as well. In case of *Campylobacter* strains, ciprofloxacin and tetracycline resistance was high, regardless of their origin (animal, food or human) [20].

Resistance has a great impact on treatment methods and makes it increasingly difficult to treat economically significant, common, and in many cases, zoonotic diseases. The importance of finding alternatives to antibiotics are not only financial but also environmental, as the spread of resistance could have a detrimental effect on the natural world including humans, animals, and the ecosystem.

2.1.3. Solutions

To conquer the complex problem of resistance, steps must be taken to decrease the use and misuse of antibiotics worldwide not only in veterinary, but also in human medicine. To support prudent use of these agents in veterinary medicine, antibiotics have been grouped into 4 categories (Category A, B, C and D) by the Antimicrobial Advice ad hoc Expert Group (AMEG) of the European Medicines Agency (EMA), according to their importance in human and veterinary medicine, and the risk of resistance development and transfer. Antibiotics belonging to Category A such as vancomycin, are to be avoided in veterinary medicine, as they only authorised for the treatment of humans and can only be used in small amounts in life-saving situations for non-food-producing animals according to the prescribing cascade. The usage of those in Category B – fluroquinolones, colistin, third and fourth generation cephalosporins – should be restricted – these can only be used for animal treatment if the less valuable antibiotics could not be used effectively or would not be effective based on susceptibility testing results. These agents are also known as highest priority critically important antibiotics (HP-CIA) according to the WHO, since they are essential in human medicine for the treatment of some severe infections. Category C antibiotics such as amoxicillin-clavulanic acid, lincosamides and pleuromutilins should be used with caution, however, can be more widespread in their use as they are less valuable for human treatment. Finally, Category D antibiotics, e.g. narrow-spectrum penicillins, tetracyclines and sulphonamides are recommended to be the first-line choices for veterinarians, but should also be used prudently [8].

Furthermore, monitoring programs about antibiotic consumption and prevalence of resistance have been implemented in several countries. There are monitoring reports, including the United Kingdom Veterinary Antimicrobial Resistance and Sales Surveillance (UK-VARSS) report, the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) report by the EMA and the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS), which measure antibiotics

sold by species weight and thus revealing the overall consumption in different species by using a unit known as the population correction unit (PCU) [15]. Furthermore, collecting data on the sales and use of antibiotics is an important measure of Regulation (EU) 2019/6 on veterinary medicinal products entering into force in January 2022. This data is going to be used for developing methods to limit the risk of AMR and to monitor the effect of previously introduced measures [17].

Maintaining proper housing conditions and hygiene in large-scale farms also play a crucial role in decreasing antimicrobial resistance, as with efficient cleaning and disinfection measures, the number of infections can significantly be reduced, leading to less need for antibiotic treatment. Studies carried out in poultry and pig farms showed that in practice, with proper use of disinfectants, there was no selection for resistance against these agents in *E. coli*. On the other hand, when cleaning and disinfection had not been carried out properly, some strains of *E. coli* were still present as a result of resistance [19].

Besides the above-mentioned measures, it is also of paramount importance to find antibiotic alternatives that can be used alone or in synergistic combination with antibiotics for the prevention and treatment of bacterial infections in order to increase the chances of successfully battling bacterial diseases. The research and use of these agents have become significant in recent years. Development of new, conventional antibiotics is a great challenge for the industry, which also supports the usage of alternative substances [21].

2.1.3.1. Antibiotic alternatives

The group of potential antibiotic alternatives include – without claim for completeness – pro-, pre- and synbiotics, organic acids, phytochemicals, enzymes, antimicrobial peptides, anti-bacterial virulence agents (e.g. quorum sensing disruptors) and bacteriophages [11, 12]. These substances might be used for the prevention and treatment of bacterial infections, alone or in synergistic combination with antibiotics, and they can also exert growth promoter activities [22].

One of the most commonly used feed additives from the above-mentioned substances are the pro-, pre- and synbiotics. Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” [23]. These non-

pathogenic microorganisms are added to food supplements or directly into foods such as yoghurt, and act in the gastrointestinal (GI) tract of the individual through binding and colonizing the intestinal cells. Their activity includes balancing and restoring the environment in the GI tract. Most commonly, bacteria included in probiotics are of intestinal origin such as *Bifidobacterium*, *Lactobacillus* and *Enterococcus* spp. [24]. Prebiotics are defined as "a group of nutrients that are degraded by gut microbiota in the colon" [25]. When used together, prebiotics and probiotics are known as synbiotics. Used in combination, they exert a synergistic effect that can increase their beneficial effects in the body [26].

Organic acids, such as butyrate, fumaric acid and lactic acid are also known for their beneficial effects on the GI tract [11, 27]. Furthermore, growth promoter effects of several organic acids (e.g. fumaric, citric, malic and benzoic acid) have been reported as well [11]. Other potential antibiotic alternatives, such as enzymes [11], antimicrobial peptides [16] and bacteriophages [28] have shown direct antibacterial effect which makes them eligible for use against bacterial infections. Antibacterial virulence agents also exert their effect directly on bacteria, however, instead of inhibiting bacterial growth or killing them, these agents can disarm pathogens through targeting their virulence mechanisms, and therefore making them unable to infect host cells [11].

Natural bioactive compounds derived from plants, also known as phytochemicals, phytobiotics or phytogenics, represent perhaps the largest group of antibiotic alternatives. They can have various beneficial effects including antioxidant and antibacterial activity, protection of barrier integrity in the GI tract, and stabilization of the GI microbiota. The main biologically active substances found in phytochemicals are polyphenols, including the group of flavonoids [12].

2.2. Flavonoids

Flavonoids are found abundantly in our surroundings, and their characteristics could potentially provide an alternative to the treatment of bacterial infections. They are polyphenols, extractable from tea, grapes, fruits, vegetables, and roots [29]. The group of flavonoids is mainly known for their antioxidant properties, however, they can also show antibacterial, antiviral, antifungal, antiparasitic, antitumor, anti-inflammatory and immunomodulatory activities. Furthermore, they have beneficial effects on the

cardiovascular system and the GI microbiota. Used together with antibiotics, they can act synergistically, leading to increased efficacy of the drug [30].

In human research, flavonoids have shown to have nutritional benefits, particularly in the geriatric population. In vascular diseases, bone health and cancer prevention, flavonoids have exhibited protective properties, their intake can reduce the risk of the previously mentioned diseases. Through their anti-inflammatory action, flavonoids enhanced bone formation and inhibition of bone reabsorption through osteoclasts and osteoblasts [31]. In small animals, such as dogs and cats, flavonoids can also be used to decrease blood pressure [32].

As a result of their strong natural antioxidant character, flavonoids might be used to reduce oxidative stress caused by bacterial infections. Antibiotics and other antimicrobial agents frequently have undesired mild or serious side-effects including for example: diarrhoea, vomiting, allergic reactions, dysbacteriosis, nephrotoxicity, ototoxicity, teratogenicity, and sometimes carcinogenic effects. In contrast to these, flavonoids, as natural compounds are preferred due to generally having much fewer known side-effects compared to synthetic antibiotics [33]. Studies have shown that by three mechanisms, flavonoids can show antibacterial activity, which are: plasma membrane damage and/or reduction of its fluidity, inhibitions of nucleic acid synthesis and inhibition of energy metabolism in bacteria [34].

Several flavonoids found in foods such as fruits, dark chocolate, and teas can occur as large polymerous molecules, called tannins. Tannins can be further divided into condensed tannins, derived tannins and hydrolysable tannins. Condensed tannins, also known as proanthocyanidins (PACs), are found in bark and have monomeric units of flavans linked through carbon-carbon and ether linkages. Tannins have health-promoting benefits including their bacterial anti-adhesion activity and effectiveness against cardiovascular diseases. Tannins are not essential in the diet and so, if the body lacks them, it does not lead to deficiency diseases [29].

2.2.1. Proanthocyanidins

PACs belong to the group of polyphenols and are classified as condensed tannins. Seeds, nuts, vegetables, plants, and berries such as blueberries, blackberries and cranberries

abundantly contain these condensed tannins as seen in Figure 1. As a result of their sweet taste and beneficial health properties, which include being antioxidant, anticancer, antimicrobial, and neuroprotective, they are often researched and used as dietary supplements [35].

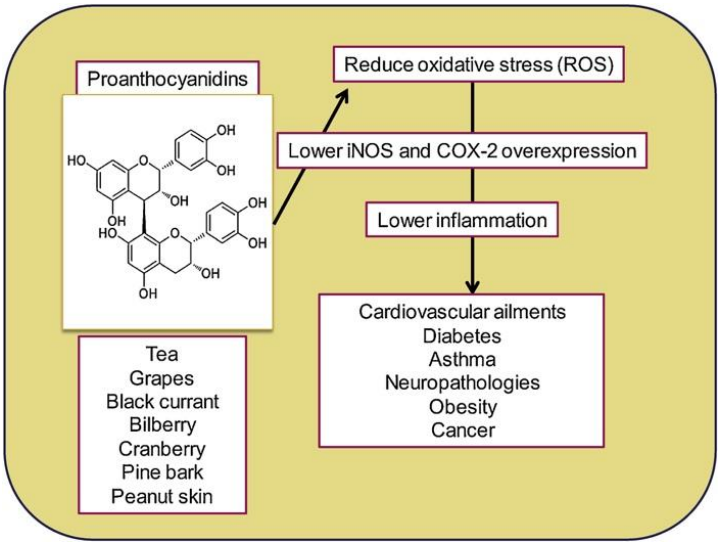

Figure 1: The basic structure, sources, and properties of proanthocyanidins [35]

Based on the arrangement and linkage between monomers, PACs can be classified into A-type (most commonly A1 and A2 compounds), and the more abundant B-type (B1, B2, B3 and B4 compounds) [35]. Figure 2 demonstrates the different structures of PACs.

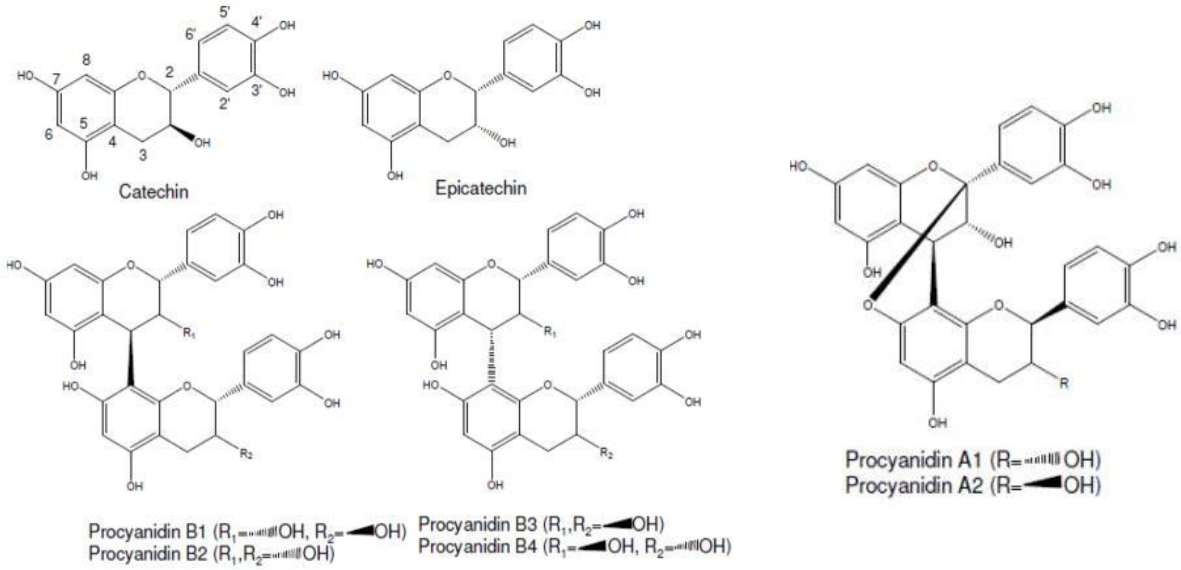

Figure 2: The structures of A and B-type proanthocyanidins [36]

These differences in their structures and linkages will also determine their polymerization ability. The first, A-type arrangement contains an extra ether linkage between the hydroxyl group and carbon-2 on the A-ring, and an interflavan bond. On the other hand, B-type PACs only have one interflavan bond between the B-ring carbon-4 with either C-ring carbon-8 or carbon-6 [36]. PACs with different structure can have diverse biological activity. Grape seed oligomeric proanthocyanidins, that were used in the current research, belong to the group of B-type proanthocyanidins [37].

2.2.1.1. Specific properties of proanthocyanidins

PACs can show various beneficial effects on the human and animal health. It has been demonstrated that lipid peroxidation, platelet aggregation, and capillary permeability can all be decreased by PACs [35]. Studies using oligomeric PACs extracted from grape seeds in human patients with chronic obstructive pulmonary disease have shown that, PACs could inhibit oxidative damage and apoptosis via exhibiting antioxidant activity. Furthermore, studies showed that PACs could have stronger antioxidant effects than those found in vitamins C and E [38]. The benefits of using PACs in these studies and as antioxidant treatment includes its easy extraction, low cost, and low toxicity levels [39].

There have been promising studies about PACs exhibiting successful antibacterial activity against pathogenic bacteria including *E. coli*. The high activity was mostly found in substances containing oligomeric compounds [40]. Furthermore, it is their inhibitory action against β-lactamase and their ability to destabilize the cell membrane of bacteria, which represents the most important antibacterial activity of PACs [41]. A further important property of PACs found in cranberry juice, is their ability to inhibit bacterial adhesion, most evidently through their A-type linkages, which was demonstrated in a human study in multi-drug resistant *E. coli.* Within the uroepithelial cells, PACs blocked the adhesion of fimbriae, which hindered the ability of bacteria to take over these cells [42].

Besides the above-mentioned abilities, anti-inflammatory, antiviral and antifungal properties of PACs have been described [43, 44, 45]. In studies exploring the effect of grape seed PACs in the airways of asthmatic patients, results showed that the PACs exhibited their anti-inflammatory effects by reducing the number of inflammatory

markers [35]. Their beneficial effects on the barrier integrity of the intestinal epithelium have also been reported in cell culture and animal models of intestinal dysfunction [46].

2.3. Porcine intestinal bacterial diseases

GI infections caused by *E. coli* and *S. enterica* are significant health problems worldwide in pigs [47]. Furthermore, both bacteria are zoonotic, and can be transmitted to humans with pork meat, which leads to a significant number of human infections with these microorganisms [48, 49, 50, 51]. This is aggravated by the fact that their strains originating from humans, animals and food of animal origin are frequently resistant to multiple antibiotics [20].

Piglet diarrhoea is a common small intestinal, infectious disease, most commonly caused by *E. coli*. For many years, antibiotics have been used to treat diseases such as this, however the abundant use and misuse of these agents has resulted in their reduced efficacy and an increase in resistance most commonly in case of neomycin, apramycin, potentiated sulphonamides and colistin [52]. *Salmonella enterica* is a zoonotic pathogen affecting intestinal epithelial cells via toll-like receptors [53]. It can also cause systemic infections with septicaemia, enterocolitis, pneumonia, and hepatitis [54]. It occurs frequently as asymptomatic infection in pigs, but even in these cases, it can be transmitted to humans through the food-chain, leading to foodborne salmonellosis [55].

3. AIM OF RESEARCH

The aims of this study were to establish an *in vitro* model of swine GI bacterial infections and to use this system for the evaluation of protective effects of grape seed PACs as potential antibiotic alternative substances.

Our model consisted of porcine intestinal epithelial cells (IPEC-J2 cell line) that were infected with bacteria of swine enteral origin *(E. coli* and *S. enterica* ser. Typhimurium). In order to investigate changes in oxidative stress and inflammatory markers of the cells, we have initially determined highest tolerable bacterial concentration that could be applied on them without causing significant viability decrease.

Afterwards, we investigated the antioxidant and anti-inflammatory effects of B-type PACs of grape seeds origin in this model. For this purpose, intracellular reactive oxygen species (IC ROS) level and interleukin-6 (IL-6) and interleukin-8 (IL-8) production of cells were determined after treatment with bacteria alone and bacteria in combination with PACs. PACs were tested at two different concentrations (50 and 100 µg/ml) against both bacteria.

4. MATERIALS AND METHODS

4.1. Chemicals and instruments

Chemicals (grape seed PACs, growth medium of cells, Neutral Red dye, 2',7' dichloro-dihydro-fluorescein diacetate (DCFH-DA) reagent, enzyme-linked immunosorbent assay (ELISA) kits) were obtained from Sigma-Aldrich (Darmstadt, Germany), tryptone soy agar (TSA) plates were supplied by Biolab Zrt. (Budapest, Hungary), and cell culture plates were purchased from Corning Inc. (Corning, NY, USA). For the measurements, EZ Read 400 Microplate Reader (Biochrom Ltd., Cambridge, UK) and Victor X2 2030 fluorometer (Perkin-Elmer Inc., Waltham, MA, USA) were used. The statistical analysis was performed with R software 3.3.2 (2016) (R Foundation for Statistical Computing, Vienna, Austria).

4.2. IPEC-J2 cell line

Dr. Jody Gookin (Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, USA) generously provided IPEC-J2 cells for the research. Cells were propagated on 37 °C, with 5% CO_2, in the 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient (DMEM/F12) until a passage number of approximately 50 for all investigations. Cells were provided an optimal environment by supplementing DMEM/F12 with fetal bovine serum (5%), insulin (5 µg/ml), transferrin (5 µg/ml), selenium (5 ng/ml), epidermal growth factor (5 ng/ml) and penicillin-streptomycin (1%) for cell culturing (full DMEM/F12). In the working solutions, plain DMEM/F12 (without supplementation) was used. The experiments were performed with the cells on 96- (Neutral Red) and 6-well (DCFH-DA, ELISA) polystyrene cell culture plates after they reached a differentiated, confluent monolayer (evaluated under light microscope).

4.3. Bacterial strains

One-one pathogenic *E. coli* and *S.* Typhimurium strains were used for the experiments, both of them originating from GI infections of pigs and obtained from the Department of Microbiology and Infectious Diseases, University of Veterinary Medicine Budapest. Bacteria were kept frozen at -80 °C, in Microbank tubes until the beginning of investigations, when they were propagated in plain DMEM/F12 for 18-24 hours at 37 °C, with 5% CO_2. Colony forming unit (CFU) counting was performed on TSA plates, which allowed us to determine the concentration of the overnight suspensions.

4.4. Cell viability determination (Neutral Red assay)

To begin with, it was important to determine the highest tolerable bacterial concentration level for both *E. coli* and *S.* Typhimurium which could be used to co-culture with the cells. Effect of bacterial suspensions at 10^4, 10^6 and 10^8 CFU/ml concentrations on IPEC-J2 cell viability was tested with Neutral Red assay based on the description by Repetto et al. [56]. Cells were cultured on 96-well microplates until a confluent monolayer was formed and then were washed with phosphate buffered saline (PBS) and plain medium in order to eliminate antibiotic residues from full DMEM/F12. This was followed by treatment with the different bacterial suspensions (both bacteria in all three concentrations) for one hour. Working suspensions of bacteria were prepared with plain DMEM/F12, which was also used as a control for the experiment. Neutral Red assay was performed 24 hours after the treatments, until which cells were incubated in full DMEM/F12. Biochrom EZ Read 400 Microplate Reader was used for the absorbance measurement on 540 nm. Based on results of the cell viability assay, bacterial suspensions at the concentration of 10^6 CFU/ml were used in further experiments in case of both strains. PACs alone did not cause any cell viability decrease when they were tested previously on IPEC-J2 cells [37].

4.5. IC ROS level determination (DCFH-DA assay)

For the determination of IC ROS level changes in cells, DCFH-DA assay was used. In this experiment, cells were cultured on 6-well microplates and similarly to the Neutral Red assay, they were washed with PBS and plain medium before the addition of working solution. For the treatments, bacteria were applied on some cells at the concentration of 10^6 CFU/ml. Another group of cells was treated with grape seed PACs alone (50 and 100 µg/ml), while there were cells receiving bacteria and PACs in combination (both bacteria with both PACs concentrations). All working solutions were prepared with plain medium and were incubated with the cells for one hour. Control cells were only treated with plain medium. Treatment groups are summarized in Table 1.

Group	PACs	Bacterium
Control	-	-
E. coli	-	*E. coli* 10^6 CFU/ml
S. Typhimurium	-	*S.* Typhimurium 10^6 CFU/ml
P50	50 μg/ml	-
P100	100 μg/ml	-
P50 + Ec	50 μg/ml	*E. coli* 10^6 CFU/ml
P100 + Ec	100 μg/ml	*E. coli* 10^6 CFU/ml
P50 + ST	50 μg/ml	*S.* Typhimurium 10^6 CFU/ml
P100 + ST	100 μg/ml	*S.* Typhimurium 10^6 CFU/ml

Table 1: Treatment groups and their abbreviation for IC ROS and IL-6, IL-8 determination.

For the detection of IC ROS, 10 μM DCFH-DA reagent was used on the cells 24 hours after treatments, until which cells were incubated in full DMEM/F12. IC ROS are able to oxidize DCFH-DA into a detectable fluorescent product: dichloro-dihydro-fluorescein (DCF) [57]. Using this dye, we were able to determine if there were elevated fluorescein values, showing an increased amount of IC ROS. For a total of 60 minutes, the reagent was added to the cells, which was followed by rinsing with medium, scraping and centrifugation for 10 minutes (at 3000 g). Using a fluorometer (Victor X2 2030) the fluorescence of the samples was determined (excitation wavelength: 480 nm, emission wavelength: 530 nm).

4.6. Interleukin level determination (ELISA method)

IL-6 and IL-8 production levels were investigated in our experiment using porcine-specific ELISA kits for IL-6 and IL-8 detection. Cells were cultured on 6-well microplates with similar experimental design as in the DCFH-DA assay (Table 1). Samples were taken from the supernatant of cells 6 hours after treatments [58] and were stored on -80°C until analysis. ELISA method was performed with the samples according to instructions by the manufacturer.

4.7. Statistical analysis

R 3.3.2 software was used for statistical analysis of the data obtained from the experiments. Mean value differences between the experimental groups were evaluated with one-way ANOVA and Tukey post-hoc test. Significance was determined if p value was lower than 0.05.

5. RESULTS

5.1. Cell viability of IPEC-J2 cells treated with bacteria (Neutral Red assay)

Treatment of IPEC-J2 cells with bacterial suspensions at 10^8 CFU/ml (regardless of the bacterial species) caused a significant ($p < 0.01$ for *E. coli* and $p < 0.001$ for *S.* Typhimurium) cell viability decrease compared to the control (Figure 3). The effect of 10^8 CFU/ml *S.* Typhimurium was significantly ($p < 0.001$) more pronounced compared to *E. coli* at the same concentration. However, lower concentrations (10^4 and 10^6 CFU/ml) of both bacteria did not alter the amount of viable IPEC-J2 cells. Based on these observations, 10^6 CFU/ml concentration was used in the further experimental steps in case of *E. coli* and *S.* Typhimurium as well.

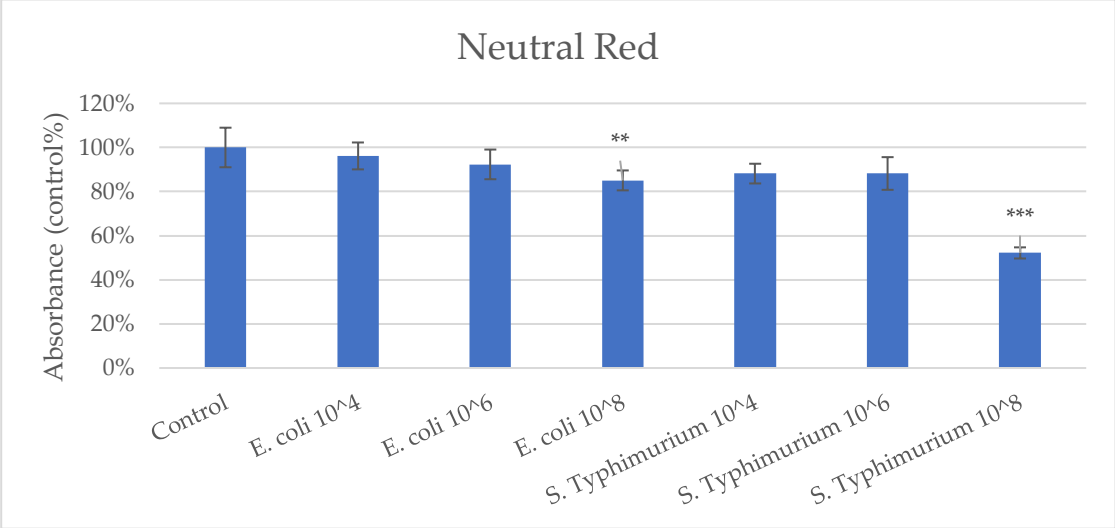

Figure 3: Viability of IPEC-J2 cells after treatment with different bacterial suspensions (average absorbance values with standard deviation, n=6)

**: $p < 0.01$; *** $p < 0.001$ (significant difference compared to control)

5.2. IC ROS level changes (DCFH-DA assay)

5.2.1. IC ROS levels in IPEC-J2 cells treated with *E. coli* and PACs

One hour treatment with *E. coli* at 10^6 CFU/ml did not cause significant increase in the IC ROS level of IPEC-J2 cells (Figure 4). As seen in Figure 4, significant ($p < 0.01$) decrease in IC ROS compared to the control was found in the IPEC-J2 cells treated with 50 µg/ml PAC (P50) on its own, where the fluorescence value was only 73.77% of the control. PACs alone, at 100 µg/ml concentration did not affect IC ROS production. When *E. coli* was used in combination with PACs at concentrations of 50 and 100 µg/ml (P50 + Ec and P100 + Ec, respectively), IC ROS levels decreased significantly ($p < 0.001$ and

p < 0.01, respectively), from 100% to 52.55% and 62.67%. These results suggest antioxidant activity of PACs that could alleviate the effects of *E. coli* treatment on IC ROS level of cells.

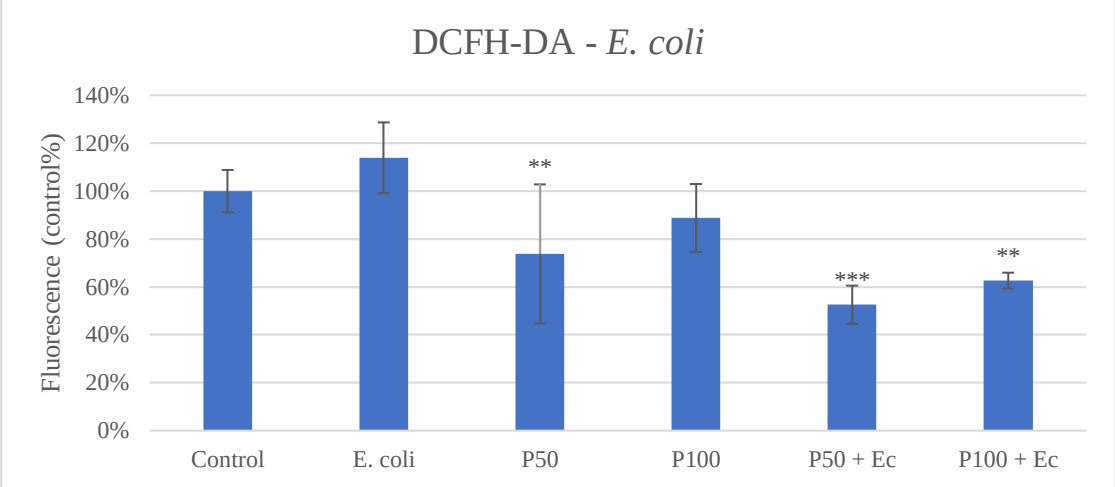

Figure 4: IC ROS amount in IPEC-J2 cells after treatment with *E. coli* and PACs
(average fluorescence values with standard deviation, n=6)
**: p < 0.01; *** p < 0.001 (significant difference compared to control)

5.2.2. IC ROS levels in IPEC-J2 cells treated with *S.* Typhimurium and PACs

Compared to the control, there was a large, significant (p < 0.05) increase of 31.21% in the fluorescence value when *S.* Typhimurium was used on its own, which means an increase in IC ROS levels of IPEC-J2 cells (Figure 5). As shown in Figure 5, PACs treatment (50 and 100 µg/ml) used in combination with *S.* Typhimurium (P50 + ST and P100 + ST, respectively) resulted in a significant (p < 0.001) decrease in fluorescence values compared to the control, with the cells exhibiting very low levels of fluorescence at 6.19% and 5.52%. These values show that PACs have been able to alleviate the effect of *S.* Typhimurium on IC ROS levels in IPEC-J2 cells. PACs alone (P50 and P100) did not alter IC ROS level of cells in this setting.

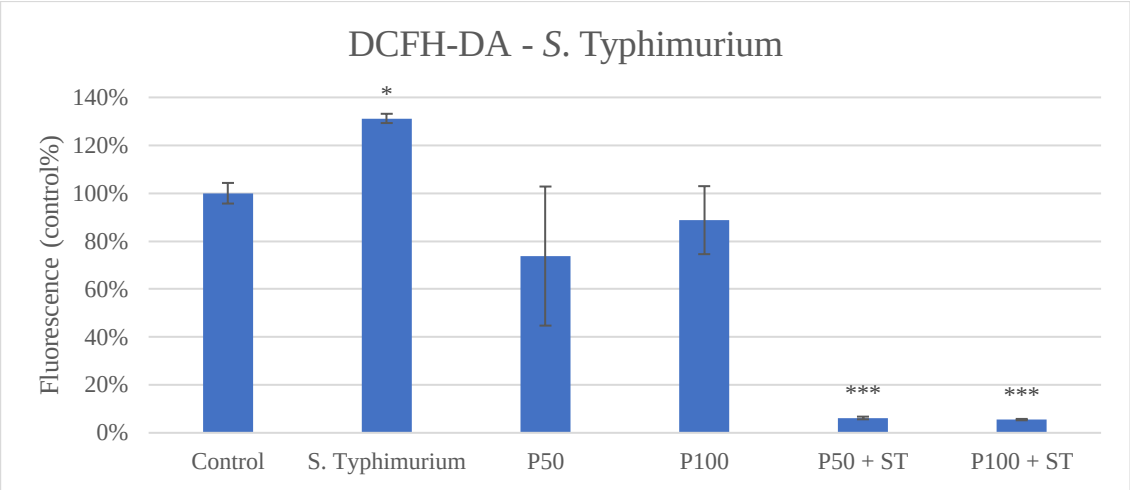

Figure 5: IC ROS amount in IPEC-J2 cells after treatment with *S.* Typhimurium and PACs (average fluorescence values with standard deviation, n=6)

*: $p < 0.05$; *** $p < 0.001$ (significant difference compared to control)

5.3. Interleukin level changes (ELISA method)

5.3.1 IL-6, IL-8 levels in IPEC-J2 cells treated with *E. coli* and PACs

In our experiments, *E. coli* treatment did not cause any detectable change in interleukin production (neither IL-6, nor IL-8) of IPEC-J2 cells. Therefore, it was not possible to test anti-inflammatory effect of PACs in this setting.

5.3.2. IL-6 levels in IPEC-J2 cells treated with *S.* Typhimurium and PACs

Figure 6 shows that there was a significant ($p < 0.05$) increase (33.33%) in the absorbance value suggesting high IL-6 levels, when using *S.* Typhimurium on its own compared to the control. The increase of IL-6 levels was shown to be alleviated with the usage of PACs at 50 and 100 µg/ml when they were applied simultaneously with bacteria (P50 + ST and P100 + ST, respectively). In these cases, absorbance values were similar to the control. PACs have therefore shown the ability to reduce the concentration of inflammatory markers increased by bacteria in IPEC-J2 cells. PACs alone (P50 and P100) did not alter IL-6 level of cells.

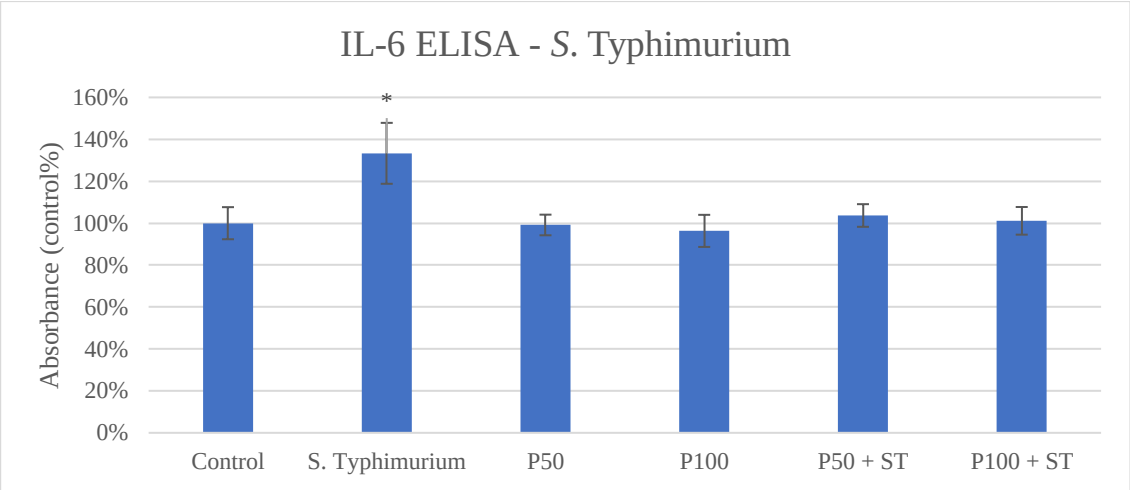

Figure 6: IL-6 amount in IPEC-J2 cells after treatment with *S.* Typhimurium and PACs (average absorbance values with standard deviation, n=6).

*: $p < 0.05$ (significant difference compared to control)

5.3.3. IL-8 levels in IPEC-J2 cells treated with *S.* Typhimurium and PACs

Similarly to the changes in IL-6 production of cells, there was a significant increase ($p < 0.05$) in the IL-8 levels expressed when *S.* Typhimurium was used on its own (Figure 7). An increase of 29.0% was found compared to the control, which could be alleviated when PACs was used in combination with the bacteria at concentrations of 50 and 100 µg/ml (P50 + ST and P100 + ST, respectively). PACs alone (P50 and P100) did not alter IL-8 level of cells.

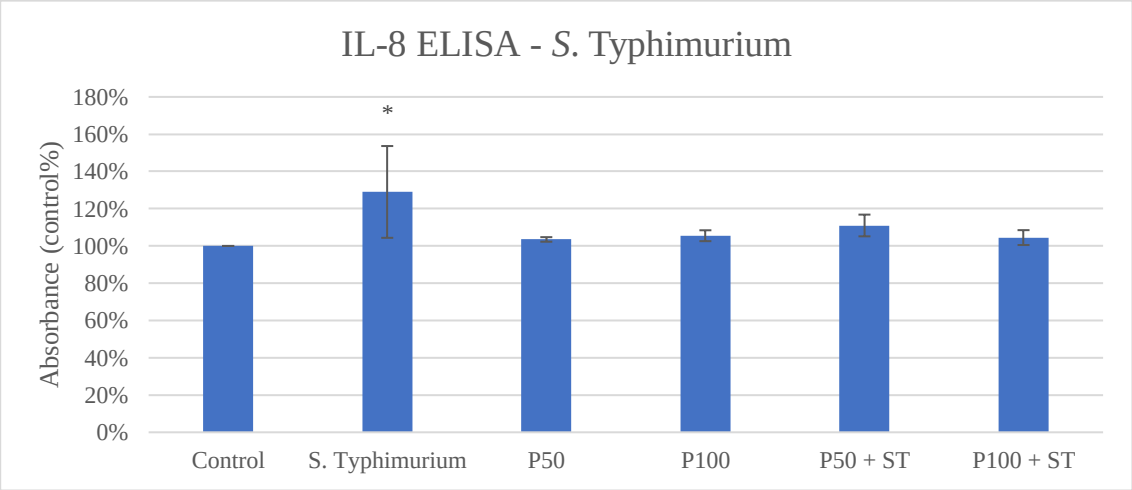

Figure 7: IL-8 amount in IPEC-J2 cells after treatment with *S.* Typhimurium and PACs (average absorbance values with standard deviations, n=6).

*: $p < 0.05$ (significant difference compared to control)

6. DISCUSSION

GI infections caused by zoonotic bacteria such as *E. coli* and *Salmonella* spp. are found abundantly in swine populations globally and pose a threat to humans through the consumption of their meat and by-products [14]. These bacteria act by destroying the primary barrier of the GI tract by causing oxidative stress, inflammation, and morphological damage in intestinal epithelial cells. Damage done to the intestinal epithelial barrier integrity can worsen the disease by leading to increased permeability and consequently, bacterial translocation and absorption of toxins [37]. Antibiotics are frequently administered to prevent and treat bacterial infections in swine, however, there is an increasing number of strains showing resistance to the applied agents, mainly as a result of their inappropriate usage [59]. Application of antibiotics in food-producing animals, such as pigs, can not only lead to higher prevalence of resistant strains in animals, but in humans as well [60]. Consequently, it is of upmost importance to decrease the consumption antibiotics, which includes finding alternative strategies for the protection of GI health [55]. It is also supported by regulations implemented in the EU to decrease the usage of antibiotics and to aid in the fight against resistance [11, 17].

Studies on potential antibiotic alternatives originating from plants such as flavonoids, including PACs are on-going [38], including our study that has shown promising effects and a potential future usage in bacterial infections. Flavonoids are favoured as they are cheap, easily obtained through extraction and have low toxicity [39]. PACs are plant-derived polyphenolic compounds which have a known antioxidant effect, that has already been proved *in vitro* and *in vivo* as well. Previously, a study at the Department of Pharmacology and Toxicology, University of Veterinary Medicine Budapest has demonstrated antioxidant effect of grape seeds PACs in IPEC-J2 cells against oxidative stress induced by bacterial endotoxins (lipopolysaccharides, LPS) of *E. coli* and *S.* Typhimurium origin [37]. The current investigation supplements previous findings with results in a more representative model of GI infections.

In this research, co-cultures composed of 10^6 CFU/ml *E. coli* or *S.* Typhimurium of swine origin with IPEC-J2 porcine intestinal epithelial cells were established using a cell viability (Neutral Red) assay. Following this, one-hour treatment with *E. coli* and *S.* Typhimurium were used in combination with grape seeds PACs to investigate if the latter can influence effect of bacteria on IC ROS level of the cells (using DCFH-DA assay).

Our results showed that PACs could significantly decrease the level of oxidative stress (IC ROS amount) in IPEC-J2 cells, which were otherwise increased by bacteria. These findings are in line with previous publications about antioxidant effects of PACs but demonstrated it in a new setting that has not been published yet. Based on the observations, it can be stated that grape seeds PACs could be beneficial in alleviating oxidative stress caused by bacterial infections in the GI tract.

Anti-inflammatory properties have also been exhibited by PACs previously through reduction in the concentration of inflammatory markers [35]. In our research, we used *E. coli* and *S.* Typhimurium to demonstrate the effect of these bacteria on the IL-6 and IL-8 levels in IPEC-J2 cells (with ELISA method). Unfortunately, treatment with *E. coli* did not cause any measurable change in the IL-6 and IL-8 levels and so, it was not possible to test anti-inflammatory effect of PACs against this bacterium. On the other hand, our results showed that on its own, *S.* Typhimurium caused a significant increase in IL-6 and IL-8 levels in IPEC-J2 cells, which was decreased when PACs (at concentrations of 50 and 100 µg/ml) were used simultaneously with the bacterium. We concluded that grape seeds PACs could successfully alleviate the effect of *S.* Typhimurium on the levels of both inflammatory markers in IPEC-J2 cells. Similarly to the antioxidant effect, our results are in accordance with the literature, and supports them with findings in a special experimental setting.

To summarize, based on the results obtained from our experiments, grape seeds PACs are promising compounds and have the potential to be used successfully as both antioxidants and anti-inflammatories against *E. coli* and *S.* Typhimurium caused GI infections in swine. The effect of *S.* Typhimurium on IPEC-J2 cells was more pronounced in our investigation than that of *E. coli*. It could be useful in the future to test the effects of PACs in similar experimental settings but with different bacterial strains and species. Also, further *in vivo* studies should be carried out to provide more information about these properties and how they could be used in veterinary and human medicine, taking into account pharmacokinetic aspects (e.g. bioavailability) of these substances that were not examined in the current investigation.

7. ABSTRACT

Nowadays, as a result of the abundant and inappropriate use of antibiotics, resistance in bacteria against these agents poses a potentially great risk in both veterinary and human medicine. The increasing prevalence of resistant strains can lead to treatment failure, which is especially important in case of infections caused by zoonotic bacteria such as *Escherichia coli* and *Salmonella enterica* ser. Typhimurium. This phenomenon can only be overcome with the 'One Health' concept, where the veterinary and human fields work together in finding solutions to decrease and optimise the usage of antibiotics and to find potential antibiotic alternatives that can help in the prevention and treatment of bacterial infections. Among many natural substances, flavonoids, including proanthocyanidins (PACs), might be used for this purpose due to their antioxidant, anti-inflammatory and antibacterial properties.

In this study, our aim was to test protective effects of grape seed PACs in an *in vitro* model of gastrointestinal infections, which consisted of bacteria *(E. coli* and *S.* Typhimurium) of swine enteral origin and porcine intestinal epithelial cells (IPEC-J2 cell line). To establish this co-culture, initially, Neutral Red assay was used to determine the highest tolerable bacterial concentration that could be applied on IPEC-J2 cells without causing significant viability decrease. Afterwards, cells were treated with either *E. coli* or *S.* Typhimurium alone or in combination with PACs (50 or 100 µg/ml), and the amount of intracellular reactive oxygen species (IC ROS) were measured with 2',7' dichloro-dihydro-fluorescein diacetate (DCFH-DA) reagent, while interleukin-6 (IL-6) and interleukin-8 (IL-8) levels were determined with enzyme-linked immunosorbent assay (ELISA).

In our experiment, 1 hour treatment with 10^6 CFU/ml *E. coli* and *S.* Typhimurium did not affect the viability of IPEC-J2 cells, therefore bacteria were used in this concentration during the further steps. PACs in 50 and 100 µg/ml concentrations exhibited the ability to reduce IC ROS level of IPEC-J2 cells when they were applied in combination with bacteria. Furthermore, *S.* Typhimurium caused an increase in IL-8 and IL-6 production of cells, which could also be alleviated with the application of PACs.

Our results showed that PACs from grape seeds were able to reduce the amount of oxidative stress and inflammatory markers in porcine intestinal epithelial cells treated

with bacteria. Based on these observations, PACs might be eligible for the prevention and treatment of intestinal bacterial infections in swine, but further *in vitro* and *in vivo* studies are necessary to support their usage.

8. ÖSSZEFOGLALÁS

Az antibiotikumok túlzott, és sok esetben nem megfelelő használata miatt a baktériumokban velük szemben kialakult rezisztencia napjaink egyik legjelentősebb problémája a humán- és állatgyógyászatban egyaránt. Rezisztens baktériumok nagy arányú jelenléte fertőzések során az antibiotikumos kezelések sikertelenségéhez vezethet, amely különösen jelentős probléma zoonotikus baktériumok, például *Escherichia coli* és *Salmonella enterica* ser. Typhimurium által okozott megbetegedések esetén. A jelenség leküzdése csak az 'Egy Egészség' ('One Health') koncepcióval lehet sikeres, amelynek lényege, hogy az állat- és humánegészségügy szereplői együttesen keresnek megoldásokat az antibiotikumok használatának csökkentésére és optimalizálására, valamint bakteriális fertőzések megelőzésére vagy kezelésére használható potenciális antibiotikum-alternatívákat. A flavonoidok közé tartozó proantocianidinok (PAC-ok) biológiailag aktív növényi vegyületek, amelyek antioxidáns, gyulladáscsökkentő és antibakteriális tulajdonságaik alapján alkalmasak lehetnek erre a célra.

Kutatásunk célja az volt, hogy megvizsgáljuk a szőlőmag eredetű PAC-ok védő hatásait egy gasztrointesztinális fertőzéseket modellező *in vitro* rendszerben, amely sertésekből izolált bélpatogén baktériumokból (*E. coli* és *S.* Typhimurium), valamint sertés eredetű bélhámsejtekből (IPEC-J2 sejtvonal) állt. Ezen ko-kultúra létrehozásához elsőként Neutral Red festéssel meghatároztuk a legnagyobb tolerálható baktériumkoncentrációt, amely a sejteken alkalmazva nem okoz szignifikáns életképesség csökkenést. Ezt követően a sejteket *E. coli*-val és *S.* Typhimurium-mal kezeltük önállóan, vagy PAC-okkal kombinálva (50 és 100 µg/ml koncentrációban), majd meghatároztuk a sejtek intracelluláris reaktív oxigén származék szintjét (IC ROS) 2',7'diklór-dihidro-fluoreszcein-diacetát (DCFH-DA) reagenssel, valamint interleukin-6 (IL-6) és interleukin-8 (IL-8) termelését enzimhez kapcsolt immunszorbens vizsgálattal (ELISA).

Egy órás kezelés során az *E. coli* és *S.* Typhimurium törzsek 10^6 CFU/ml koncentrációjú szuszpenziója nem befolyásolta az IPEC-J2 sejtek életképességét, így a további vizsgálatokhoz ilyen töménységű baktériumszuszpenziót alkalmaztunk. A PAC-ok 50 és 100 µg/ml koncentrációban, baktériumokkal együtt alkalmazva csökkenteni tudták az IPEC-J2 sejtek IC ROS szintjét. Ezen túl a *S.* Typhimurium-mal történő kezelés a sejtek IL-8 és IL-6 termelésének növekedését okozta, amely szintén enyhíthető volt PAC-ok alkalmazásával.

Eredményeink alapján elmondható, hogy a szőlőmag eredetű PAC-ok képesek csökkenteni az oxidatív stressz és a gyulladás markereinek mennyiségét baktériumokkal kezelt sertés bélhám sejtkultúrában. Ezen megfigyelések alapján a PAC-ok alkalmasak lehetnek sertések bakteriális bélfertőzéseinek megelőzésére és kezelésére, de további *in vitro* és *in vivo* vizsgálatok szükségesek alkalmazhatóságuk alátámasztására.

9. RESOURCES

[1] Palma E, Tilocca B, Roncada P (2020) Antimicrobial Resistance in Veterinary Medicine: An Overview. Int J Mol Sci 21(6) 191 https://doi.org/10.3390/ijms21061914

[2] Christaki E, Marcou M, Tofarides A (2019) Antimicrobial Resistance in Bacteria: Mechanisms, Evolution, and Persistence. UCLA Sym Bi 88(1) 26-40 https://doi.org/10.1007/s00239-019-09914-3

[3] Gálfi P, Csikó G, Jerzsele Á (2014) In: Gálfi P., Csikó G., Jerzsele Á: Állatorvosi gyógyszertan III. Robbie-Vet Kft., Budapest

[4] Cloeckaert A, Zygmunt M, Doublet B (2017) Editorial: Genetics of Acquired Antimicrobial Resistance in Animal and Zoonotic Pathogens. Front Microbiol 8:2428 https://doi.org/10.3389/fmicb.2017.02428

[5] Blake D, Hillman K, Fenlon D, Low J (2003) Transfer of antibiotic resistance between commensal and pathogenic members of the Enterobacteriaceae under ileal conditions. J Appl Microbiol 95(3) 428-438 https://doi.org/10.1046/j.1365-2672.2003.01988.x

[6] Magiorakos A, Srinivasan A, Carey R, Carmeli Y, Falagas M, Giske C, Harbarth S, Hindler J, Kahlmeter G, Olsson-Liljequist B, Paterson D, Rice L, Stelling J, Struelens M, Vatopoulos A, Weber J, Monnet D (2012) Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infec 18(3) 268-281 https://doi.org/10.1111/j.1469-0691.2011.03570.x

[7] van der Kolk J, Endimiani A, Graubner C, Gerber V, Perreten V (2019) *Acinetobacter* in veterinary medicine, with an emphasis on *Acinetobacter baumannii*. J Glob Antimicrob Re 16:59-71 https://doi.org/10.1016/j.jgar.2018.08.011

[8] European Medicines Agency (EMA) (2019) Categorisation of antibiotics in the European Union (EMA/CVMP/CHMP/682198/2017)

[9] Casaburi G, Duar R, Vance D, Mitchell R, Contreras L, Frese S, Smilowitz J, Underwood M (2019) Early-life gut microbiome modulation reduces the abundance of antibiotic-resistant bacteria. Antimicrob Resist In 8:131 https://doi.org/10.1186/s13756-019-0583-6

[10] World Health Organization (2018) Tuberculosis: Multidrug-resistant tuberculosis. https://www.who.int/news-room/q-a-detail/tuberculosis-multidrug-resistant-tuberculosis-(mdr-tb). Accessed 6 Oct 2021

[11] Hassan YI, Lahaye L, Gong MM, Peng J, Gong J, Liu S, Gay CG, Yang C (2018) Innovative drugs, chemicals, and enzymes within the animal production chain. Vet Res 49:71 https://doi.org/10.1186/s13567-018-0559-1

[12] Lillehoj H, Liu Y, Calsamiglia S, Fernandez-Miyakawa M, Chi F, Cravens R, Oh S, Gay C (2018) Phytochemicals as antibiotic alternatives to promote growth and enhance host health. Vet Res 49(1) 76 https://doi.org/10.1186/s13567-018-0562-6

[13] Burow E, Rostalski A, Harlizius J, Gangl A, Simoneit C, Grobbel M, Kollas C, Tenhagen B, Käsbohrer A (2019) Antibiotic resistance in *Escherichia coli* from pigs from birth to slaughter and its association with antibiotic treatment. Prev Vet Med 165:52-62 https://doi.org/10.1016/j.prevetmed.2019.02.008

[14] Sarrazin S, Joosten P, Van Gompel L, Luiken R, Mevius D, Wagenaar J, Heederik D, Dewulf J, Wagenaar J, Graveland H, Heederik D, Schmitt H, Luiken R, Gompel L, Mevius D, van Essen A, Gonzalez-Zorn B, Moyano G, Sanders P, Chauvin C, David J, Battisti A, Caprioli A, Dewulf J, Blaha T, Wadepohl K, Brandt M, Aarestrup F, Hald T, Duarte S, Wasyl D, Krasucka D, Biernacki B, Szumiło J, Daskalov H, Saatkamp H, Stärk K (2018) Quantitative and qualitative analysis of antimicrobial usage patterns in 180 selected farrow-to-finish pig farms from nine European countries based on single batch and purchase data. J Antimicrob Chemoth 74(3) 807-816 https://doi.org/10.1093/jac/dky503

[15] Cameron A, McAllister T (2016) Antimicrobial usage and resistance in beef production. J Anim Sci Biotechno 7:68 https://doi.org/10.1186/s40104-016-0127-3

[16] Xiao H, Shao F, Wu M, Ren W, Xiong X, Tan B, Yin Y (2015) The application of antimicrobial peptides as growth and health promoters for swine. J Anim Sci Biotechno 6:19 https://doi.org/10.1186/s40104-015-0018-z

[17] European Union (2019) Regulation (EU) 2019/6 of the European Parliament and of the Council on veterinary medicinal products and repealing Directive 2001/82/EC

[18] Gigante A, Atterbury RJ (2019) Veterinary use of bacteriophage therapy in intensively-reared livestock. Virol J 16:155 https://doi.org/10.1186/s12985-019-1260-3

[19] Maertens H, De Reu K, Meyer E, Van Coillie E, Dewulf J (2019) Limited association between disinfectant use and either antibiotic or disinfectant susceptibility of *Escherichia coli* in both poultry and pig husbandry. BMC Vet Res 15(1) 310 https://doi.org/10.1186/s12917-019-2044-0

[20] European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) (2021) The European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2018/2019. EFSA Journal 19(4) 6490 https://doi.org/10.2903/j.efsa.2021.6490

[21] Czaplewski L, Bax R, Clokie M, Dawson M, Fairhead H, Fischetti V, Foster S, Gilmore B, Hancock R, Harper D, Henderson I, Hilpert K, Jones B, Kadioglu A, Knowles D, Ólafsdóttir S, Payne D, Projan S, Shaunak S, Silverman J, Thomas C, Trust T, Warn P, Rex J (2016) Alternatives to antibiotics - a pipeline portfolio review. Lancet Infect Dis 16(2) 239-251 https://doi.org/10.1016/S1473-3099(15)00466-1

[22] Kovács D, Palkovicsné Pézsa N, Farkas O, Jerzsele Á (2021) Usage of antibiotic alternatives in pig farming. Magy Allatorvosok 143:281-292

[23] World Health Organisation (2002) Guidelines for the evaluation of probiotics in food. https://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf. Accessed: 24 June 2020

[24] Dubreuil J (2017) Enterotoxigenic *Escherichia coli* and probiotics in swine: what the bleep do we know?. Biosci Microbiota Food Health 36(3) 75-90 https://doi.org/10.12938/bmfh.16-030

[25] Davani-Davari D, Negahdaripour M, Karimzadeh I, Seifan M, Mohkam M, Masoumi S, Berenjian A, Ghasemi Y (2019) Prebiotics: Definition, Types, Sources, Mechanisms, and Clinical Applications. Front Microbiol 8(3) 92 https://doi.org/10.3390/foods8030092

[26] Pandey K, Naik S, Vakil B (2015) Probiotics, prebiotics and synbiotics - a review. J Food Sci Tech 52:7577-7587 https://doi.org/10.1007/s13197-015-1921-1

[27] Mátis G, Petrilla J, Kulcsár A, van den Bighelaar H, Boomsma B, Neogrády Z, Fébel H (2019) Effects of dietary butyrate supplementation and crude protein level on carcass traits and meat composition of broiler chickens. Arch Anim Breed 62(2) 527-536 https://doi.org/10.5194/aab-62-527-2019

[28] Grygorcewicz B, Grudziński M, Wasak A, Augustyniak A, Pietruszka A, Nawrotek P (2017) Bacteriophage-mediated reduction of *Salmonella* Enteritidis in swine slurry. Appl Soil Ecol 119:179-182 https://doi.org/10.1016/j.apsoil.2017.06.020

[29] Beecher G (2003) Overview of Dietary Flavonoids: Nomenclature, Occurrence and Intake. J Nutr 133(10) 3248S-3254S https://doi.org/10.1093/jn/133.10.3248S

[30] Karancsi Z, Balázs A, Gálfi P, Farkas O (2015) Flavonoidok – új lehetőségek az állatgyógyászatban. Magy Allatorvosok 137:695–704

[31] Schaeffer J (2013) Flavonoids' nutritional benefits. Today's geriatric medicine. 6(4)30 https://www.todaysgeriatricmedicine.com/archive/070113p30.shtml

[32] Clark JL, Zahradka P, Taylor CG (2015) Efficacy of flavonoids in the management of high blood pressure. Nutr Rev 73(12) 799-822 https://doi.org/10.1093/nutrit/nuv048

[33] Bustos P, Deza-Ponzio R, Páez P, Cabrera J, Virgolini M, Ortega M (2018) Flavonoids as protective agents against oxidative stress induced by gentamicin in systemic circulation. Potent protective activity and microbial synergism of luteolin. Food Chem Toxicol 118:294-302 https://doi.org/10.1016/j.fct.2018.05.030

[34] Cushnie T, Lamb A (2005) Antimicrobial activity of flavonoids. Int J Antimicrob Ag 26(5) 343-356 https://doi.org/10.1016/j.ijantimicag.2005.09.002

[35] Rauf A, Imran M, Abu-Izneid T, Iahtisham-Ul-Haq Patel S, Pan X, Naz S, Sanches Silva A, Saeed F, Rasul Suleria H (2019) Proanthocyanidins: A comprehensive review. Biomed Pharmacother 116:108999 https://doi.org/10.1016/j.biopha.2019.108999

[36] Rue E, Rush M, van Breemen R (2017) Procyanidins: a comprehensive review encompassing structure elucidation via mass spectrometry. Phytochem Rev 17:1-16 https://doi.org/10.1007/s11101-017-9507-3

[37] Kovács D, Karancsi Z, Farkas O, Jerzsele Á (2020) Antioxidant Activity of Flavonoids in LPS-Treated IPEC-J2 Porcine Intestinal Epithelial Cells and Their Antibacterial Effect against Bacteria of Swine Origin. Antioxidants in Veterinary Medicine 9(12) 1267 https://doi.org/10.3390/antiox9121267

[38] Lu M, Yang M, Li P, Fang H, Huang H, Chan Y, Bau D (2018) Effect of Oligomeric Proanthocyanidin on the Antioxidant Status and Lung Function of Patients with Chronic Obstructive Pulmonary Disease. In Vivo 32(4) 753-758 https://doi.org/10.21873/invivo.11304

[39] Yang L, Xian D, Xiong X, Lai R, Song J, Zhong J (2018) Proanthocyanidins against Oxidative Stress: From Molecular Mechanisms to Clinical Applications. Biomed Res Int 2018:1-11 https://doi.org/10.1155/2018/8584136

[40] Mayer R, Stecher G, Wuerzner R, Silva R, Sultana T, Trojer L, Feuerstein I, Krieg C, Abel G, Popp M, Bobleter O, Bonn G (2008) Proanthocyanidins: Target Compounds as Antibacterial Agents. J Agr Food Chem 56(16) 6959-6966 https://doi.org/10.1021/jf800832r

[41] Zhao T, Shen X, Dai C, Cui L (2018) Benefits of procyanidins on gut microbiota in Bama minipigs and implications in replacing antibiotics. J Vet Sci 19(6) 798-807 https://doi.org/10.4142/jvs.2018.19.6.798

[42] Gupta A, Dwivedi M, Mahdi A, Nagana Gowda G, Khetrapal C, Bhandari M (2011) Inhibition of adherence of multi-drug resistant *E. coli* by proanthocyanidin. Urol Res 40:143-150 https://doi.org/10.1007/s00240-011-0398-2

[43] Feldman M, Tanabe S, Howell A, Grenier D (2012) Cranberry proanthocyanidins inhibit the adherence properties of *Candida albicans* and cytokine secretion by oral epithelial cells. BMC Complem Altern M 12:6 https://doi.org/10.1186/1472-6882-12-6

[44] Rane H, Bernardo S, Howell A, Lee S (2013) Cranberry-derived proanthocyanidins prevent formation of *Candida albicans* biofilms in artificial urine through biofilm- and adherence-specific mechanisms. J Antimicrob Chemoth 69(2) 428-436 https://doi.org/10.1093/jac/dkt398

[45] Zhou P, Zhang L, Li W, Zhang S, Luo L, Wang J, Sun B (2018) *In vitro* evaluation of the anti-digestion and antioxidant effects of grape seed procyanidins according to their degrees of polymerization. J Funct Foods 49:85-95 https://doi.org/10.1016/j.jff.2018.08.001

[46] González-Quilen C, Rodríguez-Gallego E, Beltrán-Debón R, Pinent M, Ardévol A, Blay M, Terra X (2020) Health-Promoting Properties of Proanthocyanidins for Intestinal Dysfunction. Nutrients 12(1) 130 https://doi.org/10.3390/nu12010130

[47] Knecht D, Cholewinska P, Jankowska-Makosa A, Czyz K (2020) Development of Swine's Digestive Tract Microbiota and Its Relation to Production Indices — A Review. Animals 10(3) 527 https://doi.org/10.3390/ani10030527

[48] Porter S, Strain SAJ, Bagdonaite G, McDowell S, Bronckaers T, Sherrey M, Devine P, Pascual-Linaza AV, Spence N, Porter R, Guelbenzo-Gonzalo M, Davies RH, Lahuerta-Marin A (2020) Trends in *Salmonella* serovars and antimicrobial resistance in pigs and

poultry in Northern Ireland between 1997 and 2016. Vet Rec 186(5) 156 https://doi.org/10.1136/vr.105640

[49] de Azevedo EC, Martins BTF, Casas MRT, Possebon FS, Junior JPA, Nero LA, Yamatogi RS (2020) Multidrug resistance and virulence profiles of *Salmonella* isolated from swine lymph nodes. Microb Drug Resist 27(4) 562-570 https://doi.org/10.1089/mdr.2020.0120

[50] Fallah N, Ghaemi M, Ghazvini, K, Rad, M, Jamshidi A (2021) Occurrence, pathotypes, and antimicrobial resistance profiles of diarrheagenic *Escherichia coli* strains in animal source food products from public markets in Mashhad, Iran. Food Control 121:107640 https://doi.org/10.1016/j.foodcont.2020.107640

[51] Garrido V, Sánchez S, Román BS, Fraile L, Migura-García L, Grilló M-J (2020) *Salmonella* infection in mesenteric lymph nodes of breeding sows. Foodborne Pathog Dis 17(6) 411-417 https://doi.org/10.1089/fpd.2019.2708

[52] Yin H, Ye P, Lei Q, Cheng Y, Yu H, Du J, Pan H, Cao Z (2020) *In vitro* probiotic properties of *Pediococcus pentosaceus* L1 and its effects on enterotoxigenic *Escherichia coli*-induced inflammatory responses in porcine intestinal epithelial cells. Microb Pathogenesis 144:104163 https://doi.org/10.1016/j.micpath.2020.104163

[53] Brosnahan A, Brown D (2012) Porcine IPEC-J2 intestinal epithelial cells in microbiological investigations. Vet Microbiol 156(3-4) 229-237 https://doi.org/10.1016/j.vetmic.2011.10.017

[54] Hyland K, Brown D, Murtaugh M (2006) *Salmonella enterica* serovar Choleraesuis infection of the porcine jejunal Peyer's patch rapidly induces IL-1β and IL-8 expression. Vet Immunol Immunop 109(1-2) 1-11 https://doi.org/10.1016/j.vetimm.2005.06.016

[55] Fabá L, Litjens R, Allaart J, Roubos-van den Hil P (2020) Feed additive blends fed to nursery pigs challenged with *Salmonella*. Anim Sci 98(1) 1-10 https://doi.org/10.1093/jas/skz382

[56] Repetto G, del Peso A, Zurita J (2008) Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat Protoc 3:1125-1131 https://doi.org/10.1038/nprot.2008.75

[57] Wang H, Joseph JA (1999) Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radic Biol Med 27(5-6) 612–616 https://doi.org/10.1016/s0891-5849(99)00107-0

[58] Loss H, Aschenbach J, Tedin K, Ebner F, Lodemann U (2018) The Inflammatory Response to Enterotoxigenic *E. coli* and Probiotic *E. faecium* in a Coculture Model of Porcine Intestinal Epithelial and Dendritic Cells. Mediat Inflamm 2018:1-16 https://doi.org/10.1155/2018/9368295

[59] Luppi A (2017) Swine enteric colibacillosis: Diagnosis, therapy and antimicrobial resistance. Porc Health Manag 3:16 https://doi.org/10.1186/s40813-017-0063-4

[60] Hayer SS, Rovire A, Olsen K, Johnson, TJ, Vannucci F, Rendahl A, Perez A, Alvarez J (2020) Prevalence and trend analysis of antimicrobial resistance in clinical *Escherichia coli* isolates collected from diseased pigs in the USA between 2006 and 2016. Transbound Emerg Dis 67(5)1930–1941 https://doi.org/10.1111/tbed.13528

10. ACKNOWLEDGEMENTS

I would like to firstly thank the entire Department of Pharmacology and Toxicology, but mostly, my supervisor dr. Kovács Dóra for providing me with the opportunity to write my TDK and being a fantastic teacher. The laboratory work, continuous support, effort, and positive attitude throughout this process and in these last years was immensely appreciated, especially in these difficult times during COVID. Also I am grateful to dr. Ákos Jerzsele, Head of Department, for providing the opportunity to conduct this research.

My last acknowledgement is for my family, and my parents for being my biggest supporters and always believing in me. Without their love and continuous encouragement I would not be where I am today.

HuVetA

ELECTRONIC LICENSE AGREEMENT AND COPYRIGHT DECLARATION*

Name: Bella Gál

Contact information (e-mail): gal.bella1996@gmail.com

Title of document (to be uploaded): Antioxidant and anti-inflammatory effects of proanthocyanidins in porcine intestinal epithelial cell – bacterium co-culture

Publication data of document: 2021

Number of files submitted: 1 electronic (PDF), 1 printed

By accepting the present agreement the author or copyright owner grants non-exclusive license to HuVetA over the above mentioned document (including its abstract) to be converted to copy protected PDF format without changing its content, in order to archive, reproduce, and make accessible under the conditions specified below.

The author agrees that HuVetA may store more than one copy (accessible only to HuVetA administrators) of the licensed document exclusively for purposes of secure storage and backup, if necessary.

You state that the submission is your original work, and that you have the right to grant the rights contained in this license. You also state that your submission does not, to the best of your knowledge, infringe upon anyone's copyright. If the document has parts which you are not the copyright owner of, you have to indicate that you have obtained unrestricted permission from the copyright owner to grant the rights required by this Agreement, and that any such third-party owned material is clearly identified and acknowledged within the text of the licensed document.

The copyright owner defines the scope of access to the document stored in HuVetA as follows **(mark the appropriate box with an X)**:

- [X] I grant unlimited online access.
- [ ] I grant access only through the intranet (IP range) of the University of Veterinary Medicine.
- [ ] I grant access only on one dedicated computer at the Ferenc Hutÿra Library.
- [ ] I grant unlimited online access only to the bibliographic data and abstract of the document.

Please, define the **in-house accessibility of the document** by marking the below box with an **X**:

X	I grant in-house access (namely, reading the hard copy version of the document) at the Library.

If the preparation of the document to be uploaded was supported or sponsored by a firm or an organization, you also declare that you are entitled to sign the present Agreement concerning the document.

The operators of HuVetA do not assume any legal liability or responsibility towards the author/copyright holder/organizations in case somebody uses the material legally uploaded to HuVetA in a way that is unlawful.

Date: Budapest, 11 November 2022

Gál Bella

Author/copyright owner signature

HuVetA Magyar Állatorvos-tudományi Archívum – Hungarian Veterinary Archive *is an online veterinary repository operated by the Ferenc Hutÿra Library, Archives and Museum. It is an electronic knowledge base which aims to collect, organize, store documents regarding Hungarian veterinary science and history, and make them searchable and accessible in line with current legal requirements and regulations.*

HuVetA relies on the latest technology in order to provide easy searchability (by search engines, as well) and access to the full text document, whenever possible.
Based on the above, HuVetA aims to:

- *increase awareness of Hungarian veterinary science not only in Hungary, but also internationally;*
- *increase citation numbers of publications authored by Hungarian veterinarians, thus improve the impact factor of Hungarian veterinary journals;*
- *present the knowledge base of the University of Veterinary Medicine Budapest and its partners in a focussed way in order to improve the prestige of the Hungarian veterinary profession, and the competitiveness of the organizations in question;*
- *facilitate professional relations and collaboration;*
- *support open access.*

I hereby confirm that I am familiar with the content of the thesis entitled:

Antioxidant and anti-inflammatory effects of proanthocyanidins in porcine intestinal epithelial cell – bacterium co-culture

written by …… Bella Gál …… which I deem suitable for submission and defence.

Date: Budapest, 11 November 2022

Dóra Kovács DVM

Supervisor

Department of Pharmacology and Toxicology

DECLARATION

I hereby declare that the thesis entitled ..... Antioxidant and anti-inflammatory effects of proanthocyanidins in porcine intestinal epithelial cell – bacterium co-culture .....

is identical in terms of content and formal requirements to the TDK research paper submitted in ... 2021 ... (year).

Date: Budapest, 11 November 2022

Bella Gál

.....

Student name and signature

Thesis progress report for veterinary students

Name of student: Bella Gál

Neptun code of the student: IL6DGO

Name and title of the supervisor: dr. Dóra Kovács, Assistant Lecturer

Department: Department of Pharmacology and Toxicology

Thesis title: Antioxidant and anti-inflammatory effects of proanthocyanidins in porcine intestinal epithelial cell – bacterium co-culture

Consultation – 1st semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2021	02	09	Participation in experiments, discussion on the thesis	
2.	2021	03	17	Participation in experiments	
3.	2021	04	05	Participation in experiments, discussion on the thesis	
4.	2021	05	11-13	Participation in experiments	
5.	2021	06	28	Discussion on the thesis	

Grade achieved at the end of the first semester: 5 (excellent)

Consultation – 2nd semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2021	09	06-08	Participation in experiments, discussion on the thesis	
2.	2021	09	15	Participation in experiments, discussion on the thesis	
3.	2021	09	22	Discussion on the thesis	
4.	2021	10	04	Discussion on the thesis	

5.	2021	10	27	Preparation for TDK conference	

Grade achieved at the end of the second semester: 5 (excellent)

The thesis meets the requirements of the Study and Examination Rules of the University and the Guide to Thesis Writing.

I accept the thesis and found suitable to defence,

.....
dr. Dóra Kovács

Signature of the student: .....

Signature of the secretary of the Department: .....

Date of handing the thesis in...2022...11.....16.....