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In vitro investigation of the effects of flavonoids in porcine gastrointestinal infection models

PhD thesis

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List of abbreviations

AIEC – Adherent invasive *Escherichia coli*

AMEG – Antimicrobial Advice ad hoc Expert Group

AMR – Antimicrobial resistance

AP-1 – Activator protein 1

ARE – Antioxidant response element

ARGs – Antimicrobial resistance genes

ATP – Adenosine triphosphate

CFU – Colony forming unit

CLSI – Clinical and Laboratory Standards Institute

DAEC – Diffusely adherent *Escherichia coli*

DCFH-DA – 2',7'-dichlorodihydrofluorescein diacetate

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

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

EAEC – Enteroaggregative *Escherichia coli*

EC – Extracellular

ECDC – European Centre for Disease Prevention and Control

EFSA – European Food Safety Authority

EHEC – Enterohemorrhagic *Escherichia coli*

EIEC – Enteroinvasive *Escherichia coli*

ELISA – Enzyme-linked immunosorbent assay

EMA – European Medicines Agency

EPEC – Enteropathogenic *Escherichia coli*

ESBL – Extended-spectrum beta-lactamase

ESVAC – European Surveillance of Veterinary Antimicrobial Consumption

ETEC – Enterotoxigenic *Escherichia coli*

EU – European Union

FAO – Food and Agriculture Organization of the United Nations

FD4 – Fluorescein isothiocyanate-dextran (4 kDa)

FDA – Food and Drug Administration

FIC – Fractional inhibitory concentration

GI – Gastrointestinal

GSOP(s) – Grape seed oligomeric proanthocyanidins (purified)

IC ROS – Intracellular reactive oxygen species

IFN-γ – Interferon gamma

IL-1 / 1β / 6 / 8 – Interleukin-1 / 1β / 6 / 8

JAK-STAT – Janus kinases - signal transducer and activator of transcription proteins

Keap1 – Kelch-like ECH-associated protein-1

LPS – Lipopolysaccharide, bacterial endotoxin

LUT – Luteolin

MAPK – Mitogen-activated protein kinase

MDR – Multidrug-resistance/resistant

MH – Mueller-Hinton liquid broth

MIC – Minimum inhibitory concentration

MRSA – Methicillin-resistant *Staphylococcus aureus*

NADH – Nicotinamide adenine dinucleotide hydrogen

NF-κB – Nuclear factor kappa-light-chain-enhancer of activated B cells

Nrf2 – Nuclear factor erythroid 2-related factor 2

OIE – World Organisation for Animal Health (*formerly: Office International des Epizooties*)

PACs – Proanthocyanidins

PBS – Phosphate buffered saline

PDR – Pandrug-resistance

PK/PD - Pharmacokinetic/pharmacodynamic

PMA – Phorbol 12-myristate 13-acetate

PPAR – Peroxisome proliferator-activated receptor

ROS – Reactive oxygen species

STEC – Shiga toxin-producing *Escherichia coli*

TEER – Transepithelial electrical resistance

TJ – Tight junction

TLR4 – Toll-like receptor 4

TNF-α – Tumor necrosis factor alpha

TSA – Tryptone soya agar

VRE – Vancomycin-resistant enterococci

WHO – World Health Organization

XDR – Extensive drug-resistance

ZO-1 – Zonula occludens-1

1. Summary

Antimicrobial resistance (AMR) is a leading health threat of the 21st century that impacts humans, animals, and the environment inextricably. Even though the term AMR can refer to insusceptibility of any microorganisms to the drugs developed against them, it is most commonly used to describe antibiotic resistance in bacteria. The widespread presence of resistant bacteria, and especially the occurrence of multidrug-resistant (MDR) strains can remarkably reduce available treatment options against these infections, and consequently increase their mortality. Bacterial AMR has developed as the result of frequent and improper use of antibiotics on both the human and veterinary fields, and must also be tackled with coordinated, interdisciplinary measures from these sectors, under the so-called “One Health” approach. From this point of view, antibiotic usage in food-producing animals requires special attention, as it has been linked to the spread of resistance in humans mainly via the foodborne route. In pigs, gastrointestinal infections caused by *Escherichia coli* and *Salmonella* spp. are of high importance, as both pathogens are widespread, potentially zoonotic, and highly prone to develop resistance. Therefore, these infections are difficult to treat, and have public health importance due to the possible foodborne transmission, which might include the spread of resistance conferring genes as well. Among the strategies developed and supported by researchers, international organizations, and authorities to overcome AMR, the development and use of antibiotic alternatives became highly important. These substances have the potential to be effective in the prevention and treatment of bacterial infections alone, or in combination with antibiotics. Among many other conceivable alternatives, flavonoids – bioactive compounds of plant origin – represent a large, promising group with several beneficial health promoting activities. In this study, our aim was to test the efficacy of flavonoids, grape seed oligomeric proanthocyanidins (GSOP) and luteolin (LUT) in porcine gastrointestinal infection models *in vitro* to evaluate their usage as potential antibiotic alternatives for the prevention and/or treatment of swine intestinal bacterial infections.

For this purpose, firstly we tested cytotoxicity of these flavonoids on IPEC-J2 porcine intestinal epithelial cells (Neutral Red method). Then IPEC-J2 cells were treated with bacterial endotoxin of *E. coli* and *S. enterica* ser. Typhimurium origin, and we investigated the protective effects of GSOP and LUT in different concentrations against oxidative stress inflicted by the endotoxin (2’,7’-dichlorodihydrofluorescein diacetate [DCFH-DA] assay and Amplex Red method). Afterwards, minimum inhibitory concentration (MIC) values of GSOP and LUT were determined against eight-eight *E. coli* and *S*. Typhimurium field isolates of porcine origin (broth microdilution). Besides evaluating their antibacterial activity, interaction of the flavonoids was tested with three, conventionally used antibiotics. Both GSOP and LUT were combined with amoxicillin, gentamicin and enrofloxacin against the same bacteria to test their potential

synergistic, additive, or antagonistic effect in combination with the active substances (checkerboard assay). In the third phase of this study, we have infected IPEC-J2 cells with *E. coli* and *S*. Typhimurium of porcine origin and investigated protective effects of GSOP and LUT against damages caused by the bacteria. The flavonoids' antioxidant (DCFH-DA assay and Amplex Red method), anti-inflammatory (enzyme-linked immunosorbent assay [ELISA]), barrier protective (fluorescein isothiocyanate-dextran [FD4] assay) and anti-adhesive properties (colony forming unit, CFU counting) were tested in different concentrations and treatment types (i.e. pre-, parallel and post-treatment, depending on the time of flavonoid addition compared to infection).

Both GSOP and LUT proved to be safe in IPEC-J2 cells without causing cell viability decrease up to 200 µg/ml (GSOP) and 100 µg/ml (LUT) for 24 hours. Treatment with LPS of both *E. coli* and *S*. Typhimurium resulted in increased intracellular reactive oxygen species (IC ROS) levels in cells, that could successfully be alleviated with the addition of GSOP and LUT, even at the lowest tested concentrations (50 µg/ml for GSOP and 25 µg/ml for LUT). MIC values of GSOP were found to be 2048 µg/ml against all *E. coli* and *S*. Typhimurium isolates being tested, whereas LUT showed more potent bacteriostatic activity with its MIC being 256 µg/ml for all bacterial strains. In the interaction study, none of the flavonoids impacted efficacy of the tested antibiotics (average fractional inhibitory concentration [FIC] indexes between 1.0000 and 1.4375). Both GSOP and LUT showed antioxidant and anti-inflammatory effect by decreasing IC ROS, as well as interleukin-6 and interleukin-8 levels elevated by *E. coli* and *S*. Typhimurium *in vitro* infections. Amplex Red method proved not to be suitable for detecting oxidative stress in IPEC-J2 cells inflicted by LPS or bacteria. However, *E. coli* and *S*. Typhimurium infections damaged the barrier integrity of IPEC-J2, resulting in an increased permeability of the cell layer. This effect could also be counteracted by GSOP against both bacteria, while LUT was less effective against *S*. Typhimurium. GSOP remarkably inhibited the adhesion of *E. coli* to IPEC-J2 cells, and it also showed anti-adhesive activity against *S*. Typhimurium when it was applied as pre-treatment. In contrast, LUT did not show anti-adhesive property against any of the tested bacteria. In our studies, we could not establish a clear proportionality between the concentration and efficacy of flavonoids. Among the different treatment types tested, pre- and parallel treatments seemed to be slightly more effective than post-treatment, suggesting superior activity of these substances as prophylaxis compared to therapeutic usage.

Our findings have shown several beneficial effects of GSOP and LUT, and support the already existing knowledge about these flavonoids with studies on porcine GI infection models *in vitro*. Based on the obtained results, GSOP and LUT might be used in the future as feed additives in pigs against GI bacterial infections, however, further *in vitro* and *in vivo* studies should be conducted to establish their practical application.

Összefoglalás

Az antimikrobiális rezisztencia (AMR) a XXI. század egyik vezető humán- és állategészségügyi problémája, amely területek elválaszthatatlanul kapcsolódnak egymáshoz az AMR kialakulása és leküzdése szempontjából. Az AMR kifejezés magában foglalja bármely mikroorganizmusban az ellene használt kemoterapeutikummal szemben kialakuló ellenállást, de leggyakrabban a baktériumokban antibiotikumokkal szemben megjelenő rezisztenciát értjük a kifejezés alatt. Az antibiotikumokra rezisztens baktériumok széleskörű előfordulása, különösen a multirezisztens (multidrug-resistant, MDR, azaz több hatóanyaggal szemben egyaránt ellenálló) kórokozók jelenléte nagymértékben megnehezíti az általuk okozott fertőzések gyógykezelését, és a betegség mortalitásának növekedéséhez vezethet. A bakteriális AMR kialakulásának hátterében a túlzott és helytelen antibiotikum használat áll, amely a humán- és állatorvoslás területein egyaránt megfigyelhető, így a probléma visszaszorításához is elengedhetetlen a két oldal összefogása, és közös, tudományterületeken átívelő intézkedések végrehajtása (“Egy Egészség”, “One Health” megközelítés). Kiemelt figyelmet érdemel a haszonállatokban történő antibiotikum felhasználás, amely összefüggést mutat az emberekben tapasztalt rezisztens baktériumok előfordulásával, elsősorban az állati eredetű élelmiszerekkel történő terjedés következményeként. Sertésekben az *Escherichia coli* és *Salmonella* fajok által okozott gyomor-bélrendszeri fertőzések nagy jelentőséggel bírnak, mivel mindkét patogén széleskörűen előfordul, potenciálisan zoonótikus, és igen hajlamos rezisztencia kialakítására. Az általuk okozott fertőzések így gyakran nehezen kezelhetők, és közegészségügyi jelentőséggel is bírnak az élelmiszerlánccal való terjedés lehetősége miatt. Az AMR leküzdésére számos stratégia született, amelyek közé tartozik az antibiotikum alternatívák kutatása. Ezen vegyületek önállóan, vagy antibiotikumokkal kombinációban alkalmasak lehetnek bakteriális fertőzések kezelésére és/vagy megelőzésére. Az antibiotikum alternatívák nagy csoportjába sorolhatók többek között a flavonoidok, amelyek növényi eredetű bioaktív anyagok számos, emberi és állati egészségre gyakorolt jótékony hatással. Jelen kutatás célja az volt, hogy megvizsgáljuk egyes flavonoidok – szőlőmag oligomer proantocianidinek (GSOP) és luteolin (LUT) – hatékonyságát sertések zoonótikus baktériumok (*E. coli* és *S. enterica* ser. Typhimurium) okozta gyomor-bélfertőzéseit modellező *in vitro* rendszerekben, ezáltal képet kapva lehetséges alkalmazhatóságukról, mint takarmánykiegészítők sertések bakteriális bélfertőzéseinek megelőzésére és kezelésére.

Elsőként megvizsgáltuk a flavonoidok sejtéletképességre kifejtett hatását IPEC-J2 sertés vékonybélhám sejteken, hogy meghatározzuk a biztonságosan alkalmazható koncentrációikat (Neutral Red festés). Ezt követően a bélhámsejteket bakteriális (*E. coli* és *S*. Typhimurium eredetű) endotoxinnal (lipopoliszacharid, LPS) kezeltük, és vizsgáltuk a flavonoidok

antioxidáns hatását az LPS által kiváltott oxidatív stresszel szemben (dikloro-dihidro-fluoreszcein diacetát [DCFH-DA] és Amplex Red módszerek). A tesztelt flavonoidokat különböző koncentrációkban alkalmaztuk LPS-sel kombinációban, hogy vizsgáljuk a hatásuk esetleges koncentráció-függését. Ezt követően meghatároztuk a flavonoidok minimális gátló koncentrációját (MIC értékét) sertésből származó, klinikai *E. coli* és *S*. Typhimurium izolátumokban, hogy megvizsgáljuk esetleges bakteriosztatikus hatásukat (mikrohígításos módszer). Teszteltük továbbá, hogy mutatnak-e bármilyen interakciót (szinergizmus, antagonista vagy semleges hatás) három, sertésekben gyakran használt antibiotikummal (amoxicillin, gentamicin, enrofloxacin) együttesen alkalmazva (kereszthígításos módszer). A kutatás harmadik fázisában kialakítottunk egy ko-kultúra modellt, amelyben IPEC-J2 sejteket sertésből izolált *E. coli* és *S*. Typhimuriummal fertőztünk, és vizsgáltuk a GSOP és a LUT jótékony hatásait a kórokozókkal szemben. Teszteltük hatásukat a sejtek reaktív oxigéngyök (DCFH-DA és Amplex Red módszerek), valamint interleukin-6 és interleukin-8 (IL-6, -8) szintjére (enzimhez kötött immunoszorbens próba [ELISA]) a bakteriális fertőzések által okozott káros hatások esetén. Ezeken túl vizsgáltuk a GSOP és a LUT esetleges védőhatását a kórokozók által kiváltott bélbarrier károsodással szemben (fluoreszcein-izotiocianát-dextrán [FD4] próba), valamint, hogy képesek-e gátolni a baktériumok IPEC-J2 sejtekhez történő tapadását (telepformáló egység [CFU] számlálás). A flavonoidokat ebben az esetben is különböző koncentrációkban teszteltük, valamint vizsgáltuk azt is, hogy a fertőzés előtt, azzal egyidőben, vagy utólagosan alkalmazva hogyan alakul a hatásuk.

Sem a GSOP (200 µg/ml koncentrációig), sem pedig a LUT (100 µg/ml-ig) nem mutatott citotoxikus hatást IPEC-J2 sertés vékonybélhám sejteken 1, 12 és 24 órás kezelési idők alatt. A GSOP és a LUT egyaránt csökkenteni tudták az IPEC-J2 sejtekben *E. coli* és *S*. Typhimurium eredetű LPS-sel kiváltott oxidatív stresszt, a legkisebb alkalmazott koncentrációkban is (GSOP: 50 µg/ml, LUT: 25 µg/ml). A GSOP MIC értéke minden vizsgált *E. coli* és *S*. Typhimurium izolátum esetén 2048 µg/ml-nek bizonyult, míg a LUT bakteriosztatikus hatása már 256 µg/ml-es koncentrációnál megmutatkozott. Sem a GSOP, sem pedig a LUT jelenléte nem befolyásolta a tesztelt antibiotikumok (amoxicillin, gentamicin, enrofloxacin) hatékonyságát (az átlagos frakcionált gátló koncentráció [FIC] indexek értéke 1,000 és 1,4375 között alakultak). A GSOP és a LUT egyaránt csökkenteni tudták az IPEC-J2 sejteken *E. coli* és *S*. Typhimurium *in vitro* fertőzéssel kiváltott oxidatív stresszt és gyulladást. Az Amplex Red módszer nem bizonyult alkalmasnak sem az LPS, sem a baktériumok által okozott oxidatív stresszel szembeni védőhatás tanulmányozására. A fentieken túl a flavonoidok csökkenteni tudták az IPEC-J2 sejteken *E. coli* és *S*. Typhimurium által okozott bélbarrier károsodást, bár a LUT kevésbé volt hatékony *S*. Typhimuriummal szemben. A GSOP gátolni tudta a vizsgált baktériumok IPEC-J2 sejtekhez való kitapadását, *E. coli* esetén

minden kezelési típusnál, *Salmonella* fertőzésnél azonban csak előkezelésként. A LUT nem mutatott adhéziógátló hatást egyik kórokozóval szemben sem. A vizsgálataink során nem tudtunk egyértelmű összefüggést megállapítani a flavonoidok alkalmazott koncentrációja, és a tapasztalt hatások mértéke között. A kezelési típusokat összehasonlítva azonban az utókezelés kevésbé bizonyult hatékonynak, mint az elő- és egyidejű kezelések, amely alapján a tesztelt flavonoidok prevenciós céllal alkalmazva hatékonyabbak lehetnek, mint fertőzések kezeléseként.

Eredményeink a GSOP és LUT jótékony hatásairól összhangban vannak a szakirodalmi adatokkal, és kiegészítik azokat sertések gyomor-bélfertőzéseit modellező *in vitro* rendszerekben kapott információkkal. Előnyös tulajdonságaik alapján a GSOP és a LUT potenciális antibiotikum alternatívák, amelyek a jövőben takarmánykiegészítőként alkalmazva hatékonyak lehetnek sertések *E. coli* és *S*. Typhimurium által okozott gyomor-bélfertőzéseinek megelőzésére és/vagy kezelésére, gyakorlati alkalmazásukhoz azonban további *in vitro* és *in vivo* vizsgálatok szükségesek.

2. Introduction

Pig production represents one of the largest markets worldwide (Pungpian et al., 2021). The presence of pathogenic bacteria in swine herds can cause significant economic losses, therefore the control of these microorganisms is of high importance (Lückstädt and Theobald, 2011). Furthermore, food-producing animals, such as pigs, can serve as reservoirs for several foodborne pathogens, including *Escherichia coli* and *Salmonella enterica*, that are able to infect humans. Infections caused by foodborne microbes are responsible for millions of diseases and human deaths per year. It is also exacerbated by the emergence of multidrug-resistant (MDR) bacterial strains that has a negative impact on the efficacy of antibiotic treatments and might lead to increased mortality in these infections (Luppi, 2017; Heredia and García, 2018).

The widespread occurrence of bacterial antimicrobial resistance (AMR) has developed as the result of improper and excessive usage of antibiotics (Tang K.L. et al., 2017; Christaki et al., 2019). Antibacterial agents administered for the treatment of bacterial infections in humans and animals are similar in many cases (Souto et al., 2017; EFSA and ECDC, 2021). For example, antibiotics are frequently used in swine production, and many of them include drugs important in human medicine (Moredo et al., 2015). One of the bacterial diseases most frequently treated with antibiotics is the group of gastrointestinal (GI) infections (Souto et al., 2017), that are highly common in pig production (Edfors-Lilja et al., 2000). The administration of antibiotics can result in the development of resistance in both human and veterinary medicine, due to the continuous selection pressure expressed by these drugs on pathogenic, commensal, and environmental bacteria (EFSA and ECDC, 2021). Furthermore, resistant bacteria developed in food-producing animals can spread to humans through the food chain (Ter Kuile et al., 2016; Tang K.L. et al., 2017; Emes et al., 2022), and they can also confer their antimicrobial resistance genes (ARGs) to other pathogens when they get in close contact (Tóth et al., 2020). Increasing number of resistant bacteria in the microbial communities can have serious consequences on human and animal health (EFSA and ECDC, 2021).

To combat AMR, there are several strategies that are supported by national and international authorities and organizations. These include the optimization of antibiotic administration based on PK/PD (pharmacokinetic/pharmacodynamic) data, improved hygiene and infection prevention measures, as well as surveillance programs on antibiotic usage and the prevalence of resistant bacteria (WHO, 2015). Furthermore, there is an increasing need for the usage of antibiotic alternatives, i.e. substances that have direct antibacterial effect, can improve efficacy of antibiotics when administered in combination, or have a protective effect on the GI barrier that can aid the prevention and/or treatment of bacterial infections. Previously, herbs were

widely used prophylactically and therapeutically against diseases, which practice diminished simultaneously with the increasing development of synthetic drugs. However, due to the spread of AMR, there is again an increasing need for natural substances for the prevention and treatment of infectious diseases (Šikić Pogačar et al., 2016).

In vitro models of the intestine are essential for conducting research about pathogens causing GI infections (Cencic and Langerholc, 2010). The porcine intestinal epithelial cell line, IPEC-J2 provides an important tool for modeling zoonotic enteric infections due to its similarities to the porcine and human intestinal epithelium *in vivo.* IPEC-J2 can also be used for evaluating the effect of bioactive food components before their *in vivo* examination (Vergauwen, 2015).

3. Literature review

3.1. Antimicrobial resistance

3.1.1. Development of resistance

Bacterial AMR is among the major public health threats of the 21st century (Antimicrobial Resistance Collaborators, 2022) that is predicted to be responsible for the death of 10 million people annually by 2050 (Christaki et al., 2019; Antimicrobial Resistance Collaborators, 2022). The phenomenon, which can also be called antibiotic resistance occurs when bacteria have or develop an ability that cause the drugs used against infections becoming less effective (Christaki et al., 2019; Antimicrobial Resistance Collaborators, 2022). Treatment of infections with resistant bacteria is difficult, they can easily relapse, and result in high morbidity and mortality. Furthermore, if they occur in healthcare settings, they can lead to longer hospitalization and a consequential economic impact on healthcare systems (Christaki et al., 2019). It is difficult to determine the total economic cost attributable to AMR considering its burden on human, animal, and environmental health altogether, but it is estimated to be highly significant by numerous studies (Morel et al., 2020).

Types of antibiotic resistance include intrinsic, acquired, and adaptive. Intrinsic, also known as *ab ovo* resistance comes from inherent properties of bacteria (Christaki et al., 2019), and refers to those cases when the bacteria have never been and will never be sensitive to the concerned agent. These include for example the resistance of bacteria without cell wall against drugs targeting cell wall biosynthesis (e.g. beta-lactams against mycoplasmae), and the ineffectiveness of aminoglycosides – that require oxygen for their action – against bacteria occurring in anaerobic conditions (Gálfi et al., 2014). Acquired resistance means the evolutionary adaptation process of bacteria that include a previously susceptible microorganism becoming resistant to an active substance due to acquiring resistance mechanisms either by mutation or horizontal gene transfer (Cloeckaert et al., 2017; Christaki et al., 2019). The latter one can occur via transformation (i.e. obtaining a DNA fragment from a dead bacterium), transduction (i.e. transfer of the resistance conferring genetic material by a bacteriophage) and conjugation (i.e. transfer of genes on plasmids through direct contact between bacteria). Adaptive resistance is the result of gene expression modulations induced by environmental signals (e.g. pH, ion concentrations, nutrient conditions) that usually disappears if the triggering factor is removed (Christaki et al., 2019). Among these, conjugation has a significant role in the emergence of resistance (Guo et al., 2021).

Resistance appears in several forms. MDR means resistance to three or more agents from different antimicrobial classes; extensive drug-resistance (XDR) refers to the non-susceptibility

to at least one drug in all but two or fewer categories and pandrug-resistance (PDR) is the resistance to all antibiotics in all classes (Magiorakos et al., 2012). Resistance can be the result of different mechanisms. The main categories include inactivation of the active substance; modification, protection, or overproduction of the drug's target; and reduced accumulation of the antibiotic through increased efflux and/or decreased permeability (**Figure 1**) (Alav et al., 2018; Christaki et al., 2019).

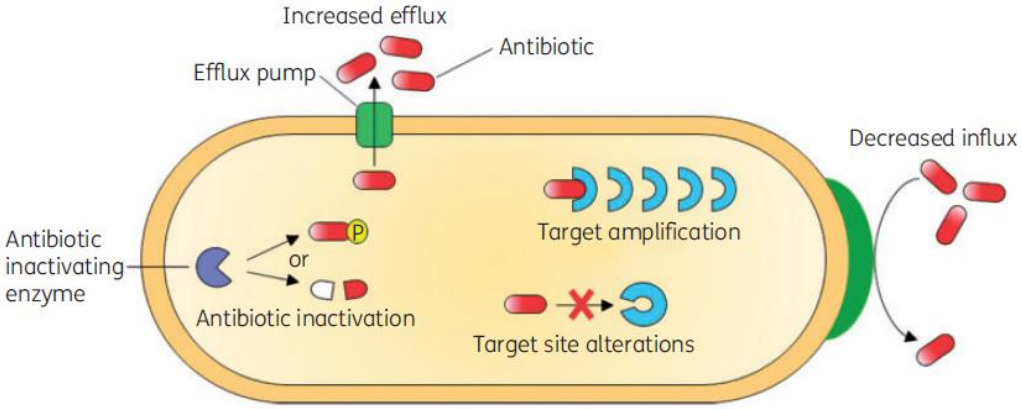

Figure 1. Main mechanisms of antibiotic resistance (Alav et al., 2018).

The emergence of AMR has been triggered by the overuse and misuse of antimicrobials in humans, animals, and agriculture (Tang K.L. et al., 2017; Christaki et al., 2019). As these areas are strongly linked to each other, the problem of AMR must be targeted interdisciplinary (Tang K.L. et al., 2017), under the so-called "One Health" approach (Christaki et al., 2019). Most common definition of this approach is as follows: "One Health is defined as a collaborative, multisectoral, and transdisciplinary approach — working at the local, regional, national, and global levels — with the goal of achieving optimal health outcomes recognizing the interconnection between people, animals, plants, and their shared environment" (Mackenzie and Jeggo, 2019).

Consumption of antimicrobials is considerably higher in food-producing animals than in humans, and it plays a role in not only the development and spread of resistance in animals, but in humans as well (Emes et al., 2022). Globally, 73% of all sold antimicrobials are used in food-producing animals (Van Boeckel et al., 2019). Resistant bacteria and ARGs from food-producing animals can be transmitted to humans through direct contact, food of animal origin and indirectly via contamination of the environment (Ter Kuile et al., 2016; Tang K.L. et al., 2017; Emes et al., 2022). Foodborne resistant bacteria can be commensal or pathogenic, and they can contaminate edible products during slaughter or further processing. They represent a threat to consumers in case of cross-contamination during cooking, or if insufficiently cooked meat is consumed. Afterwards, ARGs can be transmitted to the gut microbiota (Ter Kuile et al., 2016) that can serve as a reservoir for resistance genes and might spread them further to

pathogenic microorganisms (EFSA and ECDC, 2021) mainly via conjugation or transduction. Due to this phenomenon, currently there is a great demand to reduce the usage of antimicrobials in food-producing animals in order to decrease the occurrence of AMR in human medicine. However, it should be noted that development and spread of AMR in humans is not solely the result of antibiotic use in food-producing animals, as antibiotics used in human medicine and the agriculture also select for resistance. Furthermore, in case of some bacterium – drug combinations, it has been demonstrated that animals are unlikely to be the drivers of AMR in humans (Emes et al., 2022). Therefore, strategies created by the World Health Organization (WHO) to decelerate the spread of AMR include improvement of hygiene and optimization of antibiotic usage in both humans and animals (Tang K.L. et al., 2017).

Among the types of antibiotic use in food-producing animals, their administration as growth promoters has particular impact on the spread of resistance. Growth-promoting usage of antibiotics means their administration in subtherapeutic doses that can improve weight gain and feed conversion ratio of poultry, swine, and cattle. However, it was noted early that this practice increases the development and spread of resistance, therefore the administration of antibiotics as growth promoters have been banned for a long time in the European Union (EU), USA and Australia (Ter Kuile et al., 2016). Several countries (particularly in the EU) have made further efforts to decrease the usage of antibiotics in food-producing animals (Tang K.L. et al., 2017). The current 2019/6 EU regulation on veterinary medicinal products that came into force on the 28th January 2022 also include measures that aim to combat AMR (EU, 2019). However, antibiotics are still being used for growth promotion, prophylaxis and metaphylaxis, especially in low- and middle-income countries (Guo et al., 2021; Emes et al., 2022).

In a systematic analysis conducted on data from 204 countries in 2019, it was found that the leading pathogen associated with AMR in humans was *E. coli*, followed by *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. Altogether these were responsible for 3.57 million human deaths associated with AMR in 2019 (Antimicrobial Resistance Collaborators, 2022). Most of these bacteria belong to the so-called ESKAPE organisms (*Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter* species) that have frequent occurrence in hospital infections and a high rate of resistance (van der Kolk et al., 2019). These bacteria have been listed by the WHO as critical or high priority pathogens requiring new and effective therapeutic interventions (EMA, 2019). MDR is commonly found in microorganisms of food and animal origin, such as *E. coli*, *Salmonella*, *Campylobacter* and *Enterococcus* strains (Ter Kuile et al., 2016). These bacteria can transfer ARGs to microbes important in human health care (Ter Kuile et al., 2016), and one conjugation event can even result in the transfer of MDR when multiple ARGs occur on a single plasmid (Christaki et al., 2019).

3.1.2. Intervention strategies

The development of new antibiotics and the occurrence of bacteria resistant against them closely followed each other after the discovery of penicillin in 1928. Between 1960 and 1980, the production of new antimicrobials seemed to be adequate to keep pace with the evolution of bacteria, however, after the 1980s there was a significant decrease in the discovery of new antibiotic classes. Currently there are only a few new antibiotics under development, and a very low number of new antibiotic classes. Furthermore, there is a risk that the effective period of newly developed active substances will also be short due to fast evolution of microorganisms (Christaki et al., 2019).

Decreasing the usage of antibiotics and optimization of treatment strategies have an important potential in slowing down the development and spread of resistance (Ter Kuile et al., 2016). In a meta-analysis, it has been demonstrated that interventions that restrict the usage of antibiotics in food-producing animals result in a decrease of AMR prevalence in both animals and humans, therefore this strategy to combat AMR was found to be beneficial for both fields (Tang K.L. et al., 2017). Several international organizations, including the European Food Safety Authority (EFSA), the Food and Drug Administration (FDA), the Food and Agriculture Organization of the United Nations (FAO), the World Organisation for Animal Health (OIE) and the WHO have created guidelines to support these purposes (Ter Kuile et al., 2016). A comprehensive scientific advice that aims to promote prudent use of antibiotics in animals was prepared by the Antimicrobial Advice ad hoc Expert Group (AMEG) of the European Medicines Agency (EMA) taking into account recommendations of WHO and OIE. Based on their importance in human and veterinary medicine, and also on the risk of resistance development and transfer, antibiotics have been categorized to A, B, C and D groups, with all of them having different usage instructions in animals (EMA, 2019) (**Table 1**). This guideline provides an important bases for regulations on antibiotic usage at EU and national levels.

Table 1. Categorization of antibiotics by the Antimicrobial Advice ad hoc Expert Group (AMEG) of the European Medicines Agency (EMA, 2019). Antibiotic class examples include drugs that are important in veterinary medicine.

Category	Antibiotic class examples	Veterinary usage recommendations
Category A – Avoid	• Carboxy-, ureidopenicillins • Glycopeptides • Phosphonic acid derivates • Rifamycins	These drugs should not be used in food-producing animals, but might be given to companion animals under exceptional circumstances.
Category B – Restrict	• Fluoroquinolones • Polymyxins • Third and fourth generation cephalosporins	Use of these drugs should be considered if antibiotics in classes C and D could not be clinically effective. Their usage should be based on antibiotic susceptibility testing.
Category C – Caution	• Aminoglycosides • Aminopenicillins with beta lactamase inhibitors • First and second generation cephalosporins • Lincosamides • Macrolides • Phenicols • Pleuromutilins	Use of these drugs should be considered if antibiotics in class D could not be clinically effective.
Category D – Prudence	• Aminopenicillins without beta lactamase inhibitors • Narrow-spectrum penicillins • Sulfonamides • Tetracyclines	These drugs can be chosen as first line treatment, but still should be used prudently.

Monitoring the usage of antibiotics and the prevalence of resistance also have in important role in establishing measures against AMR and in following up their efficacy. At the EU level, these are regularly presented in the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) reports published by EMA (2021), and in the European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals, and food by the EFSA and the European Centre for Disease Prevention and Control (ECDC) (EFSA and ECDC, 2021). National competent authorities also have an important role in monitoring resistance status and the usage of antibiotics on farms, and supervising their good agricultural practice (Ter Kuile et al., 2016).

Strategies for addressing the challenge of antibiotic resistance include the following cornerstones: prevention and control of infections, including proper hygiene and vaccinations, minimizing the occurrence of unnecessary antibiotic usage, developing new antibiotics, and

improving access to second-line antibiotics where their availability is limited (Guo et al., 2021; Antimicrobial Resistance Collaborators, 2022). Optimization of the usage of already existing antibiotics (e.g. PK/PD based evidence, short term treatment at the highest authorized dosage, combinational or alternating therapies) could also be a way to tackle AMR (Ter Kuile et al., 2016; Guo et al., 2021). Furthermore, alternative, non-antibiotic approaches are being developed for targeting bacterial infections. These include for example the use of bacteriophages for the treatment of infections, immunomodulation of the host responses against specific pathogens and administration of monoclonal antibodies against bacterial strains (Christaki et al., 2019). On the veterinary field, it is indisputable that improved management, hygiene, and biosecurity measures are of utmost importance in the prevention of infections and therefore in reducing the need for antibiotic usage (Lüsckstädt and Theobald, 2011; Luppi, 2017). Furthermore, there is a great increase of research focused on finding antibiotic alternatives that can not only improve animal health, but productivity as well, thus leading to a reduced use of antibiotics in animal production. These include – without claim for completeness – phytochemicals, pre-, pro and synbiotics, enzymes, organic acids, antimicrobial peptides, and bacteriophages (Lillehoj et al., 2018).

Phytochemicals are natural bioactive substances of plant origin that possess antimicrobial, antioxidant, and immunomodulatory properties. Their main biologically active compounds are polyphenols, that occur in different composition and concentration depending on several factors, and thus can have an impact on the phytochemicals' mechanism of action (Lillehoj et al., 2018). Flavonoids are dietary polyphenols that exert various beneficial effects. Due to the emergence of AMR, the importance of research with these bioactive compounds found in plants is high (Adamczak et al., 2019). The increasing interest towards the use of flavonoids against diseases is also supported by the fact that they are synthesized by plants to provide protection against microbial infections and other environmental stressors (Biharee et al., 2020). Besides their use alone due to their beneficial properties, the combination of natural compounds and antibiotics to enhance antibacterial activity of drugs could also be an effective strategy to combat AMR (Sanhueza et al., 2017).

3.2. Flavonoids

The group of flavonoids include several thousands of ubiquitous bioactive substances found in fruits, vegetables, spices, grains, nuts, seeds, medicinal plants, propolis and honey (Cushnie and Lamb, 2005; Adamczak et al., 2019; Biharee et al., 2020). In humans, they are also consumed with olive or soybean oils, red wine, tea, and chocolate (Biharee et al., 2020). Flavonoids cannot be synthesized by humans and animals (Kumar and Pandey, 2013); they are produced as plant secondary metabolites, and show antibacterial, antiviral, antifungal, antioxidant, anti-inflammatory, neuroprotective, anticancer, immunomodulatory, antidiabetic, antithrombotic and antihyperlipidemic activities (Amin et al., 2015; Adamczak et al., 2019; Biharee et al., 2020). Another important property of flavonoids is their low systemic toxicity (Adamczak et al., 2019), which is supported by their abundant distribution in edible plants and usage in traditional medicine (Cushnie and Lamb, 2005). Amount and composition of flavonoids present vary among plant species and parts, and is also affected by environmental factors such as nutrient and water availability, sunlight, humidity, and soil type (Biharee et al., 2020). In restricted sense, the term flavonoid refers to substances comprising of a benzene ring (A), a pyran (C) and a phenyl group (B) on ring C (Rauter et al., 2018) (**Figure 2**).

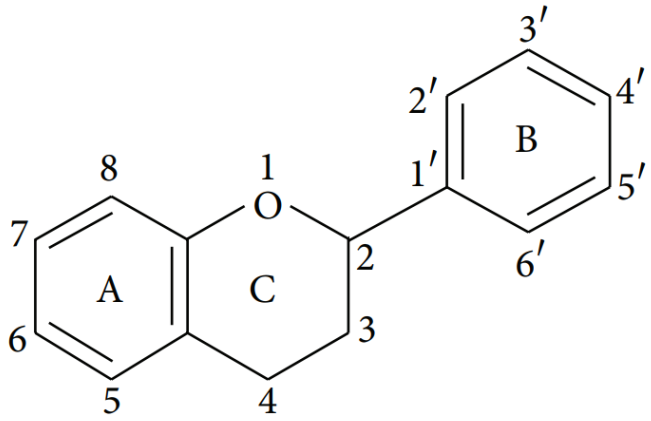

Figure 2. Core structure of flavonoids (Kumar and Pandey, 2013).

Flavonoids (in restricted sense) can be classified into 3 main classes based on their structure: (1) compounds with a 2-phenyl-3,4-dihydro-2*H*-1-benzopyran skeleton (flavans, flavanols, flavanones and leukoanthocyanidins); (2) compounds with a 2-phenyl-4*H*-1-benzopyran-4-one structure (flavones and flavonols); and (3) compounds derived from 2-phenyl-1λ^4-benzopyran-1-ylium (anthocyanidins) (Rauter et al., 2018). Major classes of polyphenols and flavonoids can be seen on **Figure 3** including classification of flavonoids tested in this study.

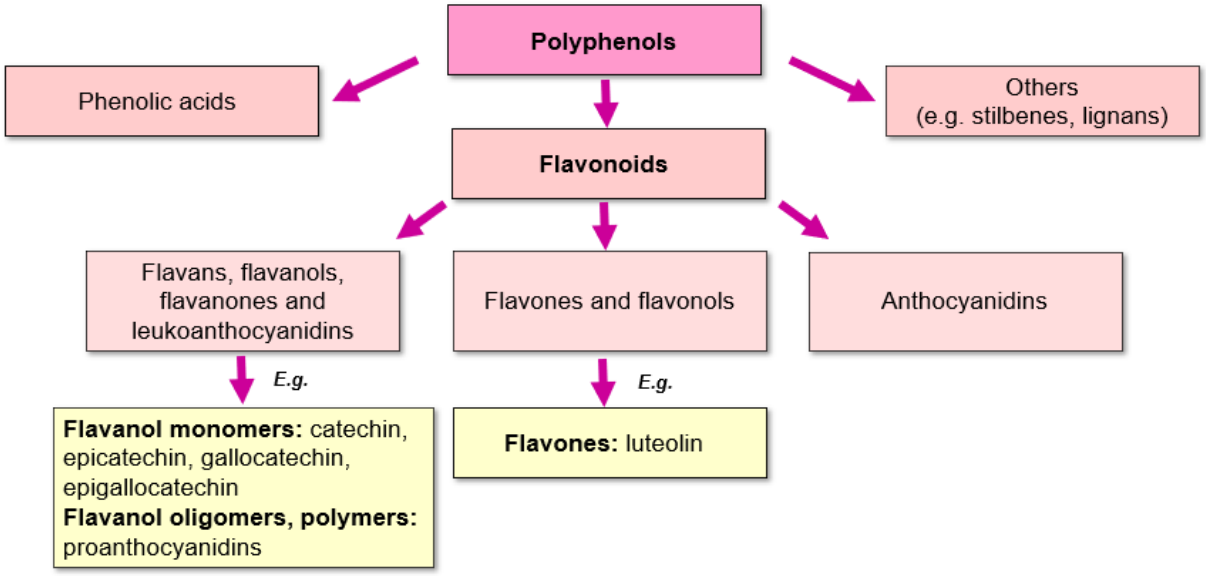

Figure 3. Main classes of polyphenols and flavonoids in restricted sense based on Belščak-Cvitanović et al., 2018 and Rauter et al., 2018. The figure includes classification of flavonoids tested in this study, proanthocyanidins and luteolin.

Diversity of the individual flavonoids' structure comes from the presence and location of hydroxyl groups, unsaturated bonds, and further functional groups (e.g. methoxy, carbonyl, olefinic) on the molecules. Furthermore, they can occur in plants in both aglycone and glycoside forms (Biharee et al., 2020). Structure of flavonoids has a significant impact on their bioavailability, metabolism, and biological activity (Kumar and Pandey, 2013). The role of flavonoids in plants include attracting pollinators, protection from pathogens, UV radiation, extreme temperature, heavy metals, and drought, and they are involved in energy transfer, photosynthesis, morphogenesis, and plant growth as well (Cushnie and Lamb, 2005; Biharee et al., 2020; González et al, 2021). They play a role in plants' defense against oxidative stress, with both inhibiting formation of reactive oxygen species (ROS) and inactivating ROS when present (González et al, 2021).

Most widely known and described property of flavonoids is their antioxidant effect in both humans and animals. It can be exerted via both scavenging ROS and interaction with enzymes related to oxidative stress. Among these properties, the former refers to the ability of flavonoids to donate hydrogen atom to free radicals, including superoxide, peroxyl, alkoxyl, and hydroxyl radicals (Kumar and Pandey, 2013). By donating hydrogen atom from a hydroxyl group, a flavonoid phenoxyl radical is formed that can react with another free radical resulting in the formation of a stable, quinone structure (**Figure 4**). The number and position of hydroxyl groups in flavonoids, especially those on ring B, have a significant impact on their ROS scavenging activity (Procházková et al., 2011; Kumar and Pandey, 2013; Hošek and Šmejkal,

2015). Furthermore, aglycone and glycoside forms show different scavenging capacity, with the former being more potent (Procházková et al., 2011; Kumar and Pandey, 2013; Hošek and Šmejkal, 2015).

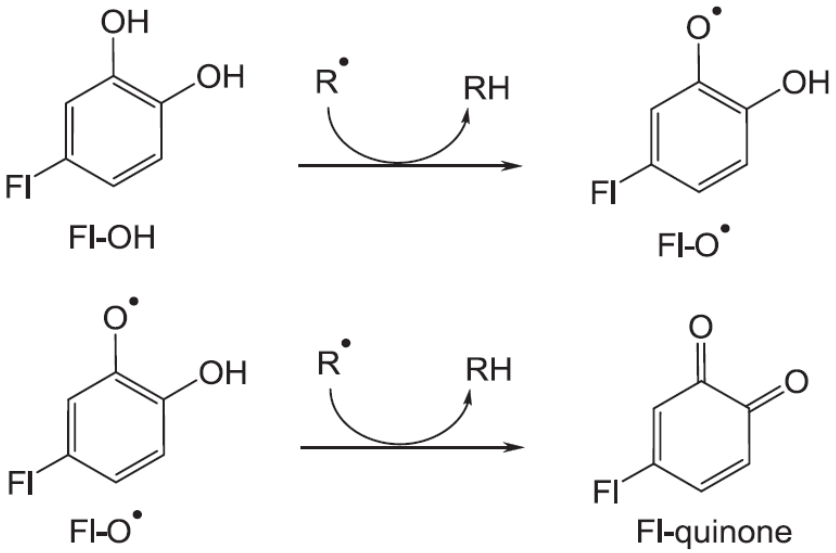

Figure 4. Free radical scavenging reactions of flavonoids (Procházková et al., 2011).

Another possible antioxidant mechanism of flavonoids is that they can chelate metals, e.g. free iron and copper, that would act as enhancers of ROS generation (Pietta, 2000) since free metal ions can participate in the Fenton reaction (Hošek and Šmejkal, 2015). In the Fenton reaction, iron(II) reacts with hydrogen peroxide resulting in a hydroxyl radical and a hydroxide ion (Bystrom et al., 2014). Besides iron, various metal ions, including copper can participate this reaction (Goldstein et al., 1993). Consequently, metal chelating activity of flavonoids can also play a role in their protective activity against free radicals (Procházková et al., 2011), and this property is also related to molecular structural elements, including the presence of hydroxyl and oxo groups in certain positions (Pietta, 2000). Besides scavenging ROS and binding metals, flavonoids can inhibit enzymes responsible for ROS generation such as microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase and NADH (nicotinamide adenine dinucleotide hydrogen) oxidase (Kumar and Pandey, 2013). They can also inhibit xanthine oxidase and protein kinase C, enzymes involved in superoxide anion production (Pietta, 2000). Furthermore, they can induce the activity of antioxidant enzymes, for example the glutathione S-transferase (Procházková et al., 2011). Several flavonoids are known to impact the nuclear factor erythroid 2-related factor 2 (Nrf2) - mediated antioxidant pathway as well (Yuan et al., 2021A), that is one of the main routes of cell defense against oxidative stress (Sova and Saso, 2018). Under physiological circumstances, Nrf2, a transcription factor, binds to the repressor protein, Keap1 (Kelch-like ECH-associated protein-1). In case of oxidative stress, Keap1 is modified and releases Nr2, which is then translocated

into the nucleus, where it can impact gene expression of enzymes involved antioxidant defense (by binding to the antioxidant response element [ARE] in the regulatory regions of target genes) (Sova and Saso, 2018). Thus, the activation of Nrf2 by flavonoids is another important underlying mechanism of their antioxidant activity and it might also be related to other beneficial effects of flavonoids. In oxidative stress, a cross talk between Nrf2 and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) has been reported, suggesting the possible role of Nrf2 in the regulation of inflammation (Paredes-Gonzalez et al., 2015). Nrf2 activity is also important in the intestines for the expression of TJ proteins and membrane assembly (Piotrowska et al., 2021).

Although antioxidant property of flavonoids is well-known, it should be noted that they can act as pro-oxidants as well depending on the circumstances (Procházková et al., 2011). The occurrence of antioxidant or pro-oxidant activity of flavonoids observed in different experimental settings can be influenced by the concentrations and treatment durations in which they are applied, as well as the type of cell cultures used for the investigation, the cells' environment and culture conditions, and the presence of other nutrients (Ju et al., 2007; Chung et al., 2009; Chedea et al., 2010).

Another advantage of flavonoids is that many of them have been shown to have anti-inflammatory effect by affecting the immune system via inflammatory cell modulation (Kumar and Pandey, 2013). This anti-inflammatory effect of flavonoids can partly be the result of their antioxidant activity (Gendrisch et al, 2021). Mechanisms behind flavonoids' anti-inflammatory activity can be the followings: suppression of immune cell activity (e.g. neutrophil granulocytes and macrophages), inhibition of inflammatory mediator production (e.g. interleukins, interferon gamma [IFN-γ] and tumor necrosis factor alpha [TNF-α]), and modulation of signaling pathways (e.g. NF-κB, peroxisome proliferator-activated receptor [PPAR], mitogen-activated protein kinase [MAPK] and AP-1 [activator protein 1]) (Yi, 2018). Flavonoids can inhibit kinase enzymes and phosphodiesterases, that are involved in immune cell activation, and the expression of other enzymes (inducible nitric oxide synthase, cyclooxygenase, and lipoxygenase) that would be responsible for the synthesis of inflammatory mediators including nitric oxide, prostanoids, leukotrienes, cytokines, chemokines, and adhesion molecules (Kumar and Pandey, 2013).

Furthermore, several flavonoids have demonstrated antibacterial properties against a wide range of bacteria, including *E. coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis*, *Enterobacter cloacae*, *K. pneumoniae*, *Proteus mirabilis*, and *P. vulgaris*. They can also be active against resistant strains, such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE). Besides their own antibacterial effect, flavonoids can act synergistically with each other

and with antibiotics as well (Cushnie and Lamb, 2005; Amin et al., 2015). Antimicrobial effect of flavonoids can be exerted through various mechanisms, including disruption of cell membrane and inhibition of virulence factors, microbial enzymes, efflux pumps, biofilm formation, cell envelop synthesis, nucleic acid synthesis, ATP (adenosine triphosphate) synthesis and bacterial motility (**Figure 5**) (Kumar and Pandey, 2013; Biharee et al., 2020). Antibacterial mechanisms of flavonoids can be different compared to conventional antibiotics. As a consequence of this, genes encoding resistance against flavonoids might not be present yet, so they could provide an effective therapeutic option (Biharee et al., 2020). Different flavonoids might target different parts of bacteria, and one substance can have more than one sites of action (Cushnie and Lamb, 2005; Kumar and Pandey, 2013; González et al., 2021). The potential pro-oxidant (ROS inducer) activity of flavonoids might also be among their antibacterial mechanisms, as it has been shown to contribute to the killing activity of bactericidal antibiotics (Dwyer et al., 2009; Dwyer et al., 2014).

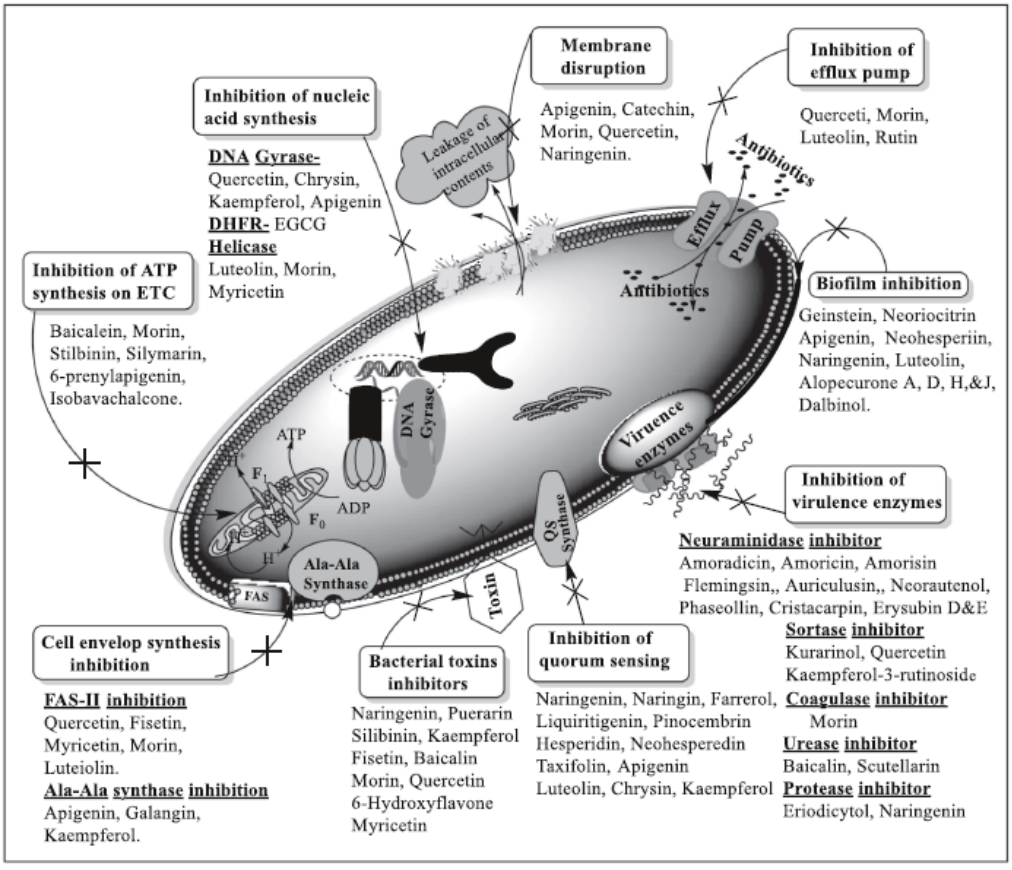

Figure 5. Possible mechanisms of action behind antibacterial effect of flavonoids, including substances that exert their activity via the mechanism shown (Biharee et al., 2020). Ala: alanine, ADP: adenosine diphosphate, ATP: adenosine triphosphate, DHFR: dihydrofolate reductase, DNA: deoxyribonucleic acid, EGCG: epigallocatechin gallate, ETC: electron transport chain, FAS-II: fatty acid synthase type II.

Further properties of phytochemicals that can be beneficial in the prevention and treatment of bacterial infections are their ability to inhibit the adhesion of bacteria to natural and synthetic surfaces (Šikić Pogačar et al., 2016; Fu et al., 2021), and their protective effect on the intestinal barrier integrity through modulating the expression of tight junction (TJ) proteins (Noda et al., 2012; Sharma et al., 2020). Besides their effect on TJs, flavonoids' barrier protective effect in the GI tract is also related to their effect on oxidative stress and the intestinal immune system, as well as their potential interaction with the GI microbiota (Wang et al., 2021).

3.2.1. Proanthocyanidins

Proanthocyanidins (PACs), also known as condensed tannins, occur naturally in fruits (e.g. berries and grape), seeds, nuts (e.g. peanut and almond), tea, cocoa, wine and some cereals (Rodríguez-Pérez et al., 2019). PACs can be presented as dimers, trimers, tetramers and pentamers (Cushnie and Lamb, 2005) of flavanol units linked together with C4-C8 or C4-C6 (B type), and possibly an additional C2-O7 (A type) bond (Gu et al., 2004). For their classification in the group of polyphenols, see **Figure 3**. Grape seed PACs contain only B-type linkages (Rodríguez-Pérez et al., 2019), and the main flavanol monomers present in them are catechin, epicatechin, epicatechin-3-*O*-gallate, epigallocatechin and gallocatechin (**Figure 6**) (Unusan, 2020). PACs can have the ability to bind bacterial endotoxin (lipopolysaccharide, LPS) as well (González-Quilen et al., 2020).

PACs possess strong antioxidant and anti-inflammatory properties, and therefore can be beneficial in oxidative stress and inflammation related medical conditions, such as cardiovascular diseases, diabetes mellitus, obesity, and cancer (Rodríguez-Pérez et al., 2019). They can directly reduce the amount of free radicals (Unusan, 2020), and can increase the activity of antioxidant enzymes, such as the glutathione peroxidase and the superoxide dismutase (Rodríguez-Pérez et al., 2019). It has been shown in numerous studies, that the antioxidant activity of PACs involves activation of the Nrf2 pathway (Liu et al., 2018; Rajput et al., 2019; Xu et al., 2019; Chen et al., 2020). However, pro-oxidant properties of PACs have also been reported previously (Chung et al., 2009; Chedea et al., 2010; Azam et al., 2004). The anti-inflammatory activity of PACs relies on modulating cell signaling pathways and decreasing the production and release of inflammatory mediators. For example, they were shown to modulate the NF-κB and MAPK pathways (Unusan, 2020). In mice and rat models, grape seed PACs could decrease the level of inflammatory mediators such as interleukin (IL) -1β, IL-6, IFN-γ and TNF-α (Rodríguez-Pérez et al., 2019). Besides the above-mentioned properties, grape seed extracts have shown antibacterial activity in several studies (Unusan, 2020) and PACs can have the ability to bind bacterial endotoxin (lipopolysaccharide, LPS) as well (González-Quilen et al., 2020).

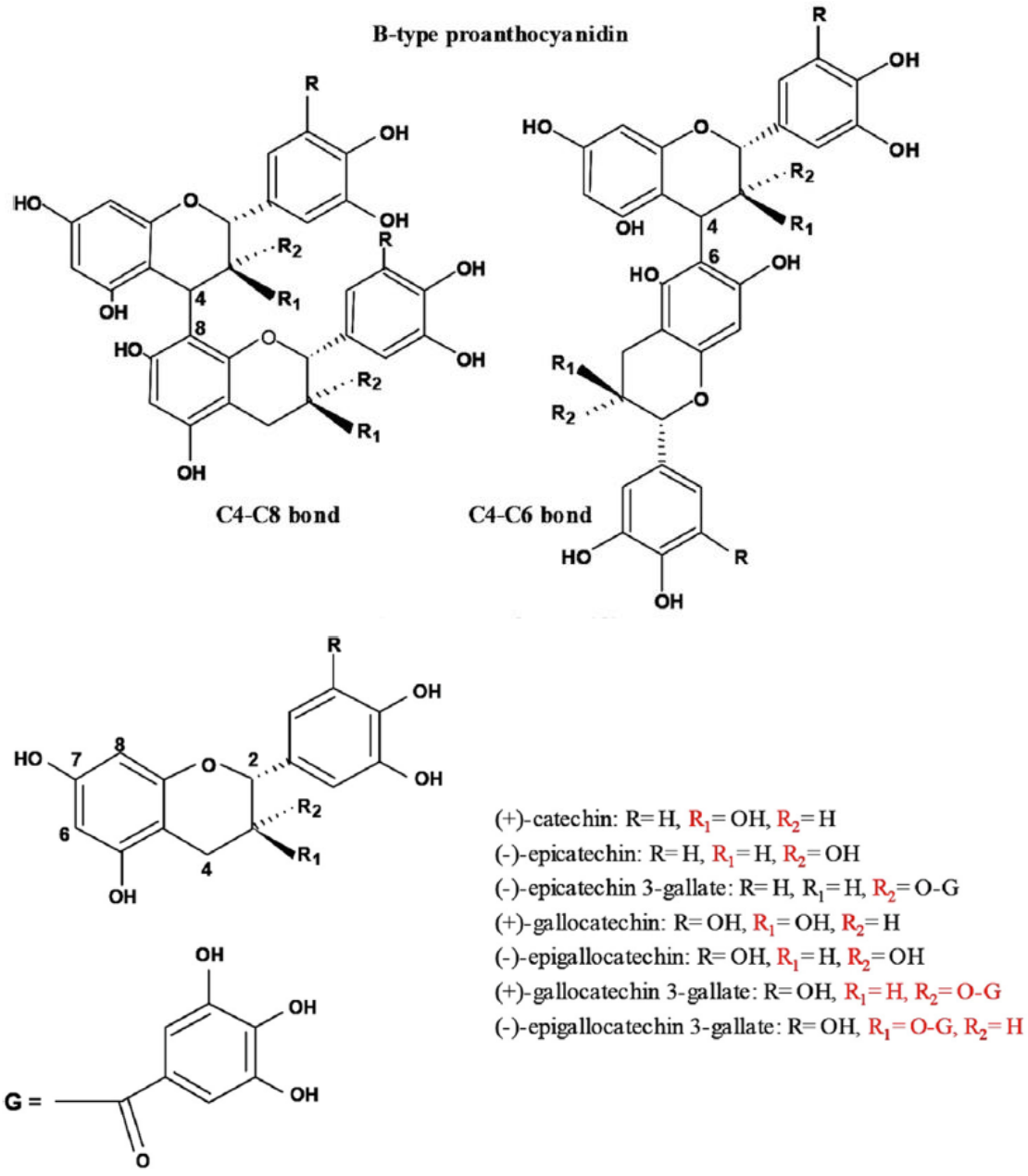

Figure 6. Chemical structure of grape seed proanthocyanidins (Unusan, 2020).

3.2.2. Luteolin

Luteolin (3',4',5,7-tetrahydroxyflavone, LUT) is a natural, abundant flavonoid found in several flowers, herbs (e.g. thyme, parsley, oregano and peppermint), vegetables (e.g. broccoli and cabbages) and spices (cardamom and anise) (Guo et al., 2020; Gendrisch et al., 2021). LUT belongs to the flavone class of flavonoids (**Figure 3**) (Adamczak et al., 2019) and contains hydroxyl groups at position 3', 4' (B ring) and 5, 7 (A ring) (Cushnie and Lamb, 2005) (**Figure 7**). Naturally, LUT is found in glycosylated form (Nabavi et al., 2015), including for example its 8- and 6-glucosides (orientin and isoorientin, respectively) that are present in buckwheat, corn silk and acai fruits (Adamczak et al., 2019).

LUT possesses several beneficial properties, such as antimicrobial, antioxidant, anti-inflammatory, antiallergic, and anticancer activities (Guo et al., 2020). There are various mechanisms behind its anti-inflammatory and antioxidant activity. For example, anti-inflammatory effect of LUT is exerted via the regulation of NF-κB, AP-1, and JAK-STAT (Janus kinases - signal transducer and activator of transcription proteins) signaling pathways. LUT is able to suppress proinflammatory mediators including IL-1β, IL-6, IL-8, and TNF-α (Gendrisch et al, 2021). Its antioxidant property comes from free radical scavenging, binding transitional metal ions, and interacting with cellular redox systems (Gendrisch et al, 2021). Similarly to PACs, LUT was able to activate the Nrf2 antioxidant pathway in several investigations (Huang et al., 2013; Pandurangan et al., 2014; Paredes-Gonzalez et al., 2015; Li et al., 2016; Kitakaze et al., 2020). ROS production contributes to the activation of MAPK pathway (Gendrisch et al, 2021), that can also be modulated by LUT (De Stefano et al., 2021). However, the potential pro-oxidant activity of LUT shown in studies (Ju et al., 2007; Wang et al., 2017) should also be mentioned. Antibacterial effect of LUT has been reported against different pathogens as well (Guo et al., 2020). Another important property of LUT that can be beneficial in Gram-negative infections is that it could inhibit LPS production of *E. coli* (Lee et al., 2010).

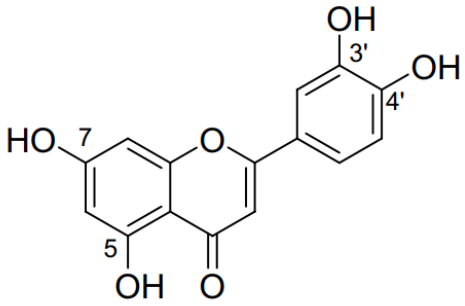

Figure 7. Chemical structure of luteolin (Lo et al., 2021).

Based on their various beneficial effects, grape seed PACs and LUT are promising candidates to be used as natural antibiotic alternatives.

3.3. Porcine gastrointestinal infections caused by *E. coli* and *Salmonella* spp.

Bacterial diarrhea is commonly diagnosed in food-producing animals, including pigs, and mostly affects them at young ages. Diarrhea in pigs is frequently caused by *E. coli* and *Salmonella* spp. infections, but as it is a multifactorial disease, predisposing factors such as viruses, parasites, inappropriate housing conditions and the susceptibility of animals also have an influence on disease prevalence and severity. Diarrhea results in loss of productivity, and a consequential economic problem in pig production (Edfors-Lilja et al., 2000). Furthermore, GI infections caused by *E. coli* and *Salmonella* spp. have public health implications (Edfors-Lilja et al., 2000), as both of them are zoonotic and can be spread to humans through the food chain. The transmission of these pathogens might include the transfer of ARGs as well that developed in food-producing animals (EFSA and ECDC, 2021).

Infections of the GI tract begins with the adhesion of bacteria to epithelial cells, followed by colonization of the intestinal epithelium, and then, in some cases invasion of cells and/or production of toxins (Reis and Horn, 2010; Luppi, 2017). The GI tract has an essential role in digestion and absorption of foods, and besides that, it constitutes a large, complex barrier surface that is constantly in contact with bacteria, bacterial products, and other antigens (Wang et al., 2021). Function of the intestinal barrier includes defending the organism from exogenous harmful substances and preventing the translocation of pathogens of the GI tract to the blood circulation (Liu et al., 2021). The barrier consists of a mucous layer, epithelial cells, TJs, commensal microbiota, immune cells, intestinal alkaline phosphatase, and antibacterial peptides (Noda et al., 2012; Ghosh et al., 2020; Wang et al., 2021). Among these, TJs includes proteins such as the zonula occludens-1 (ZO-1), occludin, and claudin-1 (Liu et al., 2021), that have an important role in regulating paracellular transport through the barrier (Noda et al., 2012). Under physiological circumstances, the intestinal barrier restricts paracellular transport of bacteria and LPS (Ghosh et al., 2020).

LPS is a constituent of Gram-negative bacteria's cell wall (Prins et al., 1994; Sampath, 2018; Nighot et al., 2019; Ghosh et al., 2020), that has a lipid A part, a carbohydrate core, and a polysaccharide O-antigen (Lepper et al., 2002; Nighot et al., 2019). LPS is an immunologically active component of the cell wall, that can induce the release of pro-inflammatory cytokines during Gram-negative infections (Lepper et al., 2002) and that is the main bacterial factor in the pathogenesis of Gram-negative septic syndrome/shock (Prins et al., 1994; Sampath, 2018). LPS is mainly recognized by the immune cells via Toll-like receptor 4 (TLR4), but it can also bind to caspases in the cytoplasm leading to inflammasome activation (Nunes-Alves, 2014). As part of the GI barrier, intestinal alkaline phosphatase has a role in detoxifying LPS

by modifying its lipid A moiety and thus inhibiting intracellular signaling that would be activated by LPS and would lead to the release of inflammatory mediators (Ghosh et al., 2020).

In the intestines, LPS can induce morphological injury and oxidative stress (Wang et al., 2015; Sundaram et al., 2020). Furthermore, during infections, bacteria and LPS present in the GI tract can disrupt TJs of the intestinal barrier (Reis and Horn, 2010; Tran et al., 2018; Nighot et al., 2019). Damages done to the intestinal barrier can lead to increased permeability, and the consequential penetration of bacteria and LPS into the systemic circulation, resulting in various intestinal and extra-intestinal disorders (**Figure 8**) (Noda et al., 2012; Ghosh et al., 2020; Liu et al., 2021). Therefore, protection of barrier integrity could have a role in the prevention and treatment of diseases associated with intestinal barrier impairment (Noda et al., 2012; Nighot et al., 2019; Wang et al., 2021).

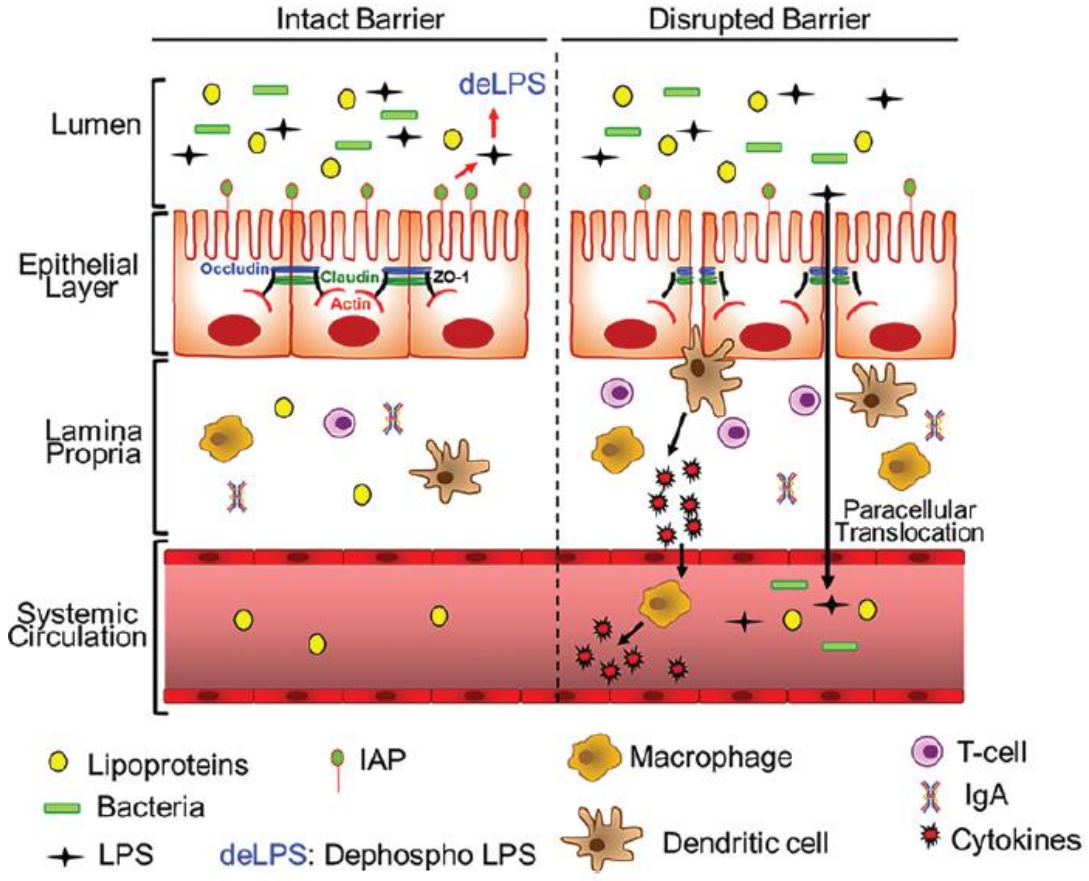

Figure 8. Main protective parts of the intestinal barrier and the consequences of its disruption. Under physiological conditions, paracellular transport of bacteria and their endotoxin (LPS) is restricted by tight junction (TJ) proteins (e.g. occludin, claudin and zonula occludens-1 (ZO-1)). Dephosphorylation (and consequential detoxication) of LPS (deLPS) is done in the lumen by intestinal alkaline phosphatase (IAP). Immune cells (e.g. macrophages, dendritic cells and T-cells) are found in the lamina propria. In case of barrier disruption (triggered by pathogenic bacteria or LPS for example), TJs are damaged, leading to paracellular transport of bacteria and LPS and a consequential immune response. Bacteria, LPS and proinflammatory cytokines produced by immune cells can also enter the systemic circulation resulting in a systemic inflammatory reaction (Ghosh et al., 2020).

If LPS is translocated to the blood circulation, it causes systemic inflammation and septic shock (Ghosh et al., 2020) through the release of inflammatory mediators such as TNF- α, IL-1 and IL-6 (Prins et al., 1994). IL-8 also belongs to the main mediators of endotoxin effect (Lepper et al., 2002). LPS can be liberated from bacteria's cell wall naturally, during their replication and lysis, leading to its highly increased biological activity. Furthermore, significant LPS release from the cell wall of bacteria can be induced by antibiotic therapy due to destruction of microorganisms which has a remarkable role in the pathogenesis of sepsis and septic shock, and the deterioration of the disease (Prins et al., 1994; Lepper et al., 2002).

E. coli is a Gram-negative bacterium, belonging to the Enterobacteriaceae family (Luppi, 2017; Heredia and García, 2018). It is a commensal and opportunistic pathogen that is found in the normal GI microbiota of both humans and animals, and that is harmless in many cases (Tran et al., 2018; Rhouma et al., 2017). Diseases are caused by pathogenic strains of *E. coli*, that harbor virulence factors and can be classified as enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC) (including a subset, called enterohemorrhagic *E. coli* [EHEC]), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), and adherent invasive *E. coli* (AIEC) (Heredia and García, 2018).

In pigs, *E. coli* can cause various diseases at several age groups, but the most common form is diarrhea at young ages (neonatal and post-weaning) (Rhouma et al., 2017; Poirel et al., 2018). ETEC is the most important pathotype in swine diseases that causes neonatal colibacillosis and post-weaning diarrhea resulting in significant economic losses due to mortality, decreased weight gain and treatment costs (Koh et al., 2008; Moredo et al., 2015; Luppi, 2017; Tran et al, 2018). These *E. coli* infections are among the most significant swine diseases worldwide (Luppi, 2017). However, ETEC can be present in swine populations without clinical signs and can be shed from healthy animals as well (Moredo et al., 2015), which cases also represent a possible source for foodborne infections. In humans, infections with *E. coli*, including ETEC strains, are common sources of diarrhea, mainly, but not only, in developing countries (Lückstädt and Theobald, 2011; Moredo et al., 2015). Symptoms of *E. coli* infections include abdominal cramps and pain, vomiting and diarrhea (Switaj et al., 2015).

E. coli is intrinsically susceptible to most antibiotics, however, it also has a significant capacity of acquiring ARGs, and as a result, MDR strains are widely observed in humans and animals worldwide. *E. coli* strains represent a major reservoir of ARGs to several antibiotic classes that are shared between humans and animal species (Poirel et al., 2018). According to the EFSA, *E. coli* is one of the most relevant antibiotic resistant bacteria in pigs in the EU, but the prevalence of resistant strains varies between countries, and is also different outside the EU.

Globally, resistance to aminopenicillins (without beta-lactamase inhibitors), tetracyclines and sulfonamides is high in *E. coli* isolates obtained from pigs, with an average 70% of them being resistant to these substances. It means that these relatively old and widely used drugs are unlikely to be effective against porcine *E. coli* infections in many countries. Furthermore, MDR *E. coli* (e.g. extended-spectrum beta-lactamase [ESBL] producers) were also found in numerous cases in the survey of EFSA with 34.2% of porcine indicator *E. coli* isolates being MDR (EFSA, 2021). Even though there are several preventive measures to control colibacillosis is pigs (e.g. improving hygiene and biosecurity, increasing immunity of piglets and administration of feed supplements), antibiotics are still widely used prophylactically and therapeutically as well against these infections (Luppi, 2017). However, the above-mentioned data about the prevalence of AMR in this bacterial species suggest decreased efficacy of antibiotic usage and supports the need for alternative strategies.

Salmonella spp. are also Gram-negative bacteria, members of the Enterobacteriaceae family. They are found naturally in the environment and in the GI tract of most animals (Heredia and García, 2018; Tran et al., 2018). *S*. enterica is a major species of *Salmonella*, that has 6 subspecies, among which subspecies *enterica* is abundant in warm-blooded animals and humans. *S. enterica* subsp. *enterica* includes the most dominant serovar, *S*. Typhimurium (Heredia and García, 2018).

Similarly to *E. coli*, *Salmonella* infections represent major enteric diseases in animals, that can result in septicemia, and can also have different clinical manifestations affecting further organs (Souto et al., 2017). In pigs, *S.* Typhimurium is the most frequently isolated *Salmonella* serovar in the EU and the United States. Infections with *S*. Typhimurium can result in enterocolitis with clinical signs such as diarrhea and dehydration. The disease usually develops in animals with poor hygiene conditions and other concurrent health problems (D'Incau et al., 2021). *Salmonella* spp. can infect animals at any ages, but weaned piglets are most commonly affected by the bacteria (Souto et al., 2017). Albeit animals usually recover from the disease, they can carry and shred the bacteria for a long time (D'Incau et al., 2021). It should also be noted that *Salmonella* infections can also be present without symptoms, and it is more common than the clinical disease (Souto et al., 2017).

Besides the widespread prevalence of *Salmonella* spp. in pigs, salmonellosis was the most common cause of foodborne diseases in 2019 and the second most frequent zoonosis of all in the EU with nearly 90,000 human cases (EFSA and ECDC, 2021). It is also among the most common foodborne infections worldwide (Heredia and García, 2018). The subspecies *enterica* is responsible for more than 99% of human salmonellosis (Heredia and García, 2018), among which *S*. Typhimurium is one of the most frequent serotypes in foodborne diseases (Sun et al.,

2020). Foodborne transmission of *S*. Typhimurium occurs via raw or undercooked eggs, dairy products, and meat (Heredia and García, 2018). The bacterium causes gastroenteritis in humans with the clinical signs of abdominal pain, nausea, vomiting and diarrhea, and it might lead to septicemia as well, resulting in systemic symptoms and possibly death (Lückstädt and Theobald, 2011; Heredia and García, 2018).

Similarly to *E. coli*, the public health impact of *Salmonella* infections is exacerbated by the presence of AMR in the pathogens (Sun et al., 2020). It was found by the EFSA and ECDC (2021) that *Salmonella* spp. isolated from food-producing animals and food of animal origin in the EU are frequently insusceptible to ampicillin, tetracyclines and sulfonamides (resistance rates in pig carcass isolates: 48.9%, 52.7%, 52.1%, respectively). Furthermore, similarly high rates of resistance against these antibiotic groups were reported about human *Salmonella* isolates during the same period of time (2018-2019). Overall, MDR was observed in 43.3% of pig carcass isolates, but it is important to note that all the collected data had high variability between countries (EFSA and ECDC, 2021). These findings support the above-described connection between the use of antibiotics in food-producing animals and the spread of resistance in humans, which can make the treatment of these infections more difficult.

It is important to note, that besides *E. coli* and *Salmonella* spp., other pathogens can also cause GI infections in swine with serious economic consequences (Varga et al., 2007). Furthermore, pig production is not the only source of *E. coli* and *Salmonella* foodborne infections, as for example, poultry products represent the most important reason of human *Salmonella* cases (Lückstädt and Theobald, 2011). Additionally, besides the above-detailed bacteria, further microbes, including viruses (e.g. norovirus), bacteria (e.g. *Campylobacter* spp., *Listeria* spp.*)* and parasites (e.g. *Giardia* spp. and *Toxoplasma gondii*) can cause foodborne illnesses as well (Switaj et al., 2015). Among these, *C. jejuni* and *C. coli* isolates from humans, food-producing animals, and food of animal origin were found to have high rate of resistance against some antibiotics, so these can also contribute to the spread of AMR. MRSA strains could be detected in meat and milk samples as well (EFSA and ECDC, 2021).

In the EU, between 2009 and 2019 the trends in resistance to different antibiotics varied (EFSA and ECDC, 2021). Prevalence of resistance against some agents were decreasing, which suggests the effectiveness of national and EU-level measures about optimizing the usage of antibiotics. However, there were still increasing trends in resistance in many countries against other agents, which supports the need for further actions to combat AMR, including the usage of antibiotic alternatives. Especially because of the import and export of live animals and animal products, AMR in one region might pose a risk to human, animal, and environmental health at places outside borders (Pungpian et al., 2021).

3.4. Cell cultures and the IPEC-J2 cell line

Cell cultures are popular tools in laboratory research, as they are widely available and easy to handle (Capes-Davis et al., 2019). They are used in various fields, such as drug and vaccine development, tissue and genetic engineering, and cancer biology (Preksha et al., 2021). Research with cell cultures is important as it can contribute to the implementation of the 3R (Replacement, Reduction and Refinement) principle that includes actions to decrease the number of live animals used in experiments and to minimize their suffering (EMA, 2016). Studies with cell lines can replace *in vivo* experiments at some extent. Generally, cell cultures can be comprised of primary cells, directly isolated from tissues, or immortalized/cancerous cell lines (Preksha et al., 2021). Primary cell cultures have limited availability, repeatability and shorter life span compared to cell lines, and they are more challenging to work with (León-Rodríguez et al., 2019; Preksha et al., 2021). However, they are more reflective of the *in vivo* circumstances than cell lines (Welser, 2015). Cell lines are usually of tumor origin, infected with viral agents or otherwise altered to have a transformed phenotype (Stacey, 2012). They are generally highly proliferative and easy to culture, but they can differ from the original tissues genetically and phenotypically (Welser, 2015).

Among others, cell cultures are important in GI research (Preksha et al., 2021). They can be used for investigating bioavailability and toxicology of food components and pharmaceutics. Furthermore, they provide an important opportunity to test the beneficial effects of phytochemicals, including their antioxidant and anti-inflammatory properties, as well as their interactions with the intestinal barrier and microbiota. Although the extrapolation of *in vitro* findings to *in vivo* should be done carefully, *in vitro* models of the GI tract have an essential role in research (León-Rodríguez et al., 2019). There are a wide range of *in vitro* and *ex vivo* models that have been developed to study the GI tract (Rahman et al., 2021). Currently, cell-based studies are mainly conducted in two-dimensional cultures including one or more cell types (León-Rodríguez et al., 2019). In 2D intestinal cell cultures, cells proliferate until they form a confluent monolayer covering the whole surface of the cell culture flask. Their growth has the following phases: lag (no growth after seeding), log (exponential growth), and stationary (constant cell number due to nutrient deprivation and cell-to-cell contact inhibition) (Preksha et al., 2021). To mimic the GI tract more closely, cell cultures containing intestinal cells and immune cells together are also used (León-Rodríguez et al., 2019). Additionally, three-dimensional cell cultures are gaining interest (León-Rodríguez et al., 2019; Preksha et al., 2021), as well as further *in vitro* and *ex vivo* systems that can be used for modeling the GI tract (e.g. organoids, Ussing chamber, Everted Sac, microfluidic gut-on-chip). All these have advantages and disadvantages that impact their usage for different purposes (Rahman et al., 2021).

In GI research, commonly used human intestinal cell lines include HT-29, T84, and Caco-2, that are derived from the colon and contain carcinoma or adenocarcinoma cells. HuTu-80 is the only widely available human cell line that is originated from the duodenum, but it is also a cancerous cell line (Brosnahan and Brown, 2012). In contrast to the above-mentioned cell lines, IPEC-J2 is unique in terms of originating from the jejunum and being non-transformed (Brosnahan and Brown, 2012). IPEC-J2 is a permanent porcine intestinal epithelial cell line that was isolated from the jejunum a neonatal, unsuckled piglet in 1989 by Helen Berschneider at the University of North Carolina (Berschneider, 1989). These cells can divide and grow for an infinite number of passages (Vergauwen, 2015). Advantage of IPEC-J2 is that by being a non-transformed, non-tumorigenic cell line, it represents normal cells more appropriately than transformed cell lines (**Figure 9**) (Geens and Niewold, 2011; Vergauwen, 2015).

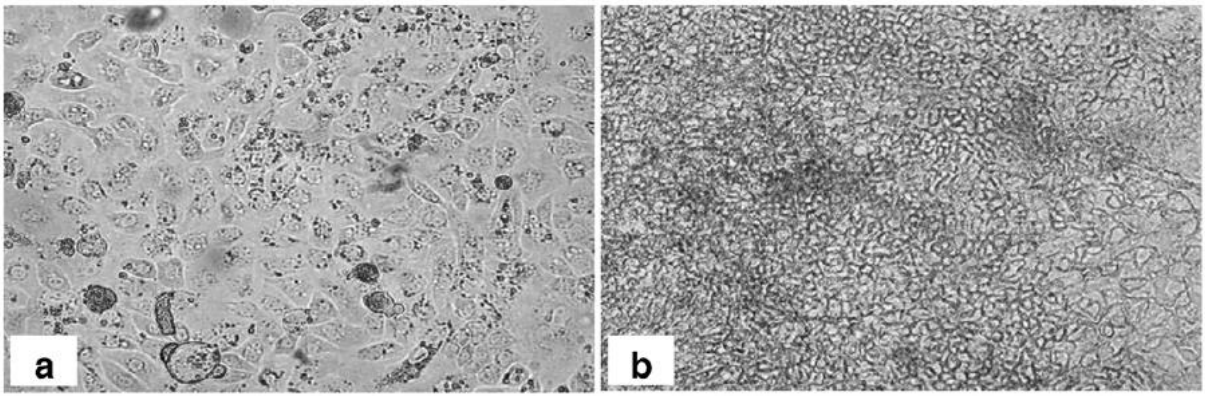

Figure 9. Growth morphology differences of IPEC-J2 (a), a non-tumorigenic intestinal epithelial cell line, and Caco-2 (b), the human colonic adenocarcinoma cell line (Cencic and Langerholc, 2010).

Main strength of IPEC-J2 is its morphological and functional similarity to epithelial cells *in vivo* (Vergauwen, 2015). These cells grow in a confluent monolayer and are able to become polarized with spontaneous differentiation, resulting in high transepithelial electrical resistance (TEER), under appropriate culture conditions. They express TJ proteins (e.g. claudin-1, occludin and ZO-1), form TJs and microvilli, but they do not produce mucus. Toll-like receptors and several cytokines have also been reported to be expressed by IPEC-J2 cells, including IL-6, IL-8 and TNF-α (Schierack et al., 2006; Brosnahan and Brown, 2012; Vergauwen, 2015).

There are two more porcine intestinal epithelial cell lines, IPEC-1 (ileal and jejunal, non-transformed cell line from day old piglet) and IPI-2I (ileal, transformed cell line from adult boar) (Brosnahan and Brown, 2012), but IPEC-J2 is the most widely characterized and used. IPEC-1 and IPEC-J2 are both spontaneously immortalized, but IPEC-J2 is morphologically and functionally more differentiated. For example, IPEC-J2 cells show more microvilli, as well as higher DNA, ATP and protein content than IPEC-1 (Nossol et al., 2015).

IPEC-J2 cells are widely used for investigating the following phenomena: infections with various human and animal pathogens (e.g. *E. coli*, *S*. Typhimurium, *Chlamydia* spp., rotavirus, vesicular stomatitis virus); effects of mycotoxins; adhesive and anti-inflammatory properties of probiotics; and effects of food components on several intestinal parameters (e.g. barrier permeability, immune response, inflammation) (Brosnahan and Brown, 2012; Vergauwen, 2015).

Besides being used in porcine specific studies, findings on IPEC-J2 cells may provide information about the human intestine as well. The reason behind this is, that among all non-primates, the GI tract's weight, size, anatomy, and physiology in pigs is the most conform to humans. Furthermore, the species also show similarities in their diet, therefore, pigs can serve as reliable GI models for human research (Geens and Niewold, 2011; Vergauwen, 2015). Among all cell lines of non-human origin, IPEC-J2 mimics human conditions most closely (Vergauwen, 2015). Compared to rodents and their cell lines (e.g. IEC-6 and IEC-18, non-transformed cell lines from rat small intestine and ileum, respectively [Zakrewski et al., 2013]), that are also used in studies with human relevance, pigs and the IPEC-J2 mimics human GI structure and function more closely (Geens and Niewold, 2011; Brosnahan and Brown, 2012).

Due to the above-mentioned similarities in the human and porcine GI tracts, IPEC-J2 is an important tool for investigating zoonotic pathogens, such as *E. coli* and *S*. Typhimurium. In numerous studies, IPEC-J2 cells were used to test the adhesive properties of *E. coli* strains. Besides that, it has also been reported that infection with ETEC reduces TEER of IPEC-J2 monolayers. *S*. Typhimurium can invade IPEC-J2 cells and can trigger the production of IL-8, TNF-α and β-defensins in them (Schierack et al., 2006; Brosnahan and Brown, 2012). In these studies, IPEC-J2 cells were demonstrated to provide an excellent model for studying enteric infections (Schierack et al., 2006; Brosnahan and Brown, 2012), including zoonotic pathogens. However, it should be noted that *in vitro* and *ex vivo* models cannot fully reflect the complex *in vivo* system of the GI tract (Rahman et al., 2021).

4. Aims of research

In this study, our aim was to test flavonoids, grape seed oligomeric proanthocyanidins (GSOP) and luteolin (LUT) for their potential beneficial effects *in vitro*, in models of porcine gastrointestinal infections caused by potentially zoonotic bacteria (*E. coli* and *S. enterica* ser. Typhimurium). Firstly, we investigated the impact of these flavonoids on cell viability to determine their concentrations that can be used safely on IPEC-J2 cells. Then we treated IPEC-J2 cells with bacterial endotoxin (lipopolysaccharide, LPS) of *E. coli* and *S*. Typhimurium origin and investigated the antioxidant effect of flavonoids (GSOP and LUT) against oxidative stress caused by LPS. In case of Gram-negative infections, LPS plays an important role in worsening the symptoms, especially when it is released in high amount during antibiotic therapy (Prins et al., 1994; Lepper et al., 2002). Both GSOP and LUT were applied in different concentrations in combination with LPS to evaluate potential correlation between their concentration and the observed activity.

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

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

The obtained results are not only important on the field of veterinary medicine, but might be extrapolated to public health, due to zoonotic potential of the investigated pathogens. As resistant bacteria threaten animal and public health as well, it is inevitable that the two fields work together under the "One Health" approach to combat AMR (WHO, 2015).

5. Materials and methods

5.1. Chemicals and instruments used in the study

Grape seed oligomeric proanthocyanidins (GSOPs, Reference Standard of the United States Pharmacopeia; main components: procyanidin B1, procyanidin B2, gallic acid, catechin, epicatechin and epicatechin-3-*O*-gallate; 0.988 mg of purified grape seeds oligomeric proanthocyanidins per mg of material on the anhydrous basis) were obtained from Sigma-Aldrich (Darmstadt, Germany).

Supplier of most other chemicals used in this study (luteolin [LUT]; dimethyl sulfoxide [DMSO]; LPS [suitable for cell culture, derived from *Salmonella enterica* ser. Typhimurium, *Escherichia coli* O111:B4 and *E. coli* O127:B8]; growth medium of IPEC-J2 cells [Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient, DMEM/F12]; Neutral Red dye; 2',7'-dichlorodihydrofluorescein diacetate [DCFH-DA] reagent; enzyme-linked immunosorbent assay [ELISA] kits; fluorescein isothiocyanate–dextran 4 kDa [FD4] dye; Triton X-100) was Sigma-Aldrich (Darmstadt, Germany) as well. Amplex Red Hydrogen Peroxide/Peroxidase Assay Kits were ordered from Thermo Fisher Scientific (Waltham, MA, USA). Mueller-Hinton liquid broth (MH), tryptone soya agar (TSA), ChromoBio Coliform and ChromoBio Salmonella Plus Base selective agars were obtained from Biolab Zrt. (Budapest, Hungary).

Cell culture plates were purchased from Corning Inc. (Corning, NY, USA), while microplates used for studies on bacterial strains were supplied by VWR International (Radnor, PA, USA).

For absorbance measurement, EZ Read 400 Microplate Reader (Biochrom Ltd, Cambridge, United Kingdom) and SpectraMax iD3 (Molecular Devices, San José, CA, USA) were used, while fluorescence was measured with Victor X2 2030 fluorometer (PerkinElmer Inc., Waltham, MA, USA) and SpectraMax iD3. Statistical analysis of the obtained data was conducted with R software (R Foundation for Statistical Computing, Vienna, Austria).

5.2. Studies on IPEC-J2 cells

5.2.1. Cell culture conditions

Experiments were performed on the IPEC-J2 porcine intestinal epithelial cell line, that originates from the jejunum of a neonatal, unsuckled piglet. IPEC-J2 cells were kindly provided by Dr. Jody Gookin (Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, USA). Cells were cultured 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) containing the following supplementations: 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 (100-100 IU/ml) for cell culturing (full DMEM/F12). Experiments were performed with IPEC-J2 cells at a passage number of approximately 50 and working solutions were prepared with plain DMEM/F12 without supplements. Cells were seeded onto 96- and 6-well polystyrene cell culture plates (tissue culture treated, without coating) for the cell viability and ROS assays (DCFH-DA, Amplex), respectively, and were incubated until forming a differentiated, confluent monolayer which was regularly inspected under light microscope. Seeding density was approx. 10^4 cells/well on 96-well plates and 10^5 cells/well on 6-well plates (Corning Inc.). Experiments were started around 2 or 5 days after seeding in case of 96- and 6-well plates, respectively. Cell culture medium was changed every other day during culturing.

5.2.2 Cell viability assay

Possible cytotoxic effect of purified GSOP and LUT at different concentrations and incubation time periods was tested with Neutral Red method based on the description of Repetto et al. (2008). Viable cells have the ability of incorporating the supravital dye in their lysosomes (Repetto et al., 2008), therefore a higher number of viable cells are shown with increased absorbance values in this assay. GSOP was applied on IPEC-J2 cells at the concentrations of 50, 100 and 200 µg/ml, for 1, 12 and 24 hours each. Similar incubation times were used in case of LUT, which was tested at 25, 50 and 100 µg/ml concentrations. Both GSOP and LUT were dissolved in plain DMEM/F12 medium for the experiment and applied on cells being cultured on 96-well plates. For the complete dissolution of LUT, DMSO was added to the working solutions at 2.5, 5 and 10% for 25, 50 and 100 µg/ml of LUT, respectively. The effect of DMSO on cell viability has been tested in preliminary experiments and it did not alter cell viability at the concentrations used in our experiments. Treatment with plain medium for 1 hour was used as control in the cell viability assay. Ratio of living cells was determined after the end of last treatments (24 hours) by absorbance measurement with Biochrom EZ Read 400 Microplate Reader (at 540 nm wavelength). The experiment was performed with 6 replicates per treatment group.

5.2.3. Determination of IC ROS levels

To provoke oxidative stress, all lipopolysaccharides (*S*. Typhimurium and both *E. coli* origin) were applied on IPEC-J2 cells at 10 µg/ml concentration (Farkas et al., 2015). For determination of their potential antioxidant activity, both GSOP and LUT were added to the cells in combination with all types of LPS each, the former at 50, 100 and 200 µg/ml, while the latter at 25, 50 and 100 µg/ml concentrations. Effects of both GSOP and LUT on the amount of intracellular reactive oxygen species (IC ROS) alone were also tested at the same concentrations. Working solutions were incubated with the cells for 1 hour on 6-well plates. Cells treated only with plain medium served as control. To detect the amount of IC ROS, 10 µM DCFH-DA dye was used. IC ROS can oxidize DCFH-DA to a detectable fluorescent product, dichloro-fluorescein (Wang and Joseph, 1999), therefore elevated fluorescence values are proportional to the increased amount of IC ROS. The method is not specific to certain types of ROS as various free radicals are able to oxidize DCFH-DA resulting in the quantification of overall oxidative stress in cells (Wang and Joseph, 1999). The dye was added to the cells for 60 minutes, followed by rinsing with medium, scraping and centrifugation for 10 minutes (at 3000 g). Victor X2 2030 fluorometer was used to determine fluorescence of the samples (excitation wavelength: 480 nm, emission wavelength: 530 nm). The experiment was performed with 6 replicates per treatment group.

5.2.4. Determination of EC H_2O_2 levels

Besides measuring IC ROS level of cells, changes in extracellular (EC) H_2O_2 concentration in the cell supernatants have also been investigated after treatments with GSOP and LPS performed as described in 5.2.3. Based on the results of the investigation with GSOP, this experiment has not been conducted with LUT as the method was not found to be suitable for quantifying oxidative stress in IPEC-J2 cells caused by LPS. All types of LPS and GSOP were added to cells at similar concentrations and treatment times as in case of the IC ROS measurement. Plain medium was used as control on the untreated cells. For quantification of EC H_2O_2, Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit was used following instructions of the manufacturer. In this assay, Amplex Red reagent can react with H_2O_2 resulting in a fluorescent product, resorufin (Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit user manual). Consequently, increased fluorescence values are proportional with higher amount of H_2O_2 in the samples. Fluorescence intensity of the samples was measured with Victor X2 2030 fluorometer (excitation wavelength: 560 nm, emission wavelength: 590 nm). The experiment was performed with 6 replicates per treatment group.

5.3. Studies on bacterial strains

5.3.1. Origin and culturing of bacteria

MICs of GSOP and LUT were determined in 16 bacterial strains, including 8 *E. coli* and 8 *S*. Typhimurium strains isolated from the gastrointestinal tract or mesenteric lymph nodes of pigs. Afterwards, in the interaction studies, 4 *E. coli* and 4 *S*. Typhimurium from the same bacterial strains were included. Strains were stored at -80 °C in MH supplemented with 20% sterile glycerol before the experiments. 24 hours prior to MIC determination and interaction studies, bacteria were propagated in MH at 37 °C.

5.3.2. MIC determination

For determination of the antibacterial activity of GSOP and LUT, broth microdilution method was performed according to Clinical and Laboratory Standards Institute (CLSI) guideline M07-A10 (CLSI, 2015). GSOP and LUT were dissolved in DMSO for the investigation. Two-fold dilution of the solutions were prepared with MH broth on 96-well microtiter plates with the final concentrations of flavonoids being set to 4096, 2048, 1024, 512, 256, 128, 64, 32, 16 and 8 µg/ml (**Figure 10**). 24-hour cultures of the 8 *E. coli* and 8 *S*. Typhimurium bacterial strains were centrifuged for 10 minutes (at 3000 g), then washed and resuspended in physiological saline in order to achieve optical density of 0.1 at 600 nm, which is considered as equal to 10^8 colony forming units (CFUs) in 1 milliliter of the suspension and a standard of 0.5 on the MacFarland scale. Bacterial suspensions were then diluted to 10^6 CFU/ml and spread on TSA plates for control CFU counting. Inoculation of the suspensions on plates containing GSOP or LUT resulted in a final 10^5 CFU/ml concentration of bacteria. Each row on the microplates contained a different bacterial strain. This was followed by 24 hours of incubation at 37 °C and the evaluation of MIC values with the unaided eye. After determination of the MIC for each strain separately, MIC_{50} and MIC_{90} values (i.e. MIC that inhibits 50 or 90% of isolates, respectively) were calculated for both *E. coli* and *Salmonella*.

5.3.3. Interaction studies

Following MIC determinations of GSOP and LUT, interaction studies with checkerboard microdilution were performed to test if any of the flavonoids show synergistic, additive, or antagonistic effect with 3 highly effective and commonly used antibiotics (amoxicillin, gentamicin and enrofloxacin). 8 bacterial strains were used in this part of the study, including 4 *E. coli* and 4 *S*. Typhimurium strains from the isolates used in 5.3.2. MIC values of amoxicillin, gentamicin and enrofloxacin against these bacteria have been previously determined at the Department of Pharmacology and Toxicology, University of Veterinary Medicine Budapest.

Preparation of checkerboard microdilution method for the interaction studies was similar to MIC determination described in 5.3.2. except for the wells of the 96-well microtiter plates containing the combination of GSOP and LUT with antibiotics in different concentration ratios. Two-fold dilution of GSOP or LUT was prepared from the first column to the seventh column, while the antibiotics were diluted from the first row until the seventh row. The eights column and row only contained one of the substances alone. Column nine served as positive control, while column ten as negative control. In case of each tested bacterial strain, two-fold dilution of the substances were prepared to include concentrations at least two-fold higher and four-fold lower than the corresponding MIC values. See **Figure 11** for example.

Final step was the inoculation of bacteria similarly to phase 5.3.2. except for each well of one plate containing the same bacteria in this part. During the evaluation of interactions, bacterial growth was checked with the unaided eye similarly to MIC determination, followed by fractional inhibitory concentration (FIC) index calculation for all bacterial strains based on the followings:

$FIC_{flavonoid} = MIC_{flavonoid}$ in combination / $MIC_{flavonoid}$ alone

$FIC_{antibiotic} = MIC_{antibiotic}$ in combination / $MIC_{antibiotic}$ alone

$FIC_{index} = FIC_{flavonoid} + FIC_{antibiotic}$

An average of the obtained FIC indexes for all combinations were then calculated for *E. coli* and *S*. Typhimurium, and then evaluated as follows (Jerzsele and Pásztiné-Gere, 2015):

- < 0.5: synergy
- 0.5-1: partial synergy or additive
- 1-4: neutral
- > 4: antagonism

Figure 10. Final concentrations of grape seed proanthocyanidins (GSOPs) and luteolin (LUT) in the minimum inhibitory concentration (MIC) determination with the application of positive (+) and negative controls (-). Values are expressed in µg/ml.

	1	2	3	4	5	6	7	8	9	10	11	12
1	4096	2048	1024	512	256	128	64	32	16	8	+	-
2	4096	2048	1024	512	256	128	64	32	16	8	+	-
3	4096	2048	1024	512	256	128	64	32	16	8	+	-
4	4096	2048	1024	512	256	128	64	32	16	8	+	-
5	4096	2048	1024	512	256	128	64	32	16	8	+	-
6	4096	2048	1024	512	256	128	64	32	16	8	+	-
7	4096	2048	1024	512	256	128	64	32	16	8	+	-
8	4096	2048	1024	512	256	128	64	32	16	8	+	-

Figure 11. Final concentrations of luteolin (LUT) and gentamicin in the interaction study with the application of positive (+) and negative controls (-). Values are expressed in µg/ml, the first and second values representing concentration of LUT and gentamicin, respectively.

	1	2	3	4	5	6	7	8	9	10	11	12
1	512 / 8	256 / 8	128 / 8	64 / 8	32 / 8	16 / 8	8 / 8	0 / 8	+	-		
2	512 / 4	256 / 4	128 / 4	64 / 4	32 / 4	16 / 4	8 / 4	0 / 4	+	-		
3	512 / 2	256 / 2	128 / 2	64 / 2	32 / 2	16 / 2	8 / 2	0 / 2	+	-		
4	512 / 1	256 / 1	128 / 1	64 / 1	32 / 1	16 / 1	8 / 1	0 / 1	+	-		
5	512 / 0.5	256 / 0.5	128 / 0.5	64 / 0.5	32 / 0.5	16 / 0.5	8 / 0.5	0 / 0.5	+	-		
6	512 / 0.25	256 / 0.25	128 / 0.25	64 / 0.25	32 / 0.25	16 / 0.25	8 / 0.25	0 / 0.25	+	-		
7	512 / 0.125	256 / 0.125	128 / 0.125	64 / 0.125	32 / 0.125	16 / 0.125	8 / 0.125	0 / 0.125	+	-		
8	512 / 0	256 / 0	128 / 0	64 / 0	32 / 0	16 / 0	8 / 0	0 / 0	+	-		

5.4. Studies on IPEC-J2 cells – bacterium co-culture

5.4.1. Cell culture conditions

Culture condition of IPEC-J2 cells before the experiments was similar as in 5.2.1. For the investigations, cells were seeded onto 96- (Neutral Red), 24- (adhesion) or 6-well (DCFH-DA, Amplex, ELISA) polystyrene cell culture plates (tissue culture treated, without coating), or 12-well polyester membrane inserts (tissue culture treated, without coating, pore size: 0.4 µm) (FD4 assay). In case of 96- and 6-well plates, seeding and culturing was performed as detailed in 5.2.1. On the 24-well plates, seeding density was approx. 10^5 cells/well (Corning Inc.) and an average of 4 days culturing was necessary, with culture medium replacement every second day. For 12-well inserts, cells were seeded with the density of 10^5 cells/well (Corning Inc.) and experiments could be started circa 7 days later (culture medium change every other day).

5.4.2. Origin and culturing of bacteria

One-one *E. coli* and *S*. Typhimurium strains - originated from GI infections of pigs - were used in the experiments. Bacteria were kept frozen at -80°C until the beginning of investigations, when they were propagated in plain DMEM/F12 for 18-24 hours at 37 °C, with 5% CO_2 to mimic culture conditions of IPEC-J2 cells. Concentration of the overnight bacterial suspensions was determined with CFU counting.

5.4.3. Co-culture establishment

To determine maximum tolerable concentration of bacteria for co-culturing with IPEC-J2 cells, cell viability assay was performed with different amounts of bacteria. IPEC-J2 cells were cultured in full DMEM/F12 on 96-well microplates until a confluent monolayer was formed. Prior to bacterial infection, the medium was removed, cells were washed with phosphate buffered saline (PBS) and incubated in plain DMEM/F12 in order to eliminate antibiotic residues remaining from full DMEM/F12. Both bacterial strains were added to the cells at the concentrations of 10^4, 10^6 and 10^8 CFU/ml that were prepared by dilution with plain medium based on the results of CFU counting. Control cells received only plain medium. Treated and control cells were incubated for 1 hour (37 °C, 5% CO_2), when the supernatants were removed, cells were washed with PBS and then received full DMEM/F12 to prevent bacterial overgrowth. Ratio of living cells was determined 24 hours later with Neutral Red method (Repetto et al., 2008). Absorbance values, which correlate with the number of viable cells, were measured with SpectraMax iD3 (on 540 nm). The experiment was performed with 6 replicates per treatment group. Based on results of the cell viability assay and the relevant literature (Klingspor et al., 2015; Loss et al., 2018), bacterial suspensions with the concentration of 10^6 CFU/ml were used in further experiments in case of both strains.

5.4.4. Experimental design

For all investigations in the co-culture, similar experimental design and treatment groups were used. Cells were cultured in full DMEM/F12 until reaching a confluent monolayer in each well, and then were washed with PBS and incubated in plain DMEM/F12 before all experiments (to remove antibiotic residues). Afterwards, some of them were infected with bacteria at the concentration of 10^6 CFU/ml without previous, parallel, or subsequent GSOPs or LUT supplementation. Other cells received GSOPs treatment (50 and 100 µg/ml) or LUT treatment (25 and 50 µg/ml) 1 hour prior, together, or 1 hour after the bacterial infection (10^6 CFU/ml). Plain medium served as untreated control in all cases. The effect of GSOPs and LUT alone in the above-mentioned concentrations has also been tested in case of the ELISA and FD4 assays. Treatment groups of the experiment are summarized in **Table 2**. All treatments were applied on cells for 1 hour, which was followed by rinsing with PBS and adding antibiotic containing DMEM/F12 on them to prevent bacterial overgrowth in cases when further incubation was necessary.

Table 2. Treatment groups in the co-culture experiments. *Treatments only included in the ELISA and FD4 assay.

	Grape seed oligomeric proanthocyanidins (GSOPs)	Luteolin (LUT)	Bacterium
Control	-	-	-
GSOPs*	50/100 µg/ml	-	-
LUT*	-	25/50 µg/ml	-
E. coli	-	-	*E. coli* 10^6 CFU/ml
S. Typhimurium	-	-	*S*. Typhimurium 10^6 CFU/ml
GSOPs pre-treatment *E. coli*	50/100 µg/ml GSOPs 1 hour prior to infection	-	*E. coli* 10^6 CFU/ml
GSOPs pre-treatment *S*. Typhimurium	50/100 µg/ml GSOPs 1 hour prior to infection	-	*S*. Typhimurium 10^6 CFU/ml
LUT pre-treatment *E. coli*	-	25/50 µg/ml LUT 1 hour prior to infection	*E. coli* 10^6 CFU/ml
LUT pre-treatment *S*. Typhimurium	-	25/50 µg/ml LUT 1 hour prior to infection	*S*. Typhimurium 10^6 CFU/ml
GSOPs parallel treatment *E. coli*	50/100 µg/ml GSOPs together with infection	-	*E. coli* 10^6 CFU/ml
GSOPs parallel treatment *S*. Typhimurium	50/100 µg/ml GSOPs together with infection	-	*S*. Typhimurium 10^6 CFU/ml
LUT parallel treatment *E. coli*	-	25/50 µg/ml LUT together with infection	*E. coli* 10^6 CFU/ml
LUT parallel treatment *S*. Typhimurium	-	25/50 µg/ml LUT together with infection	*S*. Typhimurium 10^6 CFU/ml
GSOPs post-treatment *E. coli*	50/100 µg/ml GSOPs 1 hour after infection	-	*E. coli* 10^6 CFU/ml
GSOPs post-treatment *S*. Typhimurium	50/100 µg/ml GSOPs 1 hour after infection	-	*S*. Typhimurium 10^6 CFU/ml
LUT post-treatment *E. coli*	-	25/50 µg/ml LUT 1 hour after infection	*E. coli* 10^6 CFU/ml
LUT post-treatment *S*. Typhimurium	-	25/50 µg/ml LUT 1 hour after infection	*S*. Typhimurium 10^6 CFU/ml

5.4.5. Determination of IC ROS levels

To investigate potential antioxidant effect of GSOPs and LUT in the IPEC-J2-bacterium co-culture, DCFH-DA assay was used. For the assay, cells were cultured on 6-well plates, and the above-described treatments (5.4.4.) were performed on them, followed by 24 hours of incubation in antibiotic-containing medium. For detecting the amount of IC ROS, 10 µM DCFH-DA dye was applied on cells for 1 hour, then cells were rinsed, scraped and centrifugated (10 minutes, 3000 g). After centrifugation, fluorescence of the obtained supernatants was measured with SpectraMax iD3 (excitation wavelength: 485 nm, emission wavelength: 535 nm). IC ROS can oxidize DCFH-DA to the fluorescent dichlorofluorescein (Wang and Joseph, 1999), therefore higher amount of IC ROS is proportionally shown via increased fluorescence values. The experiment was performed with 6 replicates per treatment group.

5.4.6. Determination of EC H_2O_2 levels

For the investigation of oxidative stress in the co-culture, EC H_2O_2 levels have also been measured besides detection of IC ROS in case of GSOPs. However, taking into account the findings of the experiment with GSOPs, this investigation has not been performed with LUT as the method was found to be inappropriate for quantifying oxidative stress in IPEC-J2 cells caused by bacterial infection. Cells were cultured on 6-well plates for the assay and treated according to 5.4.4 (except for the LUT treatments). Following 24 hours of incubation after treatments, Amplex Red Hydrogen Peroxide/Peroxidase Assay was performed with the cell supernatants according to instructions of the manufacturer. Fluorescence measurement was performed with SpectraMax iD3 (excitation wavelength: 560 nm, emission wavelength: 590 nm) and increased fluorescence values meant proportionally higher EC H_2O_2 concentrations. The experiment was performed with 6 replicates per treatment group.

5.4.7. Determination of IL-6, -8 levels

To determine interleukin production of cells affected by bacteria, GSOPs and LUT, cells were cultured on 6-plate wells and the previously detailed experimental settings (5.4.4.) were followed. Samples were taken from the cell supernatants 6 hours after the end of treatments (Loss et al., 2018; Karancsi et al., 2020; Loss et al., 2020) for IL-6 and IL-8 measurement with porcine-specific IL-6 and IL-8 ELISA kits following instructions of the manufacturer. At the end of the protocol, absorbance measurement of the samples was performed with SpectraMax iD3 (on 450 nm). Higher absorbance values indicated an increased amount of interleukins in the samples. The experiment was performed with 6 replicates per treatment group.

5.4.8. Barrier integrity

To evaluate effect of bacteria, GSOPs and LUT on barrier integrity of the cell layer, IPEC-J2 cells were grown on 12-well membrane inserts for performing the treatments specified in section 5.4.4. Structure of the inserts is demonstrated on **Figure 12**. Afterwards, 0.25 mg/ml FD4 tracer dye was applied on them (i.e. in the apical compartment of wells), and samples were taken 3 and 24 hours later from the basolateral compartment (all sampling times measured from the end of treatment). Amount of FD4 in the samples (i.e. ratio of dye that could penetrate through the cell layer) was detected by fluorescent method with SpectraMax iD3 (excitation wavelength: 485 nm, emission wave-length: 535 nm). Higher fluorescence values indicated increased paracellular permeability as a result of barrier integrity disruption. The experiment was performed with 6 replicates per treatment group. TEER values were measured prior to the experiment to evaluate formation of a confluent, differentiated cell monolayer.

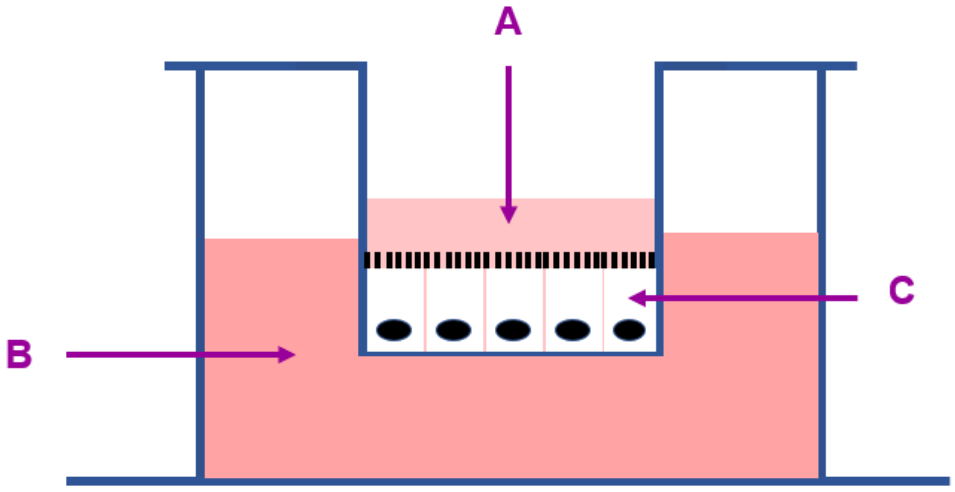

Figure 12. Structure of the inserts used for cell culturing in the barrier integrity assay. A: apical compartment, B: basolateral compartment, C: IPEC-J2 cell monolayer on permeable membrane.

5.4.9. Bacterial adhesion

To determine potential anti-adhesive effect of GSOPs and LUT, cells cultured on 24-well plates were treated in the above-mentioned manner (5.4.4.). After removal of the supernatants (i.e. bacteria not attached to IPEC-J2 cells) and washing with PBS, cells were lysed with 1% Triton X for 30 minutes on a shaker to release adhered and invaded bacteria (Polewski et al., 2016). A serial dilution was then prepared from the homogenized suspensions in each well and inoculated on selective agar plates (ChromoBio Coliform for *E. coli* and ChromoBio Salmonella Plus for *S*. Typhimurium) for overnight incubation, followed by CFU counting on the next day. The experiment was performed with 4 replicates per treatment group.

5.5. Statistics

Statistical analysis of data obtained in the cell culture experiments was performed with R 3.3.2 (2016) software. Mean values of different experimental groups were compared with one-way ANOVA and Tukey post hoc test. Results were interpreted as significant if p value was lower than 0.05. No statistical analysis was performed in case of studies with bacterial strains (MIC determination and interaction studies).

6. Results

6.1. Effects of grape seed proanthocyanidins and luteolin on IPEC-J2 cells

6.1.1. Cell viability assay

Purified GSOP did not show any negative effect on viability of IPEC-J2 cells in any of the applied concentrations and treatment durations. Measured absorbance values, which show correlation with the amount of viable cells, did not differ significantly between the untreated control and GSOP-treated cells in case of the shortest treatment period (1 h, with all concentrations) and in case of 50 and 100 µg/ml GSOP applied for 12 h. GSOP 200 µg/ml treatment for 12 h and all concentrations given for 24 h resulted in significantly increased absorbance values compared to the control ($p < 0.001$ in all cases except GSOP 50 µg/ml for 24 h: $p < 0.01$). Results of GSOP in the cell viability assay are shown in **Figure 13**.

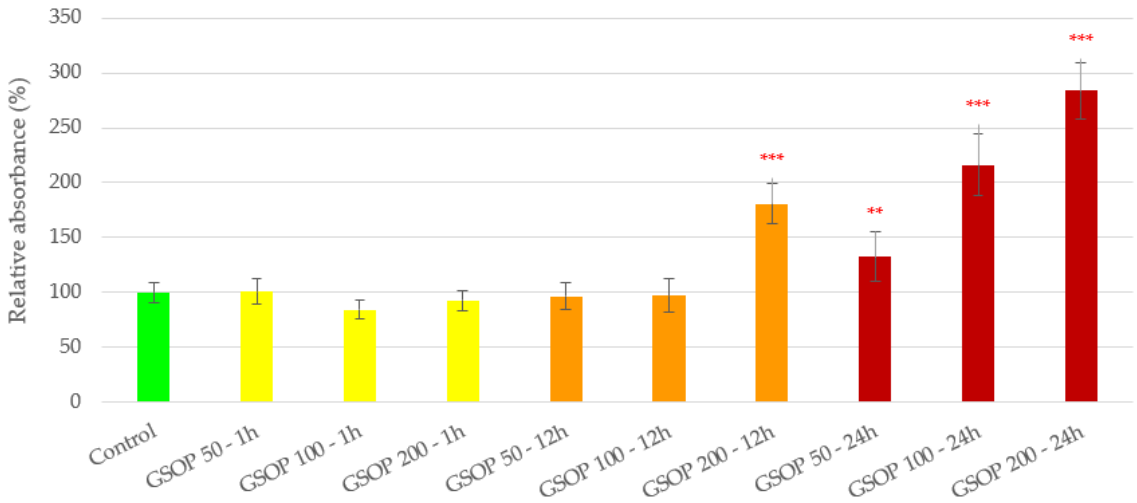

Figure 13. Viability of IPEC-J2 cells after treatment with purified grape seed oligomeric proanthocyanidins (GSOP). Control: untreated (plain medium only); GSOP 50 - 1h: 50 µg/ml GSOP treatment for 1 hour; GSOP 100 - 1h: 100 µg/ml GSOP treatment for 1 hour; GSOP 200 - 1h: 200 µg/ml GSOP treatment for 1 hour; GSOP 50 - 12h: 50 µg/ml GSOP treatment for 12 hours; GSOP 100 - 12h: 100 µg/ml GSOP treatment for 12 hours; GSOP 200 - 12h: 200 µg/ml GSOP treatment for 12 hours; GSOP 50 - 24h: 50 µg/ml GSOP treatment for 24 hours; GSOP 100 - 24h: 100 µg/ml GSOP treatment for 24 hours; GSOP 200 - 24h: 200 µg/ml GSOP treatment for 24 hours. Data are shown as means with standard deviations, and expressed as relative absorbance, considering the mean value of control as 100%. n=6/group. Significant difference compared to the untreated control: **$p < 0.01$, ***$p < 0.001$; asterisk in red: higher values than control.

Similarly to GSOP, treatment with LUT did not have any negative influence on IPEC-J2 cell viability. Treatment with 25 µg/ml LUT did not significantly alter absorbance (and therefore ratio of viable cells in the culture) regardless of treatment duration. However, all other treatments (i.e., higher concentrations of LUT applied for 1, 12 and 24 h) could significantly increase measured absorbance values ($p < 0.001$). Results of LUT in the cell viability assay are shown in **Figure 14**.

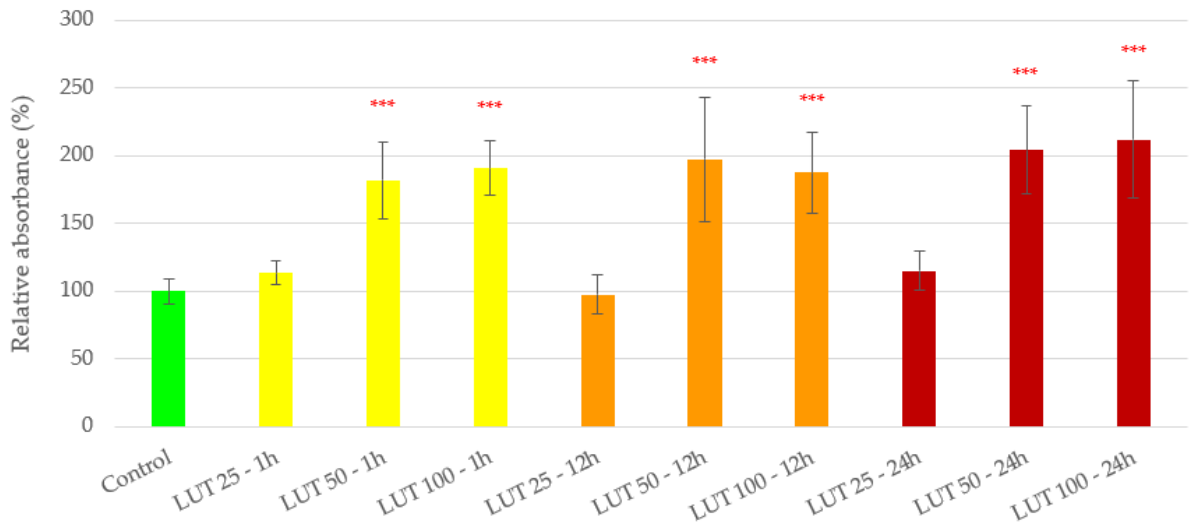

Figure 14. Viability of IPEC-J2 cells after treatment with luteolin (LUT). Control: untreated (plain medium only); LUT 25 - 1h: 25 µg/ml LUT treatment for 1 hour; LUT 50 - 1h: 50 µg/ml LUT treatment for 1 hour; LUT 100 - 1h: 100 µg/ml LUT treatment for 1 hour; LUT 25 - 12h: 25 µg/ml LUT treatment for 12 hour; LUT 50 - 12h: 50 µg/ml LUT treatment for 12 hours; LUT 100 - 12h: 100 µg/ml LUT treatment for 12 hours; LUT 25 - 24h: 25 µg/ml LUT treatment for 24 hours; LUT 50 - 24h: 50 µg/ml LUT treatment for 24 hours; LUT 100 - 24h: 100 µg/ml LUT treatment for 24 hours. Data are shown as means with standard deviations, and expressed as relative absorbance, considering the mean value of control as 100%. n=6/group. Significant difference compared to the untreated control: ***$p < 0.001$; asterisk in red: higher values than control.

6.1.2. IC ROS levels

Treatment with all three lipopolysaccharides caused oxidative stress in IPEC-J2 cells, resulting in significantly increased amount of intracellular ROS compared to the untreated control ($p < 0.001$). GSOP alone either did not change (50 and 100 µg/ml) or significantly decrease (200 µg/ml, $p < 0.001$) the amount of IC ROS in IPEC-J2 cells. When GSOP was combined with LPS, GSOP was able to alleviate harmful effect of the endotoxin in all combinations (i.e., all concentrations of GSOP combined with all types of LPS) except GSOP 100 µg/ml + *S.* Typhimurium LPS. In most cases, IC ROS levels of the combinations were similar to the control (GSOP 50 µg/ml + *S.* Typhimurium LPS; GSOP 100 µg/ml + *E. coli* O111:B4 LPS) or significantly lower (GSOP 200 µg/ml + *S.* Typhimurium LPS; GSOP 50 µg/ml + *E. coli* O111:B4 LPS; GSOP 200 µg/ml + *E. coli* O111:B4 LPS; GSOP in all concentrations + *E. coli* O127:B8 LPS). Results of GSOP in the DCFH-DA assay are shown in **Figure 15**.

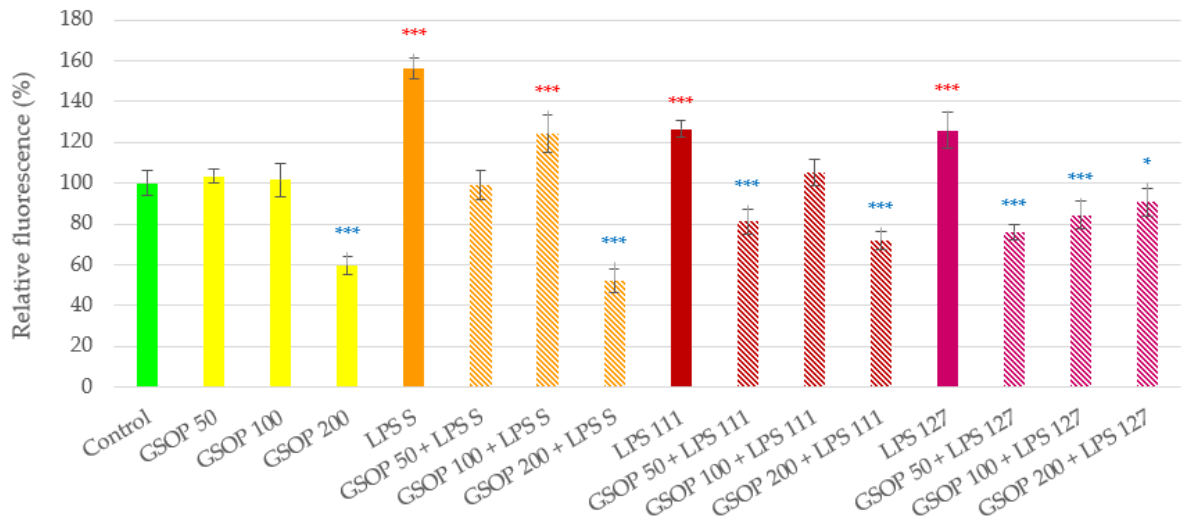

Figure 15. Amount of intracellular reactive oxygen species after treatment with bacterial endotoxin (LPS), purified grape seed oligomeric proanthocyanidins (GSOP) and their combinations. Control: untreated (plain medium only); GSOP 50: 50 µg/ml GSOP; GSOP 100: 100 µg/ml GSOP; GSOP 200: 200 µg/ml GSOP; LPS S: *S.* Typhimurium endotoxin 10 µg/ml; GSOP 50 + LPS S: 50 µg/ml GSOP + *S.* Typhimurium endotoxin 10 µg/ml; GSOP 100 + LPS S: 100 µg/ml GSOP + *S.* Typhimurium endotoxin 10 µg/ml; GSOP 200 + LPS S: 200 µg/ml GSOP + *S.* Typhimurium endotoxin 10 µg/ml; LPS 111: *E. coli* O111:B4 endotoxin 10 µg/ml; GSOP 50 + LPS 111: 50 µg/ml GSOP + *E. coli* O111:B4 endotoxin 10 µg/ml; GSOP 100 + LPS 111: 100 µg/ml GSOP + *E. coli* O111:B4 endotoxin 10 µg/ml; GSOP 200 + LPS 111: 200 µg/ml GSOP + *E. coli* O111:B4 endotoxin 10 µg/ml; LPS 127: *E. coli* O127:B8 endotoxin 10 µg/ml; GSOP 50 + LPS 127: 50 µg/ml GSOP + *E. coli* O127:B8 endotoxin 10 µg/ml; GSOP 100 + LPS 127: 100 µg/ml GSOP + *E. coli* O127:B8 endotoxin 10 µg/ml; GSOP 200 + LPS 127: 200 µg/ml GSOP + *E. coli* O127:B8 endotoxin 10 µg/ml. Data are shown as means with standard deviations, and expressed as relative fluorescence, considering the mean value of control as 100%. n=6/group. Significant difference compared to the untreated control: *$p < 0.05$, ***$p < 0.001$; asterisk in red: higher values, in blue: lower values than control.

LUT treatment alone on IPEC-J2 cells at 25 and 100 µg/mL did not influence IC ROS production; however, 50 µg/ml LUT treatment resulted in a significantly lower ROS level compared to the control ($p < 0.001$). All concentrations of LUT showed potent antioxidant activity against both *E. coli* and *S*. Typhimurium LPS treatments. IC ROS amount of the cells treated with LUT + LPS combinations were either similar (all concentrations of LUT + *S*. Typhimurium LPS; LUT 50 µg/ml + *E. coli* O111:B4 LPS; LUT 25 µg/ml + *E. coli* O127:B8 LPS) or significantly lower ($p < 0.01$ or lower) than the untreated control (LUT 25 µg/ml + *E. coli* O111:B4 LPS; LUT 100 µg/ml + *E. coli* O111:B4 LPS; LUT 50 µg/ml + *E. coli* O127:B8 LPS; LUT 100 µg/ml + *E. coli* O127:B8 LPS). The applied *Salmonella* LPS resulted in a higher IC ROS production increase compared to LPS of *E. coli* origin. As a consequence, antioxidant activity of LUT in combination with *Salmonella* LPS could reduce IC ROS amount to the control level, while when applied together with *E. coli* LPS, LUT was able to decrease IC ROS level below the control values. Results of LUT in the DCFH-DA assay are shown in **Figure 16**.

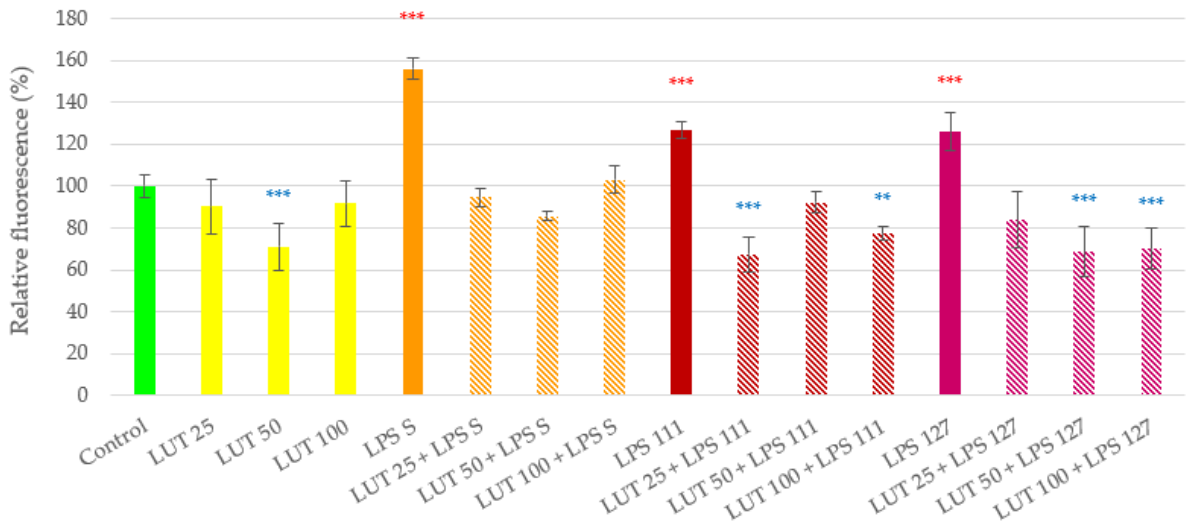

Figure 16. Amount of intracellular reactive oxygen species after treatment with bacterial endotoxin (LPS), luteolin (LUT) and their combinations. Control: untreated (plain medium only); LUT 25: 25 µg/ml LUT; LUT 50: 50 µg/ml LUT; LUT 100: 100 µg/ml LUT; LPS S: *S*. Typhimurium endotoxin 10 µg/ml; LUT 25 + LPS S: 25 µg/ml LUT + *S*. Typhimurium endotoxin 10 µg/ml; LUT 50 + LPS S: 50 µg/ml LUT + *S*. Typhimurium endotoxin 10 µg/ml; LUT 100 + LPS S: 100 µg/ml LUT + *S*. Typhimurium endotoxin 10 µg/ml; LPS 111: *E. coli* O111:B4 endotoxin 10 µg/ml; LUT 25 + LPS 111: 25 µg/ml LUT + *E. coli* O111:B4 endotoxin 10 µg/ml; LUT 50 + LPS 111: 50 µg/ml LUT + *E. coli* O111:B4 endotoxin 10 µg/ml; LUT 100 + LPS 111: 100 µg/ml LUT + *E. coli* O111:B4 endotoxin 10 µg/ml; LPS 127: *E. coli* O127:B8 endotoxin 10 µg/ml; LUT 25 + LPS 127: 25 µg/ml LUT + *E. coli* O127:B8 endotoxin 10 µg/ml; LUT 50 + LPS 127: 50 µg/ml LUT + *E. coli* O127:B8 endotoxin 10 µg/ml; LUT 100 + LPS 127: 100 µg/ml LUT + *E. coli* O127:B8 endotoxin 10 µg/ml. Data are shown as means with standard deviations, and expressed as relative fluorescence, considering the mean value of control as 100%. n=6/group. Significant difference compared to the untreated control: **$p < 0.01$, ***$p < 0.001$; asterisk in red: higher values, in blue: lower values than control.

6.1.3. EC H_2O_2 levels

In contrast to IC ROS levels, none of the LPS treatments resulted in a significant elevation of EC H_2O_2 concentrations of cell supernatants. A possible explanation of this observation could be that oxidative stress caused by LPS in IPEC-J2 cells is manifested by an increase of the amount of ROS other than H_2O_2. GSOP treatment at 100 and 200 µg/ml concentrations resulted in a significant decrease of H_2O_2 levels when used alone as well as when applied in combination with all types of LPS ($p < 0.001$ in all cases except for the 100 µg/ml GSOP + *E. coli* O111:B4 LPS combination, where $p < 0.01$ value was obtained). Therefore, this assay was considered to be inappropriate for investigating antioxidant effect of flavonoids against oxidative stress caused by bacterial endotoxin in IPEC-J2 cells, and as a result, it has not been performed with LUT. Data obtained with GSOP are shown on **Figure 17**.

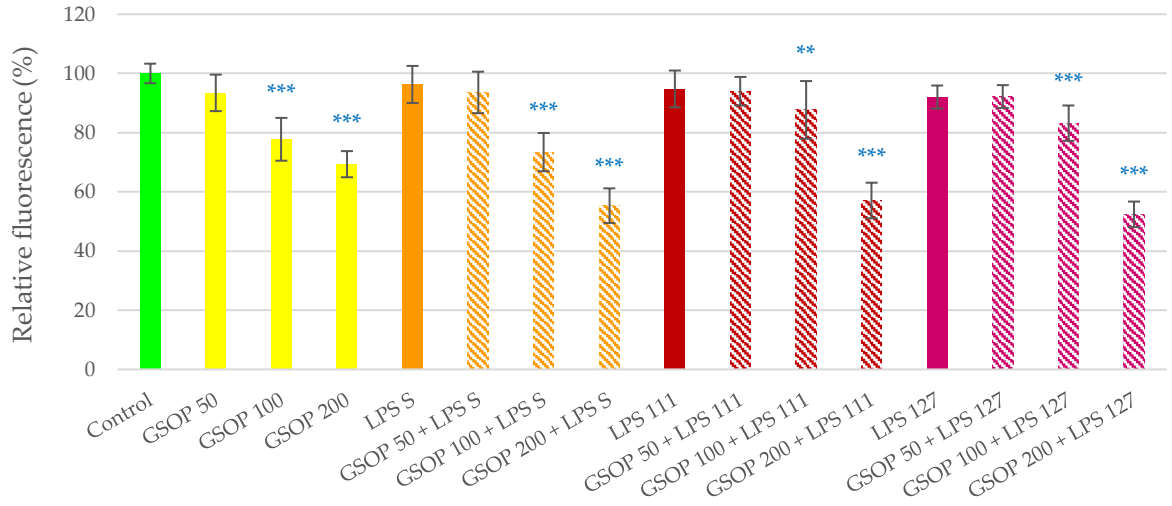

Figure 17. Amount of extracellular H_2O_2 after treatment with bacterial endotoxin (LPS), purified grape seed oligomeric proanthocyanidins (GSOP) and their combinations. Control: untreated (plain medium only); GSOP 50: 50 µg/ml GSOP; GSOP 100: 100 µg/ml GSOP; GSOP 200: 200 µg/ml GSOP; LPS S: *S.* Typhimurium endotoxin 10 µg/ml; GSOP 50 + LPS S: 50 µg/ml GSOP + *S.* Typhimurium endotoxin 10 µg/ml; GSOP 100 + LPS S: 100 µg/ml GSOP + *S.* Typhimurium endotoxin 10 µg/ml; GSOP 200 + LPS S: 200 µg/ml GSOP + *S.* Typhimurium endotoxin 10 µg/ml; LPS 111: *E. coli* O111:B4 endotoxin 10 µg/ml; GSOP 50 + LPS 111: 50 µg/ml GSOP + *E. coli* O111:B4 endotoxin 10 µg/ml; GSOP 100 + LPS 111: 100 µg/ml GSOP + *E. coli* O111:B4 endotoxin 10 µg/ml; GSOP 200 + LPS 111: 200 µg/ml GSOP + *E. coli* O111:B4 endotoxin 10 µg/ml; LPS 127: *E. coli* O127:B8 endotoxin 10 µg/ml; GSOP 50 + LPS 127: 50 µg/ml GSOP + *E. coli* O127:B8 endotoxin 10 µg/ml; GSOP 100 + LPS 127: 100 µg/ml GSOP + *E. coli* O127:B8 endotoxin 10 µg/ml; GSOP 200 + LPS 127: 200 µg/ml GSOP + *E. coli* O127:B8 endotoxin 10 µg/ml. Data are shown as means with standard deviations, and expressed as relative fluorescence, considering the mean value of control as 100%. n=6/group. Significant difference compared to the untreated control: **$p < 0.01$, ***$p < 0.001$; asterisk in blue: lower values than control.

6.2. Effects of grape seed proanthocyanidins and luteolin on bacterial strains

6.2.1. Antibacterial activity

In this study, antibacterial activity of GSOP against the investigated *E. coli* and *S*. Typhimurium strains was found only at high concentrations. GSOP could inhibit growth of all isolates with a MIC of 2048 µg/ml. As the bacteriostatic activity of GSOP was observed at the same concentration in the case of all isolates, obtained MIC_{50} and MIC_{90} values of GSOP were both 2048 µg/ml. However, LUT showed a more potent bacteriostatic effect and was able to inhibit all tested bacteria at 256 µg/ml concentration (MIC). Similarly to GSOP, MIC of LUT was similar against all strains; therefore, MIC_{50} and MIC_{90} values of LUT proved to be 256 µg/ml against the investigated *E. coli* and *Salmonella* field isolates. Obtained MIC_{50} and MIC_{90} values are shown in **Table 3**.

Table 3. Minimum inhibitory concentration (MIC) values of grape seed oligomeric proanthocyanidins (GSOPs) and luteolin (LUT) against the tested bacterial strains. n=8 strains for both bacteria.

Bacteria	GSOP		LUT	
	MIC_{50}	**MIC_{90}**	**MIC_{50}**	**MIC_{90}**
E. coli	2048 µg/ml	2048 µg/ml	256 µg/ml	256 µg/ml
S. Typhimurium	2048 µg/ml	2048 µg/ml	256 µg/ml	256 µg/ml

6.2.2. Interactions with antibiotics

In the interaction studies, both GSOP and LUT were tested in combination with different antibiotics to evaluates if they show synergistic, additive, neutral or antagonistic effect with the active substances against the investigated bacterial strains. All antibiotics used in these investigations (amoxicillin, gentamicin and enrofloxacin) are highly effective, bactericidal agents that are among the most valuable drugs for the treatment of *E. coli* and *S*. Typhimurium infections. Obtained FIC indexes of GSOP against *E. coli* ranged from 1.0000 to 1.0625 depending on the used antibiotic, while the values were between 1.0625 and 1.2891 for *S*. Typhimurium. All these indexes represented neutral effect, meaning that the application of GSOP did not influence efficacy of amoxicillin, gentamicin and enrofloxacin, neither positively, nor negatively. Obtained MIC and FIC values of GSOP with the tested substances can be seen in **Tables 4, 6** and **8**.

In case of LUT, FIC indexes ranged between 1.0313 and 1.4375 for *E. coli*, and between 1.03125 and 1.1250 for *S*. Typhimurium. Similarly to GSOP, LUT showed neutral effect on the activity of amoxicillin, gentamicin and enrofloxacin, i.e. it did not decrease or increase activity of the tested antibiotics either. Results of the interaction studies with LUT can be seen is **Tables 5, 7** and **9**.

Table 4. Minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) values of amoxicillin and grape seed oligomeric proanthocyanidins (GSOP) in different bacterial strains. MIC values are expressed as µg/ml.

Amoxicillin + GSOP					
Strain	**MIC GSOP**	**MIC amoxicillin**	**MIC GSOP + amoxicillin***	**FIC index GSOP + amoxicillin**	**Average FIC index GSOP + amoxicillin**
E. coli	2048	32	512 / 16	0.75	1.0000
E. coli	2048	64	1024 / 32	1	
E. coli	2048	16	1024 / 8	1	
E. coli	2048	64	512 / 64	1.25	
S. Typhimurium	2048	64	1024 / 32	1	1.0625
S. Typhimurium	2048	64	512 / 64	1.25	
S. Typhimurium	2048	64	1024 / 32	1	
S. Typhimurium	2048	64	1024 / 32	1	
**First and second values indicate concentration of GSOP and amoxicillin, respectively, in case of their combinational usage.*					

Table 5. Minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) values of amoxicillin and luteolin (LUT) in different bacterial strains. MIC values are expressed as µg/ml.

Amoxicillin + LUT					
Strain	**MIC LUT**	**MIC amoxicillin**	**MIC LUT + amoxicillin***	**FIC index LUT + amoxicillin**	**Average FIC index LUT + amoxicillin**
E. coli	256	32	128 / 16	1	1.0313
E. coli	256	64	128 / 32	1	
E. coli	256	16	128 / 2	0.625	
E. coli	256	64	128 / 64	1.5	
S. Typhimurium	256	64	128 / 32	1	1.1250
S. Typhimurium	256	64	128 / 64	1.5	
S. Typhimurium	256	64	128 / 32	1	
S. Typhimurium	256	64	128 / 32	1	
**First and second values indicate concentration of LUT and amoxicillin, respectively, in case of their combinational usage.*					

Table 6. Minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) values of gentamicin and grape seed oligomeric proanthocyanidins (GSOP) in different bacterial strains. MIC values are expressed as µg/ml.

Gentamicin + GSOP					
Strain	**MIC GSOP**	**MIC gentamicin**	**MIC GSOP + gentamicin***	**FIC index GSOP + gentamicin**	**Average FIC index GSOP + gentamicin**
E. coli	2048	1	1024 / 0.5	1	1.0625
E. coli	2048	1	1024 / 1	1.5	
E. coli	2048	1	1024 / 0.5	1	
E. coli	2048	1	512 / 0.5	0.75	
S. Typhimurium	2048	4	1024 / 0.5	0.625	1.2891
S. Typhimurium	2048	4	1024 / 8	2.5	
S. Typhimurium	2048	4	64 /4	1.03125	
S. Typhimurium	2048	4	1024 / 2	1	
**First and second values indicate concentration of GSOP and gentamicin, respectively, in case of their combinational usage.*					

Table 7. Minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) values of gentamicin and luteolin (LUT) in different bacterial strains. MIC values are expressed as µg/ml.

Gentamicin + LUT					
Strain	**MIC LUT**	**MIC gentamicin**	**MIC LUT + gentamicin***	**FIC index LUT + gentamicin**	**Average FIC index LUT + gentamicin**
E. coli	256	1	128 / 0.25	0.75	1.4375
E. coli	256	1	128 / 0.5	1	
E. coli	256	1	128 / 2	2.5	
E. coli	256	1	128 / 1	1.5	
S. Typhimurium	256	4	128 / 2	1	1.0625
S. Typhimurium	256	4	128 / 1	0.75	
S. Typhimurium	256	4	128 / 2	1	
S. Typhimurium	256	4	128 / 4	1.5	
**First and second values indicate concentration of LUT and gentamicin, respectively, in case of their combinational usage.*					

Table 8. Minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) values of enrofloxacin and grape seed oligomeric proanthocyanidins (GSOP) in different bacterial strains. MIC values are expressed as µg/ml.

Enrofloxacin + GSOP					
Strain	**MIC GSOP**	**MIC enrofloxacin**	**MIC GSOP + enrofloxacin***	**FIC index GSOP + enrofloxacin**	**Average FIC index GSOP + enrofloxacin**
E. coli	2048	0.125	1024 / 0.0625	1	1.0624
E. coli	2048	0.03125	1024 / 0.03125	1.5	
E. coli	2048	0.03125	1024 / 0.0156	1	
E. coli	2048	0.0625	1024 / 0.0156	0.7496	
S. Typhimurium	2048	0.0625	1024 / 0.0156	0.7496	1.2499
S. Typhimurium	2048	0.0625	1024 / 0.03125	1	
S. Typhimurium	2048	0.25	1024 / 0.0625	0.75	
S. Typhimurium	2048	0.03125	1024 / 0.0625	2.5	
**First and second values indicate concentration of GSOP and enrofloxacin, respectively, in case of their combinational usage.*					

Table 9. Minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) values of enrofloxacin and luteolin (LUT) in different bacterial strains. MIC values are expressed as µg/ml.

Enrofloxacin + LUT					
Strain	**MIC LUT**	**MIC enrofloxacin**	**MIC LUT + enrofloxacin***	**FIC index LUT + enrofloxacin**	**Average FIC index LUT + enrofloxacin**
E. coli	256	0.125	128 / 0.0625	1	1.0625
E. coli	256	0.03125	128 / 0.0156	1	
E. coli	256	0.03125	128 / 0.03125	1.25	
E. coli	256	0.0625	128 / 0.03125	1	
S. Typhimurium	256	0.0625	128 / 0.03125	1	1.0313
S. Typhimurium	256	0.0625	128 / 0.03125	1	
S. Typhimurium	256	0.25	64 / 0.125	0.75	
S. Typhimurium	256	0.03125	128 / 0.03125	1.25	
**First and second values indicate concentration of LUT and enrofloxacin, respectively, in case of their combinational usage.*					

6.3. Effects of grape seed proanthocyanidins and luteolin on IPEC-J2 cells – bacterium co-culture

6.3.1. Co-culture establishment

As the first step of this study, the highest tolerable bacterial concentration that could be co-cultured with IPEC-J2 cells without significant reduction in cell viability was determined. For this purpose, Neutral Red dye was applied on IPEC-J2 cells after being treated with 10^4, 10^6 and 10^8 CFU/ml bacteria for 1 h. The results of the assay can be seen in **Figure 18.** Bacterial suspensions of *E. coli* and *S*. Typhimurium at the concentrations of 10^4 and 10^6 CFU/ml did not alter viability of IPEC-J2 cells. Suspensions of 10^8 CFU/ml of both bacteria significantly decreased the ratio of viable IPEC-J2 cells in the culture. Based on these results, and in accordance with the relevant literature (Klingspor et al., 2015; Loss et al., 2018), 10^6 CFU/ml bacterial suspensions were used in further experiments.

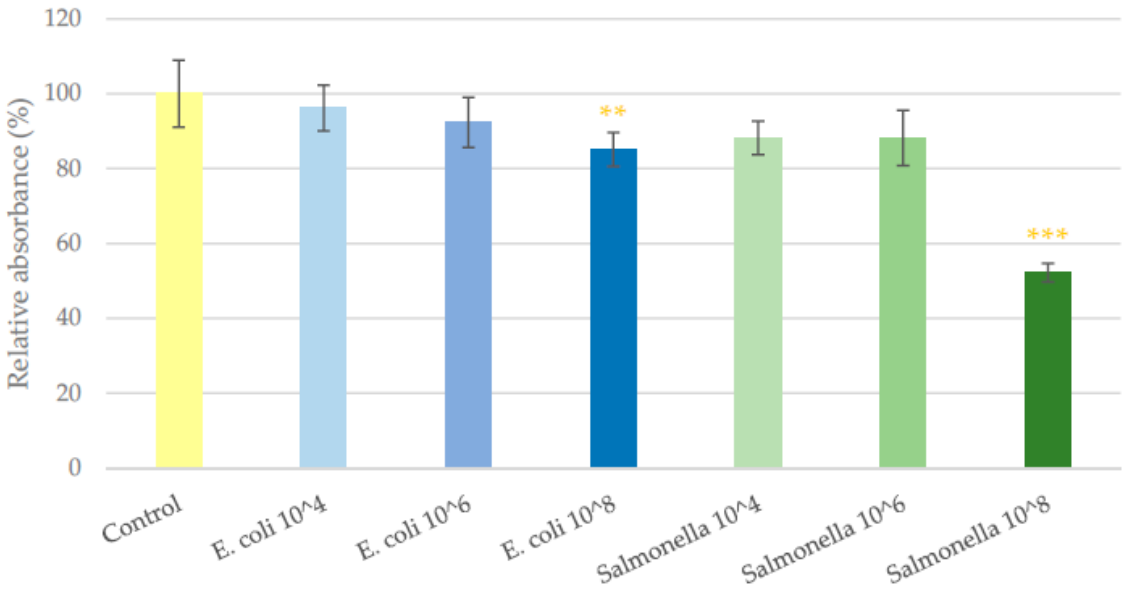

Figure 18. Viability of IPEC-J2 cells after one hour treatment with bacterial suspensions. Control – untreated (plain medium only); *E. coli* 10^4, 10^6, 10^8 – treatment with 10^4, 10^6 or 10^8 CFU/ml *E. coli*, respectively; *S*. Typhimurium 10^4, 10^6, 10^8 – treatment with 10^4, 10^6 or 10^8 CFU/ml *S*. Typhimurium, respectively. Data are shown as means with standard deviation, and expressed as relative absorbance, considering the mean value of control as 100%. n = 6/group. Significant difference: ** $p < 0.01$, *** $p < 0.001$, asterisk in yellow: compared to the untreated control.

6.3.2. IC ROS levels

To determine the potential antioxidant effect of GSOPs and LUT in *E. coli* and *S*. Typhimurium infections, changes in the intracellular reactive oxygen species (IC ROS) level of cells were investigated after the addition of bacteria alone and in combination with different GSOP and LUT treatments. After 1 hour of treatment with 10^6 CFU/ml *E. coli*, IC ROS levels increased significantly compared to the untreated control, which was significantly alleviated by the administration of GSOPs regardless of the concentration and time of GSOPs addition. There was no difference between the efficacy of GSOPs at lower and higher concentrations (pre-treatment: $p = 0.88$, parallel treatment: $p = 0.64$, post-treatment: $p = 0.93$); however, parallel treatment of GSOPs with bacterial infection showed a more pronounced effect than pre- or post-treatment when their activity was compared in similar concentrations ($p < 0.001$ in all comparisons). The results are shown in **Figure 19**.

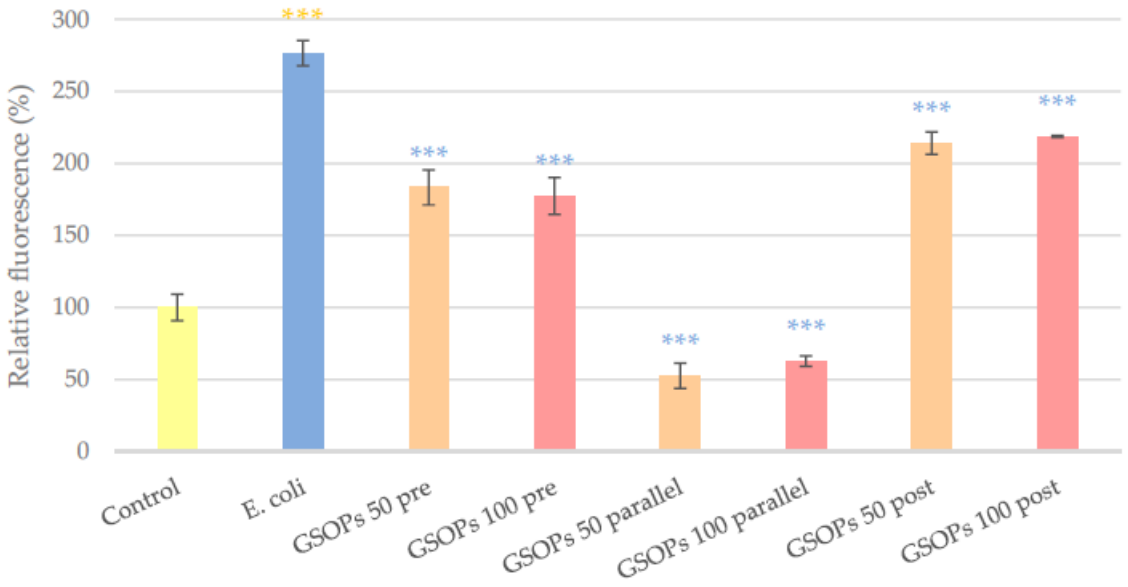

Figure 19. Intracellular reactive oxygen species level of IPEC-J2 cells after one hour treatment with *Escherichia coli* and grape seed oligomeric proanthocyanidins (GSOPs). Control – untreated (plain medium only); *E. coli* – treatment with 10^6 CFU/ml *E. coli*; GSOPs 50, 100 pre – pre-treatment before *E. coli* infection with 50 and 100 µg/ml GSOPs, respectively; GSOPs 50, 100 parallel – parallel treatment of *E. coli* infection with 50 and 100 µg/ml GSOPs, respectively; GSOPs 50, 100 post – post-treatment after *E. coli* infection with 50 and 100 µg/ml GSOPs, respectively. Data are shown as means with standard deviation, and expressed as relative fluorescence, considering the mean value of control as 100%. n = 6/group. Significant difference: *** $p < 0.001$, asterisk in yellow: compared to the untreated control, in blue: compared to *E. coli* treatment.

Similarly to *E. coli*, application of *S.* Typhimurium on the cells for 1 h resulted in the elevation of IC ROS levels, which was significantly decreased by pre-, parallel and post-treatment with GSOPs. Against *Salmonella*, all treatment types were similarly effective in comparison to each other in similar concentrations (p values between 0.06 and 0.99) and there was no dose-related difference either (pre-treatment: p = 0.55, parallel treatment: p = 0.72, post-treatment: p = 0.17) in the activity of GSOPs. The results are presented in **Figure 20**.

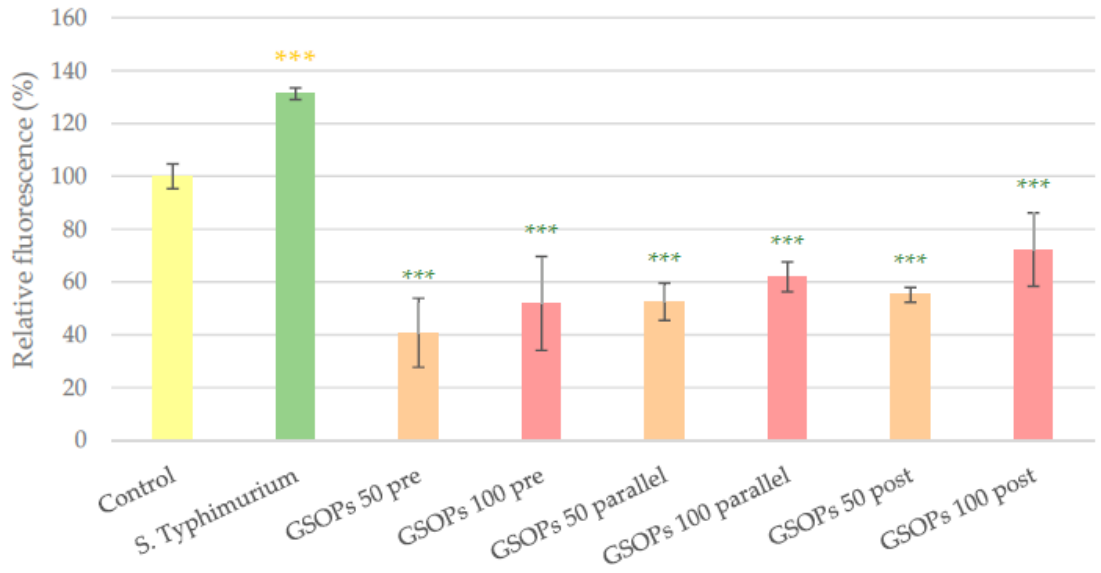

Figure 20. Intracellular reactive oxygen species level of IPEC-J2 cells after one hour treatment with *Salmonella* Typhimurium and grape seed oligomeric proanthocyanidins (GSOPs). Control – untreated (plain medium only); *S.* Typhimurium – treatment with 10^6 CFU/ml *S.* Typhimurium; GSOPs 50, 100 pre – pre-treatment before *S.* Typhimurium infection with 50 and 100 µg/ml GSOPs, respectively; GSOPs 50, 100 parallel – parallel treatment of *S.* Typhimurium infection with 50 and 100 µg/ml GSOPs, respectively; GSOPs 50, 100 post – post-treatment after *S.* Typhimurium infection with 50 and 100 µg/ml GSOPs, respectively. Data are shown as means with standard deviation, and expressed as relative fluorescence, considering the mean value of control as 100%. n = 6/group. Significant difference: *** $p < 0.001$, asterisk in yellow: compared to the untreated control, in green: compared to *S.* Typhimurium treatment.

Similarly to GSOPs, LUT showed potent antioxidant effect by decreasing IC ROS levels elevated due to bacterial infection. In case of *E. coli*, LUT in both applied concentrations and with all treatment types could significantly reduce oxidative stress in IPEC-J2 cells. There was no concentration related difference in the activity of LUT in case of pre- ($p = 1.00$) and parallel ($p = 0.63$) treatments, however, in case of post-treatment, higher concentration of LUT showed more pronounced activity ($p < 0.001$). Among the treatment types, parallel treatment was the most effective in case of 25 µg/ml LUT ($p < 0.001$ in comparison to pre- and post-treatment as well). For 50 µg/ml LUT, both parallel and post-treatment had higher activity than pre-treatment ($p < 0.001$ in both comparisons). Results of the assay can be seen in **Figure 21**.

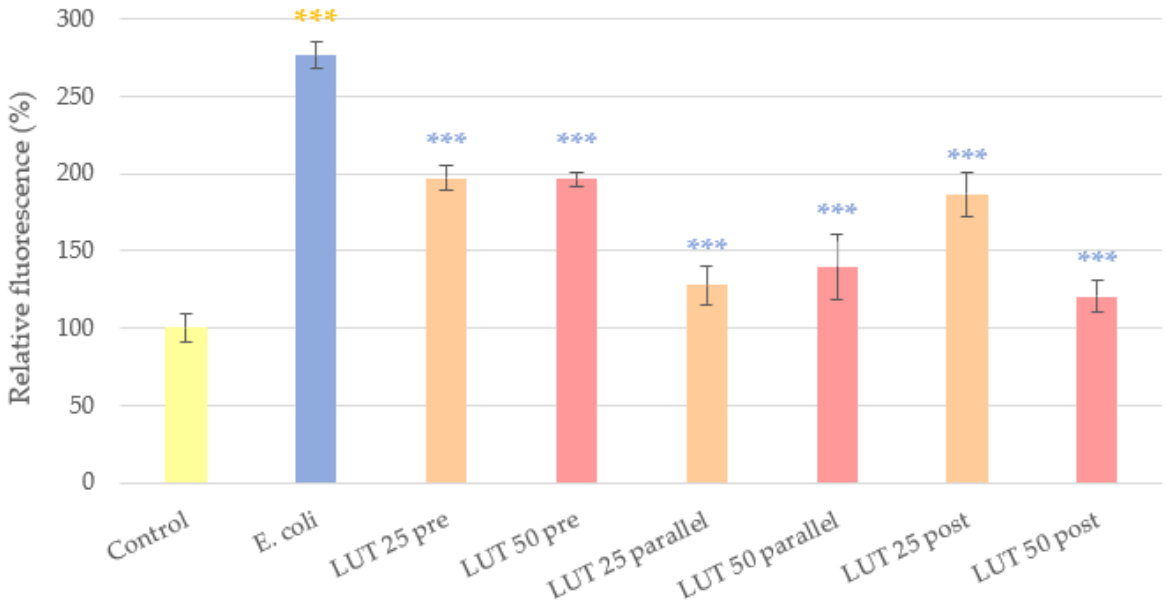

Figure 21. Intracellular reactive oxygen species level of IPEC-J2 cells after one hour treatment with *Escherichia coli* and luteolin (LUT). Control – untreated (plain medium only); *E. coli* – treatment with 10^6 CFU/ml *E. coli*; LUT 25, 50 pre – pre-treatment before *E. coli* infection with 25 and 50 µg/ml LUT, respectively; LUT 25, 50 parallel – parallel treatment of *E. coli* infection with 25 and 50 µg/ml LUT, respectively; LUT 25, 50 post – post-treatment after *E. coli* infection with 25 and 50 µg/ml LUT, respectively. Data are shown as means with standard deviation, and expressed as relative fluorescence, considering the mean value of control as 100%. n = 6/group. Significant difference: *** $p < 0.001$, asterisk in yellow: compared to the untreated control, in blue: compared to *E. coli* treatment.

When LUT was applied with *S*. Typhimurium, it was able to alleviate oxidative stress caused by bacteria regardless of the treatment type and concentration. There was no difference neither in the efficacy of the different treatment types, nor in the activity of higher and lower concentrations (p values between 0.093 and 0.99 were obtained when comparing different treatment types in similar concentrations and different concentrations in similar treatment types). Results are shown on **Figure 22**.

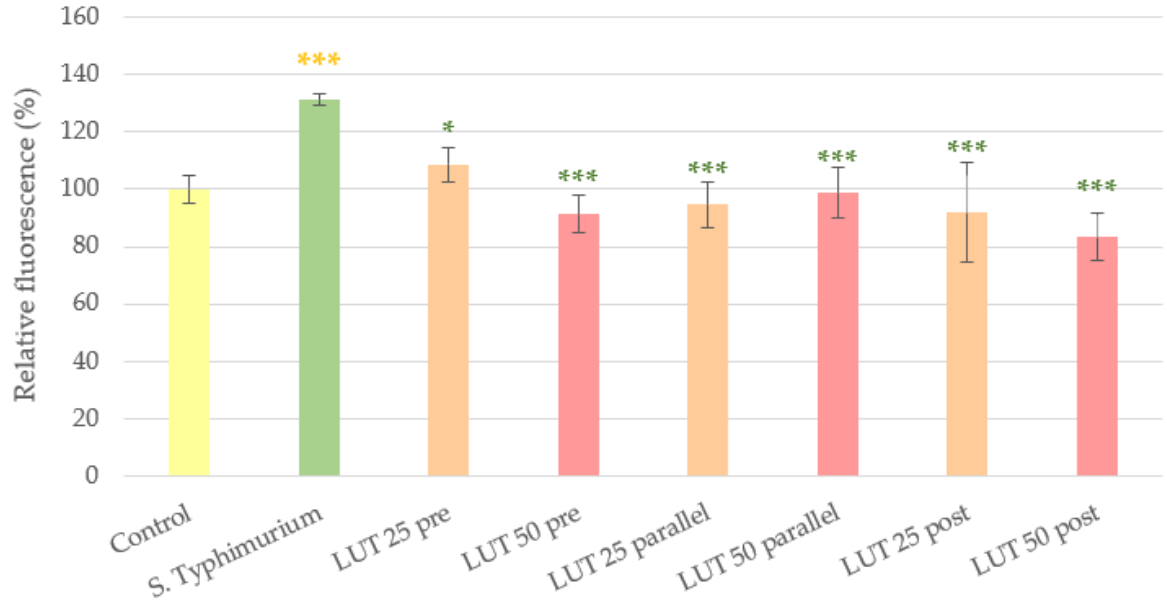

Figure 22. Intracellular reactive oxygen species level of IPEC-J2 cells after one hour treatment with *Salmonella* Typhimurium and luteolin (LUT). Control – untreated (plain medium only); *S*. Typhimurium – treatment with 10^6 CFU/ml *S*. Typhimurium; LUT 25, 50 pre – pre-treatment before *S*. Typhimurium infection with 25 and 50 µg/ml LUT, respectively; LUT 25, 50 parallel – parallel treatment of *S*. Typhimurium infection with 25 and 50 µg/ml LUT, respectively; LUT 25, 50 post – post-treatment after *S*. Typhimurium infection with 25 and 50 µg/ml LUT, respectively. Data are shown as means with standard deviation, and expressed as relative fluorescence, considering the mean value of control as 100%. n = 6/group. Significant difference: * < 0.05, *** p < 0.001, asterisk in yellow: compared to the untreated control, in green: compared to *S*. Typhimurium treatment.

6.3.3. EC H_2O_2 levels

On the contrary, *E. coli* and *S*. Typhimurium infection did not elevate EC H_2O_2 levels in the cell supernatants (**Figure 23**). These findings were in accordance with our results obtained after LPS treatment (5.1.3.) and suggested the role of ROS other than H_2O_2 in oxidative damage inflicted by bacteria and bacterial endotoxin in IPEC-J2 cells. Consequently, Amplex Red method was found to be unsuitable for the investigation of antioxidant effect of GSOP and LUT in the IPEC-J2 – bacterium co-culture.

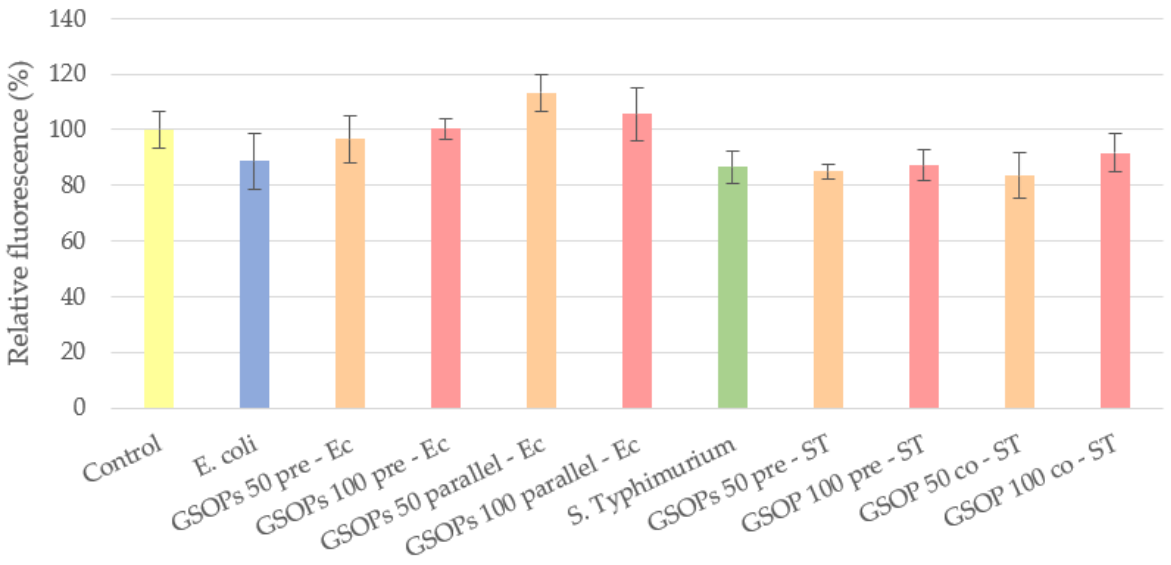

Figure 23. Amount of extracellular H_2O_2 in IPEC-J2 cells after one hour treatment with *Escherichia coli*, *Salmonella* Typhimurium, and grape seed oligomeric proanthocyanidins (GSOPs). Control – untreated (plain medium only); *E. coli* – treatment with 10^6 CFU/ml *E. coli*; GSOPs 50, 100 pre – Ec – pre-treatment before *E. coli* infection with 50 and 100 µg/ml GSOPs, respectively; GSOPs 50, 100 parallel – Ec – parallel treatment of *E. coli* infection with 50 and 100 µg/ml GSOPs, respectively; *S.* Typhimurium – treatment with 10^6 CFU/ml *S.* Typhimurium; GSOPs 50, 100 pre – ST – pre-treatment before *S.* Typhimurium infection with 50 and 100 µg/ml GSOPs, respectively; GSOPs 50, 100 parallel – ST – parallel treatment of *S.* Typhimurium infection with 50 and 100 µg/ml GSOPs, respectively. Data are shown as means with standard deviation, and expressed as relative fluorescence, considering the mean value of control as 100%. n = 6/group.

6.3.4. Interleukin-6,8 levels

For the evaluation of anti-inflammatory properties of GSOPs and LUT, interleukin-6 (IL-6) and interleukin-8 (IL-8) levels of IPEC-J2 cells were measured. Treatment with *E. coli* significantly elevated levels of both IL-6 and IL-8 (**Figure 24**), while GSOPs and LUT alone did not influence production of these interleukins (data not shown). In case of IL-6, parallel and post-treatments with GSOPs were similarly able to decrease production of the inflammatory mediator and there was no difference in the activity of the applied concentrations either (parallel treatment: $p = 0.79$, post-treatment: $p = 0.99$). For IL-8, GSOPs pre-treatments also resulted in a significant alleviation of the effect of *E. coli*, but from the post-treatments, only the higher concentration of GSOP were shown to be effective and its activity was lower than pre- and parallel treatments. Efficacy of pre- and parallel treatments were not impacted by the applied concentrations ($p = 0.99$ for both). Results are demonstrated in **Figure 24**.

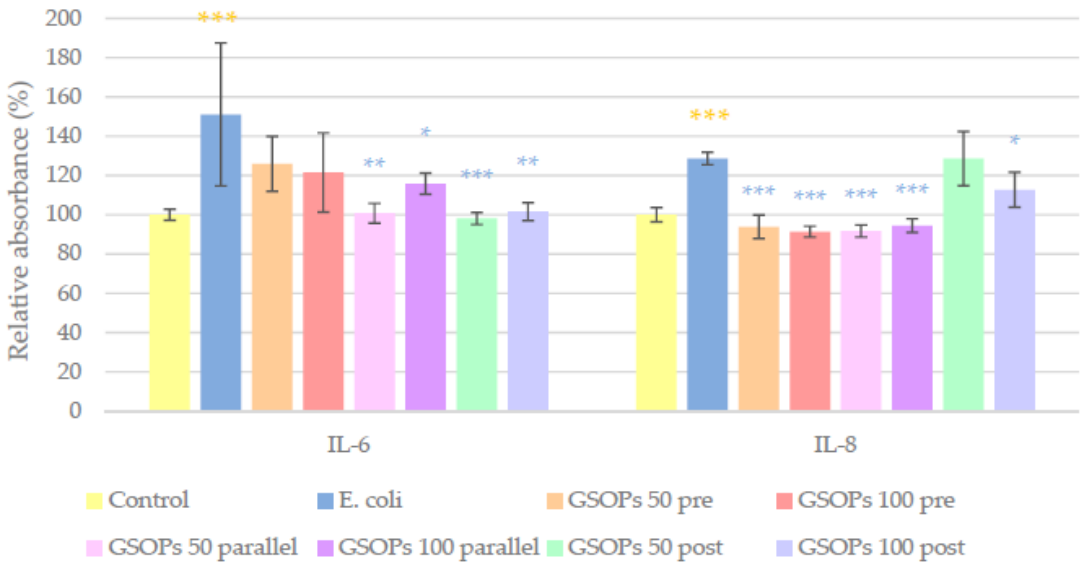

Figure 24. Interleukin-6 (IL-6) and interleukin-8 (IL-8) levels of IPEC-J2 cells after one hour treatment with *Escherichia coli* and grape seed oligomeric proanthocyanidins (GSOPs). Control – untreated (plain medium only); *E. coli* – treatment with 10^6 CFU/ml *E. coli*; GSOPs 50, 100 pre – pre-treatment before *E. coli* infection with 50 and 100 µg/ml GSOPs, respectively; GSOPs 50, 100 parallel – parallel treatment of *E. coli* infection with 50 and 100 µg/ml GSOPs, respectively; GSOPs 50, 100 post – post-treatment after *E. coli* infection with 50 and 100 µg/ml GSOPs, respectively. Picogram levels of the mediators were detected. Data are shown as means with standard deviation, and expressed as relative absorbance, considering the mean value of control as 100%. n = 6/group. Significant difference: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, asterisk in yellow: compared to the untreated control, in blue: compared to *E. coli* treatment.

Similarly to the effect of *E. coli*, *S.* Typhimurium caused a significant increase in the levels of the investigated cytokines (IL-6 and IL-8). All types and concentrations of GSOP treatments could similarly decrease IL-6 levels, while in the case of IL-8, GSOPs at 50 µg/ml concentration were not effective when applied before or after bacterial infection. There was no difference in the activity of 50 and 100 µg/ml GSOPs when used as pre-treatment ($p = 0.99$). The results can be seen in **Figure 25**.

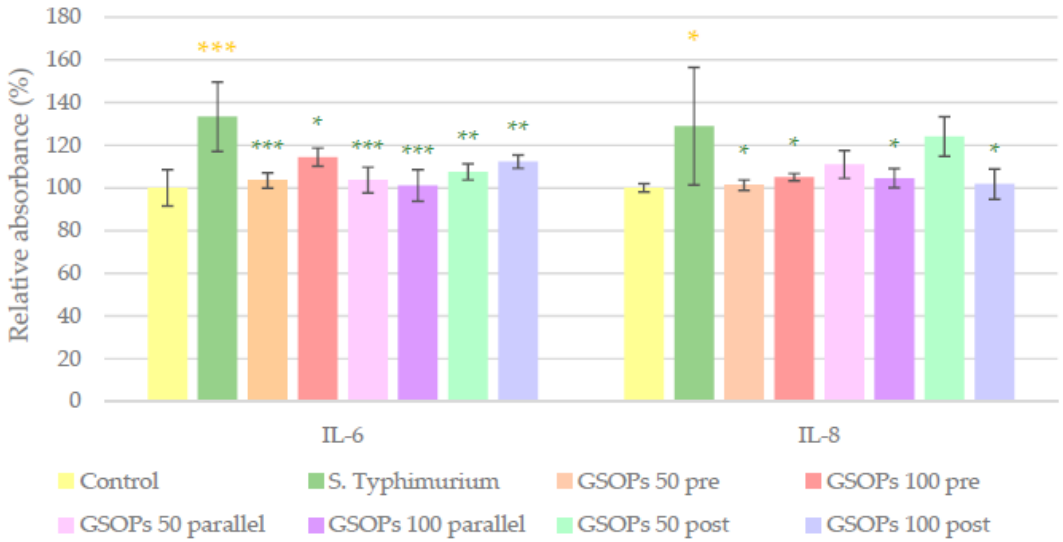

Figure 25. Interleukin-6 (IL-6) and interleukin-8 (IL-8) levels of IPEC-J2 cells after one hour treatment with *Salmonella* Typhimurium and grape seed oligomeric proanthocyanidins (GSOPs). Control – untreated (plain medium only); *S.* Typhimurium – treatment with 10^6 CFU/ml *S.* Typhimurium; GSOPs 50, 100 pre – pre-treatment before *S.* Typhimurium infection with 50 and 100 µg/ml GSOPs, respectively; GSOPs 50, 100 parallel – parallel treatment of *S.* Typhimurium infection with 50 and 100 µg/ml GSOPs, respectively; GSOPs 50, 100 post – post-treatment after *S.* Typhimurium infection with 50 and 100 µg/ml GSOPs, respectively. Picogram levels of the mediators were detected. Data are shown as means with standard deviation, and expressed as relative absorbance, considering the mean value of control as 100%. n = 6/group. Significant difference: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, asterisk in yellow: compared to the untreated control, in green: compared to *S.* Typhimurium treatment.

LUT showed high efficacy in decreasing interleukin production of cells elevated by *E. coli*. The administration of LUT reduced IL-6 level of cells in all applied concentrations and treatment types, and there were no differences between the efficacy of LUT treatments (p values between 0.99 and 1.00 were obtained when comparing different treatment types in similar concentrations and different concentrations in similar treatment types). In terms of IL-8 production, all LUT treatments could alleviate the effect of *E. coli* at a similar level except for post-treatment with 50 µg/ml. Obtained data are shown on **Figure 26**.

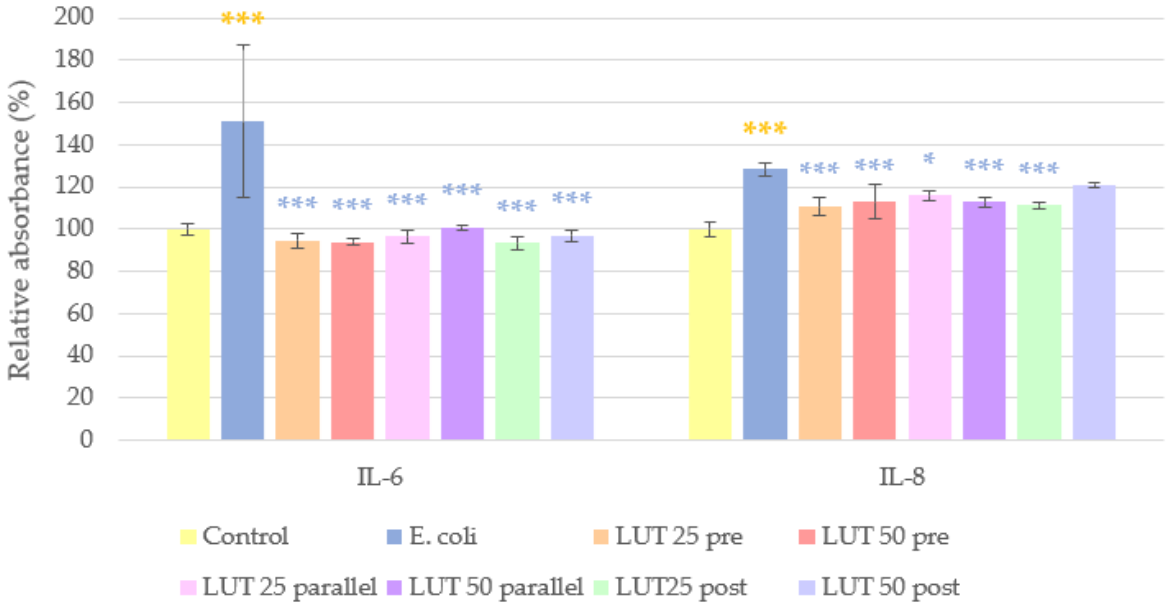

Figure 26. Interleukin-6 (IL-6) and interleukin-8 (IL-8) levels of IPEC-J2 cells after one hour treatment with *Escherichia coli* and luteolin (LUT). Control – untreated (plain medium only); *E. coli* – treatment with 10^6 CFU/ml *E. coli*; LUT 25, 50 pre – pre-treatment before *E. coli* infection with 25 and 50 µg/ml LUT, respectively; LUT 25, 50 parallel – parallel treatment of *E. coli* infection with 25 and 50 µg/ml LUT, respectively; LUT 25, 50 post – post-treatment after *E. coli* infection with 25 and 50 µg/ml LUT, respectively. Picogram levels of the mediators were detected. Data are shown as means with standard deviation, and expressed as relative absorbance, considering the mean value of control as 100%. n = 6/group. Significant difference: * $p < 0.05$, *** $p < 0.001$, asterisk in yellow: compared to the untreated control, in blue: compared to *E. coli* treatment.

Similarly to its effect against *E. coli*, LUT in both tested concentrations and all treatment forms significantly decreased IL-6 levels triggered by *S*. Typhimurium. All of these treatments had similar efficacy (p values between 0.50 and 1.00 were obtained when comparing different treatment types in similar concentrations and different concentrations in similar treatment types). However, only pre-treatment with 25 µg/ml LUT and parallel treatment with 50 µg/ml LUT had significant effect on IL-8 levels. Data are demonstrated on **Figure 27**.

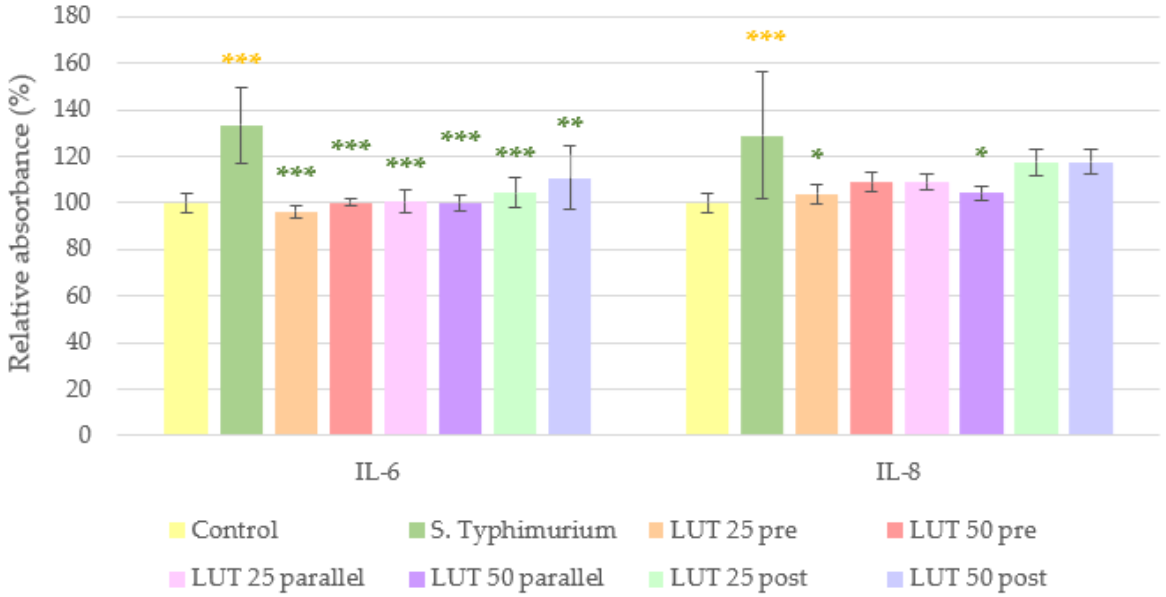

Figure 27. Interleukin-6 (IL-6) and interleukin-8 (IL-8) levels of IPEC-J2 cells after one hour treatment with *Salmonella* Typhimurium and luteolin (LUT). Control – untreated (plain medium only); *S*. Typhimurium – treatment with 10^6 CFU/ml *S*. Typhimurium; LUT 25, 50 pre – pre-treatment before *S*. Typhimurium infection with 25 and 50 µg/ml LUT, respectively; LUT 25, 50 parallel – parallel treatment of *S*. Typhimurium infection with 25 and 50 µg/ml LUT, respectively; LUT 25, 50 post – post-treatment after *S*. Typhimurium infection with 25 and 50 µg/ml LUT, respectively. Picogram levels of the mediators were detected. Data are shown as means with standard deviation, and expressed as relative absorbance, considering the mean value of control as 100%. n = 6/group. Significant difference: * $p < 0.05$, *** $p < 0.001$, asterisk in yellow: compared to the untreated control, in green: compared to *S.* Typhimurium treatment.

6.3.5. Barrier integrity

Protective effect of GSOPs and LUT on the barrier integrity of IPEC-J2 cells was tested via the penetration of a tracer dye through the cell layer. Changes in paracellular permeability were more apparent 24 h after treatment compared to the measurement performed after only 3 h, possibly due to a delayed toxic action of bacterial infection. GSOPs and LUT alone did not influence barrier integrity of the cell layer (data not shown). After 24 h, cells treated with *E. coli* showed significantly higher paracellular permeability compared to the untreated control, meaning a pronounced destruction of their barrier integrity due to bacterial infection. The deteriorating effect of bacteria was significantly alleviated by the application of GSOPs in all treatment groups and concentrations, and there was no difference between their efficacy (p values between 0.18 and 1.00 were obtained when comparing different treatment types in similar concentrations and different concentrations in similar treatment types). The results are shown in **Figure 28**.

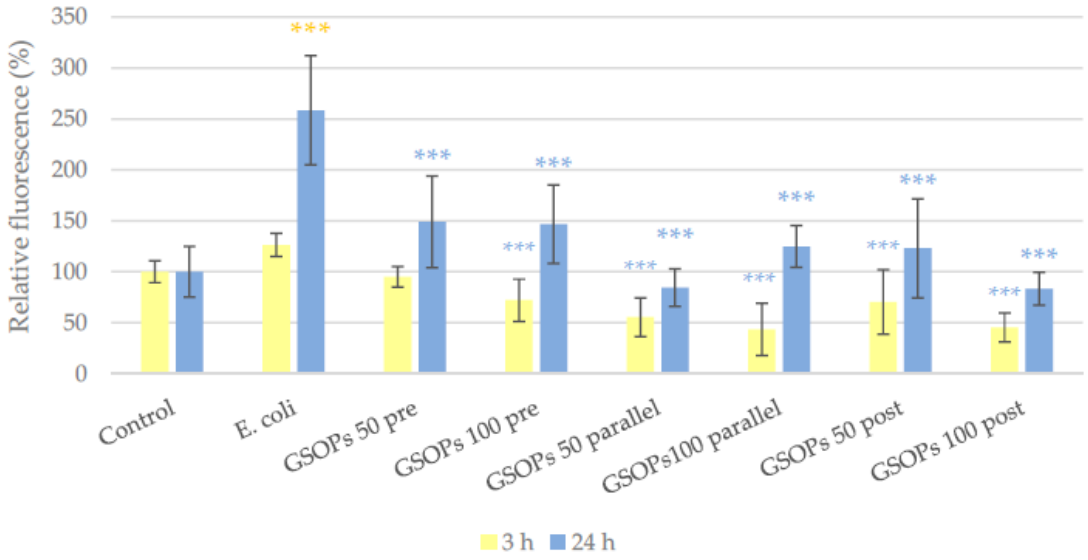

Figure 28. Paracellular permeability of IPEC-J2 cells 3 and 24 hours after one hour treatment with *Escherichia coli* and grape seed oligomeric proanthocyanidins (GSOPs). Control – untreated (plain medium only); *E. coli* – treatment with 10^6 CFU/ml *E. coli*; GSOPs 50, 100 pre – pre-treatment before *E. coli* infection with 50 and 100 µg/ml GSOPs, respectively; GSOPs 50, 100 parallel – parallel treatment of *E. coli* infection with 50 and 100 µg/ml GSOPs, respectively; GSOPs 50, 100 post – post-treatment after *E. coli* infection with 50 and 100 µg/ml GSOPs, respectively. Data are shown as means with standard deviation, and expressed as relative fluorescence, considering the mean value of control as 100%. n = 6/group. Significant difference: *** $p < 0.001$, asterisk in yellow: compared to the untreated control, in blue: compared to *E. coli* treatment.

In the experiment with *S*. Typhimurium, 24 hours after treatment, significantly increased paracellular permeability was observed in cells infected with bacteria. However, GSOPs could prevent barrier integrity impairment when applied as pre-treatment in both concentrations,

parallel treatment in 50 µg/ml and post-treatment in 100 µg/ml. In case of pre-treatment, efficacy of the two concentrations did not differ from each other (p = 0.96), and they were similarly effective as other treatment types with the same concentration (50 µg/ml pre-parallel: p = 0.99, 100 µg/ml pre-post: p = 0.46) Results are visible in **Figure 29**.

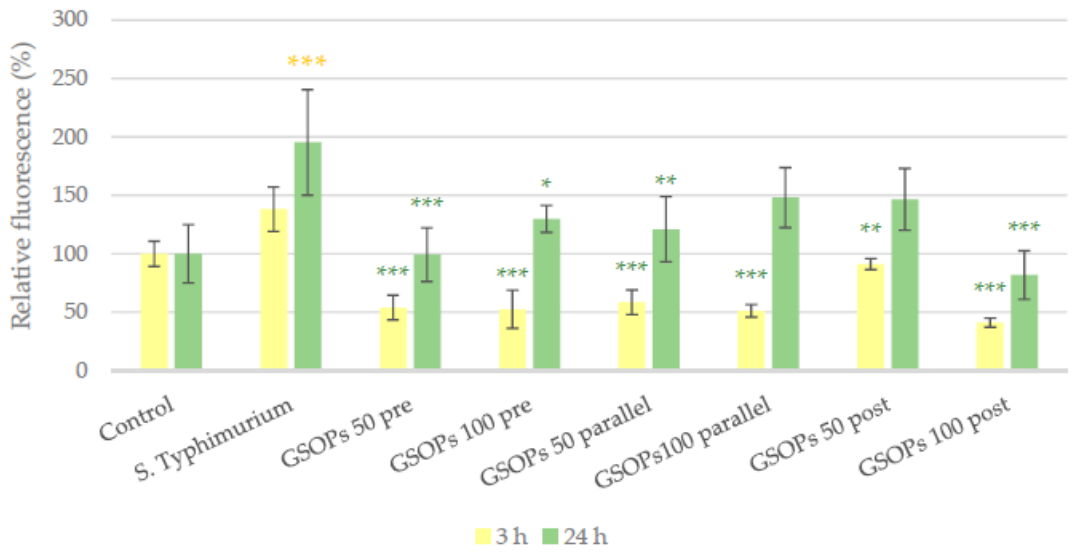

Figure 29. Paracellular permeability of IPEC-J2 cells 3 and 24 hours after one hour treatment with *Salmonella* Typhimurium and grape seed oligomeric proanthocyanidins (GSOPs). Control – untreated (plain medium only); *S.* Typhimurium – treatment with 10^6 CFU/ml *S.* Typhimurium; GSOPs 50, 100 pre – pre-treatment before *S.* Typhimurium infection with 50 and 100 µg/ml GSOPs, respectively; GSOPs 50, 100 parallel – parallel treatment of *S.* Typhimurium infection with 50 and 100 µg/ml GSOPs, respectively; GSOPs 50, 100 post – post-treatment after *S.* Typhimurium infection with 50 and 100 µg/ml GSOPs, respectively. Data are shown as means with standard deviation, and expressed as relative fluorescence, considering the mean value of control as 100%. n = 6/group. Significant difference: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, asterisk in yellow: compared to the untreated control, in green: compared to *S.* Typhimurium treatment.

When LUT was applied together with *E. coli*, it could significantly decrease barrier damage caused by bacteria in all treatment forms except for pre-treatment with 50 µg/ml LUT. In case of the significantly active LUT treatments, there were no concentration or treatment type related difference in their efficacy (p values between 0.45 and 1.00 were obtained when comparing different treatment types in similar concentrations and different concentrations in similar treatment types). However, in the study with *S.* Typhimurium, only post-treatment with 50 µg/ml LUT was able to significantly alleviate barrier integrity impairment. Results are demonstrated on **Figure 30** and **Figure 31**.

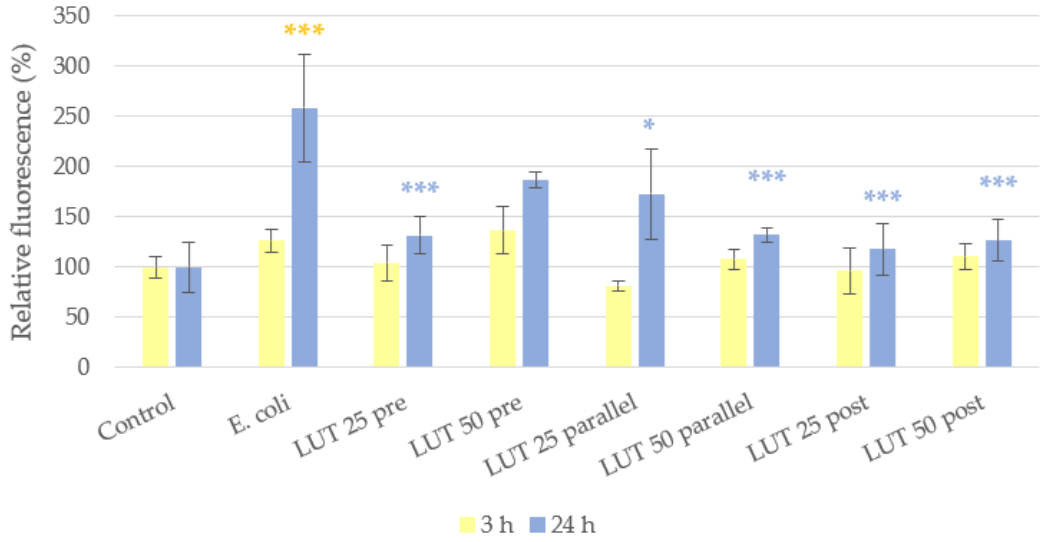

Figure 30. Paracellular permeability of IPEC-J2 cells 3 and 24 hours after one hour treatment with *Escherichia coli* and luteolin (LUT). Control – untreated (plain medium only); *E. coli* – treatment with 10^6 CFU/ml *E. coli*; LUT 25, 50 pre – pre-treatment before *E. coli* infection with 25 and 50 µg/ml LUT, respectively; LUT 25, 50 parallel – parallel treatment of *E. coli* infection with 25 and 50 µg/ml LUT, respectively; LUT 25, 50 post – post-treatment after *E. coli* infection with 25 and 50 µg/ml LUT, respectively. Data are shown as means with standard deviation, and expressed as relative fluorescence, considering the mean value of control as 100%. n = 6/group. Significant difference: * $p < 0.05$, *** $p < 0.001$, asterisk in yellow: compared to the untreated control, in blue: compared to *E. coli* treatment.

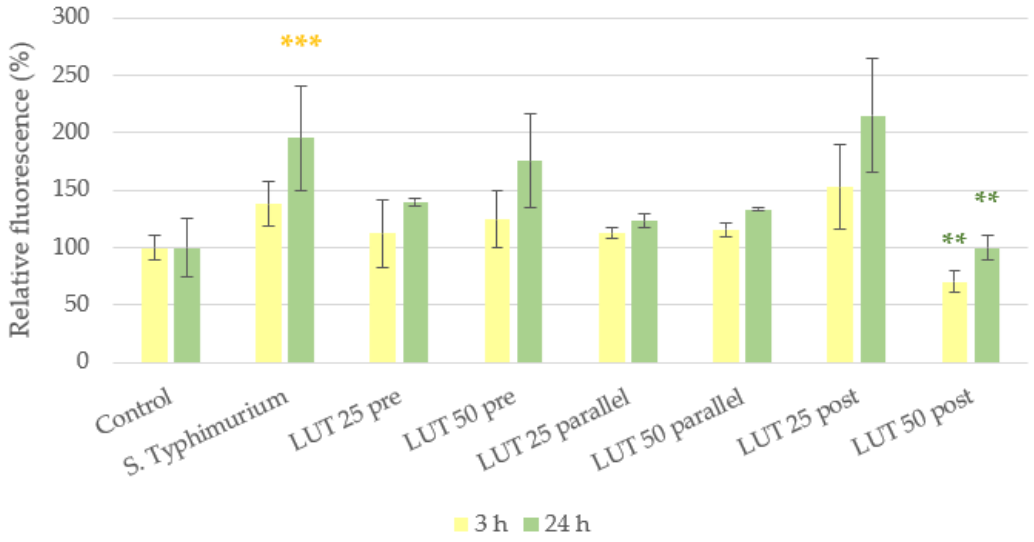

Figure 31. Paracellular permeability of IPEC-J2 cells 3 and 24 hours after one hour treatment with *Salmonella* Typhimurium and luteolin (LUT). Control – untreated (plain medium only); *S.* Typhimurium – treatment with 10^6 CFU/ml *S.* Typhimurium; LUT 25, 50 pre – pre-treatment before *S.* Typhimurium infection with 25 and 50 µg/ml LUT, respectively; LUT 25, 50 parallel – parallel treatment of *S.* Typhimurium infection with 25 and 50 µg/ml LUT, respectively; LUT 25, 50 post – post-treatment after *S.* Typhimurium infection with 25 and 50 µg/ml LUT, respectively. Data are shown as means with standard deviation, and expressed as relative fluorescence, considering the mean value of control as 100%. n = 6/group. Significant difference: ** $p < 0.01$, *** $p < 0.001$, asterisk in yellow: compared to the untreated control, in green: compared to *S.* Typhimurium treatment.

6.3.6. Bacterial adhesion

To determine potential anti-adhesive effect of GSOPs and LUT, the amount of bacteria attached to IPEC-J2 cells was tested with CFU counting. When no flavonoid treatment was applied, the average number of adhered bacteria was $9x10^5$ CFU and $2x10^5$ CFU in case of *E. coli* and *S*. Typhimurium, respectively. In the experiment with GSOPs, more pronounced effect of the flavonoid was observed in case of *E. coli* than for *S*. Typhimurium. The addition of GSOPs resulted in significant reduction (43.62 - 75.12%) of the amount of *E. coli* adhered to IPEC-J2 cells in all treatment groups except for post-treatment with 50 µg/ml GSOPs. Among the treatments that showed significant anti-adhesive activity, there was no difference between the two concentrations in similar treatment type (pre-treatment: $p = 0.90$, parallel treatment: $p = 1.00$), or similar concentrations in different treatment types either (p values between 0.12 and 0.92). For *Salmonella*, only pre-treatment with GSOPs showed significant anti-adhesive activity, with the bacterial count reduction being over 50%. These two treatments had similar efficacy ($p = 1.00$). Results are demonstrated in **Table 10**.

Table 10. Reduction in the amount of bacteria adhered to IPEC-J2 cells by one hour treatment with grape seed oligomeric proanthocyanidins (GSOPs). GSOPs 50, 100 pre – pre-treatment before bacterial infection with 50 and 100 µg/ml GSOPs, respectively; GSOPs 50, 100 parallel – parallel treatment of bacterial infection with 50 and 100 µg/ml GSOPs, respectively; GSOPs 50, 100 post – post-treatment after bacterial infection with 50 and 100 µg/ml GSOPs, respectively. Data are shown as bacterial count reduction compared to the mean value of control (attached bacteria without flavonoid treatment) that was considered as 100%. n=4/group. Significant difference compared to the untreated control: * $p < 0.05$*, ** $p < 0.01$, *** $p < 0.001$.

Treatment	*E. coli*		*S.* Typhimurium	
	Reduction	p value	Reduction	p value
GSOPs 50 pre	-62.35%	p < 0.001***	-51.14%	p < 0.05*
GSOPs 100 pre	-75.12%	p < 0.001***	-57.55%	p < 0.05*
GSOPs 50 parallel	-43.62%	p < 0.05*	-24.03%	p = 0.16
GSOPs 100 parallel	-44.25%	p < 0.01**	-30.66%	p = 0.11
GSOPs 50 post	-23.27%	p = 0.46	-5.38%	p = 0.34
GSOPs 100 post	-56.35%	p < 0.001***	-17.39%	p = 0.21

In contrast to GSOPs, LUT did not show anti-adhesive activity against the tested bacterial strains. Slight reduction in the amount of adhered bacteria was observed, however, none of these effects were significant in any of the treatment groups, regardless of the concentration of LUT and the time of addition (pre-, parallel or post-treatment). Results can be seen in **Table 11**.

Table 11. Reduction in the amount of bacteria adhered to IPEC-J2 cells by one hour treatment with luteolin (LUT). LUT 25, 50 pre – pre-treatment before bacterial infection with 25 and 50 µg/ml LUT, respectively; LUT 25, 50 parallel – parallel treatment of bacterial infection with 25 and 50 µg/ml LUT, respectively; LUT 25, 50 post – post-treatment after bacterial infection with 25 and 50 µg/ml LUT, respectively. Data are shown as bacterial count reduction compared to the mean value of control (attached bacteria without flavonoid treatment) that was considered as 100%. n=4/group.

Treatment	*E. coli*		*S.* Typhimurium	
	Reduction	p value	Reduction	p value
LUT 25 pre	-42.41%	p = 0.418	-23.75%	p = 0.970
LUT 50 pre	-39.70%	p = 0.492	-27.50%	p = 0.942
LUT 25 parallel	-3.93%	p = 1.000	-5.00%	p = 1.000
LUT 50 parallel	-30.83%	p = 0.748	-8.75%	p = 1.000
LUT 25 post	-36.31%	p = 0.591	-10.00%	p = 1.000
LUT 50 post	-13.55%	p = 0.996	-10.00%	p = 1.000

7. Discussion

Foodborne diseases, that occur through the contamination of food with bacteria, viruses, and parasites, represent a major public health threat globally (Qian et al., 2020), that is even exacerbated by the spread of AMR (Switaj et al., 2015). *E. coli* and *Salmonella* spp. are important foodborne pathogens, in which the prevalence of resistance is high including isolates from both humans and animals (Souto et al., 2017). Due to the widespread occurrence of resistant and MDR bacterial strains in pigs, there is a significant need for alternative infection control strategies, including vaccines, improved management practices and feed additives (Luppi, 2017). Enhancing gut health could be an effective way to protect animals from intestinal pathogens, and consequently to maintain productivity (Lückstädt and Theobald, 2011). In this study, beneficial effects of flavonoids, GSOP and LUT were tested as potential antibiotic alternatives for the prevention and treatment of porcine GI bacterial infections. A large number of publications is available about the health benefits of flavonoids, however, our study was the first to test them in porcine GI infection models *in vitro*.

As the first step, we demonstrated that GSOP and LUT were not cytotoxic to IPEC-J2 cells when used up to 200 and 100 µg/ml, respectively, for a maximum of 24 hours. This is in accordance with previous findings of procyanidins tested on porcine intestinal epithelial cell lines (Chedea et al., 2018; Yan et al., 2020) and this is the first report about the effects of LUT on these cells. In the cell viability assay, elevated absorbance values – suggesting an increase in the number of viable cells – have also been measured in case of some treatments. Similarly to this finding, cell viability increase was reported previously as an effect of PACs (Galarraga-Vinueza et al., 2020) and LUT (Quan et al., 2019). Bioactive properties of PACs were suggested to be in the background of this observation (Galarraga-Vinueza et al., 2020). However, unchanged cell viability has also been reported in other studies after treatment with PACs and LUT (Sung et al., 2015; Chedea et al., 2018; Liu et al., 2020; Yan et al., 2020). These discrepancies might be due to differences in the tested cells, as well as in the origin and concentration of flavonoids, but further studies would be necessary to reveal the exact underlying mechanisms.

Antioxidant effect is the most widely known property of flavonoids, that has been widely described. Several studies focus on the role of grape seed PACs in oxidative stress, and they have been shown both *in vitro* and *in vivo* to be more potent antioxidants than vitamin C, E and β-carotene (Rodríguez-Pérez et al., 2019). In pigs, antioxidant activity of PACs has been described in numerous *in vivo* investigations (Hao et al., 2015; Chedea et al., 2018; Taranu et al., 2019), and their beneficial effect against LPS-induced oxidative stress has also been reported in Caco-2 cells (Wu et al., 2018) and Wistar rats (Gil-Cardoso et al., 2019). In our

study, we could demonstrate antioxidant effect of GSOPs in LPS-treated IPEC-J2 cells and IPEC-J2 cells infected with *E. coli* and *S*. Typhimurium. Our results are in accordance with the literature data and demonstrates antioxidant activity of GSOPs in different experimental settings that has not been used previously for this purpose.

Protective effect of LUT against oxidative stress also seemed to be higher than vitamin C under some circumstances (Bustos et al., 2018). Antioxidant effect of LUT has already been described in the literature, but none of these studies were conducted in pigs or cells of porcine origin. In the studies of Yuan et al. (2021A) and Yuan et al. (2021B), LUT showed antioxidant effect against oxidative damage caused by ethanol and polybrominated diphenyl ether in human colonic adenocarcinoma (Caco-2) cells. Kang et al. (2011) demonstrated it in cisplatin-damaged kidney cells, Al-Megrin et al. (2020) in lead acetate-treated liver tissues, and Bustos et al. (2018) in gentamicin-induced ROS generation in human leukocytes and rat whole blood. LUT could also inhibit ROS production increased by LPS in RAW264.7 murine macrophage cells (Zhang et al., 2018). Our study was the first to investigate the effect of LUT in oxidative stress generated by LPS or Gram-negative bacterial infection of IPEC-J2 cells, and our findings of LUT's potent antioxidant activity are in line with the above-described results. The observed antioxidant effect of GSOP and LUT can be beneficial in alleviating oxidative stress caused by Gram-negative bacteria and their endotoxin in the GI tract.

We have also aimed to investigate changes in the EC H_2O_2 levels of IPEC-J2 cells with Amplex Red method after treatment with LPS, bacteria and flavonoids. Previously, this assay was shown to be appropriate for demonstrating oxidative stress triggering effect of the mycotoxin deoxynivalenol in IPEC-J2 cells (Pomothy et al., 2021). Furthermore, the assay has already been used to detect antioxidant effect of LUT in human blood (Soares et al., 2019). However, in our study, the tested endotoxins and bacterial isolates did not affect H_2O_2 concentration in IPEC-J2 cells' supernatants, therefore, this method was found to be inapplicable for detecting oxidative stress caused by LPS and bacteria in IPEC-J2 cells.

Studies about the antibacterial activity of flavonoids frequently report controversial results. These discrepancies might be due to different assays used that involve non-identical broth and agar types, well sizes, bacterial species, and incubation periods, as well as different solvents used for dissolution of flavonoids. Furthermore, the source of flavonoids (i.e. commercial or natural) might also impact their properties (Cushnie and Lamb, 2005). Susceptibility of standard bacterial strains and clinical isolates can also vary (Adamczak et al., 2019). For example, in the study of Bustos et al. (2018), LUT showed inhibitory action against reference strains of *E. coli* and *S. aureus* at 125 µg/ml, however, this concentration was not enough to reduce growth of clinical isolates from the same bacterial species. It is suggested that there is

a relationship between the structure of flavonoids and their antimicrobial effect (González et al, 2021), however, it has not yet been fully clarified (Adamczak et al., 2019). Generally, it is thought that at least one hydroxyl group on ring A is necessary for their activity, and the presence of further -OH groups increases this effect. In contrast, glycosyl groups on the molecules can reduce their efficacy (Adamczak et al., 2019). In the anti-MRSA efficacy of flavonoids, hydroxyl groups at positions 2',4',5,7 are thought to have an important role, however, methoxy groups decrease their antibacterial activity (Cushnie and Lamb, 2005). Adamczak et al. (2019) tested the antibacterial activity of several flavonoids and found moderate or relatively low efficacy for all substances. In their study, flavonoids were more effective against Gram-negative bacteria (*E. coli* and *P. aeruginosa*) in comparison to Gram-positives (*Enterococcus faecalis* and *S. aureus*). Comparing structure and activity of the tested compounds, they concluded that generally neither the number of hydroxyl groups on their A and B rings, nor sugar groups on the molecules affected the obtained MIC values, which was in contrast with previous findings. Skroza et al. (2019) also concluded that the antibacterial activity of phenolic compounds was unrelated to the number of -OH groups in their molecule.

In our study, MIC values of GSOP were found to be 2048 µg/ml against eight-eight *E. coli* and *S*. Typhimurium field isolates of porcine origin. In the literature, there are various MIC values reported about PACs, depending on the origin of the flavonoid and species of bacteria tested. Margetis et al. (2015) observed bacteriostatic effect of cranberry PAC at only 250 µg/ml against human clinical *E. coli* isolates. Shan et al. (2007) found that procyanidin B2 (consisting of (epi)-catechin monomers only) isolated from cinnamon stick exhibited bacteriostatic effect with MIC values of 625 µg/ml against *S. aureus* and 1250 µg/ml against *E. coli*. The latter is in accordance with the results of Tang C. et al. (2017), who found that B-type oligomeric procyanidins (extracted from lotus seedpod) inhibited growth of two ETEC strains at 800 and 1200 µg/ml. However, Alshaibani et al. (2017) found much higher MIC values (18,000 µg/ml) when they tested cranberry PACs against EPEC and ETEC strains. These MIC values cover a wide range of concentrations, but the low number of isolates tested per study limits interpretation of these findings. Furthermore, in several cases, antibacterial effect of plant and fruit extracts are tested, that contain numerous bioactive compounds, therefore their observed activity might be a result of the interaction of more than one compounds. For example, in the study of Kupnik et al. (2021), different extracts of pomegranate showed inhibitory activity against Gram-positive and Gram-negative bacteria, including *E. coli*, *P. aeruginosa*, and *S. aureus*, but PACs represented only a small proportion in the total phenolic content of extracts.

MIC values of LUT in our study were found to be 256 µg/ml against porcine *E. coli* and *S*. Typhimurium isolates. This value is in accordance with the study of Sanhueza et al. (2017), where LUT could inhibit the growth of *E. coli* strains with MIC values between 200 and 300

μg/ml. Antibacterial activity of LUT was thought to be the result of cell membrane damage and enzyme inhibition. Slightly higher MIC value was found by Adamczak et al. (2019). In this study LUT showed antibacterial effect against *E. coli* at 500 μg/ml. MIC value of LUT was similar against *S. aureus* and *P. aeruginosa*, but it could only inhibit growth of *E. faecalis* at 1000 μg/ml. Antibacterial activity of LUT glucosides (orientin and isoorientin) did not differ from the original molecule. Lower MIC values of LUT were found by studies on different bacterial species. In the study of Guo et al. (2020), LUT showed antibacterial activity against *Trueperella pyogenes* strains with MIC values of only 78 μg/ml. They suggested that multiple mechanisms of action resulted in the observed effect of LUT including cell wall and membrane damage, nucleic acid synthesis inhibition, and an impact on protein expression and energy metabolism of bacteria. Furthermore, Qian et al. (2020) demonstrated potent antibacterial effect of LUT against *S. aures* (MIC: 16–32 μg/ml) and *Listeria monocytogenes* (MIC: 32–64 μg/ml) that was exerted via cell membrane disruption. They have also observed significant inhibitory effect on biofilm formation of the flavonoid. In contrast, Huang et al. (2017) found high MIC values of LUT against *E. coli* ATCC25922 and *Salmonella* Typhimurium C7731 strains, with the former being 2500 μg/ml, and the latter 1250 μg/ml.

For both GSOP and LUT, it can be concluded that their antibacterial activity highly depends on the species of bacteria tested, as well as the types of isolates (e.g. human or animal origin, reference strains or clinical isolates). Most studies are conducted with bacteria obtained from human patients, therefore our study complements the already existing knowledge with information on porcine-origin bacteria. Generally, it can be assumed that GSOPs and LUT possess antibacterial effect against porcine *E. coli* and *S*. Typhimurium isolates, and in our study, LUT seemed to be more potent against both pathogens. However, their MIC values are significantly higher than those of clinically used antibiotics. A summary about the activity of PACs and LUT against *E. coli* and *Salmonella* strains can be seen in **Table 12**.

Table 12. Minimum inhibitory concentrations (MICs) of proanthocyanidins (PACs) and luteolin (LUT) in *Escherichia coli* and *Salmonella* spp. obtained in our study and previous investigations.

Tested substance	Bacterial strain	MIC value	Reference
PACs from cranberry	*E. coli*	250 µg/ml	Margetis et al. (2015)
B-type oligomeric procyanidins from lotus seedpod	*E. coli*	800-1200 µg/ml	Tang C. et al. (2017)
Procyanidin B2 from cinnamon	*E. coli*	1250 µg/ml	Shan et al. (2007)
PACs from grape seeds	***E. coli***	**2048 µg/ml**	**Current study**
PACs from cranberry	*E. coli*	18,000 µg/ml	Alshaibani et al. (2017)
PACs from grape seeds	***S.* Typhimurium**	**2048 µg/ml**	**Current study**
LUT	***E. coli***	**256 µg/ml**	**Current study**
LUT	*E. coli*	200-300 µg/ml	Sanhueza et al. (2017)
LUT	*E. coli*	500 µg/ml	Adamczak et al. (2019)
LUT	*E. coli*	2500 µg/ml	Huang et al. (2017)
LUT	***S.* Typhimurium**	**256 µg/ml**	**Current study**
LUT	*S.* Typhimurium	1250 µg/m	Huang et al. (2017)

There is an increasing number of studies investigating interactions between antibiotics and plant extracts, or their bioactive compounds. If some compounds can potentiate the effect of clinically used antibiotics, it might pose a therapeutic solution against resistant strains. Amin et al. (2015) investigated synergism between flavonoids (rutin, morin and quercetin) and several antibiotics (ampicillin, amoxicillin, cefixime, ceftriaxone, vancomycin, methicillin, cephradine, erythromycin, imipenem, sulfamethoxazole/trimethoprim, ciprofloxacin, and levofloxacin) against MRSA strains. Additive effect was found in some cases when one or two flavonoids were combined with antibiotics, but synergism only occurred in case of the combination of all 3 flavonoids with antibiotics. Cell wall damage was suggested to be the underlying mechanism of their action. Similar observations were made in another study with MRSA strains, where LUT showed additive effect in combinations with some antibiotics (ampicillin, cephradine, ceftriaxone, imipenem, and methicillin), but synergism occurred only when LUT and quercetin together were combined with the same active substances (Amin et al, 2016). In the study of Sanhueza et al. (2017), grape pomace extract showed synergistic activity with different antibiotics (ampicillin, fluoroquinolones, tetracycline, and chloramphenicol) against *S. aureus* and *E. coli*. However, it should be noted that the tested grape pomace extract contained several phenolic compounds, among which quercetin, gallic acid, protocatechuic acid and LUT were the most abundant substances. The same can be

concluded based on the study of Trabelsi et al. (2020), where pomegranate extract enriched with total oligomer flavonoids - containing rutin, LUT, gallic acid, and ellagic acid - showed synergism with amoxicillin against penicillin-resistant *E. coli* and *S. aureus*. It is also mentioned by Skroza et al. (2019) that the antimicrobial effect of plant extracts is frequently the result of interactions among several substances present in the extract.

In our study, GSOP and LUT did not show synergistic, additive, or antagonistic effect with any of the tested antibiotics (amoxicillin, gentamicin, enrofloxacin) against clinical isolates of *E. coli* and *S*. Typhimurium of porcine origin. This is in accordance with the study of Bustos et al. (2018), where LUT showed synergistic activity with gentamicin against a reference strain of *S. aureus*, and additive effect against a reference *E. coli* strain, however, it could not increase the efficacy of gentamicin against clinical strains of the same bacteria that showed resistance to gentamicin. Furthermore, LUT was not synergistic with gentamicin against *T. pyogenes* clinical isolates from cows with mastitis (Zhang et al., 2019). On the other hand, Eumkeb et al. (2012) could detect synergism between LUT and amoxicillin in four *E. coli* isolates resistant to amoxicillin. They proposed three mechanisms, that might explain the potential of LUT in reversing resistance. These included inhibition of protein and peptidoglycan synthesis, inhibition of β-lactamase enzyme activity, and alteration of membrane permeability. LUT has also shown synergism with amoxicillin, oxacillin, and gentamicin against MRSA strains (Joung et al., 2016). Maisuria et al. (2019) investigated the synergism of cranberry PACs with several antibiotics (gentamicin, kanamycin, tetracycline, azithromycin, sulfamethoxazole, trimethoprim, nitrofurantoin, and fosfomycin) against one *E. coli*, one *P. mirabilis*, and two *P. aeruginosa* strains. In most antibiotic-pathogen combinations, cranberry PACs could potentiate the effect of the active substance, however, some differences were also observed (e.g. PACs could increase the effect of fosfomycin against *P. mirabilis*, but did not show this activity in case of *E. coli*). Therefore, it was concluded that potentiating effect of PACs is specific. PACs' mechanism of action was reported to be the suppression of selective membrane permeability and multidrug efflux pumps (Maisuria et al., 2019). Efflux pump inhibition – as a potential resistance elimination mechanism – has also been suggested about LUT (Zhang et al., 2019).

Similarly to the antibacterial activity of flavonoids, interaction studies of antibiotics with phytochemicals show controversial results. The observed discrepancies can be due to differences in the species and original host of the tested bacteria, type of the isolates (clinical or reference strain), as well as in the source of flavonoids. Generally, these investigations are conducted on low number of isolates, that further limits interpretation of the results. However, a neutral effect between flavonoids and antibiotics – seen in our studies with bacteria of porcine origin – means the lack of synergism as well as the lack of unfavorable interactions, that could

also be an advantage, as it would not limit simultaneous administration of these phytochemicals with antibiotics.

The antibiotics tested in our study have different primary targets in bacteria, but increased ROS production – as an additional killing effect – was shown to be involved in the bactericidal activity of all these antibiotic classes (Dwyer et al., 2009; Dwyer et al. 2014). Alteration in the amount of ROS in bacteria might also occur during exposure to flavonoids, taking into account their potential antioxidant and pro-oxidant activity reported in eukaryotic cells (see above). Assuming that GSOP and LUT acted as antioxidants in bacteria during our interaction studies, it is possible that they decreased the antibiotics' ROS-based killing activity, but this could have been counteracted by other flavonoid mechanisms (e.g. cell wall and cell membrane damage, efflux pump inhibition) that improve efficacy of the tested antibiotics, resulting in the overall absence of interactions. Although it was not investigated in our study, it is also interesting to note that antioxidants might inhibit the development of resistance that evolves as a result of ROS-induced mutagenesis in bacteria that are not killed by the ROS increase due to antibiotic exposure (Pribis et al., 2019). Contrary to this, if the flavonoids acted as pro-oxidants in our study, they could have improved the antibiotics' killing efficacy by further ROS induction, resulting in additive or synergistic effect, which was not observed in our study. A possible explanation of this could be that primarily, bacteriostatic activity can be evaluated with the checkerboard assay, which can occur at different concentration ranges compared to bactericidal effect. It cannot be excluded that at higher, bactericidal concentrations, additive or synergistic interactions would have been observed. Furthermore, some of the antibiotics had high MIC values against the tested strains, i.e. those isolates were resistant and could have mutations at the primary targets of antibiotics, which can diminish ROS inducer effect of drugs as well, as suggested by Dwyer et al. 2014.

Intestinal epithelial cells are thought to have only a secondary role in immunity, however, they produce important signaling molecules that control the immune response (Schierack et al., 2006). In our study, treatment of porcine intestinal epithelial cells with *E. coli* and *S*. Typhimurium resulted in an increased IL-6 and IL-8 production, that could successfully be alleviated with the addition of GSOP and LUT. Anti-inflammatory effect of PACs from various sources has already been described in intestinal cell culture models. Apple procyanidins could decrease IL-8 release in Caco-2 cells treated with PMA (phorbol 12-myristate 13-acetate) (Yoshioka et al., 2008), as well as the gene expression of NF-κB and TNF-α induced by LPS (Wu et al., 2018). Cranberry procyanidins could also reduce IL-6 and TNF-α concentration of Caco-2/15 cells (a stable clone of parent Caco-2 cells) elevated by LPS (Denis et al., 2015). Anti-inflammatory effect of grape seed PACs has been investigated in several mouse and rat models of intestinal dysfunction, and their administration resulted in a lower amount of

inflammatory mediators (e.g. IL-1β, IL-6, TNF-α and IFN-γ) (González-Quilen et al., 2020). Interestingly, Wu et al. (2022) proposed that protective effects of grape seed PACs in intestinal inflammation (induced by LPS in mice) can be exerted via modulation of the GI microbiota and composition of bile acids. Our study was the first to investigate anti-inflammatory effect of GSOPs in IPEC-J2 cells infected with bacteria, and we could demonstrate potential of GSOP in decreasing IL-6 and IL-8 levels elevated by the *E. coli* and *S*. Typhimurium. These results are in accordance with the above-mentioned data and supplements the already existing knowledge about anti-inflammatory effect of GSOP in intestinal cells.

Similarly to GSOP, anti-inflammatory effect of LUT has been reported previously in studies with *in vitro* and *in vivo* models of GI diseases. Boeing et al. (2020) demonstrated anti-inflammatory properties of LUT in the GI tract of mice, in case of irinotecan (an agent used for the treatment of colorectal cancer) – induced intestinal damage: the flavonoid was able to decrease IL-1β, IL-6 and TNF-α concentrations in the duodenum and colon samples. LUT could also decrease IL-8 production of HT-29 cells stimulated with TNF-α (Kim et al., 2005), or with the mixture of TNF-α and IFN-γ (Nunes et al., 2017), as well as LPS-induced IL-8 expression of Caco-2 cells in a co-culture with macrophages (Nishitani et al. 2013). Furthermore, LUT could inhibit NF-κB signaling and proinflammatory gene expression in IEC-18 cells treated with LPS (Kim and Jobin, 2005). In high-fat diet-fed rats, LUT decreased TNF-α levels of the colon, plasma and liver, and IL-6 levels of the plasma and liver. Interestingly, it was suggested that the observed anti-inflammatory effect of LUT might be related to alterations in the GI microbiota caused by the flavonoid (Sun et al., 2021). In line with the above-mentioned information, we could also observe anti-inflammatory activity of LUT by decreasing IL-6 and IL-8 levels in IPEC-J2 cells. Our study was the first to investigate this effect of LUT in IPEC-J2 cells infected with *E. coli* and *S*. Typhimurium. Even though the current discussion focused on the role of PAC and LUT in regulating inflammation in GI models, it is worth mentioning that their anti-inflammatory effect has also been demonstrated in other cell types, for example in LPS-treated murine macrophages (Zhang et al., 2018; Zhang et al., 2020).

As mentioned previously, protection of the intestinal barrier integrity has an important role in the prevention and treatment of diseases. Several phytochemicals have shown the ability to alleviate inflammation-mediated TJ disruption (Ghosh et al., 2020). PACs from different sources have been tested for their barrier protective activity in different *in vitro* and *in vivo* models of intestinal dysfunction (González-Quilen et al., 2020). The effect of PACs on intestinal barrier integrity is thought to be the result of several mechanisms, including their antioxidant and anti-inflammatory activity (Unusan, 2020). PACs from Granny Smith apple could increase the expression of occludin and ZO-1 in Caco-2 cells after LPS treatment (Wu et al., 2018). Cocoa procyanidins were also able to decrease barrier permeability in Caco-2 cells damaged

with dextran sodium sulfate (Bitzer et al., 2015). In the study of Gil-Cardoso et al. (2019), grape seed PACs increased ZO-1 expression in the GI tract of rats fed with high energy diet. In our study, we could also observe barrier protective effect of GSOP, and this was the first time to demonstrate it in IPEC-J2 cells infected with *E. coli* and *S*. Typhimurium.

In rats, LUT was able to counteract the deteriorating effect of high-fat diet on the intestinal barrier by increasing the expression of TJ proteins, such as ZO-1, occludin, and claudin-1 (Liu et al., 2021). Furthermore, LUT could to alleviate intestinal barrier damage caused by ethanol and polybrominated diphenyl ether in human colonic adenocarcinoma (Caco-2) cells, shown by an increase in TEER value and an upregulation of ZO-1, occludin, and claudin-1 (Yuan et al., 2021A; Yuan et al., 2021B). Li et al. (2021) also observed barrier protective function of LUT in TNF-α and IFN-γ treated Caco-2 cells, through the increase in TEER values and expression of occludin, claudin-1 and ZO-1. LUT could also decrease barrier permeability in high-fat diet-fed rats (Sun et al., 2021). In our study, LUT showed protective effect against barrier damage caused by *E. coli*, but it was less effective in case of *S*. Typhimurium infection. The reason of this observation might be the difference in the effect of *S*. Typhimurium compared to *E. coli*, or the difference between the effect of LUT on the two bacteria. We have investigated barrier protective activity of GSOP and LUT through the penetration of a fluorescent dye across the cell layer, but it would worth examining the exact mechanisms behind the observed beneficial effects.

Adhesion of bacteria to epithelial cells is a preliminary, fundamental step in the development of GI infections (Edfors-Lilja et al., 2000; Reis and Horn, 2010). Several studies have shown the preventive effect of cranberry-derived PACs against urinary tract infections (Rodríguez-Pérez et al., 2019; González de Llano et al., 2020). Besides other possible mechanisms, it is thought to be the result of anti-adhesive properties of cranberry polyphenols and their metabolites that prevent the adherence of pathogens to uroepithelial cells by directly inhibiting bacterial adhesins and inducing non-specific defense in the kidney. Anti-adhesive activity can be exhibited by A-type procyanidins (e.g. those in cranberry), but has not been observed in case of B-type structure (González de Llano et al., 2020). Besides findings about the urinary tract, cranberry PACs inhibited the adherence of *E. coli* isolates to buccal epithelial cells (Margetis et al., 2015), and vaginal epithelial cells (Gupta et al., 2007). Furthermore, cranberry PACs could inhibit the invasion of *S*. Typhimurium in HeLa (human cervical carcinoma) cells in the study of Harmidy et al. (2011). They have also observed anti-adhesive activity of PACs against EPEC, and suggested that the observed effects against both pathogens are more likely to be the consequence of PACs' effect on the host cells instead of directly affecting bacterial virulence (Harmidy et al., 2011). In our investigation, grape seed PACs, that contain only B-type linkages, showed potent anti-adhesive activity against *E. coli* in IPEC-J2 cells. This is in

contrast to the above-mentioned statement of González de Llano et al. (2020) suggesting anti-adhesive activity of A-type procyanidins only. This discrepancy might be due to the effect of other components in GSOPs, or due to differences in the pathogens and host cells. However, GSOPs only inhibited the adhesion of *S*. Typhimurium in case of pre-treatment, assuming the importance of host cell alteration in the anti-adhesive action of PACs, as suggested by Harmidy et al. (2011). Pre-treatment with plant extracts was also found to be the more effective than parallel or post-treatment by Šikić Pogačar et al. (2016).

There are reports about the anti-adhesive activity of LUT as well. In the study of Fu et al. (2021), LUT could inhibit the adherence and biofilm formation of *Candida albicans* and *E. faecalis* on glass surfaces in concentrations lower than those that showed growth inhibitory effect against the tested microorganisms. LUT also decreased adhesion and invasion of uropathogenic *E. coli* to human bladder epithelial cells (Shen et al., 2014). In the study of Šikić Pogačar et al. (2016), thyme ethanolic extract, thyme post-hydrodistillation residue and olive leaf extract – all of them containing luteolin 7-*O*-glucoside among other phytochemicals – could inhibit the adhesion of *C. jejuni* to both abiotic surfaces and porcine small intestinal epithelial cells. For their anti-adhesive activity, lower concentrations were enough compared to MIC values of the extracts. Furthermore, they found that pre-treatment with the extract was more effective than parallel or post-treatment, between which post-treatment was the least effective. In contrast to previous publications, in our investigation, LUT did not exert anti-adhesive activity in IPEC-J2 cells against neither *E. coli*, nor *S*. Typhimurium. This discrepancy might be the result of different bacterial strains and host cells being tested. However, adhesion is just the first step of infections, therefore it is important to evaluate the ability of feed additives to reduce or eliminate the infections altogether (Spitzer et al., 2016).

In our investigation with IPEC-J2 cells, we could not establish a clear concentration dependence of the activity of flavonoids. In many cases, higher concentrations of GSOP and LUT were more potent, however, in other study parts, the results seemed to the opposite (**Table 13**). A possible explanation of this finding is that there might be nonlinear dose effects of flavonoids as stated by Kay et al. (2012). In many cases, the lowest applied concentrations (i.e. 50 µg/ml for GSOP and 25 µg/ml for LUT) had significant beneficial effects on the tested parameters in IPEC-J2 cells, which is a great advantage in terms of their possible future usage as feed additives, taking into consideration limited financial opportunities of several farms. However, it should be noted that concentrations necessary for the bacteriostatic effect of these flavonoids were much higher than the amounts found to be effective in cell culture studies. These concentrations might not be profitable in case of the use of flavonoid as feed additives, and it might also affect sensory properties of the feed after mixing. Therefore, achieving the concentrations effective on IPEC-J2 cells seems to be a more reasonable goal *in vivo*.

Table 13. Differences in the activity of grape seed proanthocyanidins (GSOP) and luteolin (LUT) when applied at various concentrations (50-200 µg/ml for GSOP, and 25-100 µg/ml for LUT) and treatment types (pre-, parallel, post-treatment). Observations of similar efficacy are not included.

	GSOP	LUT
Antioxidant effect - LPS	Pronounced activity, but there was no clear proportionality between concentration and efficacy.	Pronounced activity, but here was no clear proportionality between concentration and efficacy.
Antioxidant effect – Co-culture	*E. coli* Parallel treatment was more effective than pre- or post-treatments.	*E. coli* Higher concentration showed higher efficacy in case of post-treatment. Parallel treatment was the most effective in lower concentrations. Pre-treatment was the least effective in higher concentrations.
Anti-inflammatory effect	*E. coli* Pre-treatment was not effective in case of IL-6. For IL-8, post-treatment was only effective in higher concentration, and it showed the lowest efficacy. *S.* Typhimurium In case of IL-8, the lower concentration was only effective as pre-treatment.	*E. coli* For IL-8, the higher concentration was not effective as post-treatment. *S.* Typhimurium There was no clear proportionality between concentration/treatment type and efficacy for IL-8.
Barrier protection	*S.* Typhimurium There was no clear proportionality between concentration/treatment type and efficacy.	*E. coli* The higher concentration was not effective as pre-treatment. *S.* Typhimurium Only post-treatment with higher concentration was effective.
Anti-adhesive effect	*E. coli* Pronounced activity, except for post-treatment, where only the higher concentration was effective. *S.* Typhimurium Only pre-treatment was effective.	-

Efficacy of different treatment types (pre-, parallel, and post-treatment) also varied between tests, flavonoids, and bacterial species (**Table 13**). Post-treatments did not seem to be effective in more cases than pre- or parallel treatments. Regarding *E. coli*, parallel treatments had significant activity in all tests with GSOPs and LUT as well. These suggest that the presence of flavonoids in the GI tract before, or simultaneously with bacterial infection (i.e. preventive usage), might be more effective compared to their usage after infection (i.e. therapeutic usage). It is in accordance with the finding of Spitzer et al. (2016), who concluded that *in vivo*, feed additives can be present before pathogenic bacteria reach the intestines, and this might be needed for them to modify physiology of the epithelial cell. In this context, it is beneficial that GSOP and LUT did not show any interaction with the tested antibiotics, therefore their presence in the GI tract presumably do not alter efficacy of simultaneously administered amoxicillin, gentamicin and enrofloxacin.

In terms of comparing the activity of GSOP and LUT in our studies, both flavonoids had significant antioxidant and anti-inflammatory activity, but LUT showed more potent bacteriostatic effect with 8-fold lower MIC values than GSOP (even though this concentration might still be too high to be reasonably achieved in the GI tract *in vivo*). In contrast, LUT was less effective in protecting barrier integrity of IPEC-J2 cells against *S*. Typhimurium infection, and contrary to GSOP, it showed no anti-adhesive effect against any of the tested bacteria. Structural similarities of GSOP and LUT might explain their common effects, while differences in their skeleton could be responsible for the observed alterations. For example, hydroxyl groups can be found on ring A and B of both flavonoids, and these have a role antioxidant (mainly ROS scavenging) and anti-inflammatory action of flavonoids (Hošek and Šmejkal, 2015). Furthermore, hydroxyl groups at position 5, 7 (ring A) and 4’ (ring B) – present in both GSOP and LUT – are correlated to the antibacterial activity of flavonoids against certain bacteria (Shamsudin et al., 2022). However, a hydroxyl group at position 5’ (ring B) might decrease antibacterial efficacy (Shamsudin et al., 2022), and it is present only in GSOP, but not in LUT. The opposite can be mentioned about the double bond between C2 and C3: it is involved in exerting antibacterial effect (Shamsudin et al., 2022), and it is found in LUT, but not in GSOP. These structural differences might explain lower MIC values (i.e. higher antibacterial activity) of LUT compared to GSOP. Regarding better anti-adhesive and barrier protective effects of GSOP, they might originate from the ability of PACs to adhere to intestinal epithelial cells that can increase TEER values in Caco-2 cells (Deprez et al., 2001). Attachment of PACs to the cell layer might also contribute to their anti-adhesive action against bacteria.

Generally, antibacterial potency of phytochemical is lower than that of antibiotics. For future usage, structural alteration of these substances (e.g. through halogenation or substitution with functional groups) might be useful to increase their efficacy (Cushnie and Lamb, 2005). Furthermore, their low water solubility, susceptibility to environmental factors, poor permeability, relative chemical instability, rapid release, and mainly low bioavailability pose a challenge in reaching effective *in vivo* concentrations (González et al., 2021). Oral bioavailability of flavonoids is low, only 5-10% of their amount is absorbed from the small intestines, but they can exert their antimicrobial properties in the intestines (Adamczak et al., 2019). Bioavailability of PACs highly depend on their degree of polymerization. The term oligomeric PACs refers to a lower degree of polymerization, i.e. the presence of 2-4 monomers (Unusan, 2020). However, the alteration of flavonoids in the GI tract can also result in their decreased activity. To overcome these problems, many advanced delivery systems (e.g. nanoparticles, liposomes, micelles, emulsions) has been developed to improve bioavailability and modify release of flavonoids. Chemical modification of their structure, for example the generation of flavonoid prodrugs, could aid these purposes as well (González et al., 2021). Glycoside forms of flavonoids can also have better bioavailability compared to their aglycone version, but their biological activity might be weaker (Kumar and Pandey, 2013).

It should also be mentioned, that even though flavonoids likely to have minimal toxicity, an *in vivo* evaluation of their possible side effects would be necessary before their practical application (Cushnie and Lamb, 2005). In our study, GSOP and LUT did not show cytotoxic effect on IPEC-J2 porcine intestinal epithelial cells in any of the tested concentrations and treatment lengths, which are promising preliminary results that support their safe usage.

Limitations of the current study included relatively low number of bacterial isolates and antibiotics tested, as well as all experimental settings being *in vitro*. It could be recommended to conduct further experiments with more bacterial strains, including other bacterial species as well, and to test possible interactions of GSOP and LUT with further drugs (not only antibiotics) and feed additives, that might occur in the diet simultaneously. Furthermore, *in vivo* efficacy and dose confirmation studies would also be necessary to establish their practical application. Therefore, the actual usage of these flavonoids as feed additives should depend on further *in vitro* and *in vivo* studies, as well as financial calculations about the economic effect of their usage in pork production.

To conclude our findings, both GSOP and LUT showed several beneficial effects *in vitro*, in models of porcine GI infections. Based on these properties, they might be used in the future for the prevention and/or treatment of bacterial GI infections of swine caused by *E. coli* and *S*. Typhimurium. Their use as feed additives could reduce the occurrence of GI diseases, and consequently the need for antibiotics. Another potential indication of their use could be the simultaneous administration with antibiotics to counteract oxidative stress, inflammation and barrier damage caused by bacteria, especially in case of LPS release due to killing effect of antibiotics. Furthermore, flavonoids as antioxidants might inhibit development of resistance that occurs due to ROS-induced mutagenesis, which could be an additional benefit of GSOP and LUT co-administration with antibiotics, even though this effect has not been tested in the current study. It would also worth investigating potential beneficial effects of GSOP and LUT in other species, including humans. Altogether their future use might replace antibiotics at some extent and improve treatment options of GI infections, thus decreasing the spread of resistance and improving human and animal health. GSOP and LUT are extensively studied and easily available substances, therefore the development of feed additives containing any of them would be more time- and cost-effective in comparison to newly synthesized or discovered substances. The models we used in our studies could also be beneficial in the future for testing drug and feed additive candidates.

8. Overview of new scientific results

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

Main findings of the study are as follows:

1. GSOP (up to 200 µg/ml) and LUT (up to 100 µg/ml) did not show cytotoxic effect on IPEC-J2 cells when applied for 1, 12 and 24 hours.

2. GSOP and LUT were able to decrease oxidative stress (intracellular reactive oxygen species levels) in IPEC-J2 cells triggered by LPS of *E. coli* (O111:B4, O127:B8) and *S*. Typhimurium origin, as well as oxidative stress inflicted by *E. coli* and *S*. Typhimurium *in vitro* infections.

3. In certain treatment types, GSOP and LUT showed anti-inflammatory effect (decreased interleukin-6 and interleukin-8 levels) and barrier protective activity in IPEC-J2 cells in case of inflammation and barrier integrity damage caused by *E. coli* and *S*. Typhimurium *in vitro* infections.

4. GSOP could inhibit the adhesion of *E. coli* and *S*. Typhimurium to IPEC-J2 cells, while LUT did not show anti-adhesive activity against any of the tested bacteria.

5. GSOP inhibited the growth of *E. coli* and *S*. Typhimurium field isolates of porcine origin with minimum inhibitory concentration (MIC) values of 2048 µg/ml, while LUT showed bacteriostatic effect against the same bacteria at 256 µg/ml concentrations. The addition of GSOP and LUT did not influence activity of conventionally used antibiotics (amoxicillin, gentamicin, enrofloxacin) against the same strains (average FIC indexes between 1.0000 and 1.4375).

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

9. References

Adamczak A., Ożarowski M., Karpiński TM.: **Antibacterial Activity of Some Flavonoids and Organic Acids Widely Distributed in Plants**, J. Clin. Med., 9(1). 109, 2019.

Al-Megrin W.A., Alkhuriji A.F., Yousef A.O.S., Metwally D.M., Habotta O.A., Kassab R.B., Moneim A.E.A., El-Khadragy M.F.: **Antagonistic Efficacy of Luteolin against Lead Acetate Exposure-Associated with Hepatotoxicity is Mediated via Antioxidant, Anti-Inflammatory, and Anti-Apoptotic Activities**, Antioxidants, 9. 10, 2020.

Alav I., Sutton J.M., Rahman K.M.: **Role of bacterial efflux pumps in biofilm formation**, J. Antimicrob. Chemother., 73(8). 2003-2020, 2018.

Alshaibani D., Zhang R., Wu V.C.: **Antibacterial characteristics and activity of *Vaccinium macrocarpon* proanthocyanidins against diarrheagenic *Escherichia coli***, J. Funct. Foods, 39. 133–138, 2017.

Amin M.U., Khurram M., Khan T.A., Faidah H.S., Ullah Shah Z., Ur Rahman S., Haseeb A., Ilyas M., Ullah N., Umar Khayam S.M., Iriti M.: **Effects of Luteolin and Quercetin in Combination with Some Conventional Antibiotics against Methicillin-Resistant *Staphylococcus aureus***, Int. J. Mol. Sci., 17(11). 1947, 2016.

Amin M.U., Khurram M., Khattak B., Khan J.: **Antibiotic additive and synergistic action of rutin, morin and quercetin against methicillin resistant *Staphylococcus aureus***, BMC Complement. Altern. Med., 15. 59, 2015.

Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit user manual https://assets.thermofisher.com/TFS-Assets/LSG/manuals/mp22188.pdf

Antimicrobial Resistance Collaborators: **Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis**, Lancet, 399(10325). 629-655, 2022.

Azam S., Hadi N., Khan N.U., Hadi S.M.: **Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: implications for anticancer properties**, Toxicol. In Vitro, 18. 555–561, 2004.

Belščak-Cvitanović A., Durgo K., Huđek A., Bačun-Družina V., Komes D.: **1 - Overview of polyphenols and their properties**, Editor(s): Charis M. Galanakis, Polyphenols: Properties, Recovery, and Applications, Woodhead Publishing, 2018.

Berschneider H.M.: **Development of normal cultured small intestinal epithelial cell lines which transport Na and Cl**, Gastroenterology, 96. A41, 1989.

Biharee A., Sharma A., Kumar A., Jaitak V.: **Antimicrobial flavonoids as a potential substitute for overcoming antimicrobial resistance**, Fitoterapia, 146. 104720, 2020.

Bitzer Z.T., Glisan S.L., Dorenkott M.R., Goodrich K.M., Ye L., O'Keefe S.F., Lambert J.D., Neilson A.P.: **Cocoa procyanidins with different degrees of polymerization possess distinct activities in models of colonic inflammation**, J. Nutr. Biochem., 26. 827–831, 2015.

Boeing T., de Souza P., Speca S., Somensi L.B., Mariano L.N.B., Cury B.J., Ferreira Dos Anjos M., Quintão N.L.M., Dubuqoy L., Desreumax P., da Silva L.M., de Andrade S.F**.: Luteolin prevents irinotecan-induced intestinal mucositis in mice through antioxidant and anti-inflammatory properties**, Br. J. Pharmacol., 177(10). 2393-2408, 2020.

Brosnahan A.J., Brown D.R.: **Porcine IPEC-J2 intestinal epithelial cells in microbiological investigations**, Vet. Microbiol., 156(3-4). 229-237, 2012.

Bustos P.S., Deza-Ponzio R., Páez P.L., Cabrera J.L., Virgolini M.B., Ortega M.G.: **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, 2018.

Bystrom L.M., Guzman M.L., Rivella S.: **Iron and reactive oxygen species: friends or foes of cancer cells?**, Antioxid. Redox. Signal., 20(12). 1917-24, 2014.

Capes-Davis A., Bairoch A., Barrett T., Burnett E.C., Dirks W.G., Hall E.M., Healy L., Kniss D. A., Korch C., Liu Y., Neve R.M., Nims R.W., Parodi B., Schweppe R.E., Storts D.R., Tian F.: **Cell Lines as Biological Models: Practical Steps for More Reliable Research**, Chem. Res. Toxicol., 32(9). 1733–1736, 2019.

Cencic A., Langerholc T.: **Functional cell models of the gut and their applications in food microbiology--a review**, Int. J. Food Microbiol., 141(1). S4-14, 2010.

Chedea V.S., Braicu C., Socaciu C: **Antioxidant/prooxidant activity of a polyphenolic grape seed extract**, Food Chem., 121(1). 132-139, 2010.

Chedea V.S., Braicu C., Chirilă F., Ogola H.J., Pelmuş R.Ş., Călin L.G., Socaciu C.: **Antioxidant/Prooxidant and antibacterial/probacterial effects of a grape seed extract in complex with lipoxygenase**, Biomed. Res. Int., 313684, 2010.Chedea V.S., Palade L.M., Marin D.E., Pelmus R.S., Habeanu M., Rotar M.C., Gras M.A., Pistol G.C., Taranu I.: **Intestinal Absorption and Antioxidant Activity of Grape Pomace Polyphenols**, Nutrients, 10. 588, 2018.

Chen L., Hu S.L., Xie J., Yan D.Y., Weng S.J., Tang J.H., Wang B.Z., Xie Z.J., Wu Z.Y., Yang L.: **Proanthocyanidins-Mediated Nrf2 Activation Ameliorates Glucocorticoid-Induced Oxidative Stress and Mitochondrial Dysfunction in Osteoblasts**, Oxid. Med. Cell. Longev., 9102012, 2020.

Christaki E., Marcou M., Tofarides A.: **Antimicrobial Resistance in Bacteria: Mechanisms, Evolution, and Persistence**, UCLA Sym. Bi., 88(1). 26-40, 2019.

Chung W.G., Miranda C.L., Stevens J.F., Maier C.S.: **Hop proanthocyanidins induce apoptosis, protein carbonylation, and cytoskeleton disorganization in human colorectal adenocarcinoma cells via reactive oxygen species**, Food Chem. Toxicol., 47(4). 827-36, 2009.

Clinical and Laboratory Standards Institute (CLSI): **Guideline M07-A10 Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically**. In Approved Standard, 10th ed.; CLSI: Wayne, PA, USA, 2015.

Cloeckaert A., Zygmunt M., Doublet B.: **Editorial: Genetics of Acquired Antimicrobial Resistance in Animal and Zoonotic Pathogens**, Front. Microbiol., 8. 2428, 2017.

Corning Inc.: **Surface Areas and Guide for Recommended Medium Volumes for Corning® Cell Culture Vessels**. https://www.corning.com/catalog/cls/documents/application-notes/CLS-AN-209.pdf

Cushnie T.P., Lamb A.J.: **Antimicrobial activity of flavonoids**, Int. J. Antimicrob. Agents, 26(5). 343-356, 2005.

De Stefano A., Caporali S., Di Daniele N., Rovella V., Cardillo C., Schinzari F., Minieri M., Pieri M., Candi E., Bernardini S., Tesauro M., Terrinoni A.: **Anti-Inflammatory and Proliferative Properties of Luteolin-7-O-Glucoside**, Int. J. Mol. Sci., 22(3). 1321, 2021.

Denis M.C., Desjardins Y., Furtos A., Marcil V., Dudonné S., Montoudis A., Garofalo C., Delvin E., Marette A., Levy E.: **Prevention of oxidative stress, inflammation and mitochondrial dysfunction in the intestine by different cranberry phenolic fractions**, Clin. Sci., 128(3). 197-212, 2015.

Deprez S., Mila I., Huneau J.F., Tome D., Scalbert A.: **Transport of proanthocyanidin dimer, trimer, and polymer across monolayers of human intestinal epithelial Caco-2 cells**, Antioxid. Redox Signal., 3(6). 957-67, 2001.

Dwyer D.J., Kohanski M.A., Collins J.J.: **Role of reactive oxygen species in antibiotic action and resistance**, Curr. Opin. Microbiol., 12(5). 482-489, 2009.

Dwyer D.J., Belenky P.A., Yang J.H., MacDonald I.C., Martell J.D., Takahashi N., Chan C.T., Lobritz M.A., Braff D., Schwarz E.G., Ye J.D., Pati M., Vercruysse M., Ralifo P.S., Allison K.R., Khalil A.S., Ting A.Y., Walker G.C., Collins J.J.: **Antibiotics induce redox-related physiological alterations as part of their lethality**, Proc. Natl. Acad. Sci. U S A, 111(20), E2100-9. 2014.

D'Incau M., Salogni C., Giovannini S., Ruggeri J., Scali F., Tonni M., Formenti N., Guarneri F., Pasquali P., Alborali G.L.: **Occurrence of *Salmonella* Typhimurium and its monophasic variant (4, [5],12:i:-) in healthy and clinically ill pigs in northern Italy**, Porc. Health Manag., 7(1). 34, 2021.

Edfors-Lilja I., Wallgren P., Axford R.F.E., Bishop S.C., Nicholas F.W., Owen J.B.: ***Escherichia coli* and *Salmonella* diarrhoea in pigs**, Breeding for disease resistance in farm animals, 2nd ed. Wallingford: CABI Publishing, 253267, 2000.

Emes D., Naylor N., Waage J., Knight G.: **Quantifying the Relationship between Antibiotic Use in Food-Producing Animals and Antibiotic Resistance in Humans**, Antibiotics, 11. 66, 2022.

Eumkeb G., Siriwong S., Thumanu K.: **Synergistic activity of luteolin and amoxicillin combination against amoxicillin-resistant *Escherichia coli* and mode of action**, J. Photochem. Photobiol. B., 117. 247-253, 2012.

European Food Safety Authority (EFSA) Panel on Animal Health and Welfare (AHAW): **Scientific Opinion on the assessment of animal diseases caused by bacteria resistant to antimicrobials: Swine**. EFSA J., 19(12). 7113, 2021.

European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC): **The European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2018/2019**, EFSA J., 19(4). 6490, 2021.

European Medicines Agency (EMA) (2016) **Guideline on the principles of regulatory acceptance of 3Rs (replacement, reduction, refinement) testing approaches** (EMA/CHMP/CVMP/JEG-3Rs/450091/2012)

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

European Medicines Agency (EMA), European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) (2021) **Sales of veterinary antimicrobial agents in 31 European countries in 2019 and 2020** (EMA/58183/2021)

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**

Farkas O., Palócz O., Pászti-Gere E., Gálfi P.: **Polymethoxyflavone Apigenin-Trimethylether Suppresses LPS-Induced Inflammatory Response in Nontransformed Porcine Intestinal Cell Line IPEC-J2**, Oxid. Med. Cell. Longev., 673847, 2015.

Fu Y., Wang W., Zeng Q., Wang T., Qian W.: **Antibiofilm Efficacy of Luteolin Against Single and Dual Species of *Candida albicans* and *Enterococcus faecalis***, Front. Microbiol., 12. 715156, 2021.

Galarraga-Vinueza M.E., Dohle E., Ramanauskaite A., Al-Maawi S., Obreja K., Magini R., Sader R., Ghanaati S., Schwarz F.: **Anti-inflammatory and macrophage polarization effects of Cranberry Proanthocyanidins (PACs) for periodontal and periimplant disease therapy**, J. Periodont. Res., 55. 821–829, 2020.

Gálfi P., Csikó G., Jerzsele Á.: **Állatorvosi gyógyszertan III**, Robbie-Vet Kft., Budapest, 2014.

Geens M.M., Niewold T.A.: **Optimizing culture conditions of a porcine epithelial cell line IPEC-J2 through a histological and physiological characterization**, Cytotechnology, 63(4). 415-423, 2011.

Gendrisch F., Esser P.R., Schempp C.M., Wölfle U.: **Luteolin as a modulator of skin aging and inflammation**, Biofactors, 47(2). 170-180, 2021.

Ghosh S.S., Wang J., Yannie P.J., Ghosh S.: **Intestinal Barrier Dysfunction, LPS Translocation, and Disease Development**, J. Endocr. Soc., 4(2). bvz039, 2020.

Gil-Cardoso K., Comitato R., Ginés I., Ardévol A., Pinent M., Virgili F., Terra X., Blay M.: **Protective Effect of Proanthocyanidins in a Rat Model of Mild Intestinal Inflammation and Impaired Intestinal Permeability Induced by LPS**, Mol. Nutr. Food Res., 63. 1800720, 2019.

Goldstein S., Meyerstein D., Czapski G.: **The Fenton reagents**, Free Radic. Biol. Med., 15(4). 435-45, 1993.

González A., Casado J., Lanas Á.: **Fighting the Antibiotic Crisis: Flavonoids as Promising Antibacterial Drugs Against *Helicobacter pylori* Infection**, Front. Cell. Infect. Microbiol., 11. 709749, 2021.

González de Llano D., Moreno-Arribas M.V., Bartolomé B.: **Cranberry Polyphenols and Prevention against Urinary Tract Infections: Relevant Considerations**, Molecules, 25(15). 3523, 2020.

González-Quilen C., Rodríguez-Gallego E., Beltrán-Debón R., Pinent M., Ardévol A., Blay M.T., Terra X.: **Health-Promoting Properties of Proanthocyanidins for Intestinal Dysfunction**, Nutrients, 12. 130, 2020.

Gu L., Kelm M.A., Hammerstone J.F., Beecher G., Holden J., Haytowitz D., Gebhardt S., Prior R.L.: **Concentrations of Proanthocyanidins in Common Foods and Estimations of Normal Consumption**, J. Nutr., 134. 613–617, 2004.

Guo K., Zhao Y., Cui L., Cao Z., Zhang F., Wang X., Feng J., Dai M.: **The Influencing Factors of Bacterial Resistance Related to Livestock Farm: Sources and Mechanisms**, Front. Anim. Sci., 2. 650347, 2021.

Guo Y., Liu Y., Zhang Z., Chen M., Zhang D., Tian C., Liu M., Jiang G.: **The Antibacterial Activity and Mechanism of Action of Luteolin Against *Trueperella pyogenes***, Infect. Drug Resist., 13. 1697-1711, 2020.

Gupta K., Chou M.Y., Howell A., Wobbe C., Grady R., Stapleton A.E.: **Cranberry Products Inhibit Adherence of P-Fimbriated *Escherichia coli* to Primary Cultured Bladder and Vaginal Epithelial Cells**, J. Urol., 177. 2357–2360, 2007.

Hao R., Li Q., Zhao J., Li H., Wang W., Gao J.: **Effects of grape seed procyanidins on growth performance, immune function and antioxidant capacity in weaned piglets**, Livest. Sci., 178. 237–242, 2015.

Harmidy K., Tufenkji N., Gruenheid S.: **Perturbation of Host Cell Cytoskeleton by Cranberry Proanthocyanidins and Their Effect on Enteric Infections**, PLoS ONE, 6. e27267, 2011.

Heredia N., García S.: **Animals as sources of foodborne pathogens: A review**, Anim. Nutr., 4(3). 250-255, 2018.

Hošek J., Šmejkal K.: **Flavonoids as Anti-inflammatory Agents**. In: Parnham M. (eds) Encyclopedia of Inflammatory Diseases. Birkhäuser, Basel. 2015.

Huang C.C., Gao X., Sun T.T., Yu L., Guo Y., Hong W., Zhang D., Liu M.: **The antimicrobial activity of luteolin against four bacteria *in vitro***, Chin. J. Vet. Sci., 37(8). 1558–1561, 2017.

Huang C.S., Lii C.K., Lin A.H., Yeh Y.W., Yao H.T., Li C.C., Wang T.S., Chen H.W.: **Protection by chrysin, apigenin, and luteolin against oxidative stress is mediated by the Nrf2-dependent up-regulation of heme oxygenase 1 and glutamate cysteine ligase in rat primary hepatocytes**, Arch. Toxicol., 87(1). 167-78, 2013.

Jerzsele Á., Pásztiné-Gere E.: **Evaluating synergy between marbofloxacin and gentamicin in *Pseudomonas aeruginosa* strains isolated from dogs with otitis externa**, Acta Microbiol. Immunol. Hung., 62(1). 45–55, 2015.

Joung D.K., Kang O.H., Seo Y.S., Zhou T., Lee Y.S., Han S.H., Mun S.H., Kong R., Song H.J., Shin D.W., Kwon D.Y.: **Luteolin potentiates the effects of aminoglycoside and β-lactam antibiotics against methicillin-resistant *Staphylococcus aureus in vitro***, Exp. Ther. Med., 11(6). 2597-2601, 2016.

Ju W., Wang X., Shi H., Chen W., Belinsky S.A., Lin Y.: **A critical role of luteolin-induced reactive oxygen species in blockage of tumor necrosis factor-activated nuclear factor-kappaB pathway and sensitization of apoptosis in lung cancer cells**, Mol. Pharmacol., 71(5). 1381-8, 2007.

Kang K.P., Park S.K., Kim D.H., Sung M.J., Jung Y.J., Lee A.S., Lee J.E., Ramkumar K.M., Lee S., Park M.H., Roh S.G., Kim W.: **Luteolin ameliorates cisplatin-induced acute kidney injury in mice by regulation of p53-dependent renal tubular apoptosis**, Nephrol. Dial. Transplant., 26. 814–822, 2011.

Karancsi Z., Móritz A.V., Lewin N., Veres A.M., Jerzsele Á., Farkas O.: **Beneficial Effect of a Fermented Wheat Germ Extract in Intestinal Epithelial Cells in case of Lipopolysaccharide-Evoked Inflammation**, Oxid. Med. Cell. Longev., 1482482, 2020.

Kay C.D., Hooper L., Kroon P.A., Rimm E.B., Cassidy A.: **Relative impact of flavonoid composition, dose and structure on vascular function: A systematic review of randomised controlled trials of flavonoid-rich food products**, Mol. Nutr. Food Res., 56. 1605–1616, 2012.

Kim J.A., Kim D.K., Kang O.H., Choi Y.A., Park H.J., Choi S.C., Kim T.H., Yun K.J., Nah Y.H., Lee Y.M.: **Inhibitory effect of luteolin on TNF-alpha-induced IL-8 production in human colon epithelial cells**, Int. Immunopharmacol., 5(1). 209-217, 2005.

Kim J.S., Jobin C.: **The flavonoid luteolin prevents lipopolysaccharide-induced NF-kappaB signalling and gene expression by blocking IkappaB kinase activity in intestinal epithelial cells and bone-marrow derived dendritic cells**, Immunology, 115(3). 375-87, 2005.

Kitakaze T., Makiyama A., Yamashita Y., Ashida H.: **Low dose of luteolin activates Nrf2 in the liver of mice at start of the active phase but not that of the inactive phase**, PLoS ONE, 15(4). e0231403, 2020.

Klingspor S., Bondzio A., Martens H., Aschenbach J.R., Bratz K., Tedin K., Einspanier R., Lodemann, U.: ***Enterococcus faecium*** **NCIMB 10415 modulates epithelial integrity, heat shock protein, and proinflammatory cytokine response in intestinal cells**, Mediat. Inflamm., 304149, 2015.

Koh S.Y., George S., Brözel V., Moxley R., Francis D., Kaushik R.S.: **Porcine intestinal epithelial cell lines as a new *in vitro* model for studying adherence and pathogenesis of enterotoxigenic *Escherichia coli***, Vet. Microbiol., 130(1-2). 191-197, 2008.

Kumar S., Pandey A.K.: **Chemistry and biological activities of flavonoids: an overview**, Sci. World J., 162750, 2013.

Kupnik K., Primožič M., Vasić K., Knez Ž., Leitgeb M.: **A Comprehensive Study of the Antibacterial Activity of Bioactive Juice and Extracts from Pomegranate (*Punica granatum* L.) Peels and Seeds**, Plants, 10(8). 1554, 2021.

Lee K.A., Moon S.H., Kim K.T., Mendonca A.F., Paik H.D.: **Antimicrobial Effects of Various Flavonoids on *Escherichia coli* O157:H7 Cell Growth and Lipopolysaccharide Production**, Food Sci. Biotechnol., 19(1). 257-261, 2010.

León-Rodríguez M.D.C.P., Guyot J.P., Laurent-Babot C.: **Intestinal *in vitro* cell culture models and their potential to study the effect of food components on intestinal inflammation**, Crit. Rev. Food Sci. Nutr., 59(22). 3648-3666, 2019.

Lepper P.M., Held T.K., Schneider E.M., Bölke E., Gerlach H., Trautmann M.: **Clinical implications of antibiotic-induced endotoxin release in septic shock**, Intensive Care Med., 28(7). 824-833, 2002.

Li B.L., Zhao D.Y., Du P.L., Wang X.T., Yang Q., Cai Y.R.: **Luteolin alleviates ulcerative colitis through SHP-1/STAT3 pathway**, Inflamm. Res., 70(6). 705-717, 2021.

Li Y., Shen L., Luo H.: **Luteolin ameliorates dextran sulfate sodium-induced colitis in mice possibly through activation of the Nrf2 signaling pathway**, Int. Immunopharmacol., 40. 24-31, 2016.

Lillehoj H., Liu Y., Calsamiglia S., Fernandez-Miyakawa M., Chi F., Cravens R., Oh S., Gay C.: **Phytochemicals as antibiotic alternatives to promote growth and enhance host health**, Vet. Res., 49(1). 76, 2018.

Liu B., Jiang H., Lu J., Baiyun R., Li S., Lv Y., Li D., Wu H., Zhang Z.: **Grape seed procyanidin extract ameliorates lead-induced liver injury via miRNA153 and AKT/GSK-3β/Fyn-mediated Nrf2 activation**, J. Nutr. Biochem., 52. 115-123, 2018.

Liu M., Cheng C., Li X., Zhou S., Hua J., Huang J., Li Y., Yang K., Zhang P., Zhang Y., Tian, J.: **Luteolin alleviates ochratoxin A induced oxidative stress by regulating Nrf2 and HIF-1 pathways in NRK-52E rat kidney cells**, Food. Chem. Toxicol., 141. 111436, 2020.

Liu X., Sun R., Li Z., Xiao R., Lv P., Sun X., Olson M.A., Gong Y.: **Luteolin alleviates non-alcoholic fatty liver disease in rats via restoration of intestinal mucosal barrier damage and microbiota imbalance involving in gut-liver axis**, Arch. Biochem. Biophys., 711. 109019, 2021.

Lo S., Leung E., Fedrizzi B., Barker B.: **Syntheses of mono-acylated luteolin derivatives, evaluation of their antiproliferative and radical scavenging activities and implications on their oral bioavailability**, Sci. Rep., 11. 12595, 2021.

Loss H., Aschenbach J.R., Ebner F., Tedin K., Lodemann U.: **Inflammatory Responses of Porcine MoDC and Intestinal Epithelial Cells in a Direct-Contact Co-culture System Following a Bacterial Challenge**, Inflammation, 43. 552-567, 2020.

Loss H., Aschenbach J.R., Tedin K., Ebner F., Lodemann U.: **The inflammatory response to enterotoxigenic *E. coli* and probiotic *E. faecium* in a coculture model of porcine intestinal epithelial and dendritic cells**, Mediat. Inflamm., 9368295, 2018.

Luppi A.: **Swine enteric colibacillosis: diagnosis, therapy and antimicrobial resistance**, Porc. Health Manag., 8. 3-16, 2017.

Lückstädt C., Theobald P.: **Control of *E. coli* and *Salmonella* in growing-finishing pigs through the use of potassium diformate (KDF) – European case studies**, Ninth International Conference on the Epidemiology and Control of Biological, Chemical and Physical Hazards in Pigs and Pork, 2011.

Mackenzie J.S., Jeggo M.: **The One Health Approach-Why Is It So Important?** Trop. Med. Infect. Dis., 4(2). 88, 2019.

Magiorakos A.P., Srinivasan A., Carey R.B., Carmeli Y., Falagas M.E., Giske C.G., Harbarth S., Hindler J.F., Kahlmeter G., Olsson-Liljequist B., Paterson D.L., Rice L.B., Stelling J., Struelens M.J., Vatopoulos A., Weber J.T., Monnet D.L**.: Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance**, Clin. Microbiol. Infect., 18(3). 268-281, 2012.

Maisuria V.B., Okshevsky M., Déziel E., Tufenkji N.: **Proanthocyanidin Interferes with Intrinsic Antibiotic Resistance Mechanisms of Gram-Negative Bacteria**, Adv. Sci., 6(15). 1802333, 2019.

Margetis D., Roux D., Gaudry S., Messika J., Bouvet O., Branger C., Ponnuswamy P., Oufella H.A., Dreyfuss D., Denamur E., Ricard J.D.: **Effects of Proanthocyanidins on Adhesion, Growth, and Virulence of Highly Virulent Extraintestinal Pathogenic *Escherichia coli* Argue for Its Use to Treat Oropharyngeal Colonization and Prevent Ventilator-Associated Pneumonia**, Crit. Care Med., 43(6). e170–e178, 2015.

Moredo F.A., Piñeyro P.E., Márquez G.C., Sanz M., Colello R., Etcheverría A., Padola N.L., Quiroga M.A., Perfumo C.J., Galli L., Leotta G.A.: **Enterotoxigenic *Escherichia coli* Subclinical Infection in Pigs: Bacteriological and Genotypic Characterization and Antimicrobial Resistance Profiles**, Foodborne Pathog. Dis., 12(8). 704-711, 2015.

Morel C.M., Alm R.A., Årdal C., Bandera A., Bruno G.M., Carrara E., Colombo G.L., de Kraker M.E.A., Essack S., Frost I., Gonzalez-Zorn B., Goossens H., Guardabassi L., Harbarth S., Jørgensen P.S., Kanj S.S., Kostyanev T., Laxminarayan R., Leonard F., Hara G.L., Mendelson M., Mikulska M., Mutters N.T., Outterson K., Baňo J.R., Tacconelli E., Scudeller L., GAP-ON€ network: **A one health framework to estimate the cost of antimicrobial resistance**, Antimicrob. Resist. Infect. Control, 9(1). 187, 2020.

Nabavi S.F., Braidy N., Gortzi O., Sobarzo-Sanchez E., Daglia M., Skalicka-Woźniak K., Nabavi S.M. **Luteolin as an anti-inflammatory and neuroprotective agent: A brief review**, Brain Res. Bull., 119. 1-11, 2015.

Nighot M., Rawat M., Al-Sadi R., Castillo E.F., Nighot P., Ma T.Y.: **Lipopolysaccharide-Induced Increase in Intestinal Permeability Is Mediated by TAK-1 Activation of IKK and MLCK/MYLK Gene**, Am. J. Pathol., 189(4). 797-812, 2019.

Nishitani Y., Yamamoto K., Yoshida M., Azuma T., Kanazawa K., Hashimoto T., Mizuno M.: **Intestinal anti-inflammatory activity of luteolin: role of the aglycone in NF-κB inactivation in macrophages co-cultured with intestinal epithelial cells**, Biofactors, 39(5). 522-533, 2013.

Noda S., Tanabe S., Suzuki T.: **Differential effects of flavonoids on barrier integrity in human intestinal Caco-2 cells**, J. Agric. Food Chem., 60(18). 4628-4633, 2012.

Nossol C., Barta-Böszörményi A., Kahlert S., Zuschratter W., Faber-Zuschratter H., Reinhardt N., Ponsuksili S., Wimmers K., Diesing A.K., Rothkötter HJ.: **Comparing Two Intestinal Porcine Epithelial Cell Lines (IPECs): Morphological Differentiation, Function and Metabolism**, PLoS ONE, 10(7). e0132323, 2015.

Nunes C., Almeida L., Barbosa R.M., Laranjinha, J.: **Luteolin suppresses the JAK/STAT pathway in a cellular model of intestinal inflammation**, Food Funct., 8(1). 387–396, 2017.

Nunes-Alves C.: **New LPS receptors discovered**, Nat. Rev. Microbiol., 12. 658, 2014.

Pandurangan A.K., Ananda Sadagopan S.K., Dharmalingam P., Ganapasam S.: **Luteolin, a bioflavonoid inhibits azoxymethane-induced colorectal cancer through activation of Nrf2 signaling**, Toxicol. Mech. Methods, 24(1). 13-20, 2014.

Paredes-Gonzalez X., Fuentes F., Jeffery S., Saw C.L., Shu L., Su Z.Y., Kong A.N.: **Induction of NRF2-mediated gene expression by dietary phytochemical flavones apigenin and luteolin**, Biopharm. Drug Dispos., 36(7). 440-51, 2015.

Pietta P.G.: **Flavonoids as Antioxidants**, J. Nat. Prod., 63(7). 1035–1042, 2000.

Piotrowska M., Swierczynski M., Fichna J., Piechota-Polanczyk A.: **The Nrf2 in the pathophysiology of the intestine: Molecular mechanisms and therapeutic implications for inflammatory bowel diseases**, Pharmacol. Res., 163. 105243, 2021.

Poirel L., Madec J-Y., Lupo A., Schink A-K., Kieffer N., Nordmann P., Schwarz S.: **Antimicrobial resistance in *Escherichia coli***, Microbiol. Spectr., 6(4). ARBA-0026-2017, 2018.

Polewski M.A., Krueger C.G., Reed J.D., Leyer G.: **Ability of cranberry proanthocyanidins in combination with a probiotic formulation to inhibit *in vitro* invasion of gut epithelial cells by extra-intestinal pathogenic *E. coli***, J. Func. Foods, 25. 123-134, 2016.

Pomothy J.M., Gatt K., Jerzsele Á., Gere E.P.: **The impact of quercetin on a porcine intestinal epithelial cell line exposed to deoxynivalenol**, Acta Vet. Hung., 68(4). 380-386, 2021.

Preksha G., Yesheswini R., Srikanth C.V.: **Cell culture techniques in gastrointestinal research: Methods, possibilities and challenges**, Indian J. Pathol. Microbiol., 64(S1). 52-57, 2021.

Pribis J.P., García-Villada L., Zhai Y., Lewin-Epstein O., Wang A.Z., Liu J., Xia J., Mei Q., Fitzgerald D.M., Bos J., Austin R.H., Herman C., Bates D., Hadany L., Hastings P.J., Rosenberg S.M. Gamblers: **An Antibiotic-Induced Evolvable Cell Subpopulation Differentiated by Reactive-Oxygen-Induced General Stress Response**, Mol. Cell., 74(4). 785-800.e7, 2019.

Prins J.M., van Deventer S.J., Kuijper E.J., Speelman P.: **Clinical relevance of antibiotic-induced endotoxin release**, Antimicrob. Agents Chemother., 38(6). 1211-8, 1994.

Procházková D., Boušová I., Wilhelmová N.: **Antioxidant and prooxidant properties of flavonoids**, Fitoterapia, 82(4). 513-23, 2011.

Pungpian C., Lee S., Trongjit S., Sinwat N., Angkititrakul S., Prathan R., Srisanga S., Chuanchuen R.: **Colistin resistance and plasmid-mediated mcr genes in *Escherichia coli* and *Salmonella* isolated from pigs, pig carcass and pork in Thailand, Lao PDR and Cambodia border provinces**, J. Vet. Sci., 22(5). e68, 2021.

Qian W., Liu M., Fu Y., Zhang J., Liu W., Li J., Li X., Li Y., Wang T.: **Antimicrobial mechanism of luteolin against *Staphylococcus aureus* and *Listeria monocytogenes* and its antibiofilm properties**, Microb. Pathog., 142. 104056, 2020.

Quan H., Dai X., Liu M., Wu C., Wang D.: **Luteolin supports osteogenic differentiation of human periodontal ligament cells**, BMC Oral Health, 19. 229, 2019.

Rahman S., Ghiboub M., Donkers J.M., van de Steeg E., van Tol E.A.F., Hakvoort T.B.M., de Jonge W.J.: **The Progress of Intestinal Epithelial Models from Cell Lines to Gut-On-Chip**, Int. J. Mol. Sci., 22(24). 13472, 2021.

Rajput S.A., Sun L., Zhang N.Y., Khalil M.M., Ling Z., Chong L., Wang S., Rajput I.R., Bloch D.M., Khan F.A., Shaukat A., Qi D.: **Grape Seed Proanthocyanidin Extract Alleviates AflatoxinB_1-Induced Immunotoxicity and Oxidative Stress via Modulation of NF-κB and Nrf2 Signaling Pathways in Broilers**, Toxins, 11(1). 23, 2019.

Rauter A.P., Ennis M., Hellwich K.H., Herold B.J., Horton D., Moss G.P., Schomburg I.: **Nomenclature of flavonoids (IUPAC Recommendations 2017)**, Pure Appl. Chem., 90(9). 1429–1486, 2018.

Reis R.S.d., Horn F.: **Enteropathogenic *Escherichia coli, Salmonella, Shigella* and *Yersinia*: cellular aspects of host-bacteria interactions in enteric diseases**, Gut Pathog., 2. 8, 2010.

Repetto G., del Peso A., Zurita J.L.: **Neutral red uptake assay for the estimation of cell viability/cytotoxicity**, Nat. Protoc., 3. 1125–1131, 2008.

Rhouma M., Fairbrother J.M., Beaudry F., Letellier A.: **Post weaning diarrhea in pigs: risk factors and non-colistin-based control strategies**, Acta Vet. Scand., 59(1). 31, 2017.

Rodríguez-Pérez C., García-Villanova B., Guerra-Hernández E., Verardo V.: **Grape Seeds Proanthocyanidins: An Overview of *In Vivo* Bioactivity in Animal Models**, Nutrients, 11(10). 2435, 2019.

Sampath V.: **Bacterial endotoxin-lipopolysaccharide; structure, function and its role in immunity in vertebrates and invertebrates**, Agric. Nat. Resour., 52(2). 115-120, 2018.

Sanhueza L., Melo R., Montero R., Maisey K., Mendoza L., Wilkens M.: **Synergistic interactions between phenolic compounds identified in grape pomace extract with antibiotics of different classes against *Staphylococcus aureus* and *Escherichia coli***, PLoS ONE, 12(2). e0172273, 2017.

Schierack P., Nordhoff M., Pollmann M., Weyrauch K.D., Amasheh S., Lodemann U., Jores J., Tachu B., Kleta S., Blikslager A., Tedin K., Wieler L.H.: **Characterization of a porcine intestinal epithelial cell line for *in vitro* studies of microbial pathogenesis in swine**, Histochem. Cell Biol., 125(3). 293-305, 2006.

Shamsudin N.F., Ahmed Q.U., Mahmood S., Ali Shah S.A., Khatib A., Mukhtar S., Alsharif M.A., Parveen H., Zakaria Z.A.: **Antibacterial Effects of Flavonoids and Their Structure-Activity Relationship Study: A Comparative Interpretation**, Molecules, 27(4). 1149, 2022.

Shan B., Cai Y.Z., Brooks J.D., Corke H.: **Antibacterial Properties and Major Bioactive Components of Cinnamon Stick (*Cinnamomum burmannii*): Activity against Foodborne Pathogenic Bacteria**, J. Agric. Food Chem., 55(14). 5484–5490, 2007.

Sharma S., Tripathi P., Sharma J., Dixit A.: **Flavonoids modulate tight junction barrier functions in hyperglycemic human intestinal Caco-2 cells**, Nutrition, 78. 110792, 2020.

Shen X.F., Ren L.B., Teng Y., Zheng S., Yang X.L., Guo X.J., Wang X.Y., Sha K.H., Li N., Xu G.Y., Tian H.W., Wang X.Y., Liu X.K., Li J., Huang N.: **Luteolin decreases the attachment, invasion and cytotoxicity of UPEC in bladder epithelial cells and inhibits UPEC biofilm formation**, Food Chem. Toxicol., 72. 204-211, 2014.

Šikić Pogačar M., Klančnik A., Bucar F., Langerholc T., Smole Možina S.: **Anti-adhesion activity of thyme (*Thymus vulgaris* L.) extract, thyme post-distillation waste, and olive (*Olea europea* L.) leaf extract against *Campylobacter jejuni* on polystyrene and intestine epithelial cells**, J. Sci. Food Agric., 96(8). 2723-2730, 2016.

Skroza D., Šimat V., Smole Možina S., Katalinić V., Boban N., Generalić Mekinić I.: **Interactions of resveratrol with other phenolics and activity against foodborne pathogens**, Food Sci. Nutr., 7(7). 2312-2318, 2019.

Soares T., Rodrigues D., Sarraguça M., Rocha S., Lima J., Ribeiro D., Fernandes E., Freitas M.: **Optimization of Experimental Settings for the Assessment of Reactive Oxygen Species Production by Human Blood**, Oxid. Med. Cell. Longev., 7198484, 2019.

Souto M.S., Coura F.M., Dorneles E.M., Stynen A.P., Alves T.M., Santana J.A., Pauletti R.B., Guedes R.M., Viott A.M., Heinemann M.B., Lage A.P.: **Antimicrobial susceptibility and phylotyping profile of pathogenic *Escherichia coli* and *Salmonella enterica* isolates from calves and pigs in Minas Gerais, Brazil**, Trop. Anim. Health Prod., 49(1). 13-23, 2017.

Sova M., Saso L.: **Design and development of Nrf2 modulators for cancer chemoprevention and therapy: a review**, Drug Des. Devel. Ther., 12. 3181-3197, 2018.

Spitzer F., Speiser S., Vahjen W., Zentek J.: **Effect of different feed ingredients and additives on IPEC-J2 cells challenged with an enterotoxigenic *Escherichia coli* strain**, Cytotechnology, 68(4). 1463-1471, 2016.

Stacey G.: **Current developments in cell culture technology**, Adv. Exp. Med. Biol., 745. 1-13, 2012.

Sun H., Wan Y., Du P., Bai L.: **The Epidemiology of Monophasic *Salmonella* Typhimurium**, Foodborne Pathog. Dis., 17(2). 87-97, 2020.

Sun W.L., Yang J.W., Dou H.Y., Li G.Q., Li X.Y., Shen L., Ji H.F.: **Anti-inflammatory effect of luteolin is related to the changes in the gut microbiota and contributes to preventing the progression from simple steatosis to nonalcoholic steatohepatitis**, Bioorg. Chem., 112. 104966, 2021.

Sundaram T.S., Giromini C., Rebucci R., Baldi A.: **Omega-3 Polyunsaturated Fatty Acids Counteract Inflammatory and Oxidative Damage of Non-Transformed Porcine Enterocytes**, Animals, 10. 956, 2020.

Sung J., Lee S.: **Anti-Inflammatory Activity of Butein and Luteolin Through Suppression of NFκB Activation and Induction of Heme Oxygenase-1**, J. Med. Food., 18. 557–564, 2015.

Switaj T.L., Winter K.J., Christensen S.R.: **Diagnosis and Management of Foodborne Illness**, Am. Fam. Physician., 92(5). 358-365, 2015.

Tang C., Xie B., Sun Z.: **Antibacterial activity and mechanism of B-type oligomeric procyanidins from lotus seedpod on enterotoxigenic *Escherichia coli***, J. Funct. Foods, 38. 454–463, 2017.

Tang K.L., Caffrey N.P., Nóbrega D.B., Cork S.C., Ronksley P.E., Barkema H.W., Polachek A.J., Ganshorn H., Sharma N., Kellner J.D., Ghali W.A.: **Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: a systematic review and meta-analysis**, Lancet Planet Health, 1(8). 316-327, 2017.

Taranu I., Marin D.E., Palade M., Pistol G.C., Chedea V.S., Gras M.A., Rotar C.: **Assessment of the efficacy of a grape seed waste in counteracting the changes induced by aflatoxin B1 contaminated diet on performance, plasma, liver and intestinal tissues of pigs after weaning**, Toxicon, 162. 24–31, 2019.

Ter Kuile B.H., Kraupner N., Brul S.: **The risk of low concentrations of antibiotics in agriculture for resistance in human health care**, FEMS Microbiol. Lett., 363(19), fnw210, 2016.

Trabelsi A., El Kaibi M.A., Abbassi A., Horchani A., Chekir-Ghedira L., Ghedira K.: **Phytochemical Study and Antibacterial and Antibiotic Modulation Activity of *Punica granatum* (Pomegranate) Leaves**, Scientifica (Cairo), 8271203, 2020.

Tran T.H.T., Everaert N., Bindelle J.: **Review on the effects of potential prebiotics on controlling intestinal enteropathogens *Salmonella* and *Escherichia coli* in pig production**, J. Anim. Physiol. Anim. Nutr., 102(1). 17-32, 2018.

Tóth A.G., Csabai I., Maróti G., Jerzsele Á., Dubecz A., Patai Á.V., Judge M.F., Nagy S.Á., Makrai L., Bányai K., Szita G., Solymosi N.: **A glimpse of antimicrobial resistance gene diversity in kefir and yoghurt**, Sci Rep., 10(1). 22458, 2020.

Unusan N.: **Proanthocyanidins in grape seeds: An updated review of their health benefits and potential uses in the food industry**, J. Funct. Foods, 67. 103861, 2020.

Van Boeckel T.P., Pires J., Silvester R., Zhao C., Song J., Criscuolo N.G., Gilbert M., Bonhoeffer S., Laxminarayan R.: **Global trends in antimicrobial resistance in animals in low- and middle-income countries**, Science, 365(6459). eaaw1944, 2019.

Van der Kolk J., Endimiani A., Graubner C., Gerber V., Perreten V.: ***Acinetobacter* in veterinary medicine, with an emphasis on *Acinetobacter baumannii***, J. Glob. Antimicrob., 16. 59-71, 2019.

Varga J., Tuboly S., Mészáros J.: **A háziállatok fertőző betegségei (Állatorvosi járványtan II.)**, 2. kiadás, Mezőgazda Kiadó, Budapest, 2007.

Vergauwen H.: **The IPEC-J2 Cell Line.** In: Verhoeckx K., Cotter P., López-Expósito I., Kleiveland C., Lea T., Mackie A., Requena T., Swiatecka D., Wichers H., editors. The Impact of Food Bioactives on Health: *in vitro* and *ex vivo* models. Chapter 12. Springer; 2015.

Wang M., Zhao H., Wen X., Ho C.T., Li S.: **Citrus flavonoids and the intestinal barrier: Interactions and effects**, Compr. Rev. Food Sci. Food Saf., 20(1). 225-251, 2021.

Wang H., Joseph J.A.: **Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader**, Free Radic. Biol. Med., 27. 612-616, 1999.

Wang Q., Wang H., Jia Y., Pan H., Ding H.: **Luteolin induces apoptosis by ROS/ER stress and mitochondrial dysfunction in gliomablastoma**, Cancer Chemother. Pharmacol., 79(5). 1031-1041, 2017.

Wang X., Liu Y., Li S., Pi D., Zhu H., Hou Y., Shi H., Leng W.: **Asparagine attenuates intestinal injury, improves energy status and inhibits AMP-activated protein kinase signaling pathways in weaned piglets challenged with *Escherichia coli* lipopolysaccharide**, Br. J. Nutr., 114. 553–565, 2015.

Welser J.: **Primary Cells Versus Cell Lines**. 2015. https://sciencellonline.com/blog/primary-cells-versus-cell-lines/

World Health Organization (WHO): **Global action plan on antimicrobial resistance**. 2015. https://apps.who.int/iris/handle/10665/193736

Wu H., Luo T., Li Y.M., Gao Z.P., Zhang K.Q., Song J.Y., Xiao J.S., Cao Y.P.: **Granny Smith apple procyanidin extract upregulates tight junction protein expression and modulates oxidative stress and inflammation in lipopolysaccharide-induced Caco-2 cells**, Food. Funct., 9. 3321–3329, 2018.

Wu Y., Mo R., Zhang M., Zhou W., Li D.: **Grape Seed Proanthocyanidin Alleviates Intestinal Inflammation Through Gut Microbiota-Bile Acid Crosstalk in Mice**, Front. Nutr., 8. 786682, 2022.

Xu M., Niu Q., Hu Y., Feng G., Wang H., Li S.: **Proanthocyanidins Antagonize Arsenic-Induced Oxidative Damage and Promote Arsenic Methylation through Activation of the Nrf2 Signaling Pathway**, Oxid. Med. Cell. Longev., 8549035, 2019.

Yan F., Zhao L., Chen W., Lu Q., Tang C., Wang C., Liu R**.: Comparison of the inhibitory effects of procyanidins with different structures and their digestion products against acrylamide-induced cytotoxicity in IPEC-J2 cells**, J. Funct. Foods, 72. 104073, 2020.

Yi Y.S.: **Regulatory Roles of Flavonoids on Inflammasome Activation during Inflammatory Responses**, Mol. Nutr. Food Res., 62(13). 1800147, 2018.

Yoshioka Y., Akiyama H., Nakano M., Shoji T., Kanda T., Ohtake Y., Takita T., Matsuda R., Maitani T.: **Orally administered apple procyanidins protect against experimental inflammatory bowel disease in mice**, Int. Immunopharmacol., 8(13-14). 1802-7, 2008.

Yuan J., Che S., Zhang L., Ruan Z.: **Reparative Effects of Ethanol-Induced Intestinal Barrier Injury by Flavonoid Luteolin via MAPK/NF-κB/MLCK and Nrf2 Signaling Pathway**, J. Agric. Food Chem., 69(14). 4101–4110, 2021A.

Yuan J., Che S., Ruan Z., Song L., Tang R., Zhang L.: **Regulatory effects of flavonoids luteolin on BDE-209-induced intestinal epithelial barrier damage in Caco-2 cell monolayer model**, Food Chem. Toxicol., 150. 112098, 2021B.

Zakrewski S.S., Richter J.F., Krug S.M., Jebautzke B., Lee I.F., Rieger J., Sachtleben M., Bondzio A., Schulzke J.D., Fromm M., Günzel D.: **Improved cell line IPEC-J2, characterized as a model for porcine jejunal epithelium**, PLoS One, 8(11). e79643, 2013.

Zhang B.C., Li Z., Xu W., Xiang C.H., Ma Y.F.: **Luteolin alleviates NLRP3 inflammasome activation and directs macrophage polarization in lipopolysaccharide-stimulated RAW264.7 cells**, Am. J. Transl. Res., 10(1). 265-273, 2018.

Zhang D., Gao X., Song X., Zhou W., Hong W., Tian C., Liu Y., Liu M.: **Luteolin Showed a Resistance Elimination Effect on Gentamicin by Decreasing MATE mRNA Expression in *Trueperella pyogenes***, Microb. Drug Resist., 25(4). 619-626, 2019.

Zhang L.; Chen J.; Liao H.; Li C.; Chen M.: **Anti-inflammatory effect of lipophilic grape seed proanthocyanidin in RAW264.7 cells and a zebrafish model**, J. Funct. Foods, 75. 104217, 2020.

10. Own scientific publications

10.1. Publications related to the topic of the present dissertation

10.1.1. Full text papers in peer-reviewed journals

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

Kovács D., Karancsi Z., Farkas O., Jerzsele Á.: **Antioxidant activity of flavonoids in LPS-treated IPEC-J2 porcine intestinal epithelial cells and their antibacterial effect against bacteria of swine origin**, Antioxidants, 9(12). 1267, 2020.

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

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

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

10.1.2. Conference presentations

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

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

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

10.2. Publications not related to the topic of the present dissertation

10.2.1. Full text papers in peer-reviewed journals

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

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

Somogyi Z., Mag P., Kovács D., Kerek Á., Szabó P., Makrai L., Jerzsele Á.: **Synovial and Systemic Pharmacokinetics of Florfenicol and PK/PD Integration against *Streptococcus suis* in Pigs**, Pharmaceutics, 14(1). 109, 2022.

Dobos A.B., Kovács D., Lajos Z., Jerzsele Á.: ***Corynebacterium urealyticum* okozta fertőző megbetegedések társállatokban - Irodalmi összefoglaló és esetismertetés**, Magyar Állatorvosok Lapja, 143. 547-562, 2021.

Kardos G., Roza E., Kovács D., Jerzsele Á.: **Antibakteriális szerek gyakorlati alkalmazása szarvasmarhában 2. rész: Tőgygyulladások, lábvégbetegségek - Irodalmi összefoglaló**, Magyar Állatorvosok Lapja, 143. 387-400, 2021.

Essősy M., Fodor I., Ihnáth Z., Jerzsele Á., Karancsi Z., Kovács D., Szalai K.V., Szentmiklósi D.: **A butirát tápkiegészítő hatásának vizsgálata nagyüzemi brojlerházityúk-állományban II.**, Magyar Állatorvosok Lapja, 143, 2. 107–118, 2021.

Kerek Á., Sterczer Á., Somogyi Z., Kovács D., Jerzsele Á.: **Investigation of the environmental presence of multidrug-resistant bacteria at small animal hospitals in Hungary**, Acta Veterinaria Hungarica, 68(4). 387-392, 2021.

Roza E., Kardos G., Kovács D., Jerzsele Á.: **Antibakteriális szerek gyakorlati alkalmazása szarvasmarhában 1. rész: Légzőszervi és enterális kórképek: Irodalmi összefoglaló**, Magyar Állatorvosok Lapja, 143(2). 67–78, 2021.

Jerzsele Á., Kovács D., Manczur F.: **Toraszemid, csúcshatású diuretikum kutyák pangásos szívelégtelenségének kezelésére**, Kamarai Állatorvos, 15(1). 22-23, 2020.

Essősy M., Fodor I., Ihnáth Z., Karancsi Z., Kovács D., Szalai K.V., Szentmiklósi D., Jerzsele Á.: **Az antibiotikum-mentes brojler-házityúk-hizlalás lehetőségei, különös tekintettel a pre- és probiotikumok használatára I.**, Magyar Állatorvosok Lapja, 142(7). 397–407, 2020.

Jerzsele Á., Somogyi Z., Szalai M., Kovács D.: **A fermentált búzacsíra-kivonat hatása brojlercsirkék mesterséges *Salmonella* Typhimurium fertőzésére**, Magyar Állatorvosok Lapja, 142(2). 77–85, 2020.

Jerzsele Á., Kovács D., Somogyi Z.: **Az oklacitinib, egy új Janus-kinázgátló hatóanyag kutyák atópiás bőrgyulladásának és egyéb, viszketéssel járó megbetegedéseinek kezelésére : Irodalmi összefoglaló**, Magyar Állatorvosok Lapja, 139(9). 545–552, 2017.

Jerzsele Á., Somogyi Z., Kovács D., Gál J.: **A féregellenes kezelések jelentősége és lehetőségei az intenzív baromfitartásban**, Magyar Állatorvosok Lapja, 139(7). 397–404, 2017.

Submitted, under review:

Karancsi Z., Kovács D., Csikó Gy., Palócz O., Jerzsele Á., Gálfi P., Farkas O.: **Effect of hydroxylated and methylated flavonoids on cytochrome P450 activity in porcine intestinal epithelial cells.** Submitted to: Acta Veterinaria Hungarica.

Medek N., Karancsi Z., Kovács D., Jerzsele Á.: **Therapeutic effect of cannabidiol in the treatment of canine osteoarthritis – a placebo controlled, double-blinded clinical trial**. Submitted to: Veterinary Research Communications.

10.2.2. Conference presentations

Kovács D., Karancsi Z., Lubov B., Farkas O.: **Flavonoidok hatásának vizsgálata a CYP450 enzimrendszerre sertés bélhám sejtkultúrán**. Magyar Szabadgyök-Kutató Társaság X. Kongresszusa, Szeged, Hungary, 2019.

Farkas O., Karancsi Z., Kovács D., Lubov B., Csikó G., Palócz O.: **Effect of flavonoids on cytochrome P450 activity in porcine intestinal epithelial cells – *in vitro* interaction study**, Journal Of Veterinary Pharmacology And Therapeutics, 41. 142-143, Meeting Abstract: P12.6, 2018.

Kovács D., Palócz O., Karancsi Z., Csikó Gy., Farkas O.: **Flavonoidok hatása a citokróm P450 enzimrendszerre sertés bélhám sejtkultúrán**. MTA Akadémiai Beszámolók, Budapest, Hungary, 2016.

10.3. Supervision of theses

Bella Gál: **Antioxidant and anti-inflammatory effects of proanthocyanidins in porcine intestinal epithelial cell – bacterium co-culture**. TDK thesis. Supervisor: Kovács D., Budapest, 2021.

Móritz Alma Virág: **Luteolin védőhatásának *in vitro* vizsgálata bakteriális eredetű bélhámsejt-károsodás esetén**. TDK thesis. Supervisors: Farkas O. and Kovács D., Budapest, 2020.

Lucas Ellis Hunter: **Drug absorption modelling in porcine intestinal epithelial cell line**. TDK thesis. Supervisors: Karancsi Z. and Kovács D., Budapest, 2020.

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