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Evaluation of probiotics on porcine intestinal epithelial cells

Ph. D. thesis

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List of abbreviations

AMPs – Antimicrobial peptides

AP-1 – activator protein-1

ARE – Antioxidant response element

B. cereus – Bacillus cereus

B. licheniformis – Bacillus licheniformis

B. subtilis – Bacillus subtilis

Caco-2 – Colon carcinoma cell line

cAMP – Cyclic adenosine monophosphate

CAT – Catalase

CFTR – Cystic fibrosis transmembrane regulator

CFU – Colony forming unit

cGMP – Cyclic guanosine monophosphate

CT – Connective tissue

DAPI – 4′,6-diamidin-2-phenylindol

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

DCs – Dendritic cells

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

DNA – Deoxyribonucleic acid

DON – Deoxynivalenol

E. coli – Escherichia coli

E. faecium – Enterococcus faecium

EcN – *Escherichia coli* strain Nissle 1917

EDEC – Edema disease *E. coli*

EHEC – Enterohemorrhagic *E. coli*

ELISA – Enzyme-linked immunosorbent assay

EPEC – Enteropathogenic *E. coli*

ETEC – Enterotoxigenic *E. coli*

ExPEC – Extraintestinal pathogenic *E. coli*

FAO – Food and Agriculture Organization of the United Nations

FD4 – Fluorescein isothiocyanate-dextran

GA – glutaraldehyde

GIT – Gastrointestinal tract

GPx – Glutathione peroxidase

GRAS – Generally recognized as safe

GSH – Glutathione

GSSG – glutathione disulfide

GTP – Guanosine-5'-triphosphate

HIF-1α – hypoxia-inducible factor-1α

Hsps – Heat shock proteins

HT29 – Human colorectal adenocarcinoma

IAP – Intestinal alkaline phosphatase

IC – Intracellular

IEC-18 – Intestinal epithelial cell-18

IEC-6 – Intestinal epithelial cell-6

IECs – Intestinal epithelial cells

IELs – Intraepithelial lymphocytes

IgA – Immunoglobulin A

IL-1 – Interleukin-1

IL-10 – Interleukin-10

IL-1β – Interleukin-1β

IL-6 – Interleukin-6

IL-8 – Interleukin-8

iNOS – Inducible nitric oxide synthase

IPEC1 – Intestinal porcine epithelial cell line-1

IPEC-J2 – Intestinal porcine epithelial cell line J2

IPI-2I – Ileal porcine intestinal

Keap1 – Kelch-like ECH-associated protein-1

L. reuteri – *Lactobacillus reuteri*

L. rhamnosus – *Lactobacillus rhamnosus*

LAB – Lactic acid bacteria

LMWB – Low-molecular-weight bacteriocins

L-NAME – NG-nitro-L-arginine methyl ester

LPS – Lipopolysaccharide

LT – Heat-labile toxin

MAMP – Microbial-associated molecular patterns

MAPK – Mitogen-activated protein kinase

MDA – Malondialdehyde

MH – Mueller-Hinton liquid broth

Mn-SOD – Manganese superoxide dismutase

MRS – De Man, Rogosa, Sharpe broth

MUC – Mucin

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

NOS – Nitric oxide synthase

Nrf2 – Nuclear factor erythroid 2-related factor 2

NRU – Neutral Red Uptake

PAMP – Pathogen-associated molecular pattern

PB – Phosphate buffer

pBD1 – Porcine β-defensin 1

pBD2 – Porcine β-defensin 2

PBS – Phosphate buffered saline

PFA – Paraformaldehyde

PIE – Porcine intestinal epitheliocyte

PKC – Protein kinase C

PRRs – Pattern recognition receptors

PWD – Post-weaning diarrhea

ROS – Reactive oxygen species

S. Typhimurium – *S. enterica* serovar Typhimurium

SCS – Spent culture supernatant

SD – Standard deviation

SOD – Superoxide dismutase

STAT3 – Signal transducer and activator of transcription 3

STb – Heat-stable enterotoxin b

STEC – Shiga toxin-producing *E. coli*

STs –Heat-stable toxins

TEER – Transepithelial electrical resistance

TEM – Transmission electron microscope

TJs – Tight junctions

TLR – Toll-like receptor

TNF-α – Tumor necrosis factor alpha

TSB – Tryptone soya broth

WHO – World Health Organization

ZO-1 – Zonula occludens 1

Summary/Összefogalalás

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

First, we tested the effect of probiotic spent culture supernatants on the cell viability of intestinal porcine epithelial cell line J2 (IPEC-J2), then these cells were treated with LPS (of *S.* Typhimurium or *E. coli* origin) and the effect against oxidative stress induced by LPS was examined. Next, the antibacterial activity of the supernatant was determined against eight *E. coli* and eight *S.* Typhimurium field isolates of porcine origin. Afterwards, IPEC-J2 cells were infected with *E. coli* or *S.* Typhimurium of porcine origin and the effects of probiotic bacteria on barrier function, immune response, oxidative stress homeostasis and adhesion inhibition of pathogens were tested.

Cell viability of IPEC-J2 cells was either not affected (*Bacillus subtilis* supernatant and all probiotic bacterial suspensions) or was increased (*Enterococcus faecium*, *Lactobacillus rhamnosus*, *Bacillus licheniformis* supernatants). *Bacillus licheniformis* and *Bacillus subtilis* supernatants could counteract oxidative stress induced by LPS deriving from *S.* Typhimurium or by LPS of *E. coli* origin. Moreover, *Enterococcus faecium* and *Lactobacillus rhamnosus* were effective in reducing oxidative stress evoked by LPS of *S.* Typhimurium origin. Interestingly, none of the probiotic spent culture supernatants showed any antibacterial effect.

Pre-, co-, and post-treatment with *Enterococcus faecium* and *Lactobacillus rhamnosus* could significantly counteract damage caused by *S.* Typhimurium and *E. coli* in barrier integrity, however this could not be observed in the case of *Bacillus licheniformis* and *Bacillus subtilis*.

Pre-treatment with *Enterococcus faecium*, pre-, and post-treatment with *Lactobacillus rhamnosus*, all treatment combination with *Bacillus licheniformis* and pre-treatment with *Bacillus subtilis* could significantly reduce elevated IL-6 levels induced by *S.* Typhimurium. In addition, pre-, and co-treatment with *Enterococcus faecium* and all treatment combinations with *Lactobacillus rhamnosus* could also decrease elevated IL-8 production evoked by *S.* Typhimurium. All treatment combinations with all examined probiotic bacteria could prevent both *S.* Typhimurium and *E. coli* induced oxidative stress. Furthermore pre-, co-, and post-treatment with *Enterococcus faecium*, *Lactobacillus rhamnosus*, *Bacillus licheniformis* and *Bacillus subtilis* could significantly inhibit the adhesion of *E. coli*, while the same treatment with *Enterococcus faecium*, *Lactobacillus rhamnosus* and *Bacillus licheniformis* showed also significant inhibition properties against *S.* Typhimurium.

Our results help to address and deepen our understanding of probiotic action on intestinal porcine epithelial cells and serve as a basis for both human and swine *in vivo* research and application.

Összefoglalás

A világ népességének növekedésével egyidejűleg nő az igény az állati eredetű élelmiszerek, és ezen belül a sertéshús iránt is. A sertések *Escherichia coli* (*E. coli*) és *Salmonella* törzsek által kiváltott emésztőrendszeri megbetegedése súlyos gazdasági károkat okozhat és gyakran antibiotikumos kezelést igényel. A múltban a sertéságazat nagymértékben az antibiotikumok profilaktikus és metafilaktikus alkalmazására támaszkodott a bélrendszeri betegségek leküzdése során. Az antibiotikumok nem körültekintően történő alkalmazása azonban antibiotikum-rezisztencia kialakulásához vezethet, valamint antibiotikummaradványok jelenhetnek meg az élelmiszerláncban, ezzel veszélyeztetve az emberek egészségét is. Következésképpen a sertéságazat számára kulcsfontosságúvá vált, hogy olyan takarmány-adalékanyagokat keressen, amelyek hozzájárulhatnak a bélrendszer egészségéhez. A probiotikumok ígéretes jelöltek erre a célra. A probiotikumok hatása összetett, a pontos mechanizmusukat széles körben tanulmányozták, de még mindig sok nyitott kérdés maradt. A probiotikus baktériumok által kifejtett jótékony hatások között szerepel a kórokozók tapadásának gátlása, a hősokkfehérjék stimulálása, a citokintermelés megváltoztatása, antioxidáns tulajdonságok és a barrierfunkció fokozása. Kutatásunk során négy probiotikum; *Enterococcus faecium, Lactobacillus rhamnosus, Bacillus licheniformis és Bacillus subtilis*, illetve felülúszóik hatását vizsgáltuk bélfertőzést modellező *in vitro* rendszerben. A bélfertőzést két, gazdasági szempontból is fontos sertés patogénnel *E. colival* és *S. enterica* serovar Typhimuriummal (*S.* Typhimurium), illetve S. Typhimurium vagy *E. coli* eredetű LPS-sel váltottuk ki.

Először a probiotikumok, illetve felülúszóik hatását vizsgáltuk sertés bélhámsejtek (IPEC-J2) életképességére, majd a sejteket *S.* Typhimurium vagy *E. coli* eredetű LPS-sel kezeltük, és az LPS által kiváltott oxidatív stressz elleni hatást vizsgáltuk. Ezt követően a felülúszók antibakteriális hatását vizsgáltuk sertés eredetű klinikai *E. coli* és *S*. Typhimurium izolátumokkal szemben. A kutatás következő fázisában az IPEC-J2 sejteket sertésből izolált *E. coli*val vagy *S*. Typhimuriummal fertőztük meg, és vizsgáltuk a probiotikus baktériumok hatását a barrier funkcióra, az immunválaszra, az oxidatív stressz homeosztázisra és a kórokozók adhéziójának gátlására.

Az IPEC-J2 sejtek életképességét a probiotikumok és felülúszóik vagy nem befolyásolták (*Bacillus subtilis* felülúszó, illetve *Enterococcus faecium, Lactobacillus rhamnosus, Bacillus licheniformis, Bacillus subtilis* baktériumok), vagy növelték (*Enterococcus faecium* felülúszó, *Lactobacillus rhamnosus* felülúszó, *Bacillus licheniformis* felülúszó). A *Bacillus licheniformisból* és *Bacillus subtilisből* készült felülúszó ellensúlyozta a *S.* Typhimurium és *E. coli* eredetű LPS által kiváltott oxidatív stresszt. Az *Enterococcus faeciumból* és a *Lactobacillus rhamnosusból* készült felülúszó pedig a *S.* Typhimurium eredetű LPS indukálta oxidatív stresszt csökkentette. Várakozásunkkal ellentétben egyik probiotikus felülúszó sem mutatott antibakteriális hatást.

Az *Enterococcus faeciummal* és a *Lactobacillus rhamnosusszal* végzett elő-, egy- és utóidejű kezelés szignifikánsan csökkentette a *S.* Typhimurium és az *E. coli* által a barrier integritásában okozott károsodást. Az *Enterococcus faeciummal* történő előkezelés, a *Lactobacillus rhamnosusszal* történő elő-, és utókezelés, a *Bacillus subtilisszal* történő előkezelés, valamint a *Bacillus licheniformisszal* történő összes kezeléstípus csökkentette a *S.* Typhimurium által kiváltott IL-6 növekedést. Továbbá az *E. faeciummal* történő elő-, és egyidejű kezelés, valamint a *L. rhamnosus*szal történő összes kezeléstípus megakadályozta a *S.* Typhimurium által okozott IL-8 növekedést. Az összes vizsgált probiotikus baktériummal végzett kezelési kombináció mind a *S.* Typhimurium, mind az *E. coli* által kiváltott oxidatív stresszt csökkentette. Továbbá az *Enterococcus faeciummal*, *Lactobacillus rhamnosusszal*, *Bacillus licheniformisszal* és *Bacillus subtilisszal* végzett elő-, egy- és utóidejű kezelés jelentősen gátolni tudta az *E. coli* adhézióját, míg az *Enterococcus faeciummal*, *Lactobacillus rhamnosusszal* és *Bacillus licheniformisszal* végzett kezelés az *S.* Typhimuriummal szemben is jelentős gátló hatást mutatott.

Eredményeink hozzájárulnak a probiotikumok sertés bélhámsejt tenyészeteken vizsgált hatásmechanizmusának megértéséhez, valamint alapul szolgálhatnak mind a humán -, mind a sertésegészségügyben *in vivo* kutatásokhoz és a lehetséges gyakorlati alkalmazáshoz.

1 Introduction

According to estimations the number of people will reach 9 billion by 2050 and simultaneously with the growth of human population also the demand for food of animal origin, including pork meat, rises (Markowiak and Śliżewska, 2018). In pork production the desired growth performance can only be reached with a healthy gastrointestinal tract, which enables better digestion of feed and more efficient absorption of nutrients. All these improve performance parameters and result in a return of investment for swine producers. Harmful microorganisms can enter and colonize the pig gastrointestinal tract (GIT) even under normal farming conditions and cause an imbalance in the microbial ecosystem (*dysbiosis*). Pathogens produce toxic compounds that may lead to bloating, diarrhea, constipation, ulcer or even poisoning. Under such circumstances nutrients cannot be absorbed efficiently and consequently the growth performance of pigs decreases (Liao and Nyachoti, 2017). Intestinal diseases caused by *Salmonella* and *E. coli* spp may lead to significant economic loss in the swine industry. Enterotoxogenic *Escherichia coli* (ETEC) strains play a significant role in the development of neonatal and post-weaning diarrhea that often leads to growth retardation, requires antibiotic therapy and might also result in the death of animals (Dubreuil, 2017). *Salmonella* spp infection may occur in any life phase of the animal, however weaning pigs are more at risk (Souto et al., 2017). Enterocolitis, diarrhea, dehydration are clinical manifestations in ill pigs, however *Salmonella* infections without clinical signs are more common. Pigs mostly recover from the disease, however they can remain carriers and might shed the bacteria for several months (D'Incau et al., 2021). Even asymptomic *Salmonella* infections are dangerous, because they pose a risk to human health through the contamination of pork products (Fabà et al., 2020; Kovács et al., 2020). Furhermore, both *E. coli* and *Salmonella* are zoonotic and if they enter the food chain they also pose a threat to human health (Kovács et al., 2022; Zimmerman et al., 2012).

From the 1950s on the swine industry started to use antibiotics not only for treatment of diseases but also for growth promoting purposes in subtherapeutical doses. However, the misuse of antibiotics leads to the emergence of antibiotic resistance and residues in the human food chain may appear thus also threatening human health (Liao and Nyachoti, 2017). Therefore, in a few countries (EU, USA) the use of antibiotics for growth promoting purposes has been banned, however in other countries they are still applied in subtherapeutical dosis in order to prevent diarrhea and promote growth performance (Bajagai et al., 2016; Liao and Nyachoti, 2017). In the European Union, and so also in Hungary, the use of antibiotics for growth promoting purposes has been banned in 2006 (*Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition (Text with EEA relevance)*, 2003), moreover, the new EU regulation on veterinary

medicines (2019/6 of the European Parliament and of the Council of 11 December 2018 on veterinary medicinal products and repealing Directive 2001/82/EC) further restricts the application of antibiotics in veterinary medicine ("EUR-Lex - 32019R0006 - EN - EUR-Lex,"). However, according to the One Health concept, antimicrobial resistance is not only a concern for the veterinary sector, but it also affects humans and the natural environment that animals and humans share and as this, it is considered to be one of the biggest health challenges nowadays (Guardabassi et al., 2020; Liao and Nyachoti, 2017). Any option that can reduce the spread of resistance is crucial for human health so that antibiotic treatment can remain effective (Kovács et al., 2022; Palma et al., 2020).

Following poultry, pork is the second most frequently consumed meat in the world ("Global meat consumption by type 1990-2021"), the demand from consumers' side is high therefore it has become an important research issue for the swine industry to seek for natural feed additives that are capable of contributing to the health of the GIT and with the application of which similar growth performance can be reached as with growth promoting antibiotics. Finding feed additives capable of maintaining the health of the GIT without the use of antibiotics is pivotal for the swine industry (supporting sustainable and profitable pork production) and for human health as well. (Bajagai et al., 2016; Kovács et al., 2022; Liao and Nyachoti, 2017; Markowiak and Śliżewska, 2018). Among phytochemicals, prebiotics, organic acids, enzymes, antimicrobial peptides, anti-bacterial virulence drugs and minerals, probiotics are promising candidates to replace growth promoting antibiotics in swine farming (Hassan et al., 2018; Kovács et al., 2021). Probiotic action is complex, the exact mechanisms have been widely studied, but still need to be elucidated. Among the beneficial effects exerted by probiotic bacteria are inhibition of pathogen adhesion, stimulation of heat shock proteins, alteration of cytokine production, antioxidant properties and enhancement of barrier function (Kovács et al., 2021; Liao and Nyachoti, 2017).

2 Literature review

2.1 The role of the intestinal barrier

The main role of the intestine is the absorption of nutrients and water, however at the same time it also serves as a barrier separating the content of the lumen from the rest of the body. The is constantly exposed to diverse microorganisms and nutrient components and has to fulfill several functions, such as restricting interaction with bacteria (both commensal and pathogenic), detoxifying bacterial endotoxins, regulating nutrient uptake, limiting transport of toxic componds and bacteria, initiating immune response, preventing growth of pathogens, simultaneously (Abreu, 2010; Ghosh et al., 2020). A multilayer GIT barrier system operates in order to satisfy the needs of these many functions. Four layers provide together a complete physical and functional barrier, parts of which are the following: (1) luminal intestinal alkaline phosphatase (IAP), (2) the mucus layer, (3) single layer of columnar epithelial cells - with intraepithelial mucin producing goblet cells, and (4) the antibacterial proteins and immunoglobulin A (IgA) (**Figure 1**). IAP is secreted by intestinal epithelial cells and detoxifies bacterial endotoxin lipopolysaccharide (LPS) by removing phosphate groups. The inactivation of LPS prevents downstream intracellular (IC) signaling and the transcription of proinflammatory cytokines and thus intestinal inflammation is reduced. The mucus layer consists of an inner and an outer layer, the inner one is thinner and prevents the penetration of bacteria, while the outer one is thicker and looser, and it is the place where commensal bacteria reside. With the adherence of commensal bacteria, the entry of pathogens can be restrained. The mucous layer is a network of proteins with mucin (MUC 2) being the major glycoprotein secereted. Depletion of the mucus layer leads to disrupted intestinal barrier function (Ghosh et al., 2020).

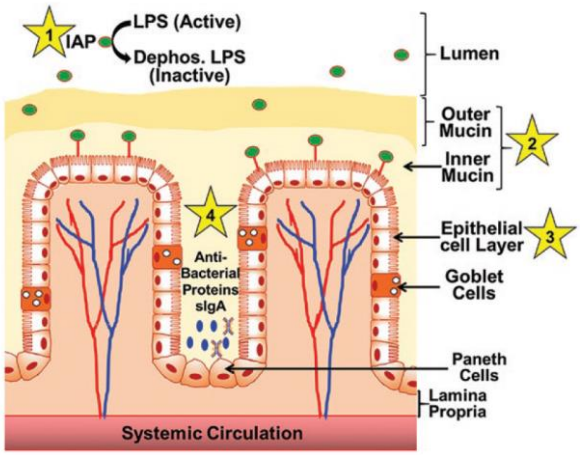


Figure 1: The multiple layers of the intestinal barrier. The intestinal barrier is composed of four layers (indicated by numbers 1-4). Layer 1: intestinal alkaline phosphatase (IAP). Layer 2: mucin layer. Layer 3: single layer of epithelial cells, Layer 4: antibacterial proteins and IgA (Ghosh et al., 2020).

The third part of the barrier, the intestinal epithelial layer, is composed of several cell types i.e., intestinal epithelial cells (IECs), goblet cells, enteroendocrine cells, Paneth cells, follicle associated epithelial cells, M cells and epithelial stem cells. Goblet cells are responsible for mucus production, enteroendocrine cells produce hormones, while the role of Paneth cells is the secretion of antimicrobial peptides or lectins. Follicle associated epithelial cells and M cells overlie the Peyer's patches, which are mucosal lymphoid tissues. Beneath the epithelial layer lies the own loose connective tissue (CT) of the mucous membrane (lamina propria mucosae) in which CT cells, stromal cells, B cells, T cells, macrophages and dendritic cells reside. Intraepithelial lymphocytes (IELs) and few dendritic cells are found between the IECs, which enables them to sample the content of the intestinal lumen (Abreu, 2010). IECs are structurally and functionally polarized, forming an apical surface facing the intestinal lumen and a basolateral surface facing the lamina propria. This polarized structure is maintained by junctional complexes that are localized at the most apical part of the lateral membrane and consist of three components, tight junctions (TJs), adherens junctions and desmosomes (**Figure 2 A**)(Abreu, 2010; Tsukita et al., 2001). The intestinal epithelium serves as a selective barrier that enables the translocation of nutrients, electrolytes and water from the lumen to the systemic circulation, restricts however the passage of harmful content (microorganisms, toxins). Two mechanisms – paracellular and transcellular pathways – are involved in this selective transport process (**Figure 2 B**). The transcellular pathway is regulated mainly by selective transporters, while the paracellular transport is regulated by the junctional complexes. TJs are made up of proteins such as claudins, occludin and junctional adhesion molecule (JAM) and serve as paracellular barriers to control the transport of ions, water and solutes through the paracellular pathway (Ghosh et al., 2020; Tsukita et al., 2001).

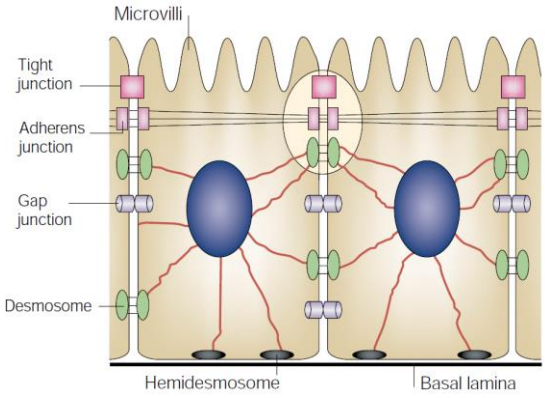


Figure 2 A: **Junctional complexes sealing epithelial cells.** Junctional complexes indicated in circle are located at the most apical part of lateral membranes (Tsukita et al., 2001).

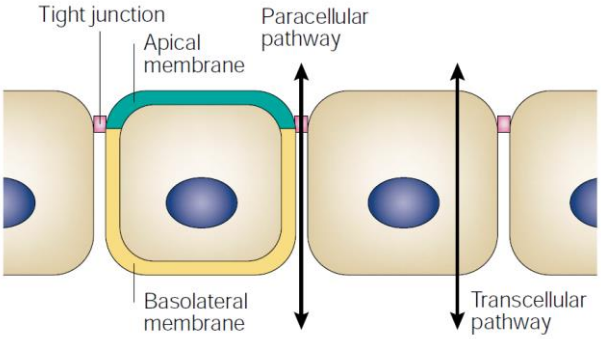


Figure 2 B: Pathways across epithelial cells. Materials cross epithelial cells through paracellular and transcellular partways (Tsukita et al., 2001).

Antimicrobial peptides (AMPs) secreted by Paneth cells and IgA secreted by immune cells establish the fourth layer of the intestinal barrier (Ghosh et al., 2020). AMPs are antibacterial, antiviral, and antiparasitic by nature and exert their effect by peptide-mediated membrane disruption. AMPs consist of two peptide families, namely cathelicidins and defensins. The expression of the latter can be induced by bacterial products or proinflammatory cytokines (Mair et al., 2014). IgA can bind to various substrates, incuding microogranisms, toxins and immune complexes and promotes their removal (Ghosh et al., 2020).

2.1.1 Consequences of barrier dysfunction

Impairment of any of the constituents of the barrier results in its dysfunction, however, increased paracellular transport due to damage of the TJs is considered to be the most important one (Ghosh et al., 2020). Reactive oxygen species, cytokines and toxins rupture the TJs and thus compromise barrier integrity of the intestinal epithelium (Seth et al., 2008). Disruption of the epithelial barrier (also known as “leaky gut”) is one of the crucial causes of diarrhea (F. Yang et al., 2015). Under these circumstances bacterial derived LPS can translocate into systemic circulation and initiates a cascade of intracellular signaling, in which the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) plays a key role. NFκB induces the transcription of several genes that are responsible for immune and stress responses (Oeckinghaus and Ghosh, 2009). In this case the translocation of NFκB to the nucleus leads to the transcription and production of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) that result in tissue inflammation. Local intestinal inflammation is responsible for several gastrointestinal diseases. In addition, increased inflammation also affects other organs, such as the liver, adipose tissue, muscles and artery, thus contributing to the development of extraintestinal disorders, e.g. insulin resistance, fatty liver diseases and atherogenesis (**Figure 3**) (Ghosh et al., 2020). Therapeutic and prophylactic treatments against several diseases aim at strengthening the operation of tight junction proteins in order to decrease intestinal permeability (F. Yang et al., 2015).

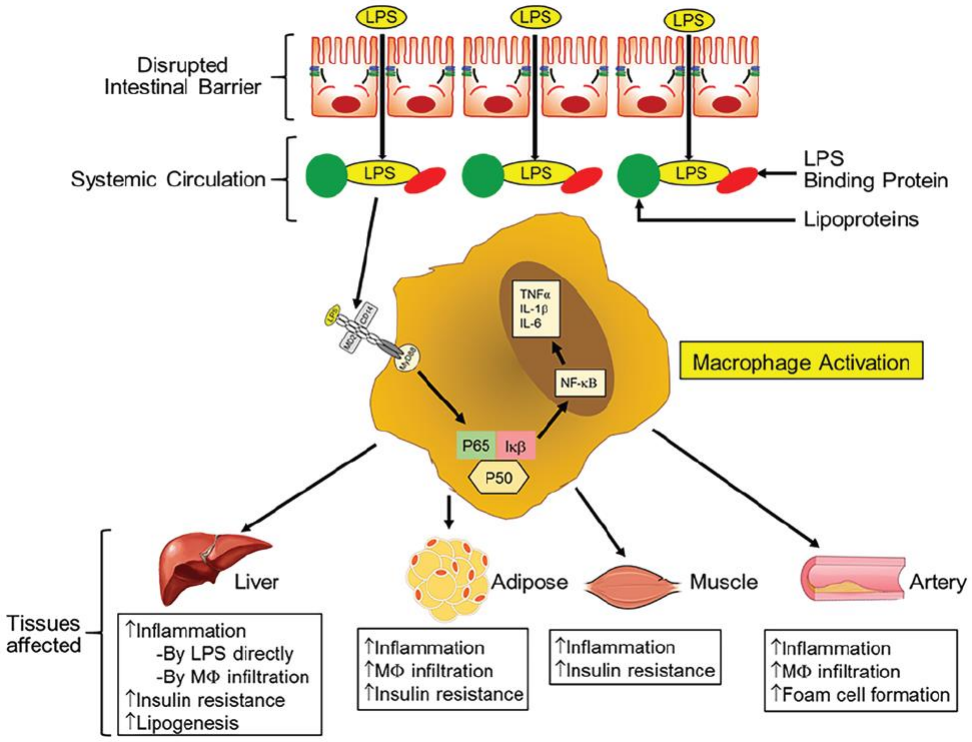


Figure 3: Consequences of impaired barrier integrity. If the intestinal barrier is disrupted LPS enters systemic circulation, associates with lipoproteins and LPS bindig protein and binds to TLR4 triggering intracellular signalling. With the activation of NFκB proinflammatory cytokines are produced leading to increased tissue inflammation. LPS reaching the liver activates macrophages and Kupffer cells. Increased inflammation results in hepatic insulin resistance and lipogenesis. Inflitrations of macrophages into adipose tissue causes inflamed adipose tissue and insulin resistance, thus contributing to the development of diabetes. In skeletal muscles inflammation also contributes to insulin resistance. In the artery infiltration of activated macrophages leads to artherosclerotic plaque development (Ghosh et al., 2020).

2.1.2 Consequences of oxidative stress

An imbalance between prooxidants and antioxidants characterized by the excessive production of ROS is referred to as oxidative stress (Lykkesfeldt and Svendsen, 2007). This imbalance can lead to damage of important biomolecules and cells – commonly described as oxidative damage (Reuter et al., 2010). Reactive oxygen species (ROS) derive from the partial reduction of O_2 and are produced as byproducts of normal cellular metabolism. ROS include compounds such as superoxide ($O_2^{\cdot -}$) hydroxyl radicals ($HO^{\cdot}$), hydroperoxyl radical ($HO_2^{\cdot}$), lipid hydroperoxides, singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), nitric oxide ($NO^{\cdot}$) and peroxynitrite ($ONOO^-$) (Bhattacharyya et al., 2014; Carocho and Ferreira, 2013). Several endogenous and exogenous factors lead to the formation of ROS. Besides the respiratory chain in the mitochondria, various intracellular enzymes (NADPH oxidase, xanthine oxidase, lipooxigenases, myeloperoxidase, nitric oxidase synthase) are also generators of endogenous ROS. Transition metals (e.g., Fe^{2+}, Cu^{+}) also contribute to $HO^{\cdot}$ generation via the Fenton reaction. Among the exogenous factors of ROS production are air

pollutants, tobacco smoke, radiation, food, nutrients, drugs, xenobiotics and chemical agents (e.g., heavy metals) (Bhattacharyya et al., 2014). Furthermore, immune reactions may also contribute remarkably to ROS generation during infections and autoimmune responses (Lykkesfeldt and Svendsen, 2007). The presence of pathogens can activate the epithelium, neutrophils, and macrophages in multiple ways. (Dubreuil, 2017). Foreign microorganisms can cause inflammation during which the immune system gets activated. Inflammatory cells are recruited to the site of damage leading to a respiratory burst, that is characterized by increased oxygen uptake and (as a consequence of the former) increased release and accumulation of ROS. In addition, inflammatory cells produce soluble metabolites (arachidonic acid, cytokines, and chemokines) that further recruit inflammatory cells to the site of damage resulting in increased reactive species production. ROS can modulate various transcription factors, e.g. nuclear factor κB (NF-κB), signal transducer and activator of transcription 3 (STAT3), hypoxia-inducible factor-1α (HIF-1α), activator protein-1 (AP-1), nuclear factor of activated T cells, and nuclear factor erythroid 2-related factor 2 (Nrf2), which mediate immediate cellular stress responses. Oxidative stress-induced inflammation might cause the induction of cyclo-oxygenase-2, inducible nitric oxide synthase (iNOS) and the abnormal expression of inflammatory cytokines (TNF, interleukin-1 [IL-1], IL-6) and chemokines (interleukin-8 [IL-8]) (Reuter et al., 2010).

ROS are highly reactive with proteins, lipids, carbohydrates, and nucleic acids within cells, causing oxidative damage. Oxidation of deoxyribonucleic acid (DNA) leads to base misincorporations, mutations, single or double DNA strand breaks while protein oxidation causes malfunctioning of enzymes and damage of cellular and TJ proteins, the latter leading to increased gut permeability (Bhattacharyya et al., 2014; Lykkesfeldt and Svendsen, 2007). The unsaturated fatty acid part of lipids is prone to oxidation, ROS abstracts hidrogen from fatty acids forming conjugated dienes, which than react with molecular oxygen and form lipid peroxil radicals. Lipid peroxil radicals may easily oxidise neighbouring lipids initiating a chain reaction. Lipid oxidation compromises cell integrity and due to the chain reaction they commence, oxidative damage is propagated. Organisms have adapted to ROS production and developed defence startegies that include both enzymatic and non-enzymatic antioxidant elements aiming to maintain balance between prooxidants and antioxidants. Superoxide dismutase, catalase and glutathione peroxidase are part of the enzymatic defense system and vitamin C, vitamin E and glutathione (GSH) are represenatatives of the non-enzymatic antioxidants (Lykkesfeldt and Svendsen, 2007).

During the pig production process five main factors can induce oxidative stress: (1) birth, (2) weaning stress, (3) mycotoxin pollution in feed, (4) feeding environment and (5) social factors. During parturition many changes (such as spontaneous respiration outside the uterus, ambient temperature, humidity, lighting, and noise) occur, that trigger the respiratory system

in the mitochondria and other physiological metabolic systems of newborn piglets to produce large amounts of ROS. At birth oxidative damage was characterized by an (1) increased level of malondialdehyde (MDA) (a marker of blood lipid oxidation) and (2) decreased activities of antioxidant enzymes (glutathione peroxidase (GPx) and superoxide dismutase (SOD)), confirming that (1) large amounts of ROS are produced at birth and that (2) the weak antioxidant systems cannot handle ROS excess. Oxidative stress at weaning was represented by elevated MDA and protein hydroxyl (a marker of protein oxidative damage) levels. The response to weaning oxidative stress is a complex process, affected by many factors and with multiple signaling mechanisms and also intestinal microorganisms being involved. Mixed mycotoxins (including aflatoxin B1, deoxynivalenol, ochratoxin, and fumatoxin) reduced blood SOD activity in piglets. Environmental and social factors, such as feeding density, fighting, pig house hygiene, heat/cold stress, transportation stress, and *E. coli* infection can also induce oxidative stress in pigs. Blood protein hydroxyl levels in high-density pigs were significantly increased. In addition, high-density feeding also leads to factors (such as house temperature rise, fighting, harmful gas accumulation and bacterial infection) that can further contribute to large amounts of ROS and oxidative damage (Hao et al., 2021). In growing pigs, heat stress decreased GPx activity and increased glutathione disulfide (GSSG)-to- GSH ratio (markers of oxidative stress) (Liu et al., 2016). Some of the stressors inducing oxidative stress can also alter the immune system at systemic and local levels including the gastrointestinal tract. Heat-stress e.g. causes changes in the barrier function (by increasing permeability) coincidently with gut inflammation in pigs. Under heat-shock myeloperoxidase activity (a marker of neutrophil activation) was increased in porcine gut. Mycotoxin pollution in feed promotes altered intestinal proinflammatory cytokines production and changes barrier function (through increasing permeability) in pigs. Deoxynivalenol (DON) induced pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β expression in the jejunum and ileum (Lee et al., 2016). Infectious stress, induced by some enteric pathogens, might cause inflammatory diarrhea by up-regulating pro-inflammatory cytokines. Heat-labile enterotoxins of ETEC e.g. activate B-cells and alter cytokine secretion of monocytes (Fairbrother et al., 2005).

Oxidative stress can contribute to the development of numerous disorders and gastrointestinal diseases, including atherosclerosis, cancer, peptic ulcer and inflammatory bowel disease (Bhattacharyya et al., 2014; Lykkesfeldt and Svendsen, 2007). Moreover, increased ROS production seems to be involved in the development of enteritis, sepsis and pneumonia in pigs (Lykkesfeldt and Svendsen, 2007). The GIT is also a main source of reactive oxygen species and if the barrier function is disrupted the intestine becomes even more vulnerable to oxidative stress (Bhattacharyya et al., 2014). In a piglet model gut injury was induced by deoxycholate and elevated nitrite (end products of $NO^{\bullet}$) levels were measured in luminal lavages, indicating that nitric oxide was released in response to gut injury. However,

when nitric oxide synthase (NOS) was inhibited by NG-nitro-L-arginine methyl ester (L-NAME) permeability was increased, suggesting that NO• seems to have also a role in the functional repair of the epithelial barrier. (Lykkesfeldt and Svendsen, 2007; Miller et al., 1993) NO• also plays a role in oxidative stress in sepsis. In pigs, LPS administration increased NO• production in the portal ciculation. With the inhibition of iNOS sepsis-induced oxidative damage could be reduced. Pneumonia caused by *Actinobacillus pleuropneumoniae* was characterized by reduced ascobate levels, indicating that oxidative stress related mechanisms might be involved. (Lykkesfeldt and Svendsen, 2007).

In intensive pig production oxidative stress is prevalent and causes a hazard to animal health. Pigs use energy to counteract oxidative damage, which results in growth retardation, decrease of production perfomance and thus in economic loss. Nutritional measures (supplements containing antioxidant compounds) have a potential to reduce or prevent oxidative stress related diseases. (Hao et al., 2021). It needs to be emphasized that increased ROS production may lead to numerous disorders, however, if cellular ROS concentration is maintained at a proper level, ROS play an important role in regulating cell signalling pathways (Wang et al., 2017a). Oxidative stress might lead to oxidative damage, however, oxidative stress is not neccesarily associated with oxidative damage. Therefore, markers of oxidative stress should be interpreted in correlation with oxidative damage (Lykkesfeldt and Svendsen, 2007).

2.2 Probiotics

The original word “probiotic” derives from Greek and means “for life”. The application of probiotics looks far back into the past. Fermented milk is supposed to be the first food that contained living microorganisms as mentioned in the Old Testament (Genesis 18: 8). The definition of probiotics developed with time, in 1965 Lilley and Stillwell, in 1972 Sperti and in 1989 Fuller gave new, more appropriate definitions to probiotics (Fuller, 1992). Nowadays the definition of The World Health Organization (WHO)/ Food and Agriculture Organization of the United Nations (FAO) is accepted. According to the WHO/FAO probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Hill et al., 2014). Probiotics can be classified as follows: (1) bacterial or non-bacterial probiotics, (2) spore forming or non-spore forming probiotics, (3) multi-species or single species probiotics, (4) allochthonous or autochthonous probiotics (Bajagai et al., 2016). Microorganisms to be used as probiotics need to meet specific safety, functionality, and technological usability criteria. Probiotics should be isolated from the species for which they are intended to be used, because it is supposed that beneficial effects are species specific (Markowiak and Śliżewska, 2018). Promising candidates have a history of safe use, are not

associated with infective diseases, have no adverse effects, and lack genes responsible for antibiotic resistance. Acid and bile salt tolarance is also preferable, since they need to survive the passage through the GIT. Probiotics should withstand the circumstances applied during the feed production process, resistance to high temperature and pressure is prefered (Markowiak and Śliżewska, 2018; Teneva-Angelova et al., 2018). Microorganisms from many genera including *Bacillus*, *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Pediococcus* and *Streptococcus* are used as bacterial probiotics, however most probiotic bacteria belong to the group of lactic acid-producing bacteria and originate from the intestine. (Dubreuil, 2017; Liao and Nyachoti, 2017; Nithya and Halami, 2013). In **Table 1** the most frequently applied bacterial probiotics in animal feed supplements are summarized. (Liao and Nyachoti, 2017; Markowiak and Śliżewska, 2018).

Table 1: Most frequently used probiotic bacteria in animal feed supplements.

Lactobacillus	*Bifidobacterium*	Other lactic acid bacteria	Other bacteria
L. brevis	*B. animalis*	*Enterococcus faecalis*	*Bacillus cereus*
L. casei	*B. longum*	*Enterococcus faecium*	*Bacillus licheniformis*
L. crispatus	*B. pseudolongum*	*Lactococcus lactis*	*Bacillus subtilis*
L. farciminis	*B. thermophilum*	*Leuconostoc citreum*	*Propionibacterium freudenreichi*
L. fermentuma		*Leuconostoc lactis*	
L. murinus		*Leuconostoc mesenteroides*	
L. gallinarium		*Pediococcus acidilactici*	
L. paracasei		*Pediococcus pentosaceus*	
L. pentosus		*Streptococcus infantarius*	
L. plantarum		*Streptococcus salivarius*	
L. reuteri		*Streptococcus thermophilus*	
L. rhamnosus		*Sporolactobacillus inulinus*	
L. salivarius			

Lactic acid bacteria (LAB) are Gram-positive, catalase-negative, non-spore-forming, nonmotile, nonrespiring, acid-resistant, anaerobic to aerotolarant cocci or rod-shaped bacteria, which produce lactic acid as the principal end product of their carbohydrate fermentation (Teneva-Angelova et al., 2018). Within the group of LAB *Lactobacillus* is the largest genus and their utility is related to their generally recognized as safe (GRAS) status (De Angelis and Gobbetti, 2016). Enterococci are also part of LAB and most of their physiological properties (being Gram-positive, non-spore-forming, catalase-negative) are also similar to LAB (Klein, 2003). On the one hand Enterococci are widely used as probiotics to enhance the microbial balance of the intestine but on the other hand Enterococci are nosocomial pathogens causing bacteraemia, endocarditis, urinary tract, and other infections and the multi-drug resistant strains of Enterococci raise serious concerns (Franz et al., 1999; Miller et al., 2014).

Bacillus species are rod-shaped, Gram-positive, aerobic or facultative anaerobe, endospore-forming bacteria and are found everywhere in the environment, including soil,

water, air (Achi and Halami, 2016). Although they are not part of the commensal microbiota, they are also attractive probiotic candidates thanks to their spore forming properties, which them enable to resist during the transit through the GIT (Nithya and Halami, 2013; Pahumunto et al., 2021). Some *Bacillus* strains form biofilms which enable them to protect themselves against the different conditions present in the gut and contribute to their good survival rate in the GIT (Hernandez-Patlan et al., 2019). Further advantages of spores are good reproducibility, high viability, and stability during storage and feed preparation processes (Larsen et al., 2014; Luise et al., 2022). Among various *Bacillus* species, *Bacillus subtilis (B. subtilis)*, *Bacillus licheniformis (B. licheniformis)*, and *Bacillus cereus* (*B. cereus)* are used for animal feed (Larsen et al., 2014). However, among *Bacillus* species, pathogenic members can also be found, which raises general concern about their use as probiotics (Hong et al., 2008). The production of enterotoxins and the possible transfer of antibiotic resistance genes might further contribute to their limited use (Luise et al., 2022).

Adhesion inhibition of pathogenic bacteria, modulation of the immune system, and enhancement of the GIT barrier function are some of the beneficial effects exerted by probiotics that have been proved in several *in vitro* (summarized in **Table 2**) and *in vivo* (summarized in **Table 3**) experiments. Probiotics also exert a beneficial effect on the production performance and on the reproductive parameters of pigs (**Table 4**). As summarized in **Table 4** probiotics may increase the daily weight gain, the daily feed intake and the feed conversion ratio in pigs (Liao and Nyachoti, 2017). Supplementation with probiotics improved meat color, marbling, tenderness, flavor and juiciness (Ahasan et al., 2015). Some probiotic bacteria also improved littersize, the quality and quantity of colostrum and milk, furthermore the viability and the weight of piglets were also increased, however the incidence of diarrhea was decreased (Alexopoulos et al., 2004a, 2004b; Böhmer et al., 2006; Zeyner and Boldt, 2006).

Table 2. The effect of probiotics on porcine epithelial cells in *in vitro* experiments

Probiotic strain	Cell-line	Pathogen	Adhesion inhibition	Immune modulation	Barrier integrity	Other*	Reference
Lactobacillus reuteri LR-1 /	IPEC-1	ETEC	x	x	x		(Wang et al., 2016)
Lactobacillus rhamnosus GG	IPEC-J2	ETEC	x		x		(Liu et al., 2015)
Lactobacillus johnsonii	IPEC-J2	ETEC	x		x		(Liu et al., 2015)
Lactobacillus rhamnosus ATCC 7469	IPEC-J2	ETEC		x	x		(Zhang et al., 2015)
Enterococcus faecium NCIMB 10415	IPEC-J2	ETEC		x	x		(Klingspor et al., 2015)
Enterococcus faecium NCIMB 10415	IPEC-J2	ETEC			x		(Lodemann et al., 2015)
Enterococcus faecium (HDRsEf1)	IPEC-J2	ETEC K88	x	x	x		(Tian et al., 2016)
Lactobacillus reuteri ATCC 53608 and *Bacillus licheniformis* ATCC 10716	IPEC-J2	*S.* Typhimurium		x			(Skjolaas et al., 2007)
E. coli Nissle 1917	IPEC-J2	*S.* Typhimurium	x				(Schierack et al., 2011)
Lactobacillus plantarum ZLP001	IPEC-J2	ETEC	x			x	(Wang et al., 2018)
Lactobacillus reuteri I5007	IPEC-J2	LPS *E. coli* 055:B5		x	x		(F. Yang et al., 2015)

*: production of antimicrobial substances, production of host defence peptides (HDP) and alteration of redox homeostasis

Table 3. The effect of probiotics on pigs in *in vivo* experiments

Probiotic strain	Pathogen	Adhesion inhibition	Immune modulation	Barrier integrity	Other*	Reference
Enterococcus faecium 18C23	*Escherichia coli* K88ac and K88MB	x				(Jin et al., 2000)
Pediococcus acidilactici	ETEC			x		(Lessard et al., 2009)
Pediococcus acidilactici	ETEC K88	x	x			(Daudelin et al., 2011)
Lactobacillus sobrius DSM 16698		x				(Konstantinov et al., 2008)
Lactobacillus plantarum	ETEC K88			x		(Yang et al., 2014)
Lactobacillus rhamnosus ATCC 7469	ETEC K88		x		x	(Li et al., 2012)
Bacillus licheniformis	ETEC		x	x		(Yang et al., 2016)
Bacillus subtilis	ETEC		x	x		(Yang et al., 2016)
Lactobacillus reuteri TMWI.656	ETEC	x			x	(Y. Yang et al., 2015)

*: microbial diversity, inhibition of enterotoxin production

Table 4. The effect of probiotics on the growth and reproductive performance of pigs.

Subjects	Probiotic strain	Time of administration	Main outcome	Reference
114 sucking piglets	*Enterococcus faecium* DSM 10663 NCIMB 10415	From birth to weaning (24 ±3,2 days).	Lower incidence of diarrhea, higher daily weight gain.	(Zeyner and Boldt, 2006)
33 sows	*Enterococcus faecium* DSM 7134	From the 90th day of pregnancy to the 28th day of lactation.	Higher feed consumption, offspring size and weight gain.	(Böhmer et al., 2006)
26 gestating sows, 153 sucking piglets	*Enterococcus faecium* NCIMB 10415	17 weeks (sow), 6 weeks (piglets).	Lower death rate during lactation (sow), lower incidence of post weaning diarrhea (piglets).	(Taras et al., 2006)
15 weaning piglets	*2 Lactobacillus murinus strains+ Lactobacillus salivarius subsp. salivarius* or *Lactobacillus pentosus or Pediococcus pentosaceous.*	30 days 6 days treatment with probiotics, on day 6 infection with *Salmonella*.	Lower incidence, duration and severity of diarrhea, decreased *Salmonella* shedding. Improved clinical signs of *Salmonella* infection.	(Casey et al., 2007)
sows and piglets	*E. faecium* NCIMB 10415, *B. cereus* toyoi	6 weeks	Lower incidence of diarrhea, no effect on weight gain.	(Simon et al., 2003)
96 growing-finishing pigs	*Bacillus subtilis, Clostridium butyricum*	10 weeks	Improved growth permformance, increased average daily gain and improved apparent total tract digestibility of nutrients.	(Meng et al., 2010)
90 piglets (35-40 days old)	*Bacillus subtilis* MA 139	28 days	Enhanced daily gain and feed conversion. Increased Lactobacilli shedding and decreased *E. coli* shedding.	(Guo et al., 2006)
neonatal piglets	*Bifidobacterium longum* (AH1206)	18 days	No effect on weight gain, lower feed consumption.	(Herfel et al., 2013)

109 gilts	*Bacillus licheniformis, Bacillus subtilis* (BioPlus 2B)	14 days prior to the expected farrowing up to the weaning day	Improved litter health and performance (decreased incidence of diarrhea in piglets, decrease in pre-weaning mortality, increased piglet body weight at weaning), decrease of sow weight loss during suckling period, improved milk parameters (higher milk fat and protein content)	(Alexopoulos et al., 2004a)
54 weaned piglets	*Bacillus licheniformis, Bacillus subtilis* (BioPlus 2B)	Weaning, growing/finishing stage	Lower morbidity and mortality, improved weight gain, feed conversion and carcass quality.	(Alexopoulos et al., 2004b)

2.2.1 Mechanism of probiotic action

Probiotic action is complex and similarly to the term probiotics, also the classification of probiotic action has evolved over the years. Nowadays several classifications of modes of actions exist. Oelschlager for example distinguishes three modes of actions, namely (1) immunomodulation, (2) direct effect on other microorganisms, (3) effect on microbial and host products, while according to Sánchez, probiotics exert their beneficial effects through four mechanisms: (1) interference with pathogens, (2) improvement of epithelial barrier function, (3) immunomodulation, (4) influence on other organs. Liao classified probiotic action in five groups as follows: (1) modulation of the gut microbiota, (2) modulation of host immune response, (3) diarrhea reduction and antitoxin effect, (4) modulation of nutrient digestibility, (5) other actions (Liao and Nyachoti, 2017; Oelschlaeger, 2010; Sánchez et al., 2017). Many of the probiotic actions have an influence on other ones, which makes their classification difficult.

Probiotics might modulate the gut microbiota either through (1) competitive exclusion or through (2) direct antimicrobial inhibition. Competitive exclusion indicates that probiotics compete with pathogens either for adhesions sites on IECs or for nutrients present in the GIT. With the adhesion of probiotic bacteria to IECs the access of pathogens is limited or even excluded and since it is suggested that harmful bacteria need to adhere to the gut in order to exert harmful effects the development of infection can be prevented (**Figure 4**). If the colonization of pathogenic bacteria to the intestinal mucosa is restricted, nutrients and immunoglobulins of the colostrum can be absorbed more effectively, which is of upmost importance after birth (Dowarah et al., 2017). In addition to adhesive ability to intestinal cells probiotic bacteria might bind to each other (auto-aggregate) or to pathogenic bacteria (co-aggregate) (Monteagudo-Mera et al., 2019). Auto-aggregates form a barrier that prevents colonization of pathogens, however by binding pathogens into co-aggregates biofilm forming processes of pathogenic bacteria that are often involved in infection can be inhibited (**Figure 4**) (Monteagudo-Mera et al., 2019; Pahumunto et al., 2021). *Lactobacillus sobrius* could co-aggregate with ETEC and thus promoting pathogen removal (Roselli et al., 2007). As a results of probiotics competing with pathogens for nutrients, energy sources and limited substances the growth of pathogens might be suppressed (Liao and Nyachoti, 2017; Oelschlaeger, 2010). Contrary to almost all bacteria, iron is not essential for Lactobacilli. However, *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* are capable of binding ferric hydroxid thus making it unavailable to pathogens. Probiotic *Escherichia coli* strain

Nissle 1917 (EcN) encodes seven different iron uptake systems which renders it more competitive for the uptake of limited iron resource (Oelschlaeger, 2010).

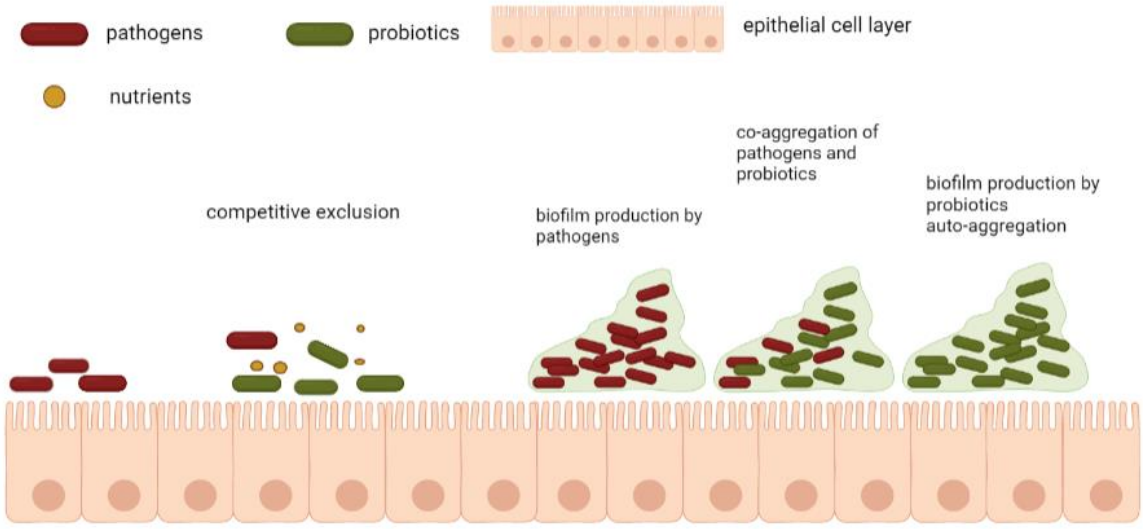


Figure 4: Inhibition of pathogens by competitive exclusion and biofilm production. Probiotics compete with pathogens for adhesion sites or for nutrients. Probiotics form auto-aggregates thus preventing colonization of pathogens or inhibit biofilm processes of pathogenic bacteria by forming co-aggregates. Illustration was made with biorender software tool ("BioRender").

Direct antimicrobial inhibition means that some probiotic bacteria are capable of producing bacteriostatic and bactericidal substances with organic acids, hydrogen peroxide, antioxidants, antibiotics (reuterin), bacteriocins, microcins and deconjugated bile acids being the most important ones. Among probiotic bacteria, lactic acid bacteria represent a significant group and these organisms ferment carbohydrates (e.g., lactose) to short chain fatty acids such as lactic and acetic acid. The production of acidic compounds results in the decrease of luminal pH that pathogenic bacteria cannot tolerate (Liao and Nyachoti, 2017). Lactobacilli can also produce low-molecular-weight bacteriocins (LMWB) which are antimicrobial peptides and one of their representatives —Abp118 — has been proved to protect mice against infection with pathogen *Listeria monocytogenes*. Reuterin is produced by *Lactobacillus reuteri* (*L. reuteri*) strain ATCC55730 and is known as a broad- spectrum antibiotic active against both Gram-positive and Gram-negative bacteria and against yeast, fungi, protozoa and viruses as well. Microcines are peptides, can be synthetized by many probiotics and possess a narrow activity spectrum. Deconjugated bile acids are derivatives

of bile salts with stronger antimicrobial activity (Oelschlaeger, 2010). *Bacillus* species are known to produce a wide range of antimicrobial substances. *B. subtilis* produces subtilin, entianin, ericin, surfactin, iturin, mycosubtilin, fengycin, bacilysin, bacitracin, while *B. licheniformis* is known to produce lichenicidins and bacitracin (Achi and Halami, 2016). The substances produced by probiotics do not only decrease the number of pathogens but also affect bacterial metabolism and toxin production (Yirga, 2015).

Stressful conditions deriving from the environment (like weaning, high temperature and humidity, changes in energy balance and diet) and weakening of the host defence may evoke oxidative stress. Probiotics may modulate the redox status of the host through multiple ways: (1) chelating metal ions, (2) decomposing ROS with their own antioxidant enzymes, (3) producing metabolites with antioxidant capacity, (4) regulating cell signalling pathways, (5) regulating the host's enzymes producing ROS and (6) regulating the intestinal microbiota of the host. If metal ions are captured by chelators the catalysis of oxidation reactions can be prevented. *Streptococcus thermophilus* 821, *Lactobacillus casei* KCTC 3260 and *Lactobacillus helveticus* CD6 have shown Fe^{2+} or Cu^{2+} chelating ability. Superoxide dismutase (SOD) is part of the antioxidant enzymatic defence of probiotic bacteria, it catalyzes the breakdown of superoxide into hydrogen peroxide and water and plays a key role in the regulation of ROS levels (Wang et al., 2017a). *Lactobacillus fermentum* strains E-3 and E-18 express manganese superoxide dismutase (Mn-SOD) and increased resistance to several ROS have been shown (Kullisaar et al., 2002). Glutathione, butyrate, and folate are substances with antioxidant activity and can be produced by certain probiotics. Folate production was proved for *Lactobacillus helveticus,* while *Lactobacillus fermentum strains* E-3 and E-18 contain remarkable levels of GSH and *Clostridium butyricum* strain MIYAIRI 588 is a butyrate-producing probiotic (Wang et al., 2017a). In addition to its antioxidant properties butirate has been proved to increase the expression of tight junction proteins and thus conferring to the maintenance of the intestinal barrier integrity (Ma et al., 2012). Probiotic bacteria can exert their protective effect against oxidative stress through the regulation of the nuclear factor erythroid 2-related factor 2— Kelch-like ECH-associated protein-1 — antioxidant response element (Nrf2-Keap1-ARE), the NFκB, the mitogen-activated protein kinase (MAPK) and the protein kinase C (PKC) pathways. If ROS levels are low, Nrf2 is kept inactive by its inhibitor Keap1. Keap1 is redox sensitive and if the level of free radicals rises Keap1 undergoes a change in conformation and Nrf2 gets

activated, translocates to the nucleus and binds to antioxidant response element (ARE) sequences inducing the transcription of antioxidant enzymes and detoxifying proteins. *Lactobacillus plantarum* FC225 has been effective in promoting NRf 2 expression and thus improved superoxide anion radical scavenging in mice. Using intestinal porcine epithelial cell line-1 (IPEC-1), H_2O_2 induced oxidative stress could be alleviated by *Bacillus amyloliquefaciens* by regulating Nrf2 expressions, causing a decrease in ROS levels. In case of inflammation, ROS can mediate the activation of NFκB and the successive expression of inflammatory cytokines. LPS-induced inflammation was prevented by *Bacillus* spp. strain LBP32 in RAW 264.7 macrophages through the inhibition of NFκB and ROS production. MAPKs and PKC can be activated by various stimuli and are involved in a variety of pathways that regulate response to stress (Wang et al., 2017a). MAPKs are also involved in the induction of heat shock proteins (Hsps). *Lactobacillus johnsonii* and *Lactobacillus reuteri* strains could stimulate the sythesis of Hsp27, which can bind to cytoskeleton protein F-actin and stabilise the TJ complex (Dubreuil, 2017). In colon carcinoma cell line (Caco-2) secreted compounds of (*Lactobacillus rhamnosus*) *L. rhamnosus* GG could protect the barrier function from H_2O_2 induced oxidative stress in a PKC- and MAPK-dependent mechanism (Seth et al., 2008). Probiotics can increase the antioxidase activity of the host. *Lactobacillus fermentum* could elevate serum SOD and glutathione peroxidase (GPx), hepatic catalase (CAT), muscle SOD, and Cu and Zn-SOD levels. *Bacillus amyloliquefaciens* SC06 raised CAT and GSH gene expressions and CAT activity in IPEC-1 cells. Dysbiosis is characterized by the abnormal proliferation of harmful bacteria, leading to increased endotoxin levels in the blood and thus conferring to oxidative stress. If probiotic bacteria regulate the intestinal microbiota through competitive exclusion, consumption of nutrient sources, and production of antimicrobial substances they contribute to decreased oxidative stress (Wang et al., 2017a). Antimicrobial and antioxidant properties of probiotics are summarized in **Figure 5**.

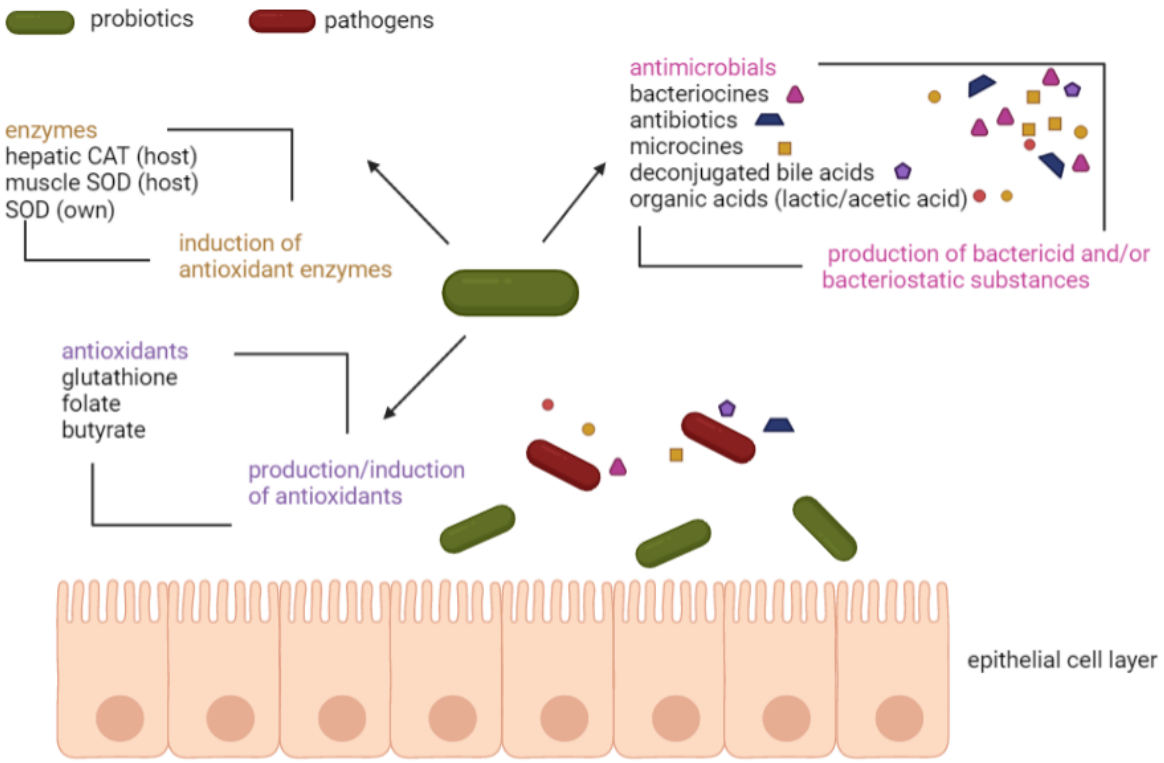


Figure 5: **Antimicrobial and antioxidant properties of probiotics.** Probiotics exert antioxidant activity by (1) inducing the host's (e.g., hepatic CAT, muscle SOD) and/or their own antioxidant enzymes (e.g., SOD) and/or by (2) producing/inducing metabolites (e.g., glutathione, folate, and butyrate) with antioxidant capacity. Probiotics exert antimicrobial effect through the production of bacteriostatic and bactericid substances including organic acids, bacteriocins, antibiotics, microcines, deconjugated bile acids. Illustration was made with biorender software tool ("BioRender").

Probiotics exert their immunomodulatory effect by influencing both innate and acquired immunity. The main target cells are IECs and gut associated immune cells. Modulation of the immune system can basically be reached in two ways: (1) the adherence of the probiotics themselves to IECs and (2) the release of soluble molecules triggering the signalling cascade (Oelschlaeger, 2010). Dendritic cells (DCs) take up probiotic bacteria through direct or M-cell mediated sampling and interact with T and B cells. Probiotics, like other bacteria, possess conserved microbial-associated molecular patterns (MAMP) which interact with pattern recognition receptors (PRRs) found on the membrane surface of IECs and DCs. This interaction plays a pivotal role in the maturation of antigen presenting cells and determines the immune response which can be effector or regulatory. Probiotics triggering the regulatory response are particularly important in inflammatory diseases

(Sánchez et al., 2017). Pathogen-induced inflammation activates the immune system mainly through the NFkB and MAPK signalling pathways and consequently various proinflammatory cytokines such as IL-6, IL-8 and TNF-α are synthetized. Probiotic bacteria can alter the expression of cytokines in epithelial cells either through decreasing the production of proinflammatory cytokines (e.g. IL-6 , IL-8) or through increasing the secretion of anti-inflammatory cytokines, e.g. interleukin-10 (IL-10) (Bahrami et al., 2011; Carey and Kostrzynska, 2013). *Lactobacillus reuteri* could successfully inhibit the expression of proinflammatory cytokines IL-6 and TNF-α induced by ETEC and was able to increase the production of anti-inflammatory cytokine IL-10 (Dubreuil, 2017). Probiotics can also induce the release of defensins from epithelial cells (Cerdó et al., 2019). Defensins are small antimicrobial peptides, have an important role in the innate immune defence and exert their antimicrobial effects by peptide-mediated membrane disruption. In pigs, two types of β-defensins have been described up to date, porcine β-defensin 1 (pBD1) and porcine β-defensin 2 (pBD2) (Veldhuizen et al., 2007). Defensins can be induced by bacterial products or pro-inflammatory cytokines (Mair et al., 2014). *L. acidophilus*, *L. fermentum*, *L. paracasei* subsp. paracasei, *Pediococcus pentosaceus*, and *E. coli* Nissle 1917 were able to induce human β-defensin-2 gene expression in Caco-2 cells (Cerdó et al., 2019).

Probiotics can enhance the barrier function of epithelial cells through the modulation of cytoskeletal and tight junctional proteins and through the promotion of mucus production (Cerdó et al., 2019). In IPEC-1 cell line *Lactobacillus sobrius* could prevent barrier disruption caused by ETEC by maintaining the appropriate localization of zona occludens 1 (ZO-1), occludin, and F-actin, and by disabling the decrease of occludin amount (Roselli et al., 2007). Mucin expression was increased by *Lactobacillus* species in Caco-2 and human colorectal adenocarcinoma (HT29) cell lines thus preventing *E. coli* adhesion (Cerdó et al., 2019). The effect of probiotics on immune modulation and on barrier enhancement is summarized in **Figure 6**.

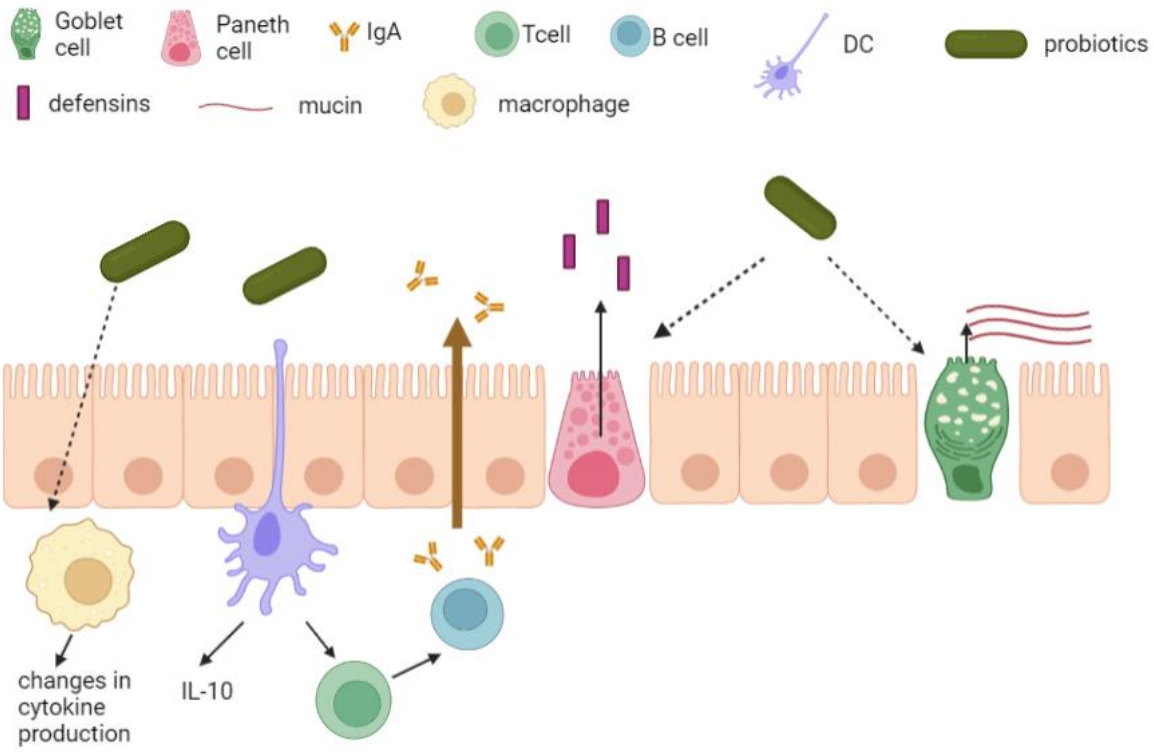


Figure 6: Modulation of the immune system and enhancement of the barrier function by probiotics. Dendritic cells take up probiotic bacteria and interact with T and B cells, resulting in the secretion of IgA. Probiotics alter the expression of cytokines by decreasing the expressions of proinflammatory cytokines and by increasing the expression of anti-inflammatory cytokines (e.g., IL-10). Probiotics induce the release of defensins (antimicrobial peptides) from Paneth-cells. Probiotics contribute to the enhancement of the barrier function of epithelial cells through the promotion of mucus production. Illustration was made with biorender software tool ("BioRender") based on (Cerdó et al., 2019)

The diarrhea reducing and anti-toxin effect of probiotics are rather a combination of the already mentioned modes of actions. Pathogenic bacteria produce enterotoxins that stimulate epithelial cells to secrete fluid to the lumen, resulting in diarrhea which is one of the major problems in post weaning piglets (Liao and Nyachoti, 2017). Enterotoxins can be neutralized in a direct or an indirect way. *Lactobacillus acidophilus* has been proved to bind aflatoxin (Pop et al., 2022). Probiotics can remove aflatoxins through biodegradation or bioadsorption mechanisms. During biodegradation metabolites are produced, which might have an adverse effect on the host. Bioadsorption means the direct binding of the toxin, which is related to the many polysaccharide like cell wall components of probiotic bacteria enabling to bind toxins through weak non-covalent interactions (**Figure 7**) (Afshar et al., 2020). The many substances (organic acids, antioxidants, bacteriocins) produced by

probiotic bacteria contribute to the neutralization of enterotoxins in an indirect way. These rather have an effect on the toxin producing pathogen than on the toxin itself. Bacteriocins produced by Lactobacilli permeate the outer membrane of Gram-negative bacteria and inactive them. The presence of organic acids may support this process (Yirga, 2015). Moreover, in an *in vitro* study organic acids produced by Lactobacilli could prevent shiga toxin expression in *E. coli* O157:H7 (Liao and Nyachoti, 2017). The effect of toxins might also be counteracted with the binding of probiotics to epithelial cell oligosaccharide receptors as demonstrated in **Figure 7** (Monteagudo-Mera et al., 2019).

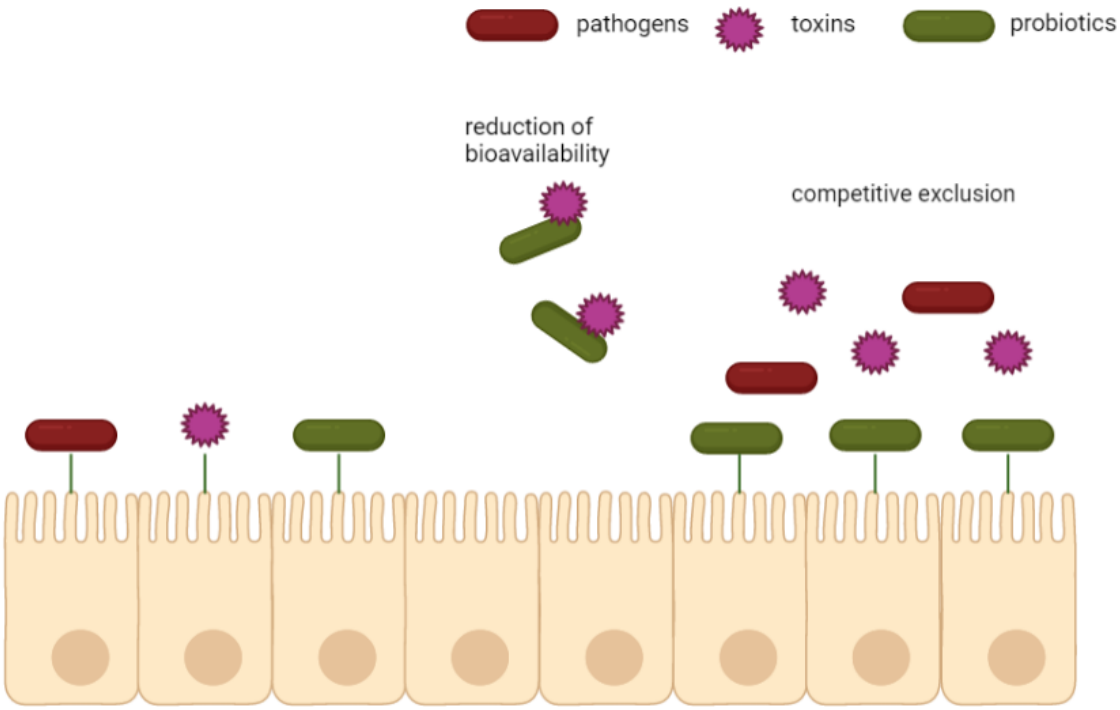


Figure 7: Antitoxin effect of probiotic bacteria. Probiotic bacteria bind toxins on their cell wall thus reducing the bioavailability of the toxin or bind to epithelial cell oligoszaccharide receptors resulting in the competitive excluson of the toxin. Illustration was made with biorender software tool (“BioRender”) based on (Hernandez-Patlan et al., 2019; Monteagudo-Mera et al., 2019)

Probiotics also proved to modulate the digestibility of nutrients. *B. subtilis* and *Clostridium butyricum* improved crude protein and energy digestibility in pigs, while *Lactobacillus reuteri* and *Lactobacillus plantarum* increased apparent total tract digestibilities of nitrogen and energy. The effect of probiotics on the digestibility of nutrients is partly related to the fact that probiotics can induce the production of digestive enzymes.

Furthermore, probiotics also alter absorption and secretion properties of the gut. In pigs villi leghts in the duodenum, jejunum, and ileum have been improved upon feeding with *Enterococcus faecium* (*E. faecium)* (Liao and Nyachoti, 2017).

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

Escherichia coli (*E. coli*) and *Salmonella* spp. are two major representatives among the challenges that affect the intestinal tract of swine.

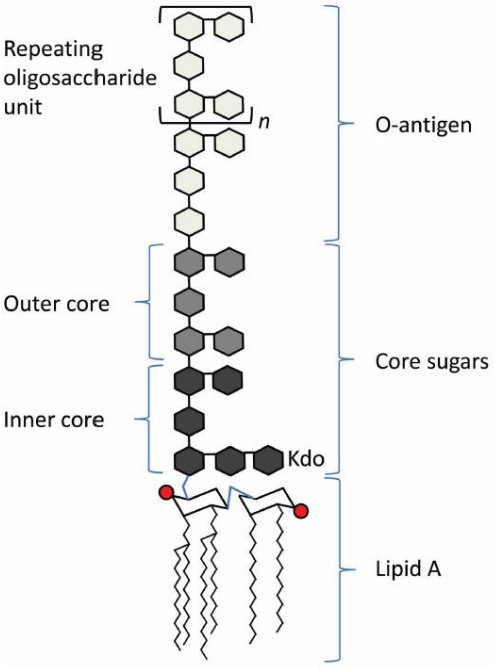
E. coli are Gram-negative peritrichously flagellated facultatively anaerobic rods, belonging to the family *Enterobacteriaceae* (Luppi, 2017). Among the species, normal inhabitants of the GIT as well as causative agents of intestinal and extraintestinal diseases in swine can also be found. *E. coli* causes a wide range of diseases in pigs, including neonatal diarrhea, postweaning diarrhea, edema disease, septicemia, coliform mastitis, urinary tract infection and polyserositis. These diseases require antibiotic therapy and ultimately result in the death of animals or cause significant economic loss due to morbidity, growth retardation, cost of treatment, vaccinations and feed supplements. Based on the virulence mechanism, *E. coli* are classified to the following pathotypes: (1) enterotoxigenic *E. coli* (ETEC), (2) Shiga toxin-producing *E. coli* (STEC) including the edema disease (EDEC) and enterohemorrhagic *E. coli* (EHEC*),* (3) enteropathogenic *E. coli* (EPEC) and (4) extraintestinal pathogenic *E. coli* (ExPEC). ETEC is the most important pathotype in pigs that causes secretory diarrhea in neonatal and post weaning piglets (Zimmerman et al., 2012). Neonatal and post-weaning diarrhea outbreaks are usually recurrent in swine herds with many pigs being affected and are important causes of death occurring worldwide. ETEC causing neonatal and post-weaning diarrhea enter the animal by ingestion and may proliferate and colonize in the gastrointestinal tract. Whether the infection results in a disease depends on predisposing environmental conditions and host factors. Weaning is a critical life phase in piglets when host defence is weakened rendering the animals more susceptible to diseases. Susceptibility to some ETEC strains decreases with the age (Luppi, 2017). ETEC does not damage intestinal cells, it disrupts the homeostasis of the cell, thus causing diarrhea. Pathogenesis starts with the attachment of ETEC to epithelial cells with the help of fimbrial adhesins (fimbriae) and then enterotoxins are produced (Zimmerman et al., 2012). ETEC responsible for neonatal diarrhea posses F4 (k88), F5 (k99), F6 (987P)

and F41 fimbriae, while post-weaning ETEC strains carry F4 and F18 fimbriae (Luppi, 2017). These fimbriae are not only essential for adhesion, but also induce the secretion of IL-6 and IL-8 proinflammatory cytokines. However, also flagellin can evoke the production of the same proinflammatory cytokines. ETEC produce two major classes of enterotoxins: (1) heat-labile (LT) and (2) heat-stable toxins (STs) that differ in their mode of action, but both result in diarrhea (Zimmerman et al., 2012). Most ETEC strains causing neonatal diarrhea produce STa, while post weaning ETEC strains produce one or more of STa, STb and LT (Luppi, 2017). Binding of LT enterotoxin to its receptor (ganglioside GM1) results in the activation of adenylate cyclase, intracellular cAMP levels increase, cystic fibrosis transmembrane regulator (CFTR) gets activated and finally Cl^- and HCO_3^- are secreted. Heat stable enterotoxins are subdivided to STa and STb. If STa binds to its receptor (guanylyl cyclase C glycoprotein receptor), intracellular cGMP rises, cGMP-dependent protein kinase II is activated, leading to the phosphorylation of CFTR and ultimately resulting in Cl^- and HCO_3^- secretion. Binding of STb enterotoxin also causes Cl^- and HCO_3^- secretion, however through different mechanisms, involving binding to sulfatide on the surface of epithelial cells, activation of GTP-binding regulatory protein, increase in Ca^{2+} level, activation of different protein kinases and finally activation of CFTR (Dubreuil, 2017). Regardless of which type of toxin induced the electrolyte imbalance, hypersecretion leads to dehydration and eventual death (Luppi, 2017). Moreover, all enterotoxins are involved in tight junction opening, thus contributing to increased permeability and leakage (Dubreuil, 2017).

Enteric diseases in animal production are also often associated with *Salmonella* spp. Salmonellosis can be manifested in multiple symptoms, including diarrhea, abortion, pneumonia, septic arthritis, meningitis and gangrene of distal extremities (Souto et al., 2017). Disease in swine is mostly related to *Salmonella choleraesuis* variety *kunzendorf* or *S. enterica* serovar Typhimurium. Weaned pigs are most susceptibe to salmonellosis, disease in adults and suckling pigs is rare. Disease most frequently develops in pigs with weakened immune system and cause enterocolitis, diarrhea and dehydration. Watery yellow diarrhea is the first clinical sign in pigs infected with *S.* Typhimurium. Then pigs have fever, get dehydrated and decrease their feed intake. Most pigs totally recover, however shedding can last for at least five month. About 10^7 *S.* Typhimurium bacteria per gram of intestinal content are thought to cause lesions in pigs, however lower numbers might be enough, if intestinal defences are weakened. Invasion is a requirement for pathogenesis and can occur

at several epithelial cell types, including enterocytes, M cells, goblet cells and the Peyer's patches. Bacteria attach to receptors found on epithelial cells, vacuoles are formed and then transported through the cytoplasm towards the lamina propria and are finally secreted via exocytosis. Meanwhile the enterocytes undergo damage and during invasion several new proteins are synthethised that aim at enhancing the intracellular survival of the bacteria. One of the key features of salmonellosis is early intestinal inflammation, which is characterized by (1) increase in interleukin-1 beta and interleukin-18, (2) activation of phosphokinase C and (3) activation of NF-κB and phosphokinase C, the ultimate resulting in the basolateral secretion of IL-8, a chemokine responsible for promoting the transepithelial migration of neutrophils into the intestinal lumen (Zimmerman et al., 2012).

E. coli and *Salmonella* might also exert their harmful effect via one of their cell wall components, namely LPS. LPS is a cell wall component of Gram-negative bacteria and it is a well-characterized pathogen-associated molecular pattern (PAMP) and is composed of three parts, (1) lipid A molecule (endotoxin), (2) core sugar, and (3) O antigen (**Figure 8 A**) (Maeshima and Fernandez, 2013). The toxicity of LPS is attributable to lipid-A, which binds to Toll-like receptor-4 (TLR4), a member of the Toll-like receptor family. PAMPs are recognized by different TLRs and LPS is recognized specifically by TLR4. Binding of LPS to TLR4 initiates downstream intracellular signaling leading to the activation of NF-κB and resulting in the transciption of proinflammatory cytokines like TNFα, IL-1β, and IL-6 and tissue inflammation (**Figure 8 B**) (Ghosh et al., 2020; Maeshima and Fernandez, 2013). The GIT is constantly exposed to LPS and the disruption of the intestinal barrier facilitates the paracellular transport of LPS into the systemic circulation. LPS is correlated with systemic inflammation, septic shock and is thought to be responsible for the development of diverse diseases (Ghosh et al., 2020).



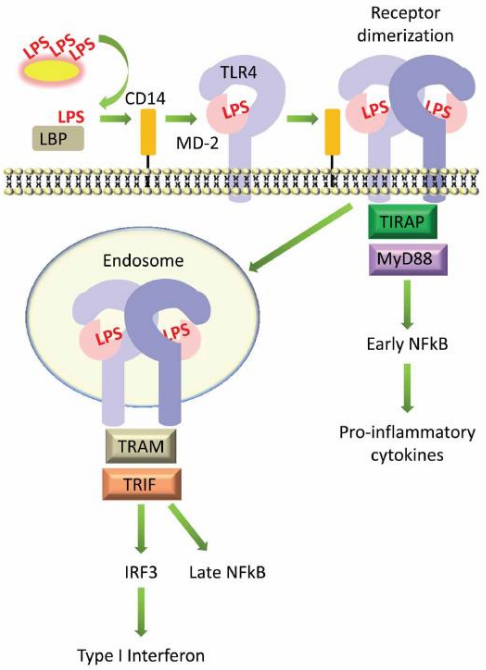


Figure 8 A: **Structure of LPS**: LPS is composed of 3 parts: (1) lipid A molecule (endotoxin), (2) a core sugar consisting of 3-deoxy-D-*manno*-oct-2-ulosonicacid (Kdo) and (3) the O antigen consisting of repeating oligosaccharide units. The letter n indicates the number of repeating units (Maeshima and Fernandez, 2013).

Figure 8 B: **LPS-induced signalling pathway**: LPS binds to LPS binding protein (LBP) and is passed to CD14, which then presents LPS to the TLR4-MD-2 receptor complex. TLR4-MD-2 receptors dimerize and recruit adaptor proteins TIRAP and MyD88, which activate NFκB. Alternatively, the TLR4-MD-2 complex is internalized, TRIF and TRAM adaptor proteins are recruited, resulting in the delayed activation of NFκB and activation of IRF3 and production of type I interferons (Maeshima and Fernandez, 2013).

Neonatal and post-weaning colibacillosis is often treated with antibiotics. Under-dosing frequently occurs if antibiotics are administered orally thus contributing to the selection of resistant bacteria. Antimicrobial resistance to several antibiotics (e.g., apramycin, neomycin, trimethoprim-sulfonamide and colistin) has been proved for ETEC strains causing post-weaning diarrhea (PWD), in addtition multidrug resistance has also been described (Luppi, 2017). *Salmonella* spp. isolated from pigs have also been proved to be resistant to multiple antibiotics, such as tetracycline, sulfonamide compounds, ampicillin,

trimethoprim-sulfamethoxazole, streptomycin, and chloramphenicol, and also multidrug resistance occurs frequently (Souto et al., 2017).

The emergence of resistant *E. coli* and *Salmonella* strains in pigs is of great concern to animal and human health as well. Resistant strains limit the type of antibiotics that can be used in veterinary medicine. Moreover since among *E. coli* and *Salmonella* spp zoonotic strains can be found which may be passed to humans via direct contact with animals or through the food chain, also the type of antibiotics that can be used for the treatment of humans gets limited (Souto et al., 2017).

2.4 Intestinal models and the IPEC-J2 cell line

Appropriate intestinal models are necessary for both the food/feed and the pharmaceutical industry in order to study new food/feed ingredients and drugs. Furthermore, models of the intestine enable to elucidate the effect of pathogenic bacteria, which is of great importance, since the mechanisms of interactions between foodborne pathogens, mammalian host and intestinal microbiota are unknown. *In vitro* cell culture systems are essential tools for biological experiments to study normal physiological and biochemical processes and with some limitations conclusions can also be made for *in vivo* circumstances (Cencic and Langerholc, 2010; Verhoeckx et al., 2015). Cell culture models provide reproducible and consistent experimental results, further contributing to the advantages of their use (Verhoeckx et al., 2015). Moreover, they correspond to the 3R concept, according to which experiments conducted on animals should be reduced, replaced and refined (Flecknell, 2002). They will never fully replace *in vivo* experiments (due to their limited complexity) but provide basics for further *in vivo* investigations. *In vitro* cell models should resemble to *in vivo* conditions in as many aspects as possible, e.g., in case of the GIT, many factors of the complex gut ecosystem should be considered. Expression of tight junction proteins is essential; without them the epithelial barrier cannot develop. Moreover, response to environmental factors is also crucial. The origin of the cell line is also pivotal since tumorogenic cells proliferate differently and their response to environmental stimuli may change. In most *in vitro* studies of the gut, tumorogenic Caco-2, T84 and HT-29 cell lines deriving from human colon have been used (Cencic and Langerholc, 2010). The most widely used non-transformed rodent lines are intestinal epithelial cell-6 (IEC-6) from rat small intestine and intestinal epithelial cell-18 (IEC-18), from rat ileum (Cencic and Langerholc,

2010; Zakrzewski et al., 2013). Four pig intestinal cell lines exist: (1) intestinal porcine epithelial cells (IPEC-1) from pig ileum and jejunum, (2) intestinal porcine epithelial cells-jejunum (IPEC-J2) from pig jejunum, (3) ileal porcine intestinal (IPI-2I) cells; (4) porcine intestinal epitheliocyte (PIE) cells (Roselli et al., 2017; Zakrzewski et al., 2013).

IPEC-J2 cell line was isolated from neonatal piglet's mid-jejunum in 1989 by Helen Berschneider at the University of North Carolina. It is unique in two aspects: (1) it is non-transformed (compared to the porcine IPI-2I cell line) and (2) derives from the small intestine (compared to colon-derived cell lines HT-29, T84, and Caco-2) (Brosnahan and Brown, 2012). Due to the similarities between the pig and human intestine (in size, weight, anatomy, physiology), the IPEC-J2 cell line is not only important for mimicking the GIT of swine but conclusions can also be made for humans (Guilloteau et al., 2010; Verhoeckx et al., 2015). It mimics human physiology more closely than rodent-derived cell lines (IEC-6 or IEC-18), which has a relevance in studying zoonotic enteric infections that also affect humans. Furthermore it enables to study porcine derived infections with high specificity (Brosnahan and Brown, 2012; Verhoeckx et al., 2015). The IPEC-J2 cell line is well-characterized, single cell monolayers are formed, consisting exclusively of epithelial cells. When cultivated on 0.4 mm pore-size Transwell1 filters cells are polarized forming an apical and a basolateral surface and junctional complexes. Tight junction proteins, including claudin-3, -4 and occludin are localized at the apicolateral membrane. Microvilli of different length and width can be found on the apical surface (Brosnahan and Brown, 2012). As it is obvious in **Figure 10.** IPEC-J2 cells develop intercellular junctions with associated tonofilaments. Their cytoplasm also contains several mitochondria, free ribosomes and moderately electron dense granules. If confluency is reached the transepithelial electrical resistant (TEER) values ─which are indicators of functional integrity─ are between 1,200–2,200 Ω and 4,900–6,500 Ω, depending on the type of surface used for cultivation (Schierack et al., 2006). Cytoskeletal proteins (indicators of epithelial origin) as well as several molecules related to immune and inflammatory responses (IL-6, IL-8, TNF-α, pBD1, pBD2) and Toll-like receptors (responsible for the recognition of PAMPs; TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR8, TLR9, TLR10) are expressed (Brosnahan and Brown, 2012).

The IPEC-J2 cell line is a widely used tool for studying interactions with pathogens (including *Salmonella enterica* and *E. coli*), the effects of probiotic applications (Klingspor et

al., 2015; Palócz et al., 2016; Tian et al., 2016) and other substances (for example plant derived subtances) (Karancsi et al., 2020; Kovács et al., 2022).

Studies demonstrated that *Salmonella enterica* serovar Typhimurium invades and replicates in IPEC-J2 cells and the growth phase of *S.* Typhimurium seems to be a factor affecting the efficiency of invasion. Bacteria in the mid-log phase showed to be more efficient than bacteria in the stationary phase of growth. Virulence factors have also been shown to influence invasion of *S.* Typhimurium into IPEC-J2 cells. *S.* Typhimurium with a defective LPS core was less efficient in invading into IPEC-J2 cells as the wild-type strain. In response to *S.* Typhimurium challenge IPEC-J2 cells have shown to produce IL-8 and TNF-α, moreover pBDs were also expressed (Brosnahan and Brown, 2012). In addition, expression of TLRs was also induced by LPS from *S.* Typhimurium (Arce et al., 2010).

Studies have revealed that EPEC form attaching and effacing lesions on IPEC-J2 cells, while the adhesion of ETEC to IPEC-J2 cells is mediated by F4 fimbriae (Brosnahan and Brown, 2012; Devriendt et al., 2010). Pathophysiological challenge with F4+ ETEC strain increased apical and basolateral IL-6 and IL-8 cytokine secretion in IPEC-J2 cells. Moreover, ETEC infection has also been shown to reduce the TEER in IPEC-J2 cells (Brosnahan and Brown, 2012). The role of toxins in the adherence of bacteria to IPEC-J2 cells was also studied, revealing that LT of ETEC is able to increase bacterial adherence to IPEC-J2 cells and shiga toxin also contributes to the adherence of EHEC O157:H7 to IPEC-J2 cells, however heat-stable enterotoxin b (STb) from ETEC associates with IPEC-J2 cells (Brosnahan and Brown, 2012).

Studies on the effect of probiotic bacteria on IPEC-J2 cells focus mainly on the adhesion properties and on the inhibition of pathogen evoked inflammatory responses. Pre- and coincubation with *E. faecium* NCIMB 10415 could prevent the decrease in TEER and the increase in IL-8 expression induced by ETEC in IPEC-J2 cells (Klingspor et al., 2015). *L. rhamnosus* counteracted enhancement in TNF-α concentration evoked by F4+ ETEC in IPEC-J2 cells (Zhang et al., 2015). *L. plantarum* ZLP001 inhibited ETEC adhesion to IPEC-J2 cells in a concentration-dependent manner (Wang et al., 2018).

Despite the fact that IPEC-J2 cell line is an *in vitro* model, it is still a relevant tool to provide information under standardized, regulated settings when investigating a limited number of factors (Verhoeckx et al., 2015).

3 Significance and aim of the study

Our study aimed at investigating the potential beneficial effects of four different probiotic species (*E. faecium, L. rhamnosus, B. licheniformis and B. subtilis*) on the prerequisites of a healthy gastrointestinal tract in an *in vitro* model using porcine intestinal epithelial (IPEC-J2) cells. Gastrointestinal infection was modelled with pathogenic bacteria *E. coli* and *S. enterica* ser. Typhimurium or LPS of S. Typhimurium or *E. coli* origin. *E. coli* and *S. enterica* ser. Typhimurium were chosen due to the fact that they are two economically important swine pathogens, they induce a wide range of gastrointestinal diseases and both of them are zoonotic.

Firstly, we aimed to determine the optimal treatment conditions for our experiments. The impact of spent culture supernatants and bacterial cells on the viability of IPEC-J2 cells was tested.

Secondly, our goal was to examine whether cell-free bacterial spent culture supernatants (SCSs) of *E. faecium*, *L. rhamnosus*, *B. licheniformis* and *B. subtilis* can achieve beneficial effects. Therefore IPEC-J2 cells were challenged with three different types of LPS, namely *S.* Typhimurium LPS, *E. coli* 111:B4 LPS and *E. coli* 127:B8 LPS and treated with the SCSs of *E. faecium*, *L. rhamnosus*, *B. licheniformis* and *B. subtilis.* The effect on IC ROS production was measured using DCFH-DA method and we determined the antioxidant capacity of the spent culture supernatants. Moreover, the potential antibacterial effect of the SCSs were tested against *E. coli* and *S*. Typhimurium field isolates of porcine origin.

The third objective of our study was to evaluate the *in vitro* probiotic potential of four probiotic candidates *E. faecium*, *L. rhamnosus*, *B. licheniformis* and *B. subtilis* against pathogen-induced damages using bacteria. Therefore, we established a co-culture modell, in which gastrointestinal infection was modelled using *E. coli* and *S*. Typhimurium of porcine origin. Different treatment conditions were applied, pre-treatment, co-treatment and post-treatment. Pre-treatment meant that the probiotic bacterium was added one hour before the addition of the pathogenic bacterium to IPEC-J2 cells, during co-treatment probiotic and pathogenic bacteria were added at the same time and in the post-treatment assay,

pathogenic bacteria were added prior to the addition of the probiotic bacteria. These different treatment regimens were included in the study in order to evaluate the probiotics' action as preventive or therapeutic agents. The effects on paracellular permeability, inflammatory response, IC ROS production, and adhesion inhibition were investigated using IPEC-J2 cell line. The objectives of the study and performed experiments are summarized in **Table 5**.

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

Table 5: Overview of the performed studies

Objective of the study	Main scientific question	Measured parameters
Determination of optimal treatment conditions	Optimal treatment time and concentration using probiotic SCSs and bacterial suspensions	Cell viability of IPEC-J2 cells (NR method)
Determination of probiotic SCSs' effect on IPEC-J2 cells	• Antioxidant effect against *S.* Typhimurium/ *E. coli* 111/ *E. coli* 127 LPS evoked oxidative stress.	IC ROS (DCFH-DA method)
	• Antimicrobial activity against *E. coli* and *S.* Typhimurium field isolates	Microdilution
Determination of probiotics' effect using IPEC-J2—bacterium co-culture modell	Effect of pre/co/post treatment with probiotics on:	
	• barrier integrity	Paracellular permeability (FD4 method)
	• proinflammatory cytokine secretion	IL-6, IL-8 (ELISA method)
	• IC ROS production	IC ROS (DCFH-DA method)
	• *E.coli*/*S.* Typhimurium adhesion inhibition.	CFU counting on *E. coli* or *S.* Typhimurium selective agar.

4 Materials and methods

4.1 Chemicals and instruments used in the study

LPS of *S*. Typhimurium, *E. coli* O111:B4 and *E. coli* O127:B8 origin; 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; paraformaldehyde; glutaraldehyde; normal donkey serum; 4′,6-diamidin-2-phenylindol (DAPI)-containing mounting medium; primary antibodies (rabbit anti-occludin) and epoxy resin were purchased from Sigma-Aldrich (Darmstadt, Germany). De Man, Rogosa, Sharpe (MRS) broth, tryptone soy broth (TSB), 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). Supplements for DMEM/F12 medium (fetal bovine serum [FBS], insulin, transferrin, selenium, epidermal growth factor [EGF] and penicillin-streptomycin) were acquired from Biocenter Ltd., Szeged, Hungary. Secondary antibodies (Donkey anti rabbit Alexa488) were purchased from Jackson (Netherland).

Microplates were supplied by VWR International (Radnor, PA, USA) and cell culture plates were purchased from Corning Inc. (Corning, NY, USA). Filters of 0.22 µm pore size were purchased from VWR.

Absorbance measurement was performed with EZ Read 400 Microplate Reader (Biochrom Ltd, Cambridge, United Kingdom) and SpectraMax iD3 (Molecular Devices, San José, CA, USA). Fluorescence measurements were carried out with Victor X2 2030 fluorometer (PerkinElmer Inc., Waltham, MA, USA) and SpectraMax iD3 (Molecular Devices).

Microscopic assessment was carried out with a Leica SP2 laser scanning confocal microscope (Münster, Germany) and a JEOL JEM-1011 transmission electron microscope (Tokyo, Japan) equipped with a Mega-View-III digital camera and a Soft-Imaging-System (SIS, Germany). Ultrathin sections for electron microscopy were made with Reichert ultramicrotome (Ultrostain II, Leica, Germany).

4.2 Light- and electron microscopy

The culture medium was aspirated off and 600 µl of fixative was added in each well. For light microscopy, the applied fixation solution contained only 4% paraformaldehyde (PFA), while for transmission electron microscopic (TEM) assessment we used a mixture of 0.1% glutaraldehyde (GA) and 4% PFA for 20 min at 4°C for the time of fixation. After 20 minutes the samples were washed twice with PBS (pH 7.4). For fluorescent immunocytochemistry cells were permeabilized with PBS containing 0.5% triton and blocked with 10% normal donkey serum in PBS 0.1% triton. We incubated the cells with primary antibodies (rabbit anti-occludin - 1:100 for occludin) for 2 hours, then washed several times with 0.1M PBS, and secondary antibody was applied for 1 hour (Donkey anti rabbit Alexa488). After washing, we coverslipped the cells in DAPI-containing mounting medium and examined the cells with a Leica SP2 laser scanning confocal microscope.

For examination with TEM, cells were postfixed with 1% OsO_4, washed with 0.1 M PB, dehydrated in ascending ethanol series, and embedded in epoxy resin. 60 nm thin sections were cut on a Reichert ultramicrotome, mounted on 300 mesh copper slot grids, contrasted with lead citrate, and examined with a JEM-1011 transmission electron microscope equipped with a Mega-View-III digital camera and a Soft-Imaging-System.

4.3 Studies on IPEC-J2 cells using LPS and SCSs

4.3.1 Bacterial culture and spent culture supernatant

The applied bacterial strains were *Enterococcus faecium* NCIMB 10415, *Lactobacillus rhamnosus* DSM7133, *Bacillus licheniformis* DSM 5749 and *Bacillus subtilis* DSM 5750 isolated from swine. The bacteria were acquired from our research partner Dr. Zoltán Kerényi (Hungarian Dairy Experimental Institute Ltd., Mosonmagyaróvár, Hungary). *Enterococcus faecium* NCIMB 10415 and *Lactobacillus rhamnosus* DSM7133 were grown in MRS broth, while *Bacillus licheniformis* DSM 5749 and *Bacillus subtilis* DSM 5750 were grown in TSB broth. Inoculation was accomplished with a stationary culture of a probiotic strain (1% inoculum). The bacteria were placed into the incubator and were grown for 24 hours at 37°C and sub-cultured twice prior to experiments. Spent culture supernatants were prepared by centrifugation of the bacterial suspension at 3000g at 5°C for 10 minutes. The pH values of SCSs were set to 7 using NaOH (1M). SCSs were then passed through a sterile 0.22 µm pore size filter unit.

Different concentrations of SCSs (3%, 6%, 12%, 24%) were prepared. For dilution, DMEM/F12 medium without supplementation was used (plain DMEM/F12).

4.3.2 Cell line and culture conditions

The IPEC-J2 epithelial cell line was a kind gift from Dr. Jody Gookin's Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, USA. The cells were grown and maintained in a complete medium consisting of 10 ml of DMEM/F12 in a 1:1 ratio. This was supplemented with 5% fetal bovine serum (FBS), 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 5 ng/ml epidermal growth factor (EGF) and 1% penicillin-streptomycin. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 (Schierack et al., 2006). Cells with passage numbers 49–52 were used for our experiments. For cell viability determination with the Neutral Red Uptake (NRU) method, cells were cultured onto a 96-well plate. For intracellular ROS determination, cells were grown on 6-well culture plates. In each case, cells were cultured until confluency was reached.

4.3.3 Assessment of cell viability

SCSs of *E. faecium*, *L. rhamnosus*, *B. licheniformis* and *B. subtilis* were prepared in different concentrations (3%, 6%, 12%, 24%) as described in section 4.2.1. The influence of different SCS concentrations and different incubation periods on the viability of IPEC-J2 cells was tested with Neutral Red method based on the description of Repetto et al (Repetto et al., 2008). IPEC-J2 cells were seeded onto a 96-well plate and incubated with SCS of different concentrations for 1, 2, 4 and 24 h, respectively. Treatment with plain medium for 1 h was used as control in the experiment. Viability of IPEC-J2 cells was measured after 24 h. Absorbance was measured with Biochrom EZ Read 400 Microplate Reader (at a wavelength of 540 nm). The experiment was performed with 6 replicates per treatment group.

The influence of LPS of *S*. Typhimurium, *E. coli* O111:B4 and *E. coli* O127:B8 origin applied in different concentrations was tested by our research group previously (Karancsi et al., 2020).

4.3.4 Assessment of IC ROS levels

To evaluate the effect of SCSs of *E. faecium, L. rhamnosus*, *B. subtilis and B. licheniformis* on the intracellular ROS production of IPEC-J2 cells, the DCFH-DA method was used. The DCFH-DA dye is oxidized to the highly fluorescent form dichloro-fluorescein (DCF) by

intracellular ROS (Wang and Joseph, 1999). Oxidative stress was evoked by LPS of *S.* Typhimurium, and LPS of *E. coli* 111 or *E. coli* 127 at 10 µg/ml concentrations in IPEC-J2 cells. In order to determine their potential antioxidant activity, SCSs of *E. faecium, L. rhamnosus*, *B. subtilis or B. licheniformis* respectively were added to the cells together with either type of LPS and incubated for 1 hour. Moreover, the effects of SCSs alone on the amount of intracellular reactive oxygen species were tested. Cells treated with plain medium were used as a negative control. After the treatment, solutions were discarded, and plain medium was added to the cells. For the detection, cells were washed with PBS after 24 h, and DCFH-DA reagent (40 mM) was added to them. After one hour, the reagent was removed, cells were washed twice with phenol-free plain DMEM/F12 (2 ml) and were scratched (with a cell scraper) and lysed (by pipetting up and down). The lysed cells were then pipetted into an Eppendorf tube and centrifuged for 10 min at 4 °C at 4500 rpm. Then, 100 µl of supernatant from each sample was added to a 96-well plate. A Spectramax iD3 instrument was used to measure the fluorescence at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. The experiment was performed with 6 replicates per treatment group.

4.3.5 Assessment of antibacterial activity

The antibacterial activities of SCSs of *E. faecium, L. rhamnosus*, *B. subtilis and B. licheniformis* were determined against 7 *E. coli* and 7 *S.* Typhimurium strains isolated from the GIT or mesenteric lymph nodes of pigs using the broth microdilution methods. Antibacterial potential of the SCSs of probiotic strains was also tested against *E. coli* and *S.* Tyhimurium strains used in our co-culture experiments. Bacterial isolates were stored at -80 °C in Mueller-Hinton liquid broth (MH) supplemented with 20% sterile glycerol or on Microbank beads at −80 °C before the experiments. Eighteen - twenty-four hours prior to the determination of antibacterial activity of probiotic SCSs, the 8 *E. coli* and 8 *S. Typhimurium* strains were propagated in TSB broth at 37 °C. After 18-24 hours cultures of pathogenic bacterial strains were centrifuged for 10 min at 3000 g and 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 ml 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 agar plates for control CFU counting. Spent culture supernatants of probiotic bacteria were prepared as described in section 4.3.1. A series of two-fold

dilutions were prepared from the SCSs with TSB or MRS (MRS for *E. faecium* and *L. rhamnosus* and TSB for *B. subtilis* and *B. licheniformis*) broth on 96-well microtiter plates. SCSs were inoculated with pathogenic bacteria to reach a final concentration of 10^5 CFU/ml and incubated for 24 hours at 37°C. Evaluation of turbidity was performed with unaided eye.

4.4 Studies on IPEC-J2 cells — bacterium co-culture

4.4.1 Bacterial culture

S. Typhimurium and *E. coli* originated from gastrointestinal infections in pigs and were isolates from clinical samples in Hungary (obtained from the Department of Microbiology and Infectious Diseases, University of Veterinary Medicine Budapest). Identification was verified by the Department of Microbiology and Infectious Diseases. *E. coli* expresses F4 fimbriae and produces both heat-stable (STa and STb) and heat-labile (LT) enterotoxins. *Enterococcus faecium* NCIMB 10415, *Lactobacillus rhamnosus, Bacillus licheniformis* and *Bacillus subtilis* were acquired from the Hungarian Dairy Research Institute Ltd. and were also swine intestine isolates. All six bacterial strains were preserved on Microbank beads at −80 °C.

Cell suspensions were prepared by suspending microbeads in plain DMEM/F12 (without supplementation). Incubation was performed for 18–24 h at 37 °C in the presence of 5% CO_2/95% air atmosphere in order to mimic culture conditions of IPEC-J2 cells. In previous experiments, *E. faecium, L rhamnosus, B. licheniformis*, *B. subtilis*, *E. coli*, and *S.* Typhimurium were shown to grow to 10^8 CFU/ml under these circumstances. For cell viability measurements, *E. faecium* suspensions of 10^8, 10^6, 10^4 CFU/ml and *L. rhamnosus*, *B. licheniformis* and *B. subtilis* suspensions of 10^8 CFU/ml were used. For the determination of intracellular ROS, paracellular permeability, IL-6 and IL-8 the applied concentration of *L. rhamnosus*, *Bacillus licheniformis* and *Bacillus subtilis* suspensions was 10^8 CFU/ml and the applied concentration of *E. faecium* suspension was 10^7 or 10^8 CFU/ml. *E. faecium* suspension of 10^7 CFU/ml was diluted from the stock solutions (*E. faecium* 10^8 CFU/ml) and *E. coli* and *S.* Typhimurium suspensions were diluted from the stock solutions (*E. coli* 10^8 CFU/ml, *S Typhimurium* 10^8 CFU/ml) to 10^6 CFU/ml using plain DMEM/F12 medium (free of antibiotics) as a dilution reagent.

4.4.2 Cell line and culture conditions

IPEC-J2 cell were cultured as described in section 4.3.2 with the following supplementation. For IL-6 and IL-8 determination, cells were grown on 6-well polystyrene culture plates (tissue culture treated, without coating). For adhesion inhibition, assays cells were seeded onto 24-well polystyrene cell culture plates (tissue culture treated, without coating). For the measurement of paracellular permeability, cells were cultured on 12-well polyester membrane cell culture inserts (tissue culture treated, without coating, pore size: 0.4 µm). In each case, cells were cultured until confluency was reached.

In order to remove the remaining antibiotics before starting the treatment of IPEC-J2 cells with the different treatment solutions (described in Section 4.4.1) IPEC-J2 cells were washed twice with phosphate buffered saline (PBS) then DMEM/F12 without antibiotics was added to each well, and cells were incubated for 30 min at 37°C.

4.4.3 Assessment of cell viability

The influence of different *E. faecium, L. rhamnosus, B. licheniformis and B. subtilis* bacterial suspension concentrations and different incubation periods on the viability of IPEC-J2 cells was tested with the neutral red uptake method based on the description of Repetto et al. (Repetto et al., 2008). *E. faecium, L. rhamnosus, B. licheniformis and B. subtilis* suspensions of different concentrations were prepared as described in section 4.4.1. IPEC-J2 cells were seeded onto a 96-well plate and incubated with *E. faecium, L. rhamnosus, B. licheniformis and B. subtilis* suspensions of different concentrations (10^8, 10^6, 10^4 CFU/ml) for 1, 2, 4 and 24 h, respectively (37 °C, 5% CO_2). Treatment with plain medium for 1 h was used as a control in the experiment. The viability of IPEC-J2 cells was measured after 24 h. Absorbance was detected with a Spectramax iD3 instrument at a wavelength of 540 nm. The experiment was performed with 6 replicates per treatment group.

The influence of *E. coli* and *S.* Typhimurium suspensions applied in different concentrations and for different incubation periods was tested by our research group previously (Kovács et al., 2022).

4.4.4 Experimental setup

For our DCFH-DA, ELISA, FD4, adhesion assay experiments, IPEC-J2 cells were incubated for 1 h with the pathogen strain *E. coli* or *S.* Typhimurium, respectively. Control cells received

plain DMEM/F12 medium. As a positive control, IPEC-J2 cells were mono-incubated with only *E. coli* (10^6 CFU/mL) or *S.* Typhimurium (10^6 CFU/mL), respectively. For pre-treatment assays, cells were pre-incubated with *E. faecium*, *L. rhamnosus*, *B. licheniformis* or *B. subtilis* respectively for 1 h before the addition of the pathogen strain. For co-treatment experiments, the pathogen strain (*E. coli* or *S.* Typhimurium) and *E. faecium*, *L. rhamnosus*, *B. licheniformis* or *B. subtilis* respectively were added at the same time to IPEC-J2 cells. In our post-treatment assay, IPEC-J2 cells were incubated with *E. faecium*, *L. rhamnosus*, *B. licheniformis* or *B. subtilis* respectively for 1 h after the treatment with the pathogen strains (*E. coli* or *S*. Typhimurium). Bacterial infections were performed with *E. coli* or *S.* Typhimurium at a concentration of 10^6 CFU/mL. The applied tolerable pathogen concentration was based on our previous investigations (Kovács et al., 2022). *E. faecium* suspensions were applied either in a 10^7 or 10^8 CFU/ ml concentration and *L. rhamnosus*, *B. licheniformis* or *B. subtilis* suspensions were applied in 10^8 CFU/ ml concentration based on our cell viability experimental results. IPEC-J2 cells were also mono-incubated with *E. faecium* (both 10^8 and 10^7 CFU/ml) and *L. rhamnosus*, *B. licheniformis* or *B. subtilis* 10^8 CFU/ml) respectively. If further incubation was needed after the treatments, cells were washed with PBS and DMEM/F12 supplemented with antibiotics. Moreover, 1% penicillin-streptomycin was added to prevent the growth of bacteria. The applied treatment solutions in our experiments are summarized in **Table 6** and **Figure 9** shows the timeline of our experimental setup.

Table 6: Applied treatment solutions in our experiments

Type of treatment	Applied probiotic strain and concentration	Applied pathogen strain and concentration
Pre-addition *E. faecium* + *S.* Typhimurium	*E. faecium* 10^7 or 10^8 CFU/ml prior to infection	*S.* Typhimurium 10^6 CFU/ml
Co-addition *E. faecium* + *S.* Typhimurium	*E. faecium* 10^7 or 10^8 CFU/ml at the same time with infection	*S.* Typhimurium 10^6 CFU/ml
Post-addition *E. faecium* + *S.* Typhimurium	*E. faecium* 10^7 or 10^8 CFU/ml after infection	*S.* Typhimurium 10^6 CFU/ml
Pre- addition *E. faecium* + *E. coli*	*E. faecium* 10^7 or 10^8 CFU/ml prior to infection	*E. coli* 10^6 CFU/ml
Co-addition *E. faecium* + *E. coli*	*E. faecium* 10^7 or 10^8 CFU/ml at the same time with infection	*E. coli* 10^6 CFU/ml
Post-addition *E. faecium* + *E. coli*	*E. faecium* 10^7 or 10^8 CFU/ml after infection	*E. coli* 10^6 CFU/ml
E. faecium 10^7 (mono-incubation)	*E. faecium* 10^7 CFU/ml	-
E. faecium 10^8 (mono-incubation)	*E. faecium* 10^8 CFU/ml	-
Pre-addition *L. rhamnosus* + *S.* Typhimurium	*L. rhamnosus* 10^8 CFU/ml prior to infection	*S.* Typhimurium 10^6 CFU/ml
Co-addition *L. rhamnosus* + *S.* Typhimurium	*L. rhamnosus* 10^8 CFU/ml at the same time with infection	*S.* Typhimurium 10^6 CFU/ml
Post-addition *L. rhamnosus* + *S.* Typhimurium	*L. rhamnosus* 10^8 CFU/ml after infection	*S.* Typhimurium 10^6 CFU/ml
Pre- addition *L. rhamnosus* + *E. coli*	*L. rhamnosus* 10^8 CFU/ml prior to infection	*E. coli* 10^6 CFU/ml
Co-addition *L. rhamnosus* + *E. coli*	*L. rhamnosus* 10^8 CFU/ml at the same time with infection	*E. coli* 10^6 CFU/ml
Post-addition *L. rhamnosus* + *E. coli*	*L. rhamnosus* 10^8 CFU/ml after infection	*E. coli* 10^6 CFU/ml
L. rhamnosus (mono-incubation)	*L. rhamnosus* 10^8 CFU/ml	
Pre-addition *B. licheniformis* + *S.* Typhimurium	*B. licheniformis* 10^8 CFU/ml prior to infection	*S.* Typhimurium 10^6 CFU/ml
Co-addition *B. licheniformis* + *S.* Typhimurium	*B. licheniformis* 10^8 CFU/ml at the same time with infection	*S.* Typhimurium 10^6 CFU/ml
Post-addition *B. licheniformis* + *S.* Typhimurium	*B. licheniformis* 10^8 CFU/ml after infection	*S.* Typhimurium 10^6 CFU/ml
Pre- addition *B. licheniformis* + *E. coli*	*B. licheniformis* 10^8 CFU/ml prior to infection	*E. coli* 10^6 CFU/ml
Co-addition *B. licheniformis* + *E. coli*	*B. licheniformis* 10^8 CFU/ml at the same time with infection	*E. coli* 10^6 CFU/ml
Post-addition *B. licheniformis* + *E. coli*	*B. licheniformis* 10^8 CFU/ml after infection	*E. coli* 10^6 CFU/ml
B. licheniformis (mono-incubation)	*B. licheniformis* 10^8 CFU/ml	
Pre-addition *B. subtilis* + *S.* Typhimurium	*B. subtilis* 10^8 CFU/ml prior to infection	*S.* Typhimurium 10^6 CFU/ml
Co-addition *B. subtilis* + *S.* Typhimurium	*B. subtilis* 10^8 CFU/ml at the same time with infection	*S.* Typhimurium 10^6 CFU/ml
Post-addition *B. subtilis* + *S.* Typhimurium	*B. subtilis* 10^8 CFU/ml after infection	*S.* Typhimurium 10^6 CFU/ml
Pre- addition *B. subtilis* + *E. coli*	*B. subtilis* 10^8 CFU/ml prior to infection	*E. coli* 10^6 CFU/ml
Co-addition *B. subtilis* + *E. coli*	*B. subtilis* 10^8 CFU/ml at the same time with infection	*E. coli* 10^6 CFU/ml
Post-addition *B. subtilis* + *E. coli*	*B. subtilis* 10^8 CFU/ml after infection	*E. coli* 10^6 CFU/ml
B. subtilis (mono-incubation)	*B. subtilis* 10^8 CFU/ml	
S. Typhimurium (mono-incubation)	-	*S.* Typhimurium 10^6 CFU/ml
E. coli (mono-incubation)	-	*E. coli* 10^6 CFU/ml

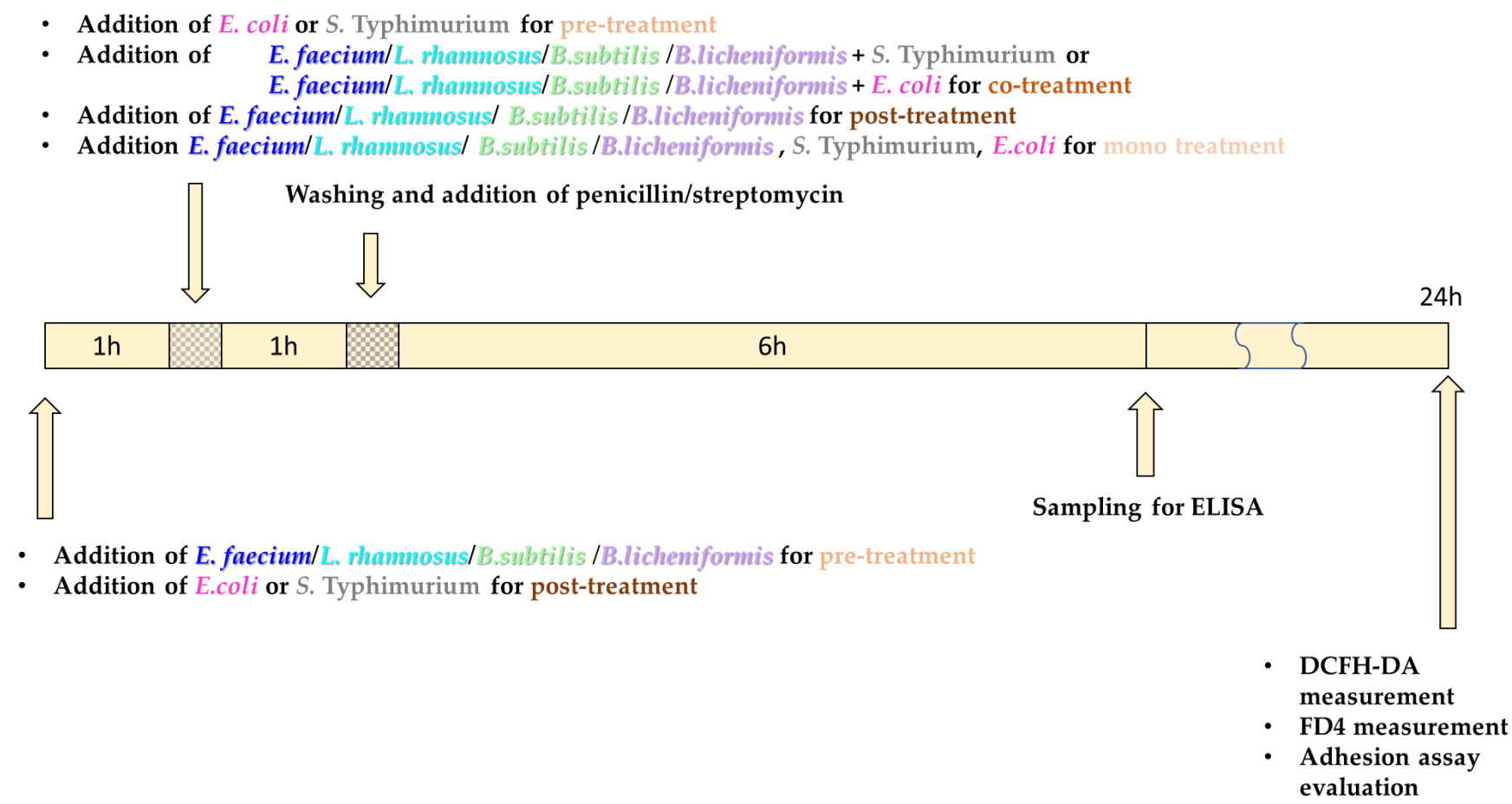


Figure 9: Experimental setup

4.4.5 Assessment of IC ROS levels

To evaluate the effect of *E. faecium, L. rhamnosus*, *B. subtilis and B. licheniformis* on the intracellular ROS production of IPEC-J2 cells, the DCFH-DA method was used. In IPEC-J2 cells, inflammation was evoked by *E. coli* or *S.* Typhimurium, respectively. *E. faecium, L. rhamnosus*, *B. subtilis or B. licheniformis* respectively was added as pre-, co-, or post-treatment. Moreover, the effect of *E. faecium, L. rhamnosus, B. subtilis* or *B. licheniformis* respectively alone on the amount of intracellular reactive oxygen species was tested. Cells treated with plain medium were used as a negative control and cells treated with either *E. coli* or *S.* Typhimurium served as positive controls. After the treatment, the treatment solutions were discarded and plain medium containing 1% penicillin-streptomycin was added. Measurement was carried out as described in section 4.3.4. The experiment was performed with 6 replicates per treatment group.

4.4.6 Assessment of IL-6 and IL-8 levels

For the enzyme-linked immunosorbent assay (ELISA) experiments cells were seeded onto six-well culture plates and pre-, co-, and post-treatments were performed as described in the experimental setup (4.4.4) section. After the removal of treatment solutions, IPEC-J2 cells were incubated with cell culture medium and cell supernatants were collected after 6 hours. IL-6 and IL-8 secretion was determined by porcine-specific ELISA Kits (Sigma-Aldrich, Darmstadt, Germany) according to the manufacturer's instructions. The experiment was performed with 6 replicates per treatment group.

4.4.7 Assessment of barrier integrity

The effect of *E. faecium* / *L. rhamnosus* / *B. subtilis* / *B. licheniformis* and *E. coli* or *S.* Typhimurium on the paracellular permeability of IPEC-J2 cells was evaluated with fluorescein isothiocyanate–dextran (FD4) tracer dye. Prior to treatments, transepithelial electrical resistance (TEER) values of IPEC-J2 cells were measured to check the development of a differentiated, confluent monolayer. Mono-, pre-, co-, and post-treatments were performed as described in the experimental setup (4.4.4) section. After treatment, the cells were washed with PBS, and FD4 (dissolved in fenol free DMEM/F12 medium) at a final concentration of 0.25 mg/ml was added to the apical layer cells. To the basolateral chamber, phenol-free DMEM/F12 medium was added. Cells were incubated at 37 °C (5% CO_2). Samples of 100 µl were taken from the basolateral chamber after 24 h. The fluorescent signal was measured with a Spectramax iD3 instrument using 485 nm excitation and 535 nm emission wavelength. The experiment was performed with 6 replicates per treatment group.

4.4.8 Assessment of adhesion inhibition

In order to evaluate the inhibitory effect of *E. faecium*, *L. rhamnosus*, *B. subtilis* or *B. licheniformis* respectively on *E. coli* or *S.* Typhimurium adhesion to IPEC-J2 cells, *E. faecium*, *L. rhamnosus, B. subtilis* or *B. licheniformis* respectively was added as pre-, co-, or post-treatment. As control, cells treated with only *E. coli* or *S.* Typhimurium were used. IPEC-J2 cells were incubated for 1 h and then washed to remove unbound bacteria. The lysis of cells was performed with 500 µl 0.1% Triton X-100. Viable *E. coli* and *S.* Typhimurium counts were determined by serial dilution and plating on ChromoBio Coliform (for *E. coli*) or ChromoBio *Salmonella* Plus Base (for *S.* Typhimurium) agar. Adhesion was calculated as a control percentage. Adhering *E. coli* and *S.* Typhimurium were normalized to the control. The experiment was performed with 4 replicates per treatment group.

4.5 Statistical analysis

In order to make data comparable to data from other measurements control % was used. Mean concentration value of the controll cells was considered as 100% and values of different treatment groups were compared to this. In the case of all measured parameters mean values and standard deviation (SD) were calculated in all treatment groups. Normal distribution and homogeneity of variance of the data was checked using R 4.0.4 software (R Foundation for Statistical Computing, Vienna, Austria) package. Both criteria were met in all cases, therefore differences among the mean values of different experimental groups were evaluated with one-way ANOVA and Tukey post-hoc test using R 4.0.4 software. The results were interpreted as significant if the *p*-value was lower than 0.05. For the assessment of cell viability, IC ROS, IL-6/IL-8 and barrier integrity 6 replicates per treatment group, while for the assessment of adhesion inhibition 4 replicates per treatment group were used.

5 Results

5.1 Results of microscopic assessment

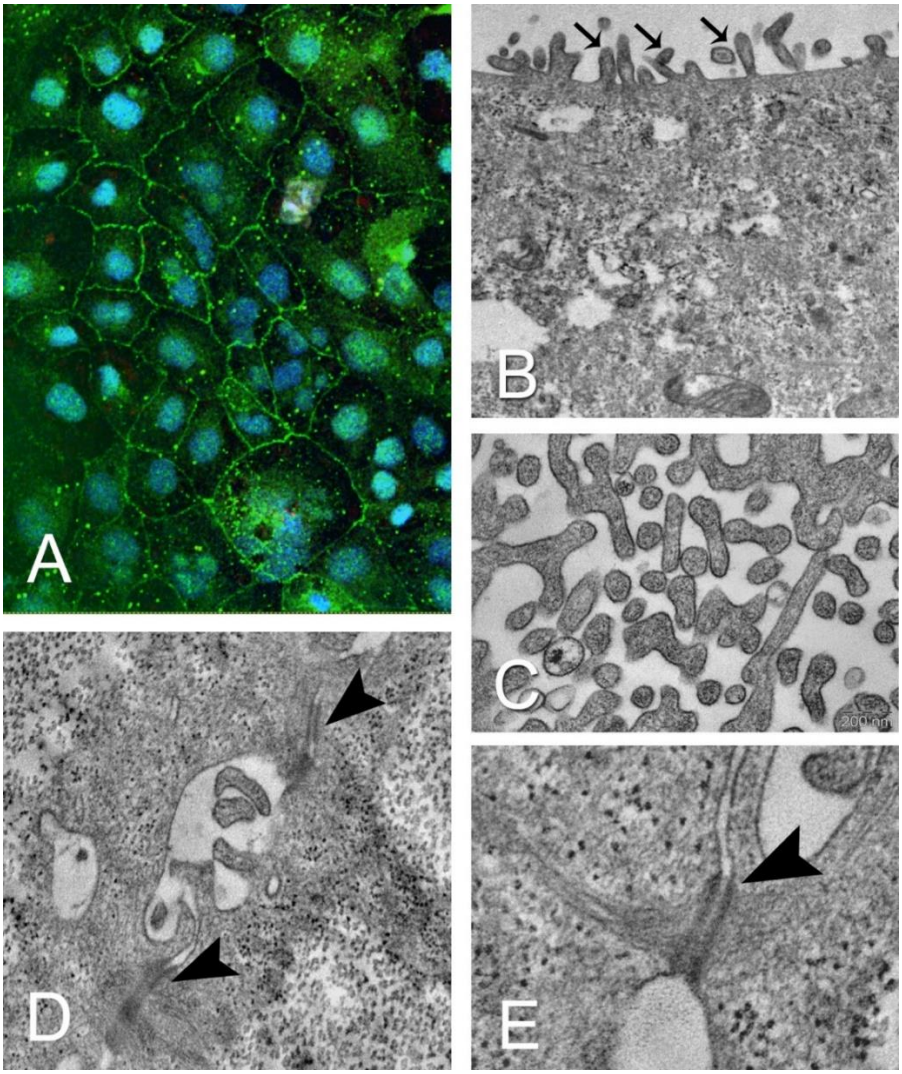


Figure 10: Light- and electron microscopic structure of the IPEC-J2 cells *in vitro*. A. Immunofluorescence confocal microscopic visualization of the IPEC-J2 cell layer show the typical *in vitro* epithelial cell morphology, as revealed by the presence of the membrane-bound protein, occludin. (blue: DAPI-nuclear DNA, green occludin). Note the intense fluorescent label at the cell membrane, suggesting the abundant presence of occluding junctions between cells. **B, C.** Transmission electron microscopy showed, that IPEC-J2 cells develop microvilli on their surface (arrows), with obviously recognizable microfilaments in the core, likely representing actin. **D,E.** Presence of transmembrane junctions between neighboring cells. Black arrowheads point to putative desmosomes with typical laminar organization, while the white arrowhead points to an occluding junction. Note the tonofilaments that are anchored at the desmosomes. Scale bars: A: 25 µm, B,D: 500 nm , C,E: 200 nm

At the beginning of our experiments, we wanted to verify the microscopic and ultrastructural architecture of *in vitro* IPEC-J2 cells in our cell culture. Therefore, we first examined the cells at the light microscopic level. Routine immunofluorescence labeling was used to detect proteins located at the cell membrane junctional complexes, and then the overall morphology of the cells was validated by confocal laser scanning microscopy (**Figure 10 A**). We could confirm, that our experimental cell culture contained cells with the expected and required epithelial cell morphology - also reported by other research groups (Schierack et al., 2006). Further examination of these cells by transmission electron microscopy confirmed that these IPEC-J2 epithelial cells had microvilli on their cell surface and the presence of characteristic cell-junctional structures between cells (i.e.: tight junctions and desmosomes) was validated (**Figure 10 B-E**).

5.2 Results with SCSs and LPS

5.2.1 Assessment of cell viability

In order to determine the effect of *E. faecium, L. rhamnosus, B. licheniformis and B. subtilis* spent culture supernatants on the viability of IPEC-J2 cells, the Neutral Red Uptake method was used.

5.2.1.1 Results with Enterococcus faecium NCIMB 10415 SCS

In the case of treatment for 1 hour measured absorbance values, which are in correlations with the number of viable cells, showed a significant difference between cells treated with SCS and untreated control cells ($p<0.001$ in all cases except Ef 24%: $p<0.01$). Moreover, groups treated with 3%, 6% and 12% SCS were different from the control at a higher significance level ($p<0.001$). Treatment of 2 hours also showed significant increase in cell viability in case of each SCS concentration, though at different significance levels ($p<0.001$ for Ef 3%, $p<0.01$ for Ef 6% and Ef 12%, $p<0.05$ for Ef 24%). Four hours treatment resulted in significant elevation in the viability of IPEC-J2 cells ($p<0.001$ for Ef 3%, Ef 6% and Ef 12%). The absorbance values of samples treated with 3%, 6% and 12% SCS were more than double of control samples. Contrarily, treatment with 24% SCS for 4 hours caused no significant alteration in the number of living cells compared to the control. While 3% and 6% treatment for 24 hours caused a significant elevation in the number of living enterocytes ($p<0.001$), the effect of 12% SCS and 24% SCS was not significant. Spent culture

supernatants did not show any decrease in the viability of IPEC-J2 cells in any of the applied concentrations and treatment times (**Figure 11**).

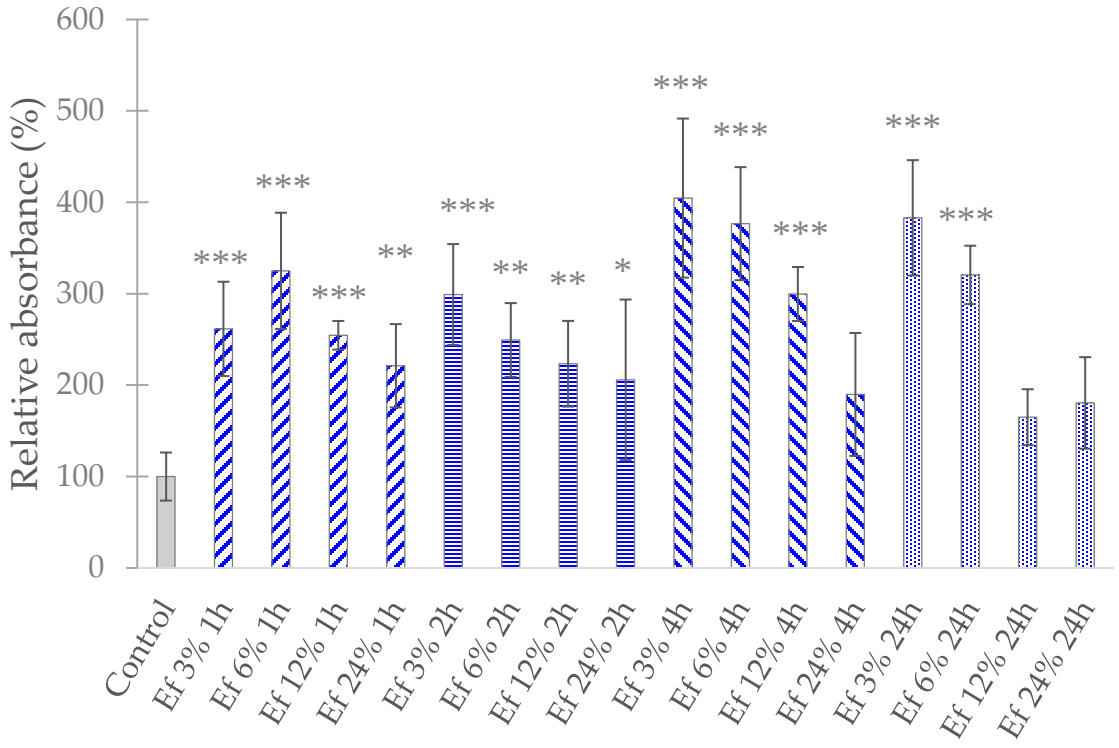


Figure 11. Viability of IPEC-J2 cells after treatment with *E. faecium* (Ef) NCIMB 10415 supernatant. Control: plain cell culture medium treatment for 1 h; **Ef 3% 1h**: 3% SCS treatment for 1h; **Ef 6% 1h**: 6% SCS treatment for 1h; **Ef 12% 1h**: 12% SCS treatment for 1h; **Ef 24% 1h**: 24% SCS treatment for 1h; **Ef 3% 2h**: 3% SCS treatment for 2h; **Ef 6% 2h**: 6% SCS treatment for 2h; **Ef 12% 2h**: 12% SCS treatment for 2h; **Ef 24% 2h**: 24% SCS treatment for 2h; **Ef 3% 4h**: 3% SCS treatment for 4h; **Ef 6% 4h**: 6% SCS treatment for 4h; **Ef 12% 4h**: 12% SCS treatment for 4h; **Ef 24% 4h:** 24% SCS treatment for 4h; **Ef 3% 24h**: 3% SCS treatment for 24h; **Ef 6% 24h**: 6% SCS treatment for 24h; **Ef 12% 24h**: 12% SCS treatment for 24h; **Ef 24% 24h**: 24% SCS treatment for 24h. 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: *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$; in grey: compared with the control.

5.2.1.2 Results with Lactobacillus rhamnosus DSM 7133 SCS

Treatement with 3%, 6%, 12% SCSs for 1 hour significantly increased cell viability compared to untreated control cells (p<0.001 for Lrh 3% and Lrh 6%; p<0.05 for Lrh 12%). Moreover, IPEC-J2 cells treated with 3%, 6% SCS were different from the control at a higher significance level (p<0.001). When IPEC-J2 cells were treated for 2 hours, treatment with

3% and 6% SCSs significantly increased the viability of IPEC-J2 cells compared to untreated control cells ($p<0.001$). However, treatment for 2 hours using 12% and 24% SCSs did not cause any significant change in cell viability. Similarly, when treating cells for 4 hours 3% and 6% SCS significantly increased cell viability as compared to the untreated control cells ($p<0.001$), while 12% and 24% did not cause any change. In the case of treatment for 24 hours only 3% SCS increased significantly the cell viability compared to untreated control cells ($p<0.001$), while the other applied SCS concentrations (6%, 12% and 24%) did not alter the cell viability (**Figure 12**).

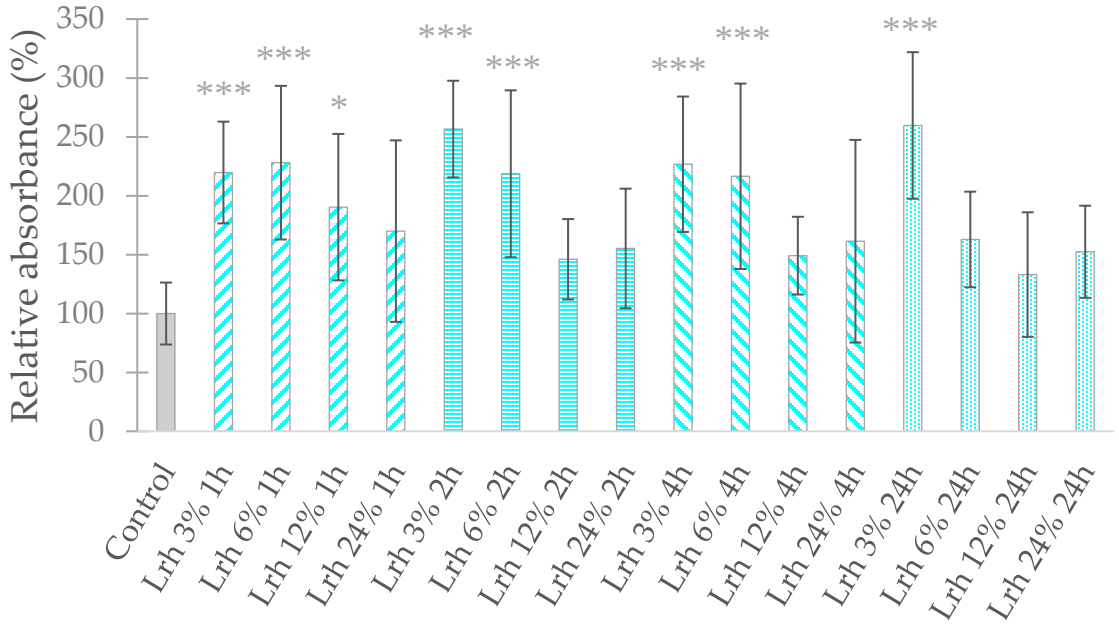


Figure 12. Viability of IPEC-J2 cells after treatment with *L. rhamnosus* (Lrh) DSM 7133 supernatant. Control: plain cell culture medium treatment for 1 h; **Lrh 3% 1h**: 3% SCS treatment for 1h; **Lrh 6% 1h**: 6% SCS treatment for 1h; **Lrh 12% 1h**: 12% SCS treatment for 1h; **Lrh 24% 1h**: 24% SCS treatment for 1h; **Lrh 3% 2h**: 3% SCS treatment for 2h; **Lrh 6% 2h**: 6% SCS treatment for 2h; **Lrh 12% 2h**: 12% SCS treatment for 2h; **Lrh 24% 2h**: 24% SCS treatment for 2h; **Lrh 3% 4h**: 3% SCS treatment for 4h; **Lrh 6% 4h**— 6% SCS treatment for 4h; **Lrh 12%** 4h: 12% SCS treatment for 4h; **Lrh 24% 4h**: 24% SCS treatment for 4h; **Lrh 3% 24h**: 3% SCS treatment for 24h; **Lrh f 6% 24h**: 6% SCS treatment for 24h; **Lrh 12% 24h**: 12% SCS treatment for 24h; **Lrh 24% 24h**: 24% SCS treatment for 24h. 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: * $p < 0.05$; *** $p < 0.001$; in grey: compared with the control.

5.2.1.3 Results with Bacillus licheniformis DSM 5749 SCS

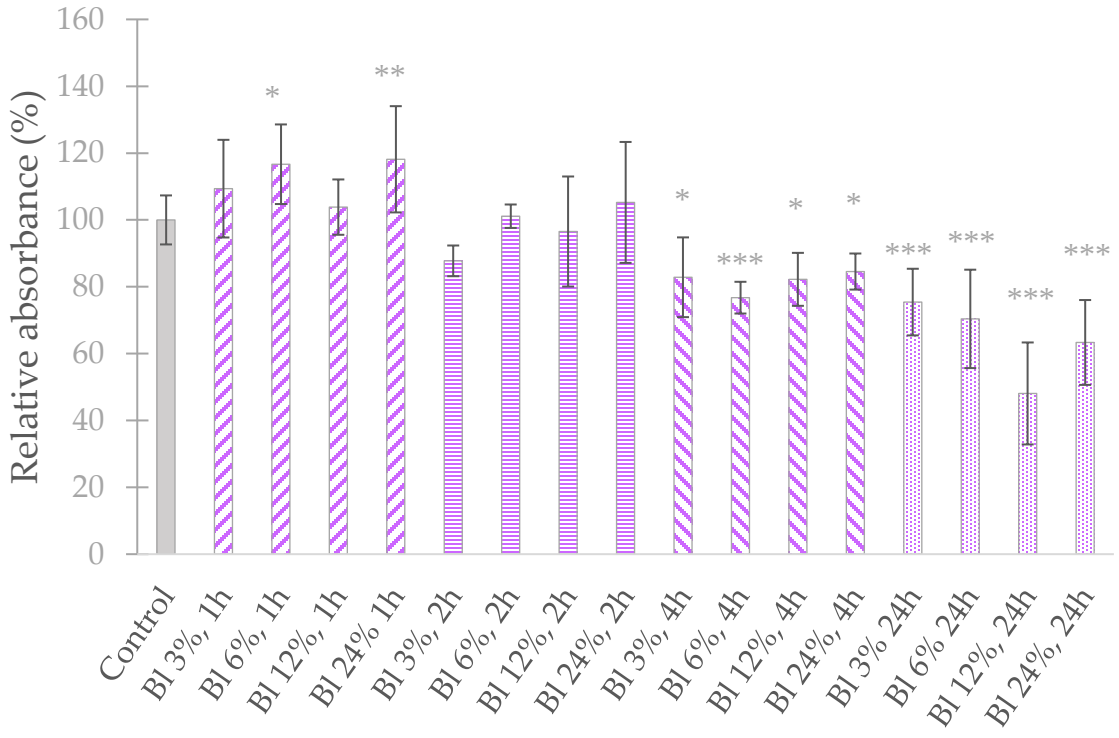


Figure 13. Viability of IPEC-J2 cells after treatment with *B. licheniformis* (Bl) DSM 5749 supernatant. Control: plain cell culture medium treatment for 1 h; **Bl 3% 1h**: 3% SCS treatment for 1h; **Bl 6% 1h**: 6% SCS treatment for 1h; **Bl 12% 1h**: 12% SCS treatment for 1h; **Bl 24% 1h**: 24% SCS treatment for 1h; **Bl 3% 2h**: 3% SCS treatment for 2h; **Bl 6% 2h**: 6% SCS treatment for 2h; **Bl 12% 2h**: 12% SCS treatment for 2h; **Bl 24% 2h**: 24% SCS treatment for 2h; **Bl 3% 4h**: 3% SCS treatment for 4h; **Bl 6% 4h**— 6% SCS treatment for 4h; **Bl 12%** 4h: 12% SCS treatment for 4h; **Bl 24% 4h**— 24% SCS treatment for 4h; **Bl 3% 24h**: 3% SCS treatment for 24h; **Bl f 6% 24h**: 6% SCS treatment for 24h; **Bl 12% 24h**: 12% SCS treatment for 24h; **Bl 24% 24h**: 24% SCS treatment for 24h. 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: *p < 0.05; *p ≤<0.01; *** p < 0.001; in grey: compared with the control.

When IPEC-J2 cells were treated for 1 h, 6% and 24 % SCSs significantly increased the cell viability as compared to untreated control cells (p<0.05 for Bl 6%; p<0.01 for Bl 24%). However, treatment with 3% and 12 % SCSs did not cause any alteration in cell viability. When IPEC-J2 cells were treated for 2 hours none of the applied SCSs concentrations caused any change in cell viability compared to the untreated control cells. When IPEC-J2 cells were treated for 4 and for 24 hours each of the applied SCS concentrations resulted in a significant decrease in cell viability compared to control cells, though at different

significance levels (p<0.001 for Bl 6% for 4 h and Bl 3%, B l6%, Bl 12%, Bl 24% for 24h; p<0.05 for Bl 3%, Bl 12%, Bl 24% for 24 h) (**Figure 13**).

5.2.1.4 Results with Bacillus subtilis DSM 5750 SCS

Treatment of IPEC-J2 cells with 3%, 6%, 12% and 24% SCSs for 1, 2 and 4 hours did not cause any significant change in cell viability compared to the untreated contorl cells. Treating IPEC-J2 cells with 3% SCS for 24 hours did not result in an alteration of cell viability compared to the control, however the treatment with 6%, 12% and 24% SCS significantly decreased the cell viability compared to the control (p<0.001) (**Figure 14**).

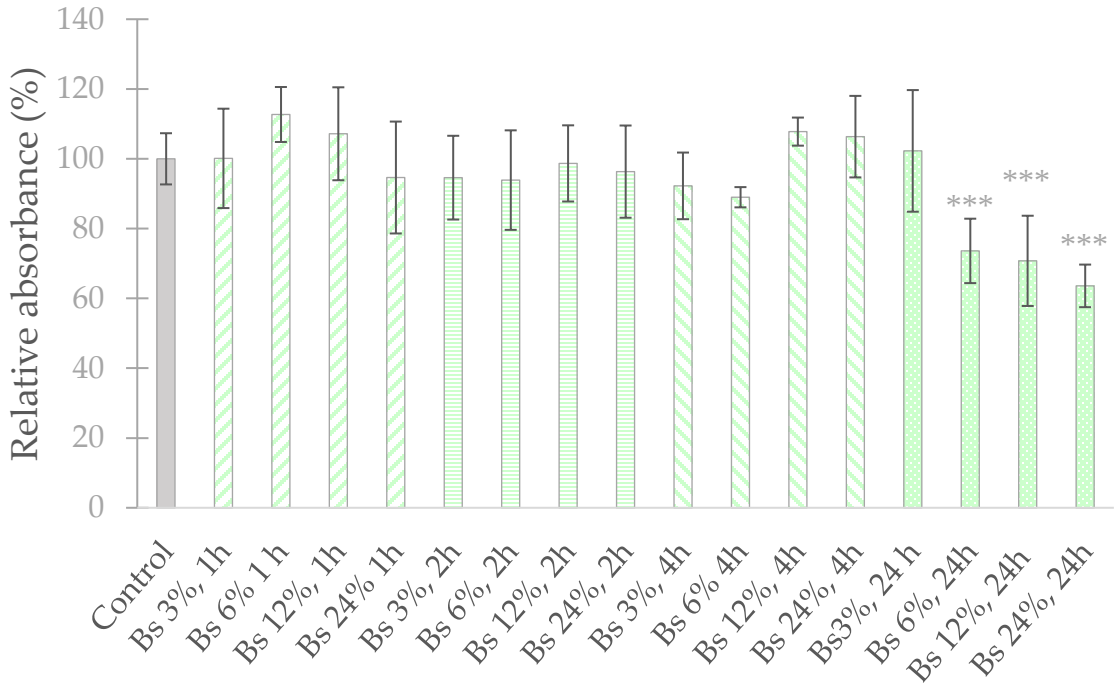


Figure 14. Viability of IPEC-J2 cells after treatment with *B. subtilis* (Bs) DSM 5750 supernatant. Control: plain cell culture medium treatment for 1 h; **Bs 3% 1h**: 3% SCS treatment for 1h; **Bs 6% 1h**: 6% SCS treatment for 1h; **Bs 12% 1h**: 12% SCS treatment for 1h; **Bs 24% 1h**: 24% SCS treatment for 1h; **Bs 3% 2h**: 3% SCS treatment for 2h; **Bs 6% 2h**: 6% SCS treatment for 2h; **Bs 12% 2h**: 12% SCS treatment for 2h; **Bs 24% 2h**: 24% SCS treatment for 2h; **Bs 3% 4h**: 3% SCS treatment for 4h; **Bs 6% 4h**— 6% SCS treatment for 4h; **Bs 12%** 4h: 12% SCS treatment for 4h; **Bs 24% 4h**— 24% SCS treatment for 4h; **Bs 3% 24h**: 3% SCS treatment for 24h; **Bs f 6% 24h**: 6% SCS treatment for 24h; **Bs 12% 24h**: 12% SCS treatment for 24h; **Bs 24% 24h**: 24% SCS treatment for 24h. 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: *** $p < 0.001$; in grey: compared with the control.

5.2.2 Assessment of IC ROS levels

5.2.2.1 Effect of Enterococcus faecium NCIMB 10415 SCS on the IC ROS production of IPEC-J2 cells

In order to characterize the intracellular redox state of the IPEC-J2 cells the DCFH-DA method was used.

All three types of LPS (*S.* Typhimurium, *E. coli* 111 and *E. coli* 127) caused an increase in the fluorescence compared to the control (p<0.001) (**Figure 15**).

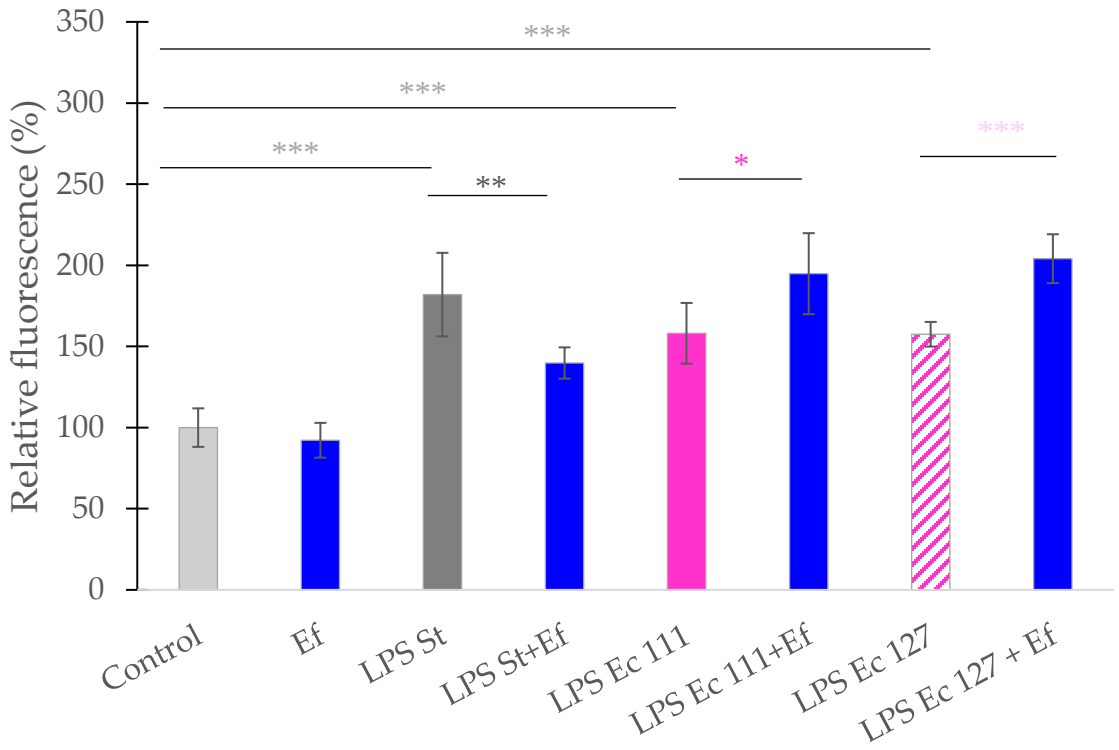


Figure 15: Amount of intracellular ROS after treatment with bacterial endotoxin (LPS), *E. faecium* (Ef) NCIMB 10415 SCS and their combinations. Control: plain cell culture medium treatment; **Ef**: *E. faecium* SCS; **LPS St**: *S*. Typhimurium endotoxin 10 µg/ml; **LPS St+Ef:** *E. faecium* SCS+ *S*. Typhimurium endotoxin 10 µg/ml; **LPS Ec 111**: *E. coli* O1111:B4 endotoxin 10 µg/ml; **LPS Ec 111+Ef**: *faecium* SCS+ *E. coli* O1111:B4 endotoxin 10 µg/ml; **LPS Ec 127**: *E. coli* O127:B8 endotoxin 10 µg/ml; **LPS Ec 127+Ef**: *E. faecium* SCS+ *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: *** $p < 0.001$, in light grey: compared with the untreated control. ** $p < 0.01$, in dark grey: compared with treatment with LPS St. * $p < 0.05$, in pink: compared with treatment with LPS Ec 111. *** $p < 0.001$, in light rose: compared with treatment with LPS Ec 127.

When IPEC-J2 cells were treated with only *E. faecium* SCS no significant change in the fluorescence could be observed. Samples treated with *S.* Typhimurium LPS and *E. faecium* SCS significantly reduced the amount of reactive oxygen species in IPEC-J2 cells compared to samples only treated with *S.* Typhimurium LPS ($p<0.01$). However, samples treated with *E. coli* 111 LPS and *E. faecium* SCS significantly increased ROS compared to samples only treated with *E. coli* 111 LPS ($p<0.05$). The same could be obeserved in the case of combined treatment with *E. coli* 127 LPS and *E. faecium* SCS, in this case ROS production of IPEC-J2 cells was further increased compared to cells only treated with *E. coli* 127 LPS ($p<0.001$) (**Figure 15**).

5.2.2.2 Effect of Lactobacillus rhamnosus DSM 7133 SCS on the IC ROS production of IPEC-J2 cells

No significant change in the fluorescence could be observed when IPEC-J2 cells were treated with only *L. rhamnosus* SCS. Treatment with *S.* Typhimurium LPS and *L. rhamnosus* SCS significantly reduced the amount of reactive oxygen species in IPEC-J2 cells compared to samples only treated with *S.* Typhimurium LPS ($p<0.001$). When IPEC-J2 cells were treated with *E. coli* 111 LPS and *L. rhamnosus* SCS no change in ROS production could be observed as compared to samples only treated with *E. coli* 111 LPS. Treatment with *E. coli* 127 LPS and *L. rhamnosus* SCS further increased the ROS production of IPEC-J2 cells compared to cells only treated with *E. coli* 127 LPS ($p<0.001$) (**Figure 16**).

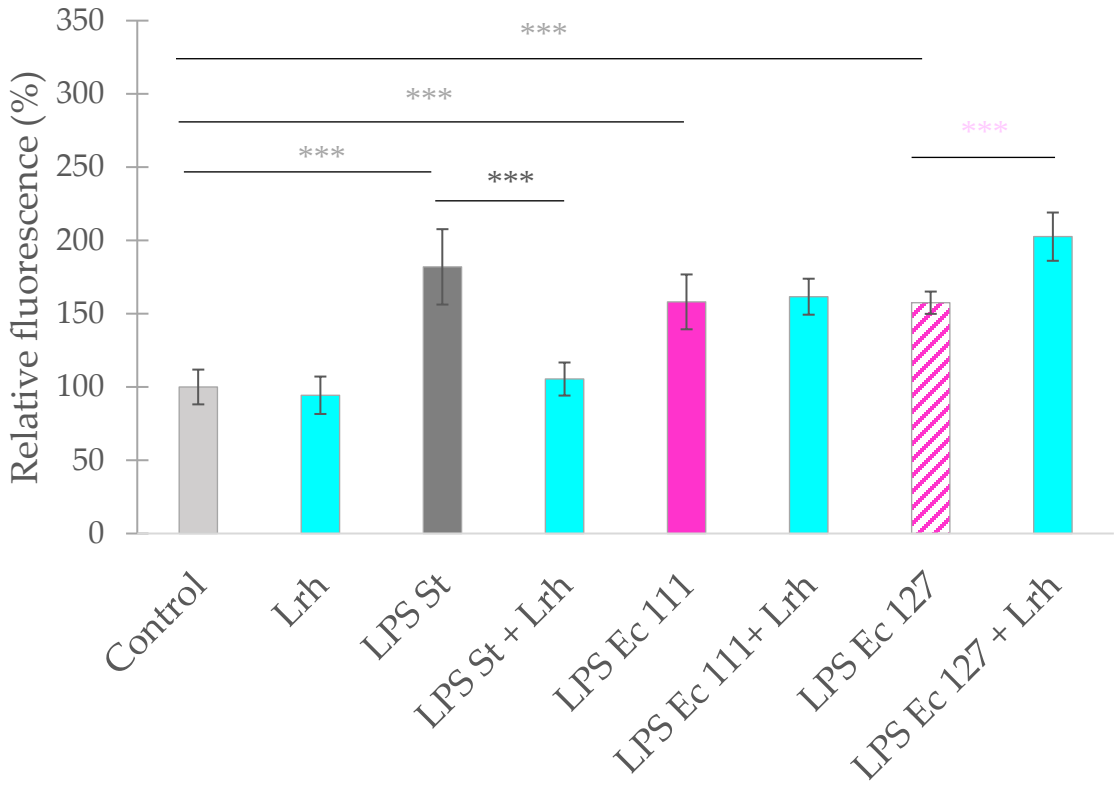


Figure 16: Amount of intracellular ROS after treatment with bacterial endotoxin (LPS), *L. rhamnosus* (Lrh) SCS and their combinations. Control: plain cell culture medium treatment; **Lrh**: *L. rhamnosus* SCS; **LPS St**: *S*. Typhimurium endotoxin 10 µg/ml; **LPS St+Lrh**: *L. rhamnosus* SCS+ *S*. Typhimurium endotoxin 10 µg/ml; **LPS Ec 111**: *E. coli* O1111:B4 endotoxin 10 µg/ml; **LPS Ec 111+Lrh** *L. rhamnosus* SCS+ *E. coli* O1111:B4 endotoxin 10 µg/ml; **LPS Ec 127**: *E. coli* O127:B8 endotoxin 10 µg/ml; **LPS Ec 127+Lrh**: *L. rhamnosus* SCS+ *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: *** $p < 0.001$, in light grey: compared with the untreated control. *** $p < 0.001$, in dark grey: compared with treatment with LPS St. *** $p < 0.001$, in light rose: compared with treatment with LPS Ec 127.

5.2.2.3 Effect of Bacillus licheniformis DSM 5749 SCS on the IC ROS production of IPEC-J2 cells

No significant change in the fluorescence could be observed when IPEC-J2 cells were treated with only *B. licheniformis* SCS. Treatment with *S.* Typhimurium LPS and *B. licheniformis* SCS significantly reduced the amount of reactive oxygen species in IPEC-J2 cells compared to samples only treated with *S.* Typhimurium LPS ($p<0.001$). Similarly, the treatment of IPEC-J2 cells with *E. coli* 111 LPS in combination with *B. licheniformis* SCS and *E. coli* 127 LPS in combination with *B. licheniformis* SCS respectively, also resulted in

a decreased ROS production of IPEC-J2 cells compared to cells only treated with *E. coli* 111 LPS and *E. coli* 127 LPS respectively (p<0.001) (**Figure 17**).

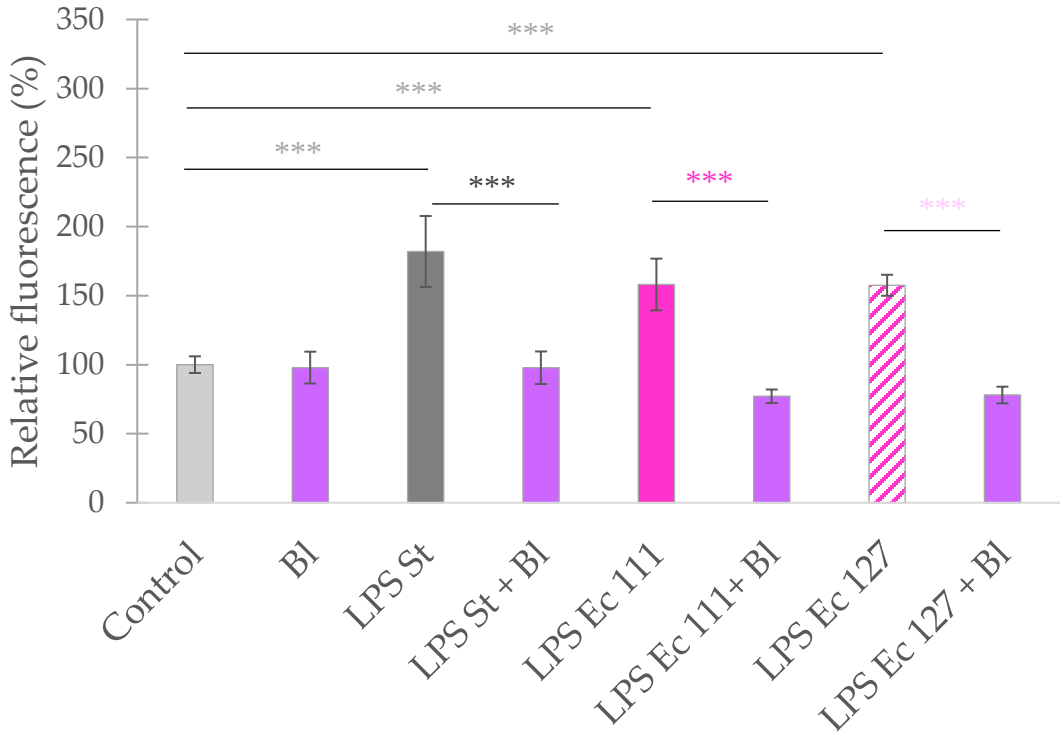


Figure 17: Amount of intracellular ROS after treatment with bacterial endotoxin (LPS), *B. licheniformis* (Bl) DSM 5749 SCS and their combinations. Control: plain cell culture medium treatment; **Bl**: *B. licheniformis* SCS; **LPS St**: *S*. Typhimurium endotoxin 10 µg/ml; **LPS St+Bl**: *B. licheniformis* SCS+ *S*. Typhimurium endotoxin 10 µg/ml; **LPS Ec 111**: *E. coli* O1111:B4 endotoxin 10 µg/ml; **LPS Ec 111+Bl**: *B. licheniformis* SCS+ *E. coli* O1111:B4 endotoxin 10 µg/ml; **LPS Ec 127**: *E. coli* O127:B8 endotoxin 10 µg/ml; **LPS Ec 127+Bl**: *B. licheniformis* SCS+ *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: *** $p < 0.001$, in light grey: compared with the untreated control. *** $p < 0.001$, in dark grey: compared with treatment with LPS St. *** $p < 0.001$, in pink: compared with treatment with LPS Ec 111. *** $p < 0.001$, in light rose: compared with treatment with LPS Ec 127.

5.2.2.4 Effect of Bacillus subtilis DSM 5750 SCS on the IC ROS production of IPEC-J2 cells

Treatment with *B. subtilis* SCS did not cause a significant change in the ROS production of IPEC-J2 cells. When IPEC-J2 cells were treated with *S.* Typhimurium LPS and *B. subtilis* SCS the ROS porduction was significantly reduced as compared to samples only treated with *S.* Typhimurium LPS (p<0.001). Also the treatment with *E. coli* 111 LPS in combination

with *B. subtilis* SCS and *E. coli* 127 LPS in combination with *B. subtilis* SCS respectively could significantly decrease the production of reactive oxigen species in IPEC-J2 cells compared to cells only treated with *E. coli* 111 LPS and *E. coli* 127 LPS respectively ($p<0.001$) (**Figure 18**).

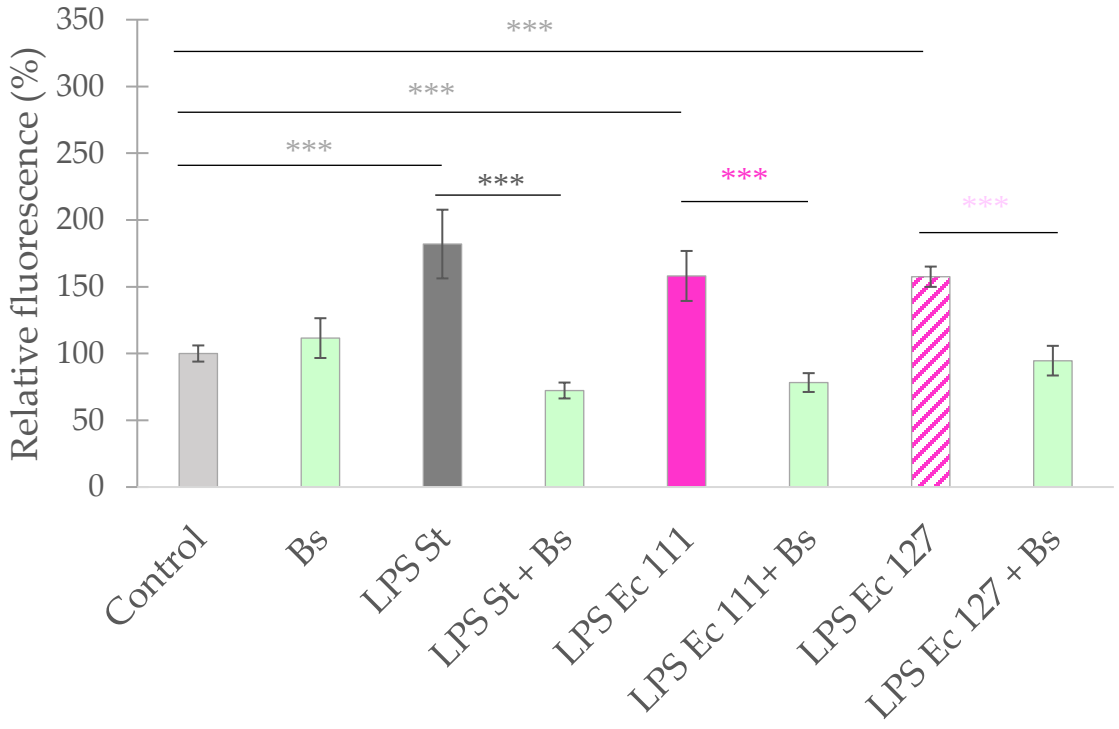


Figure 18: Amount of intracellular ROS after treatment with bacterial endotoxin (LPS), *B. subtilis* (Bs) SCS and their combinations. Control: plain cell culture medium treatment; **Bs**: *B. subtilis* SCS; **LPS St**: *S*. Typhimurium endotoxin 10 µg/ml; **LPS St+Bs**: *B. subtilis* SCS+ *S*. Typhimurium endotoxin 10 µg/ml; **LPS Ec 111**: *E. coli* O1111:B4 endotoxin 10 µg/ml; **LPS Ec 111+Bs**: *B. subtilis* SCS+ *E. coli* O1111:B4 endotoxin 10 µg/ml; **LPS Ec 127**: *E. coli* O127:B8 endotoxin 10 µg/ml; **LPS Ec 127+Bs**: *B. subtilis* SCS+ *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: *** $p < 0.001$, in light grey: compared with the untreated control. *** $p < 0.001$, in dark grey: compared with treatment with LPS St. *** $p < 0.001$, in pink: compared with treatment with LPS Ec 111. *** $p < 0.001$, in light rose: compared with treatment with LPS Ec 127.

5.2.3 Assessment of antibacterial activity

SCSs of *E. faecium*, *L. rhamnosus*, *B. licheniformis* and *B. subtilis* did not show any antibacterial activity against the investigated *E. coli* and *S*. Typhimurium strains. Even the highest SCS concentrations were unable to inhibit the growth of any of the isolates.

5.3 Results with bacteria

5.3.1 Assessment of cell viability

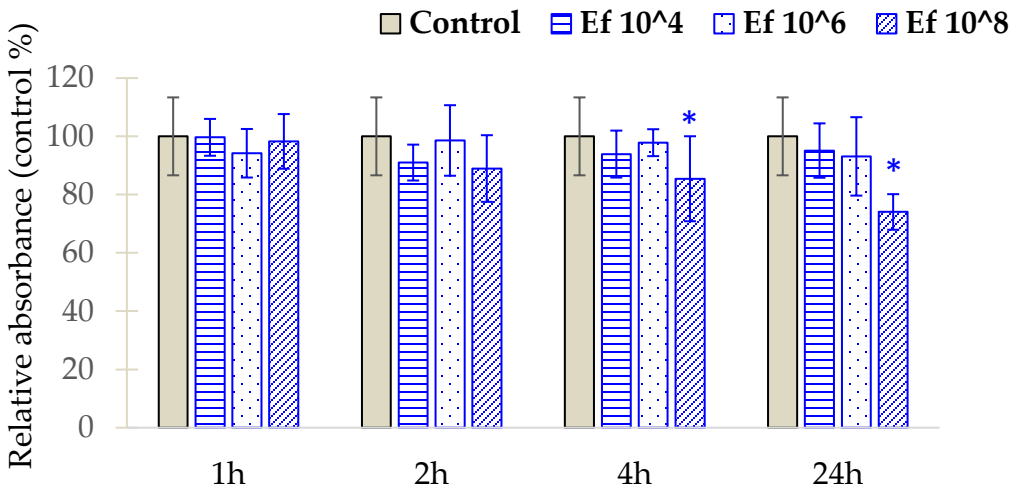


Figure 19. Viability of IPEC-J2 cells after treatment with *E. faecium* (Ef) NCIMB 10415 for different times. **Control**: plain cell culture medium treatment for 1 h; **1 h, Ef 10^4**: treatment for 1 h with *Ef* suspension of 10^4 CFU/ml; **1 h, Ef 10^6**: treatment for 1 h with *Ef* suspension of 10^6 CFU/ml; **1 h, Ef 10^8**:treatment for 1 h *Ef* suspension of 10^8 CFU/ml; **2 h, Ef 10^4**:treatment for 2 h with *Ef* suspension of 10^4 CFU/ml; **2 h, Ef 10^6**: treatment for 2 h with *Ef* suspension of 10^6 CFU/ml; **2 h, Ef 10^8**: treatment for 2 h with *Ef* suspension of 10^8 CFU/ml; **4 h, Ef 10^4**: treatment for 4 h with *Ef* suspension of 10^4 CFU/ml; **4 h, Ef 10^6**: treatment for 4 h with *Ef* suspension of 10^6 CFU/ml; **4 h, Ef 10^8**: treatment for 4 h with *Ef* suspension of 10^8 CFU/ml; **24 h, Ef 10^4**: treatment for 24 h with *Ef* suspension of 10^4 CFU/ml; **24 h, Ef 10^6**: treatment for 24 h with *Ef* suspension of 10^6 CFU/ml; **24 h, Ef 10^8**: treatment for 24 h with *Ef* suspension of 10^8 CFU/ml. 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: $^*p < 0.05$ in grey: compared with the control.

In order to determine the effect of *E. faecium, L. rhamnosus, B. licheniformis and B. subtilis* suspensions on the viability of IPEC-J2 cells, the neutral red uptake method was used. *E. faecium* suspensions of a 10^8 CFU/ml concentration significantly reduced the viability of IPEC-J2 cells when they were applied for 4 and 24 h ($p<0.05$) (**Figure 19**). Any other

treatment concentrations and treatment times did not cause any significant change in the viability of IPEC-J2 cells as compared to the control. In the case of *L. rhamnosus, B. licheniformis and B. subtilis* none of the treatment times resulted in a significant change in the viability of IPEC-J2 cells as compared to the control (**Figure 20**).

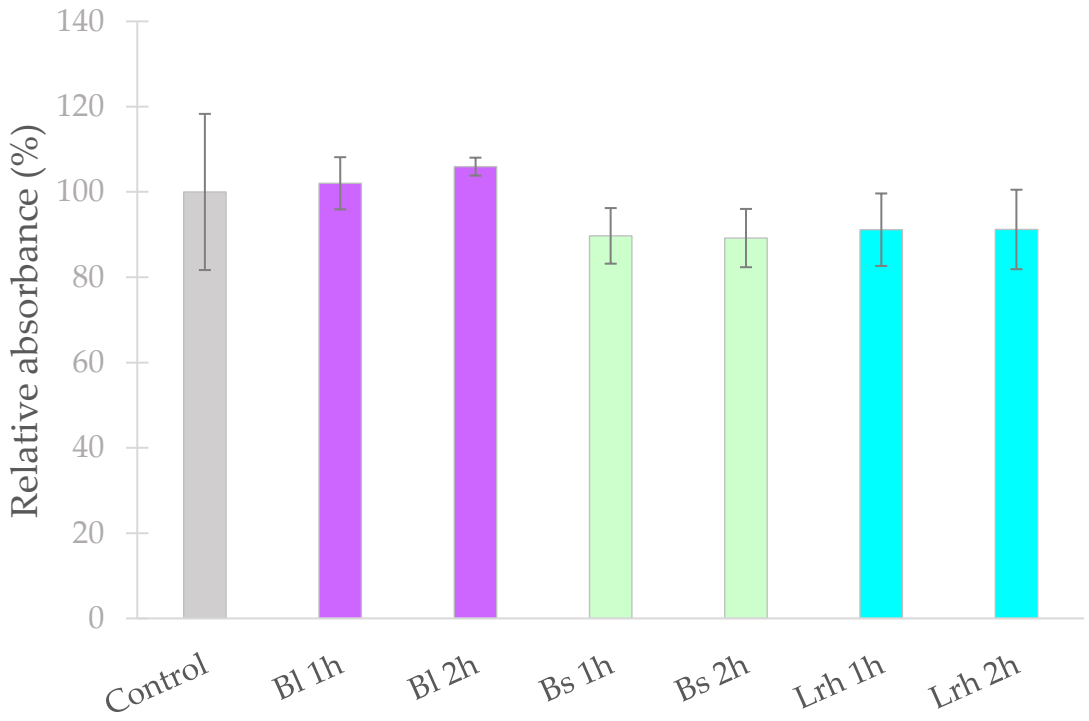


Figure 20. Viability of IPEC-J2 cells after treatment with *B. licheniformis* (Bl), *B. subtilis* (Bs) and *L. rhamnosus* (Lrh) for 1 and 2 hours respectively. Control: plain cell culture medium treatment for 1 h; **Bl 1h**: treatment for 1 h with *B. licheniformis* suspension of 10^8 CFU/ml; **Bl 2h**: treatment for 2 h with *B. licheniformis* suspension of 10^8 CFU/ml; **Bs 1h**: treatment for 1 h with *B. subtilis* suspension of 10^8 CFU/ml; **Bs 2h**: treatment for 2 h with *B. subtilis* suspension of 10^8 CFU/ml, **Lrh 1h**: treatment for 1 h with *L. rhamnosus* suspension of 10^8 CFU/ml, **Lrh 2h**: treatment for 2 h with *L. rhamnosus* suspension of 10^8 CFU/ml. Data are shown as means with standard deviations and expressed as relative absorbance, considering the mean value of control as 100%. n = 6/group.

5.3.2 Assessment of barrier integrity

5.3.2.1 Results with Enterococcus faecium NCIMB 10415

After 24 h of pathogen exposure, the epithelial cell layer was partially disrupted. The fluorescence intensity measured in the basolateral compartment significantly increased (compared to the untreated control samples) when IPEC-J2 cells were treated with *S.* Typhimurium ($p<0.001$) (**Figure 21**) or *E. coli* ($p<0.01$) (**Figure 22**).

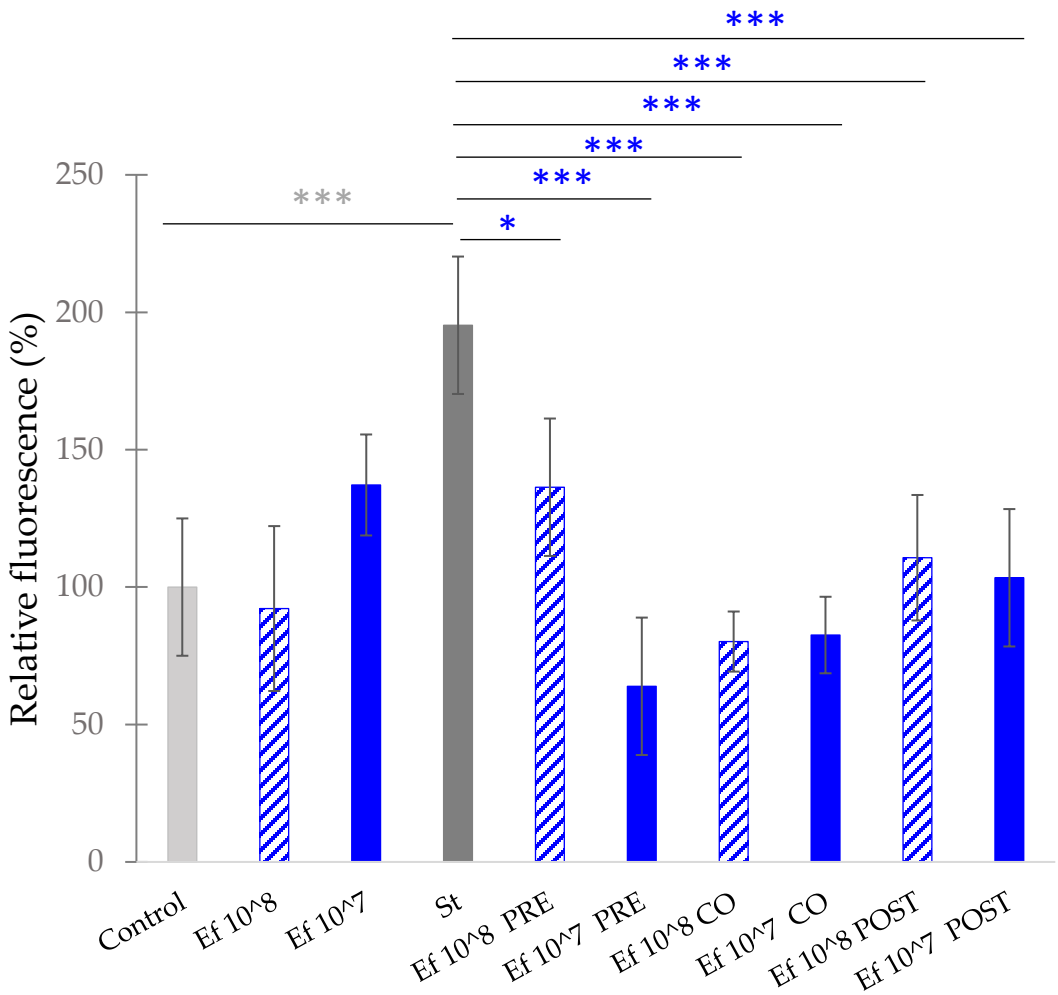


Figure 21. Effect of *E. faecium* (Ef) on the paracellular permeability of IPEC-J2 cells treated with *S.* Typhimurium. *Ef* was added 1 h before (pre-treatment), at the same time (co-treatment) and 1 h after (post-treatment) the addition of *S.* Typhimurium. Detection of the FD4 dye was performed 24 after the treatment of *S.* Typhimurium. **Control**: plain cell culture medium treatment; **Ef 10^8**: *Ef* 10^8 CFU/ml; **Ef 10^7**: *Ef* 10^7 CFU/ml**; Ef 10^8 PRE**: pre-treatment with *Ef* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Ef 10^7 PRE**: pre-treatment with *Ef* 10^7 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Ef 10^8 CO**: co-treatment with *Ef* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Ef 10^7 CO**: co-treatment with *Ef* 10^7 CFU/ml + *S.* Typhimurium 10^6 CFU/ml. **Ef 10^8 POST**: post-treatment with *Ef* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Ef 10^7 POST**: post-treatment with *Ef* 10^7 CFU/ml + *S.* Typhimurium 10^6 CFU/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: *** $p < 0.001$; in grey: compared with the untreated control. ***p* < 0.05; **** $p < 0.001$, in blue: compared with treatment with *S.* Typhimurium.

The treatment with *E. faecium* alone, in two different concentrations (10^8 CFU/ml or 10^7 CFU/ml), did not result in the alteration of fluorescence intensity (**Figure 21**). Pre-treatment, co-treatment and post-treatment with *E. faecium* significantly decreased the presence of FD4 tracer in the basolateral chamber, when cells were exposed to *S.* Typhimurium ($p<0.001$ in all cases except Ef 10^8 PRE: $p<0.05$) (**Figure 21**). The same effect could be observed when IPEC-J2 cells were challenged by *E. coli* ($p<0.001$) **(Figure 22**).

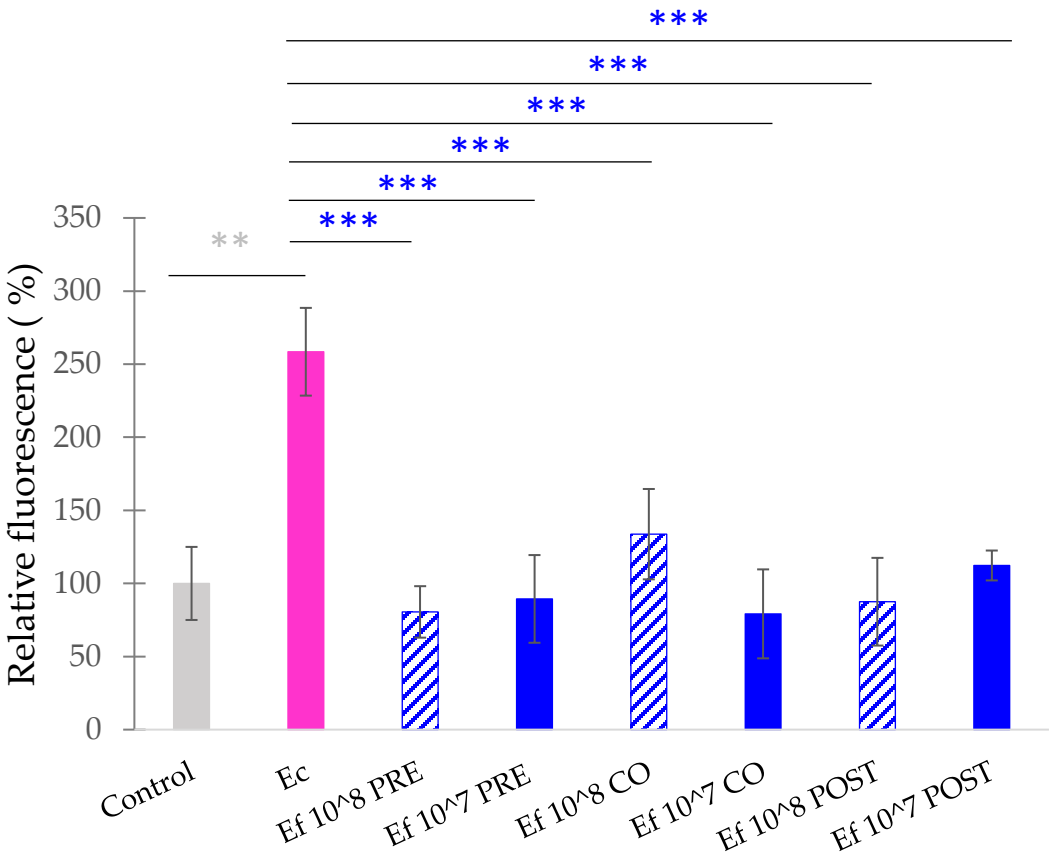


Figure 22. Effect of *E. faecium* (Ef) on the paracellular permeability of IPEC-J2 cells treated with *E. coli*. *Ef* was added 1 h before (pre-treatment), at the same time (co-treatment) and 1 h after (post-treatment) the addition of *E. coli*. Detection of the FD4 dye was performed 24 after the treatment of *E. coli*. **Control**: plain cell culture medium treatment; **Ec**: *E. coli* 10^6 CFU/ml**; Ef 10^8 PRE**: pre-treatment with *Ef* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Ef 10^7 PRE**: pre-treatment with *Ef* 10^7 CFU/ml + *E. coli* 10^6 CFU/ml; **Ef 10^8 CO**: co-treatment with *Ef* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Ef 10^7 CO**: co-treatment with *Ef* 10^7 CFU/ml + *E. coli* 10^6 CFU/ml; **Ef 10^8 POST**: post-treatment with *Ef* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Ef 10^7 POST**: post-treatment with *Ef* 10^7 CFU/ml + *E. coli* 10^6 CFU/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: *** $p < 0.001$; in grey: compared with the untreated control. *** $p < 0.001$, in blue: compared with treatment with *E. coli*.

5.3.2.2 Results with Lactobacillus rhamnosus DSM 7133

The treatment with *L. rhamnosus* alone resulted in a significant decrease of fluorescence intensity compared with the control (p<0.001) (**Figure 23**). Pre-treatment, co-treatment and post-treatment with *L. rhamnosus* significantly decreased the presence of FD4 tracer in the basolateral chamber, when cells were exposed to *S.* Typhimurium (**Figure 23**) or *E. coli* (**Figure 24**) respectively (p<0.001).

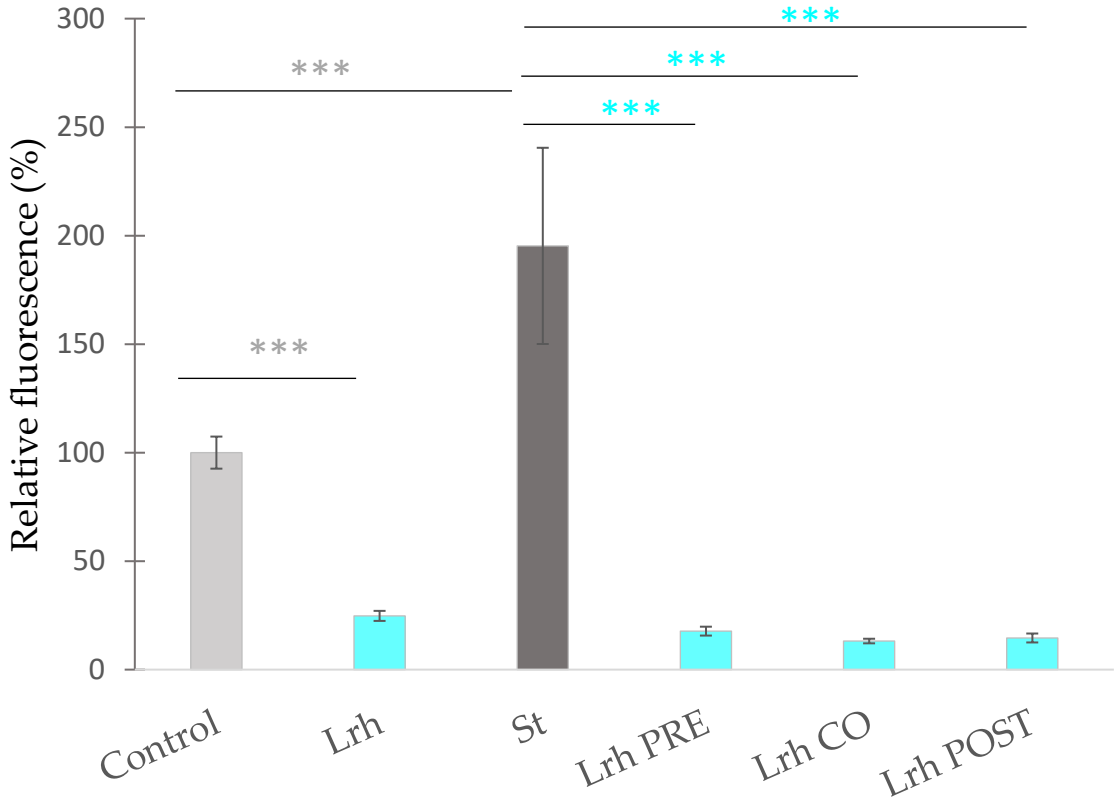


Figure 23. Effect of *L. rhamnosus* (Lrh) on the paracellular permeability of IPEC-J2 cells treated with *S.* Typhimurium. *L. rhamnosus* was added 1 h before (pre-treatment), at the same time (co-treatment) and 1 h after (post-treatment) the addition of *S.* Typhimurium. Detection of the FD4 dye was performed 24 after the treatment of *S.* Typhimurium. **Control**: plain cell culture medium treatment; **Lrh**: *L. rhamnosus* 10^8 CFU/ml**;** **Lrh PRE**: pre-treatment with *L. rhamnosus* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Lrh CO**: co-treatment with *L. rhamnosus* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Lrh POST**: post-treatment with *L. rhamnosus* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/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: *** $p < 0.001$; in grey: compared with the untreated control. *** $p < 0.001$, in turquoise: compared with treatment with *S.* Typhimurium.

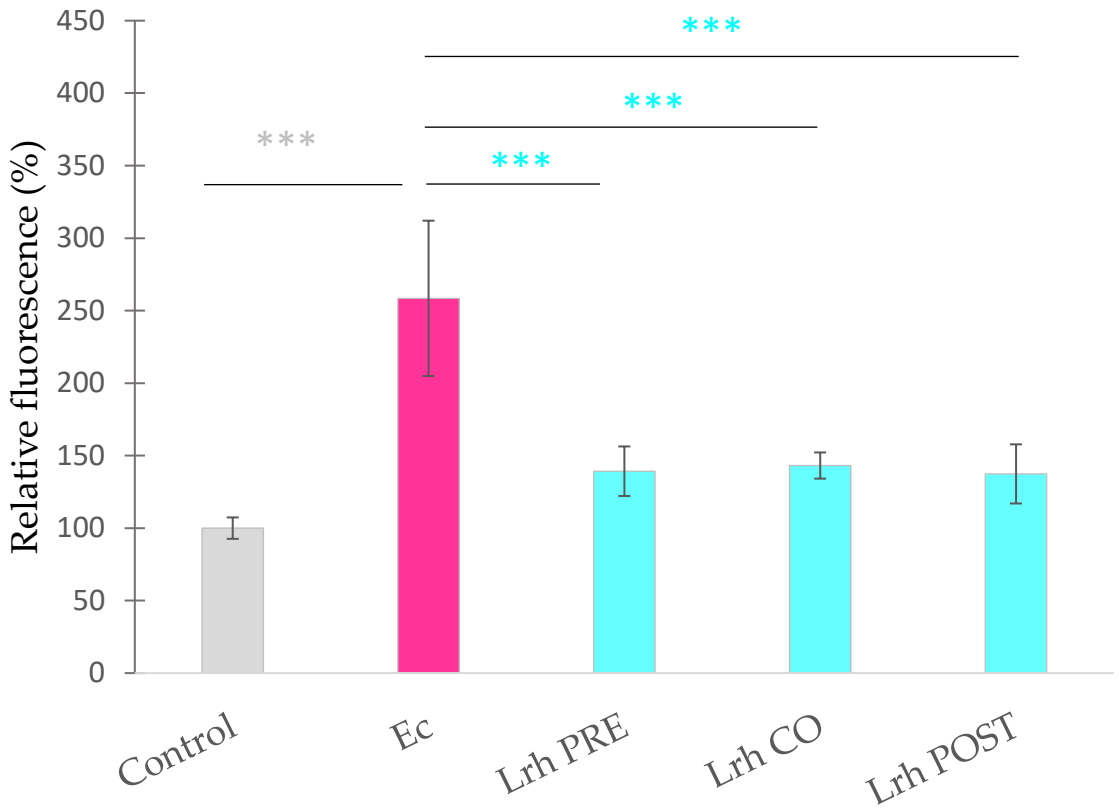


Figure 24. Effect of *L. rhamnosus* on the paracellular permeability of IPEC-J2 cells treated with *E. coli*. *L. rhamnosus* was added 1 h before (pre-treatment), at the same time (co-treatment) and 1 h after (post-treatment) the addition of *E. coli*. Detection of the FD4 dye was performed 24 after the treatment of *E. coli*. **Control**: plain cell culture medium treatment; **Lrh**: *L. rhamnosus* 10^8 CFU/ml**; Lrh PRE**: pre-treatment with *L. rhamnosus* 10^8 CFU/ml + *E. coli*.10^6 CFU/ml; **Lrh CO**: co-treatment with *L. rhamnosus* 10^8 CFU/ml + *E. coli*.10^6 CFU/ml; **Lrh POST**: post-treatment with *L. rhamnosus* 10^8 CFU/ml + *E. coli*.10^6 CFU/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: *** $p < 0.001$; in grey: compared with the untreated control. *** $p < 0.001$, in turquoise: compared with treatment with *E. coli*.

5.3.2.3 Results with Bacillus licheniformis DSM 5749

After 24 h of pathogen exposure, the epithelial cell layer was partially disrupted. Fluorescence intensity measured in the basolateral compartment significantly increased (compared with untreated control samples) when IPEC-J2 cells were treated with *S.* Typhimurium ($p<0.05$) (**Figure 25**) or *E. coli* ($p<0.05$) (**Figure 26**). The treatment with *B. licheniformis* alone did not result in the alteration of fluorescence intensity (**Figure 25**).

None of the treatments could significantly decrease the presence of FD4 tracer in the basolateral chamber. However, in the cases of co- and post-treatment with *B. licheniformis*, fluorescence intensity was further significantly increased ($p<0.001$ for Bl CO; $p<0.01$ for Bl POST) compared with the fluorescence when IPEC-J2 cells were challenged by *E. coli* (**Figure 26**).

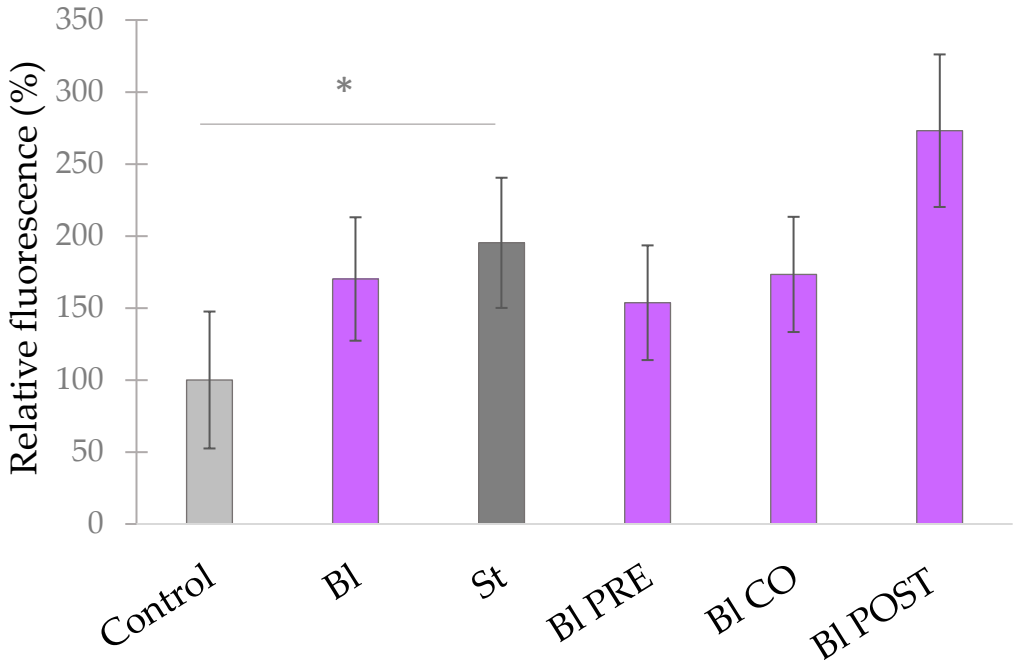


Figure 25. Effect of *B. licheniformis* (Bl) on the paracellular permeability of IPEC-J2 cells treated with *S.* Typhimurium. *B. licheniformis* was added 1 h before (pre-treatment), at the same time as (co-treatment), and 1 h after (post-treatment) the addition of *S.* Typhimurium. Detection of the FD4 dye was performed 24 h after the treatment of *S.* Typhimurium **Control**: plain cell culture medium treatment; **St**: *S.* Typhimurium 10^6 CFU/ml; **Bl**: treatment with *B. licheniformis* 10^8 CFU/ml; **Bl PRE**: pre-treatment with *B. licheniformis* 10^8 CFU/ml + *S.* Typhimurium CFU/ml; **Bl CO**: co-treatment with *B. licheniformis* 10^8 CFU/ml *S.* Typhimurium 10^6 CFU/ml; **Bl POST**: post-treatment with *B. licheniformis* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/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: * $p < 0.05$; in grey: compared with the untreated control.

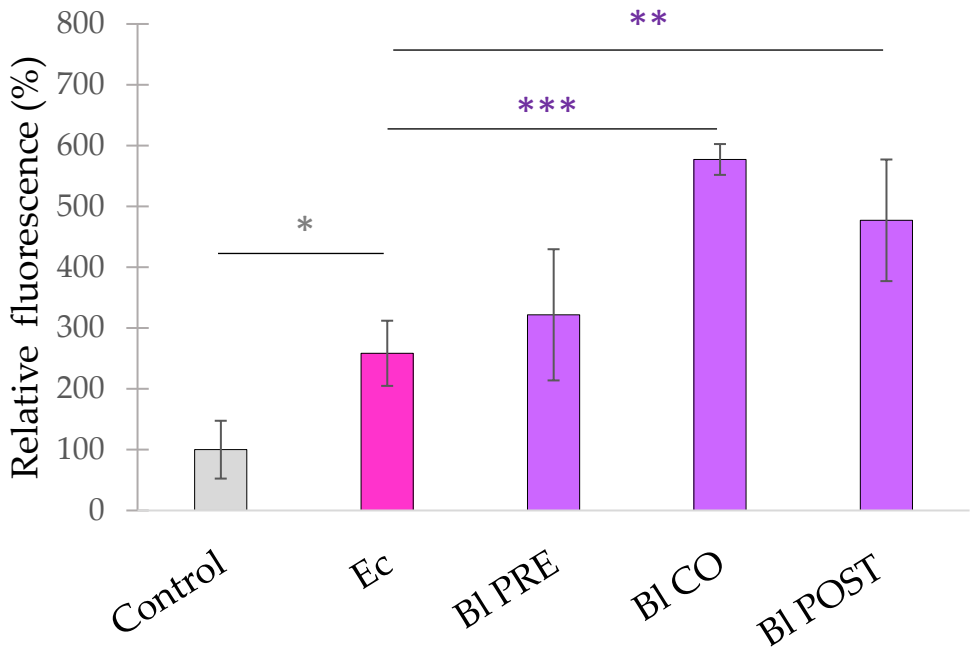


Figure 26. Effect of *B. licheniformis* (Bl) on the paracellular permeability of IPEC-J2 cells treated with *E. coli*. *B. licheniformis* was added 1 h before (pre-treatment), at the same time as (co-treatment), and 1 h after (post-treatment) the addition of *E. coli*. Detection of the FD4 dye was performed 24 h after the treatment of *E. coli.* **Control**: plain cell culture medium treatment; **Ec**: *S.* Typhimurium 10^6 CFU/ml; **Bl PRE**: pre-treatment with *B. licheniformis* 10^8 CFU/ml + *E. coli* CFU/ml; **Bl CO**: co-treatment with *B. licheniformis* 10^8 CFU/ml *E. coli* 10^6 CFU/ml; **Bl POST**: post-treatment with *B. licheniformis* 10^8 CFU/ml + *E. coli* 10^6 CFU/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: * $p \leq 0.05$; in grey: compared with the untreated control. ** $p < 0.01$; *** $p < 0.001$, in purple: compared with treatment with *E. coli*.

5.3.2.4 Results with Bacillus subtilis

Treatment with *B. subtilis* alone caused an increase in paracellular permeability compared with the control ($p<0.001$) (**Figure 27**). Pre-, co-, and post-treatments further increased ($p<0.001$) the fluorescence signal measured in the basolateral compartment compared with the fluorescence intensity increase induced by *S.* Typhimurium (**Figure 27**) or *E. coli* (**Figure 28**).

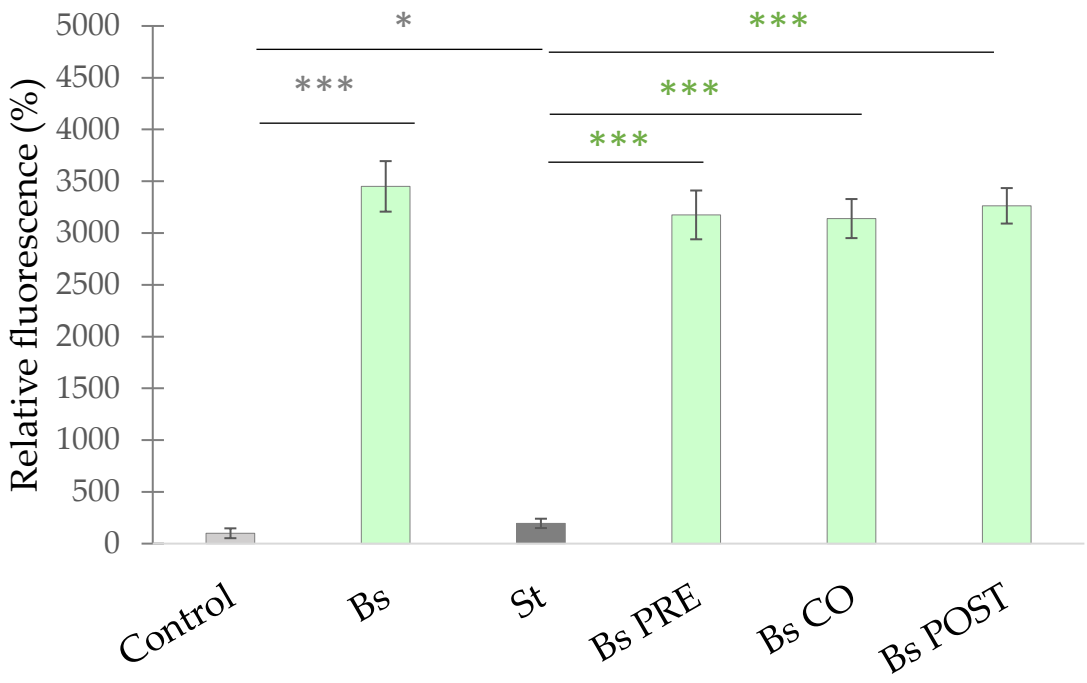


Figure 27. Effect of *B. subtilis* (Bs) on the paracellular permeability of IPEC-J2 cells treated with *S.* Typhimurium. *B. subtilis* was added 1 h before (pre-treatment), at the same time as (co-treatment), and 1 h after (post-treatment) the addition of *S.* Typhimurium. Detection of the FD4 dye was performed 24 h after the treatment of *S.* Typhimurium. **Control**: plain cell culture medium treatment; **St**: *S.* Typhimurium 10^6 CFU/ml; **Bs**: treatment with *B. subtilis* 10^8 CFU/ml; **Bs PRE**: pre-treatment with *B. subtilis* 10^8 CFU/ml + *S.* Typhimurium CFU/ml; **Bs CO**: co-treatment with *B. subtilis* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Bs POST**: post-treatment with *B. subtilis* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/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: * $p < 0.05$, *** $p < 0.001$ in grey: compared with the untreated control. *** $p < 0.001$, in green: compared with treatment with *S.* Typhimurium.

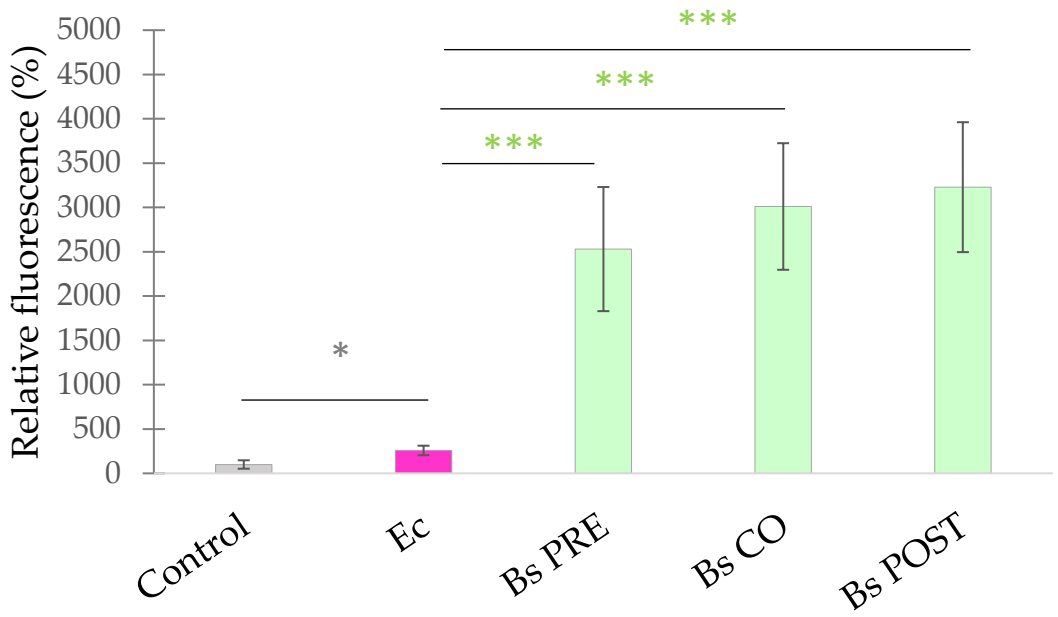


Figure 28. Effect of *B. subtilis* (Bs) on the paracellular permeability of IPEC-J2 cells treated with *E. coli*. *B. subtilis* was added 1 h before (pre-treatment), at the same time as (co-treatment), and 1 h after (post-treatment) the addition of *E. coli*. Detection of the FD4 dye was performed 24 h after the treatment of *E. coli*. **Control**: plain cell culture medium treatment; **Ec**: *E. coli* 10^6 CFU/ml; **Bs**: treatment with *B. subtilis* 10^8 CFU/ml; **Bs PRE**: pre-treatment with *B. subtilis* 10^8 CFU/ml + *E. coli* CFU/ml; **Bs CO**: co-treatment with *B. subtilis* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Bs POST**: post-treatment with *B. subtilis* 10^8 CFU/ml + *E. coli* 10^6 CFU/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: * $p < 0.05$ in grey: compared with the untreated control. *** $p < 0.001$, in green: compared with treatment with *E. coli.*

5.3.3 Assessment of IL-6 and IL-8 levels

5.3.3.1 Results with Enterococcus faecium

Infection of intestinal epithelial cells with *S.* Typhimurium significantly induced the secretion of IL-6 compared to the controls (i.e., non-infected cells) ($p<0.05$). In comparison, treatment with only the probiotic strain did not result in a significant change in IL-6 secretion, even if *E. faecium* was applied at a concentration of 10^8 CFU/ml or 10^7 CFU/ml. The pre-treatment with *E. faecium* 10^8 CFU/ml caused a significant decrease in IL-6 production as compared to the IL-6 secretion induced by *S.* Typhimurium ($p<0.05$). However, the co-treatment of *S.* Typhimurium and *E. faecium* at 10^8 CFU/ml did not alter the IL-6 secretion compared to the IL-6 secretion evoked by *S.* Typhimurium. The pre-treatment and the co-treatment with *E.*

faecium 10^7 CFU/ml failed to significantly decrease IL-6 secretion compared to the IL-6 production induced *by S.* Typhimurium (**Figure 29**).

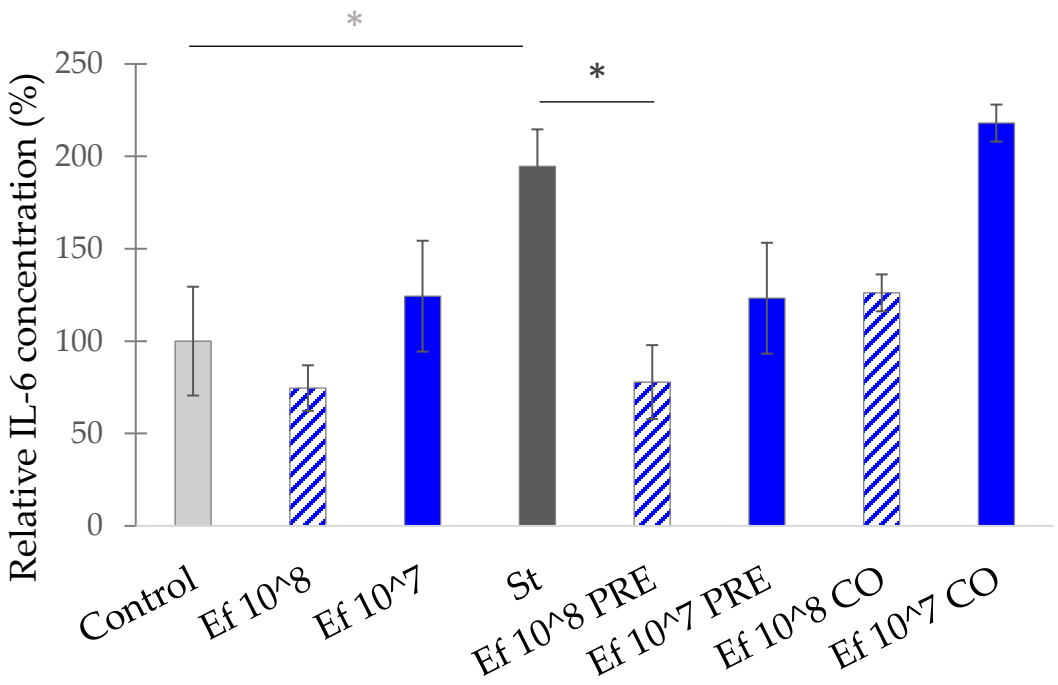


Figure 29. IL-6 levels of IPEC-J2 cells after treatment with *S.* Typhimurium (St) and *E. faecium* (Ef). *E. faecium* was added 1 h before (pre-treatment) or at the same time (co-treatment) of the addition of S. Typhimurium. *E. faecium* was added in 10^8 CFU/ml or in 10^7 CFU/ml concentration. **Control**: plain cell culture medium treatment; **St**: *S.* Typhimurium 10^6 CFU/ml; **Ef 10^8**: *E. faecium* 10^8 CFU/ml; **Ef 10^7**: *E. faecium* 10^7 CFU/ml**; Ef 10^8 PRE**: pre-treatment with *E. faecium* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Ef 10^7 PRE**: pre-treatment with *E. faecium* 10^7 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Ef 10^8 CO**: co-treatment with *E. faecium* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Ef 10^7 CO**: co-treatment with *E. faecium* 10^7 CFU/ml + *S.* Typhimurium 10^6 CFU/ml. Data are shown as means with standard deviations and expressed as relative IL-6 concentration, considering the mean value of control as 100%. n = 6/group. Significant difference: * $p < 0.05$ in light grey: compared with the untreated control, * $p < 0.05$, in dark grey: compared with treatment with *S.* Typhimurium.

Infection of IPEC-J2 cells with *S.* Typhimurium also increased the secretion of IL-8 ($p<0.001$). Treatment with the probiotic strain itself did not result in a significant change in IL-8 secretion, regardless of the applied concentration. Pre-treatment and co-treatment with

E. faecium, applied at a concentration of 10^8 CFU/ml, significantly reduced the secretion of IL-8 compared to the amount of IL-8 secretion when IPEC-J2 cells were challenged by *S.* Typhimurium ($p<0.001$). Pre-treatment and co-treatment with *E. faecium*, applied at a concentration of 10^7 CFU/ml, failed to decrease the IL-8 secretion in comparison to the secretion observed when cells were treated with *S.* Typhimurium itself (**Figure 30**).

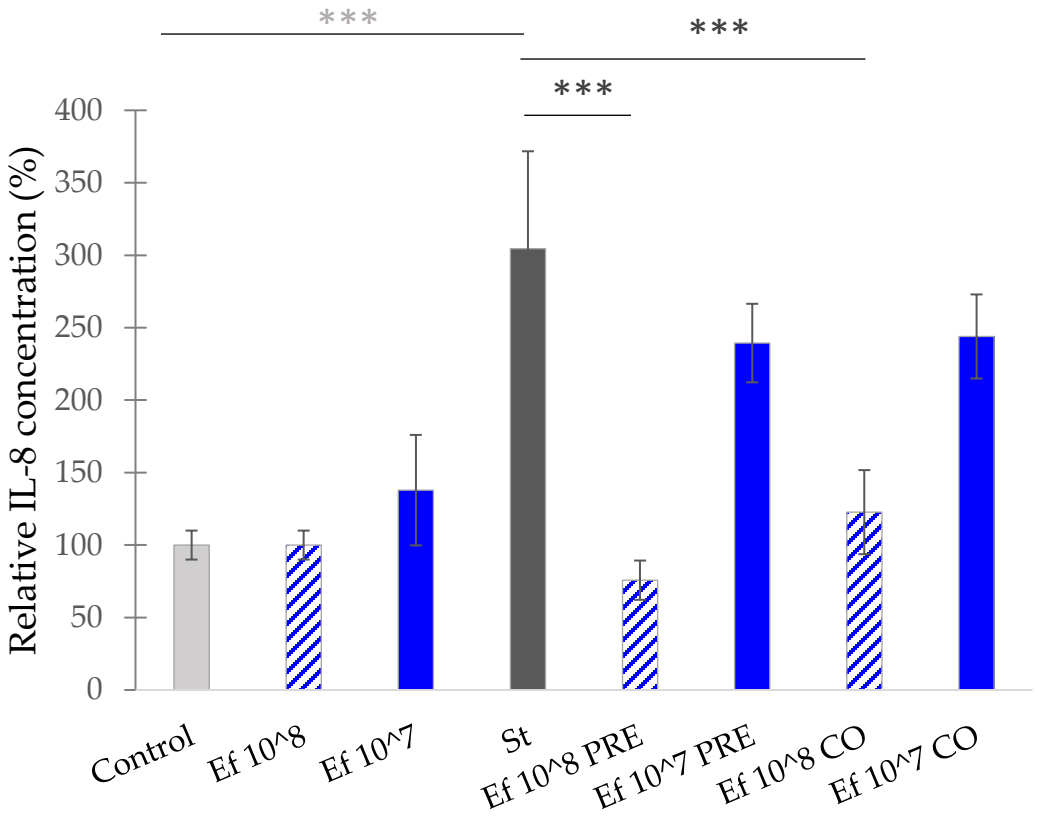


Figure 30. IL-8 levels of IPEC-J2 cells after treatment with *S.* Typhimurium (St) and *E. faecium* (Ef). *E. faecium* was added 1 h before (pre-treatment) or at the same time (co-treatment) of the addition of *S.* Typhimurium. *E. faecium* was added in 10^8 CFU/ml or in 10^7 CFU/ml concentration. **Control**: plain cell culture medium treatment; **St**: *S.* Typhimurium 10^6 CFU/ml; **Ef 10^8**: *E. faecium* 10^8 CFU/ml; **Ef 10^7**: *E. faecium* 10^7 CFU/ml; **Ef 10^8 PRE**: pre-treatment with *E. faecium* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Ef 10^7 PRE**: pre-treatment with *E. faecium* 10^7 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Ef 10^8 CO**: co-treatment with *E. faecium* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Ef 10^7 CO**: co-treatment with *E. faecium* 10^7 CFU/ml + *S.* Typhimurium 10^6 CFU/ml. Data are shown as means with standard deviations and expressed as relative IL-8 concentration, considering the mean value of control as 100%. n = 6/group. Significant difference: *** $p < 0.001$ in light grey: compared with the untreated control, *** $p < 0.001$, in dark grey: compared with treatment with *S.* Typhimurium.

IL-6 secretion was induced significantly by *E. coli* in comparison to the control cells ($p<0.05$). Neither pre-treatment nor co-treatment with *E. faecium* could compensate for the IL-6 elevation induced by *E. coli* (**Figure 31**).

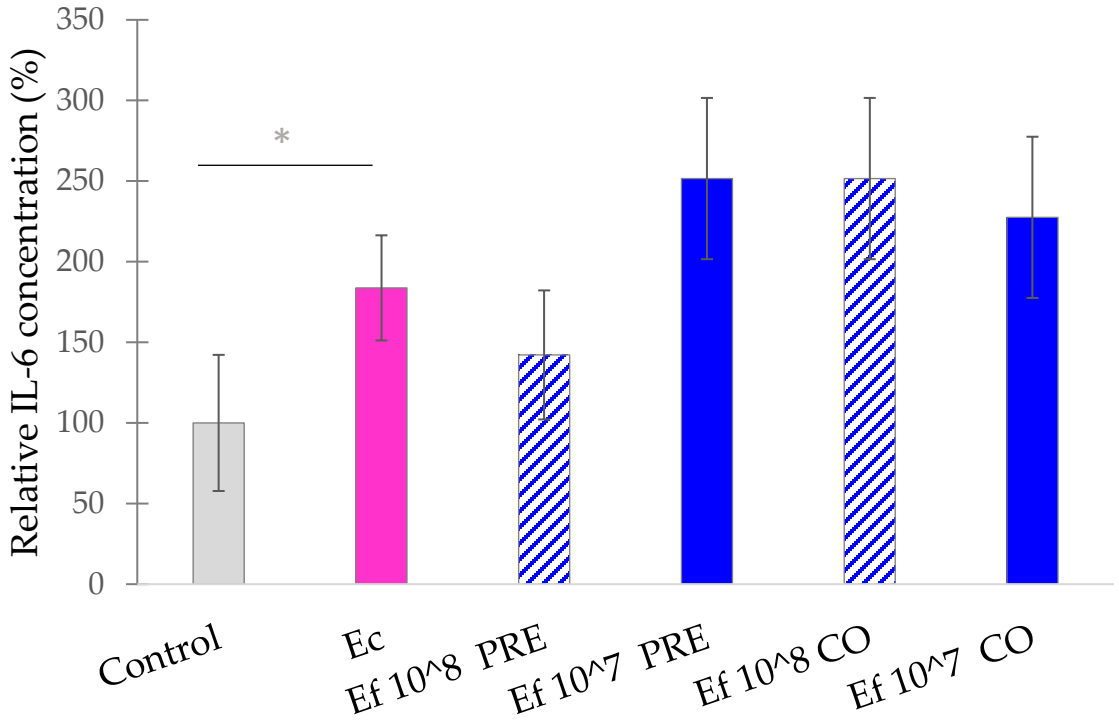


Figure 31. IL-6 levels of IPEC-J2 cells after treatment with *E. coli* (Ec) and *E. faecium* (Ef). *E. faecium* was added 1 h before (pre-treatment) or at the same time (co-treatment) of the addition of *E. coli*. *E. faecium* was added in 10^8 CFU/ml or in 10^7 CFU/ml concentration. **Control**: plain cell culture medium treatment; **Ec**: *E. coli* 10^6 CFU/ml; **Ef 10^8 PRE**: pre-treatment with *E. faecium* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Ef 10^7 PRE**: pre-treatment with *E. faecium* 10^7 CFU/ml + *E. coli* 10^6 CFU/ml; **Ef 10^8 CO**: co-treatment with *E. faecium* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Ef 10^7 CO**: co-treatment with *E. faecium* 10^7 CFU/ml + *E. coli* 10^6 CFU/ml. Data are shown as means with standard deviations and expressed as relative IL-6 concentration, considering the mean value of control as 100%. n = 6/group. Significant difference: * $p < 0.05$ in light grey: compared with the untreated control.

Also IL-8 secretion was induced significantly by *E. coli* compared to the control cells ($p<0.05$). Pre-treatment and co-treatment with *E. faecium*, applied at a concentration of 10^8 CFU/ml further increased the secretion of IL-8 ($p<0.05$ for Ef 10^8 PRE and $p<0.01$ for Ef

10^8 CO). The pre-treatment and co-treatment with *E. faecium*, applied at a concentration of 10^7 CFU/ml, failed to cause any significant effect on IL-8 secretion (**Figure 32**).

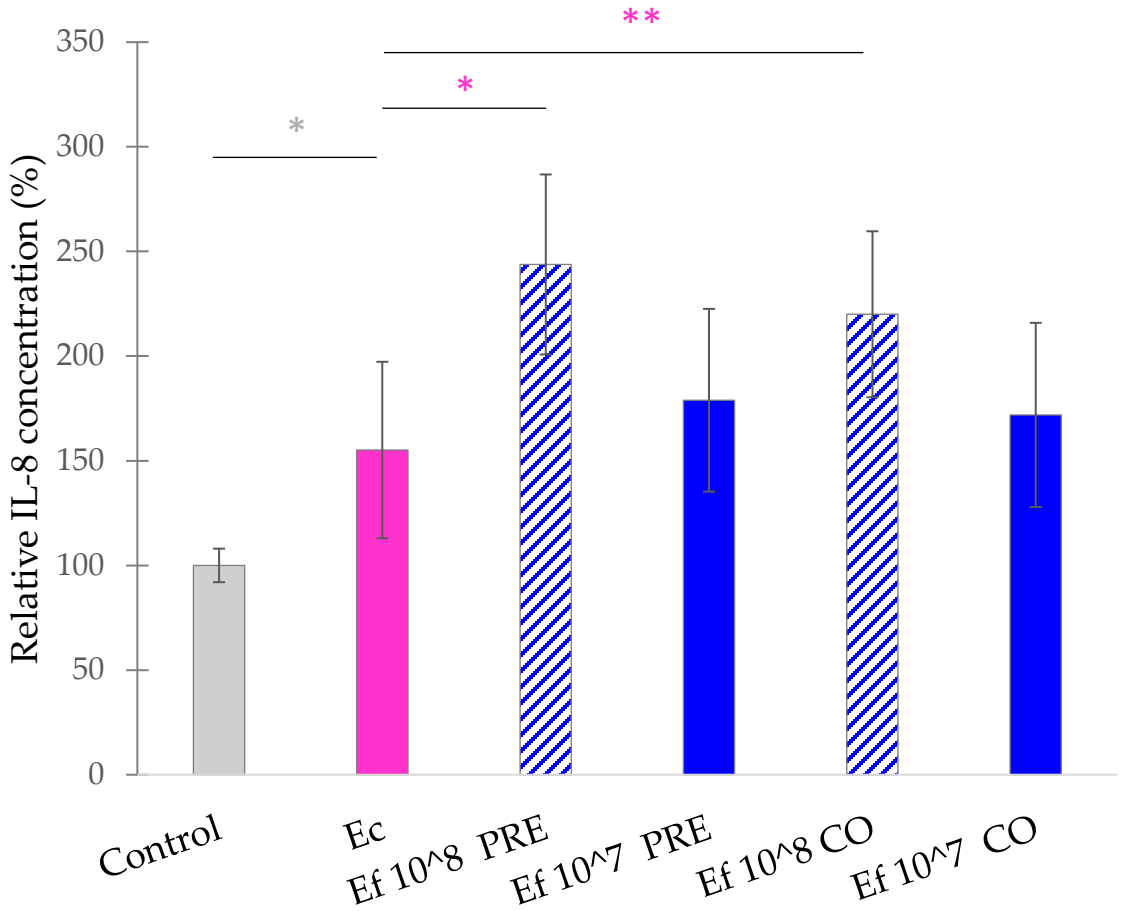


Figure 32. IL-8 levels of IPEC-J2 cells after treatment with *E. coli* (Ec) and *E. faecium* (Ef). *E. faecium* was added 1 h before (pre-treatment) or at the same time (co-treatment) of the addition of *E. coli*. *E. faecium* was added in 10^8 CFU/ml or in 10^7 CFU/ml concentration. **Control**: plain cell culture medium treatment; **Ec**: *E. coli* 10^6 CFU/ml; **Ef 10^8 PRE**: pre-treatment with *E. faecium* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Ef 10^7 PRE**: pre-treatment with *E. faecium* 10^7 CFU/ml + *E. coli* 10^6 CFU/ml; **Ef 10^8 CO**: co-treatment with *E. faecium* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Ef 10^7 CO**: co-treatment with *E. faecium* 10^7 CFU/ml + *E. coli* 10^6 CFU/ml. Data are shown as means with standard deviations and expressed as relative IL-8 concentration, considering the mean value of control as 100%. n = 6/group. Significant difference: * $p < 0.05$ in light grey: compared with the untreated control, * $p < 0.05$; ** $p < 0.01$, in pink: compared with treatment with *E. coli*.

5.3.3.2 Results with Lactobacillus rhamnosus

Treatment with *L. rhamnosus* alone did not alter the secretion of IL-6 in IPEC-J2 cells compared to the control. The pre-treatment and the post-treatment with *L. rhamnosus* 10^8 CFU/ml caused a significant decrease ($p<0.05$ for Lrh PRE and $p<0.01$ for Lrh POST) in IL-6 production as compared with the IL-6 secretion induced by *S.* Typhimurium, however the co-treatment with *L. rhamnosus* failed to decrease the IL-6 production compared with the IL-6 secretion induced by *S.* Typhimurium (**Figure 33**).

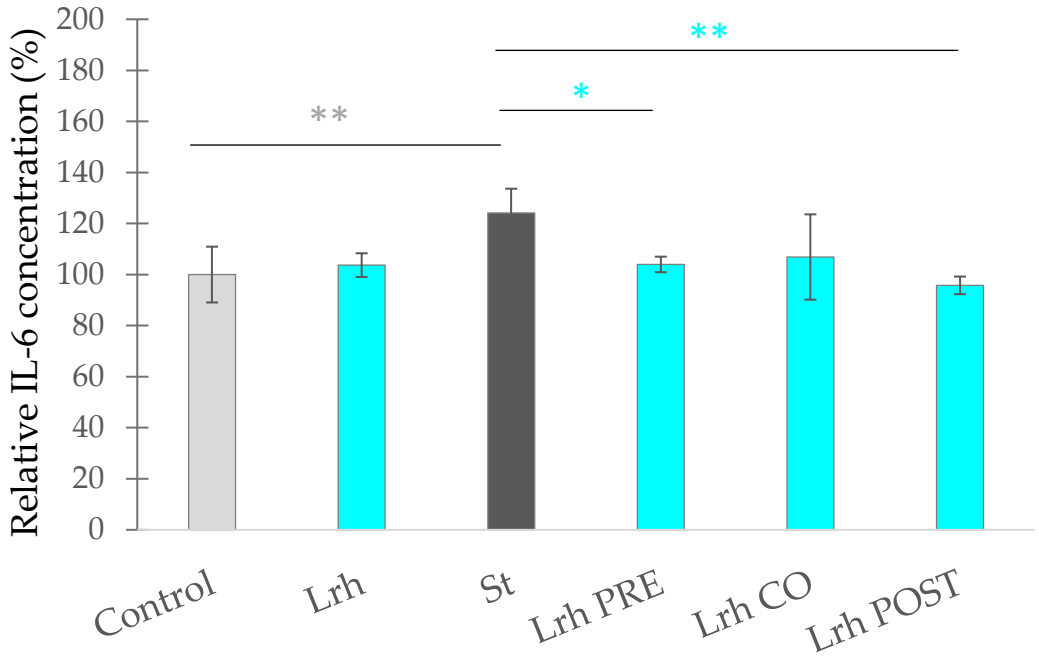


Figure 33. IL-6 levels of IPEC-J2 cells after treatment with *S.* Typhimurium (St) and *L. rhamnosus* (Lrh). *L. rhamnosus* was added 1 h before (pre-treatment), at the same time as (co-treatment), or 1 h after (post-treatment) the addition of *S.* Typhimurium. *L. rhamnosus* was added in 10^8 CFU/ml and *S.* Typhimurium was added in 10^6 CFU/ml concentration. **Control**: plain cell culture medium treatment; **St**: *S.* Typhimurium 10^6 CFU/ml; **Lrh**: *L. rhamnosus* 10^8 CFU/ml; **Lrh PRE**: pre-treatment with *L. rhamnosus* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Lrh CO**: co-treatment with *L. rhamnosus* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Lrh POST**: post-treatment with *L. rhamnosus* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml. Data are shown as means with standard deviations and expressed as relative IL-6 concentration, considering the mean value of control as 100%. n = 6/group. Significant difference: ** $p < 0.01$, in grey: compared with the untreated control. * $p \leq 0.05$; ** $p < 0.01$, in blue: compared with treatment with *S.* Typhimurium.

Treatment with *L. rhamnosus* alone did not result in a significant change in IL-8 secretion as compared with the control. All three treatment combinations (pre-, co-, and post-treatment) could signidicantly decrease the IL-8 secretion of IPEC-J2 cells compared with the IL-8 secretion induced by *S.* Typhimurium ($p<0.001$) (**Figure 34**).

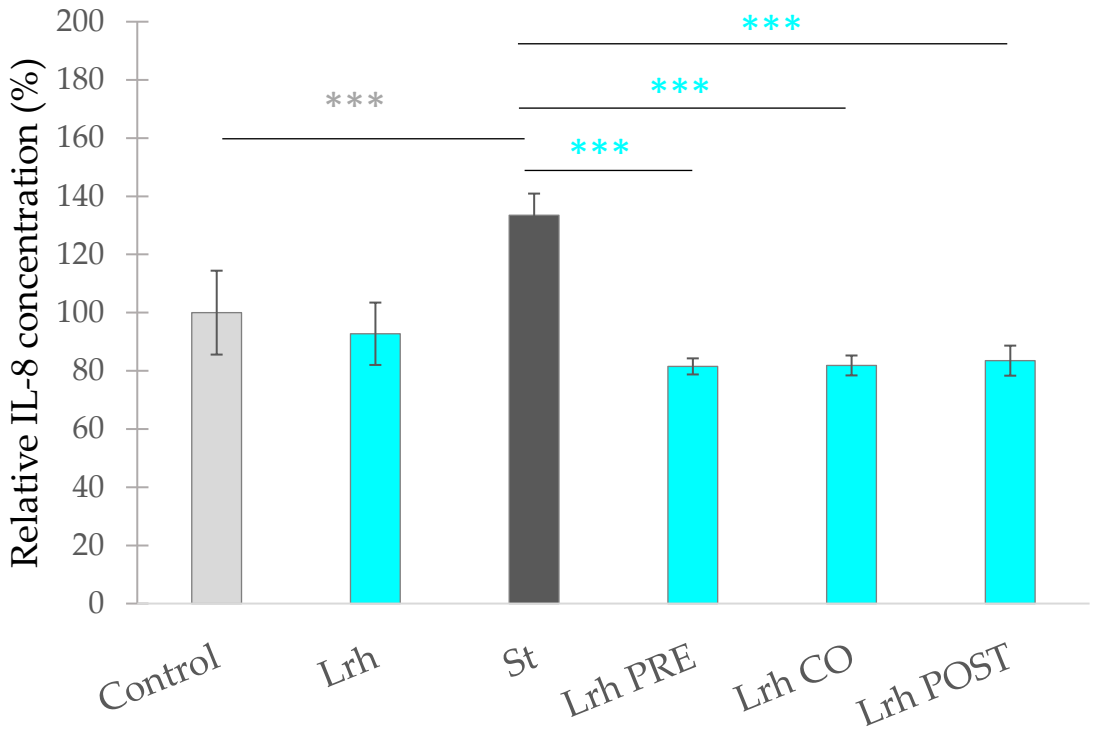


Figure 34. IL-8 levels of IPEC-J2 cells after treatment with *S.* Typhimurium (St) and *L. rhamnosus* (Lrh). *L. rhamnosus* was added 1 h before (pre-treatment), at the same time as (co-treatment), or 1 h after (post-treatment) the addition of *S.* Typhimurium. *L. rhamnosus* was added in 10^8 CFU/ml and *S.* Typhimurium was added in 10^6 CFU/ml concentration. **Control**: plain cell culture medium treatment; **St**: *S.* Typhimurium 10^6 CFU/ml; **Lrh**: *L. rhamnosus* 10^8 CFU/ml; **Lrh PRE**: pre-treatment with *L. rhamnosus* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Lrh CO**: co-treatment with *L. rhamnosus* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Lrh POST**: post-treatment with *L. rhamnosus* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml. Data are shown as means with standard deviations and expressed as relative IL-8 concentration, considering the mean value of control as 100%. n = 6/group. Significant difference: *** $p < 0.001$, in grey: compared with the untreated control. *** $p < 0.001$, in blue: compared with treatment with *S.* Typhimurium.

All three treatment combination (pre-, co-, and post-treatment with *L. rhamnosus*) failed to significantly alter the IL-6 secretion induced by *E. coli* (**Figure 35**).

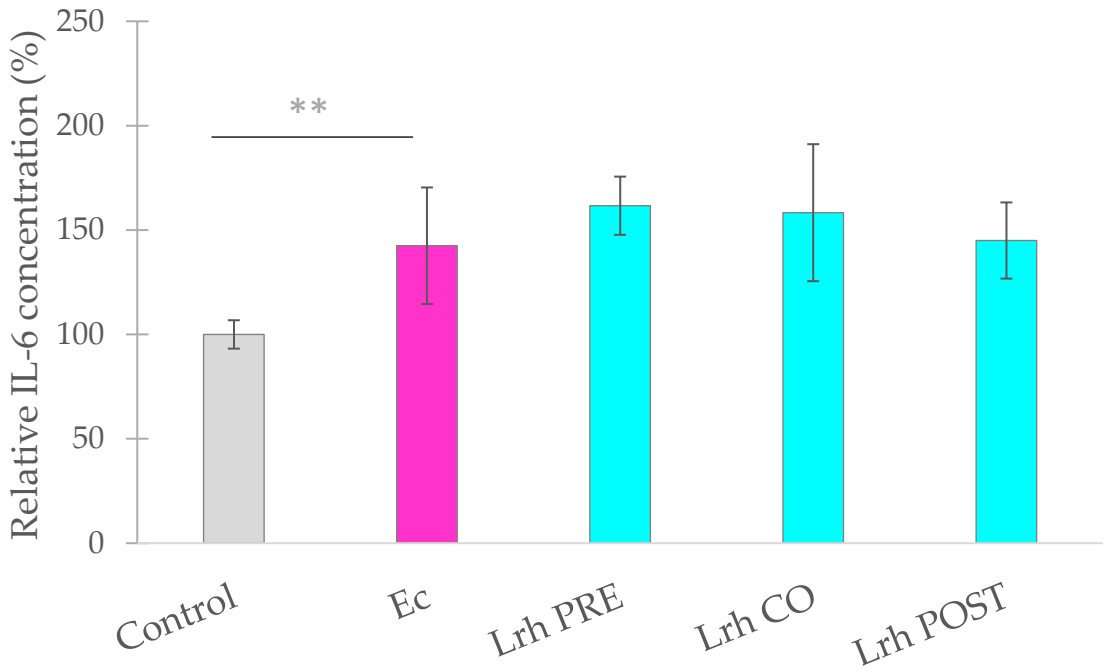


Figure 35. IL-6 levels of IPEC-J2 cells after treatment with *E. coli* (Ec) and *L. rhamnosus* (Lrh). *L. rhamnosus* was added 1 h before (pre-treatment), at the same time as (co-treatment), or 1 h after (post-treatment) the addition of *E. coli*. *L. rhamnosus* was added in 10^8 CFU/ml and *E. coli* was added in 10^6 CFU/ml concentration. **Control**: plain cell culture medium treatment; **Ec**: *E. coli* 10^6 CFU/ml; **Lrh PRE**: pre-treatment with *L. rhamnosus* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Lrh CO**: co-treatment with *L. rhamnosus* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Lrh POST**: post-treatment with *L. rhamnosus* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; Data are shown as means with standard deviations and expressed as relative IL-6 concentration, considering the mean value of control as 100%. n = 6/group. Significant difference: ** $p < 0.01$, in grey: compared with the untreated control.

5.3.3.3 Results with Bacillus licheniformis and Bacillus subtilis

Infection of intestinal epithelial cells with *S.* Typhimurium significantly induced the secretion of IL-6 compared with control ($p<0.001$). In addition the treatment with *B. subtilis* alone also resulted in significant IL-6 secretion compared with the control ($p<0.001$). In comparison, treatment with only *B. licheniformis* did not result in a significant change in IL-6 secretion compared with the control. The pre-treatment with both *B. subtilis* 10^8 CFU/ml and *B. licheniformis* 10^8 CFU/ml caused a significant decrease in IL-6 production as compared with the IL-6 secretion induced by *S.* Typhimurium ($p<0.001$). The co- and post-treatments with *B. licheniformis* 10^8 CFU/ml also reduced the IL-6 secretion ($p<0.001$ for Bl CO and $p<0.05$ for Bl post); however, the co- and post-treatments with *B. subtilis* 10^8 CFU/ml failed

to significantly decrease IL-6 secretion compared with the IL-6 production induced by *S.* Typhimurium (**Figure 36**).

Infection of IPEC-J2 cells with *S.* Typhimurium also triggered the secretion of IL-8 ($p<0.01$). Treatment with *B. licheniformis* alone also resulted in a significant rise in IL-8 secretion compared with the control ($p<0.001$). However, the treatment with *B. subtilis* alone did not result in a significant change in IL-8 secretion compared with the control. With the exception of post-treatment with *B. licheniformis*, all other treatment combinations did not alter the IL- 8 secretion induced by *S.* Typhimurium. Post-treatment with *B. licheniformis* further increased the IL-8 secretions compared with the amount of IL-8 secretion when IPEC-J2 cells were challenged by *S.* Typhimurium ($p<0.001$) (**Figure 37**).

None of the pre-, co-, and post-treatments with *B. licheniformis* and *B. subtilis* had any significant effect on the IL-6 elevation induced by *E. coli* (**Figure 38**). IL-8 secretion was induced significantly by *E. coli* compared with control cells ($p<0.05$) and pre-treatment with *B. licheniformis* 10^8 CFU/ml further increased the secretion of IL-8 ($p<0.001$). Pre-treatment with *B. subtilis* and co- and post-treatments with both probiotic bacteria failed to cause any significant effect on IL-8 secretion (**Figure 39**).

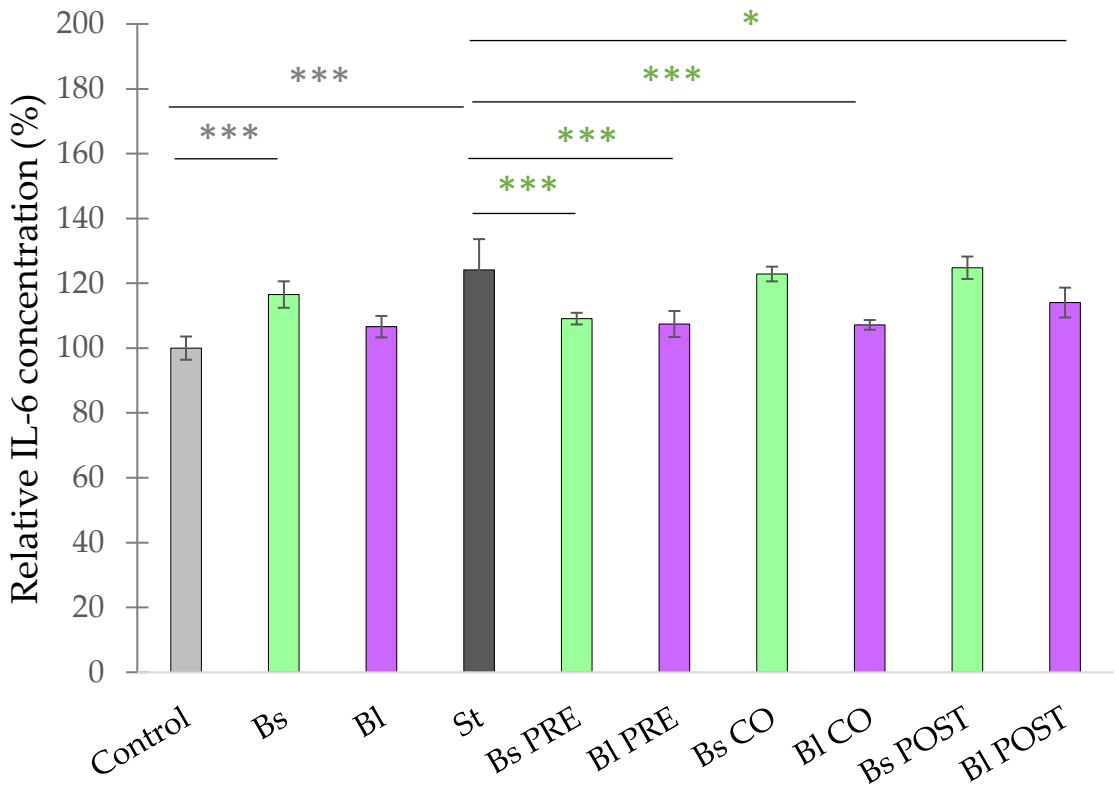


Figure 36. IL-6 levels of IPEC-J2 cells after treatment with *S.* Typhimurium (St), *B. licheniformis* (Bl), and *B. subtilis* (Bs). *B. licheniformis* and *B. subtilis* were added 1 h before (pre-treatment), at the same time as (co-treatment), or 1 h after (post-treatment) the addition of *S.* Typhimurium. *B. licheniformis* and *B. subtilis* were added in 10^8 CFU/ml and *S.* Typhimurium was added in 10^6 CFU/ml concentration. **Control**: plain cell culture medium treatment; **St**: *S.* Typhimurium 10^6 CFU/ml; **Bs**: *B. subtilis* 10^8 CFU/ml; **Bl**: *B. licheniformis* 10^8 CFU/ml; **Bs PRE**: pre-treatment with *B. subtilis* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Bl PRE**: pre-treatment with *B. licheniformis* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Bs CO**: co-treatment with *B. subtilis* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Bl CO**: co-treatment with *B. licheniformis* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Bs POST**: post-treatment with *B. subtilis* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Bl POST**: post-treatment with *B. licheniformis* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml. Data are shown as means with standard deviations and expressed as relative IL-6 concentration, considering the mean value of control as 100%. n = 6/group. Significant difference: *** $p < 0.001$, in grey: compared with the untreated control. * $p < 0.05$; *** $p < 0.001$, in green: compared with treatment with *S.* Typhimurium.

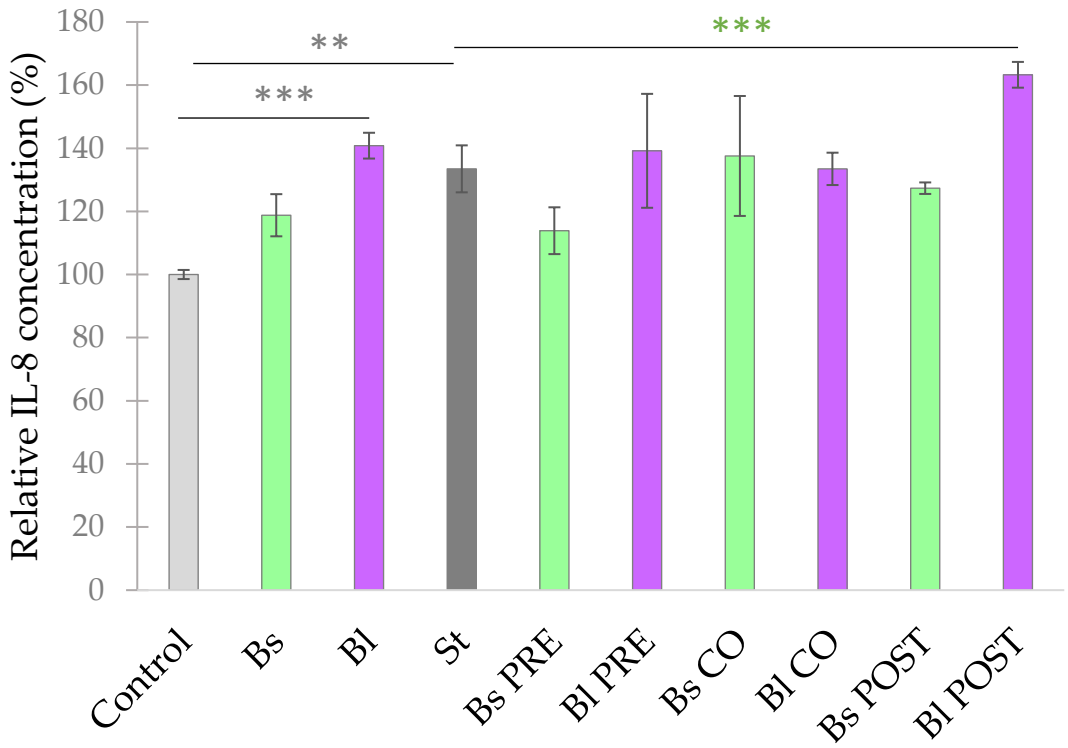


Figure 37. IL-8 levels of IPEC-J2 cells after treatment with *S.* Typhimurium (St), *B. licheniformis* (Bl), and *B. subtilis* (Bs). *B. licheniformis* and *B. subtilis* were added 1 h before (pre-treatment), at the same time as (co-treatment), or 1 h after (post-treatment) the addition of *S.* Typhimurium. *B. licheniformis* and *B. subtilis* were added in 10^8 CFU/ml and *S.* Typhimurium was added in 10^6 CFU/ml concentration. **Control**: plain cell culture medium treatment; **St**: *S.* Typhimurium 10^6 CFU/ml; **Bs**: *B. subtilis* 10^8 CFU/ml; **Bl**: *B. licheniformis* 10^8 CFU/ml; **Bs PRE**: pre-treatment with *B. subtilis* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Bl PRE**: pre-treatment with *B. licheniformis* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Bs CO**: co-treatment with *B. subtilis* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Bl CO**: co-treatment with *B. licheniformis* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Bs POST**: post-treatment with *B. subtilis* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Bl POST**: post-treatment with *B. licheniformis* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml. Data are shown as means with standard deviations and expressed as relative IL-8 concentration, considering the mean value of control as 100%. n = 6/group. Significant difference: ** $p < 0.01$, *** $p < 0.001$, in grey: compared with the untreated control *** $p < 0.01$, in green: compared with treatment with *S.* Typhimurium.

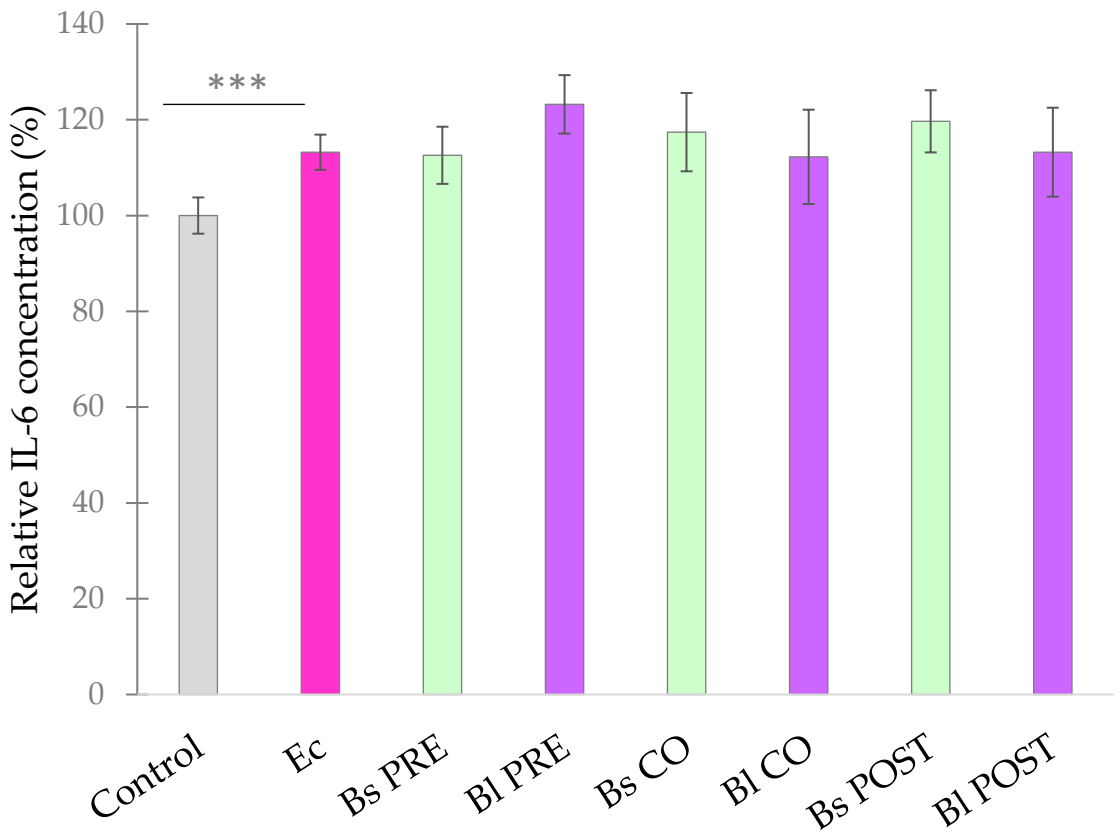


Figure 38. IL-6 levels of IPEC-J2 cells after treatment with *E. coli*, (Ec) *B. licheniformis* (Bl), and *B. subtilis* (Bs). *B. licheniformis* and *B. subtilis* were added 1 h before (pre-treatment), at the same time as (co-treatment), or 1 h after (post-treatment) the addition of *E. coli*. *B. licheniformis* and *B. subtilis* were added in 10^8 CFU/ml and *E. coli* was added in 10^6 CFU/ml concentration. **Control**: plain cell culture medium treatment; **Ec**: *E. coli* 10^6 CFU/ml; **Bs PRE**: pre-treatment with *B. subtilis* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Bl PRE**: pre-treatment with *B. licheniformis* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Bs CO**: co-treatment with *B. subtilis* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Bl CO**: co-treatment with *B. licheniformis* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Bs POST**: post-treatment with *B. subtilis* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Bl POST**: post-treatment with *B. licheniformis* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml. Data are shown as means with standard deviations and expressed as relative IL-6 concentration, considering the mean value of control as 100%. n = 6/group. Significant difference: *** $p < 0.001$ in grey: compared with the untreated control.

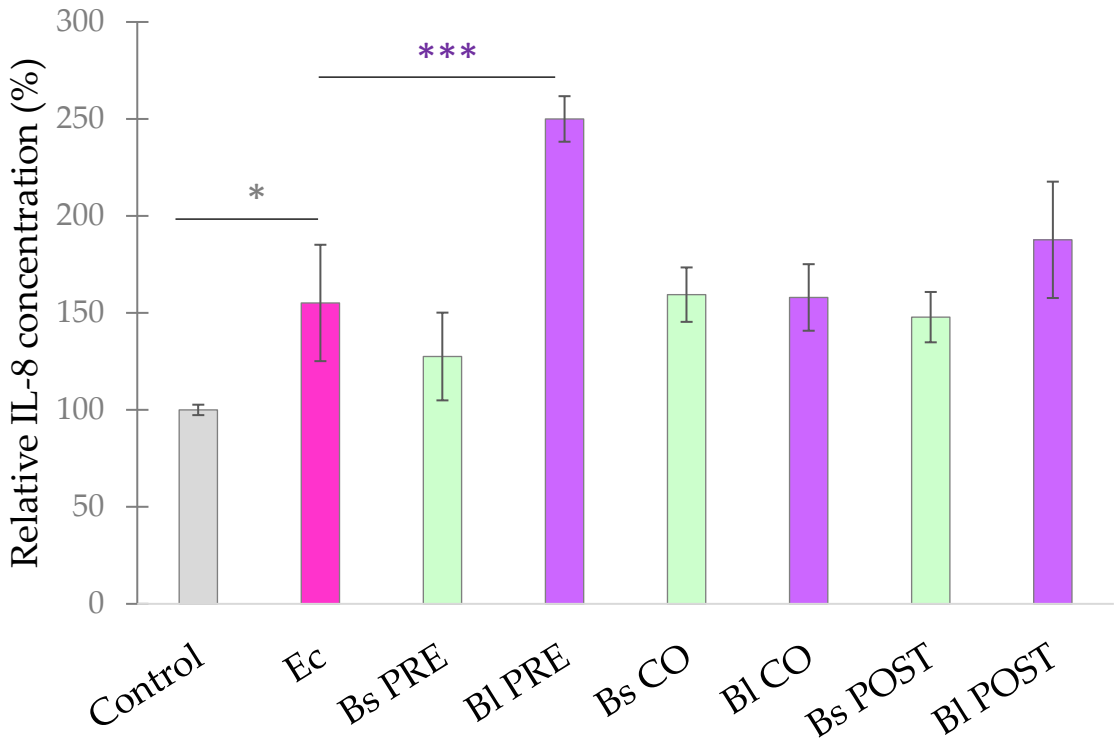


Figure 39. IL-8 levels of IPEC-J2 cells after treatment with *E. coli* (Ec), *B. licheniformis* (Bl), and *B. subtilis* (Bs). *B. licheniformis* and *B. subtilis* were added 1 h before (pre-treatment), at the same time as (co-treatment), or 1 h after (post-treatment) the addition of *E. coli*. *B. licheniformis* and *B. subtilis* were added in 10^8 CFU/ml and *E. coli* was added in 10^6 CFU/ml concentration. **Control**: plain cell culture medium treatment; **Ec**: *E. coli* 10^6 CFU/ml; **Bs PRE**: pre-treatment with *B. subtilis* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Bl PRE**: pre-treatment with *B. licheniformis* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Bs CO**: co-treatment with *B. subtilis* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Bl CO**: co-treatment with *B. licheniformis* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Bs POST**: post-treatment with *B. subtilis* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Bl POST**: post-treatment with *B. licheniformis* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml. Data are shown as means with standard deviations and expressed as relative IL-8 concentration, considering the mean value of control as 100%. n = 6/group. Significant difference: * $p < 0.05$, in grey: compared with the untreated control. *** $p < 0.001$, in purple: compared with treatment with *E. coli*.

5.3.4 Assessment of IC ROS levels

5.3.4.1 Results with Enterococcus faecium

In order to characterize the intracellular redox state of the IPEC-J2 cells, the DCFH-DA method was used. Treatment with *S.* Typhimurium caused an increase in the fluorescence compared to the control ($p<0.001$). All three treatment combinations (i.e., pre-treatment, co-treatment and post-treatment with *S.* Typhimurium and *E. faecium* in two different concentrations) resulted in a decreased amount of ROS ($p<0.001$ in all cases except Ef 10^7 CO: $p<0.01$). When IPEC-J2 cells were treated with only *E. faecium* 10^8 CFU/ml, a decrease in fluorescence could be observed compared to the control ($p<0.001$) (**Figure 40**).

Treatment with *E. coli* caused an increase in the fluorescence compared to the control ($p<0.001$). The pre-treatment with *E. faecium* significantly reduced the amount of reactive oxygen species in the cells compared with samples only treated with *E. coli*. Both applied concentrations (10^8 CFU/ml and 10^7 CFU/ml) of *E. faecium* resulted in a significant decrease in reactive oxygen species ($p<0.001$). The same could be observed in the case of co-treatments and post-treatments ($p<0.001$) (**Figure 41**).

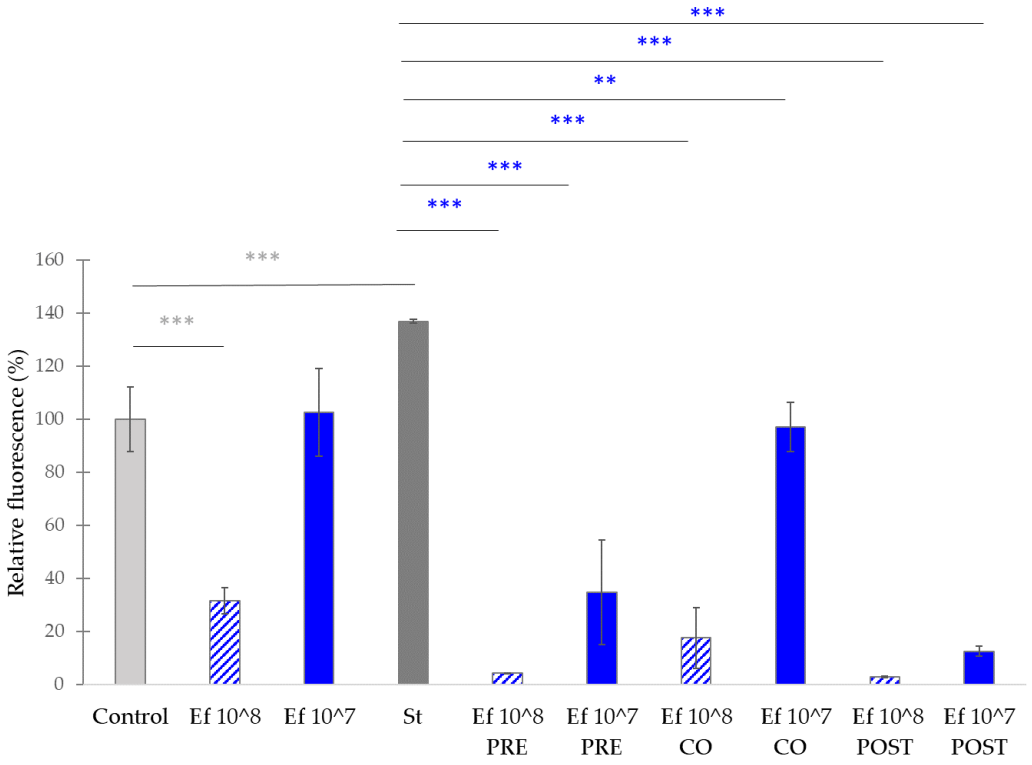


Figure 40. Amount of intracellular ROS after treatment with *S.* Typhimurium (St) and *E. faecium* (Ef) and their combinations. *E. faecium* was added 1 h before (pre-treatment), at the same time (co-treatment) or after (post-treatment) the addition of *S.* Typhimurium. *E. faecium* was added in 10^8 CFU/ml or in 10^7 CFU/ml concentration. **Control**: plain cell culture medium treatment; **St**: *S.* Typhimurium 10^6 CFU/ml; **Ef 10^8**: *E. faecium* 10^8 CFU/ml; **Ef 10^7**: *Ef* 10^7 CFU/ml; **Ef 10^8 PRE**: pre-treatment with *E. faecium* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Ef 10^7 PRE**: pre-treatment with *E. faecium* 10^7 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Ef 10^8 CO**: co-treatment with *E. faecium* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Ef 10^7 CO**: co-treatment with *E. faecium* 10^7 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Ef 10^8 POST**: post-treatment with *E. faecium* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Ef 10^7 POST**: post-treatment with *E. faecium* 10^7 CFU/ml + *S.* Typhimurium 10^6 CFU/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: *** $p < 0.001$, in grey: compared with the untreated control. ** $p < 0.01$,*** $p < 0.001$, in dark blue: compared with treatment with *S.* Typhimurium.

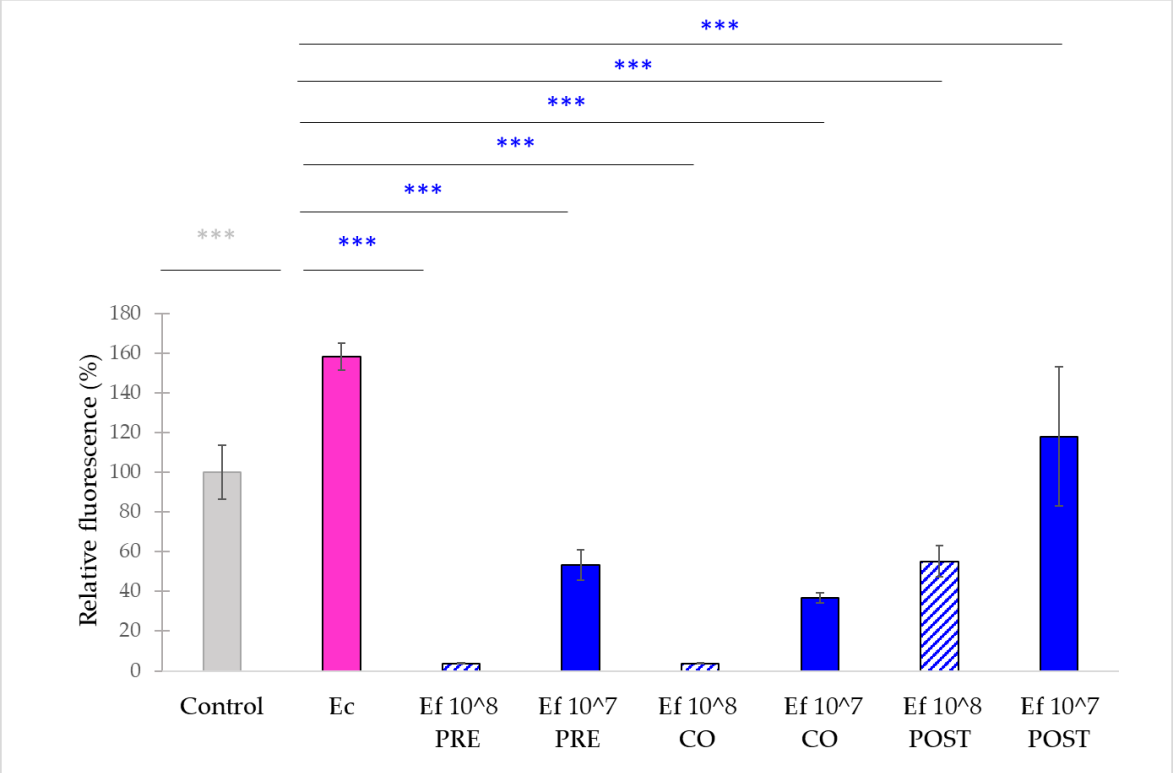


Figure 41. Amount of intracellular ROS after treatment with *E. coli* (Ec) and *E. faecium* (Ef). *E. faecium* was added 1 h before (pre-treatment), at the same time (co-treatment) or after (post-treatment) the addition of *E. coli*. *E. faecium* was added in 10^8 CFU/ml or in 10^7 CFU/ml concentration. **Control**: plain cell culture medium treatment; **Ec**: *E. coli* 10^6 CFU/ml; **Ef 10^8 PRE**: pre-treatment with *E faecium* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Ef 10^7 PRE**: pre-treatment with *E. faecium* 10^7 CFU/ml + *E. coli* 10^6 CFU/ml; **Ef 10^8 CO**: co-treatment with *E. faecium* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Ef 10^7 CO**: co-treatment with *E. faecium* 10^7 CFU/ml + *E. coli* 10^6 CFU/ml; **Ef 10^8 POST**: post-treatment with *E. faecium* 10^6 CFU/ml + *E. coli* 10^6 CFU/ml; **Ef 10^7 POST**: post-treatment with *E. faecium* 10^7 CFU/ml + *E. coli* 10^6 CFU/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: *** $p < 0.001$, in grey: compared with the untreated control. ******* $p < 0.001$, in dark blue: compared with treatment with *E. coli*.

5.3.4.2 Results with Lactobacillus rhamnosus

When IPEC-J2 cells were treated with only *L. rhamnosus*, a decrease in fluorescence could be observed compared to the control (p<0.001). Pre-treatment, co-treatment and post-treatment with *S.* Typhimurium and *L. rhamnosus* resulted in a decreased amount of ROS as compared to cells only challenged by *S.* Typhimurium (p<0.001) (**Figure 42**). The same could be observed, when IPEC-J2 cells were treated with *E. coli*. All three treatment

combination resulted in decresed ROS levels as compared to samples only treated with *E. coli* (p<0.001) (**Figure 43**)

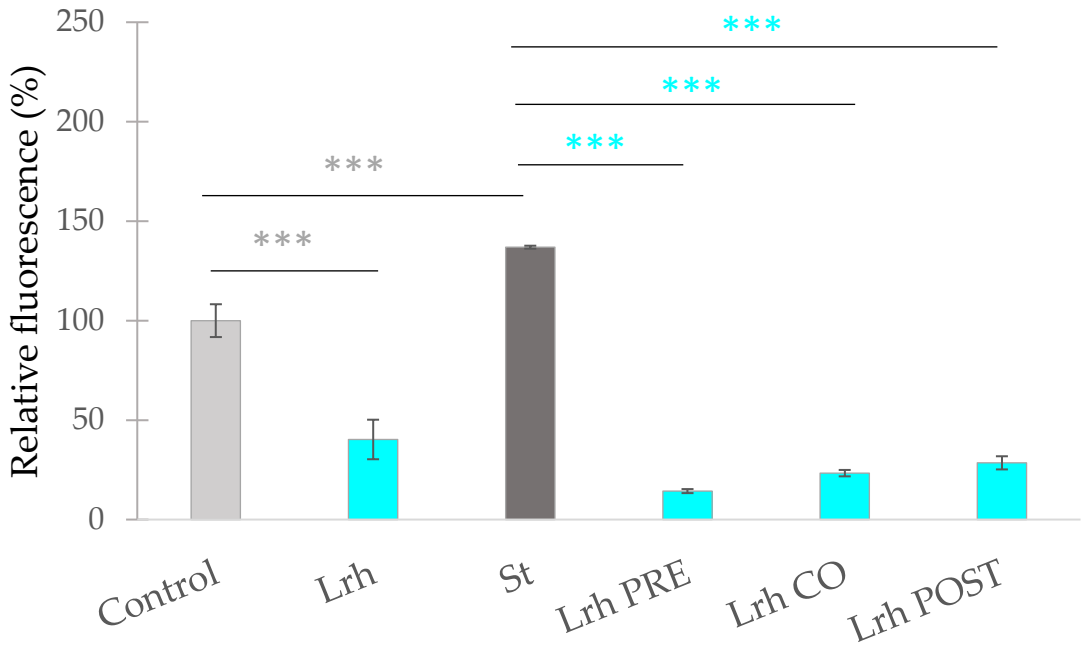


Figure 42. Amount of intracellular ROS after treatment with *S.* Typhimurium (St) and *L. rhamnosus* (Lrh) and their combinations. *L. rhamnosus* was added 1 h before (pre-treatment), at the same time (co-treatment) or after (post-treatment) the addition of *S.* Typhimurium. *L. rhamnosus* was added in 10^8 CFU/ml concentration. **Control**: plain cell culture medium treatment; **Lrh**: *L. rhamnosus* 10^8 CFU/ml, **St**: *S.* Typhimurium 10^6 CFU/ml; **Lrh PRE**: pre-treatment with *L. rhamnosus* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Lrh CO**: co-treatment with *L. rhamnosus* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Lrh POST**: post-treatment with *L. rhamnosus* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/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: *** $p < 0.001$, in grey: compared with the untreated control. *** $p < 0.001$, in light blue: compared with treatment with *S.* Typhimurium.

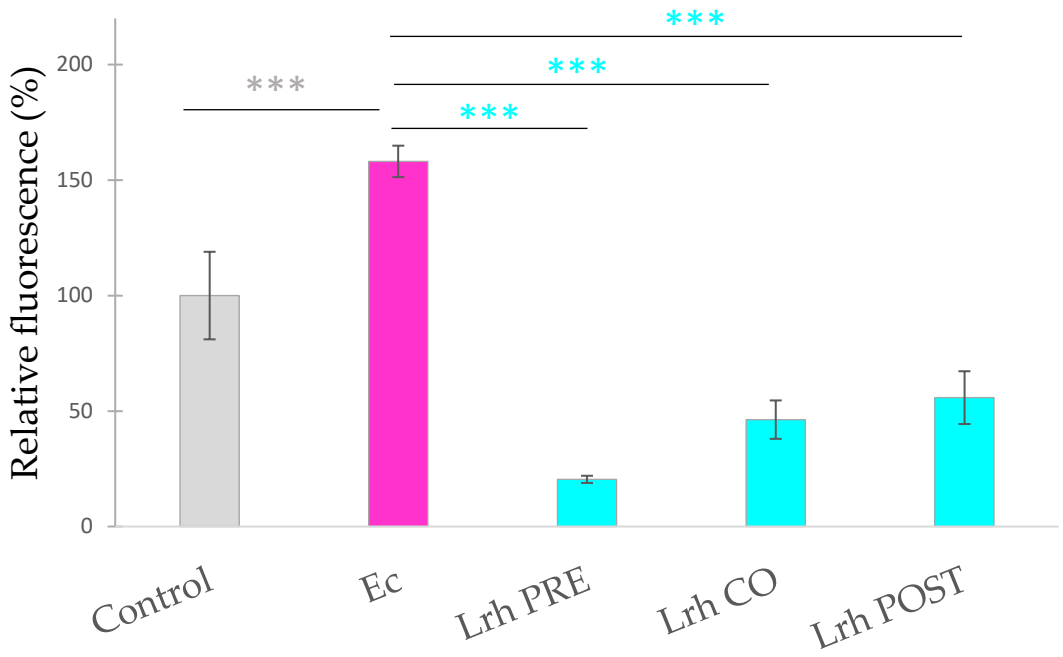


Figure 43. Amount of intracellular ROS after treatment with *E. coli* (Ec) and *L. rhamnosus* (Lrh) and their combinations. *L. rhamnosus* was added 1 h before (pre-treatment), at the same time (co-treatment) or after (post-treatment) the addition of *E. coli*. *L. rhamnosus* was added in 10^8 CFU/ml concentration. **Control**: plain cell culture medium treatment; **Lrh**: *L. rhamnosus* 10^8 CFU/ml, **Ec**: *E. coli* 10^6 CFU/ml; **Lrh PRE**: pre-treatment with *L. rhamnosus* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Lrh CO**: co-treatment with *L. rhamnosus* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Lrh POST**: post-treatment with *L. rhamnosus* 10^8 CFU/ml + *E. coli* 10^6 CFU/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: *** $p < 0.001$, in grey: compared with the untreated control. *** $p < 0.001$, in light blue: compared with treatment with *E. coli*.

5.3.4.3 Results with Bacillus licheniformis and Bacillus subtilis

Treatment with *B. subtilis* alone significantly decreased the fluorescence compared with the control ($p<0.001$); however, when IPEC-J2 cells were treated with only *B. licheniformis*, no significant effect compared with the control could be observed. Pre-, co-, and post-treatment with both probiotic bacteria resulted in a decreased amount of ROS compared with ROS production induced by *S.* Typhimurium ($p<0.001$) (**Figure 44**).

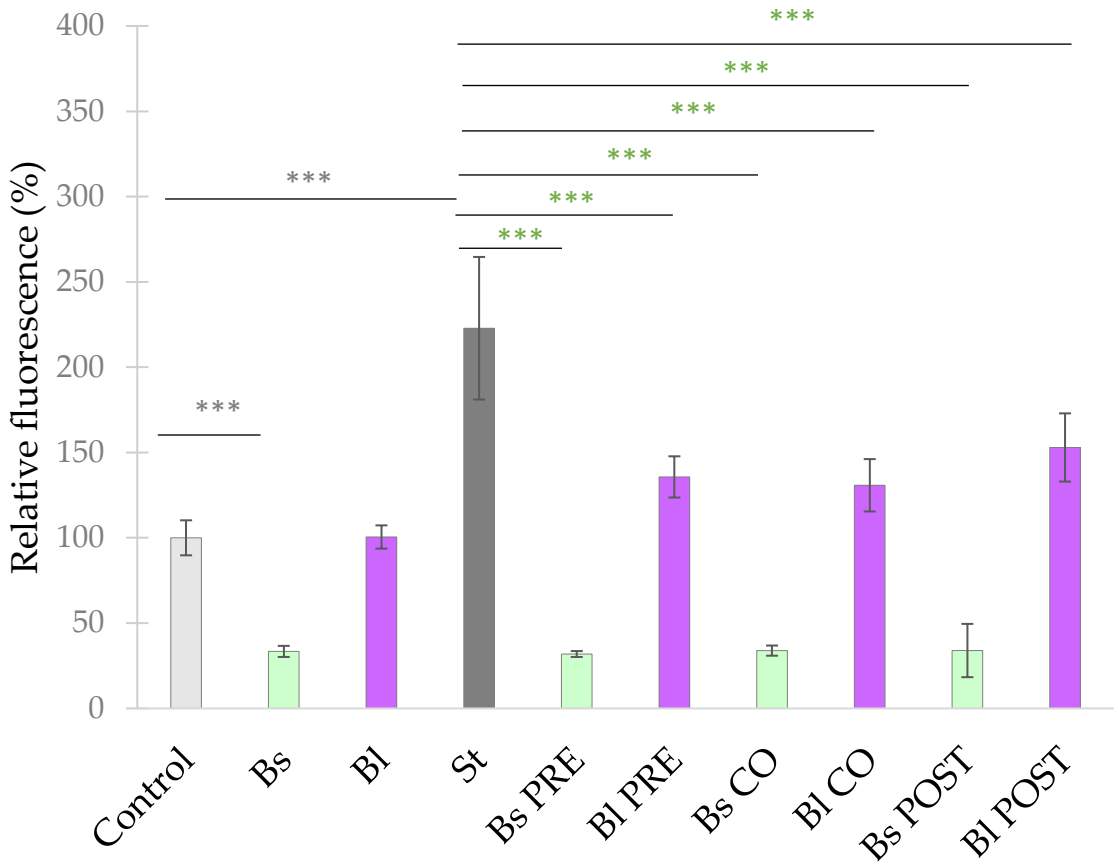


Figure 44. Amount of intracellular ROS after treatment with *S.* Typhimurium (St), *B. licheniformis* (Bl), and *B. subtilis* (Bs) and their combinations. *B. licheniformis* and *B. subtilis* were added 1 h before (pre-treatment), at the same time as (co-treatment), or after (post-treatment) the addition of *S.* Typhimurium. **Control**: plain cell culture medium treatment; **St**: *S.* Typhimurium 10^6 CFU/ml; **Bs**: *B. subtilis* 10^8 CFU/ml; **Bl**: *B. licheniformis* 10^8 CFU/ml; **Bs PRE**: pre-treatment with *B. subtilis* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Bl PRE**: pre-treatment with *B. licheniformis* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Bs CO**: co-treatment with *B. subtilis* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Bl CO**: co-treatment with *B. licheniformis* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Bs POST**: post-treatment with *B. subtilis* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/ml; **Bl POST**: post-treatment with *B. licheniformis* 10^8 CFU/ml + *S.* Typhimurium 10^6 CFU/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: *** $p < 0.001$, in grey: compared with the untreated control. *** $p < 0.001$, in green: compared with treatment with *S.* Typhimurium.

Pre-, co-, and post-treatment with both probiotic bacteria significantly reduced the amount of reactive oxygen species in the cells compared with samples only treated with *E. coli* ($p<0.001$) (**Figure 45**).

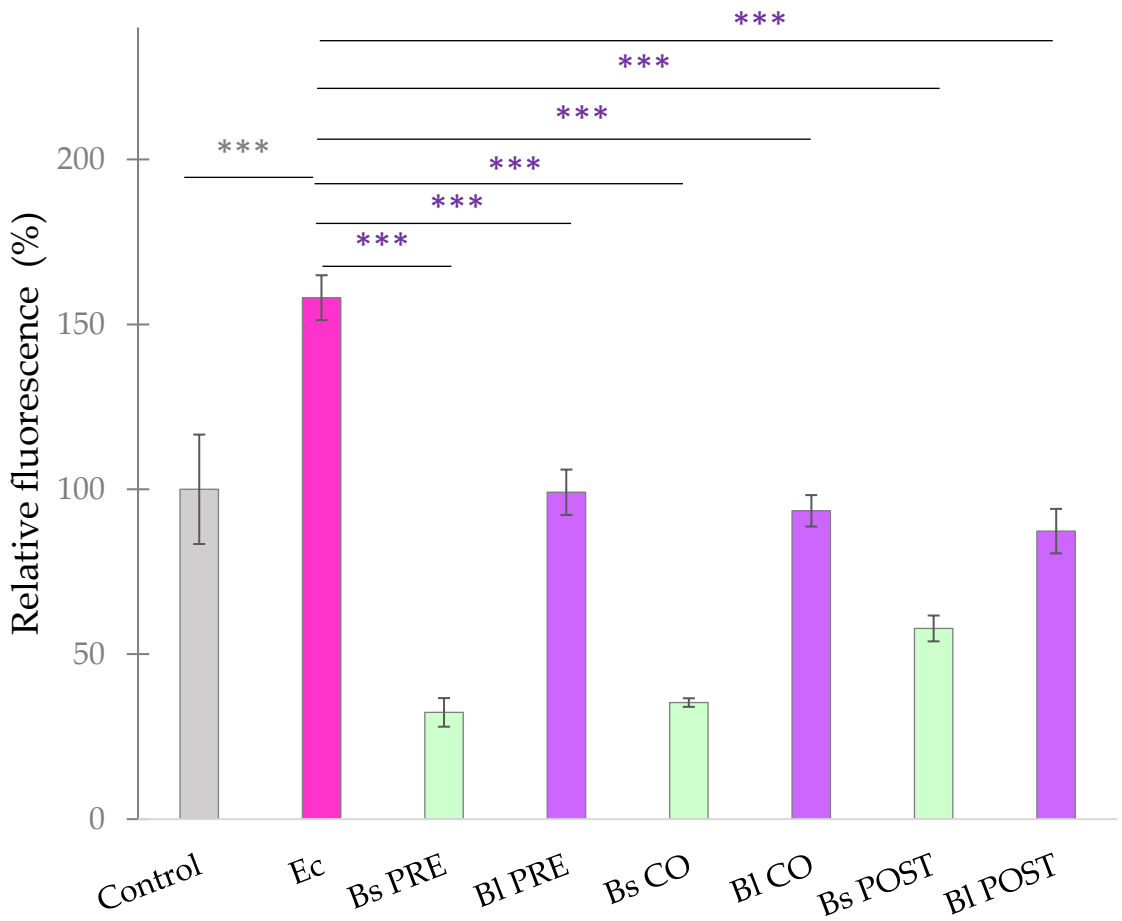


Figure 45. Amount of intracellular ROS after treatment with *E. coli* (Ec), *B. licheniformis* (Bl), and *B. subtilis* (Bs) and their combinations. *B. licheniformis* and *B. subtilis* were added 1 h before (pre-treatment), at the same time as (co-treatment), or after (post-treatment) the addition of *E. coli*. **Control**: plain cell culture medium treatment; **Ec**: *E. coli* 10^6 CFU/ml; **Bs PRE**: pre-treatment with *B. subtilis* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Bl PRE**: pre-treatment with *B. licheniformis* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Bs CO**: co-treatment with *B. subtilis* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Bl CO**: co-treatment with *B. licheniformis* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Bs POST**: post-treatment with *B. subtilis* 10^8 CFU/ml + *E. coli* 10^6 CFU/ml; **Bl POST**: post-treatment with *B. licheniformis* 10^8 CFU/ml + *E. coli* 10^6 CFU/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: *** $p < 0.001$, in grey: compared with the untreated control. *** $p < 0.001$, in purple: compared with treatment with *E. coli*.

5.3.5 Assessment of adhesion inhibition

5.3.5.1 Results with Enterococcus faecium

E. faecium was able to inhibit the adhesion of both *S.* Typhimurium and *E. coli* in all treatment combinations ($p<0.001$). When IPEC-J2 cells were exposed to *S.* Typhimurium, only a minor difference could be found in the effect of adhesion between the different treatment (pre-, co- and post-) conditions. *S.* Typhimurium adhesion was reduced by 87.06% in the case of pre-treatment, by 88.79% in the co-treatment assay, and by 87.64% in the post-treatment. When IPEC-J2 cells were challenged by *E. coli*, pre-treatment with *E. faecium* had the highest inhibitory effect, followed by co-treatment, while post-treatment showed the lowest inhibitory effect. *E. coli* adhesion was decreased by 73.79% in the case of pre-treatment, by 72.13% in the co-treatment assay and by 62.35% in the post-treatment (**Table 7**).

L. rhamnosus was able to inhibit the adhesion of both *S.* Typhimurium ($p<0.001$) and *E. coli* ($p<0.001$ for pre-treatment and post-treatment; $p<0.05$ for co-treatment) in all treatment combinations. When IPEC-J2 cells were challenged by *S.* Typhimurium pre- and co-treatment had almost the same inhibitory effect on the adhesion of *S.* Typhimurium, while post-treatment showed to be slightly less effective. *S.* Typhimurium adhesion was reduced by 96.33% in the case of pre-treatmnet, by 96.76 % in the case of co-treatment and by 91.02 % in the case of post-treatment. When IPEC-J2 cells were exposed to *E. coli* pre-treatment showed the highest inhibitory effect, while co-treatment was the less effective. *E. coli* adhesion was reduced by 90.80 % in the case of pre-treatment, by 34.92% in the case of co-treatment and by 74.63% in the case of post-treatment (**Table 7**).

B. licheniformis was able to inhibit the adhesion of both *S.* Typhimurium and *E. coli* in all treatment combinations ($p<0.001$). When IPEC-J2 cells were challenged by *S.* Typhimurium, pre-treatment with *B. licheniformis* had the highest inhibitory effect, followed by post-treatment, while co-treatment showed the lowest inhibitory effect. *S.* Typhimurium adhesion was reduced by 99.77% in the case of pre-treatment, by 99.65% in the post-treatment assay, and by 99.64% in the co-treatment. When IPEC-J2 cells were exposed to *E. coli*, pre-treatment and co-treatment had almost the same effect, while post-treatment had a lower inhibition effect. *E. coli* adhesion was reduced by 76.37% in the case

of pre-treatment, by 76.89% in the co-treatment assay, and by 49.90% in the post-treatment (**Table 7**).

All treatment combinations with *B. subtilis* could inhibit *E. coli* adhesion to IPEC-J2 cells ($p<0.001$). Pre-treatment with *B. subtilis* was the most effective, followed by co- and post-treatment. *E. coli* adhesion was reduced by 98.57% in the case of pre-treatment, by 97.26% in the co-treatment assay, and by 91.26 % in the post-treatment. However, when IPEC-J2 cells were challenged by *S.* Typhimurium, none of the treatment combinations with *B. subtilis* were able to inhibit the adhesion of the pathogenic bacterium (**Table 7**).

Table 7: Inhibitory effect of *E. faecium*, *L. rhamnosus* , *B. licheniformis* and *B. subtilis* on *S.* Typhimurium or *E. coli* adhesion to IPEC-J2 cells. *S.* Typhimurium or *E. coli* adhesion inhibition was determined upon incubation with *E. faecium/ L. rhamnosus*/ *B. licheniformis*/ *B. subtilis* added 1 h before (pre-treatment), at the same time (co-treatment) and 1 h after (post-treatment) the addition *S.* Typhimurium or *E.coli*. *E. faecium/ L. rhamnosus*/*B. licheniformis*/ *B. subtilis* was added in 10^8 CFU/ml. **PRE**: pre-treatment with *E. faecium/ L. rhamnosus*/*B. licheniformis*/ *B. subtilis* 10^8 CFU/ml + *S.* Typhimurium or *E.coli* 10^6 CFU/ml; **CO**: co-treatment with *E. faecium/ L. rhamnosus*/*B. licheniformis*/ *B. subtilis* 10^8 CFU/ml + *S.* Typhimurium or *E.coli* 10^6 CFU/ml; **POST**: post-treatment with *E. faecium/ L. rhamnosus*/*B. licheniformis*/ *B. subtilis* 10^8 CFU/ml + *S.* Typhimurium or *E.coli* 10^6 CFU/ml. Data are shown as bacterial count reduction compared to the mean value of control (attached bacteria without probiotic treatment) that was considered as 100%. n=4/group. Significant difference compared to the untreated control: * $p < 0.05$, *** $p < 0.001$.

Treatment	*S.* Typhimurium		*E.coli*	
	Reduction	p value	Reduction	p value
E. faecium PRE	-87.06%	p<0.001***	-73.79%	p<0.001***
E. faecium CO	-88.79%	p<0.001***	-72.13%	p<0.001***
E. faecium POST	-87.64%	p<0.001***	-62.35%	p<0.001***
L. rhamnosus PRE	-96.33%	p<0.001***	-90.80%	p<0.001***
L. rhamnosus CO	-96.76%	p<0.001***	-34.92%	p<0.05*
L. rhamnosus POST	-91.02%	p<0.001***	-74.63%	p<0.001***
B. licheniformis PRE	-99.77%	p<0.001***	-76.37%	p<0.001***
B. licheniformis CO	-99.64%	p<0.001***	-76.89%	p<0.001***
B. licheniformis POST	-99.65%	p<0.001***	-49.9%	p<0.001***
B. subtilis PRE	-52.49%	p=0.05	-98.57%	p<0.001***
B. subtilis CO	-22.53%	p=0.39	-97.26%	p<0.001***
B. subtilis POST	-30.92%	p=0.24	-91.26%	p<0.001***

6 Discussion

Intestinal diseases caused by *E. coli* and Salmonella spp. may lead to significant economic loss in food-producing animals and may also pose a threat to human health as (1) both bacteria are zoonotic, (2) they may contaminate pork products in the food chain, and (3) they may develop resistance to antibiotics, thus contributing to the transmission of antimicrobial resistance (Dubreuil, 2017; Kovács et al., 2022; Zimmerman et al., 2012). Finding alternative feed additives capable of maintaining the health of the gastrointestinal tract without the use of antibiotics has become an important issue to all food animal-producing sectors—including the swine industry (Alagawany et al., 2021). A healthy gut has four prerequisites: (1) proper barrier function, (2) intestinal immune fitness, (3) oxidative stress homeostasis, and (4) microbiota balance (Chalvon-Demersay et al., 2021; Luise et al., 2022). Probiotics have been shown to exert beneficial effects on the above-mentioned preconditions; however, the effect of these probiotics is strain/species-dependent (Dubreuil, 2017; Liu et al., 2015; Oelschlaeger, 2010; Roselli et al., 2017; F. Yang et al., 2015). In this study beneficial effects of four probiotic candidates of porcine origin, *E. faecium*, *L. rhamnosus*, *B. licheniformis*, *B. subtilis*, were tested as potential feed additives capable of strenghtening the GIT and thereby preventing or contributing to the treatment of gastrointestinal bacterial infections in swine. Our study was the first to comprehensively test the effect of *E. faecium*, *L. rhamnosus*, *B. licheniformis*, *B. subtilis* on intracellular ROS production, inflammatory cytokine response, paracellular permeability and adhesion inhibition of *E. coli* and *S.* Typhimurium in porcine *in vitro* epithelial cell model.

As the first step, we demonstrated the effect of *E. faecium*, *L. rhamnosus*, *B. licheniformis* and *B. subtilis* and their cell free supernatants on the viability of IPEC-J2 cells. According to these results we could select appropriate treatment conditions for our further investigations. In the case of SCS the appropriate treatment time was set at 1h and the applied concentration was set at 6%, and for our experiments using bacteria, we decided to apply 10^8 CFU/ ml concentration and 1 hour treatment time. Our results are summarized in **Table 8**. In most of the cases the treatment of IPEC-J2 cells with SCS or bacteria did not result in any significant change in cell viability. Similarly, no effect on cell viability has also been reported for *L. johnsonii* and *L. reuteri* (Liu et al., 2015). However, in some cases increased absorbance values were measured, indicating an increase in the number of viable

cells. Increased cell viability was also reported for probiotic strain *Clostridium tyrobutyricum* previously (Xiao et al., 2018). Furthermore, some treatment conditions also resulted in decreased cell viability. A decreased number of viable cells was also reported for probiotic strain *Lactobacillus rhamnosus* GG upon twelve hours incubation period (Liu et al., 2015). Longer incubation periods might be preferable for reaching high concentrations of bacterial products that can contribute to exerting antimicrobial effect against pathogens, however the secretion of such products might have an adverse effect on IPEC-J2 cells (Muñoz-Quezada et al., 2013). Strain-, and species-specific effects of probiotics have been reported by many authors, however to our knowledge our results are the first proving with the use of Neutral Red Uptake method that probiotics affect the viability of IPEC-J2 cells in a strain/species-specific manner. Also, the applied treatment time and treatment concentration might contribute to the different effects on cell viability, however further studies would be necessary to determine the exact time-, and concentration dependence of the applied probiotic bacteria and their SCSs on the number of viable IPEC-J2 cells.

Our second objective was to examine whether cell-free bacterial spent culture supernatants of *E. faecium*, *L. rhamnosus*, *B. licheniformis* and *B. subtilis* can achieve beneficial effects. Therefore, we aimed to elucidate the antimicrobial and antioxidant effect of SCSs of *E. faecium*, *L. rhamnosus*, *B. licheniformis* and *B. subtilis.* Antioxidant properties are one of the many beneficial effects that probiotics might exert (Wang et al., 2017a). As summarized in **Table 9** we have demonstrated that SCSs of *E. faecium*, *L. rhamnosus*, *B. licheniformis* and *B. subtilis* could remarkably reduce ROS generation induced by *S.* Typhimurium derived LPS. Moreover, SCSs of B. *licheniformis* and *B. subtilis* could also counteract ROS generation evoked by *E. coli* 111 and *E. coli* 127 derived LPS. Antioxidant capacity of spent culture supernatant have been proved for other probiotics; SCS of *Bifidobacterium animalis* 01 has been found to scavenge hydroxyl radicals and superoxide anion *in vitro*, moreover it has also been shown to enhance antioxidase activites of mice *in vivo*. Cell-free extract of *Lactobacillus helveticus* CD6 has shown to exert antioxidant properties through chelating Fe^{2+} ions (Wang et al., 2017a).

Table 8: Summary of cell viability measurements using NRU method. Fonts in **green** indicate those treatment conditions, which significantly increased cell viability (compared with control cells), fonts in grey indicate those treatment conditions, which did not have any effect on cell viability and **blue** indicates those treatment conditions, which significantly reduced cell viability (compared with control cells).

Probiotic strain	Cell viability using SCS	Cell viability using bacteria
E. faecium	[3%, 1h]; [6%, 1h]; [12%, 1h]; [24%, 1h]; [3%, 2h]; [6%, 2h]; [12%, 2h]; [24%, 2h]; [3%, 4h]; [6%, 4h]; [12%, 4h]; [24%, 4h]; [3%, 24h]; [6%,2 4h]; [12%, 24h]; [24%,2 4h]	[10^4 CFU/ml, 1h]; [10^6 CFU/ml, 1h]; [10^8 CFU/ml, 1h]; [10^4 CFU/ml, 2h]; [10^6 CFU/ml, 2h]; [10^8 CFU/ml, 2h] [10^4 CFU/ml, 4h]; [10^6 CFU/ml, 4h]; [10^4 CFU/ml, 24h]; [10^6 CFU/ml, 24h]; [10^8 CFU/ml, 4h]; [10^8 CFU/ml, 24h]
L. rhamnosus	[3%, 1h]; [6%, 1h]; [12%, 1h]; [3%, 2h]; [6%, 2h]; [3%, 4h]; [6%, 4h]; [3%, 24h] [24%, 1h]; [12%,2h] [24%, 2h]; [12%,4h]; [24%,4h]; [6%,24h]; [12%,24h]; [24%,24h];	[10^8 CFU/ml, 1h]; [10^8 CFU/ml, 2h]
B. licheniformis	[6%, 1h]; [24%, 1h]; [3%, 1h]; [12%,1h]; [3%,2h]; [6%,2h]; [12%,2h]; [24%,2h]; [3%, 4h]; [6%, 4h]; [12%, 4h]; [24%, 4h]; [3%, 24h]; [6%, 24h]; [12%, 24h]; [24%, 24h];	[10^8 CFU/ml, 1h]; [10^8 CFU/ml, 2h]
B. subtilis	[3%, 1h]; [6%, 1h]; [12%, 1h]; [24%, 1h]; [3%, 2h]; [6%, 2h]; [12%, 2h]; [24%, 2h]; [3%, 4h]; [6%, 4h]; [12%, 4h]; [24%, 4h] [3%, 24h]; [6%, 24h]; [12%, 24h]; [24%, 24h]	[10^8 CFU/ml, 1h]; [10^8 CFU/ml, 2h]

Our results suggest that the antioxidant capacity of SCSs of *B. licheniformis* and *B. subtilis* is independent of the type of LPS used. However, SCS of *L. rhamnosus* did not have any significant effect on *E. coli* 111 LPS induced ROS production and when challenged with *E. coli* 127 derived LPS ROS production was further increased. Also SCSs of *E. faecium* further increased ROS production evoked by *E. coli* 111 and *E. coli* 127 derived LPS. Taken together our results suggest that SCSs of probiotic bacteria may effect the intracellular ROS production of IPEC-J2 cells in a species-specific manner. The type of LPS used to evoke oxidative stress seems also to be an influencing factor, suggesting that probiotics use different strategies to combat the deleterious effect of different pathogens. Species-dependent probiotic properties have also been shown when investigating other probiotic properties, e. g. antibacterial or adherence properties. Distinct effects on different pathogens

has also been proved for *B. breve* CNCM I-4035 supernatant (Muñoz-Quezada et al., 2013). Due to the limitation of the DCFH-DA method (that measures the total ROS content) we cannot determine the exact mechanism of how probiotic bacteria derived SCSs exert their oxidative stress decreasing capacity, but compounds with antioxidant properties (e.g. glutathione, butyrate, and folate) might have a direct antioxidative effect (Wang et al., 2017b). Folate production is rather typical for Bifidobacteria, however also other probiotic species e.g. *Lactococcus lactis Streptococcus thermophilus* and *Lactobacillus helveticus* have shown folate producing characteristics (Rossi et al., 2011; Wang et al., 2017b). An *in vivo* study conducted on rats revealed that a multispecies probiotic mixture (containing *Lactobacillus acidophilus*, *L. casei*, *L. salivarius*, *Lactococcus lactis*, *Bifidobacterium bifidum*, and *B. lactis)* enhanced the synthesis of GSH both locally (in the pancreas) and systemically (Lutgendorff et al., 2008). Furthermore, high ROS levels might induce the transcription of antioxidant enzymes and detoxifying proteins via the Nrf2-Keap1-ARE, NFκB, MAPK and PKC pathways. Hydrogen peroxide induced oxidative stress in IPEC-1 cell line could be reduced by *Bacillus amyloliquefaciens* via regulating Nrf2 expressions and resulting in decreased ROS levels (Wang et al., 2017b). To reveal the exact underlying mechanisms further studies addressing to measure the contituents (e.g., hydrogen peroxide content, glutathione redox ratio, activity of superoxide dismutase) of total antioxidant capacity more specifically would be necessary. If dietery antioxidants behave as prooxidants or antioxidants depends on their concentration and the nature of surrounding molecules. Ascorbic acid is considered to be an antioxidant, however if Fe^{3+} is present in the surrounding, ascorbic acid combines with F^{3+}, resulting Fe^{2+}. Later might further react with H_2O_2, leading to increased $HO^{\cdot}$ levels and thus indirectly contributing to the prooxidant effect through the elevated $HO^{\cdot}$ concentration. Also α-tocopherol, certain flavonoids and phenolics can become proxidants depending on the environment in which they are inserted (Carocho and Ferreira, 2013). In our case, it is supposed that probiotic SCSs might contain antioxidant components. LPS is a cell wall component of Gram-negative bacteria, however bacteria belonging to different genera differ in their LPS type. LPSs can differ in their O-antigen, size, composition, and lipid A component. Furthermore, the lipid A part of LPS also differs among bacterium strains. The evoked immune response depends on the structure of LPS's lipid A part (Farhana and Khan, 2022). In our experiments three different LPSs were used that differ in their structure and since the structure of LPS influences the immune response (including

the induction of proinflammatory cytokines) that further confers to oxidative stress in an indirect way, it may be hypothesized that the different types of LPS establish distinct oxidative stress environments (characterized by different ROS composition and concentration) in the IPEC-J2 cells. As mentioned before, if an antioxidant substance behaves as prooxidant depends on the redox state of the surrounding environment (Carocho and Ferreira, 2013). The SCS of *E. faecium* and *L. rhamnosus* most probably contain components with antioxidant properties, that (depending on the different environmental composition) might act as prooxidants or antioxidants. However, further experiments (including the qualitative and quantitative determination of SCS compositions and the selective determination of ROS types) would be necessary to support these assumptions.

Table 9: Summary of the effects of SCSs on ROS production induced by different types of LPS. — in **green**: indicates no change in ROS production (compared with the untreated control) **— in black**: indicates no change in ROS production (compared with treatment with only LPS of *E. coli* 111 B:4 origin), ↓ **in black**: indicates decrease in ROS production (compared with treatment with LPS derived from *S.* Typhimirium, *E. coli* 111 B:4 or *E. coli* 127 B:8 respectively), ↑ **in black**: indicates increase in ROS production (compared with treatment with LPS derived from *S.* Typhimirium, *E. coli* 111 B:4 or *E. coli* 127 B:8 respectively)

Applied probiotic species	Probiotic alone	+ LPS *St*	+ LPS *E. coli* 111	+ LPS *E. coli* 127
E. faecium	—	↓	↑	↑
L. rhamnosus	—	↓	—	↑
B. licheniformis	—	↓	↓	↓
B. subtilis	—	↓	↓	↓

None of the cell-free spent culture supernatants were able to exert antimicrobial activity against the tested *E. coli* and *S.* Typhimurium strains. Probiotics produce organic acids and/or proteinaceous compounds that remain active in acidic pH and these may be responsible for the antimicrobial effect (Muñoz-Quezada et al., 2013). Spent culture supernatants of probiotic bacteria were prepared after 24 hours incubation, because it is suggested that incubation time might contribute to higher concentrations of inhibitory compounds (Muñoz-Quezada et al., 2013). Our results suggest that no componds with antimicrobial properties were produced that would have been able to inhibit the growth of the tested pathogenic bacteria or the concentration of inhibitory substances was not high enough to inhibit the growth of tested pathogenic bacteria. However, in the case of lactic acid producing bacteria (*E. faecium* and *L. rhamnosus)* neutralization of the pH might have led to the loss of antimicrobial capacity. At low pH organic acids are present in non-dissociated forms which enables them to penetrate into the hydrophobic cell membranes of

pathogens. Antimicrobial effect of *L. rhamnosus* against *S.* Typhimurium was attributed to lactic acid (Muñoz-Quezada et al., 2013). Further studies would be necessary to exclude pH neutralizing effects on antimicrobial activity. Others found that cell-free supernatant of *L. plantarum* was able to inhibit the growth of *C. difficile* and inhibitions was independent of pH neutralization (Fijan and Fijan, 2016). However, the inhibitory capacity *L. paracasei* CNCM I-4034 supernatants against *S.* typhi CECT 725 was completely lost when supernatant was neutralized. Similarily, not neutralised supernatants of *L. rhamnosus* CNCM I-4036 inhibited the growth of *S.* typhi CECT 725 and *E. coli* ETEC CECT 515, however after neutralizations inhibition effects diminished (Muñoz-Quezada et al., 2013).

The third objective of our study was to evaluate the *in vitro* probiotic potential of *E. faecium*, *L. rhamnosus*, *B. licheniformis* and *B. subtilis* against pathogen-induced damages using bacteria. The effects on paracellular permeability, inflammatory response, ROS production, and adhesion inhibition were investigated. Our hypothesis was that *E. faecium*, *L. rhamnosus, B. licheniformis* and *B. subtilis* might (1) improve epithelial integrity, (2) reduce the secretion of proinflammatory cytokines, (3) alleviate the amount of reactive oxygen species, and (4) inhibit the adhesion of pathogenic bacteria. Two economically important swine pathogens, inducers of a wide range of gastrointestinal diseases in pigs, *S.* Typhimurium and *E. coli*, were chosen to challenge IPEC-J2 cells *in vitro* (Dubreuil, 2017; Pan et al., 2017; Skjolaas et al., 2007; Zimmerman et al., 2012).

Intestinal permeability is a good marker to monitor epithelial barrier function. Pathogens can disrupt barrier integrity, which leads to increased gut permeability, occurrence of diarrhea, and leaky gut syndrome (Chalvon-Demersay et al., 2021). Probiotics have been shown to enhance the intestinal barrier function. The deleterious effect of LPS causing a decrease of TJ proteins could be counteracted by pre-treatment of *L. reuteri* I5007 or its culture supernatant in IPEC-J2 cells. Furthermore, *L. reuteri* I5007 also increased the abundance of TJ proteins (claudin-1, occludin and zonula occludens-1) in newborn piglets (F. Yang et al., 2015). In our experiments, the FD4 method was used to assess the changes in the integrity and permeability of the epithelial barrier. Interestingly, *E. coli* or *S.* Typhimurium induced pathophysiological challenge resulted in a significant increase in the amount of FD4 dye measured in the basolateral compartment, indicating that these strains were able to disrupt the integrity of the barrier, in line with previous findings (Geens and Niewold, 2010).

Lipopolysaccharides or bacterial metabolites (secreted effector molecules and bacterial surface proteins) might be responsible for the disruption of the epithelial barrier. Pathogens might also induce the apoptosis of enterocytes or cause the opening of the paracellular permeation pathway (due to change or delocalization of TJ or cytoskeletal proteins), which results in increased TEER values, indicating that the barrier function has been damaged (Lodemann et al., 2015). In our experiments, *E. faecium* and *B. licheniformis* alone had no significant effect on paracellular permeability. Interestingly, *B. subtilis* alone increased, while *L. rhamnosus* decreased the paracellular permeability. Our experimental results with *E. faecium*, *L. rhamnosus* and *B. licheniformis* are in line with studies showing that the use of probiotics alone might either not affect the integrity of the epithelial barrier or enhance the barrier function (Czerucka et al., 2000; Ewaschuk et al., 2008; Lodemann et al., 2015; Otte and Podolsky, 2004; Resta-Lenert and Barrett, 2003; Sherman et al., 2005). Lactobacilli had no effect on the barrier integrity of polarized intestinal epithelia (Sherman et al., 2005). *Enterococcus faecium* per se had no effect on the barrier integrity of IPEC-J2 cells; however, on Caco-2 cells, barrier function was enhanced (Lodemann et al., 2015). In the case of *B. subtilis* alone, the increased FD4 flux indicates that the barrier function has been changed. Similars results have been found by Larsen et al., who investigated the effect of *B. subtilis* isolates on the barrier integrity of IPEC-J2 cells. TEER values (indicators of barrier integrity) dropped within the first 6 hours of treatment (Larsen et al., 2014). Other *Bacillus* species (*B. cereus* var. toyoi) have also caused the impairment of barrier integrity in the first 3 hours after exposure (Larsen et al., 2014). Barrier integrity of IPEC-J2 cells was also decreased by other probiotic species, e.g. *Enterococcus faecium* from 8 h incubation onward. (Lodemann et al., 2015). Moreover, Hosoi et al. found that two non-pathogenic *B. subtilis* species decreased TEER values of Caco-2 cells. The concentration of the bacterial suspension was 10^7 CFU/ml (which is lower than the concentration applied in our experiments) indicating that the barrier integrity damaging effect may occur even at lower concentration values (Hosoi et al., 2003). Larsen finds it difficult to explain the deleterious effect of *B. subtilis* on epithelial integrity, since their safety have been proved in animal trials (Larsen et al., 2014). According to Hosoi et al., *B. subtilis* influences the function of TJ proteins resulting in decreased TEER values (Hosoi et al., 2003). This may also explain our findings. However, to get a more complex insight of *B. subtilis*'s effect on the paracellular permeability of IPEC-J2 cells further experiments (including immunefluorescence and

quantitative ultrastructural analysis) will be needed that aim to reveal the caused changes in the structure TJ proteins and in the ultrastructure of epithelial cells. Our experiments showed that pre-treatment, co-treatment, and post-treatment with *E. faecium* and *L. rhamnosus* could also prevent the damaging effects on barrier integrity induced by *E. coli* or *S.* Typhimurium, and significantly reduce the FD4 flux. Studies on Caco-2 and T84 cells have also shown that probiotic bacteria (*L. plantarum*, *L. acidophilus*, or *L. rhamnosus*) could prevent the barrier disrupting effects of *E. coli* (Anderson et al., 2010; Sherman et al., 2005). In our experiments, neither *B. licheniformis* nor *B. subtilis* was able to counteract the increased FD4 flux elicited by *S.* Typhimurium or *E. coli*. Unexpectedly, in some treatment combinations, the FD4 flux was further increased. This inconsistency might be because of the fact that probiotic properties are species-dependent. When the effect of different probiotic bacteria (*Lactobacillus delbrueckii* ssp. bulgaricus no. 3; *Lactobacillus casei* no. 9; *Lactobacillus gasseri* no. 10; *Lactobacillus rhamnosus* OLL2838) on TNF-α-induced barrier impairment was investigated, only one strain (*Lactobacillus rhamnosus* OLL2838) was effective in counteracting the disruption of the barrier (Miyauchi et al., 2009). Results of our paracellular permeability assays are summerized in **Table 10**.

Table 10: Summary of paracellular permeability measurements using FD4 method. St: *S.* Typhimurium, **Ec**: *E. coli*, **PRE**: pre-treatment, **CO**: co-treatment, **POST**: post-treatment. **— in green**: indicates no change in paracellular permeability (compared with the untreated control), **↓ in green:** indicates decrease in paracellular permeability (compared with the untreated control), **↑ in green:** indicates increase in paracellular permeability (compared with the untreated control) **— in black**: indicates no change in paracellular permeability (compared with treatment with only *S.* Typhimurium or *E. coli*), **↓ in black**: indicates decrease in paracellular permeability (compared with treatment with only *S.* Typhimurium or *E. coli)*, **↑ in black**: indicates increase in paracellular permeability (compared with treatment with only *S.* Typhimurium or *E. coli*)

		St			Ec		
	Probiotic alone	PRE	CO	POST	PRE	CO	POST
E. faecium	—	↓	↓	↓	↓	↓	↓
L. rhamnosus	↓	↓	↓	↓	↓	↓	↓
B. licheniformis	—	—	—	—	↑	↑	↑
B. subtilis	↑	↑	↑	↑	↑	↑	↑

Pathogen-induced inflammation activates the immune system and various cytokines are synthetized. In the absence of challenge, low concentrations of proinflammatory cytokines (TNF-a, IFN-g, IL-1, IL-4, IL-6, IL-8) are indicators of immune fitness (Anderson et al., 2010; Bahrami et al., 2011; Carey and Kostrzynska, 2013; Chalvon-Demersay et al., 2021; Devriendt et al., 2010; Geens and Niewold, 2010; Kagnoff and Eckmann, 1997; Luo and

Zheng, 2016; Miyauchi et al., 2009; Resta-Lenert and Barrett, 2003; Turner et al., 2014). Previous studies have shown that probiotic bacteria can alter the expression of cytokines in epithelial cells (Bahrami et al., 2011; Carey and Kostrzynska, 2013). IL-8 is a chemoattractant cytokine that can be produced by a variety of tissue and blood cells, but one of its major functions is to attract and activate neutrophils to inflammatory regions. IL-6 is a proinflammatory cytokine and is a stimulator of acute-phase proteins (Cotton et al., 2016; Luo and Zheng, 2016; Turner et al., 2014). However, the exact mechanism by which probiotics exert their influence on cytokine production need be further investigated (Klingspor et al., 2015). In our experiments, when IPEC-J2 cells were exposed to *E. coli* or *S.* Typhimurium, both IL-6 and IL-8 synthesis were significantly increased, a result also demonstrated by many previous studies (Devriendt et al., 2010; Klingspor et al., 2015; Skjolaas et al., 2007). The pre-treatment with *E. faecium* in a concentration of 10^8 CFU/ml could abrogate the increase in both IL-6 and IL-8 secretion, while the co-incubation with *E. faecium* applied at a concentration of 10^8 CFU/ml could also significantly decrease the secretion of IL-8 when an inflammatory response was evoked by *S.* Typhimurium. Pre-, co- , and post-treatment with *L. rhamnosus* could also counteract the *Salmonella*-induced IL-8 secretion, furthermore pre-, and post treatment also decreased elevated IL-6 secretion. *Salmonella*-induced IL-8 secretion was decreased by probiotic strains *Lactobacillus reuteri* ATCC 53608, which agrees with our finding, that probiotics may attenuate the proinflammatory cytokine response upon pathophysiological challenge (Roselli et al., 2017). When IPEC-J2 cells were challenged with *E. coli*, the pre- and co-incubation with 10^8 CFU/ml *E. faecium* either did not show any effect on the production of proinflammatory cytokines (IL-6) or unexpectedly, further increased their secretion (IL-8). Pre-, co-, and post-treatment with *L. rhamnosus* also failed to decrease *E. coli*-induced IL-6 production. Others, however, found that the *E. coli* induced IL-8 elevation was reduced by *E. faecium* co-incubation (Klingspor et al., 2015; Tian et al., 2016). This inconsistency might be due (1) to the different pathogenic strains used to evoke inflammation and (2) to differences in the mode of action of various probiotic strains (Klingspor et al., 2015; Roselli et al., 2017). Inflammatory cytokine reducing effect of probiotics also depends on the pathogenic species/strain that is used to evoke inflammation. When IPEC-J2 cells and Caco-2 cells were challenged with ETEC, increase in IL-8 expression could be prevented by *E. faecium*, however no such beneficial effects could be observed when EPEC was used to induce

inflammation (Klingspor et al., 2015). Bacterial species are genetically remarkably heterogen. Genomic differences can be considerable even within different strains of the same species. It is supposed that the human and animal body would respond differently to different strains of the same species (Hakansson and Molin, 2011). When the inflammatory response was elicited by *S.* Typhimurium, all treatment combinations (pre-, co-, and post-treatment) with *B. licheniformis* could counteract the increase in IL-6 secretion. *B. licheniformis* has also been shown to decrease elevated IL-6 levels *in vivo* (Cameron and McAllister, 2019; Deng et al., 2012). However, applying *B. subtilis*, only the pre-treatment with the probiotic bacteria could abrogate the elevated IL-6 synthesis. Interestingly, increased IL-8 production induced by *S.* Typhimurium was significantly further increased by the post-treatment with *B. licheniformis*. Others found that *Salmonella*-induced IL-8 secretion was decreased by *Bacillus licheniformis* ATCC 10716 (Roselli et al., 2017). The treatment of IPEC-J2 cells with *B. licheniformis* alone significantly increased the IL-8 secretion compared with the control, while the treatment with *B. subtilis* alone raised the IL- 6 synthesis. A commensal microbe-mediated response might be similar to a pathogen-mediated response and increased proinflammatory cytokine secretions were also observed in other studies (Skjolaas et al., 2007). Oral administration of *L. reuteri* and *L. brevis* in mice induced proinflammatory cytokines IL-1β, IL-2 and TNF-α however failed to induce anti-infammatory cytokines such as IL-10 and IL-4 (Maassen et al., 2000). It is not only LPS that can induce inflammatory response, other metabolites may be involved and gram-positive bacteria might also induce inflammation (Hakansson and Molin, 2011). Our data suggest that the pre-, co-, and post-treatment with *B. licheniformis* or *B. subtilis* offered no protection effect against *E. coli*-induced IL-6 and IL-8 secretion. Unexpectedly, pre-treatment with *B. licheniformis* further increased the secretion of IL-8 synthesis induced by *E. coli*. Others, however, found that *E. coli*-induced IL-8 elevation was counteracted by probiotic bacteria (Klingspor et al., 2015; Tian et al., 2016). Similar to the resident GI microbiota, certain probiotic bacteria might be more prone to counteract pathogen-induced inflammation than others. When *Clostridium* species were compared, *Faecalibacterium prausnitzii* showed anti- inflammatory effects by blocking NFκB activation and decreasing IL-8 secretion in Caco-2 cells. (Hakansson and Molin, 2011). Furthermore, animal models demonstrated that different taxa of microorganisms in combination can enhance pathogenic effects (Hakansson and Molin, 2011). We thus suppose that also probiotic and pathogen effects

could be synergistic. Our results on the immunomodulatory effect of probiotics are summarized in **Table 11**.

Taken together our results suggest that the effect of probiotics on proinflammatory response of IPEC-J2 cells is strain/species specific and also depends on the type of cytokine examined and on the causative agent (*E. coli* or *S.* typhimurium) used to evoke inflammation. The time of addition of probiotics also seem to influence the inflammation-reducing effect, however to determine time-dependency further measurements are need.

Table 11: Summary of the results of IL-6 and IL-8 measurements. **St:** *S.* Typhimurium, **Ec**: *E. coli*, **PRE**: pre-treatment, **CO**: co-treatment, **POST**: post-treatment. 10^7: 10^7 CFU/ml, 10^8: 10^8 CFU/ml. **Orange** colours indicate the changes in IL-6 secretion (compared with untreated control cells), **blue** colours indicate the changes in IL-8 secretion (compared with untreated control cells), **pink** colours indicate the changes in IL-6 secretion (compared with treatment with *S.* Typhimurium or *E. coli*), **green** colours indicate the changes in IL-8 secretion (compared with treatment with *S.* Typhimurium or *E. coli*). —: indicates no change; ↑: indicates increased secretion; ↓ indicates decreased secretion.

	Probiotic alone		St PRE		St CO		St POST		Ec PRE		Ec CO		Ec POST	
	10^7	10^8	10^7	10^8	10^7	10^8	10^7	10^8	10^7	10^8	10^7	10^8	10^7	10^8
E. faecium	—	—	—	↓	—	—			—	—	—	—		
	—	—	—	↓	—	↓			—	↑	—			
											↑			
L. rhamnosus	—			↓	—		↓		—		—		—	
	—			↓	—	↓	↓							
B. licheniformis	—			↓	↓		↓		—		—		—	
	↑			—	—		↑		↑		—			
B. subtilis	↑			↓	—		—		—		—		—	
	—			—	—		—		—		—		—	

The measurement of ROS is a marker to monitor oxidative stress. Under oxidative stress, ROS are produced that lead to damage of proteins, lipids, DNA, and tissues (Chalvon-Demersay et al., 2021). The exact mechanism of how *E. coli* and *Salmonella* exert their oxidative stress-inducing effect is obscure, but pathogens may produce oxygen to generate an aerobic environment, thus establishing oxidative stress conditions in the intestines (Wang et al., 2021). Probiotics can exert antioxidant effects in many ways (Wang et al., 2017a). To confirm the antioxidant effect of the application of *E. faecium, L. rhamnosus, B. licheniformis and B. subtilis* as a pre-treatment, co-treatment, and post-treatment, we determined the capacity of the treatment methods for the alleviation of ROS production. In our experiments, *E. coli* and *S.* Typhimurium induced an intracellular ROS burst in IPEC-J2 cells that could

be significantly reduced by pre-, co-, and post-treatments with *E. faecium* (in both concentrations), *L. rhamnosus, B. licheniformis* and *B. subtilis*. Thus, *E. faecium, L. rhamnosus, B. licheniformis* and *B. subtilis* show powerful antioxidant properties upon pathogen challenge. With the DCFH-DA method overall ROS production is measured, therefore our results suggest a general ROS reducing effect of *E. faecium, L. rhamnosus, B. licheniformis* and *B. subtilis*, moreover, this effect was not species-specific and was independent of the causative agent (*E. coli* or *S.* Typhimurium) of oxidative stress. However, we cannot determine whether the ROS reducing effect was attributable to the probiotic bacteria itself or to subtances produced by probiotics. Our results of the antioxidant effect of probiotics are summarized in **Table 12**. Our finding agrees with other studies, where antioxidative properties of probiotic bacteria were proved. In IPEC-J2 cells beneficial effect of *L. plantarum* ZLP001 on ROS generation has been proved and using IPEC-1 cell line H_2O_2-induced oxidative stress could be ameliorated by *Bacillus amyloliquefaciens* SC06. (Wang et al., 2021).

Table 12: Summary of the intracellular ROS measurements using DCFH-DA method. **St:** *S.* Typhimurium, **Ec**: *E. coli*, **PRE**: pre-treatment, **CO**: co-treatment, **POST**: post-treatment**. — in green**: indicates no change in ROS production (compared with the untreated control), ↓ **in green:** indicates decrease in ROS production (compared with the untreated control), ↓ **in black**: indicates decrease in ROS production (compared with treatment with only *S.* Typhimurium or *E. coli*).

			St						Ec					
	Probiotic alone		PRE		CO		POST		PRE		CO		POST	
	10^7	10^8	10^7	10^8	10^7	10^8	10^7	10^8	10^7	10^8	10^7	10^8	10^7	10^8
E. faecium	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
L. rhamnosus		↓		↓		↓		↓		↓		↓		↓
B. licheniformis		—		↓		↓		↓		↓		↓		↓
B. subtilis		↓		↓		↓		↓		↓		↓		↓

It is supposed that harmful bacteria need to adhere to epithelial cells in order to exert harmful effects (Dowarah et al., 2017). If the adhesion of pathogens is inhibited, their intestinal colonization can be decreased and their pathogenic effect can be prevented (Dowarah et al., 2017; Forestier et al., 2001). The inhibition of pathogen adhesion is one of the most important properties how probiotics may exert their beneficial effects. The ability of different probiotic species to inhibit pathogen adhesion has been studied extensively. *L. plantarum* ZLP001 has been proved to inhibit ETEC adhesion to IPEC-J2 cells (Wang et al., 2018) and *E. faecium* 18C23 is capable of inhibiting the adhesion of *E. coli* F4ac to

immobilized piglet mucus (Jin et al., 2000). *B. licheniformis* KMP-9 and *B. subtilis* KMP-N004 have been found to inhibit the adhesion of non-ETEC, ETEC, *S. enterica* and *S. suis* species to IPEC-J2 cells (Pahumunto et al., 2021). Our results (summarized in **Table 13**) agree with these studies reporting that probiotics are able to inhibit pathogen adhesion. Interestingly, in our experiments the inhibition effect of *E. faecium, L. rhamnosus and B. licheniformis* was independent of the time of addition. In other words, the adhesion of both *E. coli* and *S.* Typhimurium was significantly inhibited by *E. faecium, L. rhamnosus and B. licheniformis* in the case of all three treatment conditions (pre-, co- and post-treatment). Moreover, *B. subtilis* could also inhibit the adhesion of *E. coli* and the beneficial effect was also independent of the time of addition. Similar results were reported by Forestier et al, showing that adherence of three pathogens (enteropathogenic and enterotoxigenic *E. coli* and *Klebsiella pneumoniae*) was decreased by addition of *Lactobacillus casei rhamnosus*, regardless of whether the probiotic strain was added before, during or after the incubation with the pathogen (Forestier et al., 2001). Our finding that pre-treatment could inhibit adhesion of pathogens indicates that the tested probiotic species could successfully exclude pathogenic bacteria. Furthermore, that co-treatment was capable to hamper pathogen adhesion means that examined probiotics could successfully compete with the pathogens and the successfullness of post-treatment demonstrates that investigated probiotics were also able to disrupt established pathogen colonization. Even *B. subtilis* was able to perform this beneficial effect, however only against *E. coli*. *E. faecium*, *L. rhamnosus* and *B. licheniformis* proved higher adhesion inhibition rates against ETEC than *S.* Typhimurium. Moreover *B. subtilis* even failed to inhibit adhesion of *S.* Typhimurium. Thus, it is supposed that pathogen adhesion inhibiting properties of *B. subtilis* depend on the type of applied pathogenic bacteria. Similar results have been reported by Pahumunto et al also demonstrating that the inhibition of ETEC strains by probiotic bacteria was significantly higher than that of *S. enterica* (Pahumunto et al., 2021). The presence of *E. faecium, L. rhamnosus, B. licheniformis* and *B. subtilis* may hamper the access of *E. coli* or *S. Typhimurium* to tissue receptors by steric hinderance and that may explain the decrease of adhesion of these pathogens in the presence of probiotic bacteria. Other mechanisms might also be involved. Adhesion of pathogens may be restricted also through the combined effect of probiotic bacteria and mucin. HT29 cells showed increased mucin production upon incubation with probiotics (Forestier et al., 2001). IPEC-J2 cells also secrete mucins that

might interplay with the presence of *E. faecium, L. rhamnosus, B. licheniformis* and *B. subtilis* and inhibit the adhesion of *E. coli* or *S.* Typhimurium (Forestier et al., 2001). Production of compounds with bacteriostatic and bactericid activity might play an indirect role in adhesion inhibition. Biosurfactants produced by Lactobacilli have been proved to posses inhibitory activity against several Gram positive and Gram negative species (including *E. coli* and *S.* Typhimurium) and this inhibitory activity might also contribute to the adhesion inhibiting effect of probiotic bacteria (Vignolo et al., 1993). In comparison to other species of the *Bacillus* genera, *B. subtilis* cannot produce such wide range of antimicrobial subtances and this can partly explain our experimental results (Larsen et al., 2014). Since *B. subtilis* was only able to inhibit the adhesion of *E. coli*, it may be supposed that in this case the assumed mechanisms by which *B. subtilis* exerts its adhesion inhibiting effect is not competitive exclusion, but the production of antimicrobial substances capable of inhibiting *E. coli* and unable to inhibit *S.* Typhimurium. Furthermore, tested probiotics might bind to each other forming auto-aggregates or to pathogens forming co-aggregates, with both of which the colonization of pathogens can be prevented (Monteagudo-Mera et al., 2019; Pahumunto et al., 2021).

Our results support the potential use of *E. faecium, L. rhamnosus, B. licheniformis, B. subtilis* as feed additives according to their beneficial effect being capable of inhibiting the adhesion of *E. coli* or *S. Typhimurium*. However to determine the exact mechanism how *E. faecium, L. rhamnosus, B. licheniformis* and *B. subtilis* exert their adhesion inhibiting effect further studies are needed.

Table 13: Summary of the adhesion inhibiting effect of probiotic bacteria. St: *S.* Typhimurium, **Ec**: *E. coli*, **PRE**: pre-treatment, **CO**: co-treatment, **POST**: post-treatment. **—**: indicates no change in adhesion inhibition (compared with treatment with only *S.* Typhimurium or *E. coli*), ↓: indicates decreased pathogen adhesion (compared with treatment with only *S.* Typhimurium or *E. coli*).

	St			Ec		
	PRE	CO	POST	PRE	CO	POST
E. faecium	↓	↓	↓	↓	↓	↓
L. rhamnosus	↓	↓	↓	↓	↓	↓
B. licheniformis	↓	↓	↓	↓	↓	↓
B. subtilis	–	–	–	↓	↓	↓

To conclude our findings, *E. faecium, L. rhamnosus, B. licheniformis* and *B. subtilis* have proved several beneficial effects (including antioxidant, inhibition, anti-inflammatory, barrier enhancing effects) in an *in vitro* porcine model, in which gastrointestinal infection was

evoked by either *S.* Typhimurium or *E. coli*. The use of these probiotic species addresses the challenge of finding alternative treatments that can strengthen gastrointestinal health without the use of antibiotics. Our results prove that the beneficial effects of probiotics are species dependent. In order to reach the most optimal effects, the use of these species in combination with each other or with other probiotic species as multi-strain or multi-species mixtures seems to be promising, however further investigations would be necessary to determine whether a mixture of probiotics exerts its effect through synergistic, antagonistic or additive mechanisms. Furthermore, our *in vitro* model proved to be a useful tool to examine the effects of promising probiotics and other alternative substance candidates in future investigations. Our results serve to address and deepen our understanding of probiotic action on intestinal porcine epithelial cells and serve as a basis for both human and swine *in vivo* research and application.

7 New scientific results

Our study was the first to comprehensively test protective effects of *E. faecium*, *L. rhamnosus*, *B. licheniformis* and *B. subtilis* on IPEC-J2 cells. Antioxidant capacity of bacterial SCSs was evaluated under LPS induced oxidative damage and antimicrobial activity of SCSs against several swine-derived *E. coli* and *S*. Typhimurium field isolates was investigated. Furthermore, the beneficial effects on intracellular ROS production, inflammatory cytokine response, paracellular permeability and adhesion inhibition were tested using IPEC-J2 – bacterium co-culture model.

Main findings of the study are as follows:

1. *E. faecium*, *L. rhamnosus*, *B. licheniformis* and *B. subtilis* affect the viability of IPEC-J2 cells in a species-specific manner. Spent culture supernatants of *E. faecium*, *L. rhamnosus*, *B. licheniformis* and *B. subtilis* (6% concentration for 1 hour) and bacterial suspensions of *E. faecium*, *L. rhamnosus*, *B. licheniformis* and *B. subtilis* (10^8 CFU/ml for 1 hour) did not show cytotoxic effects on IPEC-J2 cells.

2. Intracellular ROS reducing ability of SCSs of *B. licheniformis* and *B. subtilis* is independent of the type of LPS used to induce oxidative stress. Intracellular ROS reducing effects of SCSs of *E. faecium*, *L. rhamnosus* depend on the applied type of LPS used to evoke oxidative stress.

3. The effect of probiotic bacterial suspensions on barrier integrity of IPEC-J2 cells is species-specific; *L. rhamnosus* enhances, *B. subtilis* reduces, while *E. faecium and B. licheniformis* do not significantly affect barrier integrity. *E. faecium* and *L. rhamnosus* can counteract barrier damage in IPEC-J2, independently of the barrier disruptions' causative agent (*E. coli* or *S.* Typhimurium) and of the time of addition (pre-, co-, post-treatment). *B. licheniformis* and *B. subtilis* do not exert beneficial effects against barrier impairment of IPEC-J2 cells caused by *E. coli* or *S.* Typhimurium.

4. In certain treatment types, *E. faecium, L. rhamnosus, B. licheniformis* and *B. subtilis* showed anti-inflammatory effect (reduced IL-6 and IL-8 levels) in IPEC-J2 cells challenged with *S.* Typhimurium. The effect of probiotics on proinflammatory

response of IPEC-J2 cells is species-specific and also depends on the type of proinflammatory cytokine examined and on the causative agent (*E. coli* or *S.* Typhimurium) used to evoke inflammation. The time of addition of probiotics also influences the inflammation-reducing effect.

5. *E. faecium, L. rhamnosus, B. licheniformis* and *B. subtilis* have a general intracellular ROS reducing effect in IPEC-J2 cells, moreover, this effect is not species-specific and is independent of the causative agent (*E. coli* or *S.* Typhimurium) of oxidative stress.

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

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

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9 Own scientific publications

9.1 Publications related to the topic of the present dissertation

9.1.1 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.

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

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

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

9.1.2 Conference presentations

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

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

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

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

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

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

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

9.2 Publications not related to the topic of the present dissertation

9.2.1 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.

Karancsi, Z., Kovács, D., Palkovicsné Pézsa, N., Gálfi, P., Jerzsele, Á., Farkas, O.. **The Impact of Quercetin and Its Methylated Derivatives 3-o-Methylquercetin and**

Rhamnazin in Lipopolysaccharide-Induced Inflammation in Porcine Intestinal Cells. *Antioxidants*, *11*(7), 1265, 2022

Wohlert, A., Palkovicsné Pézsa, N., Móritz, A. V., Jerzsele, Á., Farkas, O., Pászti-Gere, E. **Luteolin and Chrysin Could Prevent E. coli Lipopolysaccharide-Ochratoxin A Combination-Caused Inflammation and Oxidative Stress in In Vitro Porcine Intestinal Model**, *Animals*, *12*(20), 2747, 2022.

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.

9.2.2 Conference presentations

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

9.3 Supervision of theses

Hannah Bowles: **The Effect of Probiotics on the Structure and Functionality of Enterocytes.** TDK thesis. Supervisors: Farkas O. and Palkovicsné Pézsa N., Budapest, 2018.

Bryony Gartner: **The effect of *Bacillus subtilis* and *Bacillus licheniformis* on IPEC-J2 cell line.** TDK thesis. Supervisors: Farkas O. and Palkovicsné Pézsa N., Budapest, 2019.

Tóth Zsombor Tamás: **Probiotikumok gyulladáscsökkentő és antioxidáns hatása sertés eredetű bélhámsejteken.** TDK thesis. Supervisors: Farkas O., Karancsi Z. and Palkovicsné Pézsa N., Budapest, 2019.

Kiss Renáta Noémi: **Enterococcus Faecium hatása gyulladásos citokinek és defenzinek expressziójára sertés bélhám sejteken.** TDK thesis. Supervisors: Farkas O. and Palkovicsné Pézsa N., Budapest, 2021.

Somogyi Fanni: ***Lactobacillus rhamnosus* probiotikus baktériumtörzs hatásának *in vitro* vizsgálata sertés bélfertőzés modellben.** TDK thesis. Supervisors: Farkas O. and Palkovicsné Pézsa N., Budapest, 2022.

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