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The effect of *Bacillus licheniformis* on an IPEC-J2 cell line

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ABBREVIATIONS

AMPs – Antimicrobial peptides

ARB – Antibiotic resistant bacteria

B. licheniformis – Bacillus licheniformis

CAT – Catalase

CFU – Colony forming unit

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

E. coli – Escherichia coli

ELISA – Enzyme-linked immunosorbent assay

EPEC – Enteropathogenic *E. coli*

ETEC – Enterotoxigenic *E. coli*

GIT – Gastrointestinal tract

GPx – Glutathione peroxidase

IC – Intracellular

IEC – Intestinal epithelial cell

IgA – Immunoglobulin A

IL-1 – Interleukin-1

IL-10 – Interleukin-10

IL-6 – Interleukin-6

IL-8 – Interleukin-8

IPEC1 – Intestinal porcine epithelial cell line-1

IPEC-J2 – Intestinal porcine epithelial cell line J2

IPI-2I – Ileal porcine intestinal

LPS – Lipopolysaccharide

LT – Heat-labile toxin

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

Nrf2 – Nuclear factor erythroid 2-related factor 2

NRU – Neutral Red Uptake

PBS – Phosphate buffered saline

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

PWD – Post-weaning diarrhea

ROS – Reactive oxygen species

S. Typhimurium – *Salmonella. enterica* serovar Typhimurium

SCS – Spent culture supernatant

SD – Standard deviation

SOD – Superoxide dismutase

STb – Heat-stable enterotoxin b

STEC – Shiga toxin-producing *E. coli*

STs – Heat-stable toxins

TJs – Tight junctions

TLR – Toll-like receptor

TNF-α – Tumour necrosis factor alpha

WHO – World Health Organization

ZO-1 – Zonula occludens 1

1. INTRODUCTION

Extensive antibiotic use in swine production has fostered environments conducive to the development of antibiotic-resistant bacteria (ARB) [1]. Historically, antibiotics were employed not only for therapeutic purposes but also for growth promotion and disease prevention in swine herds, which has been linked with the emergence and propagation of ARB. Evidently, these ARB can transfer to humans through various pathways, including the consumption of contaminated meat, thereby posing a significant public health threat [2].

Escherichia coli (*E. coli*) and *Salmonella* spp. are among the critical pathogens that have been associated with swine and both have zoonotic potential, presenting tangible threats to human health [3]. These pathogens, often found in swine populations, have been linked with severe diseases in humans, and their resistance to antibiotics complicates treatment options [4].

Considering the significant health risks associated with antibiotic resistance, there is widespread agreement on the pressing necessity to investigate and establish viable alternatives to antibiotics for application in swine production [5]. Probiotics, prebiotics, and synbiotics have been researched as potential alternatives or adjuncts to antibiotics, aiming to boost swine health by enhancing gut microbiota and thereby limiting the establishment of pathogenic bacteria. A "One Health" approach, recognizing the intrinsic link between animal, human, and environmental health, is paramount in addressing antibiotic resistance [6]. Collaborative efforts across veterinarians, human health practitioners, microbiologists, and policymakers are pivotal to devise and implement strategies that safeguard swine health while preserving antibiotic efficacy and securing human health against zoonotic and antibiotic-resistant infections.

Studies have underscored the efficacy of probiotics in suppressing *E. coli* and *Salmonella* by enhancing gut barrier function, producing bacteriocins, and outcompeting pathogens for nutrients and adhesion sites and thus points us in the direction of holding probiotics as an important future candidate in alternative options for antibiotics.

This study is focused on examining the capacity of the probiotic *Bacillus licheniformis* to mitigate oxidative stress and inhibit the adhesion of *E. coli* and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) in cultured porcine intestinal epithelial cells during infection.

2. Literature Review

2.1. Gut Function, Structure and Role of a Healthy Gut

The gut wall consists of four distinct layers: the outer serosa layer, the muscular layer, the submucosa, and the innermost mucosa. The mucosa itself is further divided into three layers: the muscularis mucosae, the lamina propria, and the epithelium. The intestinal epithelium acts as a selective barrier and consists of villi and Lieberkühn crypts, which significantly increase the gut's surface area, and maintain gut function via tight junctions between cells. This layer is made up of three distinct cell types—absorptive cells, goblet cells, and enteroendocrine cells (EECs). EECs, scattered throughout the mucosa, act as the primary endocrine units of the gastrointestinal tract (GIT), releasing hormones in response to luminal stimuli and affecting various gut functions [7]. Their role is not merely endocrine; EECs play a critical role in maintaining epithelial integrity, influencing cell proliferation, and even aiding in mucosal defence [8]. The apical membranes of these enterocytes are covered with microvilli and glycocalyx, which are essential for digestion. At the base of the crypts are Paneth cells, containing lysosomes and defensins, suggesting they may play a part in intestinal barrier function, although their exact function is not yet fully characterized [9]. Beyond digestion, the GIT serves as a hub for immune activity, housing gut-associated lymphoid tissue (GALT) rich in immune cells such as macrophages, dendritic cells, and lymphocytes. These cells constantly monitor gut content, promoting tolerance to beneficial microbes and guarding against pathogens. The intricate relationship between gut microbiota and GALT contributes to a balanced immune response, aiding in pathogen defence and inflammation reduction [10]. The linkage between intestinal epithelial cells is composed of numerous cytoplasmic proteins situated in the cell membrane, referred to as "apical junctional proteins", which defend against pathogens and regulate the intercellular movement of ions, solutes, and water. Four primary structures can be identified from luminal to basal. Firstly, the tight junction (TJ) incorporates four key transmembrane proteins; occludin, claudins, junctional adhesion molecules and tricellulin. Zonula occludens (ZO) proteins link these transmembrane elements to an actin framework, enabling movement between cells when it contracts. The adherent junction (AJ) is formed of alpha and beta catenins and E-cadherin. Following this, the desmosome (macula adherens) comprises a variety of proteins including desmoplakin and desmocolin, desmoglein, and finally, the sequence is concluded by the gap junction comprised of a hemichannel and connexons. The

apical protein complexes on either side of the cell are interconnected and stabilized by actin, myosin, and intermediate filaments spanning the cytoplasm. This scheme is illustrated in **Figure 1** [11, 12].

Disruption of these junctions due to disease allows harmful substances or antigens to access the mucosa and trigger immune responses, which can increase permeability to allow other toxins, allergens, and microbes to pass through. Both the proinflammatory cytokine tumour necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) exert an influence on ZO-1 by triggering the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which in turn undermines the tight junction's integrity. This is a mechanism utilised by pathogenic bacteria like *Escherichia coli* and *Salmonella Typhimurium* to weaken the barrier of the intestinal epithelial tight junction. Moreover, lipopolysaccharides (LPS) present in the cell walls of gram-negative bacteria can likewise disrupt tight junction operations via Toll like receptor 4 (TLR4) activation and inducing localized cell death [12].

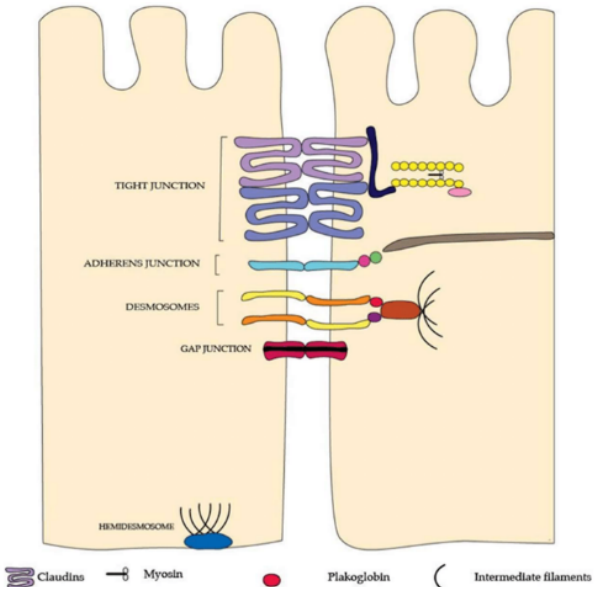

Figure 1. Schematic representation of the junctional complex in intestinal epithelium. zona ocludens (ZO) -1, myosin light chain kinase (MLCK) [11]

In the small intestine, pancreatic α-amylase and proteases break down starch and proteins respectively, which are further processed into monosaccharides and amino acids by brush border enzymes. The efficiency of enterocytes and the brush border membrane is crucial for the uptake of these dietary nutrients [13, 14]. Meanwhile, bile salts are integral for lipid digestion, by emulsifying lipids and enabling the enzymatic breakdown primarily within the jejunum, as it is the largest absorption site within the small intestines. Bile salts also help maintain a healthy gut microbiome by controlling harmful bacterial growth [14]. Brunner's glands in the small intestine regulate the flow of bile and pancreatic juice by secreting a bicarbonate buffer, which is critical in maintaining an optimal pH for enzymatic function. Alongside this, goblet cells produce mucin which forms a protective mucus layer against harmful elements in the lumen when combined with glycocalyx. In the absence of commensal bacteria, this layer is notably thinner. Factors like threonine and cystine

deficiency might restrict mucin generation [14]. Supplementing the microbiota with leucine may mitigate reduced mucus production in rotavirus-infected pigs [15].

Despite its resilience, the GIT can be perturbed by various factors. Dietary changes, stress, antibiotic use, and infections can disrupt the gut microbiota balance, leading to a state called dysbiosis. This altered state can compromise the GIT's barrier function, making it permeable to toxins and pathogens. The ramifications of this can be widespread, from local gut inflammation to systemic health issues. Research in this field has thus been fervently focused on interventions like probiotics, prebiotics, and specific nutrient supplements to bolster gut health [8].

2.2. PROBIOTICS, THEIR ROLE IN ANIMAL HEALTH

The definition of probiotics has witnessed an evolution over the years. Initially identified as live cultures that generally improve the natural balance within the gut microbiota, nowadays the term encompasses specific functions, particularly emphasizing their immunomodulatory potential [16]. A modern definition by the WHO/FAO defines probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host" [17].

Probiotics can be sorted into various categories, based on whether they are bacterial, spore-forming, multi-species, or originate from the same organism (allochthonous or autochthonous). The efficacy of probiotics is often believed to be species-specific, thus they should be isolated from the species they are intended to assist [18].

Probiotics demonstrate a broad spectrum of health-promoting activities, including the modulation of gut microbiota by utilizing mechanisms like competitive exclusion and direct antimicrobial inhibition, where they compete with pathogens for adhesion sites on IECs or for available nutrients, thereby potentially preventing infections by limiting pathogen access and adherence to the gut [19], they show promise in enhancing the intestinal barrier, immunomodulation, and influencing other organs, as well as contributing to nutrient digestibility and mitigating diarrhoea and toxin effects [20, 21]. Additionally, probiotics may form auto-aggregates, acting as a barrier against pathogenic colonization, and co-aggregates, which may inhibit pathogenic biofilm formation, both of which contribute to minimizing the impact of harmful bacteria in the GIT [22, 23].

Specific strains of probiotics have been linked to the stabilization of both gut mucosal dysfunction and abnormal intestinal permeability [18]. These strains of probiotics are

primarily sourced from the genera *Bifidobacterium* and *Lactobacillus*, are known to exhibit powerful anti-inflammatory properties. They achieve this by combating pathogenic bacteria, modulating the immune response, producing essential nutrients and neurotransmitters, and restoring the gut microbial balance [18, 21]. The intricate relationship between these probiotics and the immune system is suggested in their ability to interact with intestinal epithelial cells. These interactions may lead to the production of immune mediators which are both immune-stimulating and anti-inflammatory, with both having pivotal roles in managing a range of conditions, from inflammatory bowel disease to allergic reactions [16]. It is suspected that they modulate these diseases by producing inflammatory mediators, namely specific immunoglobulins (Ig) such as IgA and IgG, as well as cytokines such as IL-6 and IL-10 [18].

To expand on this, oral administration of certain probiotics has been linked to increased levels of IgA, an immunoglobulin that non-aggressively neutralizes bacteria and toxins—not only in the gut, but also in distant sites such as the bronchi [24] and mammary gland, implying that probiotics can exert immune effects far from their point of entry. Furthermore, these probiotics enhance the count of Paneth cells, which are pivotal in innate immunity due to their production of defensins, lysozymes, phospholipase A2, and similar peptides [25]. Dendritic cells in the intestine can bind to commensal bacteria for days, facilitating specific IgA production that shields the mucosa from harmful bacterial intrusion. These cells are confined within the gut's immune region, ensuring that the response remains localized [26].

Interestingly, probiotics have also shown potential in relieving allergies. Mechanisms include the reduction of IgE levels and the induction of a T- helper(Th)-1 immune response that favours the production of IgG over IgE [18]. *Bifidobacterium animalis subspecies lactis* has shown promise in reducing histamine release and glucose absorption during Th2-dependent allergic responses, which increases specific IgA and IgG levels and curbs eosinophilia without affecting certain parasite stages [13]. Such findings underscore the therapeutic potential of probiotics in managing allergic reactions and other Th2-dependent immune responses.

Furthermore, probiotics have a vital role within the gut's innate immune system as they engage with Toll-like receptors, thereby impacting the secretion of proinflammatory cytokines like IL-6 [27].

Certain probiotics, upon contact with dendritic cells, enhance the synthesis of the anti-inflammatory cytokine IL-10 [20]. These beneficial bacteria can also promote increased

expression of toll-like receptor 2 and mannose on dendritic cells and macrophages. Their protective role is evident as they amplify defence against *S. typhimurium* by activating the phagocytic properties of macrophages in the spleen and peritoneum [25].

Probiotic bacteria can execute direct antimicrobial inhibition by generating various substances, such as bacteriostatic and bactericidal agents. Key agents include organic acids, hydrogen peroxide, antioxidants, and specific antibiotics like reuterin, as well as bacteriocins, microcins, and deconjugated bile acids [25]. Lactic acid bacteria have shown ability to ferment carbohydrates into short-chain fatty acids that lower luminal pH, inhibiting pathogenic bacteria, and produce antimicrobial substances such as low-molecular-weight bacteriocins and reuterin, which are effective against a wide array of pathogens, including bacteria, yeast, fungi, protozoa, and viruses. Additionally, many probiotics can synthesize peptides known as microcins, which exhibit a narrow spectrum activity. Meanwhile, deconjugated bile acids, potent antimicrobial derivatives of bile salts, are also produced. *Bacillus* species are acknowledged for their capability to produce a plethora of antimicrobial substances. For instance, *B. subtilis* can produce various agents like subtilin, entianin, and bacitracin, while *B. licheniformis* is documented to produce lichenicidins and bacitracin The compounds generated by probiotics not only reduce pathogen numbers but also impact bacterial metabolism and the production of toxins.

Certain strains of *Bacillus spp.* have the capability to create biofilms, which serves as a means for them to shield against the various conditions found in the gastrointestinal tract (GIT), thereby enhancing their chances of survival within the GIT [28] **Figure 2.**

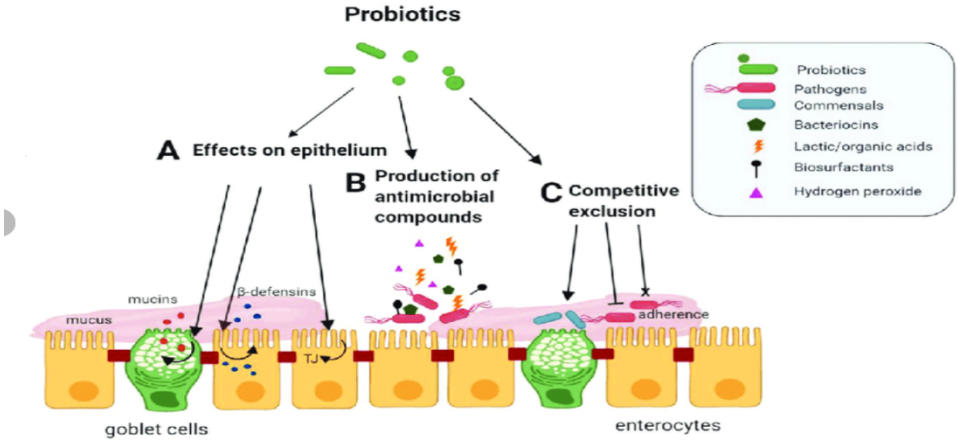

Figure 2 Probiotic mechanisms of action against enteric pathogens in the GIT. Three primary effects of probiotics against enteric pathogens in the GIT. A – Effects on epithelium, such as biofilm layer production, shield against microbial pathogens. B – Production of antimicrobial compounds (e.g. Subtilin, entianin, bacitracin, lichenicidin) directly attack pathogens, while C – Competitive exclusion prevents adherence of pathogens to the epithelial layer of the GIT [28]

One of the primary ways through which pathogenic bacteria can compromise the intestinal epithelium is by generating reactive oxygen species (ROS), resulting in oxidative stress. Fortunately, probiotics have multiple ways to counteract oxidative stress. They can bind with metal ions like Fe^{2+} and Cu^{2+} that otherwise amplify the creation of harmful radicals from hydrogen peroxide. Probiotics have natural defences against oxidative stress, such as the enzyme superoxidase dismutase, which breaks down harmful superoxide radicals. While most *Lactobacilli* don't produce the enzyme catalase, certain modified types can, enhancing the breakdown of hydrogen peroxide and lessening related intestinal issues. Additionally, probiotics boost the host's defences, increasing its production of enzymes like superoxidase dismutase, catalase, and glutathione peroxidase. Some probiotics such as *lactobacilli* also produce beneficial compounds that counteract oxidative stress. For example, certain strains enhance the host's folate levels, vital for deoxyribonucleic acid (DNA) processes and antioxidant activity. Glutathione, another antioxidant compound produced by some probiotics, works alongside other elements to reduce harmful radicals. Probiotics also have a role in modulating antioxidant pathways, including the nuclear factor erythroid 2-related factor 2 (Nrf-2) pathway. When Nrf-2 moves to the cell nucleus, it boosts the production of antioxidant and detoxifying proteins, which can serve as a marker for detecting oxidative stress. Some probiotics also have anti-inflammatory effects, as shown by a substance from *Bacillus* spp. that reduces inflammation by blocking an inflammatory pathway. Lastly, some *lactobacilli* may reduce the activity of NADPH oxidase, a primary source of harmful radicals, especially in certain immune cells [29]. More detail on the mechanisms with which pathogenic bacteria can produce ROS are explored in section 3.4.

2.2.1. *BACILLUS LICHENIFORMIS*

Bacillus licheniformis, a Gram-positive, spore-forming bacterium, has emerged as a critical player in biotechnology and health. In agriculture, *B. licheniformis* finds application as a biopesticide. Its ability to produce antifungal and antibacterial compounds can help combat plant pathogens, offering an eco-friendly alternative to chemical pesticides. In the food industry, the bacterium contributes to fermentation processes. Its proteolytic and amylolytic activities are harnessed in bread-making and brewing, enhancing product quality and shelf life [28]. The health implications of *B. licheniformis* extend to its interaction with the immune system. A 2021 study by Romo-Barrera *et al.* Offers insights into the capacity of this bacterium to stimulate the creation of macrophage extracellular traps (MET). METs are structures formed by macrophages to immobilize and neutralize pathogens. The exact

mechanisms by which *B. licheniformis* induces MET formation remain a subject of study. However, the implications are vast, offering potential therapeutic strategies against infectious agents [31]. Although they are not traditionally part of the commensal flora, there is a growing consideration for them as a potential probiotic candidate. This is due to their capability to form spores, enabling them to endure the passage through the gastrointestinal tract [23, 25]. Their innate adaptability, along with a rapid growth rate and robustness, renders them an appealing candidate for a wide range of applications [30]. Among various *Bacillus* spp. strains, *B. subtilis*, *B. licheniformis*, and *B. cereus* are used for animal feed [26].

However, not all *Bacillus* species are benign; some have been identified as pathogenic, raising general concerns about their suitability as probiotics [32]. It has also been proposed that these species might have the capacity to transfer antibiotic resistance genes while concurrently producing enterotoxins, which adds to the limitations regarding their suitability [33]. Nevertheless, *B. licheniformis* is not typically regarded as a pathogenic species, making it a suitable candidate for further study.

2.3. *E. COLI* AND *SALMONELLA* IN SWINE

The presence of *Escherichia coli (E. coli)* and *Salmonella* species in swine presents noteworthy challenges to the well-being of the intestinal tract. These two pathogens, although distinct in their effects, share a common concern. Both *E. coli* and *Salmonella* possess Lipopolysaccharide (LPS) in their cell walls, a component that can disrupt the integrity of intestinal barriers. Furthermore, LPS is associated with systemic inflammation and could potentially be linked to the development of various diseases, highlighting the gravity of their impact on swine health[34, 35].

E. coli is a diverse bacterial species with both commensal and pathogenic strains. While many strains are harmless and reside in the intestines of animals, certain pathogenic strains can cause severe diseases in swine and are zoonotic. After weaning, piglets are particularly susceptible to enterotoxigenic *E. coli* (ETEC), leading to post-weaning disease (PWD). The disease is characterized by severe watery diarrhoea, dehydration, and even death. For infants, the mortality rate can reach as high as 70%, while after weaning, it remains significantly elevated at 25% [36]. In contrast, oedema disease (ED) is a systemic condition caused by toxins produced by verotoxigenic *E. coli* (VTEC) enter the bloodstream, leading to vascular damage and oedema, especially in the eyelids, brain, and stomach [37].

ETEC's ability to colonize the piglet's intestine is primarily due to fimbrial adhesins like F4 and F18. These structures facilitate the bacteria's attachment to the intestinal wall, an essential step in pathogenesis. Vaccines targeting these adhesins have been proposed as preventive measures [34].

On the other hand, *Salmonella* is also a zoonotic pathogen. While many *Salmonella* serovars can infect pigs, *S. Typhimurium* and *S. Choleraesuis* are predominant. The latter is adapted specifically to swine and causes systemic illness, while the former is a common cause of gastroenteritis in various animals and humans [35]. *Salmonella* can spread through contaminated feed, water, equipment, or even via farm workers. Implementing stringent biosecurity measures, vaccination, and ensuring hygienic practices are essential for controlling its spread [38]. Swine are among the primary reservoirs of *S. Typhimurium* and this bacterium is responsible for enterocolitis, characterized by symptoms like diarrhoea and dehydration, and it can prove fatal in severe instances. Pigs, particularly those after weaning, display a high vulnerability to salmonellosis. Even relatively low infection levels, such as 107 *S.* Typhimurium bacteria per gram of intestinal content, can result in lesions in pigs. Predisposing factors, such as the underlying disease, weather, age, and insufficient hygiene conditions, contribute to an increased susceptibility to this condition. Infected pigs can harbour *Salmonella* in their intestines, lymph nodes, and even muscle tissue. Notably, many pigs will continue to shed the pathogen post recovery in a carrier state [39]. Consuming undercooked pork products can lead to salmonellosis in humans, marked by diarrhoea, fever, and abdominal cramps, making its prevention of great public health significance. Proper cooking, handling, and processing of pork products are vital to reduce risks [40].

The rise of antibiotic-resistant strains, driven by the excessive use of antibiotics in livestock, presents a significant and alarming public health issue. These resistant strains can complicate treatments in both animals and humans [41]. Overuse of antibiotics in swine production has led to the emergence of both multi-drug resistant *E. coli* and *S. Typhimurium* strains. This situation raises a concerning prospect, not only restricting treatment possibilities in veterinary medicine but also presenting a zoonotic risk that has the potential to constrain antibiotic use in human healthcare. Consequently, the effective management of *E. coli* and *Salmonella* in swine is of paramount importance, bearing significance not only for animal health but also for public health [42].

2.4. INFLAMMATION AND OXIDATIVE STRESS

Inflammation and oxidative stress are pivotal aspects of biology, with oxidative stress defined by an imbalance between pro-oxidants and antioxidants, leading to the overproduction of reactive oxygen species (ROS) [43]. ROS are metabolic by-products that can cause damage to biomolecules within cells, examples include superoxide (O_2^-), hydroxyl radicals (OH), and hydrogen peroxide (H_2O_2) [44, 45]. ROS generation involves both endogenous sources like NADPH oxidase and exogenous factors such as air pollutants and chemicals [45]. In the context of pig production, sources of ROS also encompass birth, weaning stress, mycotoxin contamination, environmental conditions, and social factors, all of which can lead to oxidative damage in piglets and growing pigs [46]. Oxidative stress in pigs can have severe health consequences, contributing to disorders like atherosclerosis, cancer, peptic ulcers, and inflammatory bowel disease [43, 45]. Interestingly, it's important to note that while ROS can result in oxidative damage, lower ROS levels have shown to play a physiological role in pigs and other species, as when maintained at appropriate levels they regulate several cellular functions such as cell signalling, immune response and cell proliferation [47].

However, ROS are not just by-products of cellular metabolism; they play a role in microbial persistence and inflammation. Excessive build up or inadequate clearance of ROS, especially when resulting from microbial activity from pathogenic bacteria like *E. coli* or *S.* typhimurium, leads to robust cell death and exacerbates inflammation. Infections and immune responses can indeed stimulate heightened ROS production, influencing transcription factors like NF-κB, and Nrf2 which can subsequently exacerbate inflammation, effectively initiating a persistent positive feedback loop [43, 48]. This interdependence suggests that therapeutic measures targeting one process without addressing the other might be insufficient in addressing chronic diseases [49]. This phenomenon is displayed in a recent trial by Fratta Pasini *et al*., who reported that chronic diseases such as COVID-19 treated with n-acetylcysteine showed some beneficial results but not sufficient as a curative measure [50]. Implying a need for combination therapies which could include anti-inflammatory agents alongside these antioxidants [49].

The body possesses natural defence mechanisms against oxidative stress, which primarily consist of is largely encompassed by enzymatic elements, such as glutathione peroxidases (GPX), superoxide dismutases (SOD), and catalase (CAT), as well as non-

enzymatic elements, like glutathione, vitamin C, and vitamin E, all of which show ability to reduce the effects of ROS [43]. One of these effects includes lipid peroxidation, which produces potentially toxic thermally oxidized compounds including trans fatty acids, aldehydes such as acrolein and formaldehyde, and lipid hydroperoxides, during the oxidation process, leading to oxidative stress, inflammation [51]. Knowing when to apply these antioxidant supplements is crucial to maintaining the health and production output of these animals, while remaining economical for farmers. Therefore, testing for biological markers of oxidative stress can be a useful tool in determining treatment plans [43].

Markers of oxidate stress often manifest as consistent oxidative by-products detectable in the blood [52]. Reactive oxygen molecules degrade quickly, and the proteins responsible for managing redox reactions in cells are typically restricted to specific signalling zones. Hence, it is essential to identify easily measurable indicators of oxidative damage. Four primary markers emerge as ideal for these purposes. One of these markers includes inflammatory cytokines such as, TNF-α, IL-8, IL-6, cyclooxygenase-2, cyclin A, and hypoxanthine guanine phosphoribosyl transferase. These cytokines can indicate the body's response to oxidative stress and can be detected by a standard cytokine ELISA. The second marker is malondialdehyde (MDA), a by-product of lipid peroxidation which can indicate oxidative damage to cell membranes. MDA can be quantified using a TBAR assay [51]. The third, as previously mentioned, is Nrf-2. This compound controls the expression of genes related to proteins involved in neutralizing ROS, and thus serves as another detectible marker. Its expression can be determined using antibodies specific to Nrf-2 bound DNA [48, 53]. Finally, certain ROS can be measured directly, such as extracellular H_2O_2 as detected by an Amplex red assay [54].

2.5 *IN VITRO* MODELS

In vitro models, particularly those replicating the gut environment, have garnered significant attention in the scientific community for their potential applications in drug absorption studies, food microbiology, and infectious disease research. These models aim to recreate the complex physiological environment of the gut, offering insights into various cellular and molecular interactions. The utilization of cell lines is in accordance with the values of the 3R concept, which advocates for the reduction, replacement, and refinement of experiments involving animals [55].

While static Transwell cultures have been extensively characterized and can classify molecules based on permeability, they may lack essential features of the intestinal environment, such as cellular complexity, mechanical strain, and interactions with mucus and microbes. Advanced approaches such as, organoids, microfluidic chips and intestinal slice cultures have been devised to address these discrepancies. However, the balance between model complexity, cost, and the accuracy of drug permeability predictions remains a topic of debate [47]. Human colon cell lines such as Caco-2 are highlighted for their tumorigenic nature, originating from human colon tumours. Non-transformed rodent cell lines IEC-6 and IEC-18, derived from rodents, offer an alternative to tumour cell lines. Porcine intestinal cell lines, including IPEC-1, IPEC-J2 and, IPI-2I cells of porcine origin, present a unique perspective on intestinal research [43]. These cell lines, sourced from different segments of the pig intestine, offer a diverse range of options for researchers, catering to various aspects of porcine gastrointestinal biology [56].

The IPEC-J2 cell line, derived from the jejunal epithelium of neonatal unsuckled piglets, has emerged as a pivotal *in vitro* model for studying microbial pathogenesis in swine. Being non-transformed, this cell line retains many of the physiological properties of the native intestinal epithelium, making it an attractive alternative to traditional cell lines. This is especially beneficial in studying interactions between the host epithelium and various microbial agents, including pathogens and commensals [56]. For instance, these IPEC-J2 cells exhibit a cobblestone-like morphology typical of epithelial cells. Electron microscopy has revealed the presence of tight junctions, microvilli, and other ultrastructural features that are consistent with differentiated enterocytes. Furthermore, these cells display robust barrier properties, with the expression of barrier-relevant tight junction proteins and active transport rates, all of which are ideal for studying microbial interactions and pathogenesis [56]. This cell line has been utilized in various studies to understand host-pathogen interactions, especially in the context of swine infections. One instance of this is demonstrated by Schierack *et al*., who characterized the interactions of the IPEC-J2 cell line with both commensal bacteria and key bacterial pathogens such as *E. coli Salmonella, and Chlamydia spp.*. Such studies provide insights into pathogenic mechanisms, adhesion properties, and invasion capabilities of various microbes [57].

Its physiological relevance, coupled with its non-transformed nature, makes it a preferred model for understanding host-pathogen interactions at the cellular and molecular levels. As research progresses, the IPEC-J2 cell line is poised to offer deeper insights into

the mechanisms underlying microbial pathogenesis in swine, paving the way for potential therapeutic interventions.

3. AIMS

Our goal is to evaluate the effectiveness of the probiotic *Bacillus licheniformis* as a preventive or therapeutic measure against swine gastrointestinal diseases caused by *E. coli* or *S.* Typhimurium.

Firstly, using the neutral red uptake assay, we determined optimal experimental conditions by examining the concentration at which *B. licheniformis* does not negatively impact the viability of the IPEC-J2 cells. Secondly, we evaluated the *in vitro* capabilities of *B. licheniformis* through a co-culture model simulating gastrointestinal infection with porcine-origin *E. coli* and *S.* Typhimurium. We implemented three treatment conditions: pre-treatment, where the probiotic bacterium was added one hour before introducing the pathogenic bacterium to IPEC-J2 cells; co-treatment, involving simultaneous addition of probiotic and pathogenic bacteria; and post-treatment, where pathogenic bacteria were introduced prior to the probiotic bacteria. These treatment regimens enabled us to assess the probiotics' effectiveness as preventive or therapeutic agents. We examined their impact on IC ROS production and adhesion inhibition using the IPEC-J2 cell line to gain comprehensive insights into their potential benefits. The goals are summarised in **Table 1.**

Table 1. Summary of the performed investigation.

<table>
<tr><th>Goal</th><th colspan="2">Investigation</th><th>Method</th></tr>
<tr><td>To Determine optimal treatment conditions</td><td colspan="2">Optimal treatment time and concentration of probiotic bacterial suspension</td><td>Neutral Red uptake assay</td></tr>
<tr><td rowspan="2">To Determine the effect of the probiotics on the IPEC-J2—bacterium co-culture model</td><td rowspan="2">Effect of pre/co/post treatment with probiotics on:</td><td>IC ROS production</td><td>DCFH-DA Method</td></tr>
<tr><td>E. coli/ S. Typhimurium adhesion inhibition.</td><td>CFU counting on E. coli or S. Typhimurium selective agar.</td></tr>
</table>

4. MATERIALS AND METHODS

4.1. CHEMICALS USED IN THE STUDY

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; Triton X-100 were purchased from Sigma-Aldrich (Darmstadt, Germany). ChromoBio Coliform and ChromoBio *Salmonella* Plus Base selective agars were obtained from Biolab Zrt. (Budapest, Hungary). Supplements for DMEM/F12 medium (foetal bovine serum [FBS], insulin, transferrin, selenium, epidermal growth factor [EGF] and penicillin-streptomycin) were acquired from Biocentre Ltd., Szeged, Hungary. Cell culture plates were purchased from Corning Inc. (Corning, NY, USA).

4.2. BACTERIAL CULTURE

For our research, we utilized three strains of bacteria. The probiotic strain *Bacillus licheniformis* DSM 5749 isolated from swine was obtained from the Hungarian Dairy Experimental Institute (Mosonmagyaróvár). *S.* Typhimurium and *E. coli* derived from gastrointestinal infections in pigs and were obtained and identified from clinical samples in Hungary through the Department of Microbiology and Infectious Diseases at the University of Veterinary Medicine Budapest in 2009 and 2019, respectively. *E. coli* expresses F4 fimbriae and produces both heat-stable (STa and STb) and heat-labile (LT) enterotoxins. All three strains were stored on Microbank beads at -80°C. Prior to our experiments, these bacterial strains were cultured in an incubator at 37°C for 24 hours and sub-cultured twice. For the preparation of bacterial suspensions, we suspended the beads in plain DMEM/F12 medium (pDMEM/F12), meaning no supplements were added, and incubated them for 18-24 hours at 37°C in a gas mixture consisting of 5% CO_2 and 95% air. Previous departmental experiments have confirmed that under these conditions, all three bacterial strains reach concentrations of 10^8 CFU/ml. We procured the reagents, materials, and nutrients necessary for cell culture from Merck Hungary Kft. (Merck, Darmstadt, Germany).

4.3. CELL LINE AND CULTURE CONDITIONS

The IPEC-J2 epithelial cell line was generously provided by the Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University in Raleigh, NC, USA. These cells were cultured and maintained in a complete medium composed of 10 ml of DMEM/F12 in a 1:1 ratio, supplemented with 5 μg/ml insulin, 5%

foetal bovine serum (FBS), 5 ng/ml selenium, 5 µg/ml transferrin, 5 ng/ml epidermal growth factor (EGF), and 1% penicillin-streptomycin. Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2, following the protocol described by Schierack et al. [57]. The maximum passage number of cells utilized in our experiments was 54. For cell viability assessment using the Neutral Red Uptake (NRU) method, the cells were cultured in 96-well plates. Cells were cultured in 6-well plates for intracellular ROS determination. In the case of adhesion inhibition assays, cells were seeded into 24-well cell culture plates. In each scenario, cells were cultivated until they reached confluency. To eliminate any residual antibiotics before commencing treatment with various solutions, IPEC-J2 cells underwent two washes with phosphate-buffered saline (PBS). Subsequently, pDMEM/F12 was added to each well, and the cells were incubated for 30 minutes at 37°C.

4.4. Neutral Red Uptake Assay for Cell Viability

We assessed the potential cytotoxic effect of *B. licheniformis* on IPEC-J2 cells using the Neutral Red assay. Since Neutral Red is a red dye that is only absorbed by living cells through active uptake, and does not penetrate dead cells, the amount of dye absorbed by the cells is directly proportional to the number of living cells. After removing any excess Neutral Red from the cell culture, we released the dye accumulated in living cells through an acid-alcohol extraction. For the viability test, we employed a treatment solution of *B. licheniformis* at a concentration of 10^8 CFU/ml. The bacterial suspension was prepared from the stock solution using pDMEM/F12 medium.

Following a wash with PBS, we added 100-100 µl of the bacterial treatment solutions to the IPEC-J2 cells. The control group received 100 µl of pDMEM/F12 medium. The cell cultures were then incubated for 24 hours, with six parallel measurements taken. At the end of the incubation period, we replaced the treatment solutions with phenol red-free medium and applied 100 µl of Neutral Red solution to the cells, and they were incubated for 2 hours.

After the incubation, we removed excess dye and washed the cultures with PBS. We then applied a solution consisting of 50 µl of 50% ethanol, 49% distilled water, and 1% concentrated acetic acid to the cultures. After shaking in a circular motion for 20 minutes, we extracted the accumulated dye. The absorbance of the supernatant solutions was measured at 540 nm using the Spectramax iD3 spectrophotometer (Molecular Devices, San Jose, CA, USA). Absorbance values are directly proportional to the amount of dye absorbed, and so the proportion of surviving cells in groups treated with different solution

concentrations compared to untreated cells could be determined. Similarly, the safe concentration of *E. coli* and *S.* Typhimurium (10^6 CFU/ml) for use on IPEC-J2 cells had been established in previous departmental experiments.

4.5. Experimental setup

For pre-treatment assays, cells were pre-incubated with *B. licheniformis* for 1 h before the addition of the pathogen strain. For co-treatment experiments, the pathogen strain (*E. coli* or *S.* Typhimurium) and *B. licheniformis* was added simultaneously to IPEC-J2 cells. In our post-treatment assay, IPEC-J2 cells were incubated with *B. licheniformis* for 1 h after they had already been exposed to treatment with the pathogen strains (*E. coli* or *S.* Typhimurium). A concentration of 10^6 CFU/mL was used to perform the pathogenic bacterial infections of *E. coli* or *S.* Typhimurium. *B. licheniformis* suspension was applied in 10^8 CFU/ ml concentration based on our cell viability experimental results to the IPEC-J2 cells which were also mono-incubated with *B. licheniformis* 10^8 CFU/ml). If any further incubation was required after the treatments, cells were washed with PBS, and DMEM/F12 supplemented with 1% penicillin-streptomycin to inhibit bacterial growth. A summary of the treatment solutions used in our experiments can be found in **Table 2.**

Table 2. Overview of the treatment solutions applied.

Type of treatment	Probiotic applied	Pathogen Applied
Pre-treatment *B. licheniformis* + *S.* Typhimurium	*B. licheniformis* 10^8 CFU/ml prior to infection	*S.* Typhimurium 10^6 CFU/ml
Co-treatment B. *licheniformis* + *S.* Typhimurium	*B. licheniformis* 10^8 CFU/ml simultaneously with infection	*S.* Typhimurium 10^6 CFU/ml
Post-treatment B. *licheniformis* + *S.* Typhimurium	*B. licheniformis* 10^8 CFU/ml post infection	*S.* Typhimurium 10^6 CFU/ml
Pre- treatment *B. licheniformis* + *E. coli*	*B. licheniformis* 10^8 CFU/ml prior to infection	*E. coli* 10^6 CFU/ml
Co-treatment B. *licheniformis* + *E. coli*	*B. licheniformis* 10^8 CFU/ml simultaneously with infection	*E. coli* 10^6 CFU/ml
Post-treatment *B. licheniformis* + *E. coli*	*B. licheniformis* 10^8 CFU/ml post infection	*E. coli* 10^6 CFU/ml
B. licheniformis (mono-incubation)	*B. licheniformis* 10^8 CFU/ml	
S. Typhimurium (mono-incubation)	-	*S.* Typhimurium 10^6 CFU/ml
*E. coli (*mono-incubation)	-	*E. coli* 10^6 CFU/ml

4.6. Determination of the Intracellular Redox Status of IPEC-J2 Cells

We quantified the level of ROS in the cells using a non-specific assay with dichlorofluorescein diacetate (DCF-DA). DCF-DA itself does not possess fluorescence but, upon undergoing molecular transformation in the presence of ROS, it loses its acetate group and is converted into the fluorescent compound 2',7'-dichlorofluorescein (DCF). The intensity of measurable fluorescence is directly proportional to the amount of ROS within the cells. In our experiment, IPEC-J2 cells were cultivated in a 6-well culture vessel until they formed a uniform layer. Simultaneously, we prepared bacterial suspensions, including solutions with *E. coli* and *S.* Typhimurium at a concentration of 10^6 CFU/ml, and *B. licheniformis* at a concentration of 10^8 CFU/ml. Prior to commencing the treatments, we washed the cell cultures with PBS. We employed three types of treatment: pre-treatment, concomitant treatment, and post-treatment.

During pre-treatment, cells were initially exposed to a medium containing *B. licheniformis*. After a 1-hour incubation, the probiotic solution was removed, and *E. coli* or *S.* Typhimurium was applied, followed by another 1-hour incubation. In co- treatment, both *B. licheniformis* and *E. coli* or *S.* Typhimurium treatment solutions were simultaneously administered to the cells and incubated for 1 hour. For post-treatment, *E. coli* or *S.* Typhimurium was introduced to the cells, and after 1 hour, the supernatant was replaced with a solution containing *B. licheniformis*, followed by an additional 1-hour incubation. Negative control cells received culture medium, while positive control cells were treated solely with *E. coli* or *S.* Typhimurium.

At the end of the incubation periods, we removed the suspension and replaced it with fresh DMEM/F12 medium. After a 24-hour incubation period, we removed the medium, washed the cells with PBS, and in a darkened laboratory, we dissolved DCFH-DA dye in medium free of supplements and phenol red. This dye was then applied to the cells and incubated for 1 hour. During this time, DCF entered the cells and reacted with reactive oxygen derivatives. After incubation, we performed a double wash with PBS and then collected the cells using a cell scraper, ensuring uniform scraping for 30 seconds in each well. The scraped cell debris was transferred to Eppendorf tubes, centrifuged, and 100-100 µl of the suspension was added to a 96-well cell culture vessel. This vessel was then placed

in the spectrophotometer, and two wavelengths were adjusted, 480nm to excite the resulting product and 530nm to detect the emitted light when the product returned to its ground state.

4.7. Adhesion Inhibition Assay

In the first phase of this experiment, we followed a similar procedure to the one used for assessing intracellular redox state, with the exception that the cells were cultured in 24-well plates. We applied both positive controls (*E. coli and S.* Typhimurium) and negative controls, along with pre-, co-, and post-treatment with *B. licheniformis*. After the incubation period, we removed the bacterial suspension and added a 1% solution of Triton X-100 surfactant to the cells. We gently agitated the cells in a circular motion for 30 minutes. From these cell suspensions, we prepared a series of dilutions in a 96-well culture vessel, yielding concentrated dilutions at 10^2, 10^3, 10^4, and 10^5 times. From each dilution, we plated samples of 50 µl on Petri dishes containing selective agar; ChromoBio Coliform (for *E. coli*) and ChromoBio *Salmonella* Plus Base (for *S.* Typhimurium). These dishes were then incubated at 37°C for 24 hours, and the bacterial colonies were subsequently counted.

Adhesion was calculated as a percentage relative to the control. The adhesion of *E. coli* and *S.* Typhimurium were normalized to the control. The experiment was performed with 4 replicates per treatment group.

4.8. Statistical analysis

We conducted statistical analyses using R 3.3.2 (2016). Diagnostic analysis was employed to identify and eliminate potential biases, and to ensure the normal distribution of residues. Group averages were compared using one-factor analysis of variance (ANOVA), and significance was determined based on a p-value less than 0.05.

For cell viability assessments, we utilized 6 replicates per treatment group, and 4 replicates per group for adhesion inhibition evaluations. To compare our data with measurements from other experiments, we used a control percentage (%). The mean concentration value of the control cells was set as 100%, and the values of various treatment groups were compared relative to this control. For all measured parameters, we calculated both mean values and standard deviations (SD) across all treatment groups.

5. RESULTS

5.1. CELL VIABILITY ASSAY

The neutral red method was used to determine the effect of *B. licheniformis* suspensions on the viability of IPEC-J2 cells. *B. licheniformis* did not reduce the viability of IPEC-J2 cells after 1 or 2 hours of incubation compared to control cells. **(Figure 3)**

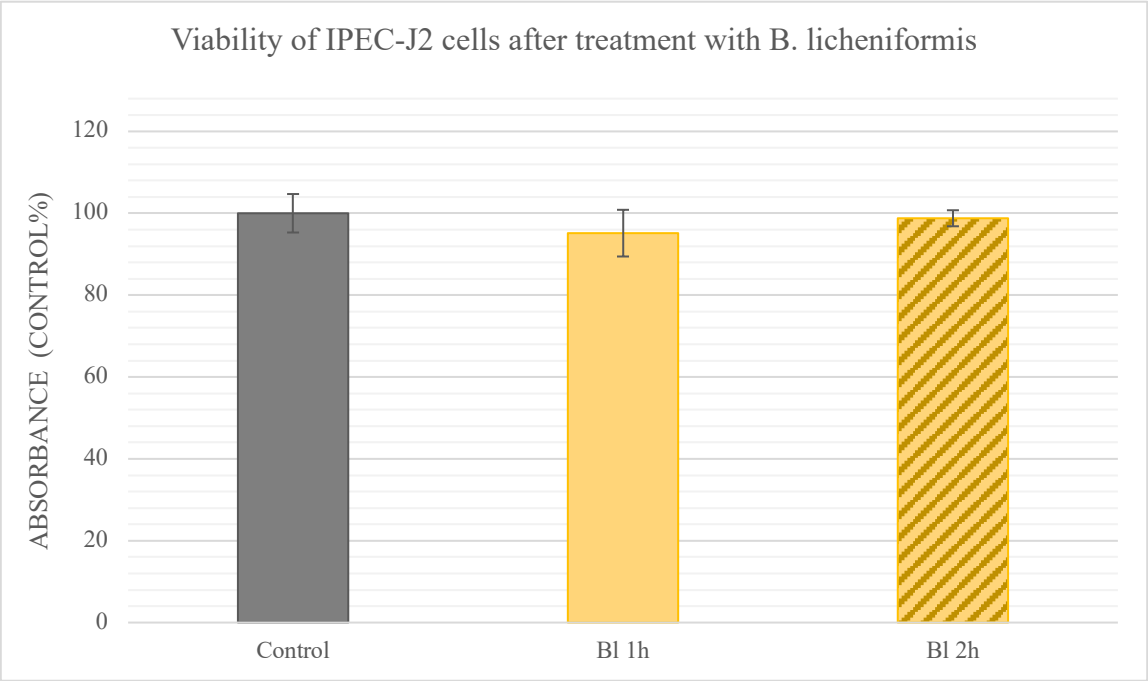

Figure 3 Viability of IPEC-J2 cells after treatment with *B. licheniformis*. **Control:** treatment with plain cell culture medium; **Bl 1 h:** treatment solution of *B. licheniformis* at a concentration of 10^8 CFU/ml for one hour; **Bl 2 h:** treatment solution of *B. licheniformis* at a concentration of 10^8 CFU/ml for two hours. The data is expressed as relative absorbance, using the mean value of the control group as the reference point (set at 100%), and presented as mean values with accompanying standard deviations.

5.2. Effect of *Bacillus licheniformis* on the Intracellular Redox State of IPEC-J2 Cells Challenged by *Salmonella* Typhimurium and *Escherichia coli*

Treatment with *S.* Typhimurium resulted in a noticeable rise in fluorescence in comparison to the control ($p<0.001$). Treatment with *B. licheniformis* alone showed no significant effect compared with the control. Significant reduction in ROS levels compared to the ROS production triggered by *S.* Typhimurium could be observed, when pre-, co-, and post- treatment with *B. licheniformis* was applied ($p<0.001$). **(Figure 4)**

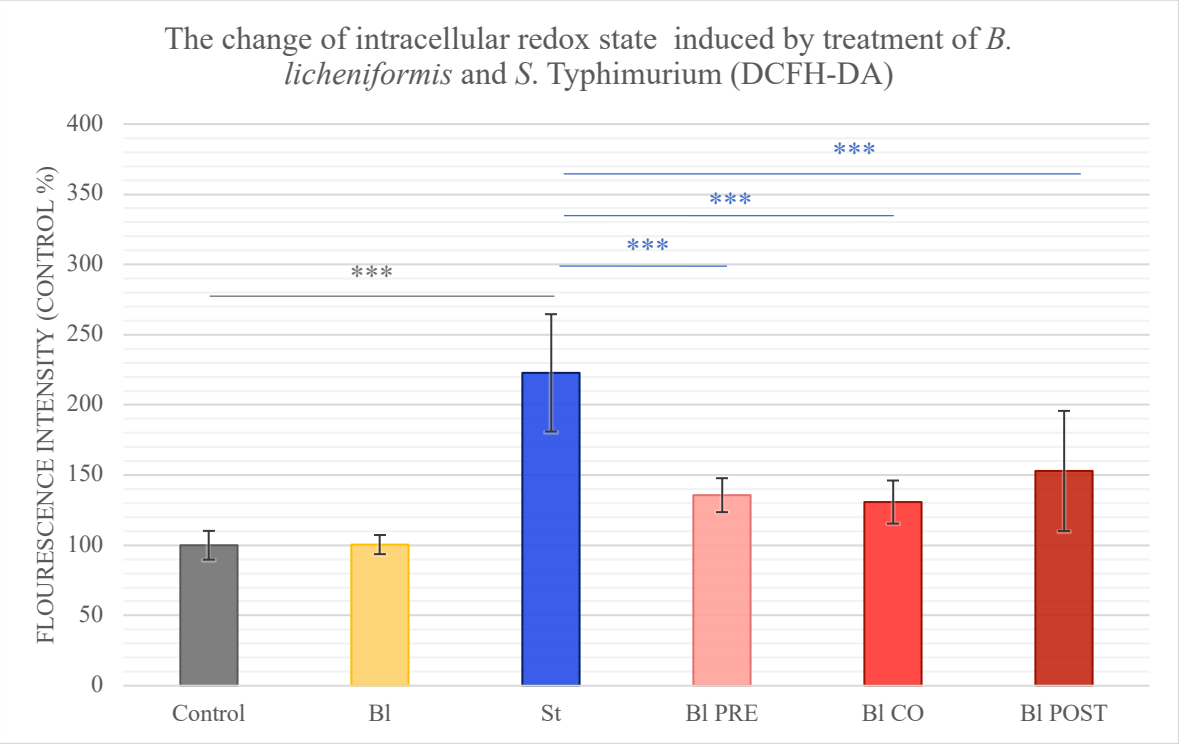

Figure 4. The level of IC ROS in IPEC-J2 cells after treatment with *S.* Typhimurium (St) and *B. licheniformis* (Bl). *B. licheniformis* was added as a pre-treatment (1 hour before), a co- treatment (at the same time as), or as a post- treatment (after) the addition of *S.* Typhimurium. **Control**: treatment with plain cell culture medium; **Bl**: treatment with *B. Licheniformis* 10^8 CFU/ml **St**: treated with *S.* Typhimurium 10^6 CFU/ml; **Bl PRE**: pre-treatment with 10^8 CFU/ml of *B. licheniformis* before *S.* Typhimurium infection; **Bl CO**: co-treatment of *S.* Typhimurium infection with 10^8 CFU/ml of *B. licheniformis* **Bl POST**: Treatment after *S.* Typhimurium infection with 10^8 CFU/ml of *B. licheniformis*. With a sample size of N = 6 per group, the data is expressed as relative fluorescence, using the mean value of the control group as the reference point (set at 100%), and presented as mean values accompanied by their respective standard deviations. Significant difference: *** $p \leq 0.001$, in grey: compared with the untreated control. *** $p \leq 0.001$, in blue: compared with treatment with *S.* Typhimurium.

Treatment with *E. coli* caused a significant increase in the fluorescence compared with the control ($p<0.001$). Pre-, co-, and post-treatment with *B. licheniformis* significantly lowered the levels of reactive oxygen species in the cells when compared to samples treated solely with *E. coli*. ($p<0.001$). **(Figure 5)**

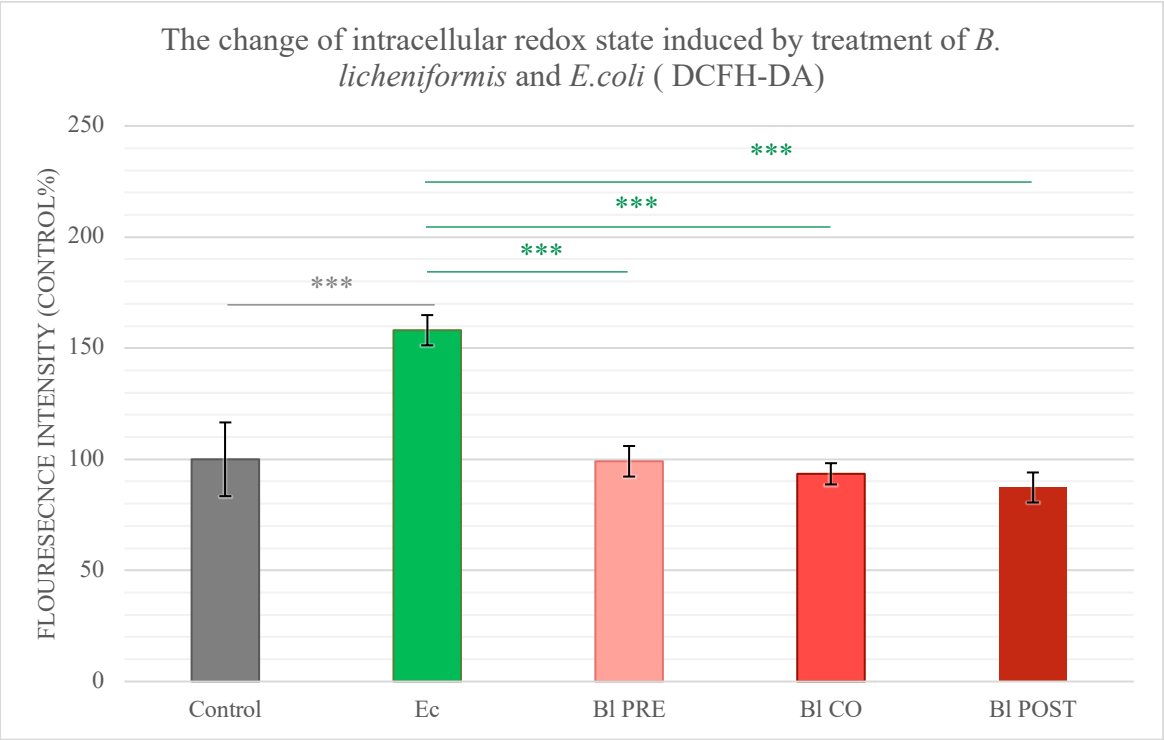

Figure 4. The level of IC ROS in IPEC-J2 cells after treatment with *E. coli* and *B. Licheniformis* (Bl). *B. Licheniformis* was added as a pre-treatment (1 hour before), a co- treatment (at the same time as), or as a post-treatment (after) the addition of *E. coli.* **Control**: treatment with plain cell culture medium; **Ec**: treated with 10^6 CFU/ml of *E. coli*; **Bl PRE**: pre-treatment with 10^8 CFU/ml of *B. Licheniformis* before E. *coli* infection; **Bl CO:** Co- treatment of *E. coli infection* with *B. Licheniformis* 10^8 CFU/ml; **Bl POST**: treatment with 10^8 CFU/ml of *B. Licheniformis* after *E. coli* infection. With a sample size of N = 6 per group, the data is expressed as relative fluorescence, using the mean value of the control group as the reference point (set at 100%), and presented as mean values accompanied by their respective standard deviations. Significant difference: *** $p \leq 0.001$, in grey: compared with the untreated control. *** $p \leq 0.001$, in green: compared with treatment with *E. coli*.

5.3. Effect of *Bacillus licheniformis* on the Adhesion of *Salmonella* Typhimurium and *E. coli* to IPEC-J2 Cells

B. licheniformis exhibited significant inhibitory effects on the adhesion of both *S.* Typhimurium and *E. coli* to IPEC-J2 cells in all treatments, with statistical significance demonstrated by p-values less than 0.01 and 0.001, respectively.

When exposed to *S.* Typhimurium, IPEC-J2 cells treated with *B. licheniformis* exhibited similar inhibitory effects amongst the different treatments. Specifically, in the pre-treatment, the adhesion of *S.* Typhimurium was reduced by 99.77%, in the co-treatment, the *S.* Typhimurium adhesion was inhibited by 99.65%, and the post treatment resulted in a reduction of adhesion of 99.64%. **(Figure 6)**

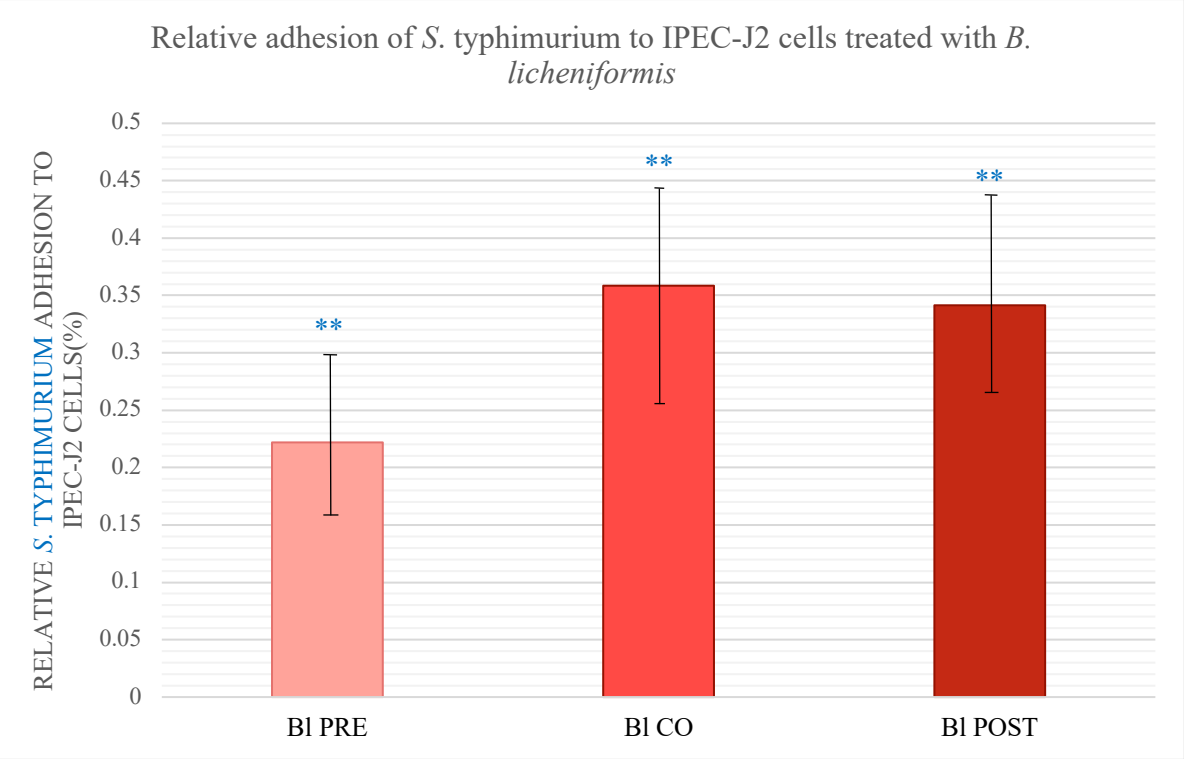

Figure 6. Inhibitory effect of *B. licheniformis* on *S.* Typhimurium adhesion to IPEC-J2 cells. *B. licheniformis* was added as a pre-treatment (1 hour before), a co- treatment (at the same time as), or as a post- treatment (after) the addition of *S.* Typhimurium. **Bl PRE**: pre-treatment with 10^8 CFU/mL of *B. licheniformis* before *S.* Typhimurium infection; **Bl CO**: co-treatment of *S.* Typhimurium infection with 10^8 CFU/ml of *B. licheniformis;* **Bl POST**: Treatment after *S.* Typhimurium infection with 10^8 CFU/ml of B. *licheniformis.* With a sample size of N = 4 per group, the data is represented as a decrease in bacterial count relative to the mean value of the control group (set at 100%). The findings are displayed as mean values, along with their corresponding standard deviations. Significant difference compared to treatment with only *S.* Typhimurium: ** $p < 0.01$.

When *E. coli* was applied to the IPEC-J2, we observed that pre-treatment and co-treatment resulted in very similar inhibitory effects, while post-treatment yielded lower adhesion inhibition. Specifically, in the pre-treatment, the adhesion of *E. coli* was reduced by 76.37%, in the co-treatment, the *E. coli* adhesion was inhibited by 76.89%, and the post treatment resulted in a reduction of adhesion of 49.90%. **(Figure 7)**

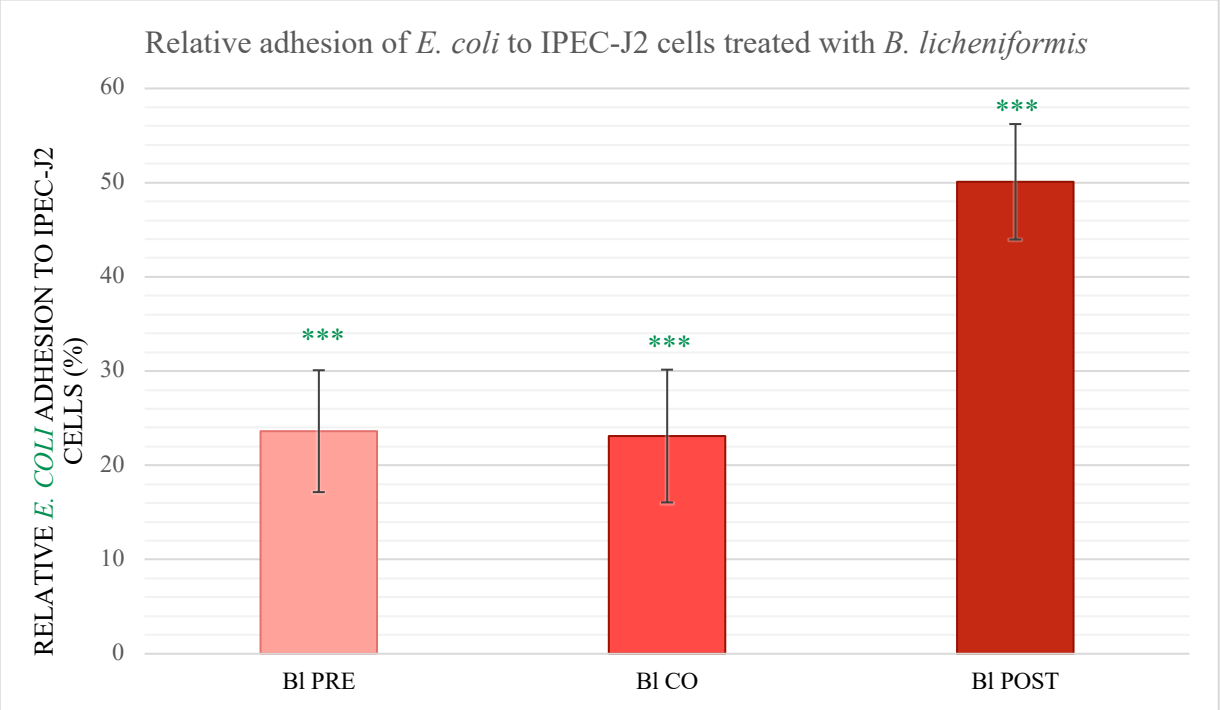

Figure 7. Inhibitory effect of *B. licheniformis* on *E. coli* adhesion to IPEC-J2 cells. *B. licheniformis* was added as a pre-treatment (1 hour before), a co- treatment (at the same time as), or as a post- treatment (after) the addition of *E.coli* **Bl PRE**: pre-treatment with 10^8 CFU/ml of *B. licheniformis* before *E.coli* infection; **Bl CO**: co-treatment of *E.coli* infection with 10^8 CFU/ml of *B. licheniformis* **Bl POST**: Treatment after *E.coli* infection with 10^8 CFU/ml *of B. licheniformis.* With a sample size of N = 4 per group, the data is represented as a decrease in bacterial count relative to the mean value of the control group (set at 100%). The findings are displayed as mean values, along with their corresponding standard deviations. Significant difference compared to treatment with only *S.* Typhimurium: *** $p < 0.001$.

6. DISCUSSION

E. coli and *S.* Typhimurium pose significant threats to both swine and human health due to both their zoonotic nature, via the consumption of contaminated pork products and direct contact with infected animals, and the fact that antibiotic resistance is becoming increasingly prevalent. These bacteria cause economic losses in the pork industry, as they cause gastrointestinal disease via mechanisms like adhesion to intestinal cells, oxidative stress induction [34, 36, 40].

The aim of our research was to assess the potential of *B. licheniformis* as probiotic *in vitro*, particularly in its ability to mitigate damage induced by pathogens. We conducted investigations to analyse its impact on cell viability, the production of ROS, and its ability to inhibit the adhesion of pathogenic bacteria. We hypothesised that *B. licheniformis* would reduce the levels of IC ROS and inhibit the adhesion of the pathogenic bacteria. To simulate these interactions, we utilized IPEC-J2 cells and challenged them with two economically significant swine pathogens, *S.* Typhimurium and *E. coli*, in a controlled *in vitro* environment [30, 32, 33].

B. licheniformis did not have an effect the viability of IPEC-J2 cells after either 1 or 2 hours of incubation in comparison to the control cells. These findings suggest that at the concentrations and durations examined in our experiments, this probiotic strain can be considered a suitable component for incorporation into our research protocols. Our results are consistent with a study conducted by Wu *et al.* where *L. plantarum* showed no detrimental effect to the viability of IPEC-J2 cells [58]. However, some probiotic bacteria have shown varying effects on the cell viability, notably, the probiotic strain *Clostridium tyrobutyricum* has been associated with increased cell viability [59], while *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* have led to decreased cell viability under conditions of higher durations and concentrations [60], emphasising that the treatment duration and concentration used may influence the variations in cell viability. Further research is required to precisely establish the time and concentration dependencies of the applied probiotic bacteria on IPEC-J2 cell viability. The investigation of the effects of the probiotics effects on cell viability is important in ensuring the reliability and interpretability of our experimental findings. Ensuring cell viability allows us to accurately assess the probiotic's impact on specific cellular processes and responses. The absence of any detrimental impact on cell viability aligns our research with physiological conditions present

in living organisms, enhancing the relevance and applicability of our findings to future *in vivo* scenarios. Additionally, the fact that *B. licheniformis* does not affect the IPEC-J2 cells, enhances the reproducibility increasing the likelihood of obtaining consistent results when replicated by other researchers [56, 57, 61].

ROS measurement serves as an essential marker for monitoring oxidative stress, with ROS production under oxidative stress causing damage to proteins, lipids, DNA, and tissues [45]. *E. coli* and *S.* Typhimurium contribute to oxidative stress in the intestines leading to inflammation and increased gut permeability [39, 46]. In the case of *E. coli* and *Salmonella*, the precise mechanism of inducing oxidative stress remains unclear. However, it is hypothesised that these pathogens create oxidative stress conditions in the intestines promoting an aerobic environment [29]. In our experiments, both *E. coli* and *S.* Typhimurium triggered a significant intracellular ROS surge in IPEC-J2 cells, which could be significantly reduced by pre-, co-, and post-treatments using *B. licheniformis*. Thus, showing the strong antioxidant effect of *B. licheniformis.* Our findings align with previous studies demonstrating the antioxidant properties of probiotic bacteria. For instance, *Bacillus amyloliquefaciens* SC06 exhibited a beneficial effect in mitigating oxidative stress induced by H_2O_2 in IPEC-1 cells [62]. Similarly, *Clostridium butyricum* was shown to alleviate oxidative damage in IPEC-J2 cells caused by enterotoxigenic *Escherichia coli* infection [63]. However, due to the limitations of the DCFH-DA method, which measures total ROS content, the exact mechanism through which probiotic bacteria reduce oxidative stress remains unclear. Some studies suggest that *Bacillus amyloliquefaciens* SC06 may enhance the expression of autophagy-related genes [64], while *Clostridium butyricum* reduces *E. coli* induced oxidative damage in IPEC-J2 cells by activating antioxidant enzymes associated with the Nrf2 antioxidant response element signalling pathway. Additionally, *Bifidobacteria, Lactobacillus* and other probiotics are known to produce folate, which having antioxidant properties, may also contribute to the antioxidative effects of probiotics [29]. The limitations of the DCFH-DA method, coupled with the relatively understudied mechanisms through which probiotic bacteria exert their antioxidant modalities, highlight the importance of future research in these areas. While the *in vitro* efficacy of *B. licheniformis* in reducing ROS levels in IPEC-J2 cells holds promise for potential *in vivo* mitigation of oxidative stress-induced damage, comprehensive investigations are warranted to fully evaluate this potential.

Additionally, *B. licheniformis* demonstrated significant inhibition of adhesion for both *S.* Typhimurium and *E.* coli, reducing adhesion by at least 44.9% and up to 99.7% for *E. coli* and *S.* Typhimurium, respectively. This finding aligns with a study by Ya Wang, which observed similar results to a lesser extent, albeit in a different cell culture model. In their study, *Lactobacillus johnsonii, Lacticaseibacillus rhamnosus*, and *Enterococcus durans* competitively suppressed the attachment of *S.* Typhimurium and *E. coli* to Caco-2 cells, resulting in a reduction of adhesion ranging from 30.73% to 55.18% [62]. Furthermore, in our study in the case of *S.* Typhimurium, the timing of *B. licheniformis* addition did not affect the level of adhesion inhibition, suggesting the involvement of multiple mechanisms. Similar outcomes were observed in another study whereby Caco-2 cells infected with *E. coli*, when exposed to *Lacticaseibacillus rhamnosus*, consistently reduced pathogen adhesion, regardless of the incubation protocol used, including coincubation, post incubation, or preincubation [65]. However, in our studies, it was seen that in the case of *E. coli* adhesion, while all treatment times resulted in significant adhesion inhibition, post-treatment with *B. licheniformis* exhibited a slightly lower inhibitory effect compared to pre- or co-treatments. The use of different cell culture models can't be disregarded when comparing findings among studies. It not only acknowledges the complexity of cell behaviour but also presents opportunities for future research and cross-study comparisons. The inhibition of pathogen adhesion holds significant importance in the context of antimicrobial therapy. By preventing pathogens from adhering to host cells, the establishment of infection will be mitigated. This not only reduces the pathogen's ability to initiate disease but also limits the release of virulence factors and toxins that cause tissue damage and harm. Furthermore, inhibiting adhesion enhances the effectiveness of the host's immune response, enabling it to better recognize and eliminate the invading pathogens [22].

Additional research is essential to substantiate our findings, involving a combination of *in vitro* investigations, such as the analysis of inflammatory cytokines and the examination of tight junction proteins to provide insight into its immunomodulatory properties and potential for reducing inflammation and its role in maintaining gut barrier integrity. Future research can explore the synergistic effects of using multiple probiotic strains or multispecies mixtures to enhance their overall impact. Extension into *in vivo* studies could provide a comprehensive validation of the outcomes observed thus far.

7. ABSTRACT

7.1. ENGLISH ABSTRACT

Modern farming continues to intensify with much higher demands for production animals. Consequently, the crowding and stress associated with intensive farming practices inherent in large-scale swine production yield a high incidence of intestinal infections caused by *E. coli* and *Salmonella* Typhimurium. These pathogens can induce oxidative stress and compromise the intestinal barrier function, resulting in diminished production, the emergence of clinical symptoms, or even mortality. For many years now, the habitual use of antibiotics for disease prevention and growth enhancement has played a significant role in the emergence and escalation of antimicrobial resistance. Currently the European Union is enforcing strict regulations on the use of antibiotics in veterinary medicine, especially on food producing animals. This has given rise to a demand for alternative treatments and dietary supplements that shall not result in resistance but positively impact animal health and productivity while preserving the integrity of the intestinal barrier.

In our experiments, we explored the efficacy of the protective actions of the probiotic bacterium *Bacillus licheniformis* (*B. licheniformis)* on IPEC-J2 cells when challenged with the pathogenic strains of *E. coli* and *S.* Typhimurium bacteria.

Firstly, by means of the Neutral Red assay, we ascertained that at a concentration of 10^8 CFU/mL, *B. licheniformis* did not impact the viability of IPEC-J2 cells; this concentration was thus employed in proceeding experiments. Subsequently, the DCFH-DA assay was used to examine the intracellular redox state of the cells. In this phase, *Bacillus licheniformis* and the pathogenic bacteria were introduced to the cells in three distinct treatment modalities: pre-treatment, co- treatment, and post-treatment.

Lastly, we evaluated the preventative efficacy of *Bacillus licheniformis* regarding inhibition of adhesion of pathogenic bacteria to the IPEC-J2 intestinal epithelial cells.

Our study's findings reveal that *Bacillus licheniformis* significantly mitigates the oxidative stress triggered by *E. coli* and *S.* Typhimurium in porcine intestinal epithelial cells. This probiotic not only preserves the integrity of the barrier function but also curtails the adhesion of pathogenic bacteria. It appears that *Bacillus licheniformis* is a strong contender for contribution to the positive effects of probiotics in animal health.

7.2. HUNGARIAN ABSTRACT ÖSSZEFOGLALÁS

A *Bacillus licheniformis* hatásának vizsgálata IPEC- J2 sejttenyészeten Az egyre intenzívebb modern állattenyésztés növeli a haszonállatok iránti keresletet. A nagyüzemi sertéstenyésztésre jellemző intenzív tenyésztési gyakorlatokhoz kapcsolódó zsúfoltság és stressz miatt gyakoriak az *Escherichia coli (E. coli)* és a *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) által okozott emésztőrendszeri megbetegedések. Ezek a kórokozók oxidatív stresszt idézhetnek elő és károsíthatják a bél barrier funkcióját, ami a termelés csökkenéséhez, klinikai tünetek megjelenéséhez vagy akár az állatok elhullásához vezethet. Az antibiotikumok betegségmegelőzésre és a hozamfokozására történő alkalmazása a múltban jelentős szerepet játszott az antimikrobiális rezisztencia kialakulásában és fokozódásában. Az Európai Unió jelenleg szigorúan szabályozza az antibiotikumok állatgyógyászatban történő felhasználhatóságát, különösen az élelmiszertermelő állatok esetében. Mindez növeli az igényt olyan takarmánykiegészítők és kezelések iránt, amelyeknem vezetnek rezisztenciához, pozitívan befolyásolják az állatok egészségét és termelékenységét, miközben megőrzik a bél barrier integritását.

Kísérleteinkben egy probiotikus baktérium, a Bacillus licheniformis *(B. licheniformis*) védőhatásának hatékonyságát vizsgáltuk *E. coli* és *S.* Typhimurium patogén baktériumtörzsekkel szemben sertés bélhámsejt (IPEC-J2) tenyészeten.

Kísérleteink első fázisában a Neutral Red módszer segítségével megállapítottuk, hogy 10^8 CFU/ml koncentrációban a *B. licheniformis* nem befolyásolja az IPEC-J2 sejtek életképességét, ezért ezt a koncentrációt alkalmaztuk a további kísérletek során. Ezt követően a DCFH-DA-módszert alkalmaztuk a sejtek intracelluláris redox állapotának vizsgálatára. Három különböző kezelési módot alkalmaztunk; a probiotikummal történő elő-, egy- és utóidejű kezelést.

Végül megvizsgáltuk, hogy a *B. licheniformis* hogyan befolyásolja a patogén baktériumok IPEC-J2 sejtekhez történő tapadását.

Eredményeink alapján a *B. licheniformis* jelentősen mérsékeli az *E. coli* és a *S.* Typhimurium által kiváltott oxidatív stresszt IPEC-J2 sertés bélhámsejtekben. Továbbá ez a probiotikum a patogén baktériumok tapadását is gátolja. A B. licheniformis tehát hozzájárulhat a probiotikumok állatok egészségére gyakorolt pozitív hatásaihoz.

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Témavezetői nyilatkozat TDK dolgozathoz

FABAS ORSOLYA DÁVIDNÉ TÉXT NICOLE

Alulírott ....., mint témavezető nyilatkozom, hogy (név).NICOLE LAWLOR....., 6..... évfolyamos hallgató „THE EFFECT OF BACILLUS LICHENIFORMIS ON AN IPEC-J2 CELL LINE" című dolgozatát átolvastam és jóváhagytam, részvételét támogatom az Állatorvostudományi Egyetem 20... . évi Tudományos Diákköri Konferenciáján. Továbbá nyilatkozom, hogy a feltöltött TDK dolgozat plágiumellenőrzésen sikeresen átesett és az esetlegesen feltárt egyezőség az Egyetemi iránymutatásoknak/szabályoknak megfelel.

Budapest, 2023 év....10.....hó 18.nap.

.....

témavezető

DECLARATION

I hereby declare that the thesis entitled **The effect of *Bacillus licheniformis* on an IPEC-J2 cell line** is identical in terms of content and formal requirements to the TDK research paper submitted in 2023 (year).

Date: 13/11/23

Nicole Lawlor

.....

Student name and signature

Thesis progress report for veterinary students

Name of student: Nicole Lawlor

Neptun code of the student: Z4IL3R

Name and title of the supervisor: Orsolya Farkas PhD, senior r. fellow

Department: Pharmacology & Toxicology | Nikolett Péric junior res. fellow

Thesis title: Effect of Bacillus licheniformis on porcine intestinal cell culture

Consultation – 1st semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2023	03	14	Experiment - cell viability mes.	
2.	2023	03	16	Paracellular permeability meas.	
3.	2023	04	03	Adhesion - colony counting	
4.	2023	04	07	Adhesion - colony counting	
5.	2023	04	24	ROS measurement	

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

Consultation – 2nd semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2023	09	11	TDK - Introduction	
2.	2023	09	18	TDK - Results, Disc.	
3.	2023	09	25	TDK - Materials, Meth.	
4.	2023	10	13	TDK - final draft	

5.	2023	10	30	Oral presentation	

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

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

I accept the thesis and found suitable to defence,

.....
signature of the supervisor

Signature of the student: Nicole Lawlor

Signature of the secretary of the department: .....

Date of handing the thesis in..... .....