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Article

Establishment of a Chicken Ileal Explant Culture for Microbial Investigation

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Abstract: Gut explant cultures represent fragments of the whole gut section with its mucosa, maintained *ex vivo*, which closely mirror the key characteristics of the organ in living animals. A major advantage of this model is its preserved polarized and layered structure, which enables essential cell-to-cell interactions. The aim of the study was an establishment of a chicken ileal explant culture to study the pathogenic effect of *Salmonella* Enteritidis pathogen to assess the compatibility of a chicken ileal explant culture with bacteria. Regarding our results, the hematoxylin and eosin-stained histology sections of a chicken ileum had a normal tissue architecture not only in the control group but also in the infected group. Gene expression analysis shows significant up-regulation of four genes (IL-22, IL-6, TNF-α and CK18), demonstrating the ability of the ileal explant culture to effectively respond to infection. Moreover, measurement of metabolic activity in ileal explants showed that metabolic activity was still relatively high after 4 h of infection, indicating good cell viability of tissue explants outside the body under conditions of high bacterial infection. In conclusion, this novel model may replicate the *in vivo* environment of the chicken intestine, incorporating all its layers and its ability to study host-pathogen interactions.

Keywords: chicken; explant culture; inflammation; qPCR; pathogen;

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1. Introduction

High production broiler chicken farming represents the largest livestock production sector in the world (Mench et al., 2021). According to a recent European Commission market report, poultry meat production in the European Union reached approximately 13.3 million tons in 2023, marking a 2.3 % increase compared to 2022 (EU Commision market report, 2023). The poultry production chain employs practices to breed large numbers of birds, reduce mortality, and enhance overall performance. Modern poultry strains are genetically selected for rapid growth, reaching a target weight of 2–2.5 kg within less than 45 days, a rate unmatched in other livestock systems (Bennett et al., 2018; Khatibjoo et al., 2018). As a result, the poultry industry is the world's number one supplier

of high-quality animal protein for human consumption. Poultry meat and eggs offer advantages over other animal-based foods, including superior protein content, balanced amino acids, and essential micronutrients (Bohrer, 2017), furthermore skinless poultry meat has lower fat levels compared to mammalian meat, due to minimal intramuscular fat. Additionally, the rapid growth, short generation interval, and omnivorous nature of meat-type poultry make them more efficient than other livestock (Korver, 2023).

However, it should be noted that all these facts about intensification of production, driven by increasing demand, often reduces the emphasis on animal health and welfare. Stressors such as temperature fluctuations, high stocking densities or infections can reduce feed intake, growth and immune response, leading to higher susceptibility to disease, because of reduced corticosteroid levels. Furthermore, immune dysfunction and the development of oxidative stress in animals can result (Abo-Al-Ela et al., 2021). *Salmonella* is a leading cause of foodborne illnesses worldwide, infecting the gastrointestinal tract and causing diarrhea, nausea, and cramps. Globally, non-typhoidal *Salmonella* particularly *Salmonella* Enteritidis and *Salmonella* Typhimurium, accounts for 93 million gastroenteritis cases and 155,000 deaths each year. The severity of salmonellosis depends on factors like strain, host age, and health status. Infections can occur with as few as 100 bacterial cells in infants, with even lower doses posing risks to immunocompromised individuals (Shaji et al., 2023).

The data clearly demonstrates that poultry has a key role in the spread of alimentary zoonoses, but representative cellular models to study host-pathogen interactions in detail are still lacking. The need for high reliability of *in vivo* studies, compliance with animal welfare requirements and complex legislation is leading to a search for alternative methods to replace animal testing in conjunction with the principles of the 3Rs (Replacement, Refinement, Reduction). *Ex vivo* gut microbiota simulation models are vital tools for evaluating the properties of functional substrates and their effects on the gastrointestinal environment and bacterial diversity. Compared to *in vivo* models, still regarded as the "gold standard" these methods are simpler, more reproducible, cost-effective, and time-efficient. Thus, *ex vivo* models are a good starting point to identify and study the real impact of supplementing animal feed with new functional ingredients (Mota de Carvalho et al., 2021). For *ex vivo* studies of host-microbe interactions in poultry, there is currently no reliable and readily available avian gut model. Conventional poultry intestinal models, consisting of primary epithelial cells, fail to mimic complex physiological processes and have plenty of serious shortcomings (Ghiselli et al., 2021a). In parallel, tissue explants more closely resemble *in vivo* tissues both in phenotype and function, offering a more accurate simulation of host-pathogen interactions (Nash & Vervelde, 2022).

The present study aimed to investigate the ability of pathogenic *Salmonella* Enteritidis to induce an inflammatory response on the intestinal mucosa of the chicken *ex vivo*, studied on ileal explant cultures to assess the compatibility of a chicken ileal explant culture with bacteria. First, the viability of the explants was assessed using the MTS assay. Following this, gene expression analysis was performed for pro-inflammatory cytokines such as interleukin (IL)-6, IL-22, and tumor necrosis factor-alpha (TNF-α), as well as genes related to epithelial integrity, including villin (VILL), cadherin-1 (CDH1), cytokeratin-18 (CK18), and mucin-2 (MUC2). Additionally, H&E-stained histology sections were examined to gain a comprehensive understanding of the enteric innate immune response.

2. Materials and Methods

2.1. Bacterial culture

Salmonella enterica subsp. *enterica* serovar Enteritidis (*S*. Enteritidis; SE) phage type PT4, was provided by assoc. prof RNDr. Ivan Rychlík, Ph.D (Veterinary Research Intitute, Brno, Czech republic). Bacteria were cultivated in LB broth (Luria–Bertani broth; Sigma-Aldrich, St. Louis, MO, USA) overnight at conditions 37 °C, 160 rpm constant shaking and the next day were inoculated into fresh LB media incubated for 4 h at the same conditions as previous. After this incubation period the bacterial culture were centrifuged (500 ×g for 10 min.; Hettich EBA 200, Tuttlingen, GER) and the pellets were washed three times with PBS (Phosphate Buffered Saline). After the last centrifugation

the bacterial culture was resuspended in DMEM/F12 media (without antibiotic and serum) for the required concentrations (10^7 CFU/well). The bacterial concentration count was quantified by measuring the optical density (OD) at wavelength 600 nm in a Synergy HTX Multi-Mode Reader spectrophotometer (Agilent, Santa Clara, CA, USA) prior to the experiments. The concentration of bacteria was previously confirmed by serial dilution and determination of colony forming units (CFU) on Mueller-Hinton agar plates. These counts were correlated with the corresponding absorbance values.

2.2. Explant isolation and treatment

The isolation of ilelal explants was performed exactly according to the procedures described in the article by Mátis et al. (2024). Briefly, a 3-week-old Ross-308 broiler chicken was humanely euthanized through decapitation under CO_2 anesthesia. After dissection of the digestive tract, a approximately 10 cm long ileal segment was collected and flushed multiple times with phosphate-buffered saline (PBS) + 1 % penicillin-streptomycin solution (Pen-Strep, Gibco, Waltham, MA, USA). The ileal segment was then longitudinally opened, and individual explant cultures were taken using disposable 1.5 mm biopsy punches with plungers (MDE, Heidelberg, Germany). These explant culture pieces were placed into 96-well plates coated with type I collagen and cultured in modified DMEM/F12 medium. The treatment of explants with enteropathogenic SE proceeded as follows: bacteria were diluted in DMEM/F12 medium to the required concentration, representing 10^7 CFU/well in the 96-well plate. After bacterial application, the plates were cultured for 4 hours under conditions of 37 °C and 5 % CO_2. After the incubation period, supernatants were collected, and medium containing gentamicin (100 ug/mL; Sigma-Aldrich, Missouri, USA) was added for 2 hours to kill the remaining extracellular bacteria. Following this incubation, the explants were harvested for total RNA isolation.

2.3. qPCR

The total RNA from each samples was isolated from the explants using RNeasy Mini Kit (Qiagen, Hilden, Germany) exactly according to the manufacturer's instructions. The quantity and purity of RNA (free of DNA and proteins), was determined at 260/280 nm, using NanoDrop 8000 (Thermo Scientific, Waltham, MA, USA). The cDNA was reverse-transcribed from RNA, using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) as outlined by the manufacturer. The qPCR analysis was performed using CFX Manager Software (CFX Manager version 2.0, Bio-Rad, Hercules, CA, USA) in a 10 μL reaction volume containing: 1 × iQ™ SYBR® Green Supermix (Bio-Rad), 0.5 μM forward and reverse primers and 40 ng/μL of cDNA. Ubiquitin was used as a reference gene for internal control. All reactions were performed in triplicate. The experimental protocol consisted of the initial denaturation at 95 ◦C for 5 min, followed by amplification - 40 cycles of 3 steps (denaturation at 94 ◦C for 30 s, annealing at 60 ◦C for 30 s, extension at 72 ◦C for 30 s), and final extension at 72 ◦C for 15 min, followed by melting curve analysis to confirm the amplification of a specific product. Relative normalized expression was calculated using the $2^{-\Delta\Delta CT}$ method. Results of the gene expression experiment conducted in triplicate were expressed as mean ± standard deviation (SD). Primers for the mRNA expression analysis are listed in Table 1.

2.4. Metabolic activity

Cell viability was investigated using the Cell Titer 96 AQueous One Solution Reagent (Promega, USA) - MTS assay. The assay was conducted in adherence to the manufacturer's instructions. Briefly, at the end of the indicated incubation time (2 h or 4 h), DMEM/F12 medium was collected from the explants and transferred to new 96-well plates. DMEM/F12 media (100 μL) was supplemented with MTS solution (20 μL/well), incubated for 1 h, and then the absorbance was measured at 490 nm with a Synergy HTX microplate reader (Agilent, USA). This colorimetric assay is used to assess cellular metabolic activity. It relies on the capability of viable cells to reduce tetrazolium compounds. During incubation with the MTS reagent, mitochondrial dehydrogenase enzymes in healthy cells convert the tetrazolium dyes in MTS into insoluble formazan, which can be measured at 490 nm. As cell viability declines, the activity of dehydrogenase enzymes diminishes, leading to a corresponding decrease in absorbance.

2.5. Hematoxylin and Eosin-staining Histology

The ileal explants were collected immediately after completing the incubation with SE. The explant cultures were fixed directly onto foam biopsy pads and placed in 8% neutral buffered formalin at room temperature for 24 hours. After fixation, the samples were trimmed and dehydrated through a series of ethanol and xylene treatments using an automatic tissue processor. The tissues were then embedded in paraffin blocks, sectioned manually, and mounted onto Superfrost+ adhesion slides (Thermo Fisher Scientific, Waltham, USA) in 3-4 μm thin sections. Routine hematoxylin and eosin-staining (H&E) was performed using an automatic staining instrument.

2.6. Statistical analysis

To determine significant differences in gene expression analyses and MTS assay -GraphPad Prism 9.0.0 software was used (GraphPad Software Inc., San Diego, USA), and one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons. The level of significance was set as p-value ≤ 0.05 considered significant (*), p-value ≤ 0.01 considered highly significant (**), p-value ≤ 0.001 (***) considered highly significant.

3. Results and Discussion

Isolation of Ileal Explants

To stop the spread of a particular disease, it is essential to understand the molecular mechanisms of the pathogen in question, ideally in its natural environment where it replicates and survives. Chicken gastrointestinal models are therefore of fundamental importance as they allow direct study of the pathogen-host relationship at the tissue level. There is growing public opposition to animal experimentation, leading to the need for stricter controls and a reduction in the number of animals used for these purposes. In this respect, the *ex vivo* models meet not only technical requirements but also bioethical considerations, as it eliminates the harmful effects of the tested substances on live animals (Duarte et al., 2021). However, there is currently no reliable and readily available avian gut model on the market for *ex vivo* studies of host-microbe interactions in poultry.

Conventional poultry gut models consisting of primary epithelial cells cannot mimic complex physiological processes and have several serious shortcomings (Ghiselli et al., 2021a). Several research teams have successfully introduced intestinal explants in poultry, but the number of publications is still limited. Explant models derived from the chicken ileum have been successfully used to test the harmful effects of mycotoxins and antifungal additives (Duarte et al., 2021), or to study the measurement of nitric oxide production and epithelial inflammatory after stimulation with bacterial lipopolysaccharides (Kallapura et al., 2015; Zhang et al., 2017). Mátis et al. (2024) used ileal explants from 21-day-old broiler chickens to analyze the protective

effect of cathelicidin-2, which belongs to the host defense peptides (HDPs). The authors pointed out the strong anti-inflammatory effect of cathelicidin-2, as it attenuated the LTA-induced increase in evaluated interleukins. In our study, we found that the protocol for obtaining ileal explants is much simpler, more accessible and less costly than working with primary cultures obtained from chicken embryos (Figure 1a-b). At the same time, this method does not require sophisticated material, technical or personnel equipment in the laboratory. In addition to these practical advantages, the explant method has attracted particular attention because it contains all the cell types and proportions normally found in epithelia. Furthermore, it allows organ fragments from a single donor to be subjected to different treatments while controlling the environmental conditions to which the tissue is exposed (Rozehnal et al., 2012). Therefore, *ex vivo* analysis using explants provides a better representation of the complex morphology of the whole organism, thus improving the reproducibility of various processes observed *in vivo* (Randall et al., 2011).

(a) (b)

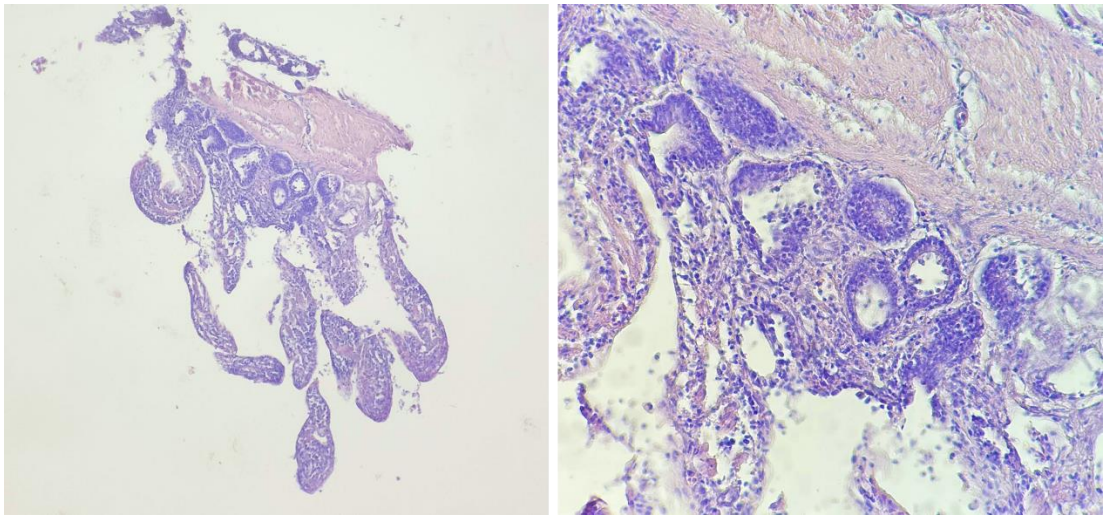
Figure 1. A procedure for isolating ileal explants from the digestive tract of poultry (**a**) Approximately a 10 cm long ileal segment, positioned 10 cm distally from the Meckel's diverticulum, was excised; (**b**) Ileal explant size 1.5 mm taken under light microscope, 10-fold magnification.

Histological analysis

The histological analysis of explants was performed to investigate the appearance of the intestine after treatment with pathogenic strain of *Salmonella* at a concentration of 10^7 CFU/well for 4 h. The use of disposable 1.5 mm biopsy punches with plungers made the routine preparation of histological samples somewhat difficult due to the small size of the tissue (Figure 2a-b). Nevertheless, we were able to obtain sections of 3-4 μm thickness, which were stained with H&E as standard. Microscopic analysis revealed numerous mucus-filled Lieberkühn's crypts in the *tunica mucosa* and several intestinal villi with a preserved epithelial layer (Figure 2a-b). Increased desquamation of the surface epithelial layer was observed in most samples, likely due to handling challenges associated with their small size. However, we did not observe any significant signs of massive epithelial destruction caused by *Salmonella* infection compared to the control group (results not shown).

Although several studies have performed morphometric analyses of intestinal explant cultures, this approach was not feasible in our case due to the miniature size of the explants used. For example, Kolf-Clauw et al. (2009) used dissected explant pieces measuring 3 x 4 mm, which allowed for detailed morphological analyses (Kolf-Clauw et al., 2009). Similarly, Duarte et al. (2021) used intestinal explants from broiler chickens measuring approximately 1 cm^2, and evaluated parameters such as villus height, villus number, crypt diameter, the diameter ratio of villus height-to-crypt, enterocyte nuclear size, apical enterocyte height and cytoplasmic vacuolization score of enterocytes at the villus tips (Duarte et al., 2021). When smaller explant cultures are used, histological analysis becomes increasingly challenging, as the intestinal villi are often prone to deformation. This is

because maintaining the organ's tubular shape requires precise positioning of explants; otherwise, eversion and deformation can occur, complicating morphological assessments (Cortez and Guedes 2023). However, when preparing explant cultures, using smaller tissue fragments is biologically advantageous due to the limitations and survival challenges of enterocytes outside the body under *ex vivo* conditions. This approach allows for more precise control over the culture environment, enhancing the viability and functionality of the enterocytes (Hughes et al., 2021).



(a) (b)

Figure 2. H&E-stained chicken ileal explant section. (**a**) Microscopic magnification = 10x; (**b**) Microscopic magnification = 40x.

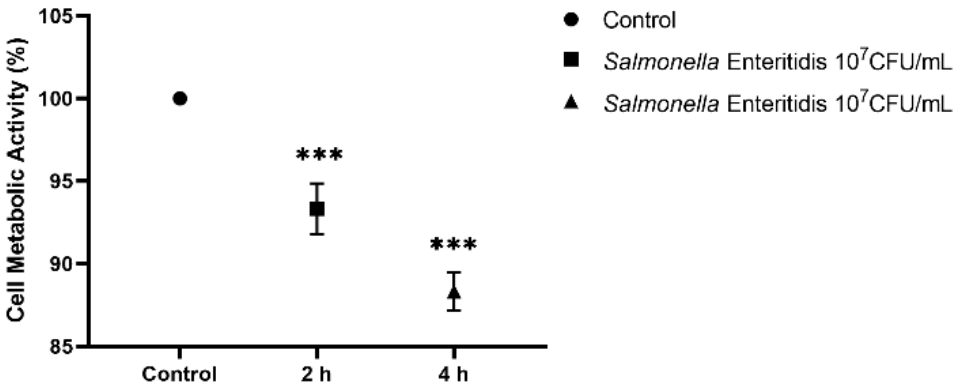


Figure 3. Metabolic activity assay of the explants after treatment. The results are presented as percentages, reflecting relative absorbance and activity, with the Control group average set as 100%.

Proliferation assay

A colorimetric MTS assay was used to assess cell viability in intestinal explants and to determine their metabolic activity under conditions of exposure to pathogenic *Salmonella* for 2 h and 4 h. Although, according to the results of the MTS assay, explants showed a significant ($p \leq 0.001$) decrease in metabolic activity two hours after SE treatment compared to the control group, the percentage of the explants was consistently high (≥ 93 %) (Figure 3). After 4 hours of induction with SE, 13 % decrease was observed compared to the control group, with the same significance as in the previous case ($p \leq 0.001$). This decrease in metabolic activity suggests that the pathogens to which the explants in the experimental group were exposed reduced cell proliferation and the metabolic activity of living cells. However, in this context, we believe that

the decrease to 87 % still indicates a modest but not critical effect. A reduction in metabolic activity below 50 % would indicate severe cell damage. The result may also indicate a specific biological response to *Salmonella* pathogens, such as activation or inhibition of certain metabolic pathways (Ghasemi et al., 2021).

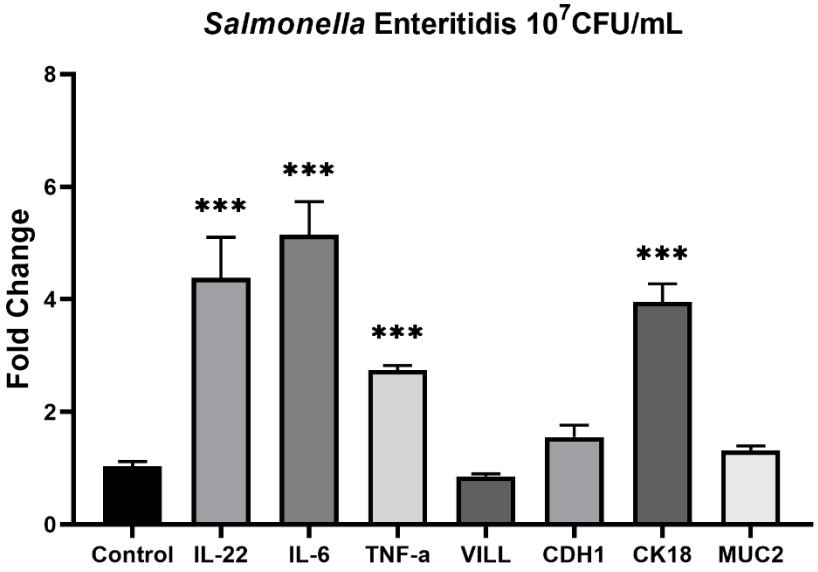


Figure 4. Gene expression analysis for selected markers. Gene expression analysis of pro-inflammatory and TJ-related genes in ileal explant cultures after 4 h incubation with SE. The level of significance was set as *p ≤ 0.05 considered significant, **p ≤ 0.01 considered highly significant, and ***p ≤ 0.001 considered highly significant. Significantly differed from the control group.

Gene expression analysis

Infection with intestinal pathogens is known to induce inflammatory processes associated with the release of pro-inflammatory cytokines or chemokines, apoptosis of epithelial cells or reduction of tight junction (TJ) proteins (Peterson, 2014). In our study, we quantified the levels of mRNAs encoding pro-inflammatory cytokines (IL-6, IL-22 and TNF-a) and molecules related to enterocyte TJ (VILL, CDH1, CK18 and MUC2) using gene expression analysis to investigate the effect of SE on intestinal explants (Figure 4). The levels of all cytokines analyzed were significantly increased ($p \leq 0.001$) compared to the control group, and cytokeratin-18 was also significantly up-regulated ($p \leq 0.001$). The obtained results are consistent with the current literature describing the pro-inflammatory effect of *Salmonella* spp. at the gene expression level in a poultry model (Swaggerty et al., 2014; Chang et al., 2020). Increased expression of CK18 may be a response to damage to the intestinal epithelium or stress conditions such as inflammation or infection. Damage may result in increased cleavage of CK18, an indicator of apoptosis or programmed cell death (Arce et al., 2014; Eguchi et al., 2014).

Table 1. Table of used primers.

Gene name	Abbreviation	Primer sequence 5′→ 3′	Reference
Interleukin-22	IL-22	F: CAGACTCATCGGTCAGCAAA R: GGTACCTCTCCTTGGCCTCT	Crhanova et al., 2011
Interleukin-6	IL-6	F: GCTACAGCACAAAGCACCTG R: GACTTCAGATTGGCGAGGAG	This study
Tumor necrosis factor-alpha	TNF-α	F: AATTTGCAGGCTGTTTCTGC R: TATGAAGGTGGTGCAGATGG	Crhanova et al., 2011
Villin	VILL	F: GAACCTCTCGTGGCACCGC R: CTCATGTCCCTGCACCTCCC	Ghiselli et al., 2021b
Cadherin-1	CDH1	F: TGAAGACAGCCAAGGGCCTG R: CTGGCGGTGGAGAGTGTGAT	Ghiselli et al., 2021b
Cytokeratin-18	CK18	F: CACAGATCCGGGAGAGCCTG R: CTCCACCGCGCTGTCATAGA	Ghiselli et al., 2021b
Mucin-2	MUC2	F: GCTGATTGTCACTCACGCCTT R: ATCTGCCTGAATCACAGGTGC	Smirnov et al., 2006
Ubiquitin	UB	F: GGGATGCAGATCTTCGTGAAA R: CTTGCCAGCAAAGATCAACCTT	De Boever et al., 2008

5. Conclusions

The chicken ileal explant model accurately mimics the *in vivo* environment of the poultry intestine by incorporating all layers of the intestine. In this study, we demonstrated the suitability of the chicken ileal explant model for the study of host-pathogen interactions by evaluating gene expression analysis for selected pro-inflammatory cytokines and TJ-related molecules, examining the intestinal epithelium using histological sections and assessing cell viability using the MTS assay. Based on our results, we suggested that this model may be used to study host-microorganism interactions at the molecular level.

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Conflicts of Interest: The authors declare no conflict of interest.

Data availability statement: Other data and files related to this study are available from the authors on request.

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