Versatile human in vitro triple coculture model coincubated with adhered gut microbes reproducibly mimics pro-inflammatory host-microbe interactions in the colon

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Abstract
The colonic epithelial barrier is vital to preserve gut and host health by maintaining the immune homeostasis between host and microbes. The mechanisms underlying beneficial or harmful host-microbe interactions are poorly understood and impossible to study in vivo given the limited accessibility and ethical constraints. Moreover, existing in vitro models lack the required cellular complexity for the routine, yet profound, analysis of the intricate interplay between different types of host and microbial cells. We developed and characterized a broadly applicable, easy-to-handle in vitro triple coculture model that combines chemically-induced macrophage-like, goblet and epithelial cells covered by a mucus layer, which can be coincubated with complex human-derived gut microbiota samples for 16 h. Comparison with a standard epithelial monolayer model revealed that triple cocultures produce thicker mucus layers, morphologically organize in a network and upon exposure to human-derived gut microbiota samples, respond via pro-inflammatory cytokine production. Both model systems, however, were not suffering from cytotoxic stress or different microbial loads, indicating that the obtained endpoints were caused by the imposed conditions. Addition of the probiotic Lactobacillus rhamnosus GG to assess its immunomodulating capacity in the triple coculture slightly suppressed pro-inflammatory cytokine responses, based on transcriptomic microarray analyses. TNF conditioning of the models prior to microbial exposure did not cause shifts in cytokines, suggesting a strong
epithelial barrier in which TNF did not reach the basolateral side. To conclude, the triple coculture model is tolerable towards manipulations and allows to address mechanistic host-microbe research questions in a stable in vitro environment.

**KEYWORDS**
coculture techniques, epithelial cells, gastrointestinal microbiome, gene expression profiling, host microbial interactions

## 1 | INTRODUCTION

The colonic mucosa constitutes a diverse yet crucial interface between the human host and its microbiome. It is composed of a specialized epithelial barrier with mainly columnar epithelial cells, intermittent goblet cells (≥25% compared to less than 10% in the small intestine), enter- endocrine cells, intraepithelial lymphocytes, an underlying lamina propria and the muscularis mucosa. The epithelial barrier separates the luminal gut content, including microbiota, from the lamina propria in order to maintain immune homeostasis. Epithelial cells express tight junction proteins to create a strong and semipermeable barrier which is covered by a mucus layer produced by goblet cells. Besides epithelial cells, intraepithelial lymphocytes also act as gatekeepers of immune homeostasis via pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and NOD-like receptors (NLRs), that recognize pathogen-associated molecular patterns (PAMPs) of microbes (for example lipopolysaccharides, flagellin and peptidoglycans) and thereby enable communication between microbiota and the underlying immune cells. The immune function of the gut is vital in the human body. After immune cell priming, the immune system must be capable to discriminate between commensal microorganisms and pathogens in order to achieve immune tolerance or immune reaction against certain antigens. Microbiota fulfill a critical role in the education and functional tuning of the immune system, particularly during the neonatal window of opportunity. The interaction between microbiota, gut barrier function and local immunity is an important driver of intestinal homeostasis. A disruption in this interaction due to genetic predisposition, environmental factors, microbiome composition or other determinants (such as age, childhood infections) may result in increased predisposition to antigen exposure, bacterial translocation or chronic inflammation. The separation of the mucosa from the in vivo environment makes it difficult to conduct mode of action studies on the impact of microbiota, microbial antigens or metabolites towards gut barrier function and immune responses. To address these difficulties, the in vitro modeling of the colonic mucosal environment using cell cultures provides an interesting opportunity to have access to the difficult accessible mucosal environment and to be able to conduct mechanistic research.

Since the first use of human cell lines in 1951, major progress has been made in the tissue culturing research field. To date, numerous models and various setups exist to mimic the gut mucosa in vitro. The cancerous Caco-2 cell line in Transwell inserts is the most widely used enterocyte model in drug permeability and absorption studies. However, variations in permeability characteristics can occur in Caco-2 cells as a result of different culturing conditions across labs, impairing reproducibility. The colon T84 cell line has similar characteristics but has been proven to be more suitable for the colon microenvironment and is valuable for host-microbe studies. Other human cell lines from colon origin such as HT29-MTX and LS-174T possess goblet-like cell properties, resulting in the secretion of mucus. The advantage of using LS-174T cells is that they mainly produce MUC2 mucin, the most abundant mucin type in the gastrointestinal tract.

Several researchers already succeeded in creating models with higher physiological relevance by coculturing Caco-2 and HT29-MTX cells and attempts have been made to include an immune compartment by adding Raji B cells to the Caco-2/HT29-MTX model. Raji B cells induce the Caco-2 cells to acquire an M cell phenotype and, notably, mucus secretion was only observed in the triple coculture model. Leonard et al. developed an inflamed intestinal mucosa model that used enterocytes seeded on top of blood-derived macrophages and dendritic cells embedded in a collagen matrix and Kämpfer et al. combined Caco-2 cells with differentiated THP-1 cells. The use of phorbol 12-myristate 13-acetate (PMA)-induced macrophage-like THP-1 cells provides an opportunity to include a part of the innate immune system in in vitro models. Although the use of cell lines has multiple advantages like cost effectiveness, easiness of use, reproducibility and the lack of ethical concerns associated with the use of animal or human tissues, the cellular responses can differ from primary cells. In addition, current cell-line based models prove inadequate to study host-microbe interactions since they do not test microbes or use single microbial strains, which is an oversimplification of the in vivo microbial complexity. The use of synthetic microbial communities was shown to be a promising strategy in a small intestinal...
host-microbe model that combines THP-1, HT29-MTX and Caco-2 cells. Moreover, more advanced models to mimic the intestinal structure are being developed. Stem cell-derived organoids, isolated from human biopsies, differentiate into all the relevant epithelial cell types and offer the possibility to study diseases at personalized level. Noel et al. developed a primary human macrophage-enteroid coculture model consisting of monocyte-derived macrophages and intestinal epithelial monolayers derived from stem cell containing crypts, that was able to investigate host responses to enteric pathogens. Microfluidic models and 3D tissue models using intestinal biopsies, on the other hand, mimic the tubular architecture of the intestine that results in a lower oxygen pressure in the lumen and allows to mimic peristaltic movement by introducing fluid flow and cyclic strain. Examples of such models include the primary human mucosal barrier model in anaerobic coculture with Faecalibacterium prausnitzii, called the gut-microbiome (GuMi) physiome platform and the primary human ileal intestine chip cocultured with human fecal-derived gut microbiota.

Although efforts have been made to create in vitro models representing the human gut physiology, existing models are often limited in volume and labor-intensive to maintain, which is not desirable for screening assays to unravel the mechanistic host response to bacteria. Technically simpler model setups, however, are lacking the cellular complexity of the epithelium in combination with immune responses, the production of a covering mucus layer and most importantly the combination with relevant microbial gut communities to mimic the host-microbe interplay.

We, therefore, developed and characterized an in vitro triple coculture model with PMA-induced macrophage-like THP-1, LS-174T goblet and T84 epithelial colon cells that can be coincubated with relevant gut microorganisms as well as complex human-derived microbiota samples in a biologically reproducible way. The added value of this model lies in (i) the integration of microbiota- and host-derived components of the gut mucosal environment, (ii) the modulatory setup that allows for mechanistic mode of action studies and (iii) the opportunity to assess the response to donor-derived microbial stimuli and gut environmental cues.

2 | MATERIALS AND METHODS

A reproducible triple coculture in vitro model consisting of PMA-induced macrophage-like THP-1, LS-174T goblet and T84 epithelial colon cells in indirect contact with adhered gut microbes was developed to permit the mechanistic study of complex microbiota-host interactions in the human gut (Figure 1).

2.1 | Human cell lines

The T84 cell line (CLS 300354) and LS-174T cell line (CLS 300392) were obtained from Cell Lines Service (Eppelheim, Germany). T84 cells were grown in Dulbecco’s modified Eagle medium/Nutrient Mixture F-12 (DMEM/F-12) containing 15 mM HEPES buffer (Gibco, Thermo Fisher Scientific, Merelbeke, Belgium), supplemented with 10% (v:v) heat-inactivated fetal bovine serum (FBSi) (Greiner Bio-One, Kremsmünster, Austria) and 1% (v:v) antibiotic antimycotic solution containing penicillin, streptomycin and amphotericin B (Merck, Overijse, Belgium). LS-174T cells were grown in minimal essential medium (MEM) containing Earle’s balanced salts (Gibco, Thermo Fisher Scientific, Merelbeke, Belgium), supplemented with 1% (v:v) non-essential amino acids solution (Gibco, Thermo Fisher Scientific, Merelbeke, Belgium), supplemented with 10% (v:v) FBSi and 1% (v:v) antibiotic antimycotic solution. The THP-1 cell line was obtained from the European Collection of Authenticated Cell Cultures (ECACC 88081201, Public

![Figure 1](image-url)
Health England, London, United Kingdom). THP-1 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 with 2 mM GlutaMAX™ (Gibco, Thermo Fisher Scientific, Merelbeke, Belgium) supplemented with 10% (v:v) FBSi and 1% (v:v) antibiotic antimycotic solution. THP-1 cells were routinely grown in suspension in a lateral 3D rotating wall vessel (RWV) bioreactor (Synthecon, Houston, USA). Passages between 48–67, 46–65, and 20–39 of T84, LS-174T, and THP-1 cells, respectively, were used, and cells were tested mycoplasma-free prior to setting up experiments using MycoAlert (Lonza, Basel, Switzerland). All three cell types were grown at 37°C, 10% CO₂ and 90% relative humidity.

### 2.2 Seeding of T84 monolayers and triple cocultures in collagen type I coated plates

A collagen type I coating was prepared in the basolateral side of 24-well plates (Corning Incorporated, New York, USA) by adding 400 µl/well from a 1% collagen type I solution from rat tail (4.5 mg/ml, Sigma-Aldrich, Overijse, Belgium) in cell culture grade H₂O (Gibco, Thermo Fisher Scientific, Merelbeke, Belgium) and incubated overnight at 4°C. Excess fluid was removed and the surface was left to dry overnight. The collagen coating was washed with 1 ml/well Dulbecco’s phosphate-buffered saline (PBS, Gibco, Thermo Fisher Scientific, Merelbeke, Belgium) before seeding cells. In the case of epithelial monolayers, T84 cells were seeded in collagen coated 24-well plates in a density of 155 500 cells/cm² in supplemented DMEM/F-12 and grown for three days until confluency was reached. For triple cocultures, THP-1, LS-174T and T84 cells were cocultured and the cells were seeded sequentially in collagen coated 24-well plates. First, THP-1 cells were seeded in a density of 15 500 cells/cm² in supplemented RPMI. To chemically differentiate THP-1 monocytes to M0 macrophage-like THP-1 cells, 100 nM phorbol 12-myristate 13-acetate (PMA, Cayman Chemical, Ann Arbor, USA) was added to the medium for 72 h, followed by 24 h of rest in medium deprived from PMA. Next, LS-174T and T84 cells were seeded in a 20:80 ratio at a density of 155 500 cells/cm² in supplemented DMEM/F-12. Triple cocultures were grown for 3 days until confluency (Figure 1). Twenty-four hours before the start of any experiment, the cell culture medium of monolayers and triple cocultures was refreshed with DMEM/F-12 with FBSi and deprived of antibiotic antimycotic solution. Triple coculture and monolayer responses were compared in the absence and presence of inflammatory (TNF) and microbial triggers in triplicates (three wells per condition) (Table 1). When TNF conditioning was assessed, 10 ng/ml recombinant human tumor necrosis factor (TNF) (Gibco, Thermo Fisher Scientific, Merelbeke, Belgium) was supplemented to the basal medium and incubated for 24 h prior to setting up the host-microbe coincubations. Host-microbe interactions were evaluated by coincubation of the triple coculture cell model with microbial biofilms composed of gut microbes (early-stage biofilms are created in our setup) with or without *Lactobacillus rhamnosus* GG (LGG) addition.

### 2.3 *L. rhamnosus* GG and simulated human gut microbiota

*L. rhamnosus* GG LMG 18243 (LGG) was plated from a −80°C glycerol stock on de Man, Rogosa, Sharp (MRS, Carl Roth, Karlsruhe, Germany) agar and grown aerobically at 37°C for 24 h. Next, one colony was picked up and transferred into 9 ml MRS broth. After overnight growth at 37°C, 10% (v:v) of the liquid culture was subcultured, at least twice, to ensure viability before coculturing with host cells. Simulated human gut microbiota (SHIME) samples were obtained from the distal colon compartment (pH 6.6-6.9, residence time 32 h) of a validated, semicontinuous in vitro model that mimics the in vivo physiological conditions and microbiota colonization along the gastrointestinal tract. Briefly, the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) was inoculated with a fecal sample of a healthy donor consuming a mixed Western diet, without a history of antibiotic or probiotic use six months prior to the study. The SHIME sample (5 ml) taken from a stable distal microbial community, was immediately diluted in autoclaved 0.1 M anaerobic phosphate buffer (pH 6.8) supplemented with 1 g/L sodium thiglycolate (Sigma-Aldrich, Overijse, Belgium) as reducing agent and flushed with N₂ gas. Short chain fatty acid (SCFA) concentrations and relative community compositions of the SHIME samples were determined and described in Supporting Information (Figures S1 and S2).

### 2.4 Setup of host-microbe cocultures with microbial biofilm

The apical part of the host-microbe coincubation was prepared by adding 75 µl of a 0.8% agar (Carl Roth, Karlsruhe, Germany)—5% porcine mucin type II-solution (Sigma-Aldrich, Overijse, Belgium) in dH₂O onto Transwell polycarbonate filters with 0.4 µm pores (Corning Incorporated, New York, USA). Prior to inoculation, flow cytometry was used to quantify intact and damaged bacterial cell counts as described by Van Nevel et al. A fresh sample of LGG culture in log phase, a SHIME sample and a mixture of
LGG and SHIME (1:1 in cell counts) were diluted to 5 × 10^5 intact cells/ml in sterile anaerobic phosphate buffer. Per well, 20 µl bacterial solution was incubated on the solidified agar-mucin layers for 2 h at 37°C, 10% CO_2 and 90% relative humidity while 1 ml DMEM/F-12 was added to the basolateral compartment (without host cells). After incubation, planktonic microbial cells were washed away with 100 µl sterile anaerobic phosphate buffer and 20 µl sterile anaerobic phosphate buffer was added on top of the mucin-adhered microbes. This procedure was tested prior to coincubation experiments to control for growth in the bacterial compartment (Figures S3 and S4). T84 monolayers and T84/LS-174T/THP-1 triple cocultures were then refreshed with 1 ml DMEM/F-12 deprived from FBSi and antibiotic antimycotic solution, which also removed the TNF conditioning medium in wells where this condition was assessed prior to host-microbe coincubations. Then, the Transwell filters with bacteria were moved to wells with monolayer and triple coculture host cells. The indirect host-microbe coincubation was maintained for 16 h at 37°C, 10% CO_2 and 90% relative humidity (Figure 1). The novel triple coculture cell model was validated and benchmarked to the traditional monolayer cell model by imaging the structure of the cell layers, measuring the mucus layer thickness, determining the cytokine production, metabolic activity, cytotoxicity and quantifying the load of intact microbial cells (Table 1). The transcriptome of host triple cocultures, unexposed to microbial stimuli, exposed simulated gut microbiota (SHIME) samples and exposed to SHIME samples + LGG addition, was determined to validate the endpoints. The host-microbe coincubations were executed in triplicates (three wells per condition) and results in the main text display the readouts of one simulated gut microbiota sample (SHIME sample). The coincubations were repeated in three independent assay, each time a SHIME sample from another donor was tested. Results of SHIME donor 1 are displayed in the main results. Results of donors 2 and 3 are available in Supporting Information (Figures S6–S9).

### 2.5 Immunofluorescence of actin skeleton and nuclei

Host cells were fixated with 4% formaldehyde (Carl Roth, Karlsruhe, Germany) for 15 min at room temperature in 24-well plates (3413, Corning Incorporated, New York, USA). Cells were washed three times with PBS and permeabilized with 0.5% Triton X-100 in PBS for 15 min. After three washing steps, 3% (w/v) bovine serum albumin in PBS was used as blocking solution overnight at room temperature. Three washing steps were performed before the F-actin staining with 10 µg/ml phalloidin-rhodamine (Sigma-Aldrich, Overijse, Belgium) in PBS was added for 60 min. Nuclei were stained with 0.1 µg/ml DAPI staining (Sigma-Aldrich, Overijse, Belgium) for 15 min. Imaging was performed after three additional washing steps with a Nikon A1R confocal microscope equipped with a Plan Fluor 40×/0.6 objective (Nikon Instruments, Amsterdam, the Netherlands) at room temperature using unidirectional Galvano scanning and a photomultiplier tube detector.

### 2.6 Determination of MUC2 mucus layer thickness with immunofluorescence

To assess mucus type 2 layer thickness, host cells seeded in 24-well plates were fixated in Carnoy’s reagent, consisting of 60% ethanol (≥99.9%, Sigma-Aldrich, Merelbeke, Belgium), 30% chloroform (≥99%, Carl Roth, Karlsruhe, Germany) and 10% glacial acetic acid.

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**Table 1** Overview of the experimental conditions used in host-microbe coincubations. (A) Conditions to assess the effect of biochemical conditioning with TNF and the subsequent impact of coincubating the probiotic LGG with biochemically stimulated host cells. (B) Conditions to assess the coincubation of host cells with simulated human gut microbiota (SHIME) samples and the effect of adding LGG to SHIME samples.

| Host cell model | TNF conditioning (pre-exposure) | LGG exposure |
|-----------------|---------------------------------|--------------|
| Monolayer (T84) | - TNF                           | - LGG        |
|                 | + TNF                           | + LGG        |
| Triple coculture (THP-1/LS-174T/T84) | - TNF                           | - LGG        |
|                 | + TNF                           | + LGG        |

| Host cell model | Exposure to simulated gut microbiota sample | LGG exposure |
|-----------------|---------------------------------------------|--------------|
| Monolayer (T84) | - SHIME                                     | - LGG        |
|                 | + SHIME                                     | + LGG        |
| Triple coculture (THP-1/LS-174T/T84) | - SHIME                                     | - LGG        |
|                 | + SHIME                                     | + LGG        |
(≥99.7%, Sigma-Aldrich, Merelbeke, Belgium) for 30 min at 4°C. After three washing steps, 3% (w:v) BSA in PBS was used as blocking solution overnight at room temperature. Blocking solution was removed, 1 µg/ml MUC2 primary polyclonal antibody (PA5-79702, Invitrogen, Carlsbad, USA) was added and incubated overnight at 4°C. After three washing steps, 5 µg/ml Alexa Fluor 488 conjugated secondary antibody, (A-11034, Invitrogen, Carlsbad, USA) was incubated for one hour at room temperature. Following three washing steps, imaging was performed with a Nikon A1R confocal microscope equipped with a Plan Fluor 40×/0.6 objective (Nikon Instruments Amsterdam, the Netherlands). In each well, z-stacks (slices every 1.25 µm) of mucus layers were recorded at four different positions. To determine the mucus layer thickness, Fiji software (https://imagej.net/Fiji) was used with an in-house developed script (macro) based on the method described by Hormel et al. 35 First, pixel noise was removed by applying a mean filter of 15 pixels. To discriminate between signal and background, the Otsu thresholding method was used which selects an intensity cutoff that maximizes the covariance between signal and background. Based on this threshold a mask was generated. 36 Background was subtracted of the image stack based on the background signal of the first frame. This image was then multiplied with the mask and an average z-intensity profile was calculated in both x- and y-directions. The intensities correspond to the presence of mucus. Assessment of mucus layer thickness in monolayers and triple cocultures (before host-microbe coincubations) was based on six wells per condition, assessment after host-microbe coincubations were based on three wells per condition.

2.7 | Cytokine quantification

Cytokine production in the basolateral medium after 16 h of host-microbe coincubation was quantified with enzyme-linked immunosorbent assays (ELISA). IL-8, IL-1β, TNF and IL-10 were quantified in technical duplicates with human uncoated ELISA kits according to manufacturer’s instructions (Invitrogen, Carlsbad, USA). Absorbances at 450 and 570 nm (background correction) were measured with a Tecan Infinite M Plex plate reader (Tecan, Mechelen, Belgium).

2.8 | Metabolic activity normalized with protein content

Metabolic and mitochondrial activity of host cells was assessed with a resazurin assay after host-microbe coincubations. Medium was refreshed with 1 ml of 0.01 mg/ml resazurin (7-Hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt, Sigma-Aldrich, Overijse, Belgium) in DMEM/F-12 (not supplemented with FBS or antibiotic antifungal solution) and incubated for 2 h at 37°C, 10% CO2 and 90% relative humidity. Metabolically active host cells are capable of reducing the resazurin substrate via mitochondrial reductase into the pink and fluorescent resorufin. Fluorescence was measured in technical triplicates in black 96-well plates at an excitation wavelength of 540 nm and an emission wavelength of 590 nm (SpectraMax M2 plate reader, Molecular Devices, Brussels, Belgium). As a negative control (abiotic control), the fluorescence of resazurin in DMEM/F-12, incubated in the absence of host cells was subtracted as a background from all fluorescent values. Metabolic activities were then calculated as percentages compared to blank monolayers (not exposed to biochemical or bacterial stimuli) and normalized with the total protein contents of monolayers or triple cocultures (not exposed to biochemical or bacterial stimuli). To determine total protein contents, host cells were washed with 1 ml PBS and stored at −20°C. Next, cells were thawed, detached with cell scrapers and dissolved in 0.1% Triton X-100 in PBS by pipetting and vortexing. Protein contents were then quantified with the Pierce BCA protein assay kit (Thermo Fisher Scientific, Merelbeke, Belgium) according to the manufacturer’s instructions. Absorbances were measured at 562 nm with a Tecan Infinite M Plex plate reader (Tecan, Mechelen, Belgium).

2.9 | Cytotoxicity

The cytotoxicity of monolayers and triple cocultures was assessed by measuring lactate dehydrogenase (LDH) in the basolateral medium after 16 h of coculturing. Quantification was done with the Pierce LDH Cytotoxicity assay kit (Thermo Fisher Scientific, Merelbeke, Belgium) in technical duplicates. Spontaneous and maximal LDH releases were measured in blank monolayers or triple coculture cells, not exposed to biochemical or bacterial stimuli. Absorbances at 490 and 680 nm (background correction) were measured with a Tecan Infinite M Plex plate reader (Tecan, Mechelen, Belgium).

2.10 | Quantification of bacterial viable cells by flow cytometry

After 16 h of host-microbe coincubations, bacterial samples were detached from agar-mucin layers as described by Tsilia et al. 37 In brief, the filters were washed with
100 µl anaerobic phosphate buffer. Next, 100 µl 0.5% Triton X-100 (Sigma-Aldrich, Overijse, Belgium) in anaerobic phosphate buffer was added and incubated for 20 min at 37°C on a rotary shaker (110 rpm) (Ika, Wilmington, NC, USA). Then, filters were washed three times with 100 µl anaerobic phosphate buffer. The washing fluid was centrifuged for 3 min at 1500 g (Eppendorf 5424, Hamburg, Germany) and the supernatant was discarded. Pellets were dissolved and subsequently diluted in filtered (0.22 µm sterile syringe filter, Sigma-Aldrich, Merelbeke, Belgium) anaerobic phosphate buffer with measured with an Accuri C6+ flow cytometer (BD, Erembodegem, Belgium), equipped with a blue (488 nm) laser and bandpass filters on the FL-1 (533/30 nm) and FL-3 (670 nm) detectors as described by Van Nevel et al.34 The flow cytometer used Milli-Q water as sheath fluid and the software for cell count registration and analyses was BD Accuri C6 Plus software (BD, Erembodegem, Belgium). Intact and damaged bacterial cell counts were measured in technical triplicate after 13 min incubation of the samples with a SYBR Green I (1000x diluted, Invitrogen, Carlsbad, USA)/propidium iodide (4 µM final concentration, Sigma-Aldrich, Merelbeke, Belgium) (SGPI) staining solution at 37°C in the dark. As a background control, 0.22 µm filtered phosphate buffer was measured.

2.11 Microarray transcriptome analysis

Triple coculture cells were lysed with LBP lysis buffer (RNA extraction kit, NucleoSpin RNA Plus, Macherey-Nagel, Düren, Germany) and homogenized by transferring the lysates to QIAshredder membranes (Qiagen, Hilden, Germany), followed by centrifugation for 2 min at 20 913 g (Eppendorf 5804 R, Hamburg, Germany). Lysates were stored at −80°C. RNA was further extracted with the NucleoSpin RNA Plus extraction kit (Macherey-Nagel, Düren, Germany). RNA concentration was determined with a Denovix DS-11 spectrophotometer (Denovix, Wilmington, USA). Next, 1 µg RNA was treated with DNase I (1 U/µl, Thermo Fisher Scientific, Merelbeke, Belgium) in a total reaction volume of 25 µl, diluted with H2O (UltraPure DEPC-Treated Water, Thermo Fisher Scientific) for 30 min at 37°C. The enzymatic reaction was stopped with 1 µl EDTA (50 mM) and an incubation of 10 min at 65°C. RNA concentration and A260/A280 ratios were measured (Denovix DS-11 spectrophotometer). The absence of genomic DNA was confirmed using a StepOnePlus real-time PCR system with GAPDH and ACTB primers (Table 2). Reactions were performed in a volume of 20 µL consisting of iTaq universal SYBR Green super mix (Bio-Rad Laboratories, Hercules, USA), 180 ng RNA template and 6 µM of each primer. Amplification conditions were initial denaturation for 2 min at 95°C, followed by 40 cycles of 15 s denaturation at 95°C and 1 min combined anneal/extension at 60°C. RNA samples that were negative for genomic DNA and with A260/A280 ratios between 1.7 and 2.1, were used as input (100 ng RNA) for RNA conversion to biotinylated DNA using the GeneChip WT PLUS Reagent Kit (Applied Biosystems, Thermo Fisher Scientific, Merelbeke, Belgium). Biotinylated DNA was hybridized to Affymetrix GeneChip Human Gene 2.1 ST Array Strips according to manufacturer’s instructions (Applied Biosystems, Thermo Fisher Scientific, Merelbeke, Belgium). Transcriptome samples of the 16 h coincubated triple coculture model with either SHIME or SHIME + LGG microbiota were compared to an untreated blank condition in triplicate.

2.12 Statistical analysis

Statistical analysis was performed in RStudio (version 3.6.1). Normality of the data was assessed with Shapiro-Wilk tests with QQ-plots. Homoscedasticity of the data was assessed with Levene’s test. The monolayer and triple coculture models were compared in the presence and absence of either TNF conditioning or microbial colonization with SHIME and/or LGG samples. To compare two groups, independent Student’s t-tests were performed when normality and homoscedasticity assumptions were met. If not, a non-parametric Wilcoxon Rank Sum test was used. Significance was considered at the α = 0.05 level.

Microarray transcriptomics data was analyzed in R version 4.1.0 on a x86_64-pc-linux-gnu platform running under Ubuntu 18.04.5 LTS. The complete analysis and the corresponding R code can be accessed on github (https://github.com/kdpaepe/Coculture_transcriptomics_AnneloreBehetarms). CEL files generated by the Affymetrix software were imported as a GeneFeatureSet using

| Gene   | Forward primer sequence, 5′-3′ | Reverse primer sequence, 3′-5′ | Reference |
|--------|-------------------------------|-------------------------------|-----------|
| GAPDH  | GAGGACCTGCTGGTTGAAGGTTGCA     | GAGGACCTGCTGGTTGAAGGTTGCA     | [78]      |
| ACTB   | GAGGACCTGCTGGTTGAAGGTTGCA     | GAGGACCTGCTGGTTGAAGGTTGCA     | [78]      |
the Oligo package (version 1.56.0) and annotated with the pd.hugene.2.1.st and expanded ChipDb package (hugene20sttranscriptcluster.db). Microarray data was pre-processed to remove technical and biological noise and to obtain a summary measure for the transcript concentration i.e., the expression value of a gene using the Robust Multi-array Average (RMA) algorithm. RMA consisted of convolution background correction, quantile normalization and summarization at gene/transcript level based on a multi-array model fit using robust median-polish for estimation.38,39 Quality control was performed both prior to and after pre-processing. As detailed in the Supporting Information, the distribution of Log2 probe intensities was assessed through diagnostic plots (Log2 scaled array pseudo-images, box plots, density plots, intensity-sequence relationships, Receiver Operator Curves (ROC), MA plots, probe-level model fit residual/weight plots), probe metrics (Relative Log Expression (RLE), Normalized Unscaled Standard Errors (NUSE), Detected Above BackGround (DABG) absence/presence calls) and Principal Component Analysis (PCA).40,41 The quality assessment measures were highly correlated and accurately indicated the control microarray loaded with a Hela cell RNA sample as outlier. Additionally, negative and positive control probes in conjunction with positive bacterial and polyA spike-ins (exogenous controls) revealed that target preparation, hybridization, washing and scanning were adequate. The latter was confirmed by endogenous positive controls (exon regions of constitutively expressed genes and the housekeeping genes ACTB, GAPDH and HMBS) and negative controls targeting intron regions included in the HuGene 2.1 ST Array chip design. Differential expression at the individual-gene-level was assessed by means of a moderated (empirical Bayes method) ANOVA with F-test statistic followed by pairwise t-tests implemented in the Limma package version 3.48.0.42,43 To increase the power to detect significant differences, non-expressed genes were filtered out based on a DABG approach comparing the main probe intensities to background probe intensities with a similar GC count through Wilcoxon signed rank-based tests.39 Differential expression analysis results were reported (Transcriptomic data table link) and summarized in volcano plots showing the p-value of a gene in function of the Log2 Fold Change (LogFC) in expression between 2 conditions. A selection of significant genes ($p < .05$) with a strong LogFC ($|\text{LogFC}| > 1$) was displayed in a heatmap. Affymetrix probe identifiers were mapped to Ensembl accession numbers, Entrez gene identifiers, RefSeq identifiers, KEGG pathway identifiers, gene symbols and gene names using the ‘hugene20sttranscriptcluster.db’ ChipDb object. In order to establish functional relations between the annotated significantly differentially expressed genes, gene ontology (GO) enrichment analysis was carried out at the ‘Molecular Function (MF)’, ‘Cellular Component (CC)’ and ‘Biological Process (BP)’ level with the topGO package version 2.44.0.44 Gene ontology terms (BP, CC, MF) were built based on the GOTERM environment from package GO.db version 3.13.3 and extracted from the gene-to-GO mapping for the Affymetrix Human Gene 2.1 ST Array Strip (hugene20sttranscriptcluster.db). To find GO terms that are enriched within the significantly differentially expressed individual genes, previously identified by Limma, a background set of non-differentially expressed genes with similar average expression strength was defined using the genefinder function from the genefilter package version 1.74.0. Enrichment of GO terms of interest (BP, MF, CC) in the significantly differentially expressed genes (Limma $P$-value < .01) compared to the background genes was determined with Fisher’s exact tests for every GO category independently and Kolmogorov-Smirnov tests in combination with a more complex elim algorithm which take the hierarchical GO dependencies into account.44 The hierarchical GO structure was pruned to remove terms with less than 10 annotated genes. Over-represented GO terms were exported (Transcriptomic data table link) and visualized in a GO graph displaying the most significant GO terms (rectangles) and their distribution in the GO hierarchy. Besides GO enrichment analysis, gene-set analysis (GSA) was performed but instead of starting from the results of a prior individual-gene-level differential expression analysis, GSA focuses on sets of related genes and aims to identify significantly up- or downregulated gene-sets. Gene-sets are pre-defined groups of genes, which are functionally related. GSA was performed with up-to-date KEGG pathway and GO based gene-sets in combination with a parametric gene randomization procedure implemented in the GAGE (generally applicable gene set enrichment for pathway analysis) package, version 2.42.0.45 Prior to GSA in GAGE, Affymetrix gene identifiers were mapped to the matching Entrez identifiers used in the KEGG and GO databases. One-directional expression changes (either up- or down-regulation) were assessed with parametric unpaired two-sample t-tests on gene sets using LogFC as per-gene statistics, followed by robust $P$-value summarization using Stouffer’s method and Benjamini and Hochberg FDR correction. The average of the individual LogFC statistics from multiple single-array based gene-set tests was displayed in bar graphs for the significant KEGG pathways and gene ontology terms ($p < .01$). FDR adjusted $P$-values were reported and bars were color-coded according to the pathway/gene ontology categories. Non-redundant gene-sets, not displaying significant overlap with other gene-sets in the core genes contributing most to the gene-set overall significance, were identified. The core genes were marked in the volcano plots obtained with Limma and the LogFC in individual genes confirmed the general trends at higher
levels (pathways, gene ontology). The non-redundant KEGG-based gene-sets were also explored with pathview version 1.32.0. The perturbed expression patterns were mapped onto the KEGG pathway view.46

Finally, results from the transcriptomics differential expression analysis were combined with functional data from mucus layer thickness, cytotoxic stress and cytokine production, flow cytometry cell counts and SCFA concentration measurements through DIABLO (Data Integration Analysis for Biomarker discovery using Latent variable approaches for Omics studies) from the mixOmics framework version 6.16.0.47 DIABLO is a latent variable multivariate dimension reduction method that aims to identify the correlated variables that best explain the categorical outcome variable of interest (triple coculture cell model treatment: Blank, SHIME or SHIME + LGG). The DIABLO model consisted of a design matrix containing the transcriptomics expression data and functional host-microbe interaction data, constructed with a link of 0.1. Global model performance was assessed by 5-fold cross validation repeated 10 times, and 2 components were selected to fit the final model based on the overall and balanced error rates for the centroids distance criterion. Next, smart feature selection was applied and the optimal number of variables was determined based on a 10 × 3-fold cross validation. The performance of the final model with 2 components, each with 6 functional, respectively, 10 transcriptomics variables was verified using the balanced classification error rate with majority vote and centroids distance. The correlations between and within variables from each dataset were computed using a similarity score that is analogous to a Pearson correlation coefficient and represented in circos plots.47,48

3 | RESULTS

3.1 | Triple cocultures morphologically organize in networks producing thicker and denser MUC2 mucus layers than T84 monocultures

The novel triple coculture model was benchmarked against the traditional monolayer cell model by imaging the structure of the cell layers, measuring the mucus layer thickness, determining the cytokine production, metabolic activity, cytotoxicity and quantifying the load of intact microbial cells. The transcriptome of host triple cocultures exposed to microbiota samples for 16 h was determined. Differences in cell morphology between monolayers and triple cocultures were assessed by immunofluorescence and confocal microscopy. T84 monolayers displayed a typical cobblestone-like pattern of intestinal epithelial cell lines with large nuclei and a lot of cytoplasm (Figure 2A, top, blue arrows). Triple cocultures showed an organization in networks initiated by interactions between the three cell types present (Figure 2A, bottom). The LS-174T cells (white arrows) accumulated in small groups and were distinguished from T84 cells by their smaller nuclei. Macrophage-like THP-1 cells (yellow arrows) were recognized by their tubular extensions lying in-between the T84 cells. Condensed chromatin in circular structures suggests the presence of apoptotic bodies in small groups and were distinguished from T84 cells by their smaller nuclei. Macrophage-like THP-1 cells (yellow arrows) were recognized by their tubular extensions lying in-between the T84 cells. Condensed chromatin in circular structures suggests the presence of apoptotic bodies and cell renewal (green arrows) in triple cocultures. In both monolayers and triple cocultures, MUC2 production was observed (Figure 2A).

The thickness of MUC2 layers in both model systems was quantitatively assessed by recording z-stacks of MUC2 immunostained samples with confocal microscopy. Significantly thicker (p = .0081) MUC2 mucus layers (82.1 ± 8.5 µm) were detected in triple cocultures compared to monolayers (65.2 ± 8.3 µm) (Figure 2B). A 3D view that was composed by all slices in a z-stack showed an even distribution of MUC2 over the triple coculture cells, whereas the distribution was less homogeneous in the case of monolayers, leaving some areas uncovered (Figure 2C).

3.2 | Monolayers and triple cocultures are robust against bacterial exposure and TNF conditioning

Host-microbe coincubations were set up to assess the responsiveness of monolayers and triple cocultures towards biochemical and microbial stimuli. As a biochemical stimulus, tumor necrosis factor (TNF) was added for 24 h to the host compartment and removed prior to combining the host with the bacterial compartment. As a microbial stimulus, simulated human gut samples from a SHIME reactor were added during host-microbe coincubations for 16 h. As both stimuli putatively elicit a stress response, we investigated whether addition of LGG to the SHIME sample would modulate that stress response.

Applying the host-microbe coincubation requires several quality control steps. First, the biochemical and microbial stimuli may induce cytotoxic stress. We therefore assessed cytotoxic stress by measuring release of lactate dehydrogenase (LDH) in the coculture basal compartment (Figure S5B). Secondly, modulated production and/or secretion of specific proteins of interest (e.g., cytokines) by the different stimuli may be affected by changes in the host cell’s metabolic activity. We therefore verified whether any of the stimuli altered the metabolic activity by measuring the reduction of resazurin to resorufin and normalizing for average...
protein contents of monolayers (157 ± 24 µg/well) or triple cocultures (244 ± 20 µg/well) (Figure S5A). Overall, none of the treatments reduced the metabolic activity compared to the blank condition or affected cell viability by inducing cytotoxicity. Thirdly, when comparing different models upon exposure to microbial stimuli, cytotoxicity or immune endpoints of interest may be affected by bacterial cell number. We therefore quantified bacterial biofilm cell counts after 16 h of coincubation with flow cytometry (Figure S5C). Addition of SHIME to LGG samples caused an additional increase in intact cell counts in triple cocultures from 6.41 ± 0.15 to 7.63 ± 0.16 log cells ($p = .00061$), even though the total bacterial load at time point zero of the host-microbe coculture was equal for all conditions (Figure S5C, right panel). TNF conditioning did

**FIGURE 2** Triple cocultures morphologically organize in networks producing thicker and denser MUC2 layers than T84 monocultures. (A) F-actin, nuclei, MUC2 mucin staining and transmission image showing morphological characteristics of T84 monolayer (top) and triple coculture (bottom) cell models as visualized by confocal microscopy. Blue arrows: epithelial T84 cells; White arrows: LS-174T cells; Yellow arrows: Macrophage-like THP-1 cells; Green arrows: apoptotic structures. (B) The thickness of MUC2 layers in monolayers versus triple cocultures prior to microbial exposure ($n = 6$). (C) Example of a cross section and top view of the MUC2 layer formed on top of the monolayer and triple coculture host cells. *$p = .0081$
not affect intact cell counts in LGG supplemented triple coculture models (6.42 ± 0.62 versus 6.57 ± 0.23 log cells, \( p = .71 \)) (Figure S5C, left panel). The same trend was observed for monolayers (\( p = .1 \)).

These quality control steps confirmed that the measured endpoints in the model systems were specifically caused by the imposed stimuli rather than alterations in cytotoxicity, metabolic activity or bacterial load. Our experimental setup to study host-microbe interactions was technically reproducible as demonstrated by three independent experiments and consistent readouts at the level of cytotoxicity (Figures S5B, S6B, and S7B), metabolic activity (Figures S5A, S6A, and S7A), bacterial counts (Figures S5C, S6C, and S7C) and cytokine measurements (Figures 3, S8, and S9).

### 3.3 Gut microbiota stimuli trigger a pro-inflammatory cytokine shift in triple cocultures, leaving the mucus layer thickness unaffected

The basal IL-8 production of blank monolayers (− SHIME − LGG, − TNF − LGG) ranged between 57.85 ± 4.06 and 64.21 ± 13.78 pg/ml (Figure 3A). The monolayer setup was unresponsive to TNF conditioning or addition of a SHIME sample as no significant difference in IL-8 production (\( p = .11 \) and \( p = .10 \), respectively) with the non-stimulated setup was observed. Triple cocultures displayed a basal IL-8 production between 82.85 ± 4.13 and 112.06 ± 3.51 pg/ml and were more responsive to external stimuli. TNF exposure tended to increase IL-8 production in the triple cocultures (Figures 3B and S10A). The addition of a SHIME sample led to a significant increase in IL-8 production in both the monolayer and triple coculture settings (Figures 3A and S10B).

The IL-10 production was also measured in the same setup. The basal IL-10 production of blank monolayers (− SHIME − LGG, − TNF − LGG) ranged between 57.85 ± 4.06 and 64.21 ± 13.78 pg/ml (Figure 3D). The monolayer setup was unresponsive to TNF conditioning or addition of a SHIME sample as no significant difference in IL-10 production (\( p = .11 \) and \( p = .10 \), respectively) with the non-stimulated setup was observed. Triple cocultures displayed a basal IL-10 production between 82.85 ± 4.13 and 112.06 ± 3.51 pg/ml and were more responsive to external stimuli. TNF exposure tended to decrease IL-10 production in the triple cocultures (Figures 3D and S10C). The addition of a SHIME sample led to a significant decrease in IL-10 production in both the monolayer and triple coculture settings (Figures 3D and S10D).

**Figure 3** Triple cocultures exhibit a pro-inflammatory cytokine shift upon exposure to a human gut microbiota sample (SHIME sample) in host-microbe coinoculations of 16 h (n = 3). (A) IL-8 concentration. (B) Relative IL-1β production. (C) Relative TNF production. (D) Relative IL-10 production. The relative IL-1β, TNF and IL-10 production were compared to the monolayer control (− LGG − TNF or − LGG − SHIME (n = 3). *\( p < .05 \).
secretion to 98.14 ± 9.65 pg/ml (p = .065) while presence of a SHIME sample caused a sharp increase in IL-8 production to 1285.18 ± 123.84 pg/ml (p < .0001). Supplementation of LGG to the SHIME microbial stimulus was able to slightly suppress the high IL-8 response (942.46 ± 681.12 pg/ml) yet no significant difference with the LGG stimulated setup (no SHIME) was observed due to high biological variation (p = .097). Similar trends were observed for relative IL-1β responses compared to the monolayer control (Figure 3B). A significant increase (p = .0030) in relative IL-1β production was observed when triple cocultures were stimulated with SHIME samples (994.04% ± 233.75%) compared to blank triple cocultures (122.11% ± 1739%), while monolayers did not respond when exposed to the same SHIME stimulus (p = .77). As opposed to the monoculture setup, the triple coculture was also able to pick up the modulatory effect from LGG supplementation in the presence of a SHIME sample as microbial stimulus. A significant increase (p = .026) in relative IL-1β production was observed (1491.93% ± 520.72%) when LGG was supplemented to the SHIME stimulated setup compared to the condition with LGG alone (344.91% ± 249.57%). Finally, quantification of relative TNF production also revealed triple cocultures to be more responsive to microbial stimuli than monoculture setups. A significant increase (p = .0030) in relative TNF production was observed for triple cocultures stimulated with SHIME samples (195.02% ± 18.39%) compared to blank triple cocultures (107.27% ± 14.96%) (Figure 3C). Changes in relative IL-10 production were not found due to the low basal production level after 16 h of host-microbe coincubations (Figure 3D).

We previously demonstrated that the triple cocultures yield a thicker mucus layer than monoculture setups (Figure 2B). In the host-microbe coinoculation experiments, we were able to confirm the increase in mucus layer thickness in blank triple cocultures as compared to blank monolayers in both the TNF (p = .014) and the SHIME (p = .031) experiment (Figure 4). As mucus forms an important component of the epithelial barrier, we also evaluated whether MUC2 mucus layer thickness in monolayers or triple coculture setups remained stable upon exposure to biochemical and microbial stimuli (Figure 4). In the presence of LGG, the difference in thickness between monolayers and triple cocultures disappeared (p = .56 in TNF experiment, p = .88 in SHIME experiment). This is suggesting that LGG can stimulate mucus production in monolayers since they are covered with thinner mucus layers at the basal state and are thus more susceptible to be affected compared to triple cocultures. The MUC2 mucus layer thickness remained stable in triple cocultures when exposed to SHIME samples (p = .66), whereas TNF

FIGURE 4 MUC2 mucus layer thickness of triple cocultures after 16 h of host-microbe coincubations in the presence of LGG increased after TNF conditioning but not in the presence of SHIME samples (n = 3). *p < .05
conditioning and exposure to LGG significantly expanded the MUC2 layer ($p = .019$) (Figure 4). Monolayers did not ($p = .1$) change in mucus layer thickness in the presence of SHIME samples amended with LGG. TNF conditioning of monolayers, on the other hand, resulted in thicker mucus layers compared to unstimulated monolayers ($p = .0031$).

### 3.4 Transcriptional gene expression analysis suggests a controlled inflammation state in triple cocultures exposed to commensal gut microbiota

To evaluate on the impact of adding complex microbiota samples to the triple coculture model, a gene expression analysis was performed using human gene transcriptome microarrays. The comparison between triple cocultures exposed to SHIME samples and blank triple cocultures (− SHIME) revealed that SHIME samples induced a pro-inflammatory response, both at pathway level and individual gene level, in the triple coculture model (Figure 5). LGG spike-in reduced the pro-inflammatory tone compared to triple cocultures coincubated with just the SHIME sample (Figure 5). The TNF signaling pathway, amongst others, was affected by exposing triple cocultures to SHIME or SHIME + LGG samples compared to blanks (Figure 6).

### 4 DISCUSSION

We developed and characterized an in vitro triple coculture model of the colonic microenvironment. Unique assets as opposed to other existing model systems are: (i) the presence of a mucus layer overlying a simulated colon epithelium, (ii) the inclusion of an innate immune component by combining colon epithelial and goblet cells with PMA-induced macrophage-like cells and (iii) the ability to study the complex interplay between this mucosal cell coculture with human-derived gut microbiota in a timeframe of 16 h under non-cytotoxic circumstances.

The triple coculture displayed a representative expression of colon specific markers, such as monocarboxylate transporters (MCTs), and exhibited a pro-inflammatory state characterized by the activation of the TNF signaling pathway. The addition of LGG to the SHIME sample reduced the pro-inflammatory response, suggesting a beneficial effect of these bacteria in the context of gut health.

![Figure 5](image-url) Triple cocultures exposed to simulated gut microbiota (SHIME) samples display a pro-inflammatory gene expression profile compared to blank triple cocultures (− SHIME), addition of LGG to a SHIME sample reduces the pro-inflammatory tone. (A) Significantly affected KEGG pathways are colored per pathway type. (B) Volcano plots indicating core genes (colored) contributing to the significantly affected pathways. The most affected genes with the highest log2 fold changes (LogFC) are annotated.
transporter 1 (MCT1/SCL16A4) and membrane spanning 4-domain A12 (MS4A12). MCT1 increases in abundance at mRNA and protein level in human biopsy samples along the intestine and is predominantly expressed in distal colon samples. Expression of MS4A12 is limited to human colon biopsy samples. The presence of PMA-induced macrophage-like THP-1 cells, furthermore, led to a relevant expression of macrophage-specific genes, such as CD68 and CD11b (Transcriptomic data table link).

Besides, we established thicker mucus layers in our triple coculture model compared to monolayers by including mucus-producing LS-174T goblet cells in the epithelial barrier. A high MUC2 expression was confirmed at mRNA level in triple cocultures (Transcriptomic data table link). The produced gel-forming MUC2 mucin type is most abundant in the gastrointestinal tract making our model more representative than cell cultures of commonly used HT29-MTX cells, mainly secreting MUC5AC.

The inclusion of goblet cells in our triple coculture model resulted in mucus thicknesses of 83 µm up to 130 µm after coculturing with gut microbial communities. In vivo, mucus thicknesses in the colon reach up to 830 µm in rats and 480 µm in humans. The impenetrable mucus layer in human colon biopsy samples is on average 400 µm thick. In vitro, the observed mucus layers vary in thickness. Roh et al. reported thicknesses around 0.6 µm in human colonoids cultured in a 3D tissue model and Béduneau et al. reported thicknesses of a few micrometres in a Caco-2/HT29-MTX coculture. VanDussen et al. obtained 36 µm in a Transwell model culturing primary epithelial cells. The most in vivo-like mucus thickness was obtained in the in vitro colon chip model of Sontheimer-Phelps et al. reporting mucus thicknesses around 300 µm, further expanding to 500–600 µm after stimulation with prostaglandin E2. The mucus levels we observed were considerable, however, not sufficient to secure a long-term direct contact between host and microbial compartment without compromising the host cell functionality. Therefore, Transwell inserts were used to physically separate both compartments. This allowed us
to establish an indirect communication while maintaining a high viability of host cells in the presence of a dense colon microbiota under non-cytotoxic circumstances in timespans relevant for host-microbe interactions.

The exposure of both monolayers and triple cocultures to complex microbiota for 16 h did not cause any harm to the host cells. The observed decrease in normalized metabolic activities in triple cocultures could be attributed to the higher protein contents compared to monolayers. The principle of indirect interaction has been implemented to avoid viability losses and cytotoxic effects of microbes in host-microbe model systems before. Reductions in viability of only 7% were reported in the T84 cell line, 24 h after the indirect exposure to E coli MG1655 for 6 h.58 The Host-Microbiota Interaction (HMI) module, consisting of Caco-2 cells in an indirect coculture with microbial gut biofilms, was maintained without viability losses for 48 h, while the step towards direct contact led to an 80% decrease in viability after 2 h.59 An alternative microfluidic model enabling long term (more than one week) direct host-microbe interactions was developed by Kim et al. 60 LGG cells first adhered to Caco-2 cells in static circumstances followed by the introduction of a continuous medium flow, which enhanced the clearance of organic acids, removed unbound LGG cells and thereby prevented bacterial overgrowth leading to viability losses. In contrast, microbial metabolites and attached microbiota effectively persisted in our triple coculture model, without impairment of host cell viability. The indirect coculture of host cells with complex gut microbial communities can therefore be proposed as a solution to avoid cytotoxic effects in static model systems.

We tested the compatibility of our newly designed triple coculture setup with a gut microbiota component by exposure to distal colon suspension harvested from a dynamic simulator of the human gut (SHIME). We chose distal colon microbiota, these typically display proteolytic activity resulting in high levels of ammonia and branched chain fatty acids.61 The toxicity of by-products from proteolytic fermentation can affect human health.62 This was shown in vitro with Caco-2 cells where ammonia for example reduced cell viability and epithelial barrier function and in vivo, where ammonia promoted colon carcinogenesis in rats.64 To relieve cytotoxic stress, distal colon samples may require dilutions when coculturing with host cells. The experimental procedures we used, focused on the adherence of microbial cells in diluted samples, thereby preventing the accumulation of toxic metabolites for host cells and obtaining microbial concentrations comparable to in vivo situations (10^3–10^6 cells/ml mucus).65

The host-microbe coinoculations in our model showed that the presence of gut microbial communities triggered a pro-inflammatory cytokine (IL-8, IL-1β and TNF) shift in the triple cocultures which was lacking in the monolayer. Both LPS and Gram-negative bacteria induce pro-inflammatory cytokine responses in Caco-2/THP-1 cells via the TLR4 receptor signaling.20 In unstimulated T84 cells, an IL-8 production of 300 pg/ml was observed after 16 h and the production did not increase unless microbial exposure and invasion were present.66,67 Together with the fact that T84 cells were unresponsive to LPS exposure, this is a possible explanation for the lack of cytokine response in our monolayers. THP-1 macrophages on the other hand have been shown to react to LPS exposure via IL-8, IL-1β and TNF secretion and by a strong upregulation of inflammation related cytokine, COX-2 and NF-κB genes.18,68 Despite differences in setup, the cytokine levels we observed under bacterial exposure were in line with those observed in a stable Caco-2/THP-1 coculture representing a healthy controlled inflamed intestine that was not exposed to bacteria.18 The analogue but inflamed epithelial barrier model (by stimulation with 10 ng/ml LPS and IFN-γ) evoked pro-inflammatory cytokine levels that were ten times higher compared to our microbiota-exposed triple coculture.18 Next to cytokine levels, inflammation related genes were upregulated upon microbial exposure in triple cocultures (Transcriptomic data table link). Together, this demonstrates that our host-microbe triple coculture model is robust enough to promote tolerance of commensal bacteria while maintaining a physiologically controlled inflamed state.

To further validate our model, we confirmed the immunomodulatory capacity of LGG amended to the complex microbiota applied to the triple cocultures. LGG toned down the pro-inflammatory expression profile elicited by the SHIME samples, which is also reflected in decreased IL-8 levels. The immunomodulatory capacity of LGG via NF-κB modulation on intestinal epithelial cells is well-established.69 Ganguli et al.70 showed that LGG can decrease TNF mRNA expression in human fetal intestines infected with Salmonella Typhimurium or stimulated with LPS. Furthermore, IL-8 secretion was reduced by LGG in T84 cells after Salmonella Typhimurium infection. The observed suppression of NF-κB (and the subsequent IL-8 production) in our indirect contact model might be mediated by secreted soluble proteins, such as p40 and p75, that can prevent epithelial damaged induced by cytokines.71,72

The apical conditioning of epithelial barriers with TNF did not lead to shifts in cytokine responses in monolayers or triple cocultures. TNF is a key molecule implicated in the pathogenesis of inflammatory bowel diseases.73 The stimulation of epithelial cells with TNF in vitro increases IL-8 production. A 16 h stimulation of T84 cells with 10 ng/ml TNF increased IL-8 levels up to 2500 pg/ml.67 We, however, used a different approach for TNF conditioning by first applying TNF on the apical side of the
epithelial barrier, then removing TNF and subsequently coculturing the conditioned epithelial barrier with microbes for 16 h prior to measuring the cytokine response. It was previously shown that TNF-stimulated (100 ng/ml for 4 h) T84 cells in Transwell inserts, yielded 97 pg/ml IL-8 upon apical stimulation, while basolateral stimulation was more effective producing 1582 pg/ml IL-8. It follows that the absence of cytokine shifts by TNF in our triple coculture model is due to the fact that the apical conditioning did not directly reach the basolateral side of the epithelial barrier, where THP-1 macrophage-like cells are residing. Moreover, we did not use IFN-γ priming, a strategy that can be used in both Caco-2 and T84 cells to increase the responsiveness to TNF.

To conclude, we acknowledge the limitations of in vitro models and the disadvantage of using cancerous cell lines. Replacement of cell lines by primary cells in future research with the triple coculture model can offer a good strategy to assess host-microbe interactions at a personal level, especially in diseased contexts (e.g., compromised epithelial barrier function and immune deficiencies in IBD).

We also point out the lack of 16S rRNA sequencing data of the microbial community after 16 h coincubation. Due to the limited number of donors tested in the current study, it is at this stage not possible to infer shifts in the community which may, in turn, affect host cell readouts during coincubations. In future research more donors in the triple coculture model will be tested, accompanied with sequencing to assess whether inter-individual differences in the microbial community composition can differentially affect host cells readouts. This will additionally yield insights in the donor-specific microbiota response to host cells and will allow coupling of host cell and microbiota readouts. However, we highlight that the presented in vitro model can be beneficial in future mechanistic research of host-microbe interactions. The triple coculture model enables coincubations with complex microbial backgrounds and is responsive towards manipulations with biotherapeutic microbial strains or biochemical compounds to impose stress. The model can thus be used as a tool to unravel the mode of action of applied manipulations and to address research questions that require stable, controllable and flexible in vitro setups. In this perspective, the future testing of more diverse donor-derived microbial gut communities from both healthy and diseased subjects will allow us to map and modulate personalized microbiota responses at the host-microbiome level.

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DISCLOSURES
The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS
Annelore Beterams, Marta Calatayud Arroyo, and Tom Van de Wiele designed the experiments. Annelore Beterams, Laure Maes, and India Jane Wise performed the laboratory experiments. Annelore Beterams, Marta Calatayud Arroyo, Kim De Paepe, and Tom Van de Wiele wrote the manuscript. Andreja Rajkovic assisted in designing microarray experiments and provided access to the technical instruments for microarray experiments. Deby Laukens was involved in data interpretation of microarray transcriptomic data. Kim De Paepe analyzed the microarray transcriptomic data. India Jane Wise and Herlinde De Keersmaecker wrote the ImageJ script to analyze mucus thickness. All authors read and approved the manuscript.

DATA AVAILABILITY STATEMENT
The sequence data has been submitted to the NCBI (National Center for Biotechnology Information) database under BioProject accession number PRJNA742708.

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