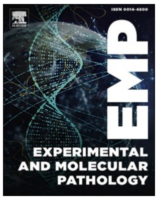
Experimental and Molecular Pathology 142 (2025) 104967

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp



Cross-feeding between beneficial and pathogenic bacteria to utilize eukaryotic host cell-derived sialic acids and bacteriophages shape the pathogen-host interface milieu

Darab Ghadimi [a,*], Regina Fölster-Holst [b], Sophia Blömer [b], Michael Ebsen [c], Christoph Röcken [d], Jumpei Uchiyama [e], Shigenobu Matsuzaki [f], Wilhelm Bockelmann [a,1]

[a] *Department of Microbiology and Biotechnology, Max Rubner-Institut, Hermann-Weigmann-Str 1, D-24103 Kiel, Germany*
[b] *Clinic of Dermatology, Venerology und Allergology, University Hospital Schleswig-Holstein, Schittenhelmstr. 7, D-24105 Kiel, Germany*
[c] *Städtisches MVZ Kiel GmbH (Kiel City Hospital), Department of Pathology, Chemnitzstr.33, 24116 Kiel, Germany*
[d] *Institute of Pathology, Kiel University, University Hospital, Schleswig-Holstein, Arnold-Heller-Straße 3/14, D-24105 Kiel, Germany*
[e] *Department of Bacteriology, Graduate School of Medicine Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan*
[f] *Department of Medical Laboratory Science, Faculty of Health Sciences, Kochi Gakuen University, Kochi, Japan*

ARTICLE INFO

Keywords:
Bacterial sialidase
Inflammation
Cytokines
Infection
Bifidobacteria
Phages

ABSTRACT

Under an inflamed-intestinal milieu, increased free sialic acids are associated with the overgrowth of some pathogenic bacterial strains. Recently, the protective immunomodulatory activity of gut bacteriophages (phages) has also been highlighted. However, the role of phages in triple reciprocal interactions between pathogenic bacteria, beneficial bacteria, and their host cell sialic acids has not been studied so far. We established a sialidase-explicit model in which beneficial and pathogenic bacteria interact through cross-feeding and competition for free sialic acid using a human triple co-culture cell model incorporating colonocytes (T84 cells), monocytes (THP-1 cells), and hepatocytes (Huh7 cells). Triple co-cultured cells were challenged with Gram-positive *Bifidobacterium bifidum* (*B. bifidum*) and Gram-negative *Pseudomonas aeruginosa* PAO1 (P. a PAO1) in the absence or presence of its KPP22 phage in two different cell culture mediums: 1) standard Dulbecco's Modified Eagle Medium (DMEM) and 2) DMEM with 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (DANA). Changes in physiological, functional, and structural health markers of stimulated cocultured cells were evaluated. The concentrations of sialic acid and pro-inflammatory cytokines in the cell culture supernatants were quantified. P. a PAO1 triggered the release of interleukin 6 and 8 (IL-6 and IL-8), accompanied by increased levels of free sialic acid, reduced viability of co-cultured cells, and disrupted the integrity of the cellular monolayer. These disruptive effects were markedly attenuated by KPP22 phage and *B. bifidum*. In addition to well-documented differences in the structure and composition of the bacterial cell walls of Gram-negative pathogenic bacteria and bifidobacteria, two distinct factors seem to be pivotal in modulating the pathogen-host interface milieu: (i) the presence of phages and (ii) the utilization of free sialic acids secreted from host cells by bifidobacteria.

1. Introduction

Host-derived sialic acids are crucial for favorable bacterial fitness *in vivo* and play a role in the initiation and development of pathogen infections. Pathogenic bacteria use sialic acids primarily as a nutrient source or as a signaling molecule to interact with their host (McDonald et al., 2016). Elevated levels of free sialic acid in the inflamed intestinal milieu cause the overgrowth of some pathogenic strains. Microbial pathogens use sialic acids to hide their antigenic sites and avoid recognition and expulsion by host immune cells (Cavalcante et al., 2021; Ghosh, 2020). Bacterial sialidases, originating from commensal and pathogenic bacteria in the human gut, promote host-microbe

---

Abbreviations: DANA, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid; DPBS, Dulbecco's Phosphate-Buffered Saline; GIT, human gastrointestinal tract; 3D, three -dimensional; Neu5Ac, N-Acetylneuraminic acid; NFkB, NF-kappaB, nuclear factor kappa B; TB, trypan blue.

* Corresponding author at: Department of Microbiology and Biotechnology, Max Rubner-Institute, Hermann-Weigmann-Straße 1, 24103 Kiel, Germany.
E-mail address: darab.ghadimi@mri.bund.de (D. Ghadimi).

[1] Retired.

https://doi.org/10.1016/j.yexmp.2025.104967
Received 22 January 2025; Received in revised form 10 April 2025; Accepted 14 April 2025

interactions on the mucosal surface. However, not all enteric commensals express the sialidases required to release free sialic acid. The sialidases encoded by intestinal bacteria vary in terms of substrate specificity and enzymatic reaction. Unlike intestinal commensals, which have their sialidases in essence for the acquisition of nutrients, some pathogens of the gastrointestinal tract (GIT) also employ sialidases to decrypt adhesin or toxin binding sites (Juge et al., 2016). This mutuality leads to an aberration in the proportion of intestinal commensals capable of modulating mucosal sialic acid levels. Maintaining a balance in the ability of intestinal commensals to produce and/or use sialic acid in the mucosal compartment is therefore crucial to intestinal homoeostasis (Juge et al., 2016).

Sialic acid, also known as [*N*-acetylneuraminic acid (Neu5Ac)], is commonly detected at the terminal location of mucins in the GIT. Because increased levels of free sialic acid in the intestinal mucosal compartment, for example, after antibiotic therapy, lead to the outgrowth of certain pathogenic bacterial strains, sialic acid catabolism in the GIT is essential during inflammation (Juge et al., 2016). In this case, intestinal inflammation and microbial dysbiosis in mice are caused by sialic acid catabolism (Huang et al., 2015).

The competition and cross-feeding between enteric pathogenic and nonpathogenic bacteria to acquire host-derived free sialic acids as a nutrient source are known. Even among nonpathogenic bacteria themselves, there is a competition to capture host-derived free sialic acids. For example, *Bifidobacterium breve* UCC2003 is capable of utilizing sialic acid released by the sialidase activity of *Bifidobacterium bifidum* (Juge et al., 2016). The association between altered levels of sialic acid homeostasis and the occurrence of intestinal inflammation and microbial dysbiosis is also known (Juge et al., 2016; Huang et al., 2015; Yu et al., 2021). For example, DANA, as a sialidase inhibitor, decreases bacterial pathogenesis and exhibits anti-inflammatory effects in *in vitro* and *in vivo* experiments (Ghosh, 2020). On the other hand, bacterial sialic acid (Neu5Ac) uptake inhibitors inhibit the growth of methicillin-resistant pathogenic bacteria (Bozzola et al., 2022). In general, the bioavailability of sialic acid derivatives in the gut has a significant influence on the modulation of intestinal microbes and host susceptibility to infection and inflammation. Alterations in homeostatic levels of sialic acid in the intestine have been associated with infectious inflammation in preclinical models (Bell et al., 2023).

Other important physiological actors in the digestive system are bacteriophages (phages), and recently the protective immunomodulatory impacts of gut phages have been illuminated. Recent *in silico*, *in vitro,* and *in vivo* studies suggest that the interactions of phages with animal and human organisms are robust and that their effects can be beneficial or deleterious to animals and humans. However, phages can mediate the specific action in mammalian cells, so their effect on these cells can vary between different phages and can also be specific to cells and tissues (Fujimoto, 2023; Górski et al., 2021; Podlacha et al., 2021; Putra and Lyrawati, 2020). Bacteriophages that target cytolytic opportunistic pathogens have been shown to mitigate liver disease and inflammation (Duan et al., 2019). In cases of intestinal barrier dysfunction, the liver is the first organ to come into contact with bacterial products and viable bacteria (Llorente and Schnabl, 2016). Bicht et al., 2021 (Bichet et al., 2021) showed that: 1) epithelial cells internalized bacteriophages; 2) the rate of uptake was influenced by size, morphology, and cell type; and 3) phages that had been accumulated internally were functional and continued to accumulate within cells over time.

Despite the data outlined above, the biological function of phages in the triple reciprocal interactions between pathogenic bacteria, beneficial bacteria, and host sialic acids of host cells has not been studied so far. The outcome of the complex quadruple interaction involving these entities is also not well understood. In particular, upon macrophage infection within the body, a genotoxic milieu is generated (Misson et al., 2023). Therefore, we hypothesized that sialic acid cross-feeding and phages within the host cell's milieu under infectious and inflamed conditions will be crucial to pathogen pathogenicity. As bacterial sialidase acts by mediating the efflux of sialic acids within intricately connected enterocytes, macrophages, and hepatocytes, we used a sialidase-explicit model where beneficial and pathogenic bacteria interact through both cross-feeding and competition for the free sialic acid of the host cell within the host cell environment. For this and to mimic the *in vivo* host eukaryotic cell milieu, we established a human triple co-culture cell model incorporating colonocytes, monocytes, and hepatocytes. Structural and physiological parameters (transepithelial electrical resistance (TEER) and cell viability), inflammatory biomarkers (interleukin-6 (IL-6) and interleukin-8(IL-8)) and pathogenicity (adhesion and invasion in host cells) were determined. The rationale for incorporating triple co-culturing of intestinal epithelial cells, macrophages, and hepatocytes is summarized as follows: (i) intestinal epithelial cells represent the first line of defense against pathogenic agents and are absorptive cells in the context of digestion. Their specific role in mucosal immune responses and inflammatory signals is crucial (Didriksen et al., 2024). (ii) Hepatocytes, as the primary cell type responsible for whole-body metabolic functions such as carbohydrate metabolism, lipid metabolism, and detoxification of xenobiotic compounds, are direct responders to microbial products (Etienne-Mesmin et al., 2016; Schulze et al., 2019). (iii) Gut macrophages are key players in intestinal immunity and tissue physiology (Muller et al., 2020; Nakagaki et al., 2018). (iv) Within the gut-liver axis, there is an intrinsic link between these three important cell types, and their contribution within this axis is key in maintaining tissue homeostasis, immunometabolism, responding to pathogens, and modulating inflammatory responses. In general, together, they oversee and regulate immune responses to pro-inflammatory nutrients and gut pro-inflammatory bacteria (Hsu and Schnabl, 2023).

2. Materials and methods

2.1. Media, reagents, kits

Merck sialic acid reagent test kit, sialidas inhibitor, N-Acetyl-2,3-dehydro-2-deoxyneuraminic acid (Sigma, Cat# D9050-10MG, synonyms: Neu5Ac2en; DANA), phorbol-12-myristate-13-acetate (PMA, Sigma Cat # P8931), and *N*-acetylneuraminic acid (Neu5Ac), ≥98 %, Cat # 110138) were purchased from Sigma Chemical (Sigma-Aldrich, Taufkirchen, Germany). 24 mm Transwell with 0.4 μm Pore Polyester Membrane Insert, Sterile (Cat #3450) and 75cm^2 U-Shaped Canted Neck Cell Culture Flask with Vent Cap (Cat # 430641 U), Gibco Dulbecco's Modified Eagle's Medium (DMEM, Cat # 11960044), Minimum Essential Medium (MEM) nonessential amino acids (Cat # 11140035), DPBS without calcium and magnesium (cat# 14190144), Fetal Bovine Serum (FBS, Cat # 16000044), Pierce chromogenes Endotoxin Quant Kit (Cat# A39552), TPP cell spatula (Cat# 99010), and Corning Small Cell Scraper (Cat# 3010) were purchased from thermo fisher (Schwerte, Germany). The 0.22 μm membrane syringe filter was purchased from Sarstedt (Nümbrecht, Germany). Human ELISA kits (IL-6 and IL-8) were obtained from abcam (Berlin, Germany).

2.2. Bacterial strains and growth conditions

Gram-negative *Pseudomonas aeruginosa* PAO1 and Gram-positive *Bifidobacterium bifidum* DSM 20456 were purchased from DSMZ (Braunschweig, Germany).

Among gram-negative opportunistic human pathogens, the risk of *P. aeruginosa* as a foodborne pathogen in the gut can be summarized as follows: It is a notorious pathogenic microorganism due to several virulence factors and is associated with hospital infections and contamination of various food groups. Antibiotic treatment-driven depletion of the intestinal microbiota (dysbiosis) facilitates colonization of the intestinal tract by multidrug-resistant *P. aeruginosa (*Li et al., *2023a)*.

The method of culture of *P. aeruginosa* PAO1 and the rationale for its use in this study have already been described in detail (Ghadimi et al., 2024a; Uchiyama et al., 2016). Bacteria counts were obtained by serially diluting overnight strain culture in PBS, plating on Luria-Bertani (LB) agar, and incubating at 37 °C to count P.a PAO1 and to confirm viability prior to inoculation with eukaryotic host cells.

Bifidobacterium bifidum was used due to its ability to use sialic acid (Bell et al., 2023). *Bifidobacterium bifidum* DSM 20456 was cultured in deMan, Rogosa and Sharpe (MRS) medium anaerobically at 37 °C (pH = 6.5). The inoculum was prepared from its frozen stock, stored at −80 °C using appropriate medium for 24 h, and grown in the medium for the next 48 h anaerobically at 37 °C. Cells were centrifuged (15 min at 5000 rpm at 4 °C) and suspended in a B. Braun isotonic saline solution of 0.9 %. The bacteria prepared in this way provided the inoculum (2×10^7 CFU/ml), which was used in an amount of 10 % (*v*/v). The number of live bacteria (CFU/ml) was determined by the total plate count after 24 and 48 h of cultivation.

2.3. Preparation of KPP22 phage supernatant

The KPP22 phage was previously characterized by Uchiyama et al. (2016) (Uchiyama et al., 2016), and was prepared according to the previously published method (Uchiyama et al., 2016). Phage titration, concentration, and removal of endotoxin were essentially based on previously published processes (Uchiyama et al., 2016; Ghadimi et al., 2023a). To perform all subsequent cell culture experiments, from a suspension of 10^9 phage KPP22 per ml, we carefully added 150 μl into the upper compartment of the inserts (10^8 PFU/ml) so that all inserts end up with 1.5 ml of medium, which will be described below.

The fate, activity, and impact of ingested lytic phages (e. g., KPP22, which is a lytic phage) within the human gut microbiota and the protective immunomodulatory activity of gut phages have been well reviewed (Sutton and Hill, 2019; Łusiak-Szelachowska et al., 2017).

2.4. Cell culture

2.4.1. Cell lines

The human colon carcinoma cell line (T84) and the human monocyte cell line THP-1 (cell stocks frozen in liquid nitrogen) were from the American Type Culture Collection (ATCC, LGC, Wesel, Germany). The human hepatocarcinoma cell line (Huh7) (our liquid nitrogen frozen cell stock) was from the Japanese Collection of Research Biosources Cell Bank (Tebubio GmbH, Offenbach, Germany). T84, THP-1, and Huh7 were maintained in standard DMEM medium supplemented with 5 % FBS and 1 % MEM non-essential amino acids. Cells were routinely split at~80 % confluence and used at passages 19–26 after thawing for experiments. Cells were kept in a humified incubator at 37 °C in a 5 % CO2 atmosphere in a Thermo Scientific Heracell 150i incubator with variable oxygen control. Cells were trypsinized with 0.25 % trypsin. Using the MycoStrip Mycoplasma Detection Kit (InvivoGen, Toulouse, France), all cell lines and cultures were examined for mycoplasma according to the manufacturer's instructions.

2.5. Setup of triple cell co-cultures

To model the cellular constituents of bidirectional crosstalk within the gut-liver axis, namely three types of cells on the gut and liver axis as frontline immune tissue (intestinal and hepatic macrophages), corning 6-well Transwell inserts with semipermeable polyester membrane supports with a diameter of 24 mm and 0.4 μm pore size were used, and triple cell co-cultures were carried out in a five-step process, in the following order: (i) The insert was inverted and THP-1 cells were seeded in a large droplet on the underside of the Transwell insert. (ii) Four hours later, the insert was reoriented and T84 cells were seeded at confluence on the inside of the insert, above the membrane. (iii) The medium was topped up on the top of the insert and in the well and the cells were incubated for 8 days to allow for the formation of the T84 monolayer. (iv) Huh7 cells were seeded in separate Transwell-suitable 6-well plates 72 h before the T84 polarization was complete. (v) Co-cultures of T84/THP-1 cell, pre-grown for 8 days, were placed directly on top of Huh7 cells in transwell-suitable 6-well plates. The assembly details of this inverted triple co-culture model are as follows. First, THP-1 cells were maintained, centrifuged, resuspended, differentiated, and seeded on the underside of inverted transwell inserts as previously described in detail (Calatayud et al., 2019; Klein et al., 2013). Briefly, one day before seeding for co-cultures, THP-1 cells were stimulated to differentiate into macrophage-like cells by adding phorbol-12-myristate-13-acetate (PMA; 25 ng/ml) and incubation in RPMI medium overnight at 37 °C and 5 % CO2. Subsequently, PMA differentiated THP-1 macrophages (100 μl = 10^5 cells per insert) were seeded on the underside of the inverted Transwell inserts and left to incubate for a period of 4 h. After this time, the Transwell inserts were placed back inside their respective wells. Subsequently, T84 was added to the apical side of Transwell at a seeding density of 7.5×10^4 cells/insert in DMEM (1.5 ml) as previously described (Ou et al., 2009; Ghadimi et al., 2012). The apical and basolateral compartments of the co-culture were refreshed every 48 h with DMEM. To achieve tight T84 monolayers, the co-culture was maintained for eight days until confluent monolayers of polarized columnar epithelium with transepithelial electrical resistance (TEER) of at least 1000 Ohm/cm2 were obtained (Ou et al., 2009). Three days before seeding for triple co-cultures, Huh7 cells (3×10^5 cells per well) were seeded at the bottom of separate 6- well transwell-suitable plates as previously described (Ghadimi et al., 2023b). To start the infectious-inflamed triple co-culture, T84/THP-1 co-culture, pre-grown for 8 days, was used to set up the triple co-culture with Huh7 cells. For this, the T84 / THP-1 co-culture (the upper chamber containing the T84/THP-1 cells) was then placed directly on top of the Huh7 cells in six-well transwell-suitable plates to initiate triple co-culture. After 24 h of triple co-culture, triple co-cultured cells were divided into eight experimental groups as follows: 1) DMEM alone control group; 2) DMEM alone + P.a PAO1 group; 3) DMEM alone + P.a PAO1 + KPP22 phage group; 4) DMEM alone + P.a PAO1 + B.bifidum group; 5) DMEM + DANA alone control group; 6) DMEM + DANA + P.a PAO1 group; 7) DMEM + DANA + P.a PAO1 + KPP22 phage group; and 8) DMEM + DANA + P.a PAO1+ B.bifidum group, as detailed hereinafter. DMEM medium was supplemented with 100 μM of DANA, a specific endogenous sialidase inhibitor, which reduces bacterial pathogenesis and exerts anti-inflammatory effects *in vitro* and *in vivo (*Yu et al., *2021)*. In summary, the setup of these experimental design groups allows for a clearer understanding of the intricate effects of P.a PAO1, KPP22 phage, *B. bifidum*, and the syntrophic metabolism of host sialic acids on cell physiology in two different media: DMEM alone and DMEM+ DANA. Therefore, we included two specific control (comparator) groups: (i) Uninfected control cells in DMEM medium alone, which serves as a baseline for cell behavior in the absence of any bacterial or phage interventions. (ii) Uninfected control cells in DMEM medium + DANA (as a positive control), which helps to account for the potential influence of the sialidase inhibitor, DANA, on the cells.

2.6. Setup of the infectious-inflamed triple cell co-cultures

For infectious-inflamed triple cell co-cultures, P.a PAO1 and/or its KPP22 phage were applied to the apical surfaces of T84 cell monolayers grown on Transwell insert membranes, as simply summarized as follows: One day before infection or experiment, P.a PAO1 was grown in Luria-Bertani (LB) medium overnight at 37°C, washed twice with DPBS, and diluted 1:10 in fresh antibiotic-free DMEM for one hour (37°C in a 5 % CO2 atmosphere). *B. bifidum* was also grown anaerobically in MRS overnight at 37°C and washed twice with DPBS. For all experiments, diluted bacterial suspensions of P. a PAO1 and *B. bifidum* were made at an optical density (OD 600) of 0.5 (~ 1×10^9 CFU/ml) in cell culture medium. Once the triple co-cultures were ready to be treated (see

Section 2.5 above), the medium was removed and the cells were washed with DPBS. Following cell washing steps, for the bacterial competition assay, 150 μl P.a PAO1 suspension with 150 μl *B. bifidum* suspension was added on top of the designated T84 cell monolayers to treat triple co-cultured cells. This resulted in a final ratio of *B. bifidum* to P. a PAO1 of 1:1. For phage treatment, 150 μl of diluted P.a PAO1 suspension (1 × 10^9 CFU/ml) with 150 μl of phage suspension (1 × 10^9 PFU/ml) were added on top of the designated T84 cell monolayers to treat triple co-cultured cells. This resulted in a final ratio of phage to P. a PAO1 of 1:1. This ratio was chosen because the ratio between phages and bacteria in the gut is 1:1 or lower (Khan Mirzaei and Deng, 2022). The transwell inserts were then submerged in DMEM or DMEM supplemented with DANA (100 μM). The final volume of the Transwell insert culture media were 1.5 ml (apical) and 2.6 ml (basolateral). The resulting (assembled) host-bacteria-phage co-cultures were then incubated in humidified conditions for six hours at 37 °C and 5 % CO_2. Parallel runs of control cells and cells exposed to P.a PAO1 without phage treatment were also carried out, and both groups underwent the same medium and wash changes. Following six hours, the cells were visually inspected and the following sections evaluated the structural and physiological functions of the cells (*e.g.*, TEER) as well as the signal molecules they secreted. Filter sterilized (0.2 μm), cell-free supernatants were collected, centrifuged (14,000 rpm, 5 min, 4 °C), frozen and kept at −80 °C until further examination. After that, cells were scraped or trypsinized, washed, and counted as detailed hereinafter.

2.7. *Measurement of the structural health of cells (TEER)*

The epithelial voltohmmeter (EVOM2) and the STX2 Chopstick Electrode Set (World Precision Instruments, USA) were used to measure the TEER values of the T84 monolayers according to the manufacturer's instructions. Briefly, the electrodes were cleaned with 70 % isopropanol alcohol, rinsed once with 1× PBS, dried, and then placed in the apical or basolateral compartments, respectively. Before each measurement, semipermeable membrane Transwell inserts with a polarized T84 membrane layer in the transwell (apical) compartment were cautiously removed from the co-culture at the conclusion of the experiment. Following the removal of the apical medium, DPBS was used twice to wash the T84 cell layer. The Transwell received 1.5 ml of DPBS and the basolateral compartment outside the transwell received 2.6 ml.

2.8. *Physiological health of cells in response to bacteria and the KPP22 phage*

The effect of treatments on the viability of triple cell co-cultures was assessed in separate experiments. When it was confirmed that T84 cells had reached confluence using TEER $\geq 1000\,\Omega$ / cm2, the monolayers of T84 cells were washed twice with 100 μl DMEM without antibiotics or supplements, and cell viability assays were performed by adding the bacterial inoculum of *B. bifidum* (approximately 1 × 10^8CFU/ml) together with P.a PAO1 (approximately 1 × 10^8 CFU/ml) in the apical chamber. Cell cultures were incubated in a humidified atmosphere with 5 % CO2 for six hours at 37 °C. Following a 6-h incubation period, T84/THP-1/Huh7 cells were detached by using tiny cell scrapers to scrape the entire culture area. Trypan blue dye exclusion was used to assess the viability of treated cells after collected cells were resuspended in DPBS. The viability of the cells in the control wells, where no stimulants were applied, was compared with that of the treated cell groups.

2.9. *Measurement of free sialic acids*

The amount of sialic acid was determined in the T84 cell supernatants. For this, subsequently, after measuring the TEER values, cells were gently scraped using sterile mini cell scrapers. Then 80 μl of cell suspensions were first used to assess cell viability, and the remainder was washed once with DPBS and then exposed to 200 μl of the appropriate lysis buffer for 2 min at room temperature. The resulting cell lysates were used and the sialic acid concentrations derived from host cells were determined as previously described (adapted for cell culture) (Jourdian et al., 1971; Weidemann et al., 2010). Briefly, cell lysates of 20 × 10 ^{6} cells were oxidized in 250 μl PBS with 5 μl of periodic acid 0.4 M at 37 °C for 90 min, followed by 15 min of boiling in 500 μl of 6 % resorcinol/2.5 mM CuSO 4/44 % HCl. After cooling for a few minutes, 500 μl of tert-butyl alcohol was added and the samples were vortexed and centrifuged for 5 min to precipitate cell debris. Immediately after spinning, the supernatants were poured into OD cuvettes and read at 630 nm. The sialic acid concentrations were calculated by comparing them with a standard curve.

2.10. *Measurement of sialidase enzyme activity in cell culture supernatants*

To determine whether bacterial sialidase activity had an effect on sialic acid-mediated pathogenesis of P.a PAO in host cells, cell culture supernatants from the same 6-h co-cultures were also evaluated for sialidase activity. For this, cell culture medium was collected and centrifuged at 15,000 *g* for 10 min at 4 °C. The supernatants were filtered through 0.22-μm syringe filters to obtain cell and debris-free supernatants. The activity of sialidase in the cell culture supernatant was determined as previously described (Li et al., 2023b). For this purpose, an aliquot (60 μl) of each supernatant was added to 40 μl of substrate (4 mM 5-bromo-4-chloro-3-indolyl-α-D-*N*-acetylneuraminic acid [Sigma, Cat# B4666]). Each reaction was then incubated at 37 °C for 1 h and absorbance at 595 nm was measured using a Tecan Genios Fluorescence, Absorbance and Luminescence Reader.

2.11. *Measurement of cytokines*

After 6 h of infection and subsequently after measuring the TEER values and cell viability of triple co-cultured cells, supernatants were collected and the levels of IL-6 and IL-8 cytokines were assessed using Abcam ELISA kits, according to the manufacturer's protocols. In addition, the pH values of the supernatants were measured. Before beginning the measurements, the pH meter (Mettler-Toledo Micro pH Electrode, Gießen, Germany) was calibrated using buffer solutions at pH 4.0, 7.0, and 9.21 (Fisher Scientific).

2.12. *Adhesion and invasion assays*

In separate experiments, co-cultured cells of T84, THP-1, and, Huh7 were developed to determine the inhibitory effect of the *B. bifidum* bacterium, the KPP22 phage and DANA on the adhesion and/or invasion of P.a PAO1 to/into T84 cells. Cell adhesion and invasion assays were carried out as previously described (Ghadimi et al., 2024b; Leneveu-Jenvrin et al., 2013). Briefly, once the triple co-cultures were ready to be treated and it was confirmed that the T84 cells had reached confluence at TEER $>1000\,\Omega$/cm2, the medium was aspirated from the triple co-cultured cells in 6-well Transwell inserts, and the cells were washed twice with 500 μl of DMEM without antibiotics or supplements. After washing, eight treatments were examined: For this, infection assays were performed by adding the bacterial inoculum of P.a PAO1 (approximately 1 × 10^8CFU/ml) in the presence or absence of the KPP22 phage (1 × 10^8 PFU/ml) together with *B. bifidum* (approximately 1 × 10^8CFU/ml) in the apical chamber. The transwell inserts containing T84/THP-1/Huh7 cells were then immersed in two different media: antibiotic-free DMEM alone or DMEM supplemented with 100 μM DANA. Cells were incubated for 6 h at 37 °C with 5 % CO2 and a humidity >95 %, to allow adhesion and invasion. After two hours of washing with PBS to remove nonadhered P.a PAO1, 250 μl of DMEM containing 150 μg/ml gentamicin was added to kill extracellular P.a PAO1 for 45 min. The following step involved carefully scraping adhered P.a PAO1 with T84 cells on the upper surface of the membrane (inside of the Transwell insert) with a mini sterile cell scraper, transferring the cells to 5 ml of barcoded sterile tubes with a pipette and centrifuging the mixture for 10 min at 5000 rpm. 0.1 % Triton X-100 was added to T84 cells for five minutes at room temperature to disrupt them. After an overnight incubation period at 37 °C, serial dilutions of the disrupted cell suspension were subsequently spread out on LB Agar plates for viable enumeration of adherent bacteria. The same technique was applied, but without gentamicin treatment, to assess the amount of P.a PAO1 invaded. The percentages of adhered or invaded P. a PAO1 to the initial numbers applied were used to calculate the adhesion and invasion rates. To evaluate the effects of P.a PAO1 on the integrity of the T84 cell monolayer after infection of the cell monolayer, TEER was measured 6 h after infection. The TEER of the infected cells was compared to those of the noninfected cells. Furthermore, the effects of co-incubation of *B. bifidum* with P.a PAO1 were also evaluated. We evaluated adhesion and invasion in T84 cell monolayers among triple co-cultured cells because the 0.4-μm membrane inserts only permit the diffusion of media and molecules secreted by bacteria; they do not allow bacterial passage through the pore, retaining bacteria, and preventing direct contact of whole bacteria with the THP-1 / Huh7 cells that are underneath (neighboring). Micron-sized particles, such as bacteria, yeast cells, colloids, and smoke particles, are removed from the suspensions using transwells with a 0.4 μm membrane insert.

2.13. Sialic acid cross-feeding assays

After establishing that the KPP22 phage and *B. bifidum* decreased the pathogenesis of P.a PAO1 and reduced the activity of sialidase in cell culture supernatants, as well as host- free sialic acid levels in cell culture supernatants, we sought to determine if inhibition of P.a PAO1 sialic acid uptake and sialic acid cross-feeding also occurred in sialic acid-rich pure cultures of bacteria without relying on host cells. Therefore, in a subset of separate experiments, we have conducted cross-feeding assays between PAO1 and *B. bifidum* in the presence and absence of heat-killed *B. bifidum* and in DMEM containing human sialic acid (Neu5Ac). For these assays, the *B. bifidum* and P.a PAO1 strains were grown, as indicated above in Section 2.2. To prepare heat-killed *B. bifidum*, it was grown anaerobically (as indicated above) in 100- ml Erlenmeyer flasks containing 50 ml of MRS broth supplemented with 0.05 % L-cysteine HCl at 37 °C for 24 h at a Whitley A45 workstation, where the atmosphere consisted of 10 % CO2, 80 % N2, and 10 % H2. After incubation, the bacterial cultures were centrifuged at 5000 ×*g* for 5 min to obtain a cell pellet. The cell pellet obtained was washed twice with sterile B. Braun isotonic saline solution 0.9 % and resuspended in B. Braun isotonic saline solution 0.9 % at a concentration of OD600 = 1.0, corresponding to approximately 2×10^9 CFU/ml. The resulting suspension was divided into two parts: The first part served as a live source of *B. bifidum*, and the second part served as a heat-killed source of *B. bifidum*. The latter was heated at 90 °C for 10 min for sialic acid cross-feeding assays. For cross-feeding assays, DMEM or DMEM supplemented with 16 mM sialic acid was inoculated with P.a PAO1 alone (10^8 CFU/ ml), P.a PAO1+ KPP22 phage (10^8 PFU/ml), P.a PAO1 + live *B. bifidum* (10^8 CFU / ml) or P.a PAO1 + heat-killed *B. bifidum* (10^8 CFU/ml), and cultures in 3 ml volume in six-well plates were incubated at 37 °C for 6 h. After incubation, the aliquots were removed, serially diluted and plated on LB agar to determine the counts of bacterial colonies of P.a PAO1. Culture supernatants were also evaluated for both the sialidase activity and sialic acid (Neu5Ac) concentration remaining in DMEM after 6 h of treatment. The levels of sialic acid in the culture supernatant were measured as described in Section 2.9. The uptake of sialic acid was calculated by sialic acid supplemented in medium minus sialic acid left after 6 h in the culture supernatant. The uptake ratios were calculated from the sialic acid uptakes divided by the sialic acid supplemented. The activity of sialidase in the culture supernatant was determined as described in Section 2.10. The sialic acid concentration (16 mM) was chosen as previously described (Li et al., 2017). The DANA concentration (100 μM; Sigma, Cat# D9050; CAS: 24967–27-9) was chosen as previously described (Heise et al., 2018).

2.14. Statistical analysis

Data analysis was performed using STATGRAPHICS Plus statistical software Version 4.1 (Statgraphics Technologies, Inc., Virginia, USA). For the data analysis of the results obtained, the mean values and standard errors of the mean (± SEM) were calculated. Data were checked for normal distribution and, subsequently, analyzed with the *t*-test (parametric) or Mann–Whitney-*U* test (nonparametric). For multiple comparisons, one-way analysis of variances (ANOVA) or the Kruskal–Wallis test was applied, depending on the normal distribution. The Dunnett test (parametric) or Dunn's test (non-parametric) served as *post hoc* tests. The significance level was set at $p < 0.05$ for all experiments. Duplicate samples per group were included in each experiment.

3. Results

3.1. Analysis of cell viability and total number of living co- cultured cells

The Trypan blue (TB) viability assessments of three-dimensional (3D) co-cultured cells, as a precise method (Piccinini et al., 2017), were included to identify the potential cytotoxic effects of treatments in stable triple cell co-culture as well as the impact of P.a PAO1 infection in the inflamed model. The viability of the uninfected control T84, THP-1, and Huh7 l cells ranged from 90 to 100 % compared to cells inoculated with P.a PAO1 (Figs. 1A-C). As expected, co-incubation of pathogenic P. a PAO1 with triple co-cultured T84, THP-1, and Huh7 cells resulted in cytotoxic effects, with disruption of the monolayers compared to uninfected cells. This was more significant for T84 cells, which showed only 36.34 % viability after 6 h (Fig. 1A), indicating that T84 cells were the most sensitive cells to P.a PAO1 infection because they faced bacteria directly. However, P.a PAO1 alone showed greater detrimental effects on Huh7 cells, resulting in only 40 to 45 % cell viability after 6 h of co-incubation (Fig. 1C). The addition of *B. bifidum*, KPP22 phage, or DANA with P.a PAO1 showed protected effects for triple co-cultured T84, THP-1, and Huh7 cell viability. The viability of THP-1, and Huh7 cells remained >89 % after the co-incubation of the combination of KPP22 phage, *B. bifidum* and DANA with P.a PAO1 for 6 h (Figs. 1 B and C).

3.2. Integrity of the intestinal epithelial barrier

To evaluate the tightness of the T84 cell monolayer in Transwell cell cultures, we examined the effectiveness of *B. bifidum*, KPP22 phage, and the sialic acid inhibitor DANA in protecting against the disruptive effects of P.a PAO1 on the integrity of the epithelial barrier. These were evaluated by measuring the TEER of polarized T84 cells after 6 h after infection. As shown in Fig. 2, at 2×10^8 CFU/ml, the adhesion of P.a PAO1 to T84 polarized cells induced a significant decrease in TEER values in T84 cell monolayers. DANA administration effectively increased TEER values in T84 cells infected with P.a PAO1. However, the combination of DANA and KPP22 phage led to significantly higher TEER values in T84 cells compared to the DANA group. The TEER level in the P.a PAO1 + *B. bifidum* group increased significantly compared to the P.a PAO1 alone group. The results obtained imply that *B. bifidum*, KPP22 phage, and DANA are capable of maintaining intestinal permeability and improving intestinal barrier function; however, findings need to be confirmed in *in vivo* studies.

3.3. Host cell's sialic acids status

Recent studies have shown that sialic acids on the surface of host cells play an important role in cellular communication between pathogens and host cells and in pathogen infection and survival (Cavalcante et al., 2021). Basal sialic acids of the human host cells are receptors for

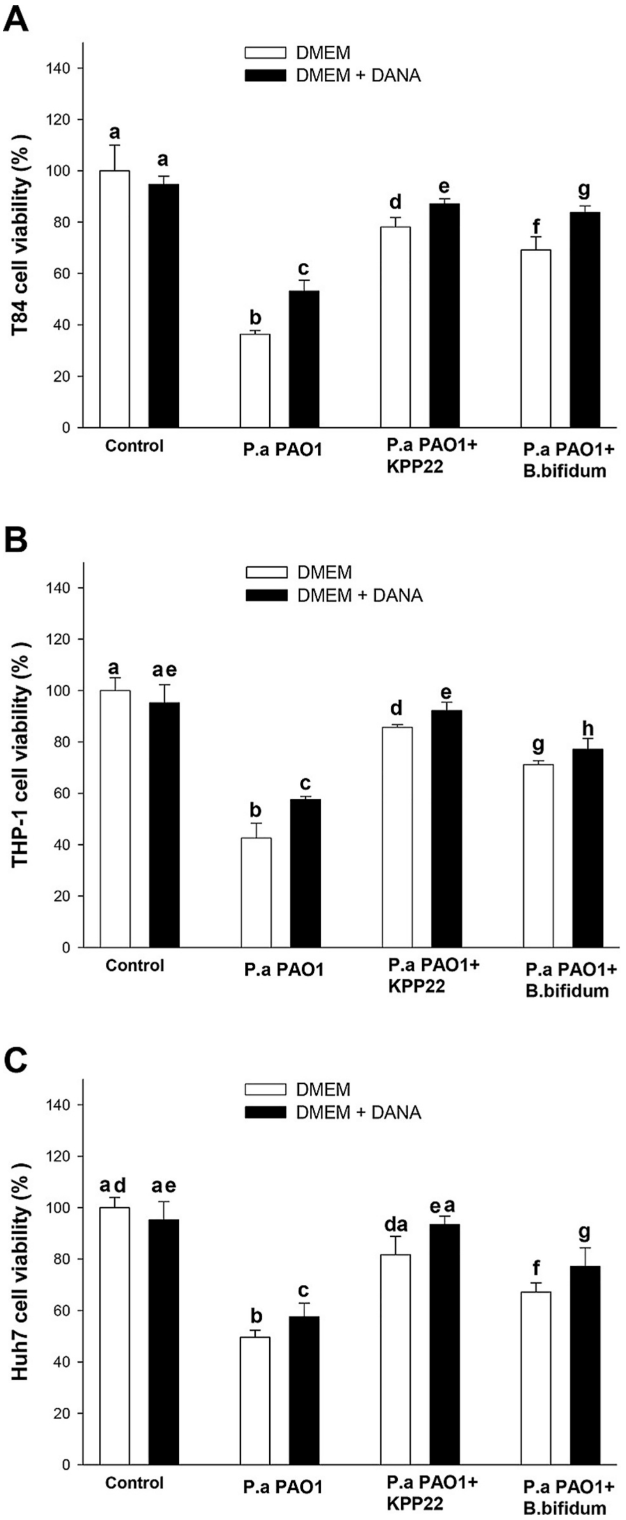


Fig. 1. Cell viability assay. The impact of P.a PAO1, KPP22 phage, and *B. bifidum* on the cell viability of T84 cells (panel A), THP-1 cells (panel B), and Huh7 cells (panel C) is shown. The seeded triple co-cultured cells were incubated in DMEM culture medium (white bars) or DMEM culture medium with 100 μM of DANA (black bars). The cells were then exposed for 6 h to the following treatments: 1) P.a PAO1 (1×10^8 CFU/ml) alone; 2) P.a PAO1 plus its KPP22 phage; and 3) P.a PAO1 plus *B. bifidum* (1×10^8 CFU/ml). In the control group, cells were incubated with only medium. The KPP22 phage was used as a specific natural phage lysing P.a PAO1. DANA was used as a specific endogenous sialidase inhibitor. The mean values of the control wells containing only cells without any bacteria were taken as 100 %. The percentage of viable active cells treated with bacteria, phage, or DANA was then calculated. Each bar represents the mean of three independent replicates (n = 3) with its corresponding standard error of the mean (SEM). Statistical comparisons were conducted using a one-way ANOVA with Tukey's post-test (A, B, and C). Data are expressed as percentage (%) cell survival compared to the control. The bars with different lowercase letters (a-g) indicate statistically significant differences between any two groups ($p < 0.05$), indicating that they are significantly different from each other. Bars with the same lowercase letters represent no significant differences between any two groups ($p < 0.05$). P.a PAO1, *Pseudomonas aeruginosa PAO1;* B.bifidum*, Bifidobacterium bifidum;* KPP22, KPP22 phage.

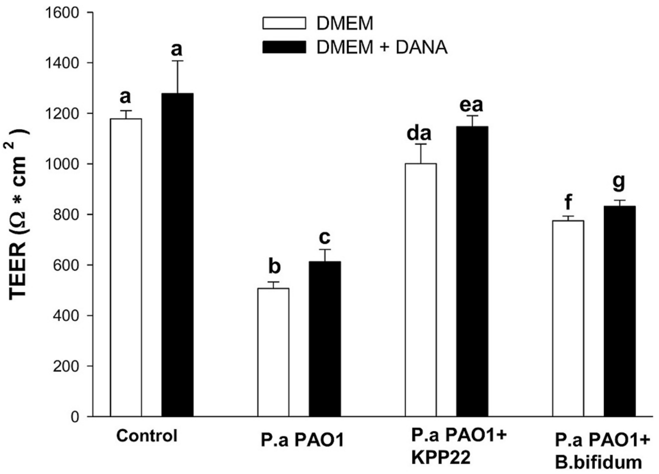


Fig. 2. Transepithelial electrical resistance (TEER) of T84 cell monolayers. The monolayers of T84 cells grown on transwell inserts were exposed to P.a PAO1 (1×10^8 CFU/ml) and *B. bifidum* (1×10^8 CFU/ml) for 6 h. The error bars indicate the standard deviation of at least three independent experiments. Data are expressed as means ±SEM. Bars with different lowercase letters represent statistically significant differences between any two groups ($p < 0.05$), indicating that they are significantly different from each other. The bars with common (same) lowercase letters represent no significant differences between any two groups ($p < 0.05$).

pathogens. On the other hand, pathogenic bacteria obtain sialic acid by scavenging pathways and engaging their sialidases, in which free sialic acid is obtained directly from human host cells and decorates their cell surfaces to escape the immune system of the host (Burzyńska et al., 2021; Kim and Kim, 2023). To determine whether disrupted TEER and cell viability, which are two good indicators of cell health and functionality, are due to the cleavage of sialic acids in the extracellular cell surface membrane, we measured the free sialic acid concentration in cell culture supernatants (Fig. 3A). In the non-inoculated control cell group, namely, when co-cultured cells were incubated for 6 h in DMEM supplemented with 100 μM DANA, the endogenous basal sialic acid concentration of the supernatant decreased (albeit not significantly) relative to the endogenous basal sialic acid concentration of the supernatant from equivalent DMEM cultures. As shown in Fig. 3A, the analyzes showed that the free sialic acid content in the supernatant of the P.a PAO1 treated cells incubated in DMEN alone (white bars) was significantly higher than in controls non-treated cells. This increase in sialic acid was completely abolished by adding the KPP22 phage and inhibited by adding *B. bifidum*. These results indicated that P.a PAO1 liberates sialic acid from the surface of host cells. When cells were incubated in DMEM plus DANA, the reduction effect of KPP22 phage on free sialic acid concentrations was significantly reinforced (black bars) compared to cells treated with KPP22 phage alone and in DMEM alone. However, treatment with DANA and *B. bifidum* additively induced a decrease in the concentration of sialic acids. These results indicate that: 1) P.a PAO1 cleaves sialic acid from the surface of host cells (here co-cultured cells); 2) KPP22 phage and *B. bifidum* are powerful biological influencers capable of modulating aberrant levels of sialylation and sialic acid levels, which are pervasive in inflammation (Rodrigues and Macauley, 2018); 3) induced free sialic acid concentrations are due to the activity of P.a PAO1 sialidase in host cell membranes; and 4) *B. bifidum* either *via* direct utilization of free sialic acids or competitive exclusion of P.a PAO1 and consequently inhibiting its sialidase activity exerts a protective effect on host cells. This interpretation is evaluated in the next section.

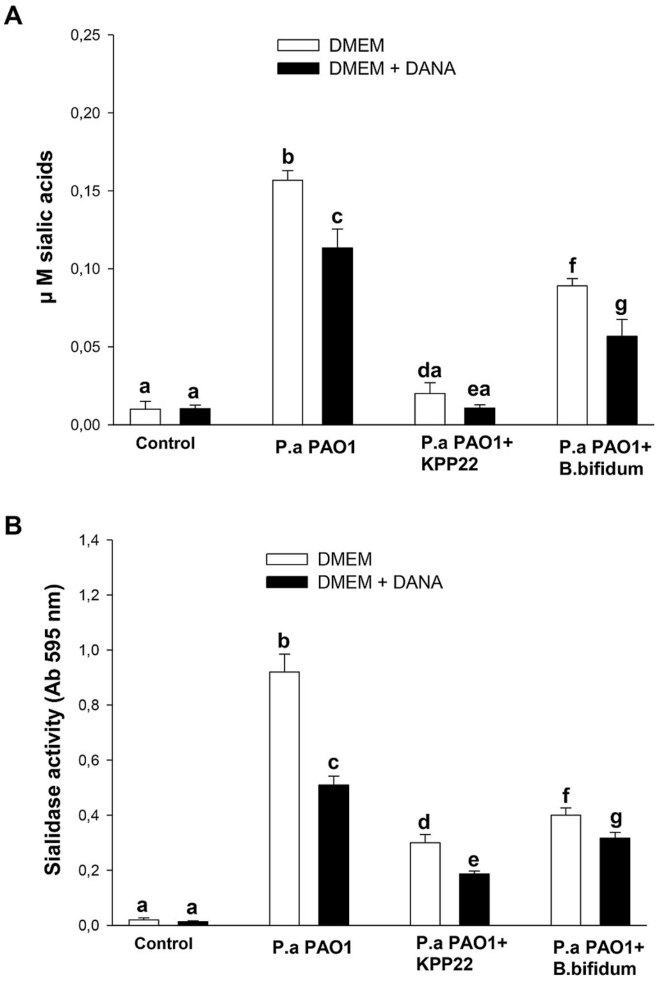


Fig. 3. Release of free sialic acid from the surface of co-cultured cells and sialidase activity in the cell culture supernatant. T84, THP-1, and Huh7 cells were co-cultured in DMEM medium or DMEM + DANA and incubated with P.a PAO1, P.a PAO1+ KPP22 phage or P.a PAO1+ *B. bifidum* for 6 h. The control group was incubated with only medium for comparison. (**A**) The amount of free sialic acid remaining in the cell culture supernatants 6 h after inoculation of P.a PAO1 and *B. bifidum* or uninoculated media control. (**B**) Sialidase activity was measured in 6-h co-cultures using supernatants from panel A. After 6 h of incubation, the activity of sialidase in the supernatants was analyzed by spectrophotometry and expressed as absorbance (Ab) at 595 nm. Both panels show the mean values of three independent experiments, with standard errors of the mean (± SEM). The bars with different lowercase letters (a-g) represent statistically significant differences between any two groups ($p < 0.05$), indicating that they differ significantly from each other. The bars with common (same) lowercase letters represent no significant differences between any two groups ($p < 0.05$). The bars represent the mean values of three independent experiments carried out in duplicate.

3.4. Sialidase activity in cell culture supernatants

Further support for the coupled contribution of pathogenic bacterial sialidase activity and the bioavailability of host-free sialic acid in cell cytotoxicity was revealed by spectrophotometric determination of supernatant sialidase activity. Supernatant sialidase activity in the supernatant of co-cultured cells is shown in Fig. 3B. When the P.a PAO1 strain was added alone to cells in DMEM or DMEM +DANA for 6 h, significantly higher absorbance values (595 nm, Ab596 nm) were observed compared to the corresponding control cells without treatment. However, significantly lower Ab596 nm absorbance values were observed in the presence of DANA *versus* DMEM. As expected, the KPP22 phage significantly abolished sialidase activity in supernatants induced by P.a PAO1. This effect was more pronounced when DANA was added to DMEM. The activity of P.a PAO1-induced sialidase was repressed significantly when *B. bifidum* was added to DMEM, and this repression

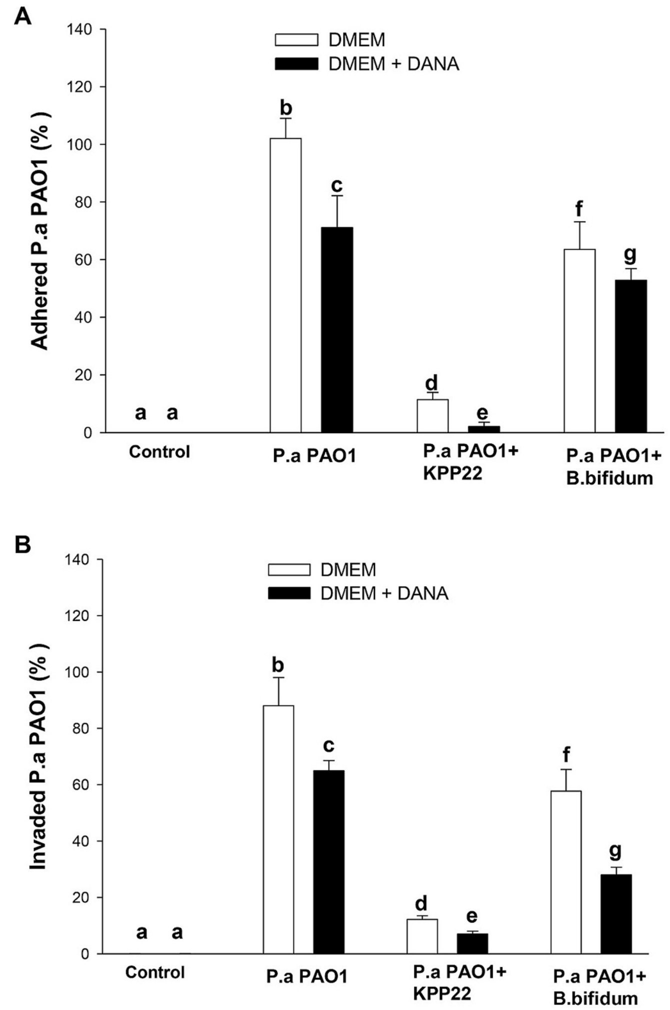


Fig. 4. Influence of DANA, the KPP22 phage, and *B. bifidum* on the host-pathogen interaction of P.a PAO1 with T84 monolayer cells. The effects of KPP22 phage (1×10^8 PFU/ml), *B. bifidum* (1×10^{8} PFU/ml), and the sialidase inhibitor DANA on P.a PAO1 (1×10^8CFU/ml) adhesion to (**A**) and invasion into (**B**) human T84 intestinal epithelial cells after 6h of infection. The error bars indicate the standard deviation of at least three independent experiments. Data are expressed as means ±SEM. The bars with different lowercase letters represent statistically significant differences between any two groups ($p < 0.05$), indicating that they differ significantly from each other. The bars with common (same) lowercase letters represent no significant differences between any two groups ($p < 0.05$).

was potentiated in the presence of DANA. These results suggest that: 1) inhibition of P.a PAO1 sialidase activity could reduce the generation and removal of free sialic acid from the surface of host cells, thus mitigating signs related to cytotoxicity (Fig. 1A-C); and 2) sialidase of P.a PAO1, not sialidase from *B. bifidum*, is the primary factor that disrupts host cell function because the addition of *B. bifidum* to the cells even repressed the values of sialidase activity induced by the P.a PAO1 bacterium in the supernatants. Interestingly, the addition of *B. bifidum* and P.a PAO1 to cells did not result in an additive increase in sialidase activity, contrary to expectations. The reason could be due to the reduced cell adhesion of P.a PAO1 because bifidobacteria (*e.g.*, *B. bifidum*) have competitive and exclusion capabilities against human pathogenic bacteria (*e.g.*, *P. aeruginosa*) (Servin, 2004; Sokolovskaya et al., 2022), in line with our results in Section 3.5. However, the catalytic mechanisms of human sialidase, bacterial sialidase, and viral sialidase are different as they work with specific substrates. Following sialic acid uptake, Gram-negative bacterial pathogens and Gram-positive bacterial nonpathogens can utilize it in multiple (different) ways depending on the chosen strain, and the presence or absence of host cells is a key factor in this context (Jennings et al., 2022; Keil et al., 2022). Our findings are in line with the results of previous studies showing that under *in vitro* and *in vivo* inflammatory conditions, there are significant positive correlations between pathogenic bacterial sialidase activity and free sialic acid concentrations (release of sialic acid from host glycans) (Huang et al., 2015), and toxin-induced host cell cytotoxicity is associated with sialidase activity (Li et al., 2011; Li and McClane, 2018).

3.5. Adhesion to and invasion into T84 cell monolayers by P.a PAO1

Because the sialic acids of eukaryotic hosts play a critical role in determining cell fate and function in response to various signals and stimuli, it is important to assess the functional consequences of reduced

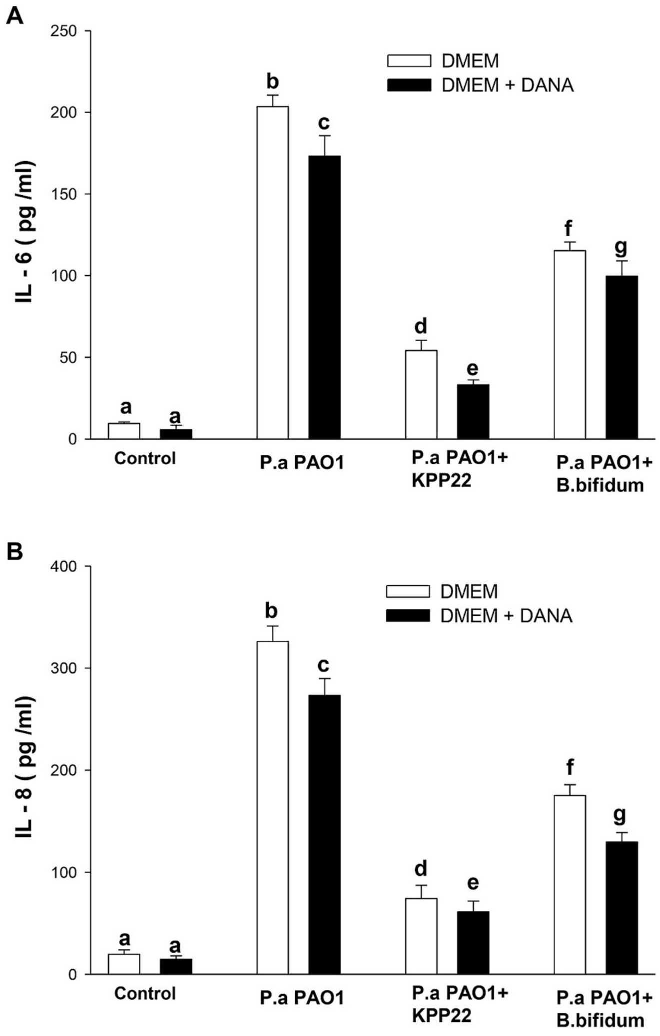


Fig. 5. Effects of DANA, KPP22 phage, and *B. bifidum* on PAO1-induced secretion of pro-inflammatory cytokines from triple co-cultured cells. The seeded triple co-cultured cells were incubated in DMEM culture medium (white bars) or DMEM culture medium with 100 μM of DANA (black bars). The upper medium of the co-culture system was treated with indicated concentrations of PAO1 (1×10^8 CFU/ml), KPP22 phage (1×10^8 PFU/ml), and *B. bifidum* (1×10^8 CFU/ml) for 6 h. In the control group, cells were incubated with only medium. The secretion of IL-6 (**A**) and IL-8 (**B**) from the co-cultured cells was determined by an ELISA assay. Bars with different lowercase letters represent statistically significant differences between any two groups ($p < 0.05$), indicating that they are significantly different from each other. The bars with common (same) lowercase letters represent no significant differences between any two groups ($p < 0.05$). Each value is presented as the mean ± SEM ($n = 3$).

levels of host sialic acids within the cell cultured milieu mediated by the KPP22 phage and *B. bifidum*. Therefore, the effectiveness of KPP22 phage, *B. bifidum*, and DANA in altering adhesion and invasion of P.a PAO1 was evaluated using a triple cell co-culture model consisting of T84, THP-1, and Huh7 cells. Post-infection growth for P.a PAO1 adhesion (Fig. 4 A) and invasion (Fig. 4 B) was determined. The adhesion of P.a PAO1 to T84 cells was more pronounced, with more adhered P.a PAO1 observed 6 h after infection. The addition of KPP22 phage, *B. bifidum*, and DANA significantly decreased the adhesion of P.a PAO1 to T84 cells compared to untreated cells (Fig. 4A). The addition of the KPP22 phage, *B. bifidum*, and DANA significantly reduced the invasiveness of P.a PAO1 in T84 cells (Fig. 4B). The KPP22 phage plus DANA was the most efficient treatment to prevent invasion by P.a PAO1 into T84 cells compared to other treatments.

P.a PAO1 alone expressed relatively high adhesion to T84 cells, with approximately 23.7 % of the applied bacteria adhering (Fig. 4A). When *B. bifidum* (1:1 to P.a PAO1) was co-added, adhesion decreased significantly compared to P.a PAO1 alone. Therefore, *B. bifidum* was chosen for competitive exclusion studies. The KPP22 phage was used as a specific natural P.a PAO1-lysing phage. DANA was used as a specific endogenous sialidase inhibitor. Combinations of treatment with DANA and KPP22 phage additively reduced the adhesion of P.a PAO1. The reason why in the KPP22 phage, *B. bifidum*, and DANA treated groups, the adhesion capacity of P.a PAO1 is reduced may be due to the decreased bioavailability of free host sialic acid for P.a PAO1, because sialic acid generally enhances the growth rate of pathogenic bacteria (Li et al., 2017), in line with our results in Section 3.7.

Table 1

Summary of the main results of the sialic acid cross-feeding in pure cultures of the P.a PAO1 and *B. bifidum* bacteria interaction model.

Treatment Groups	Variables (indices of sialic acid cross-feeding)		
	Growth yields of P. a PAO1 (log CFU/ ml)	Levels od sialidase activity in supernatants (absorbance at 595 nm [Ab595])	Sialic acid uptakes (%), namely the concentration of supplemented sialic acid, remained in DMEM after 6 h of treatments.
MP	7.35 ± 0.03 a	0.77 ± 0.06 a	0.01 ± 0.001 a
MPS	9.06 ± 0.24 b	1.47 ± 0.03 b	55.62 ± 2.95 b
MPSD	7.01 ± 0.19 c	0.40 ± 0.05 c	21.25 ± 1.77 c
MPSK	6.62 ± 0.08 d	0.103 ± 0.02 d	4.37 ± 0.94 d
MPSLB	8.08 ± 0.13 e	1.64 ± 0.05 e	82.50 ± 4.52 e
MPSHB	8.78 ± 0.09 b	1.29 ± 0.11 b	49.37 ± 0.05 b

Statistical comparisons between treatment groups (within columns*)* are indicated in lowercase letters. Different letters indicate significantly different results ($p < 0.05$) among treatment groups. The shared letters within each column indicate that there are no significant differences between the groups ($p > 0.05$, $n = 3$). M: DMEM medium; P: *Pseudomonas aeruginosa* reference strain PAO1; S: human sialic acid (Neu5Ac); D: sialidase inhibitor 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (DANA); K: KPP22 phage; LB: live *Bifidobacterium bifidum*; HB: heat-killed B.bifidum; MP: DMEM+PAO1; MPS: DMEM+PAO1+ sialic acid; MPSD: DMEM+ PAO1+ sialic acid+ DANA; MPSK: DMEM+PAO1+ sialic acid+KPP22 phage; MPSLB: DMEM+PAO1 + sialic acid+ live B.bifidum; MPSHB: DMEM+ PAO1+ sialic acid + heat-killed B.bifidum. Average plate PAO1 count measurements (±SEM), expressed in log CFU/ml, for the different treatment groups. In the fourth column, the sialic acid levels in the culture supernatant were measured as described in Section 2.9. Exogenous sialic acid uptake was calculated by sialic acid supplemented in medium minus sialic acid left after 6 h in the culture supernatant. The uptake ratios were calculated from sialic acid uptakes divided by sialic acid supplemented. Note that in the MPSLB Group, the growth yield of PAO1 is reduced, while sialidase activity and sialidase uptake in supernatants are increased. On the other hand, in the MPSHB group, those variables moved in opposite directions*,* implying that live *B. bifidum* utilized most of the available sialic acid in the culture medium.

3.6. IL-6 and IL-8 secretions

We analyzed pooled apical and basolateral media because 0.4-μm membrane inserts and barrier dysfunction allow macromolecules, such as IL-6 and IL-8, to diffuse across the monolayer barrier. Therefore, cell culture medium was harvested from the upper (apical) compartment (1.5 ml for each transwell) and the lower (basolateral) compartment (2.6 ml for each transwell). Individual collections of apical and basolateral media for each culture condition of each treatment were pooled for cytokine analysis. The concentrations of IL-6 and IL-8 in the cell-free supernatants of each experimental group are shown in Figs. 5A and B**.** IL-6 and IL-8 concentrations in non-infected controls were only 19.5 ± 2.1 pg/ml and 7.7 ± 1.3 pg/ml, respectively. Infection of T84 monolayer cells with P.a PAO1 alone caused a strong secretion of IL-6 and IL-8 compared to non-infected control cells. Cells cultured in DANA-supplemented DMEM medium exhibited a lower response to P.a PAO1. In other words, when cells were treated with DMEM + DANA, IL-6 and IL-8 secretions were significantly lower than those found in DMEM alone. The KPP22 phage and DANA additively inhibited IL-6 and IL-8 secretion induced by P.a PAO1 in triple co-cultured T84, THP-1, and Huh7 cells. When monolayers of T84 cells were treated with *B. bifidum* simultaneously with P.a PAO1, IL-6 and IL-8 secretions were markedly lower than those found after infection with P.a PAO1. The levels were approximately halved.

3.7. Assessment of sialic acid cross-feeding

Table 1 presents an overview of the main results of the sialic acid cross-feeding experiment conducted in pure cultures of P.a PAO1 and *B. bifidum* bacteria. To investigate whether P.a PAO1 and *B. bifidum* bacteria participate in a sialic acid cross-feeding interaction and to know their competitiveness within a sialic acid-rich milieu, we assessed their sialic acid utilization traits by inoculating these strains in DMEM medium supplemented with 16 mM sialic acid in the absence and presence of DANA, KPP22 phage, live *B. bifidum*, and heat-killed *B. bifidum* for 6 h. After 6 h, indices of competitiveness in sialic acid cross-feeding were evaluated including P.a PAO1 counts, sialidase activity in supernatants, and sialic acid uptake. The results show that (1) P.a PAO1 alone could grow more with sialic acid, while DANA, the KPP22 phage, and live *B. bifidum* significantly inhibited its growth. In contrast, heat-killed *B. bifidum* could not suppress sialic acid-induced P.a PAO1 counts, and (2) P.a PAO1 and *B. bifidum* bacteria achieve the strongest competitiveness in available siali acid. *B. bifidum* is the strongest competitive, while P.a PAO1 is the weakest competitive, as manifested by the sialic acid uptake ratios. In the DMEM+ P.a PAO1 + sialic acid+ live *B. bifidum* (MPSLB) group, the growth yield of P.a PAO1 is reduced, while the activity of sialidase and the uptake of sialic acid in the supernatants are increased. On the other hand, in the MPSHB group, those variables moved in opposite directions. This suggests that live *B. bifidum* utilized most of the available sialic acid in the culture medium and influenced certain aspects of sialic acid-mediated immunity, as presented in previous sections. Collectively, the results (Table 1) showed that DANA and live *B. bifidum* inhibited the transfer of medium sialic acids to P.a PAO1. Inhibition of sialic acid utilization by P.a PAO1 also reinforced KPP22 phage-mediated killing. Furthermore, in sialic acid rich pure cultures of bacteria and in the absence of host cells, sialic acid cross-feeding also occurs between P.a PAO1 and *B. bifidum*. However, when P.a PAO1 and *B. bifidum* bacteria were added together to the cultures, sialidase activity in the supernatant of bacterial cultures also increased. This indicates how the presence or absence of a host cell can influence the competitive process of sialic acid consumption. Importantly, it shows how *B. bifidum*, through a combination of sialic acid consumption and reduction in P.a PAO1 adhesion, mitigates the pathogenesis of P.a PAO1. In simpler terms, *B. bifidum* bacteria reduce the amount of bioavailable sialic acids for P.a PAO1 bacteria.

4. Discussion

We have established a triple cell co-culture model consisting of T84 cells, THP-1 macrophage-like cells, and Huh7 cells to better mimic the gut-liver axis and explore the role of bacterial sialic acid cross-feeding and phages at the pathogen-host inflammation interface. This mode aims to deepen our understanding of the role of the trinary phage-microbe-host interaction in the cellular processes involved in the defense, absorption, and metabolization of sialic acids within the gut-liver axis, both in healthy and infectiously inflamed states. To resemble *in vivo* conditions at the microhost-pathogen level and to model competition for host cell sialic acid, we chose strains of P.a PAO1 and *B. bifidum* as model pathogens and nonpathogens, respectively. Pathogens, such as P.a PAO1, that acquire sialic acids from their eukaryotic hosts, can evade the immune system. Because P.a PAO1 cannot synthesize sialic acids, it must use sialic acids that originate from host tissue. Through five sets of experiments, we can show that this model reflects several aspects of the situation of quadruple intrinsic crosstalk between pathogenic bacteria, nonpathogenic bacteria, phages, and host cells and thus may contribute to a better understanding of the relationship between P.a PAO1 and human host cells in the context of sialic acids, which both directly and indirectly mediate the host response to infection. Our results show that both P. a PAO1-induced cytokine production and P. a PAO1- arising sialic acids in the supernatant of co-cultured cells are inhibited by the KPP22 phage and *B. bifidum*, acting as bio inhibitors of sialidase activity. This suggests that the reduction in the release of sialic acids from the surface of cells mediated by KPP22 phage and *B. bifidum* plays a key role in this pathological process. They prevent P.a PAO1 from acquiring host sialic acids, enhancing the cell-mediated release of various mediators to combat infection.

We herein examined the inflammatory effects of P.a PAO1 on overall cellular health and function. We observed that P.a PAO1 induces a pro-inflammatory response in cells and disrupts the integrity of the epithelial barrier of T84 cell monolayers when co-cultured with THP-1 and Huh7 cells. The studies presented here show that P.a PAO1 alone increases IL-6 and IL-8 secretion by co-cultured T84, THP-1, and Huh7 cells. Adherence and invasion abilities are involved in stimulating IL-6 and IL-8 secretion. Toxic factors secreted by P.a PAO1 and/or host cells are required to stimulate the secretion of IL-6 and IL-8. In co-cultured T84, THP-1, and Huh7 cells, the KPP22 phage, *B. bifidum*, and DANA exhibited a protective anti-inflammatory response that modulated the pro-inflammatory immune response caused by P.a PAO1 infection.

In the cellular control of sialic acid synthesis at the enzymatic level, we observed that KPP22 phage, DANA, and *B. bifidum* decreased P.a PAO1-induced free sialic acid levels. These findings suggest that P.a PAO1 interacts with T84 cells through exposed sialic acid residues on the surfaces of host cells. Pathogenic bacteria use hydrolytic sialidases to infect mucosal surfaces, which are involved in both physiological and pathological processes. Pathogenic sialidases cleave host sialic acids, both on the extracellular surface of cells and inside cells (Keil et al., 2022).

Our results on sialidase activity in P.a PAO1-infected cell culture supernatants and bidirectional cross-feeding of host sialic acids between P.a PAO1 and *B. bifidum* are consistent with the results of previous studies showing that: 1) sialidases of pathogenic bacteria have a direct toxic effect on host cells and tissues, disrupting defensive and immunometabolic functions, and promoting the expression of inflammatory factors (Kim and Kim, 2023; Guo et al., 2022; Sudhakara et al., 2019); 2) pathogenic bacteria acquire sialic acid from their eukaryotic hosts either through the synthesis of sialidase enzymes, which cleave terminal Neu5Ac residues from host glycoconjugates, or simply by scavenging free Neu5Ac released by other bacterial species (Haines-Menges et al., 2015); 3) at the host-pathogen interface, host cells are poised to detect and respond to microbial sialidase activity with exaggerated inflammatory responses, which can be either beneficial or detrimental to the host depending on the site, stage and magnitude of the infection (Chang et al., 2012); and 4) under both *in vitro* and *in vivo* inflammatory conditions, there is a significant positive correlation between pathogenic bacterial sialidase activity and the concentration of free sialic acid (resulting from the release of sialic acid from host glycans) (Huang et al., 2015).

In our experiments, DANA exhibited moderate inhibitory activity in some cases. This lower efficacy may be due to the use of a concentration of only 100 μM, which was probably insufficient to inhibit the sialidases of both bacteria and host cells. Supporting this, in the sialic acid cross-feeding experiments conducted with pure cultures of bacteria in the absence of host cells, the impact of DANA was clearly visible. However, the somewhat lower DANA efficacy detected in our studies might be due to different assay conditions (substrates, concentrations, sample size, pH, *etc.*) and therefore does not permit direct comparison.

From a molecular perspective of pathogenicity, particularly in colonocytes, the presence of sialic acid on the surface of colonic cells has been shown to increase the adherence of pathogenic bacteria (Sakarya et al., 2010). The basis of this pathogenicity lies in the crucial roles that sialic acids play in cellular communication, pathogen infection, and survival. When attached to host surface receptors and influencing the immune system, pathogenic sialic acids can further promote infection. Therefore, pathogens must be able to inhibit or withstand host cell immune responses, including exploiting host sialic acids and perturbing bacteriostatic cytokines, to survive (Cavalcante et al., 2021).

Overall, using an *in vitro* model of the human digestive system, our results indicate that KPP22 phage and *B. bifidum* are potential biological influencers capable of modulating aberrant sialylation and sialic acid levels, which are pervasive in inflammation (Rodrigues and Macauley, 2018) and generate opposite inflammatory responses to P.a PAO1. Therefore, the beneficial protective role of endogenous bacteria, such as bifidobacteria, and appropriate bacteriophages in modulating the excessive secretion of sialidase from opportunistic pathogenic bacteria in the gut and, subsequently hampered metabolization of host sialic acid derivatives, which are key signaling molecules within and outside the gut, drives many physiological processes.

Regarding the possible mechanisms by which the KPP22 phage and *B. bifidum* can exert biocontrol against inflammation caused by P.a PAO1, healthy bacteria in the human gut, such as *B. bifidum*, are believed to act through various mechanisms. These include regulation of cytokine production, which involves the activation of mononuclear cells to produce both pro- and anti-inflammatory cytokines, enhanced secretory immunoglobulin A (IgA) secretion, production of antibacterial substances, strengthening of the intestinal barrier and competing with pathogenic microorganisms to binding to enterocytes (Ojima et al., 2020; Turroni et al., 2014; Yan and Polk, 2002). On the contrary, *P. aeruginosa* triggers the secretion of pro-inflammatory cytokines (IL-1 and IL-6) by activating the toll-like receptor 5 (TLR5), myeloid differentiation factor 88 (MyD88), and the nuclear factor κ-beta (NF-κB) pathways (Wu et al., 2021). Obviously, the structure, composition, and function of the bacterial cell walls of gram-negative pathogenic bacteria (*e.g.*, P.a PAO1) and bifidobacteria (*e.g.*, *B. bifidum*) differ, and studies that focus on these differences have been extensively reviewed elsewhere (Turroni et al., 2014; Chevalier et al., 2017).

In general, the molecular way in which how KPP22 phage and *B. bifidum* exert their effects can be summarized as follows: They can directly impact excessive inflammation by competitively excluding or lysing P.a PAO1, thus reducing the attachment of this pathogen to host cells, which is associated with inflammation (Jennings et al., 2022; Secor et al., 2020). Alternatively, they can indirectly influence microbe-host interactions. Although the exact mechanism of action is not fully understood, Pf phages have been shown to decrease the secretion of pro-inflammatory cytokines induced by lipopolysaccharide (LPS) derived from *P. aeruginosa* and activated TLR4/NF-κB (Secor et al., 2020).

The schematic presentation of sialic acid transfer from co-cultured cells to P.a PAO1 in the presence of inhibitors DANA, KPP22 phage, and *B. bifidum* can be summarized as follows: (i) P.a PAO1 induces cleavage of host sialic acids, releasing and acquiring them as a nutrient to evade the immune system or as a signaling molecule to interact with its host (Jennings et al., 2022); (ii) KPP22 phage and *B. bifidum* disable utilization of host sialic acid transfer to P.a PAO1; (iii) sialic acid biological blockade with KPP22 phage and *B. bifidum* enhances host-mediated targeting of P.a PAO1; (iv) DANA blocks sialic acid incorporation in P. a PAO1; (v) sialic acid blockade with DANA enhances phage-mediated destruction of P.a PAO1. However, it is still unclear exactly how endogenous KPP22 phage, bifidobacteria, or their soluble factors diminish the release of sialic acid within the complex quadruple interaction that encapsulates them. Although our results and model can provide some insight, much research remains to be done — at both cellular and molecular levels — in this area to extrapolate these *in vitro* results to the *in vivo* or three-dimensional tissue responses of non-transformed cells.

5. Conclusions

This study shows the biological impact of sialidase on the inflammatory response of host eukaryotic cells to the P. a PAO1.This effect is a result of the utilization of free sialic acid secreted by host cells. However, KPP22 phage, *Bifidobacterium bifidum,* and DANA blockade of IL-16 and IL-8 serve to modulate the effector and regulatory arms of the immune system, providing further insight into ways of attenuating pathogen pathogenicity. Thus, aside from the well-documented differences in the structure and composition of the bacterial cell walls of Gram-negative pathogenic bacteria (*e.g.*, *P. aeruginosa*) and bifidobacteria (*e.g.*, *B. bifidum*), two distinct factors seem to be pivotal in modulating the complex pathogen-host interface milieu: (i) the presence of phages and (ii) the ability of bifidobacteria to utilize free sialic acids secreted by host cells substantially influence the extent of bacterial infection. To validate the biological consequences of these effects on the human gut microbiota and health, additional studies are needed that incorporate *ex vivo* studies of human colon samples and host biomarkers, such as cytohistological, molecular, or biochemical measurements.

Animal study statements

Not applicable. This study has been done only in *in vitro* cell culture models.

CRediT authorship contribution statement

Darab Ghadimi: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Regina Fölster-Holst:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Sophia Blömer:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Conceptualization. **Michael Ebsen:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Conceptualization. **Christoph Röcken:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Conceptualization. **Jumpei Uchiyama:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Conceptualization. **Shigenobu Matsuzaki:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Conceptualization. **Wilhelm Bockelmann:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Informed consent statement

Not applicable.

Funding sources

The authors did not receive any type of funding for the present work.

Declaration of competing interest

The authors declare that they have no conflicts of interest with the contents of this article. The authors are not part of any associations or commercial relationships that might represent conflicts of interest in the writing of this study (*e.g.*, pharmaceutical stock ownership, consultancy, advisory board membership, relevant patents, or research funding).

Acknowledgments

The authors would like to thank A. Thoss, S. Krueger, and N. Sommer for helpful technical assistance.

Data availability

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author upon reasonable request.

References

Bell, A., Severi, E., Owen, C.D., Latousakis, D., Juge, N., 2023. Biochemical and structural basis of sialic acid utilization by gut microbes. J. Biol. Chem. 299 (3), 102989.

Bichet, M.C., Chin, W.H., Richards, W., Lin, Y.W., Avellaneda-Franco, L., Hernandez, C. A., et al., 2021. Bacteriophage uptake by mammalian cell layers represents a potential sink that may impact phage therapy. iScience 24 (4), 102287.

Bozzola, T., Scalise, M., Larsson, C.U., Newton-Vesty, M.C., Rovegno, C., Mitra, A., et al., 2022. Sialic acid derivatives inhibit SiaT transporters and delay bacterial growth. ACS Chem. Biol. 17 (7), 1890–1900.

Burzyńska, P., Sobala, Ł.F., Mikołajczyk, K., Jodłowska, M., Jaśkiewicz, E., 2021. Sialic acids as receptors for pathogens. Biomolecules 11 (6), 831.

Calatayud, M., Dezutter, O., Hernandez-Sanabria, E., Hidalgo-Martinez, S., Meysman, F. J.R., Van de Wiele, T., 2019. Development of a host-microbiome model of the small intestine. FASEB J. 33 (3), 3985–3996.

Cavalcante, T., Medeiros, M.M., Mule, S.N., Palmisano, G., Stolf, B.S., 2021. The role of sialic acids in the establishment of infections by pathogens, with special focus on Leishmania. Front. Cell. Infect. Microbiol. 11, 671913.

Chang, Y.C., Uchiyama, S., Varki, A., Nizet, V., 2012. Leukocyte inflammatory responses provoked by pneumococcal sialidase. mBio 3 (1) e00220–11.

Chevalier, S., Bouffartigues, E., Bodilis, J., Maillot, O., Lesouhaitier, O., Feuilloley, M.G. J., Orange, N., Dufour, A., Cornelis, P., 2017. Structure, function and regulation of Pseudomonas aeruginosa porins. FEMS Microbiol. Rev. 41 (5), 698–722.

Didriksen, B.J., Eshleman, E.M., Alenghat, T., 2024. Epithelial regulation of microbiota-immune cell dynamics. Mucosal Immunol. 17 (2), 303–313.

Duan, Y., Llorente, C., Lang, S., Brandl, K., Chu, H., Jiang, L., et al., 2019. Bacteriophage targeting of gut bacterium attenuates alcoholic liver disease. Nature 575 (7783), 505–511.

Etienne-Mesmin, L., Vijay-Kumar, M., Gewirtz, A.T., Chassaing, B., 2016. Hepatocyte toll-like receptor 5 promotes bacterial clearance and protects mice against high-fat diet-induced liver disease. Cell. Mol. Gastroenterol. Hepatol. 2 (5), 584–604.

Fujimoto, K., 2023. Metagenome data-based phage therapy for intestinal bacteria-mediated diseases. Biosci. Microbiota Food Health 42 (1), 8–12.

Ghadimi, D., Helwig, U., Schrezenmeir, J., Heller, K.J., de Vrese, M., 2012. Epigenetic imprinting by commensal probiotics inhibits the IL-23/IL-17 axis in an in vitro model of the intestinal mucosal immune system. J. Leukoc. Biol. 92 (4), 895–911.

Ghadimi, D., Ebsen, M., Röcken, C., Fölster-Holst, R., Groessner-Schreiber, B., Dörfer, C., Bockelmann, W., 2023a. Oral mucosal in vitro cell culture model to study the effect of Fructilactobacillus phage on the interplay between food components and Oral microbiota. Endocr Metab Immune Disord Drug Targets 23 (3), 356–374.

Ghadimi, D., Fölster-Holst, R., Röcken, C., Kaatsch, H.J., Ebsen, M., Tournebize, R., Bockelmann, W., 2023b. Endogenous ethanol-producing Bacteria interference in pathogen-host crosstalk. Endocr Metab Immune Disord Drug Targets 23 (11), 1430–1441.

Ghadimi, D., Fölster-Holst, R., Ebsen, M., Röcken, C., Dörfer, C., Uchiyama, J., Matsuzaki, S., Bockelmann, W., 2024a. Exploring the interplay between nutrients, bacteriophages, and bacterial lipases in host- and bacteria-mediated pathogenesis. Endocr Metab Immune Disord Drug Targets 24 (8), 930–945.

Ghadimi, D., Fölster-Holst, R., Blömer, S., Ebsen, M., Röcken, C., Uchiyama, J., Shigenobu, Matsuzaki S., 2024b. Intricate crosstalk between food allergens, phages, Bacteria, and eukaryotic host cells of the gut- skin axis. Yale J. Biol. Med. 97 (3), 309–324.

Ghosh, S., 2020. Sialic acid and biology of life: An introduction. Sialic acids and Sialoglycoconjugates in the biology of life. Health Dis. 1–61.

Górski, A., Borysowski, J., Międzybrodzki, R., 2021. Bacteriophage interactions with epithelial cells: therapeutic implications. Front. Microbiol. 11, 631161.

Guo, Z., Tuo, H., Tang, N., Liu, F.Y., Ma, S.Q., An, P., et al., 2022. Neuraminidase 1 deficiency attenuates cardiac dysfunction, oxidative stress, fibrosis, inflammatory via AMPK-SIRT3 pathway in diabetic cardiomyopathy mice. Int. J. Biol. Sci. 18 (2), 826–840.

Haines-Menges, B.L., Whitaker, W.B., Lubin, J.B., Boyd, E.F., 2015. Host sialic acids: a delicacy for the pathogen with discerning taste. Microbiol. Spectr. 3.

Heise, T., Langereis, J.D., Rossing, E., de Jonge, M.I., Adema, G.J., Büll, C., Boltje, T.J., 2018. Selective inhibition of sialic acid-based molecular mimicry in Haemophilus influenzae abrogates serum resistance. Cell Chem. Biol. 25 (10), 1279–1285.e8.

Hsu, C.L., Schnabl, B., 2023. The gut-liver axis and gut microbiota in health and liver disease. Nat. Rev. Microbiol. 21 (11), 719–733.

Huang, Y.L., Chassard, C., Hausmann, M., von Itzstein, M., Hennet, T., 2015. Sialic acid catabolism drives intestinal inflammation and microbial dysbiosis in mice. Nat. Commun. 6, 8141.

Jennings, M.P., Day, C.J., Atack, J.M., 2022. How bacteria utilize sialic acid during interactions with the host: snip, snatch, dispatch, match and attach. Microbiology (Reading) 168 (3), 001157.

Jourdian, G.W., Dean, L., Roseman, S., The sialic acids. XI., 1971. A periodate-resorcinol method for the quantitative estimation of free sialic acids and their glycosides. J. Biol. Chem. 246 (2), 430–435, 25.

Juge, N., Tailford, L., Owen, C.D., 2016. Sialidases from gut bacteria: a mini-review. Biochem. Soc. Trans. 44 (1), 166–175. Feb.

Keil, J.M., Rafn, G.R., Turan, I.M., Aljohani, M.A., Sahebjam-Atabaki, R., Sun, X.L., 2022. Sialidase inhibitors with different mechanisms. J. Med. Chem. 65 (20), 13574–13593.

Khan Mirzaei, M., Deng, L., 2022. New technologies for developing phage-based tools to manipulate the human microbiome. Trends Microbiol. 30 (2), 131–142.

Kim, J., Kim, B.S., 2023. Bacterial sialic acid catabolism at the host-microbe Interface. J. Microbiol. 61 (4), 369–377.

Klein, S.G., Serchi, T., Hoffmann, L., Blömeke, B., Gutleb, A.C., 2013. An improved 3D tetraculture system mimicking the cellular organisation at the alveolar barrier to study the potential toxic effects of particles on the lung. Part. Fibre Toxicol. 10, 31.

Leneveu-Jenvrin, C., Madi, A., Bouffartigues, E., Biaggini, K., Feuilloley, M., Chevalier, S., Connil, N., 2013. Cytotoxicity and inflammatory potential of two Pseudomonas mosselii strains isolated from clinical samples of hospitalized patients. BMC Microbiol. 13, 123.

Li, J., McClane, B.A., 2018. NanI Sialidase can support the growth and survival of Clostridium perfringens strain F4969 in the presence of Sialyated host macromolecules (mucin) or Caco-2 cells. Infect. Immun. 86 (2) e00547–17.

Li, J., Sayeed, S., Robertson, S., Chen, J., McClane, B.A., 2011. Sialidases affect the host cell adherence and epsilon toxin-induced cytotoxicity of Clostridium perfringens type D strain CN3718. PLoS Pathog. 7 (12), e1002429.

Li, J., Evans, D.R., Freedman, J.C., McClane, B.A., 2017. NanR regulates *nanI* Sialidase expression by Clostridium perfringens F4969, a human Enteropathogenic strain. Infect. Immun. 85 (9) e00241–17.

Li, X., Gu, N., Huang, T.Y., Zhong, F., Peng, G., 2023a. Pseudomonas aeruginosa: a typical biofilm forming pathogen and an emerging but underestimated pathogen in food processing. Front. Microbiol. 13, 1114199.

Li, J., Pradhan, A., McClane, B.A., 2023b. NanJ is the major Sialidase for Clostridium perfringens type F food poisoning strain 01E809. Infect. Immun. 91 (6), e0005323.

Llorente, C., Schnabl, B., 2016. Fast-track clearance of Bacteria from the liver. Cell Host Microbe 20 (1), 1–2.

Łusiak-Szelachowska, M., Weber-Dąbrowska, B., Jończyk-Matysiak, E., Wojciechowska, R., Górski, A., 2017. Bacteriophages in the gastrointestinal tract and their implications. Gut. Pathog. 9, 44.

McDonald, N.D., Lubin, J.B., Chowdhury, N., Boyd, E.F., 2016. Host-derived sialic acids are an important nutrient source required for optimal bacterial fitness in vivo. mBio 7 (2) e02237–15.

Misson, P., Bruder, E., Cornuault, J.K., De Paepe, M., Nicolas, P., Demarre, G., Lakisic, G., Petit, M.A., Espeli, O., Lecointe, F., 2023. Phage production is blocked in the adherent-invasive Escherichia coli LF82 upon macrophage infection. PLoS Pathog. 19 (2), e1011127.

Muller, P.A., Matheis, F., Mucida, D., 2020. Gut macrophages: key players in intestinal immunity and tissue physiology. Curr. Opin. Immunol. 62, 54–61.

Nakagaki, B.N., Vieira, A.T., Rezende, R.M., David, B.A., Menezes, G.B., 2018. Tissue macrophages as mediators of a healthy relationship with gut commensal microbiota. Cell. Immunol. 330, 16–26.

Ojima, M.N., Gotoh, A., Takada, H., Odamaki, T., Xiao, J.Z., Katoh, T., Katayama, T., 2020. Bifidobacterium bifidum suppresses gut inflammation caused by repeated antibiotic disturbance without recovering gut microbiome diversity in mice. Front. Microbiol. 11, 1349.

Ou, G., Baranov, V., Lundmark, E., Hammarström, S., Hammarström, M.L., 2009. Contribution of intestinal epithelial cells to innate immunity of the human gut–studies on polarized monolayers of colon carcinoma cells. Scand. J. Immunol. 69 (2), 150–161.

Piccinini, F., Tesei, A., Arienti, C., Bevilacqua, A., 2017. Cell counting and viability assessment of 2D and 3D cell cultures: expected reliability of the trypan blue assay. Biol. Proced Online 19, 8.

Podlacha, M., Grabowski, Ł., Kosznik-Kawśnicka, K., Zdrojewska, K., Stasiłojć, M., Węgrzyn, G., Węgrzyn, A., 2021. Interactions of bacteriophages with animal and human organisms-safety issues in the light of phage therapy. Int. J. Mol. Sci. 22 (16), 8937.

Putra, R.D., Lyrawati, D., 2020. Interactions between bacteriophages and eukaryotic cells. Scientifica (Cairo) 2020, 3589316.

Rodrigues, E., Macauley, M.S., 2018. Hypersialylation in Cancer: modulation of inflammation and therapeutic opportunities. Cancers (Basel) 10 (6), 207.

Sakarya, S., Göktürk, C., Oztürk, T., Ertugrul, M.B., 2010. Sialic acid is required for nonspecific adherence of salmonella enterica ssp. enterica serovar Typhi on Caco-2 cells. FEMS Immunol. Med. Microbiol. 58 (3), 330–335.

Schulze, R.J., Schott, M.B., Casey, C.A., Tuma, P.L., McNiven, M.A., 2019 Jul 1. The cell biology of the hepatocyte: a membrane trafficking machine. J. Cell Biol. 218 (7), 2096–2112.

Secor, P.R., Burgener, E.B., Kinnersley, M., Jennings, L.K., Roman-Cruz, V., Popescu, M., et al., 2020. Pf bacteriophage and their impact on Pseudomonas virulence, mammalian immunity, and chronic infections. Front. Immunol. 11, 244.

Servin, A.L., 2004. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol. Rev. 28 (4), 405–440.

Sokolovskaya, O.M., Tan, M.W., Wolan, D.W., 2022. Sialic acid diversity in the human gut: molecular impacts and tools for future discovery. Curr. Opin. Struct. Biol. 75, 102397.

Sudhakara, P., Sellamuthu, I., Aruni, A.W., 2019. Bacterial sialoglycosidases in virulence and pathogenesis. Pathogens 8 (1), 39.

Sutton, T.D.S., Hill, C., 2019. Gut bacteriophage: current understanding and challenges. Front. Endocrinol. (Lausanne) 10, 784.

Turroni, F., Duranti, S., Bottacini, F., Guglielmetti, S., Van Sinderen, D., Ventura, M., 2014. Bifidobacterium bifidum as an example of a specialized human gut commensal. Front. Microbiol. 5, 437.

Uchiyama, J., Suzuki, M., Nishifuji, K., et al., 2016. Analyses of short-term antagonistic evolution of Pseudomonas aeruginosa strain PAO1 and phage KPP22 (Myoviridae family, PB1-like virus genus). Appl. Environ. Microbiol. 82 (15), 4482–4491.

Weidemann, W., Klukas, C., Klein, A., Simm, A., Schreiber, F., Horstkorte, R., 2010. Lessons from GNE-deficient embryonic stem cells: sialic acid biosynthesis is involved in proliferation and gene expression. Glycobiology 20 (1), 107–117.

Wu, T., Gagnon, A., McGourty, K., DosSantos, R., Chanetsa, L., Zhang, B., Bello, D., Kelleher, S.L., 2021. Zinc exposure promotes commensal-to-pathogen transition in Pseudomonas aeruginosa leading to mucosal inflammation and illness in mice. Int. J. Mol. Sci. 22 (24), 13321.

Yan, F., Polk, D.B., 2002. Probiotic bacterium prevents cytokine-induced apoptosis in intestinal epithelial cells. J. Biol. Chem. 277 (52), 50959–50965.

Yu, S., Fan, X., Zheng, S., Lin, L., Liu, J., Pan, Y., Li, C., 2021. The sialidase inhibitor, DANA, reduces Porphyromonas gingivalis pathogenicity and exerts anti-inflammatory effects: An in vitro and in vivo experiment. J. Periodontol. 92 (2), 286–297.