

The eukaryotic host–microbe–phage–nutrition interplay determines the bacterial effectors of supplemental lysine’s health benefits: An experimental cellular senescence model of digestive conditions in older adults

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ABSTRACT

Recently, the consumption of various forms of the nutritive amino acid lysine (e.g., nutraceuticals and dietary supplements) has increased, particularly among the elderly, as it cannot be synthesized by the human body and plays a crucial role in several cellular physiological processes, including intestinal calcium and iron absorption, proteinogenesis, and fatty acid metabolism. However, the biochemical and immunological events that occur within the eukaryotic host-microbe-phage-nutrition interaction, especially when high amounts of lysine are consumed in nutraceutical or dietary supplement form by the elderly due to age-related requirements, as well as the impact on gut bacteria-derived metabolites, have not yet been fully studied. We hypothesized that (1) within an infectious-inflamed digestive milieu, the metabolism of excess extracellular lysine differs between young and senescent digestive cells, and (2) phages within the milieu exhibit alterations in host-microbe metabolite signal profiles. To address this, we examined how gut microbes and phages, key regulators of eukaryotic host physiology, influence the immunometabolism of excess lysine. By combining two models of young and senescent digestive cells, the pathogenic *P. aeruginosa* PAO1, the *Pseudomonas* phage KPP22, and the anti-inflammatory bacterium *Intestinimonas butyriciproducens*, and simulating the pathogenesis of the interactive process of excessive lysine ingestion, we developed a five-way screening approach to explore key cell readouts, including viability, integrity, and function of the epithelial barrier, as well as cellular immunometabolic responses to exogenous lysine supplementation in cultures under infectious inflammation. Our findings are consistent with the possibility that host–microbe–microbe interactions involving lysine utilization and cross-feeding may influence the effects of lysine supplementation. These effects, whether beneficial or detrimental, can depend on the bacterial community, including the presence of pathogenic or potentially beneficial bacteria, interactions with intestinal phages, and the physiological conditions and metabolic requirements of the host and individual microbial taxa.

Abbreviations: ELISA, Enzyme-Linked Immunosorbent Assay; IB, *Intestinimonas butyriciproducens*; Ki67, Antigen Ki-67; PAO1, *Pseudomonas aeruginosa* strain PAO1; PNPP, p-nitrophenylphosphate; Rpm, revolutions per minute; SASP, Senescence-Associated Secretory Phenotype; SA-β-gal, Senescence-Associated β-Galactosidase; TEER, Transepithelial Electrical Resistance; YCFAC, Yeast extract–Casitone–Fatty Acid–Carbohydrate agar; 2K6AC, 2-Keto-6-aminoheptanoate.

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1. Introduction

The reciprocal interplay among eukaryotic host cells, microbes, phages, and nutrients forms a complex and dynamic biological system with significant physiological consequences. Among micronutrients with immunomodulatory properties, lysine is a critical but incompletely studied component of this system (Balamurugan et al., 2024; Gombart et al., 2020; Saeed et al., 2025; Shi et al., 2024).

L-lysine (lysine) is an essential amino acid that cannot be synthesized by the human body and therefore must be obtained from the diet or supplements. Lysine plays an important role in cellular physiological processes, including calcium and iron intestinal absorption, proteinogenesis, and fatty acid metabolism (Tang et al., 2025). Its requirements may differ between older and younger adults, and age-related changes in nutrient absorption and metabolism may further influence lysine availability and utilization (Raffi et al., 2024; Tang et al., 2025). These considerations are particularly relevant in the context of inflammaging, a chronic, low-grade inflammatory state associated with aging (Raffi et al., 2024; Tang et al., 2025).

From an immunonutrition perspective, lysine contributes to immune cell function and metabolic pathways and may influence immune and nutritional status. Its antimicrobial activity has also been associated with potential health benefits (Abdallah et al., 2020; Li et al., 2024; Švedienė et al., 2021). Lysine absorption, digestion, and metabolism are tightly regulated and involve interactions between host cells and intestinal bacteria.

From a nutritional microbiology perspective, lysine serves as a nutrient for both pathogenic and beneficial bacteria. Pathogens can use lysine to support growth and virulence, while commensal gut bacteria can metabolize it into enzymes and metabolites, which contribute to immune function and integrity of the intestinal barrier (Li et al., 2023). (Li et al., 2023). Microbial competition for lysine may therefore influence microbiome composition and potentially limit pathogenic overgrowth. Lysine can also serve as a precursor for vitamins and other biomolecules, further linking its metabolism to systemic health (Abdallah et al., 2020; Li et al., 2024). Its multifunctional pharmacological properties and immunomodulatory effects have contributed to the use of ibuprofen-lysine (400 mg ibuprofen; equivalent to 684 mg of Ibuprofen-DL-lysine per tablet), which is marketed in Germany for the symptomatic treatment of mild to moderate acute pain and fever (Perużyńska et al., 2024).

Although the biological roles of lysine in healthy cells and younger individuals have been extensively studied, its effects within the eukaryotic host–microbe–phage–nutrition interplay in older adults, particularly under infectious or inflammatory conditions, remain poorly understood. Aging is accompanied by microbiome disturbances, increased inflammatory mediators, altered cellular and extracellular properties, loss of barrier integrity, shifts toward pro-inflammatory bacteria, and dysregulated nutrient sensing (Dodig et al., 2019; Menard et al., 2023; Pironi et al., 2023; Schmauck-Medina et al., 2022). These age-related changes may alter lysine metabolism and its physiological consequences, while age-associated gastrointestinal changes also make models based on younger adults less representative of the older gut (Menard et al., 2023).

In addition to dietary supply, lysine is produced by most bacteria from aspartate through the diaminopimelate pathway (Rodionov et al., 2003; Tang et al., 2025). Lysine deficiency or excess can affect protein synthesis, conversion to alternative biomolecules, and microbial degradation, generating metabolites that may influence immune homeostasis and immune cell responses (Driuchina et al., 2023; Tang et al., 2025). Lysine metabolism is tissue-specific and has been linked to several diseases, as evidenced by human metabolomics datasets, although the precise functions of many lysine-derived metabolites remain poorly understood (Driuchina et al., 2023). In older individuals, altered lysine availability may therefore influence gut microbial

activity, immune signaling, and phage–bacteria dynamics through interactions with lipid and glucose metabolism (Li et al., 2025; Mancinetti et al., 2024; Tan et al., 2023; Wang et al., 2024; Wunderle et al., 2024). Understanding these interactions is particularly relevant because older adults are more susceptible to infectious and inflammatory conditions (Mancinetti et al., 2024).

Another key but underexplored biological player in the lysine–host–microbe interaction is the bacteriophage. In the gastrointestinal tract, phages modulate bacterial populations and can thereby indirectly affect host immunity and metabolism, with both beneficial and detrimental consequences. For instance, temperate phages targeting multidrug-resistant *Klebsiella* have demonstrated therapeutic potential, whereas other phages may contribute to inflammation, as observed in inflammatory bowel disease (IBD) (Javaudin et al., 2021; Majzoub et al., 2024; Saeed et al., 2025; Shi et al., 2024; Tisza et al., 2025; Wahida et al., 2021). However, the mechanisms by which phages influence bacterial responses to perturbations such as antibiotics, dietary changes, or colonization by new bacteria remain unclear, particularly in older adults (Zuppi et al., 2024).

During infectious inflammation, crosstalk among absorptive enterocytes, resident intestinal macrophages, and hepatocytes contributes to the regulation of immune responses, nutrient processing, and intestinal physiology (Selvakumar et al., 2024). Lysine, as an immune nutrient and signaling molecule, can influence pathways involved in inflammation and host defense when administered enterally or parenterally (Li et al., 2025; Wang et al., 2024; Wunderle et al., 2024). However, how lysine availability interacts with phages and microbiota under these conditions, particularly in older adults, remains poorly understood.

Given these knowledge gaps, the present study aimed to: (1) evaluate the effects of nutritive lysine from nutraceutical or supplement sources on the eukaryotic host–microbe–phage–nutrition interplay under an infectious-inflammatory condition simulating the older-adult gastrointestinal tract; and (2) investigate whether lysine supplementation modulates longitudinal phage–bacteria dynamics in the elderly gut, or whether phage activity, in turn, shapes the effects of lysine on microbial and host physiology. To address these questions, we developed a high-throughput five-way screening platform integrating five components: (i) senescent eukaryotic cell models comprising co-cultured T84 enterocytes and Huh7 hepatocytes; (ii) the opportunistic pathogen *Pseudomonas aeruginosa* PAO1; (iii) its corresponding phage KPP22; (iv) the anti-inflammatory gut bacterium *Intestinimonas butyriciproducens*; and (v) nutritive lysine.

To mimic aged intestinal physiology relevant to dietary effects (Justice et al., 2024; Maduro et al., 2021), we used senescent cell models. Compared with young cells obtained at early passages (6–8), senescent cells were derived from higher passage numbers (34–37), consistent with previous reports using early-passage cells (≤ 9) as young controls and cells at ≥ 30 passages as a senescent model (Park et al., 2020; Yang et al., 2011). To more comprehensively characterize the immunometabolic responses of the experimental model, passage number was used as a criterion for defining cellular age, complemented by measurements of senescence-associated β -galactosidase (SA- β -gal) activity and p21 protein expression as biomarkers of cellular senescence (Wang et al., 2024; Yan et al., 2024). Key readouts included inflammatory mediator release, cell viability, metabolic biomarkers, and lysine competition between eukaryotic host cells and microbes. This *in vitro* model was selected to better represent the infectious-inflammatory intestinal milieu associated with aging than models based exclusively on younger cell systems. In this model, T84 cells represent absorptive and defensive intestinal epithelial cells (Okumura and Takeda, 2017), whereas Huh7 cells retain several hepatocyte-like metabolic functions relevant to nutrient and lipid metabolism (Luis et al., 2025; Ma et al., 2025). Their co-culture enables investigation of interactions between intestinal epithelial and hepatic cells under normal conditions and in response to altered nutrient availability during infectious inflammation. Intestinal epithelial cells constitute the first line of defense against

pathogenic bacteria and toxic molecules, and their barrier function is critical for intestinal homeostasis (Blyth et al., 2020). Hepatocytes, in turn, possess enzymes involved in the metabolism and clearance of drugs and their metabolites and contribute to innate immune responses and LPS signaling (Ma et al., 2025).

2. Material and methods

2.1. Reagents and kits

The following materials were purchased from Thermo fisher (Schwerte, Germany): Sterile 6 well plate 24 mm Transwell inserts with 0.4 μm Pore Polyester Membrane Insert (Cat. #3450), 75 cm^2 U-Shaped Canted Neck Cell Culture Flask with Vent Cap (Cat. #430641U), Gibco standard Dulbecco's Modified Eagle's Medium (DMEM, Cat. #11960044), Gibco custom lysine-free DMEM (Cat. #12817552 = A14431), Gibco custom lysine-free DMEM (Gibco, Cat. #12817552 = A1443101), 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), Corning Small Cell Scraper (Cat #3010), TrypLE Express (Cat. #12605010), Anaerobic Reinforced Clostridium Medium (RCM, Difco CM0149), and Yeast Casitone Fatty Acids Agar with Carbohydrates (YCFAC) (Cat # NC1383461). Endotoxin-Free Ultra-Pure Water, L-Lysin, $\geq 98\%$, cell culture grade (Cat. #L5501), NutriSelect Plus *Pseudomonas* Agar (Cat. #17208), and p-nitrophenol phosphate (pNPP) assay Kit (Cat. #MAK447) were purchased from Sigma Chemical (Sigma-Aldrich, Taufkirchen, Germany). All other materials (plasticware) and 0.22 μm membrane syringe filter used here were pyrogen- and endotoxins-free and were purchased from Sarstedt (Nümbrecht, Germany). Human ELISA kits (IL-6, IL-8, and IL-10) were obtained from abcam (Berlin, Germany) each with a sensitivity of 1.4 pg/ml, 1.8 pg/ml, and 1.4 pg/ml, respectively.

2.2. Bacterial strains and growth condition

The intestinal bacterium *Intestinimonas butyriciproducens* DSM 26243 (hereafter referred to as IB), obtained from the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures GmbH, was routinely cultured in anaerobic Reinforced Clostridium Medium (RCM, Difco) in 120-ml serum bottles sealed with butyl rubber stoppers. Cultures were incubated at 37°C in an A45 anaerobic workstation (Don Whitley Scientific) under an atmospheric condition of N₂/CO₂/H₂ (80:10:10). IB was selected as a relevant model organism because of its ability to produce butyrate, an important energy source for colonocytes, from both sugars and lysine (Bui et al., 2015; Rampanelli et al., 2025).

As a model organism for Gram-negative opportunistic human pathogens, the *Pseudomonas aeruginosa* ATCC 15692 reference strain PAO1 (hereafter referred to as PAO1) was used for this study because *P. aeruginosa* is one of the best-studied bacterial opportunistic pathogens (Martínez., 2014).

Preparations of overnight cultures were performed as previously described in detail (Uchiyama et al., 2016). Briefly, overnight cultures of the PAO1 strain were grown in Luria broth (LB) to the late logarithmic phase of growth, washed twice in Dulbecco's phosphate-buffered saline (DPBS), and resuspended at a final concentration of 1×10^9 colony-forming units (CFU/ml). Aliquots of the bacteria were then transferred into sterile cryovials and frozen with an equal volume of sterile 15% glycerol. For cell culture experiments, bacteria were thawed, centrifuged (8000 rpm for 5 min), washed twice in DPBS (pH 7.4), and resuspended in serum-free cell culture medium to a final concentration of 2×10^8 CFU/ml. The bacterial suspension was then used for cell culture experiments. Colony counts were routinely confirmed by serial dilution and plating on *Pseudomonas* isolation agar.

2.3. Preparation of *Pseudomonas* phage KPP22

Routine propagation, enumeration, and maintenance of KPP22 phages were performed essentially as previously described in detail (Uchiyama et al., 2016). Isolation, identification, and characterization of this phage have been previously carried out (Uchiyama et al., 2016). Purification, endotoxin removal, and storage of the concentrated pure phage KPP22 solution were carried out essentially as previously described in detail (Trend et al., 2018). Briefly, filter-sterilized high-titer phage preparations were purified using a single-use disposable CIMmultis QA 1 ml column (BIA separations, Sartorius) in combination with a Watson Marlow 510 U peristaltic pump. Phage was diluted 1:10 in 20 mM Tris-HCl (pH 7.5) loading buffer made in endotoxin-free ultrapure water, and 10 ml was passed through the column. The column was washed with 20 volumes of loading buffer, and then 20 mM Tris-HCl supplemented with successive increases in saline (250 mM – 1.2 M NaCl) in 2 ml volumes, which were collected as separate fractions. The phage titer in each fraction was tested as previously described (Uchiyama et al., 2016) and the fraction with the highest phage titer was selected for further purification by endotoxin depletion. Endotoxin was removed from the purified phage preparation using the EndoTrap HD 5/1 kit (Hyglos) according to the manufacturer's protocol and as previously described in detail (Trend et al., 2018). In brief, the phage preparation was diluted 1:5 in 20 mM Tris-HCl containing 450 mM NaCl and 0.125 mM CaCl₂, passing the phage solution three times through the column. Endotoxin concentrations in phage preparations after purification were evaluated using an endpoint limulus amebocyte lysate (LAL) chromogenic endotoxin assay kit (VWR, Cat. #GENSL00350C) according to the manufacturer's protocol and the DR 2800 spectrophotometer at 545 nm. Finally, the concentrated pure phage solution (1.3×10^9 PFU / ml) was stored in pyrogen-free vials (Lonza) at 4 °C for further experiments.

2.4. Step-by-step method details of cell culture and co-culture setup

2.4.1. Cell lines, routine cell maintenance, and preparation of single-cell suspension

The human colon carcinoma cell line (T84, Cat. #CCL-248) was from the American Type Culture Collection (ATCC, LGC, Wesel, Germany). The human hepatocarcinoma cell line (Huh7, CLS, Cat. #330156) was from the Japanese Collection of Research Biosources Cell Bank (Tebubio GmbH, Offenbach, Germany).

For routine cell maintenance, cells were defrosted, cultured, and maintained in 75 cm^2 flasks at 37°C with 90% humidity and 5% CO₂ in a Thermo Scientific Heracell 150i incubator with variable oxygen control. We changed the culture medium every other day and sub cultured them two times a week with a cell density of 7.5×10^5 cells/cm² for the T84 cell line and 6.5×10^5 cells/cm² for the Huh7 cell line. The T84 and Huh7 cell lines were maintained in standard maintenance DMEM medium supplemented with 5% FBS and 1% MEM non-essential amino acids to support cell growth. Both supplements were filtered with a 0.2 μm filter. Cells were routinely split at ~ 80% confluence for experiments. Cells were trypsinized with 0.25% trypsin. To minimize phenotypic changes in the cell lines resulting from extended passaging, fresh vials of the corresponding cell line were thawed and cultured before each experiment. Cell stocks stored in Gibco recovery cell culture freezing medium in cryovials in liquid-phase nitrogen were rapidly thawed and mixed with fresh DMEM medium containing 5% FBS. Cells were routinely examined microscopically each day for signs of healthy growth, including the absence of contamination, low levels of cell death, and normal growth characteristics. A 50 μl sample of cultures was taken and plated on LB agar as a basic test for bacterial contamination. All cultures were also routinely monitored for potential mycoplasma contamination using the MycoStrip Mycoplasma Detection Kit (InvivoGen, Toulouse, France) according to the manufacturer's protocols.

To model cellular aging-associated conditions, T84 and Huh7 cells at

early passages (P4–P6) were used as young cells, whereas cells at high passages (P34–P37) were used as senescent cells, consistent with previously reported passage-based criteria (Park et al., 2020; Yang et al., 2011). To further characterize the senescent phenotype, SA- β -gal activity and p21 protein expression were quantified in separately collected T84 and Huh7 cell lysates following co-culture and treatment, using commercial assay kits as described in Section 2.6.

Before seeding for any assay, cell counts were performed, and cells were only used if viability was $> 85\%$, because high cell viability is a prerequisite for intestinal functions and it is an essential facet of microbial bioprocessing in biological barriers and one of the most important indicators of intestinal function from the oxygen and nutrients perspective. Cells were maintained in a humidified incubator at 37°C under an atmosphere of $5\% \text{ CO}_2$ and $1\% \text{ oxygen}$ (hypoxia) using a Thermo Scientific Heracell 150i incubator with variable oxygen control. This hypoxic atmosphere mimics physiological conditions and provide an environment relevant to intestinal epithelial cells (IECs) and the commensal microbiota, which form a selective semipermeable barrier that allows IECs to transport essential nutrients while maintaining the separation of luminal contents from internal compartments (Muenchau et al., 2019).

2.4.2. Cell co-culture assembly procedures

Cell seeding, co-culture setup, and treatment were performed in four sequential steps, as briefly depicted in Fig. 1. (i) Single-cell suspensions of young and senescent cells from each of the two cell lines were prepared separately. (ii) Young and senescent enterocytes (T84 cells) were seeded at 2×10^6 cells per insert in two separate Transwell inserts and incubated for 2 days. (iii) At the same time, young and senescent hepatocytes (Huh7 cells) were cultured at the bottom of the wells in two separate 6-well plates. (iv) On the third day, to establish the co-cultures, inserts containing T84 cells were transferred to 6-well plates containing Huh7 cells, thereby positioning the T84 cells in the apical compartment and the Huh7 cells in the basolateral compartment without direct cell–cell contact. The co-cultures were then incubated for 24 h in a humidified incubator at 37°C under an atmosphere of $5\% \text{ CO}_2$ and 1%

oxygen (hypoxia) to allow the exchange of soluble factors between the two compartments. After 24 h of equilibration, the co-cultured cells were used for the subsequent experiments.

To develop an experimental model of infection-induced pathological inflammation that mimic the *in vivo* milieu of the aging intestinal tract (Saavedra et al., 2023), a PAO1 infection model was established using co-cultured T84 and Huh7 cells to evaluate the ability of excess lysine, the KPP22 phage, and the IB bacterium to modulate the resulting pathological inflammatory responses, as described previously (Ghadimi et al., 2024), with the following modifications: Seeding cells at high density and under hypoxic conditions has two important advantages: 1) at a density of 2×10^6 cells per insert, the high seeding density allows confluency to be reached within 48 h (Ferruzza et al., 1997); and 2) culturing T84 cells under hypoxic conditions accelerates the establishment of barrier function compared with normoxic conditions, enabling cells to reach a polarized state within 2 days after seeding. A trans-epithelial electrical resistance (TEER) of $> 330 \Omega \times \text{cm}^2$ indicates complete barrier formation (Muenchau et al., 2019). Details of inoculation of the co-cultures and the *in vitro* modeling of human leaky gut are described in the following section.

2.4.3. Inoculation on T84/ Huh7 co-cultures

Before exposure to treatments, media in the transwell inserts were aspirated. The T84 cells were then exposed to the following treatments: (1) isotonic saline, (2) PAO1 bacterium, (3) KPP22 phage, and (4) IB bacterium. To better mimic *in vivo* conditions, the concentrations of the treatments tested were equivalent to the maximum concentrations *in vivo* reported in humans or used for the *in vivo* study, that is, $150\text{--}250 \mu\text{mol/l}$ for L-lysine (Pundir et al., 2021) ($500 \mu\text{M}$ – (Jing et al., 2025)), a 1:1 ratio of phage to epithelial cell ($\sim 1:1$ ratio – (Shkoporov and Hill., 2019)), and 5.6×10^6 CFU per insert for PAO1 (1×10^7 CFU – (Dar et al., 2022)). For this, the epithelial T84 cell monolayers in the upper chamber were first inoculated with either PAO1 or IB ($28 \mu\text{l}$ of live bacterial suspension in antibiotic-free cell culture medium described above) at a final concentration of 5.6×10^6 CFU/insert. In this case, the ratio of bacteria to cells is approximately 1.61:1, which falls within the range of valid optimal ratios of bacteria to human cells in the adult body (Murali et al., 2024). Subsequently, $8 \mu\text{l}$ of KPP22 phage suspension was then slowly added to the respective inserts with a pyrogen-free syringe (21-gauge needle) at a final concentration of 1.4×10^7 PFU/insert. In this case, the ratio of phages to bacteria is about 2:1, which falls within the range of phage-to- bacteria ratios in the human gut (Marbouty et al., 2021). However, to ensure the presence of a sufficient number of phages in the culture medium and consider virion loss or low viral amplification per infected bacterium (reviewed by Sausset et al., 2020), we used slightly higher ratios.

From this point onward, for co-culture experiments, standard DMEM medium (Gibco, Cat. #11960044), which was used only for routine cell maintenance and expansion and contains $790 \mu\text{M}$ lysine, was replaced with custom lysine-free DMEM supplemented with $510 \mu\text{M}$ L-lysine and $5\% \text{ FBS}$. The rationale for this lysine treatment strategy, its nutritional relevance, and its immunometabolic outcomes are explained as follows. The purpose of this lysine treatment strategy was to elucidate the effects of excess nutritional lysine supplementation and its potential to enhance pathogenic catabolite production in an *in vitro* model of human leaky gut, particularly under conditions of cellular senescence, during which cellular metabolism undergoes profound reprogramming. To model increased extracellular lysine availability, L-lysine powder was added to custom lysine-free DMEM to achieve a final concentration of $510 \mu\text{M}$. This concentration was selected to provide a controlled experimental exposure above the physiological human plasma range while remaining within the concentration range used in previous *in vitro* studies. In healthy humans, reported serum L-lysine concentrations are generally approximately $150\text{--}250 \mu\text{M}$, although they may vary depending on nutritional and metabolic status (Pundir et al., 2021). Notably, $510 \mu\text{M}$ lysine has been used as a standard extracellular lysine concentration in

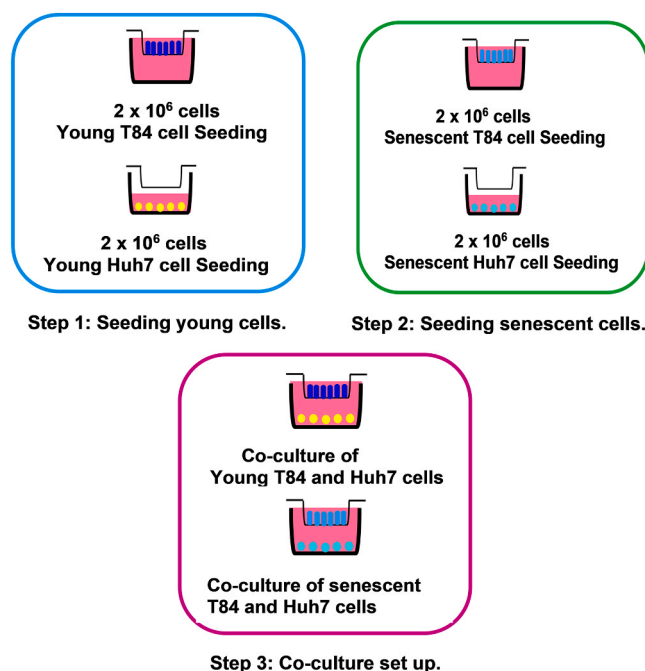


Fig. 1. Schematic illustration of the three-step experimental workflow using young and senescent intestinal T84–hepatocyte Huh7 co-cultures to evaluate immunometabolic responses to excess lysine along the gut–liver axis under infectious-inflammatory conditions.

previous cell culture experiments, providing experimental precedent for the use of approximately 510 μM lysine *in vitro* (Jing et al., 2025). Human supplementation studies have also investigated lysine supplementation in elderly individuals, demonstrating the relevance of increased dietary lysine availability in this population, although these studies do not establish 510 μM as a typical circulating plasma concentration following dietary supplementation (Flakoll et al., 2004). Experimental lysine loading can produce substantially higher circulating concentrations under specific pharmacological conditions, further demonstrating that plasma lysine levels can vary considerably according to the experimental or physiological context (Lukkarinen et al., 2000). In addition, previous experimental studies have demonstrated biological effects of lysine-containing compounds in models of gastrointestinal mucosal injury and intestinal barrier dysfunction (Cimini et al., 2015; d'Angelo et al., 2023), supporting the investigation of lysine-related effects in gastrointestinal epithelial models. Therefore, the 510 μM concentration used in the present study was intended to model increased extracellular lysine availability under controlled *in vitro* conditions rather than to reproduce a specific steady-state plasma concentration in elderly individuals. All experimental treatments were performed using custom lysine-free DMEM supplemented with defined concentrations of free L-lysine (free base), allowing precise control of extracellular lysine availability. Importantly, the lysine used in this study was L-lysine (see Section 2.1, Reagents and kits), supplied as the free amino acid (free base) rather than as a hydrochloride or acetate salt. Therefore, no additional counterions, such as chloride or acetate, were introduced into the culture medium. The free amino acid form was selected because it is commonly used in nutrient supplementation, nutrient signaling, cellular metabolism, and amino acid metabolism studies, while avoiding potential effects associated with the addition of alternative counterions.

All control cell wells received media alone. The upper and lower compartments of the cell culture inserts were then filled with 1.5 and 2.6 ml of culture medium, respectively, to achieve the final volume of medium. An uninoculated control, consisting of culture medium alone, was performed in the absence of bacteria or phage. Two additional inserts and wells were always included solely for counting cells and calculating the bacteria-to-cell ratio. Before adding bacteria and phages, the cells in these additional inserts and wells were counted to calculate the bacteria-to-cell and phage-to-cell ratios. The co-cultures were then incubated for 6 h at 37 °C in a 5% CO₂ and 1% oxygen (hypoxia) atmosphere. After 6 h of exposure incubation, the following steps were performed in order. (i) Samples (50 μl) of the apical medium were first taken and plated on LB-agar and YCFAC agar plates to count the number of PAO1 and IB CFU/ml present in the apical medium. (ii) The cell supernatants of the replicate upper and lower compartments were then collected, pooled, and centrifuged at 400 $\times$ g for 10 min at 4 °C to remove cells and debris. The cell-free supernatants obtained were then filter-sterilized (0.2 μm) and stored at -80 °C until further analysis, and batch cytokine analyses were performed. (iii) Following the removal of the supernatant from the transwell inserts, cells were washed with HBSS to remove remaining bacteria, and the TEER value was measured (Section 2.7) to test that the membrane was not broken during the experiment due to bacteria or the experimental process. (iv) Subsequently, the T84 monolayers and Huh7 cells were scraped using mini sterile cell scrapers, spun at 500 $\times$ g for 5 min, and single cell suspensions were divided into two fractions: one for analysis of cell count and viability evaluation and one for preparation of cell lysate supernatants and analysis of markers of cellular metabolic health as described in the section on cell viability assays and measurement of markers of cellular metabolic health below. Cell lysate supernatant samples were divided into two fractions: one for analysis of markers of cellular metabolic health and one backup.

2.5. Analysis of cell viability

To determine the number and viability of cells in the co-cultures after

6 h of treatment, cells were collected separately using sterile, disposable mini cell scrapers. For each treatment, cells from two transwell inserts containing T84 cells (apical compartment) and two wells containing Huh7 cells (basolateral compartment) were collected separately for each cell population. An aliquot of each cell suspension was used for the cell viability assay, while the remaining cell suspension was used to prepare cell lysates for the assessment of other parameters, including SA- β -gal activity and p21 protein expression. Separate lysates were therefore prepared for the T84 and Huh7 cell populations. Subsequently, an aliquot of each cell suspension was mixed with 0.4% (w/v) trypan blue solution, and the number of viable cells was determined using a hemocytometer. Cells that excluded the dye were considered viable, whereas cells that did not exclude the dye were considered non-viable. Each sample was counted three times using a hemocytometer.

2.6. Measurement of senescence-associated markers

Following the 6-h co-culture/treatment, SA- β -gal activity and p21 protein expression were assessed using aliquots of the separately collected T84 and Huh7 cell lysates prepared as described above. SA- β -gal activity was measured using a commercial assay kit (RayBiotech, Cat. No. MA-BGAL), according to the manufacturer's instructions. Following incubation, the reaction mixtures were transferred to semi-micro disposable cuvettes, and absorbance was measured at 420 nm using a PerkinElmer UV/VIS Spectrophotometer 550 SE. SA- β -gal activity (OD at 420 nm) was significantly higher in senescent T84 cells (late-passage cells, P34–P37; 0.44 ± 0.07) than in young T84 cells (early-passage cells; 0.15 ± 0.02 , $p = 0.0033$). Similarly, senescent Huh7 cells showed significantly higher SA- β -gal activity (0.55 ± 0.06) compared with young Huh7 cells (0.18 ± 0.09 , $p = 0.0051$). Data are presented as mean $\pm$ SEM from three biological replicates ($n = 3$), with duplicate transwell inserts/wells for each treatment condition. Statistical analysis was performed using two-way ANOVA followed by Tukey's post hoc test. These results provided additional phenotypic evidence supporting the senescence-associated phenotype of the high-passage T84 and Huh7 cells. Additionally, total cellular p21 protein expression, a complementary marker associated with the stable cell-cycle arrest characteristic of cellular senescence (Wang et al., 2024), was measured in the cell lysates using a commercial Human Total p21/CIP1/CDKN1A ELISA Kit (R&D Systems, Cat. #DYC1047-2), according to the manufacturer's instructions. Absorbance was measured at 450 nm using a PerkinElmer UV/VIS Spectrophotometer 550 SE. p21 protein expression was significantly increased in senescent cells (late-passage cells, P34–P37) compared with young cells (early-passage cells). In T84 cells, p21 protein levels increased from 171 ± 22 pg/ml in young cells to 417 ± 58 pg/ml in senescent cells ($p = 0.0049$). Similarly, p21 protein expression increased from 166 ± 19 pg/ml in young Huh7 cells to 481 ± 51 pg/ml in senescent Huh7 cells ($p = 0.0053$). Data are presented as mean $\pm$ SEM from three biological replicates ($n = 3$), with duplicate transwell inserts/wells for each treatment condition. Statistical analysis was performed using two-way ANOVA followed by Tukey's post hoc test. Overall, the increased SA- β -gal activity, together with the approximately 2.5–3-fold increase in p21 protein expression, provides additional phenotypic evidence supporting the senescent phenotype of the high-passage cells. These findings further substantiate the characterization of cellular senescence beyond the use of passage number alone.

2.7. Epithelial integrity and barrier function assessments

After 6 h of incubation, to evaluate the possible effects of the treatment conditions indicated above on the epithelial integrity and barrier function of intestinal T84 monolayers, TEER assays were performed based on the method described previously (Devriese et al., 2017) using a chopstick-type probe (STX-2) connected to a Millicell ERS-2 epithelial Ohm voltmeter (Millicell ERS-2, Merck Millipore, Darmstadt, Germany),

according to the manufacturer's instructions. Before measurements, cells were equilibrated at room temperature for 10 min. Then, the chopsticks were carefully inserted into the Transwell insert to avoid scratching the cell monolayer. Measurements were recorded after the signal stabilized. The reported TEER (T_E) was determined using the following formula: $TEER = (T_M - T_B) \times \text{area}$

The measured TEER from a T84 monolayer (T_M) was subtracted from the measured TEER from a blank Transwell insert without cells (T_B). This quantity was then multiplied by the surface area of the Transwell insert (4.67 cm^2) to determine TEER. All TEER measurements in this study are reported as $\Omega \times \text{cm}^2$. Before any assay, only cell monolayers with a TEER value above $300 \Omega \times \text{cm}^2$ were used.

2.8. Alkaline phosphatase (ALP) activity quantification

Enzyme activity of intestinal alkaline phosphatase (IAP) in the T84 cell lysate supernatants after 6 h of treatment of co-cultured cells was measured using a p-nitrophenol phosphate assay kit (Sigma) according to the manufacturer's instructions. Briefly, supernatants were collected and centrifuged at 3600 rpm at 4°C for 15 min. Subsequently, 50 μl of undiluted lysate supernatants were incubated at 37°C with 100 μl of 1 mM pNPP dissolved in 100 mM diethanolamine and 0.5 mM MgCl_2 , pH 10.5, until enzymatic reaction occurred. Absorbance was read at 405 nm using a Tecan GENios FL fluorescence plate reader.

2.9. Measurement of cytokines as biomarkers of cellular senescence

The protein secretion levels of the proinflammatory cytokines interleukin-6 (IL-6) and interleukin-8 (IL-8), as the most cited senescence-associated markers (Ayoub et al., 2026; Yue et al., 2022), and the anti-inflammatory interleukin-10 (IL-10) in medium were measured using enzyme-linked immunosorbent assays (ELISA) (all from Abcam, Berlin, Germany). The tests were performed according to the manufacturer's instructions. For each assay, three wells (technical replicates) per supernatant sample were analyzed.

2.10. Measurement of markers of cellular metabolic health

Young and senescent T84 and Huh7 cells were cultured in 6-well transwell inserts and treated as described above. After treatment with the bacterium PAO1, its KPP22 phage, the IB bacterium, or 510 μM Lys for 6 h, the cell-free supernatants were first aspirated. The cells were then quickly washed with DPBS and collected separately using disposable, sterile mini cell scrapers. After centrifugation ($3000 \times g$, 15 min), cell lysate supernatants were collected for intracellular determination of HDL-C and triglycerides using commercial kits from Thermo Fisher Scientific according to the manufacturer's instructions, except that 50 μl of supernatant was used instead of plasma, and measurements were taken photometrically at 546 nm using a biochemistry analyzer (KoneLab 20i, Thermo Fisher Scientific, USA) according to the standard procedure. The glucose content in the cell-free supernatants was also determined enzymatically using the KoneLab 20i analyzer. Triglycerides and HDL-C were measured only in hepatic Huh7 cells because the liver is a central organ in lipogenesis, gluconeogenesis, cholesterol metabolism, and detoxification (Ma et al., 2025).

2.11. Assessing lysine utilization and competition for excessively available lysine

To assess competition and determine which condition uses more lysine and how rapidly, we collected supernatants from infected and uninfected host cells and measured lysine levels in the supernatants, comparing lysine depletion rates in the presence and absence of the pathogen (PAO1), its phage, and the beneficial bacterium (IB). Briefly, the concentration of lysine remaining in the cell supernatants after 6 h of incubation was measured using an enzyme-based kit according to the

manufacturer's protocol (Fluorometric Lysine Assay Kit MAK42; Sigma-Aldrich) with a Tecan GENios Fluorescence plate reader. Lysine consumption was calculated by subtracting the final lysine concentration in the cell supernatants after 6 h of incubation from the initial lysine concentration in the supplemented DMEM.

2.12. Measurements of lysine-derived metabolites and glutamate formation

For studies of lysine-derived metabolites, an aliquot of cell-free supernatants processed for lysine analysis was used, and the concentrations of lysine-derived metabolites in the supernatants were determined in the supernatant using enzyme-based kits according to the protocols provided by the manufacturers: Homocitrulline ELISA Kit (Cat. #ab242292, Abcam, Berlin, Germany), Spermine ELISA Kit (Cat. #ABIN510549, antibodies-online GmbH, Aachen, Germany), and Glutamate Assay Kit (Cat. #MAK330; Sigma-Aldrich, Germany). 2-Keto-6-aminoheptanoate (2K6AC) is a metabolic intermediate in lysine utilization by beneficial bacteria and is not typically found in pathogens or host cells. Measuring glutamate formation and quantifying glutamate present in the culture medium is a simple, indirect but reliable proxy for 2K6AC presence and utilization (Kochi et al., 1986).

2.13. Inhibition of pathogen adherence to young and senescent intestinal T84 cell monolayers under control and treated conditions

The inhibitory effect of the IB strain on the adhesion of the pathogenic bacterium PAO1 to young and senescent T84 cells under the treatment conditions was evaluated using three different procedures: competition, exclusion, and displacement mechanisms, as previously described (Ghadimi et al., 2025). The T84 cells were cultured as described above in Section 2.4.1. The pellets of IB and PAO1 bacteria were resuspended in DMEM without antibiotics at a concentration of approximately 10^8 CFU/ml .

In the competition assay, equal volumes of IB bacterial suspension (100 μl , $5.6 \times 10^6 \text{ CFU}$ per insert) and PAO1 bacterial suspension (100 μl , $5.6 \times 10^6 \text{ CFU}$ per insert) were mixed and added to the inserts containing T84 monolayer cells followed by incubation for 1 h. In the exclusion assay, the inserts were first preincubated with IB suspension for 1 h, and then the PAO1 bacterial suspension was added to each insert. The cell cultures in the presence of bacteria were incubated for an additional 1 h. In the displacement assay, the inserts were first preincubated with PAO1 bacterial suspension for 1 h, and then, IB suspension was added to each insert. The cell cultures in the presence of bacteria were incubated for an additional 1 h. Subsequently, non-adherent bacteria and their metabolic secretion products were removed by washing, twice with DPBS, and the bound bacteria were released and lysed with 0.7 ml TrypLE Express for 10 min. When all the cells had detached, the reaction was terminated by adding 0.2 ml DMEM. The suspension containing the released bacteria was serially diluted (10-fold), spread on Pseudomonas Agar, and incubated 37°C for 24 h. The number of PAO1 bacterial colonies was counted. Cells treated with PAO1 bacteria only were used as a control. The adhesion rate was calculated with the following formula: Adhesion ability (%) = $(A_i/A_f) \times 100$, where A_i was the initial bacterial counts and A_f was the bacterial adhesion count. The percentage of adhesion inhibition was calculated as the difference between the adhesion of the pathogen in the absence and presence of the IB strain under the tested treatment conditions. Experiments were carried out in triplicate.

2.14. Statistical analysis

Data were analyzed using STATGRAPHICS Plus statistical software XIV, Version 4.1 (Statgraphics Technologies, Inc., Virginia, USA). As schematically illustrated in Fig. 1, the experimental design consisted of two co-culture conditions based on cellular senescence state: (1) young

T84 cells co-cultured with young Huh7 cells and (2) senescent T84 cells co-cultured with senescent Huh7 cells. Within each co-culture condition, six treatment conditions were examined: Control, PAO1, PAO1 + Lys, PAO1 + KPP22, PAO1 + IB, and PAO1 + IB + Lys. For each treatment condition, two transwell inserts/wells were used as technical replicates within each experiment. The duplicate technical replicates were obtained from the same cell preparation and were averaged to generate a single value for each treatment condition within each independent experiment. The complete experiment was independently repeated three times on different weeks using newly prepared cell cultures. Thus, $n = 3$ represents three independent biological experiments, and the biological experiments, rather than the duplicate technical replicates, constituted the independent observations for statistical analysis. The effects of senescence status and treatment condition on the measured outcomes were evaluated using two-way analysis of variance (ANOVA), with senescence status and treatment condition as fixed factors and their interaction included in the model. Where statistically significant effects or prespecified hypotheses warranted further examination, simple-effect pairwise comparisons were performed between young and senescent co-cultures within each treatment condition and between treatment conditions within each senescence state. The prespecified treatment comparisons were Control versus PAO1, PAO1 + Lys versus PAO1, PAO1 + KPP22 versus PAO1, PAO1 + IB versus PAO1, and PAO1 + IB + Lys versus PAO1 + Lys. p values for these prespecified comparisons were adjusted using the Holm procedure. When all six treatment conditions within a senescence state were compared, pairwise comparisons were performed separately within the young and senescent co-culture conditions using Tukey's honestly significant difference (HSD) procedure for multiple-comparison adjustment. All statistical tests were two-sided, and a p value < 0.05 was considered statistically significant. Data are presented as mean $\pm$ standard error of the mean (SEM) from three independent biological experiments ($n = 3$). Given the limited number of independent biological experiments, statistical significance was interpreted together with the magnitude and consistency of the observed effects across the three independent experiments. Normality was assessed using the Shapiro-Wilk test prior to ANOVA. Because the number of independent biological experiments was limited ($n = 3$), assessment of normality was interpreted cautiously and in conjunction with the distribution of the data and the consistency of the effects across independent experiments. In the figures, white bars represent young T84/Huh7 co-cultures, whereas black bars represent senescent T84/Huh7 co-cultures. Different lowercase letters (a, b) indicate significant differences between young and senescent co-cultures within the corresponding treatment condition, whereas different capital letters indicate significant differences among treatment conditions within the respective co-culture condition. Groups sharing the same letter are not significantly different. The statistical test and multiple-comparison procedure used for each analysis are specified in the corresponding figure legends.

3. Results

3.1. The excess of available nutritive lysine differentially affects cell viability and barrier integrity of young and senescent intestinal epithelial cells suffering from infectious inflammation

Cellular barrier function and integrity were evaluated by measuring transepithelial electrical resistance (TEER) under control and treatment conditions, and the results are summarized and compared in Table 1. In the control setting without bacteria or other treatments, the TEER value in senescent cells was significantly lower than in young cells. Compared with the control setting, after 6 h of exposure to PAO1 and/or excess available lysine (510 μ M), TEER significantly decreased, particularly in senescent cells. PAO1 significantly reduced TEER compared with control cells, and lysine further decreased TEER compared with both the control ($p = 0.0079$) and PAO1 alone ($p = 0.041$). TEER was consistently

Table 1

provides a summary and comparison of transepithelial electrical resistance (TEER) in young and senescent T84 intestinal epithelial cell monolayers co-cultured with hepatic Huh7 cells under excess-lysine conditions and an infectious, inflamed milieu.

Treatment conditions	TEER (Ω -cm ²)	
	Cellular senescence state	
	Young T84 cell monolayers	Senescent T84 cell monolayers
Control	438.23 $\pm$ 14.7 ^{Aa}	346.67 $\pm$ 8.3 ^{Ab}
PAO1	119.43 $\pm$ 7.2 ^{Ea}	93.38 $\pm$ 6.11 ^{Eb}
PAO1 + Lys	97.11 $\pm$ 3.1 ^{Fa}	75.3 $\pm$ 2.3 ^{Fb}
PAO1 + KPP22	417.30 $\pm$ 16.2 ^{Ba}	327.5 $\pm$ 9.1 ^{Bb}
PAO1 + IB	163.22 $\pm$ 10.1 ^{Da}	124.1 $\pm$ 5.1 ^{Db}
PAO1 + Lys + IB	185.44 $\pm$ 8.8 ^{Ca}	156.4 $\pm$ 3.1 ^{Cb}

Table 1. Transepithelial electrical resistance (TEER) of young and senescent T84 intestinal epithelial cell monolayers under the indicated treatment conditions. Data are presented as mean $\pm$ SEM from three independent biological experiments ($n = 3$), each performed using two technical replicates. Two transwell inserts containing T84 cells and two wells containing Huh7 cells were used as technical replicates for each treatment condition within each independent biological experiment. The technical replicates were averaged before statistical analysis and were not treated as independent biological replicates. Statistical analysis was performed using two-way ANOVA with senescence state (young vs. senescent), treatment condition (Control, PAO1, PAO1 + Lys, PAO1 + KPP22, PAO1 + IB, and PAO1 + IB + Lys), and their interaction as factors. Prespecified pairwise comparisons between treatment conditions and between young and senescent T84 cells were adjusted for multiple comparisons using the Holm procedure; when all six treatment conditions within a senescence state were compared, pairwise comparisons were performed separately within the young and senescent cell sets using Tukey's HSD procedure. Different lowercase letters (a, b) within rows indicate significant differences between young and senescent T84 cells within the corresponding treatment condition, whereas different uppercase letters (A–F) within columns indicate significant differences among treatment conditions within the respective young or senescent cell set. Groups sharing the same letter are not significantly different. A two-sided $p < 0.05$ was considered statistically significant. Letters are assigned to values in order of decreasing mean values (from highest to lowest, respectively). **Abbreviations:** IB, *Intestinimonas butyriciproducens* 26243; KPP22, PAO1-specific bacteriophage; Lys, lysine; PAO1, *Pseudomonas aeruginosa* PAO1; TEER, transepithelial electrical resistance; Control, untreated control without bacteria or other treatments.

Exact p -values for only the most biologically relevant statistically significant pairwise comparisons ($p < 0.05$) are shown below.

For young T84 cell monolayers: young Control vs. young PAO1 ($p = 0.0061$); young PAO1 vs. young PAO1 + KPP22 ($p = 0.0049$); young PAO1 vs. young PAO1 + Lys ($p = 0.027$); young PAO1 vs. young PAO1 + IB ($p = 0.024$); young PAO1 + KPP22 vs. young PAO1 + IB ($p = 0.029$); young PAO1 + KPP22 vs. young PAO1 + IB + Lys ($p < 0.0096$); and young PAO1 + IB vs. young PAO1 + IB + Lys ($p = 0.037$). Significant differences between young and senescent co-cultures were observed for Control ($p = 0.019$), PAO1 ($p = 0.025$), PAO1 + Lys ($p = 0.034$), PAO1 + KPP22 ($p = 0.018$), and PAO1 + IB + Lys ($p = 0.013$).

For senescent T84 cell monolayers: senescent Control vs. senescent PAO1 ($p = 0.0079$); senescent PAO1 vs. senescent PAO1 + KPP22 ($p = 0.0027$); senescent PAO1 vs. senescent PAO1 + Lys ($p = 0.041$); senescent PAO1 vs. senescent PAO1 + IB ($p = 0.0065$); senescent PAO1 + KPP22 vs. senescent PAO1 + IB ($p = 0.0059$); senescent PAO1 + KPP22 vs. senescent PAO1 + IB + Lys ($p < 0.0088$); and senescent PAO1 + IB vs. senescent PAO1 + IB + Lys ($p = 0.034$).

higher in young T84 cells than in senescent T84 cells under all conditions. PAO1 markedly reduced TEER in both cellular age state, and lysine caused an additional decrease. TEER in young cells remained higher than in senescent cells at baseline ($p = 0.01$), during PAO1 inoculation ($p = 0.028$), and after PAO1 + lysine treatment ($p = 0.014$).

As expected, the *Pseudomonas* KPP22 phage, as a natural lysing agent of PAO1, reversed the PAO1-induced decrease in TEER. The IB

bacterium, particularly in the presence of excess available lysine (510 μ M), caused a rescue in TEER compared with the respective PAO1-alone condition. The effect of the combination of IB and lysine was additive only. These data indicate a cytoprotective role for beneficial metabolites (e.g., glutamate) derived from lysine metabolism by IB bacteria, as reflected in Fig. 6 of the next section. This also supports previous work showing that IB can produce glutamate, particularly via amino acid metabolism (Rampanelli et al., 2025).

Regarding the assessment of the integrity and function of the epithelial barrier, we next analyzed the viability of the cells used in the model. Cellular viability in young and senescent co-cultured cells was evaluated using Trypan blue, and the results are shown in Figs. 2A and 2B. In T84 cells, treatment with PAO1 alone or PAO1 + lysine significantly decreased cell viability by 46–61% in young cells and 49–66% in senescent cells compared with the control (no bacteria) (Fig. 2A). This reduction was slightly more pronounced in senescent cells. PAO1 significantly reduced T84 viability compared with the control ($p = 0.0026$), and PAO1 + lysine further decreased viability compared with both the control ($p = 0.0019$) and PAO1 alone ($p = 0.045$). Comparison between cell types showed that senescent T84 cells were more sensitive to bacterial treatments: young cells had significantly higher viability than senescent cells during PAO1 inoculation ($p = 0.037$) and PAO1 + lysine treatment ($p = 0.024$). However, young and senescent Huh7 cells in the co-culture system showed a viability pattern similar to

T84 cells, despite not being in direct contact with bacteria (Fig. 2B). This response may be reflected to the metabolic and detoxification functions of Huh7 cells in the co-culture system.

The *Pseudomonas* KPP22 phage, a natural biological entity that lyses PAO1 bacteria, was included to clarify the role of the participants and to isolate the specific effects of lysine metabolic utilization by PAO1 bacteria on the co-cultured host cells. As anticipated, the *Pseudomonas* KPP22 phage rescued the decrease in cell viability induced by PAO1. The IB bacterium, especially in the presence of excess lysine, rescued the decrease in cell viability induced by PAO1. As can be seen in the diagram, the senescent cells benefited from this cytoprotective effect of the IB bacterium. This is due to the consumption of excess lysine in the milieu by IB bacteria and their production of more beneficial metabolites, while PAO1 bacteria had a more destructive effect on cells in the presence of excess lysine. Overall, combined analysis of epithelial barrier integrity and cell viability showed that the studied IB strain inhibited adhesion of the indicator pathogen to T84 cells, and this inhibitory effect was consistent with its own adhesion capacity. This strain, with the highest adherent activity in the presence of excess available lysine showed the strongest inhibitory activity against the indicator pathogen microorganism in each inhibition assay. However, a consistent relationship was observed between the *in vitro* cytoprotective effects and epithelial barrier integrity in T84 cell monolayers for this lysine-snatching and metabolizing strain. Notably, combining KPP22

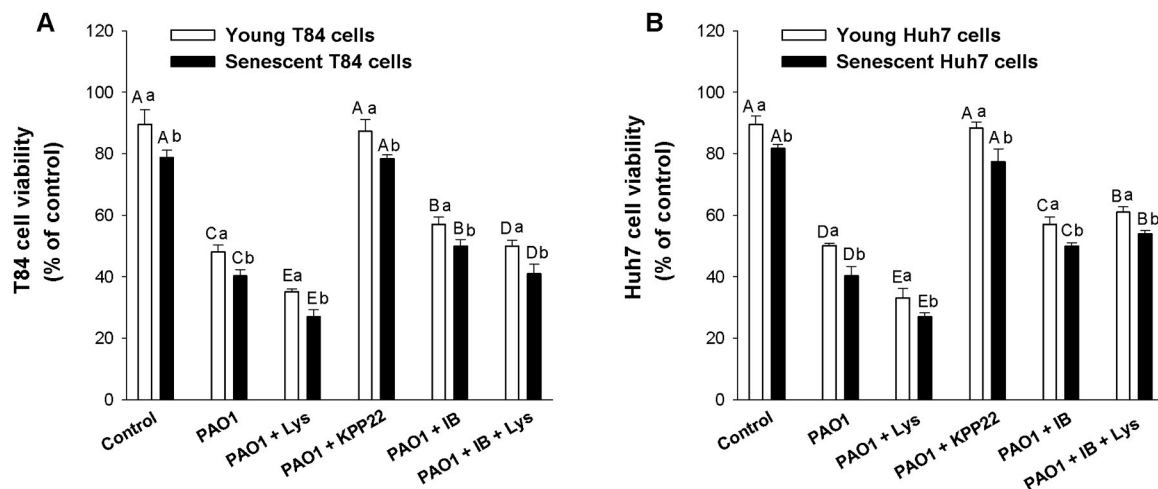


Fig. 2. Cell viability of young and senescent T84/Huh7 co-cultures under infectious-inflammatory conditions with or without lysine supplementation. The experimental design consisted of two separate co-culture sets: (1) young T84 cells co-cultured with young Huh7 cells and (2) senescent T84 cells co-cultured with senescent Huh7 cells. Within each co-culture set, six treatment conditions were examined: Control, PAO1, PAO1 + Lys, PAO1 + KPP22, PAO1 + IB, and PAO1 + IB + Lys. (A) Viability of T84 intestinal epithelial cells and (B) viability of Huh7 hepatic cells in the young and senescent co-culture sets. Data are presented as mean $\pm$ SEM from three independent biological experiments ($n = 3$) performed on different weeks using newly prepared cell cultures. Two transwell inserts containing T84 cells and two wells containing Huh7 cells were used as technical replicates for each treatment condition within each independent biological experiment. The technical replicates were averaged before statistical analysis and were not treated as independent biological replicates. Statistical analysis was performed using two-way ANOVA with senescence state (young vs. senescent), treatment condition (Control, PAO1, PAO1 + Lys, PAO1 + KPP22, PAO1 + IB, and PAO1 + IB + Lys), and their interaction as factors. Prespecified pairwise comparisons were adjusted for multiple comparisons using the Holm procedure; when all six treatment conditions within a senescence state were compared, pairwise comparisons were performed separately within the young and senescent co-culture sets using Tukey's HSD procedure. Different lowercase letters (a, b) indicate significant differences between young and senescent cells within the corresponding treatment condition, whereas different uppercase letters indicate significant differences among treatment conditions within the respective young or senescent co-culture set. Groups sharing the same letter are not significantly different. A two-sided $p < 0.05$ was considered statistically significant. White bars represent young cells, and black bars represent senescent cells. Letters are assigned to the bars in order of decreasing mean values, from highest to lowest. **Abbreviations:** IB, *Intestinimonas butyriciproducens* 26243; KPP22, PAO1-specific bacteriophage; Lys, lysine; PAO1, *Pseudomonas aeruginosa* PAO1; Control, untreated control without bacteria or other treatments. Exact p -values for only the most biologically relevant statistically significant pairwise comparisons ($p < 0.05$) are shown below. **For panel A:** young Control vs. young PAO1 ($p = 0.0026$); young PAO1 vs. young PAO1 + KPP22 ($p = 0.001$); young PAO1 vs. young PAO1 + Lys ($p = 0.0017$); young PAO1 vs. young PAO1 + IB ($p = 0.019$); young PAO1 + KPP22 vs. young PAO1 + IB ($p = 0.033$); young PAO1 + KPP22 vs. young PAO1 + IB + Lys ($p = 0.0013$); and young PAO1 + IB vs. young PAO1 + IB + Lys ($p = 0.043$). Significant differences between young and senescent co-cultures were observed for Control ($P = 0.037$), PAO1 ($p = 0.047$), PAO1 + Lys ($p = 0.042$), PAO1 + KPP22 ($p = 0.039$), and PAO1 + IB + Lys ($p = 0.034$). **For panel B:** young Control vs. young PAO1 ($p = 0.0031$); young PAO1 vs. young PAO1 + KPP22 ($p = 0.0012$); young PAO1 vs. young PAO1 + Lys ($p = 0.035$); young PAO1 vs. young PAO1 + IB ($p = 0.038$); young PAO1 + KPP22 vs. young PAO1 + IB ($p = 0.025$); young PAO1 + KPP22 vs. young PAO1 + IB + Lys ($p = 0.019$); and young PAO1 + IB vs. young PAO1 + IB + Lys ($p = 0.032$). Significant differences between young and senescent co-cultures were observed for Control ($P = 0.041$), PAO1 ($p = 0.037$), PAO1 + Lys ($p = 0.024$), PAO1 + KPP22 ($p = 0.035$), and PAO1 + IB + Lys ($p = 0.044$).

phage with its host pathogenic PAO1 bacterium and lysine would be unnecessary, as lysine and KPP22 serve distinct roles in this experimental setup. Lysine affects the metabolic activity of the bacteria and their interaction with host cells, while KPP22 is a phage that targets and kills PAO1. In our experimental design, we aimed to evaluate the specific effect of lysine on PAO1 per se, using the phage as a natural specific control to isolate the effects of PAO1. The combination of KPP22 phage, PAO1, and lysine in the medium would not provide additional insights and could confound the interpretation of lysine's role, which we sought to explore independently of the phage's activity.

3.2. Differential induction of cytokines by young and senescent yells treated with excess lysine under infectious inflammation

As immunometabolic control of cytokine production by micro-nutrients differs across health, aging, and inflammation (Ghosh and Chattopadhyay, 2026), and protein levels of the proinflammatory cytokines IL-6 and IL-8 have been suggested as the most frequently cited senescence-associated markers (Ayoub et al., 2026; Yue et al., 2022), we measured the levels of IL-6, IL-8, and IL-10. In this study, basal IL-6, IL-8, and IL-10 protein secretion by young and senescent cell cultures was used as biological indices of cellular senescence. As shown in Fig. 3 A-C, basal IL-6 and IL-8 protein secretion into cell culture supernatants by senescent cells under control (no-bacteria) conditions were higher than by younger cells. By contrast, IL-10 secretion by senescent cells was lower. In both young and senescent cell cocultures, the PAO1 pathogen bacterium alone caused a significant increase in IL-6 and IL-8 secretion (Fig. 3AB), while causing a decrease in IL-10 (Fig. 3C). This effect was reinforced in the presence of excess lysine and was more impressive in senescent cells. In contrast, IB, both alone and in the presence of excess available lysine inhibited PAO1-induced IL-6 and IL-8 secretion and restored IL-10 levels. This effect was more impressive in young cells, although senescent cells also benefited from it. These results imply that inappropriate lysine supplementation, especially in older individuals suffering from chronic infectious inflammation or repeated episodes of infectious inflammation, is not always beneficial and may even exacerbate infectious inflammation. Additionally, the status of endogenous phages and bacteria that produce beneficial metabolites from lysine are important biological actors in this context. Supporting this, adding *Pseudomonas* KPP22 phage to the culture medium abolished all the destructive effects of PAO1 bacteria on the cells. However, because 0.4 μ m pore-size Transwell inserts allow diffusion of small molecules, such as cytokines, their cellular origin (T84 cells in the apical compartment or Huh7 cells in the basolateral compartment) cannot be distinguished. Finally, these cytokines were measured as key soluble components of the senescence-associated secretory phenotype (SASP) (Flacco et al., 2026; Yue et al., 2022), as they can be readily quantified in co-culture supernatants. The observed increases in these two prominent SASP components (IL-6 and IL-8) (Ayoub et al., 2026) were accompanied by increases in complementary intracellular senescence-associated biomarkers (SA- β -gal activity and p21 expression) in separately collected cell lysates from young and senescent cells (Section 2.6).

3.3. Intestinal alkaline phosphatase activity in T84 cell lysates

Intestinal alkaline phosphatase (IAP/ALP) is a key gut mucosal defense factor and a marker of intestinal epithelial integrity. It dephosphorylates and detoxifies the endotoxin component of lipopolysaccharide (LPS), prevents bacterial translocation across the gut mucosal barrier, and thereby protects against inflammation (Goldberg et al., 2008). To assess IAP activity, T84 cells were lysed after 6 h of exposure, and the soluble fraction (cell lysate supernatant) was collected by centrifugation. IAP activity was measured in the undiluted lysate supernatant to ensure sufficient signal within the assay's linear range, reflecting the full cytoplasmic and soluble membrane-associated enzyme pool. IAP activity, measured in the undiluted lysate supernatant,

differed significantly among the six experimental groups. Compared with the control (no-bacteria conditions), ALP activity after exposure to PAO1 bacteria, particularly in lysine-supplemented DMEM medium was significantly decreased. Compared to young intestinal T84 cells, senescent intestinal T84 cells were more affected (Fig. 4). The introduction of the bacterium IB alone or in the presence of excess lysine (510 μ M) restored ALP levels compared with the respective PAO1 $\pm$ excess lysine (510 μ M) conditions. These findings suggest that, unlike PAO1, the bacterium IB produces beneficial metabolites for host cells through the consumption of lysine, as reflected by its effects on ALP activity. Moreover, when the *Pseudomonas* KPP22 phage was added to the medium, the PAO1-associated decrease in ALP activity was eliminated. Overall, the decrease in intestinal ALP activity induced by PAO1 and PAO1 + excess lysine was more pronounced in senescent cells, which appeared to benefit more from metabolites released by IB bacteria.

3.4. Positive nutritional effects of lysine on cellular metabolic health of young and senescent cells differ under infectious-inflamed milieu

To address the impact of infectious inflammation on changes in the metabolic activity of young and senescent intestinal cells, their supernatants were first analyzed for glucose consumption. As shown in Fig. 5A, the glucose level in the supernatants from young cell co-cultures under control conditions was significantly lower than that from senescent cell co-cultures. This is in line with lower glucose consumption by the latter, which exhibited lower viability and growth rates (see Fig. 2A above). The inoculation of PAO1, both alone and in the presence of excess lysine, reduced glucose levels in the supernatant of senescent cells much more than in young cells. Thus, during 6 h of incubation, young cells inoculated with PAO1 bacteria alone and together with lysine consumed 35% and 42% of the glucose present in the culture medium, respectively, while senescent cells consumed 39% and 53%, respectively. This was manifested as a drop in the glucose concentration remaining in the medium of co-cultured cells, suggesting that infection increases glucose consumption by cells, especially senescent cells. In the presence of *Pseudomonas* KPP22 phage, the glucose levels of the PAO1-alone groups were partially restored toward control levels, indicating a reduction in the infection-associated increase in glucose consumption, which is consistent with previous *in vitro* and *in vivo* studies showing that infection induces glucose consumption by host cells (Proal and VanElzakker, 2021; Tang et al., 2024). In contrast, the gut bacterium IB, both alone and in the presence of excess lysine, attenuated the metabolic responses of the co-cultured cells to PAO1, resulting in a higher supernatant glucose level than that of the groups treated with PAO1. In the PAO1 + Lys group, the remaining glucose concentration after 6 h was even lower than that of the PAO1 group alone, particularly in senescent cells. This indicates that PAO1 bacteria exerted greater stress on cells in the presence of excess lysine and that this stress consequently increased cellular glucose consumption. Accordingly, in the PAO1 + KPP22 phage group, the glucose concentration remaining after 6 h was similar to that of the control group. In the PAO1 + IB group, although three glucose-consuming organisms—PAO1, IB, and the host cells—were present, the remaining glucose concentration did not differ significantly from that of the PAO1 group alone. This suggests that IB bacteria attenuated the effects of PAO1 bacteria on cellular glucose metabolism, as also observed in the PAO1 + IB + Lys group, in which the remaining glucose concentration did not differ significantly from that of the PAO1 + IB group. Overall, the data from the six experimental treatment groups (Fig. 5A) indicate that IB bacteria utilized lysine and attenuated the disruptive effect of PAO1 bacteria on glucose metabolism, particularly in senescent cells. Notably, after 6 h, the remaining glucose concentration in the PAO1 + IB + lysine group was significantly higher than that in the PAO1 + lysine group. This suggests that PAO1 consumed lysine, induced stress in the host cells, and subsequently triggered their glucose consumption. In contrast, IB bacteria attenuated this effect.

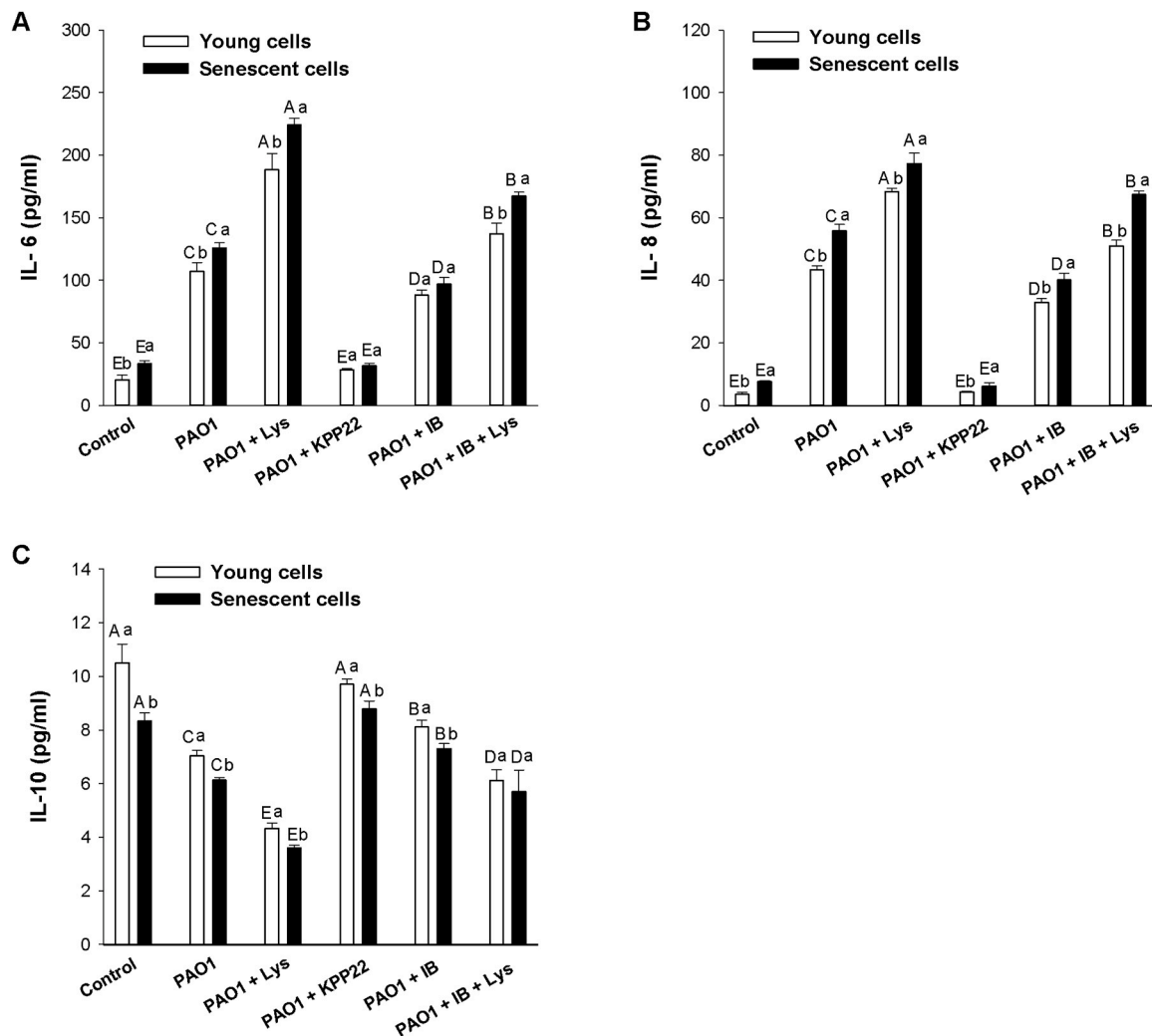


Fig. 3. Secreted cytokine levels in young and senescent T84/Huh7 co-cultures following exposure to PAO1 and the indicated treatments. (A) IL-6 and (B) IL-8, as pro-inflammatory cytokines, and (C) IL-10, as an anti-inflammatory cytokine, were measured in the culture medium of young and senescent T84/Huh7 co-cultures following 6 h of exposure to PAO1 and the indicated treatments. Cytokine concentrations were measured by ELISA and expressed as pg/ml. White bars represent young T84/Huh7 co-cultures, and black bars represent senescent T84/Huh7 co-cultures. Data are presented as mean $\pm$ SEM from three independent biological experiments ($n = 3$). Two transwell inserts containing T84 cells and two wells containing Huh7 cells were used as technical replicates for each treatment condition within each independent biological experiment. The technical replicates were averaged before statistical analysis and were not treated as independent biological replicates. Statistical analysis was performed using two-way ANOVA with senescence state (young vs. senescent), treatment condition (Control, PAO1, PAO1 + Lys, PAO1 + KPP22, PAO1 + IB, and PAO1 + IB + Lys), and their interaction as factors. Prespecified pairwise comparisons were adjusted for multiple comparisons using the Holm procedure; when all six treatment conditions within a senescence state were compared, pairwise comparisons were performed separately within the young and senescent co-culture groups using Tukey's HSD procedure. Different lowercase letters (a, b) indicate significant differences between young and senescent co-cultures within the corresponding treatment condition, whereas different capital letters indicate significant differences among treatment conditions within the respective young or senescent co-culture condition. Groups sharing the same letter are not significantly different. A two-sided $p < 0.05$ was considered statistically significant. White bars represent young cells, and black bars represent senescent cells. Letters are assigned to the bars in order of decreasing mean values, from highest to lowest. **Abbreviations:** IB, *Intestinimonas butyriciproducens* 26243; KPP22, PAO1-specific bacteriophage; Lys, lysine; PAO1, *Pseudomonas aeruginosa* PAO1; Control, untreated control without bacteria or other treatments. Exact p-values for only the most biologically relevant statistically significant pairwise comparisons ($p < 0.05$) are shown below. **For panel A:** young Control vs. young PAO1 ($p = 0.0044$); young PAO1 vs. young PAO1 + KPP22 ($p = 0.0025$); young PAO1 vs. young PAO1 + Lys ($p = 0.0023$); young PAO1 vs. young PAO1 + IB ($p = 0.017$); young PAO1 + KPP22 vs. young PAO1 + IB ($p = 0.035$); young PAO1 + KPP22 vs. young PAO1 + IB + Lys ($p = 0.0041$); and young PAO1 + IB vs. young PAO1 + IB + Lys ($p = 0.020$). Significant differences between young and senescent co-cultures were observed for Control ($p = 0.046$), PAO1 ($p = 0.032$), PAO1 + Lys ($p = 0.041$), PAO1 + KPP22 ($p = 0.049$), and PAO1 + IB + Lys ($p = 0.044$). **For panel B:** young Control vs. young PAO1 ($p = 0.004$); young PAO1 vs. young PAO1 + KPP22 ($p = 0.0027$); young PAO1 vs. young PAO1 + Lys ($p = 0.008$); young PAO1 vs. young PAO1 + IB ($p = 0.033$); young PAO1 + KPP22 vs. young PAO1 + IB ($p = 0.0019$); young PAO1 + KPP22 vs. young PAO1 + IB + Lys ($p = 0.0027$); and young PAO1 + IB vs. young PAO1 + IB + Lys ($p = 0.0241$). Significant differences between young and senescent co-cultures were observed for Control ($p = 0.039$), PAO1 ($p = 0.026$), PAO1 + Lys ($p = 0.035$), PAO1 + KPP22 ($p = 0.040$), PAO1 + IB ($p = 0.045$), and PAO1 + IB + Lys ($p = 0.037$). **For panel C:** young Control vs. young PAO1 ($p = 0.028$); young PAO1 vs. young PAO1 + KPP22 ($p = 0.0039$); young PAO1 vs. young PAO1 + Lys ($p = 0.018$); young PAO1 vs. young PAO1 + IB ($p = 0.029$); young PAO1 + KPP22 vs. young PAO1 + IB ($p = 0.039$); young PAO1 + KPP22 vs. young PAO1 + IB + Lys ($p = 0.041$); and young PAO1 + IB vs. young PAO1 + IB + Lys ($p = 0.036$). Significant differences between young and senescent co-cultures were observed for Control ($p = 0.022$), PAO1 ($p = 0.042$), PAO1 + Lys ($p = 0.041$), PAO1 + KPP22 ($p = 0.047$), and PAO1 + IB ($p = 0.034$).

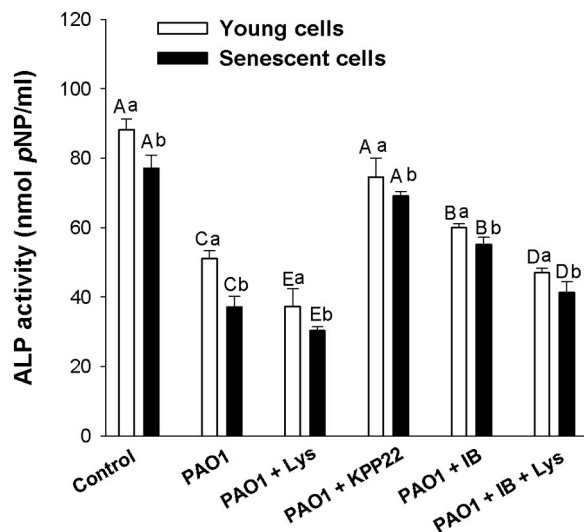


Fig. 4. Intestinal alkaline phosphatase (IAP) activity in young and senescent T84/Huh7 co-cultures following exposure to PAO1 and the indicated treatments. IAP activity was measured in cell lysate supernatants following 6 h of exposure to Control, PAO1, PAO1 + Lys, PAO1 + KPP22, PAO1 + IB, and PAO1 + IB + Lys. Lysine was provided at a concentration of 510 μ M. White bars represent young T84/Huh7 co-cultures, and black bars represent senescent T84/Huh7 co-cultures. Data are presented as mean $\pm$ SEM from three independent biological experiments ($n = 3$) performed on different weeks using newly prepared cell cultures. Two transwell inserts containing T84 cells and two wells containing Huh7 cells were used as technical replicates for each treatment condition within each independent biological experiment. The technical replicates were averaged before statistical analysis and were not treated as independent biological replicates. Statistical analysis was performed using two-way ANOVA with senescence state (young vs. senescent), treatment condition (Control, PAO1, PAO1 + Lys, PAO1 + KPP22, PAO1 + IB, and PAO1 + IB + Lys), and their interaction as factors. Prespecified pairwise comparisons were adjusted for multiple comparisons using the Holm procedure; when all six treatment conditions within a senescence state were compared, pairwise comparisons were performed separately within the young and senescent co-culture groups using Tukey's HSD procedure. Different lowercase letters (a, b) indicate significant differences between young and senescent co-cultures within the corresponding treatment condition, whereas different capital letters indicate significant differences among treatment conditions within the respective young or senescent co-culture condition. Groups sharing the same letter are not significantly different. A two-sided $p < 0.05$ was considered statistically significant. White bars represent young cells, and black bars represent senescent cells. Letters are assigned to the bars in order of decreasing mean values, from highest to lowest. **Abbreviations:** IAP, intestinal alkaline phosphatase; IB, *Intestinimonas butyriciproducens*; KPP22, PAO1-specific bacteriophage; Lys, lysine; PAO1, *Pseudomonas aeruginosa* PAO1. Exact p -values for only the most biologically relevant statistically significant pairwise comparisons ($p < 0.05$) are shown below. Young Control vs. young PAO1 ($p = 0.0064$); young PAO1 vs. young PAO1 + KPP22 ($p = 0.0022$); young PAO1 vs. young PAO1 + Lys ($p = 0.023$); young PAO1 vs. young PAO1 + IB ($p = 0.017$); young PAO1 + KPP22 vs. young PAO1 + IB ($p = 0.043$); young PAO1 + KPP22 vs. young PAO1 + IB + Lys ($p = 0.011$); and young PAO1 + IB vs. young PAO1 + IB + Lys ($p = 0.033$). Significant differences between young and senescent co-cultures were observed for Control ($p = 0.039$), PAO1 ($p = 0.028$), PAO1 + Lys ($p = 0.031$), PAO1 + KPP22 ($p = 0.040$), PAO1 + IB ($p = 0.047$), and PAO1 + IB + Lys ($p = 0.045$).

Of note, since we measured glucose concentration in the pooled apical and basolateral supernatants, it is difficult to determine precisely which cell type, T84 or Huh7, consumed more or less glucose. Additionally, we used 0.4 μ m pore-size transwell inserts, which allow glucose diffusion between the apical and basolateral compartments, further complicating this comparison. Therefore, directly attributing glucose consumption to either cell type is not straightforward. However, given that PAO1 bacteria had a more pronounced detrimental effect on the

general health of T84 cells than on Huh7 cells, it can be inferred that T84 cells likely consumed more glucose due to the stress caused by PAO1 bacteria. Importantly, unlike many enteric bacteria, *Pseudomonas aeruginosa* does not preferentially utilize glucose as a carbon source (Suresh et al., 2023). Therefore, it is unlikely that PAO1 consumed a significant amount of glucose in this experiment.

Next, cell lysate supernatants of hepatic Huh7 cells were analyzed for triglyceride (TG) and HDL-C contents. As shown in Fig. 5B, TG levels were higher in senescent than in young cells under control conditions. Treatment with PAO1 alone increased TG levels in both young and senescent cells (by 54% and 55%, respectively), while the presence of excess lysine further increased TG levels in young cells by 29% and in senescent cells by 5%. The HDL-C content (Fig. 5C) showed the opposite pattern to triglyceride content. Thus, under control conditions, the HDL-C level was significantly lower in senescent (23 mM/g) than in younger cells (28 mM/g). Treatment with PAO1 in the absence or presence of lysine decreased HDL-C levels more in younger cells (50% and 58%, respectively, compared with control) than in senescent cells (43% and 51%, respectively). Addition of KPP22 and IB reversed the effect of PAO1 $\pm$ lysine on HDL-C levels. Together, these data suggest that PAO1, particularly in the presence of excess lysine, divergently disrupts the metabolic responses of young and senescent cells. The elevated glucose uptake during infection was accompanied by a disrupted balance in cellular lipid metabolism. However, IB, both alone and in the presence of excess lysine, attenuated the effects of PAO1 under conditions in which the level of lysine in the medium was higher than normal (Fig. 5B and C). Collectively, these results suggest that excess available lysine affects the metabolic responses of young and senescent cells in a divergent manner depending on the type of bacteria and phage present within the host cell milieu. However, for the most part, the precise molecular and signaling mechanisms by which IB bacterial products affect cellular metabolic health remain to be explored.

Interestingly, in the PAO1 + Lys group, the elevated triglyceride contents were not significantly different between young and senescent cells. However, glucose consumption (Fig. 5A), driven by infection, was significantly higher in senescent cells than in young cells. The absence of a positive correlation between glucose consumption and cellular triglyceride content can be explained by several factors. Normally, increased glucose consumption can lead to higher triglyceride synthesis, as glucose can be used for energy storage in the form of lipids. However, in the context of infection, glucose may be preferentially utilized for immune responses and overcoming infection rather than for storage as triglycerides. This shift in metabolic priorities, especially in senescent cells, may explain the discrepancy between glucose consumption and triglyceride accumulation. In summary, while glucose is typically a precursor for triglyceride synthesis, infection-induced stress, particularly in senescent cells, likely redirects glucose utilization toward energy production and immune responses, rather than lipid storage.

3.5. Assessing the triple competition between the pathogenic bacterium PAO1, the beneficial bacterium IB, and host cells for the exogenous excess lysine surrounding them

As shown in Figs. 6A and 6B, the uptake of lysine (as an indirect indicator of lysine consumption) from the culture medium and the subsequent formation of glutamate by uninoculated senescent control cells are lower than those observed in young cells. Young cells in the control group (no-bacteria condition) consumed 11.72% of the lysine present in the culture medium over a period of 6 h of incubation, while senescent cells consumed 8.33%. When PAO1 was added alone to the culture medium, the concentration of lysine decreased and the concentration of glutamate increased compared to the control (Fig. 6B). This effect was more pronounced in young cells, indicating that lysine was utilized by both host cells and bacteria. During 6 h of incubation, when young cells were inoculated with PAO1 alone, the concentration of lysine in the culture medium decreased by 25.19%. In the case of

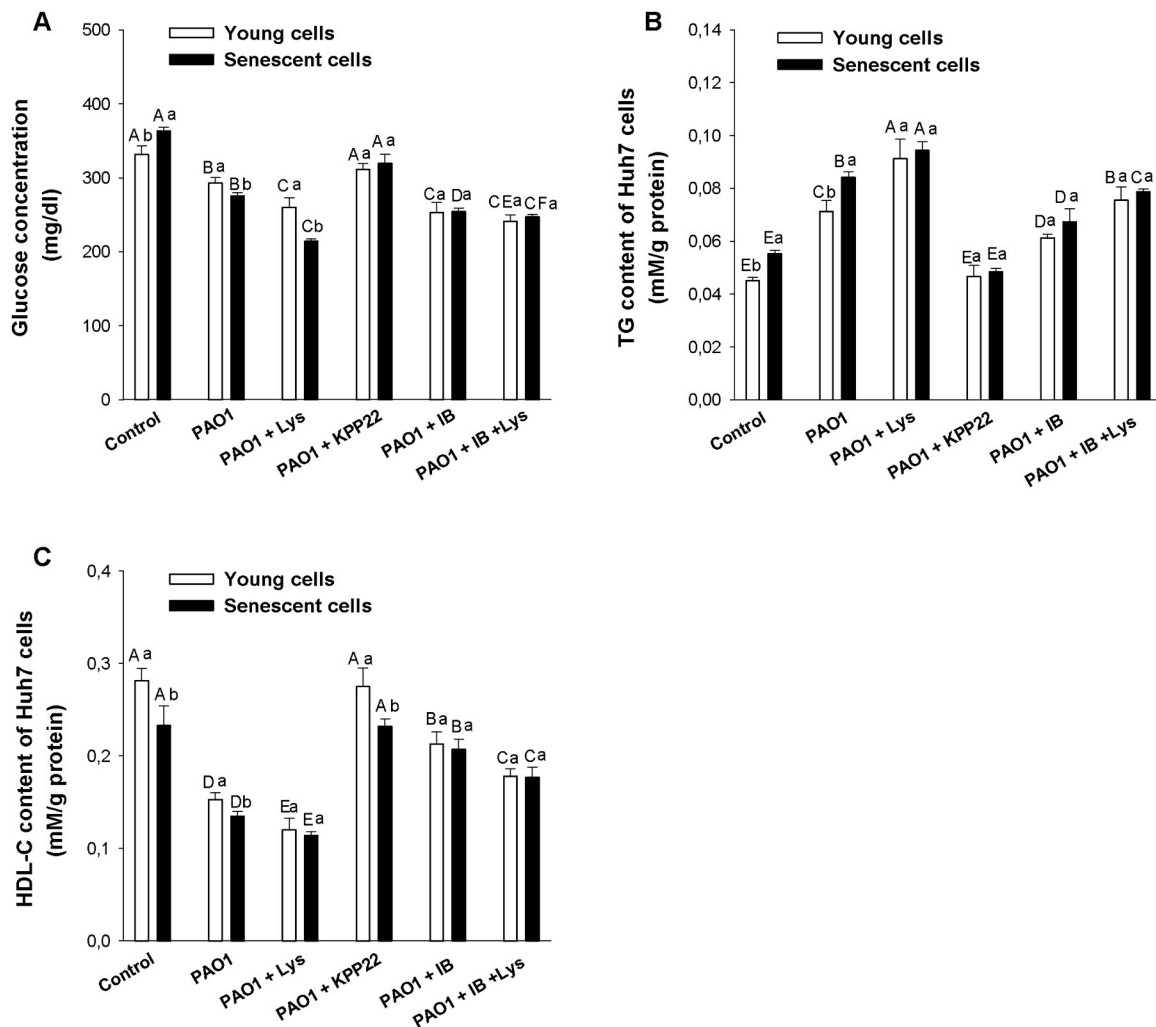
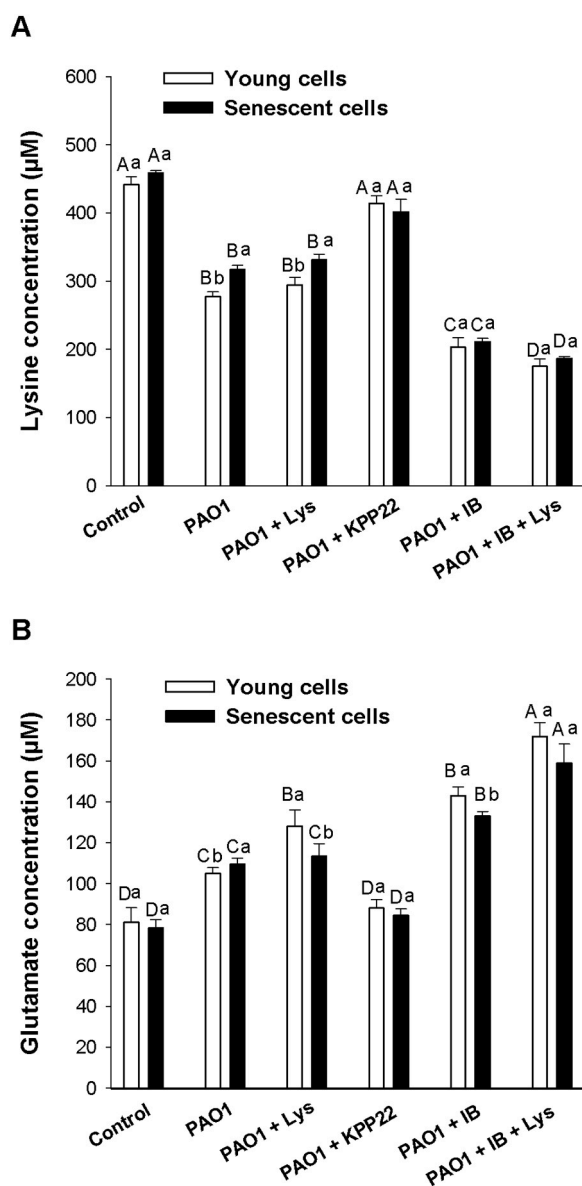


Fig. 5. Cellular health and metabolic markers in young and senescent T84/Huh7 co-cultures following exposure to PAO1 and the indicated treatments. (A) Residual glucose concentration in the culture medium after 6 h of incubation; (B) triglyceride (TG) content; and (C) high-density lipoprotein cholesterol (HDL-C) content in Huh7 cell lysate supernatants. White bars represent young T84/Huh7 co-cultures, and black bars represent senescent T84/Huh7 co-cultures. The initial glucose concentration in the culture medium was 4.5 g/L (450 mg/dL). Residual glucose concentration in panel (A) was used as an indirect indicator of glucose consumption by bacteria and host cells during the 6 h incubation period. Data are presented as mean $\pm$ SEM from three independent biological experiments ($n = 3$) performed on different weeks using newly prepared cell cultures. Two transwell inserts containing T84 cells and two wells containing Huh7 cells were used as technical replicates for each treatment condition within each independent biological experiment. The technical replicates were averaged before statistical analysis and were not treated as independent biological replicates. Statistical analysis was performed using two-way ANOVA with senescence state (young vs. senescent), treatment condition (Control, PAO1, PAO1 + Lys, PAO1 + KPP22, PAO1 + IB, and PAO1 + IB + Lys), and their interaction as factors. Prespecified pairwise comparisons were adjusted for multiple comparisons using the Holm procedure; when all six treatment conditions within a senescence state were compared, pairwise comparisons were performed separately within the young and senescent co-culture groups using Tukey's HSD procedure. Different lowercase letters (a, b) indicate significant differences between young and senescent co-cultures within the corresponding treatment condition, whereas different uppercase letters indicate significant differences among treatment conditions within the respective young or senescent co-culture condition. In panel (A), uppercase letters (A–F) indicate significant differences among treatment conditions; in panels (B) and (C), uppercase letters (A–E) indicate significant differences among treatment conditions. Groups sharing the same letter are not significantly different. A two-sided $P < 0.05$ was considered statistically significant. Letters are assigned to the bars in order of decreasing mean values, from highest to lowest. **Abbreviations:** HDL-C, high-density lipoprotein cholesterol; IB, *Intestinimonas butyriciproducens*; KPP22, PAO1-specific bacteriophage; Lys, lysine; PAO1, *Pseudomonas aeruginosa* PAO1; TG, triglycerides.

Exact p-values for only the most biologically relevant statistically significant pairwise comparisons ($p < 0.05$) are shown below. **For panel A:** young Control vs. young PAO1 ($p = 0.038$); young PAO1 vs. young PAO1 + KPP22 ($p = 0.029$); young PAO1 vs. young PAO1 + Lys ($p = 0.042$); young PAO1 vs. young PAO1 + IB ($p = 0.039$); young PAO1 + KPP22 vs. young PAO1 + IB ($p = 0.045$); and young PAO1 + KPP22 vs. young PAO1 + IB + Lys ($p = 0.021$). Significant differences between young and senescent co-cultures were observed for Control ($p = 0.039$), PAO1 ($p = 0.044$), and PAO1 + Lys ($p = 0.033$). **For panel B:** young Control vs. young PAO1 ($p = 0.031$); young PAO1 vs. young PAO1 + KPP22 ($p = 0.0093$); young PAO1 vs. young PAO1 + Lys ($p = 0.026$); young PAO1 vs. young PAO1 + IB ($p = 0.033$); young PAO1 + KPP22 vs. young PAO1 + IB ($p = 0.034$); young PAO1 + KPP22 vs. young PAO1 + IB + Lys ($p < 0.027$); and young PAO1 + IB vs. young PAO1 + IB + Lys ($p = 0.031$). Significant differences between young and senescent co-cultures were observed for Control ($p = 0.044$) and PAO1. **For panel C:** young Control vs. young PAO1 ($p = 0.0066$); young PAO1 vs. young PAO1 + KPP22 ($p = 0.0089$); young PAO1 vs. young PAO1 + Lys ($p = 0.027$); young PAO1 vs. young PAO1 + IB ($p = 0.017$); young PAO1 + KPP22 vs. young PAO1 + IB ($p = 0.041$); young PAO1 + KPP22 vs. young PAO1 + IB + Lys ($p = 0.028$); and young PAO1 + IB vs. young PAO1 + IB + Lys ($p = 0.019$). Significant differences between young and senescent co-cultures were observed for Control ($p = 0.027$), PAO1 ($p = 0.046$), and PAO1 + KPP22 ($p = 0.048$).

senescent cells, the decrease was 18.61%. This effect of PAO1 was abolished by the *Pseudomonas* KPP22 phage. When the *Pseudomonas* phage KPP22 was added to the culture medium, the lysine concentration returned to the level observed in the control cell group. This indicates that most of the lysine was utilized by PAO1. In the case of the PAO1 + IB group, namely, when the beneficial bacterium IB and PAO1 were added together to the culture medium of co-cultured cells, lysine consumption and glutamate production were particularly pronounced. This indicates that these two bacteria compete for lysine and that IB is beneficial for senescent cells.

In the case of the PAO1 + Lys group, that is, when additional lysine was added to the culture medium of cells inoculated with PAO1, lysine consumption and glutamate production increased, especially in senescent cells. In this group, although additional (excess) lysine was added to the culture medium, the lysine concentration did not increase, suggesting that PAO1 utilized/metabolized the supplemental lysine because the senescent cells were unable to absorb as much lysine as the young cells. However, this effect may predispose to the occurrence and exacerbation of infectious inflammation. This assumption is supported by the



(caption on next column)

Fig. 6. Lysine consumption and glutamate formation in young and senescent T84/Huh7 co-cultures following exposure to PAO1 and the indicated treatments. **(A)** Lysine consumption by the co-culture system was calculated as the difference between the initial lysine concentration and the lysine concentration remaining in the culture medium after 6 h of incubation. **(B)** Glutamate concentration was determined from a 100-µL aliquot of the same culture medium. White bars represent young T84/Huh7 co-cultures, and black bars represent senescent T84/Huh7 co-cultures. The initial lysine concentration in the culture medium was 500 µM for the Control, PAO1, PAO1 + KPP22, and PAO1 + IB conditions and 510 µM for the PAO1 + Lys and PAO1 + IB + Lys conditions, representing the excess-lysine conditions. Data are presented as mean ± SEM from three independent biological experiments (n = 3) performed on different weeks using newly prepared cell cultures. Two transwell inserts containing T84 cells and two wells containing Huh7 cells were used as technical replicates for each treatment condition within each independent biological experiment. The technical replicates were averaged before statistical analysis and were not treated as independent biological replicates. Statistical analysis was performed using two-way ANOVA with senescence state (young vs. senescent), treatment condition (Control, PAO1, PAO1 + Lys, PAO1 + KPP22, PAO1 + IB, and PAO1 + IB + Lys), and their interaction as factors. Prespecified pairwise comparisons between treatment conditions and between young and senescent co-cultures were adjusted for multiple comparisons using the Holm procedure; when all six treatment conditions within a senescence state were compared, pairwise comparisons were performed separately within the young and senescent co-culture groups using Tukey's HSD procedure. Different lowercase letters (a, b) indicate significant differences between young and senescent co-cultures within the corresponding treatment condition, whereas different uppercase letters (A–D) indicate significant differences among treatment conditions within the respective young or senescent co-culture condition. Groups sharing the same letter are not significantly different. A two-sided $p < 0.05$ was considered statistically significant. Letters are assigned to the bars in order of decreasing mean values (from highest to lowest, respectively). **Abbreviations:** IB, *Intestinimonas butyriciproducens*; KPP22, PAO1-specific bacteriophage; Lys, lysine; PAO1, *Pseudomonas aeruginosa* PAO1. Exact p-values for only the most biologically relevant statistically significant pairwise comparisons ($p < 0.05$) are shown below. **For panel A:** young Control vs. young PAO1 ($p = 0.027$); young PAO1 vs. young PAO1 + KPP22 ($p = 0.012$); young PAO1 vs. young PAO1 + IB ($p = 0.035$); young PAO1 + KPP22 vs. young PAO1 + IB ($p = 0.0077$); and young PAO1 + KPP22 vs. young PAO1 + IB + Lys ($p = 0.0063$). Significant differences between young and senescent co-cultures were observed for PAO1 ($p = 0.044$); and PAO1 + Lys ($p = 0.038$). **For panel B:** young Control vs. young PAO1 ($p = 0.032$); young PAO1 vs. young PAO1 + KPP22 ($p = 0.043$); young PAO1 vs. young PAO1 + Lys ($p = 0.034$); young PAO1 vs. young PAO1 + IB ($p = 0.022$); young PAO1 + KPP22 vs. young PAO1 + IB ($p = 0.044$); young PAO1 + KPP22 vs. young PAO1 + IB + Lys ($p = 0.018$); and young PAO1 + IB vs. young PAO1 + IB + Lys ($p = 0.039$). Significant differences between young and senescent co-cultures were observed for PAO1 ($p = 0.040$); and PAO1 + Lys ($p = 0.017$); PAO1 + IB ($p = 0.047$).

data from the PAO1 + KPP22 phage cultures, as well as the data from the PAO1 + IB + Lys group. Although additional lysine was added to the medium, its concentration after 6 h was even lower than that of the group PAO1 + IB. This indicates that most of the excess lysine in the culture medium was utilized by IB, and IB may even have hijacked lysine PAO1.

3.6. Metabolite outcomes of excess lysine utilization by PAO1 and IB differ

Depending on the organisms and their metabolic pathways, the metabolism of lysine by beneficial bacteria, pathogenic bacteria, or eukaryotic host cells leads to the production of different metabolites, such as widely and extensively studied microbial metabolites (e.g., SCFAs). We focused only on three important metabolites: 2-keto-6-aminohexanoate(2K6AC), homocitrulline, and spermine. With the assessment of glutamate formation as a simple, indirect, but reliable marker for the presence of 2K6AC originating from lysine utilization [Kochi et al., (1986)], it became pertinent to analyze homocitrulline and spermine in the cells used in the model. Accordingly, we measured

homocitrulline levels in cell supernatants, since homocitrulline of eukaryotic cellular origin is implicated in the pathological processes of several diseases and correlates with increased age, senescence, and inflammation (Chen et al., 2023, 2024). As shown in Fig. 7A, the addition of pathogenic PAO1 alone exacerbated the secretion of homocitrulline from senescent cells. The presence of excess lysine in the culture medium further increased the secretion of homocitrulline induced by PAO1, implying a detrimental impact of excess lysine on senescent cells during infectious inflammation, as indicated by increased homocitrulline levels in the medium, a marker of pathological processes. Young and senescent cells, in contrast, responded in an opposite manner to IB. In general, IB tended to dampen homocitrulline secretion, both at baseline and when induced by PAO1.

The pattern of spermine levels in cell culture supernatants was opposite to that of homocitrulline. As shown in Fig. 7B, pathogenic PAO1 alone significantly reduced cellular spermine release compared to the control non-bacteria conditions. This decrease was more pronounced in senescent cells, which per se already secrete lower amounts of spermine. The cause of this decrease is also the pro-inflammatory compounds produced by PAO1, as this effect was attenuated by the addition of the *Pseudomonas* KPP22 phage. In humans and mice, the polyamine spermine is essential for normal cellular functions, and its level in tissues decreased in aged mice compared to young mice and suppresses age-associated inflammation (Uemura et al., 2020). When PAO1 and IB were added to the culture medium together, the destructive effects of PAO1 were inhibited. In the presence of excess lysine, this beneficial effect of IB was further enhanced, indicating that the available lysine was utilized for the benefit of the host cells and that spermine was produced by the host cells. Although, as with other parameters, the difference between young and senescent cells in spermine release is quite evident here, overall, senescent cells benefited from this effect of IB.

3.7. Inhibitory effect of the IB strain on pathogenic bacterial adhesion under conditions of excess extracellular lysine availability

We next determined the ability of the IB strain to inhibit the adhesion of pathogenic bacteria under conditions of excess extracellular lysine availability and the indicated treatment conditions. Table 2 summarizes and compares the competition, exclusion, and displacement of PAO1 adhesion to young and senescent T84 cell monolayers by the IB strain under the experimental conditions. When IB and PAO1 were simultaneously incubated with the T84 cells, the adhesion of PAO1 was dramatically reduced in the presence of the KPP22 phage and IB. It was revealed that the adhesion of PAO1 to senescent cells under all treatment conditions was greater than that to young cells. However, Table 2 shows that pathogenic bacteria take advantage of excess lysine in the culture medium to better adhere to host cells, especially senescent cells. This difference may be due to mechanical competition triggered by immune signaling that drives the collective extrusion of bacterially infected epithelial cells, a postulated mechanism that requires detailed molecular studies. The beneficial effects of IB were enhanced additively in the presence of excess lysine, indicating that its consumption was a consequence of the reduced amount of lysine available to pathogenic bacteria, as also reflected in the lysine remaining in the medium after 6 h of treatment with PAO1 + Lys + IB (Fig. 6). Besides competition with pathogenic bacteria, the IB strain effectively excluded the adhesion of pathogenic bacteria to human intestinal epithelial cells under the indicated treatment conditions. Table 2 shows that the IB strain significantly inhibited the adhesion of PAO1 when the IB strain was incubated with the T84 cells before the pathogenic bacteria were added. Similar to the competition condition, the difference between young and senescent cells was evident here as well. Furthermore, the IB strain displaced the adhesion of pathogenic bacteria under the indicated treatment conditions. Table 2 showed that the IB strain significantly displaced the adhesion of PAO1 when the IB strain was added to the cells after PAO1.

The KPP22 phage was, as expected given its lytic activity against PAO1, the most effective treatment for the displacement of PAO1 adhesion. Overall, these results show that, depending on the type of bacteria and the condition of the host cells, the concentration of lysine and the presence of phages play important roles in the outcome of the tight competition, exclusion, and displacement mechanisms between bacteria and host cells, and consequently in host-cell immunity.

4. Discussion

This study investigated how excess extracellular lysine influences host-cell immunometabolic responses in an infectious-inflammatory co-culture model comprising age-matched young or senescent T84 and Huh7 cells, the opportunistic pathogen *Pseudomonas aeruginosa* PAO1, the PAO1-specific bacteriophage KPP22, and the beneficial bacterium *Intestinimonas butyriciproducens* (IB). Young T84 cells were co-cultured with young Huh7 cells, while senescent T84 cells were co-cultured with senescent Huh7 cells, and the responses of these two age-matched co-culture models were compared across six treatment conditions. We focused on epithelial barrier function, cell viability, inflammatory cytokine secretion, cellular metabolic markers, lysine utilization, and selected lysine-associated metabolites. Overall, the findings indicate that excess lysine availability, cellular senescence-associated changes, and exposure to PAO1, IB, and bacteriophage KPP22 jointly influenced host immunometabolic responses in this defined *in vitro* system.

4.1. Effects of excess lysine and microbial exposure on cellular and inflammatory responses

Excess extracellular lysine was associated with greater impairment of cellular responses following PAO1 exposure, with several effects being more pronounced in senescence-associated cells than in young cells. In contrast, the presence of KPP22 or IB attenuated several of the adverse effects associated with PAO1 and excess lysine. These findings indicate that the biological consequences of increased lysine availability cannot be considered independently of the microbial context in this experimental system.

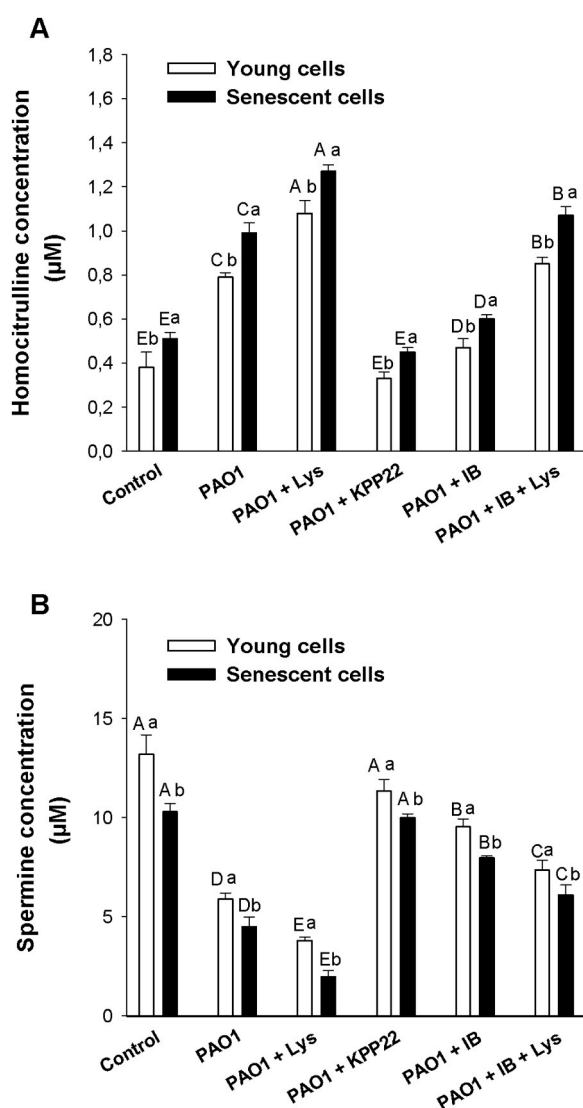
TEER measurements provided a functional assessment of epithelial barrier integrity. The observed differences between young and senescence-associated co-cultures, together with the treatment-dependent changes in TEER, indicate that cellular state influenced the response of the epithelial barrier to the experimental conditions. However, because senescent cells are intrinsically associated with altered barrier properties, the present findings should be interpreted primarily as evidence of treatment-dependent changes within the senescence-associated model rather than as a direct representation of epithelial barrier function in older adults.

The inflammatory response was further characterized by measurement of IL-6, IL-8, and IL-10. Excess lysine in the presence of PAO1 was associated with increased IL-6 and IL-8 secretion and reduced IL-10 secretion, particularly in senescence-associated cells. Conversely, KPP22 and IB were associated with attenuation of these cytokine changes. IL-6 and IL-8 are important inflammatory mediators, whereas IL-10 generally contributes to anti-inflammatory regulation and maintenance of intestinal homeostasis (Vebr et al., 2023). The elevated inflammatory cytokine levels (IL-6 and IL-8), together with increased SA- β -gal activity and p21 protein expression in senescent co-cultures, support the conclusion that the observed differences are associated with cellular senescence rather than solely with long-term culture-associated adaptations (Fan et al., 2024; Yue et al., 2022). This combination of senescence-associated biomarkers and inflammatory cytokines is consistent with established approaches for characterizing cellular senescence and the SASP (Ayoub et al., 2026; Flacco et al., 2026; Yue et al., 2022). Notably, across treatments, increased IL-6 and IL-8 levels were consistently associated with reduced cell viability and

TEER, whereas IL-10, an anti-inflammatory cytokine implicated in intestinal immune homeostasis and barrier integrity, was decreased (Vebr et al., 2023). Collectively, these findings indicate a pro-inflammatory shift accompanied by impaired cellular viability and barrier function; however, the observed associations do not establish a direct causal relationship or identify a specific underlying molecular signaling pathway.

4.2. Alterations in cellular metabolic responses

Since pro-inflammatory cytokines and pro-inflammatory bacteria within a pathological infectious-inflamed milieu disrupt biochemical events of cell-extrinsic and -intrinsic metabolism (Rampantelli et al., 2025), we measured three key markers of cellular metabolic health. The effects of excess lysine and PAO1 were also reflected in metabolic measurements. Excess lysine in the presence of PAO1 was associated with increased glucose consumption and altered intracellular triglyceride and HDL-C levels, with several changes being more pronounced in senescence-associated cells. IB attenuated some of these treatment-associated metabolic changes. These findings are consistent



(caption on next column)

Fig. 7. Extracellular homocitrulline and spermine levels in young and senescent T84/Huh7 co-cultures following exposure to PAO1 and the indicated treatments. **(A)** Extracellular homocitrulline and **(B)** extracellular spermine concentrations in the culture medium of young and senescent T84/Huh7 co-cultures following the indicated treatments. Cell culture supernatants were collected after 6 h of incubation, and metabolite concentrations were determined and expressed as μM. White bars represent young T84/Huh7 co-cultures, and black bars represent senescent T84/Huh7 co-cultures. Data are presented as mean ± SEM from three independent biological experiments (n = 3) performed on different weeks using newly prepared cell cultures. Two transwell inserts containing T84 cells and two wells containing Huh7 cells were used as technical replicates for each treatment condition within each independent biological experiment. The technical replicates were averaged before statistical analysis and were not treated as independent biological replicates. Statistical analysis was performed using two-way ANOVA with senescence state (young vs. senescent), treatment condition (Control, PAO1, PAO1 + Lys, PAO1 + KPP22, PAO1 + IB, and PAO1 + IB + Lys), and their interaction as factors. Prespecified pairwise comparisons between treatment conditions and between young and senescent co-cultures were adjusted for multiple comparisons using the Holm procedure; when all six treatment conditions within a senescence state were compared, pairwise comparisons were performed separately within the young and senescent co-culture conditions using Tukey's HSD procedure. Different lowercase letters (a, b) indicate significant differences between young and senescent co-cultures within the corresponding treatment condition, whereas different uppercase letters (A–E) indicate significant differences among treatment conditions within the respective young or senescent co-culture condition. Groups sharing the same letter are not significantly different. A two-sided $p < 0.05$ was considered statistically significant. Letters are assigned to the bars in order of decreasing mean values (from highest to lowest, respectively). **Abbreviations:** IB, *Intestinimonas butyriciproducens*; KPP22, PAO1-specific bacteriophage; Lys, lysine; PAO1, *Pseudomonas aeruginosa* PAO1. Exact p -values for only the most biologically relevant statistically significant pairwise comparisons ($p < 0.05$) are shown below. **For panel A:** young Control vs. young PAO1 ($p = 0.025$); young PAO1 vs. young PAO1 + KPP22 ($p = 0.0016$); young PAO1 vs. young PAO1 + Lys ($p = 0.033$); young PAO1 vs. young PAO1 + IB ($p = 0.022$); young PAO1 + KPP22 vs. young PAO1 + IB ($p = 0.041$); young PAO1 + KPP22 vs. young PAO1 + IB + Lys ($p < 0.0019$); and young PAO1 + IB vs. young PAO1 + IB + Lys ($p = 0.017$). Significant differences between young and senescent co-cultures were observed for Control ($p = 0.033$), PAO1 ($p = 0.027$), PAO1 + Lys ($p = 0.040$), PAO1 + KPP22 ($p = 0.028$), PAO1 + IB ($p = 0.044$), and PAO1 + IB + Lys ($p = 0.031$). **For panel B:** young Control vs. young PAO1 ($p = 0.0018$); young PAO1 vs. young PAO1 + KPP22 ($p = 0.0064$); young PAO1 vs. young PAO1 + Lys ($p = 0.038$); young PAO1 vs. young PAO1 + IB ($p = 0.013$); young PAO1 + KPP22 vs. young PAO1 + IB ($p = 0.035$); young PAO1 + KPP22 vs. young PAO1 + IB + Lys ($p = 0.037$); and young PAO1 + IB vs. young PAO1 + IB + Lys ($p = 0.042$). Significant differences between young and senescent co-cultures were observed for Control ($p = 0.0169$), PAO1 ($p = 0.033$), PAO1 + Lys ($p = 0.045$), PAO1 + KPP22 ($p = 0.046$), PAO1 + IB ($p = 0.029$), and PAO1 + IB + Lys ($p = 0.044$).

with an interaction between extracellular nutrient availability, microbial activity, and host-cell metabolism. However, the present experiments do not establish whether the observed metabolic changes result directly from lysine utilization by host cells, bacterial metabolism, inflammatory signaling, or interactions among these processes. Therefore, the metabolic findings should be interpreted as evidence of altered cellular metabolic status under the experimental conditions rather than as evidence of a defined metabolic pathway.

4.3. Lysine utilization and lysine-associated metabolites

Competition for extracellular lysine was examined by determining residual lysine concentrations after incubation. IB showed substantial lysine utilization, while differences were also observed between young and senescent cells. These observations are consistent with competition for extracellular lysine among host cells and bacteria within the co-culture environment. The addition of excess lysine altered the pattern of extracellular metabolites, including glutamate, homocitrulline, and

Table 2

shows competition, exclusion, and displacement of the pathogenic PAO1 strain adhering to young and senescent T84 cell monolayers by the IB bacterium under excess-lysine conditions.

Assay Type	Treatment conditions	Young T84 cells Adherence (%)	Senescent T84 cells Adherence (%)
Competition	PAO1 + Lys	83.7 ± 1.8 ^{Ab}	91.02 ± 1.1 ^{Aa}
	PAO1 + KPP22	29.3 ± 0.4 ^{Db}	35.01 ± 0.9 ^{Da}
	PAO1 + IB	74.3 ± 1.4 ^{Bb}	79.0 ± 1.3 ^{Ba}
	PAO1 + Lys + IB	67.5 ± 1.1 ^{Cb}	70.1 ± 0.4 ^{Ca}
Exclusion	PAO1 + Lys	89.1 ± 1.2 ^{Aa}	93.5 ± 1.1 ^{Ab}
	PAO1 + KPP22	24.2 ± 0.9 ^{Da}	30.4 ± 1.1 ^{Db}
	PAO1 + IB	63.1 ± 1.5 ^{Ca}	71.1 ± 0.8 ^{Cb}
	PAO1 + Lys + IB	80.1 ± 1.0 ^{Ba}	86.1 ± 0.4 ^{Bb}
Displacement	PAO1 + Lys	90.3 ± 1.0 ^{Aa}	94.3 ± 0.9 ^{Ab}
	PAO1 + KPP22	18.3 ± 0.7 ^{Da}	25.1 ± 0.4 ^{Db}
	PAO1 + IB	73.5 ± 1.2 ^{Ca}	80.1 ± 1.5 ^{Cb}
	PAO1 + Lys + IB	83.5 ± 0.8 ^{Ba}	88.1 ± 0.3 ^{Bb}

Table 2 legend

Table 2. Competition, exclusion, and displacement of pathogenic PAO1 adhering to young and senescent T84 cell monolayers by IB under conditions of excess lysine excess available. Three experimental approaches were used: (i) competition assay, in which an IB suspension (5.6×10^6 CFU per insert) and an equal number of PAO1 cells were mixed and co-incubated simultaneously with T84 monolayers; (ii) exclusion assay, in which T84 monolayers were preincubated with IB (5.6×10^6 CFU per insert) for 1 h before the addition of PAO1 (5.6×10^6 CFU per insert); and (iii) displacement assay, in which PAO1 (5.6×10^6 CFU per insert) was first incubated with T84 monolayers for 1 h at 37°C before the addition of IB (5.6×10^6 CFU per insert). Following incubation, transwell inserts were washed three times with sterile DPBS, and cells were lysed with sterile TrypLE Express for 10 min. Appropriate lysate dilutions were plated on Pseudomonas agar medium, and viable cell-associated PAO1 were quantified by colony counting. Adhesion of PAO1 alone to young and senescent T84 cells was assigned a value of 100% for the respective cell type. PAO1 adhesion was calculated as (test/control) $\times$ 100%. Data are presented as mean $\pm$ SEM from three independent biological experiments (n = 3). Two transwell inserts containing T84 cells and two wells containing Huh7 cells were used as technical replicates for each treatment condition within each independent biological experiment. The technical replicates were averaged before statistical analysis and were not treated as independent biological replicates. Statistical analysis was performed using two-way ANOVA with cell senescence state (young vs. senescent), assay type (competition, exclusion, and displacement), and their interaction as factors. Prespecified pairwise comparisons between young and senescent T84 cells within each assay type were adjusted for multiple comparisons using the Holm procedure. When all three assay types within a senescence state were compared, pairwise comparisons were performed separately within the young and senescent cell sets using Tukey's HSD procedure. Different lowercase letters (a, b) within rows indicate significant differences between young and senescent T84 cells within the corresponding assay type, whereas different uppercase letters (A–D) within columns indicate significant differences among assay types within the respective young or senescent cell set. Groups sharing the same letter are not significantly different. A two-sided $p < 0.05$ was considered statistically significant. Letters are assigned to values in order of decreasing mean values (from highest to lowest, respectively). **Abbreviations:** DPBS, Dulbecco's phosphate-buffered saline; IB, *Intestinimonas butyriciproducens* 26243; KPP22, PAO1-specific bacteriophage; Lys, lysine; PAO1, *Pseudomonas aeruginosa* PAO1.

Exact p-values for only the most biologically relevant statistically significant pairwise comparisons ($p < 0.05$) are shown below.

For competition in young T84 cell monolayers: young PAO1 vs. young PAO1 + Lys ($p = 0.038$); young PAO1 vs. young PAO1 + KPP22 ($p = 0.0027$); young PAO1 vs. young PAO1 + IB ($p = 0.031$); and young PAO1 vs. young PAO1 + IB + Lys ($p = 0.016$). Significant differences between young and senescent co-cultures were observed for PAO1 + Lys ($p = 0.024$), PAO1 + KPP22 ($p = 0.033$), PAO1 + IB ($p = 0.046$), and PAO1 + Lys + IB ($p = 0.039$).

For competition in senescent T84 cell monolayers: senescent PAO1 vs. senescent PAO1 + Lys ($p = 0.047$); senescent PAO1 vs. senescent PAO1 + KPP22 ($p = 0.0081$); senescent PAO1 vs. senescent PAO1 + IB ($p = 0.026$); and

senescent PAO1 vs. senescent PAO1 + IB + Lys ($p = 0.013$).

For exclusion in young T84 cell monolayers: young PAO1 vs. young PAO1 + Lys ($p = 0.031$); young PAO1 vs. young PAO1 + KPP22 ($p = 0.0020$); young PAO1 vs. young PAO1 + IB ($p = 0.024$); and young PAO1 vs. young PAO1 + IB + Lys ($p = 0.029$). Significant differences between young and senescent co-cultures were observed for PAO1 + Lys ($p = 0.028$), PAO1 + KPP22 ($p = 0.047$), PAO1 + IB ($p = 0.039$), and PAO1 + Lys + IB ($p = 0.044$).

For exclusion in senescent T84 cell monolayers: senescent PAO1 vs. senescent PAO1 + Lys ($p = 0.042$); senescent PAO1 vs. senescent PAO1 + KPP22 ($p = 0.0097$); senescent PAO1 vs. senescent PAO1 + IB ($p = 0.031$); and senescent PAO1 vs. senescent PAO1 + IB + Lys ($p = 0.033$).

For displacement in young T84 cell monolayers: young PAO1 vs. young PAO1 + Lys ($p = 0.031$); young PAO1 vs. young PAO1 + KPP22 ($p = 0.0011$); young PAO1 vs. young PAO1 + IB ($p = 0.036$); and young PAO1 vs. young PAO1 + IB + Lys ($p = 0.029$). Significant differences between young and senescent co-cultures were observed for PAO1 + Lys ($p = 0.041$), PAO1 + KPP22 ($p = 0.039$), PAO1 + IB ($p = 0.015$), and PAO1 + Lys + IB ($p = 0.045$).

For displacement in senescent T84 cell monolayers: senescent PAO1 vs. senescent PAO1 + Lys ($p = 0.036$); senescent PAO1 vs. senescent PAO1 + KPP22 ($p = 0.0054$); senescent PAO1 vs. senescent PAO1 + IB ($p = 0.041$); and senescent PAO1 vs. senescent PAO1 + IB + Lys ($p = 0.046$).

spermine. These metabolites are associated with lysine-related metabolic pathways; however, their concentrations in the shared culture medium cannot be used to assign a specific metabolic pathway or cellular origin without additional cell-specific and metabolic-flux analyses. The present findings therefore support an association between extracellular lysine availability and changes in selected lysine-associated metabolites but do not establish the complete metabolic fate of lysine in the co-culture system. Collectively, decreased spermine concentrations accompanied by increased homocitrulline and glutamate levels are consistent with alterations in lysine metabolism and may be compatible with 2K6AC formation during lysine utilization (Kochi et al., 1986). In addition, the opposing roles of spermine and homocitrulline in inflammation have been reported (Awwad et al., 2024; Chen et al., 2023). The results from the adhesion experiments further showed that IB reduced PAO1 adhesion to T84 cells under the tested conditions. The effects observed in the competition, exclusion, and displacement experiments suggest that IB can interfere with PAO1–epithelial interactions. The enhancement of these effects in the presence of lysine indicates that extracellular nutrient availability may modify this bacterial interaction. Nevertheless, the molecular mechanisms underlying these effects were not directly investigated and should therefore be considered hypothesis-generating.

4.4. Senescence-associated phenotype and cellular responses

We assessed a combination of complementary indicators of cellular senescence, including cell passage number (Park et al., 2020; Yang et al., 2011), increased levels of the most prominent SASP components IL-6 and IL-8 (Ayoub et al., 2026), and increased intracellular senescence-associated biomarkers, namely SA- β -gal activity and p21 expression (Wang et al., 2024; Yan et al., 2024). The comparison between young and high-passage cells demonstrated consistent differences in cytokine secretion and other cellular responses across the experimental conditions. These findings support the presence of a senescence-associated phenotype in the high-passage cells. Importantly, because cellular passage number alone is insufficient to establish cellular senescence, intracellular SA- β -gal activity and p21 expression were additionally assessed. Both markers were substantially higher in late-passage T84 and Huh7 cells than in their respective early-passage counterparts(cells), providing additional phenotypic evidence of senescence. In particular, p21 represents an important complementary biomarker of cellular senescence, as it plays a key role in mediating senescence-associated cell-cycle arrest (Wang et al., 2024; Yan et al., 2024). Nevertheless, the present characterization does not constitute a comprehensive molecular definition of cellular senescence. Additional

markers, including p16^{INK4a} and p53, together with assessment of proliferative activity using Ki-67 and other established senescence-associated features, could provide complementary evidence and should be incorporated in future studies.

Taken together, the different responses of young and senescence-associated cells indicate that cellular state is an important determinant of the response to the experimental combination of lysine and microbial exposure. However, these results should not be interpreted as direct evidence of physiological responses in older adults. Rather, they provide mechanistic evidence generated using an *in vitro* senescence-associated model.

4.5. Biological significance and comparison with previous work

Previous studies have extensively characterized individual aspects of cellular senescence, microbial pathogenicity, nutrient metabolism, and bacteriophage–bacteria interactions. The present study integrates these components within a defined *in vitro* co-culture system and examines their responses to altered extracellular lysine availability.

A principal finding is that the effects of excess lysine were dependent on the biological context. PAO1 exposure was associated with adverse inflammatory and immunometabolic responses, whereas IB and KPP22 attenuated several of these responses. These observations support the concept that nutrient availability and microbial composition can jointly influence host-cell responses. However, the present study does not establish a causal “lysine transfer” mechanism between individual biological compartments. Such mechanisms would require direct measurements of metabolic flux and cell-specific analyses. The findings therefore provide experimental evidence of interactions among host cells, microbial organisms, bacteriophage, and extracellular lysine within a controlled *in vitro* system. They may be relevant to understanding how nutrient availability interacts with cellular aging-associated phenotypes and microbial perturbations; however, their physiological relevance to older adults requires confirmation in more complex and physiologically representative models.

Fig. 8 provides a schematic summary of the principal findings of the present study. It illustrates the proposed interactions among host cells, PAO1, IB, KPP22, and extracellular lysine within the experimental co-culture system. The figure is intended as a conceptual representation of the observed relationships and proposed interactions rather than as evidence of directly demonstrated molecular mechanisms.

4.6. Limitations

The present study has several limitations. First, the T84–Huh7 co-culture is a reductionist *in vitro* model and does not reproduce the cellular, microbial, immune, stromal, vascular, and metabolic complexity of the human gastrointestinal tract. Accordingly, the observed responses represent interactions under defined experimental conditions rather than direct representations of human intestinal or hepatic physiology.

Second, Huh7 hepatocarcinoma cells were used as the hepatic component of the co-culture. Although this transformed human hepatic model is experimentally tractable, its phenotype and immunometabolic functions differ from those of primary human hepatocytes and may influence metabolic and inflammatory responses. Therefore, findings involving Huh7 cells should be interpreted as reflecting cellular interactions and responses relevant to the intestinal–hepatic axis rather than as a direct representation of normal human hepatic physiology. Future studies using primary hepatocytes, differentiated hepatic models, liver organoids, or organ-on-chip systems would provide valuable validation.

Third, the transwell configuration permits the diffusion of soluble mediators between the cellular compartments. Consequently, cytokine and metabolite concentrations measured in the shared culture environment cannot be unequivocally assigned to T84 or Huh7 cells.

Fourth, the experimental design focused on the PAO1-associated system and was structured to enable clear comparisons among PAO1, excess lysine, KPP22 phage, and the beneficial bacterium IB, rather than to examine all individual components in isolation. Inclusion of a KPP22 alone, a lysine-only condition, and a PAO1 + KPP22 phage + lysine group could have provided additional insight into the independent and combined contributions of lysine supplementation and KPP22 phage-mediated bacterial clearance. Therefore, the independent contribution of each component cannot be fully separated, and the findings should be interpreted within the defined experimental design.

Finally, only selected lysine-associated metabolites were measured. Important intermediates of lysine metabolism, including saccharopine and amino adipate, were not quantified. Targeted or untargeted metabolomic approaches and metabolic-flux analyses will be required to provide a more comprehensive assessment of lysine metabolism.

5. Conclusions

The present study shows that the senescent state of eukaryotic host cells and phage activity shape the immunometabolic outcome of the competition for lysine among host cells, pathogenic bacteria, and beneficial bacteria in a defined *in vitro* host–microbe–phage–nutrition system. Hence, excess lysine availability and infectious pathological inflammation may further disrupt these immunometabolic interactions and perturb lysine homeostasis. Excess lysine was associated with altered cytokine secretion and immunometabolic responses in PAO1-exposed co-cultures, while several of these effects were attenuated in the presence of IB or the PAO1-specific KPP22 phage. The responses differed between young and senescence-associated cells, indicating that cellular state influences the response to the experimental conditions. Collectively, these findings provide mechanistic evidence from an *in vitro* senescence-associated model that nutrient availability and microbial interactions can modify host-cell responses. They should not, however, be interpreted as direct evidence of physiological responses to lysine supplementation in older adults. Further studies using more physiologically complex models, including primary human cells, organoid-based systems, and appropriate *in vivo* models, are needed to

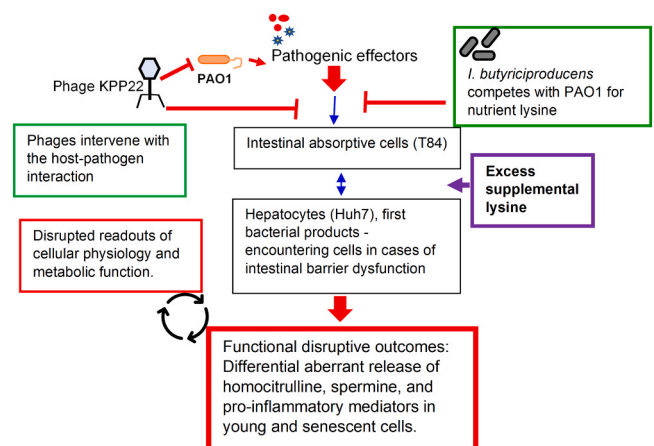


Fig. 8. Schematic diagram illustrating the sites and mechanisms associated with the inhibition of lysine flux from co-cultured host cells to pathogenic *P. aeruginosa* PAO1 in the presence of the *P. aeruginosa*-specific KPP22 phage and the beneficial bacterium IB within a complex host–microbe–phage–nutrition interaction. Excess bioavailable lysine within the infectious, inflamed gastrointestinal milieu may contribute to barrier dysfunction, initiating a vicious cycle of barrier disruption in nutrient-absorbing enterocytes and absorptive colonocytes and infectious inflammation in hepatocytes, which are essential for nutrient processing and blood filtration. The beneficial bacterium IB and *P. aeruginosa*-specific KPP22 phage intervene in this vicious cycle and improve host immunometabolic health.

determine whether and to what extent these interactions occur in the aging human gastrointestinal environment.

CRediT authorship contribution statement

Darab Ghadimi: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sophia Blömer:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation, Conceptualization. **Aysel ŞAHİN KAYA:** Writing – original draft, Validation, Supervision, Methodology, Conceptualization. **Sandra Krüger:** Writing – review & editing, Writing – original draft, Validation, Methodology, Conceptualization. **Christoph Röcken:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Conceptualization. **Heiner Schäfer:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Conceptualization. **Geraldine de Heer:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Conceptualization. **Dominik Jarczak:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Conceptualization. **Jumpei Uchiyama:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Shigenobu Matsuzaki:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Conceptualization. **Wilhelm Bockelmann:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

Author Contributions

(a) All co-authors have contributed significantly to the conception, design, execution, and/or analysis and interpretation of data; (b) they have participated in drafting, reviewing, editing, and/or revising the manuscript for intellectual content, and/or (c) approved the manuscript for publication.

Informed Consent Statement

Not applicable.

Animal study statements

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

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors declare that the licensed Writefull software was used to proofread the manuscript and improve its language and readability.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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