Current Research in Microbial Sciences 7 (2024) 100294

Contents lists available at ScienceDirect

Current Research in Microbial Sciences

journal homepage: www.sciencedirect.com/journal/current-research-in-microbial-sciences

Subinhibitory concentrations of meropenem stimulate membrane vesicle production and modulate immune response in *Bacteroides fragilis* infection

Saniya Kozhakhmetova [a,*], Ayazhan Bekbayeva [a], Elena Zholdybayeva [a], Tatyana Krivoruchko [a], Natalya Dashevskaya [a], Zhanel Mukhanbetzhanova [b], Elizaveta Vinogradova [b], Almagul Kushugulova [b], Samat Kozhakhmetov [b]

[a] National Scientific Shared Laboratory of Biotechnology, National Center for Biotechnology, Astana, Kazakhstan
[b] Laboratory of Microbiome, National Laboratory Astana, Nazarbayev University, Astana, Kazakhstan

ARTICLE INFO

Keywords:
Bacteroides fragilis
Subinhibitory concentrations
Meropenem
Membrane vesicles
Inflammatory response
Cytokine modulation
Bacterial adaptation

ABSTRACT

This study explores an adaptation mechanism of *Bacteroides fragilis* to subinhibitory concentrations of meropenem, characterized by an alteration in the production of membrane vesicles (MVs) and modulation of the host inflammatory response. Using a rat model of infection, we demonstrated a significant increase in the size of MVs accompanied by a nonsignificant increase in their number in the meropenem-treated group compared to the infected control. Both infected groups showed significantly altered hematological parameters and shifts in monocyte on day 8 (average increase of 21.5 %). At the same time, significant changes in neutrophils (decrease by 26 %) and eosinophils (increase by 3 %) were observed only in the infected group but not in the infected meropenem-treated group. On day 16, increased macrophage activation, neovascularization, and fibrosis were observed in the tissues of the antibiotic-treated group. Immunological profile analysis revealed a slight increase in the levels of pro-inflammatory cytokines (IL-5, IL-6, IFN-γ and G-CSF) on day 8 of the experiment, followed by a sharp decrease on day 16 in both infected groups compared to the negative control. At the same time, network analysis of correlations between these immunological factors showed complex changes in response to subinhibitory concentrations of meropenem. The bacterial load did not differ between the infected groups on days 8 and 16, but only in the meropenem-free group a significant decrease in the number of bacteria was observed on day 16 in all samples. These findings suggest that subinhibitory antibiotic concentrations can influence the pathophysiological progression of *B. fragilis* infection, modulating both the bacterial response and the host immune reaction, potentially leading to a more complex and chronic disease course.

1. Introduction

Bacteroides fragilis, a commensal intestinal symbiotic bacterium, produces membrane vesicles (MVs) that play a crucial role in host-microbe interactions. These MVs can carry diverse contents from their parent microorganisms and transport various inflammatory substances, including lipopolysaccharides, phospholipids, outer membrane proteins, periplasmic components, DNA, RNA, and virulence factors such as enzymes and toxins (Toyofuku et al., 2019; Toyofuku et al., 2023). *B. fragilis* exhibits both intrinsic and acquired resistance to various antibiotics. Intrinsic resistance mechanisms in B. fragilis include efflux pumps and β-lactamases, conferring resistance to many β-lactams. Additionally, *B. fragilis* can acquire resistance to tetracyclines, fluoroquinolones, and macrolides through mutations and horizontal gene transfer, further expanding its antimicrobial resistance profile. Carbapenems are among the most effective agents against highly resistant *B. fragilis* strains. Alongside metronidazole, carbapenems are considered the drugs of choice for antibacterial therapy of *B. fragilis* infections, particularly in polymicrobial intra-abdominal and soft tissue infections, due to their broad antibacterial spectrum and efficacy against both anaerobic and facultatively anaerobic resistant pathogens (Yekani et al., 2022).

Antimicrobial agents, such as antibiotics, can enhance the production and release of MVs, leading to exacerbation of inflammatory responses and cytokine modulation in experimental infections (Ribeiro de Freitas et al., 2022). *B. fragilis* MVs are characterized by an enriched

* Corresponding author at: National Scientific Shared Laboratory of Biotechnology, National Center for Biotechnology, 13/5, Kurgalzhynskoye road, Astana, 010000, Kazakhstan.
E-mail address: kozhakhmetova@biocenter.kz (S. Kozhakhmetova).

https://doi.org/10.1016/j.crmicr.2024.100294

Available online 18 October 2024

immunostimulatory cargo, including proteins, nucleic acids, and peptidoglycan, enabling them to activate a broader spectrum of innate immune receptors than their parent bacteria (Gilmore et al., 2022), which underlies their multifunctionality in immune modulation. Antibiotic exposure also increases vesicle movement and dissemination across bacterial surfaces (Bos et al., 2021).

Notably, MVs from Bacteroides strains contain surface β-lactamases capable of degrading antibiotics such as cefotaxime, potentially protecting both commensal bacteria and enteric pathogens from β-lactam antibiotics (Stentz et al., 2015). *B. fragilis* MVs exhibit both hemagglutinating and enzymatic activity (Patrick et al., 1996) and can activate a more comprehensive range of innate immune receptors compared to intact bacterial cells (Gilmore et al., 2022), indicating their unique role in immune signaling. The difference in vesicular cargo composition between enterotoxigenic and non-toxigenic bacteria exerts differential effects on the host organism (Zakharzhevskaya et al., 2017).

Furthermore, histological evidence suggests that *B. fragilis* vesicles are involved in bacterial pathogenicity, and antibiotic administration stimulates the immune system (Ribeiro de Freitas et al., 2022). Another study indicates an immunomodulatory effect of *B. fragilis* MVs by reducing IFNγ concentration (Ahmadi Badi et al. 2019). These findings demonstrate the complex interaction between antibiotics, bacterial vesicles, and host immune responses. We hypothesize that subinhibitory concentrations of meropenem induce an adaptive response in *B. fragilis*, characterized by enhanced production of MVs, leading to modulation of the host immune response and potentially influencing infection pathogenesis and antibiotic therapy efficacy.

2. Materials and methods

2.1. Object of study

The study utilized a multidrug-resistant strain of B. fragilis, designated as BFR KZ01 (BioProject: PRJNA531645; NCBI Sequence Read Archive (SRA) data available at (URL https://www.ncbi.nlm.nih.gov/sra, accession numbers SRX22081155). This strain exhibits resistance to metronidazole, tetracycline, and ciprofloxacin. The isolate was obtained from a 48-year-old male patient diagnosed with acute gangrenous perforated appendicitis complicated by peritonitis (Kozhakhmetova et al., 2021).

2.2. Cultivation of microorganisms

B. fragilis BFR KZ01 was cultivated for 48 h on Bacteroides Bile Esculin Agar (Condalab, Cat # 1031) at 37 °C under anaerobic conditions using an AnaeroGen gas-generating system (Thermo Fisher Scientific, Cat # AN0020D).

Culture purity was monitored at each stage using MALDI-TOF-MS mass spectrometry with the MALDI Biotyper 3.1 database (Bruker Daltonics). The bacterial culture was applied to a 96-well MSP chip (MSP 96 target polished steel BC, microScout Target). One microliter of matrix solution (saturated solution of α-Cyano-4-hydroxycinnamic acid (α-HCCA) containing 50 % acetonitrile and 2.5 % trifluoroacetic acid (TFA)) was applied to the culture and dried at room temperature. 40 laser pulses (60 Hz frequency) were used to obtain individual spectra. The analyzed mass-to-charge ratio range was 2000–20,000 Da.

2.3. Abdominal infection model

The rat tissue cage model for intraperitoneal abscess infection was adapted from Ribeiro de Freitas et al. (2022). This rat tissue cage model was chosen for its suitability in studying B. fragilis infections and host-pathogen interactions. The model allows for localized infection and abscess formation, mimicking clinical scenarios of intra-abdominal abscesses caused by *B. fragilis*. It provides a protected environment for bacterial growth, simulating the anaerobic conditions that B. fragilis encounters in vivo. Typical disease manifestations in this model include localized inflammation, abscess formation, and systemic immune responses, which were observed and reported in our study. While this model may not replicate all aspects of *B. fragilis* infections seen in humans, it provides valuable insights into the complex interactions between the bacteria, subinhibitory antibiotic concentrations, and the host immune system. The experimental abdominal infection model utilized 42 male Wistar rats aged 10–12 months, weighing over 450 g. All procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986 and associated guidelines, as well as EU Directive 2010/63 on the protection of animals used for scientific purposes. The study was approved by the Bioethics Committee of the National Center for Biotechnology of the Republic of Kazakhstan (Protocol No. 9, dated November 7, 2022). The animals were divided into three groups: NG (negative control, $n = 15$), intraperitoneally injected with 1.0 mL of 0.85 % NaCl. PG (positive control, $n = 14$) and EG (experimental group, $n = 13$): Perforated balls (35 mm diameter, 300 holes of 1.5 mm diameter), pre-sterilized by autoclaving, were surgically implanted into the intraperitoneal cavity via laparotomy. After a 6-week recovery period allowing for vascularization of the balls, animals were transcutaneously inoculated with 1.0 mL of *B. fragilis* BFR KZ01 (1 × 10^8 CFU/mL) in 0.85 % NaCl. The EG group additionally received subinhibitory concentrations of meropenem (MIC 1 mg/L) every 48 h for eight days. Exudate samples from the abdominal cavity and accumulated in the perforated ball holes were collected on days 8 and 16 post-inoculation.

2.4. Blood analysis

For quantitative and qualitative assessment of blood cell populations, samples were collected in single-use polypropylene tubes containing EDTA-K2 (BD Vacutainer). Erythrocyte, platelet, and leukocyte counts, along with leukocyte differential, were analyzed in peripheral blood using an automated hematology analyzer UniCel® DxH 800 (Beckman Coulter) according to the manufacturer's instructions. Blood samples from each animal were analyzed at all time points. Each sample was analyzed in triplicate.

2.5. Quantitative assessment of B. fragilis

Quantification of *B. fragilis* BFR KZ01 cells in the vascularized ball at 8- and 16-days post-inoculation was performed using real-time quantitative PCR. Primers Bfr-F (5′ - CTGAACCAGCCAAGTAGCG - 3′) and Bfr-R (5′-CGCAAACTTTCACAACTGACTTA - 3′) were used with the BioRad CFX 96 Touch amplifier (BioRad, USA) and HS-qPCR Syber Blue (2x) (Biolabmix, Cat # MHC030-400). Amplification specificity was determined through melting curve analysis. Quantitative PCR was performed on samples from each animal at all time points. Each sample was analyzed in triplicate.

2.6. Tissue analysis

Morphological evaluation of meropenem's biological effects on abdominal cavity connective tissue was conducted on tissue fragments obtained from the encapsulated ball. Samples were fixed in a 10 % buffered formalin solution. Histological Sections (3–5 μm thick) were prepared using a CUT 4062 rotary microtome (SLEE medical, Germany) and stained with hematoxylin and eosin following standard protocols. Van Gieson staining was employed for connective tissue differentiation (Gattuso et al., 2009). Microscopic examination was performed using a CELENA® X High Content Imaging System (Logos Biosystems). Three histological sections were analyzed from each animal at all time points. Each sample was analyzed in triplicate.

2.7. Transmission Electron microscopy

Transmission electron microscopy (TEM) was performed on

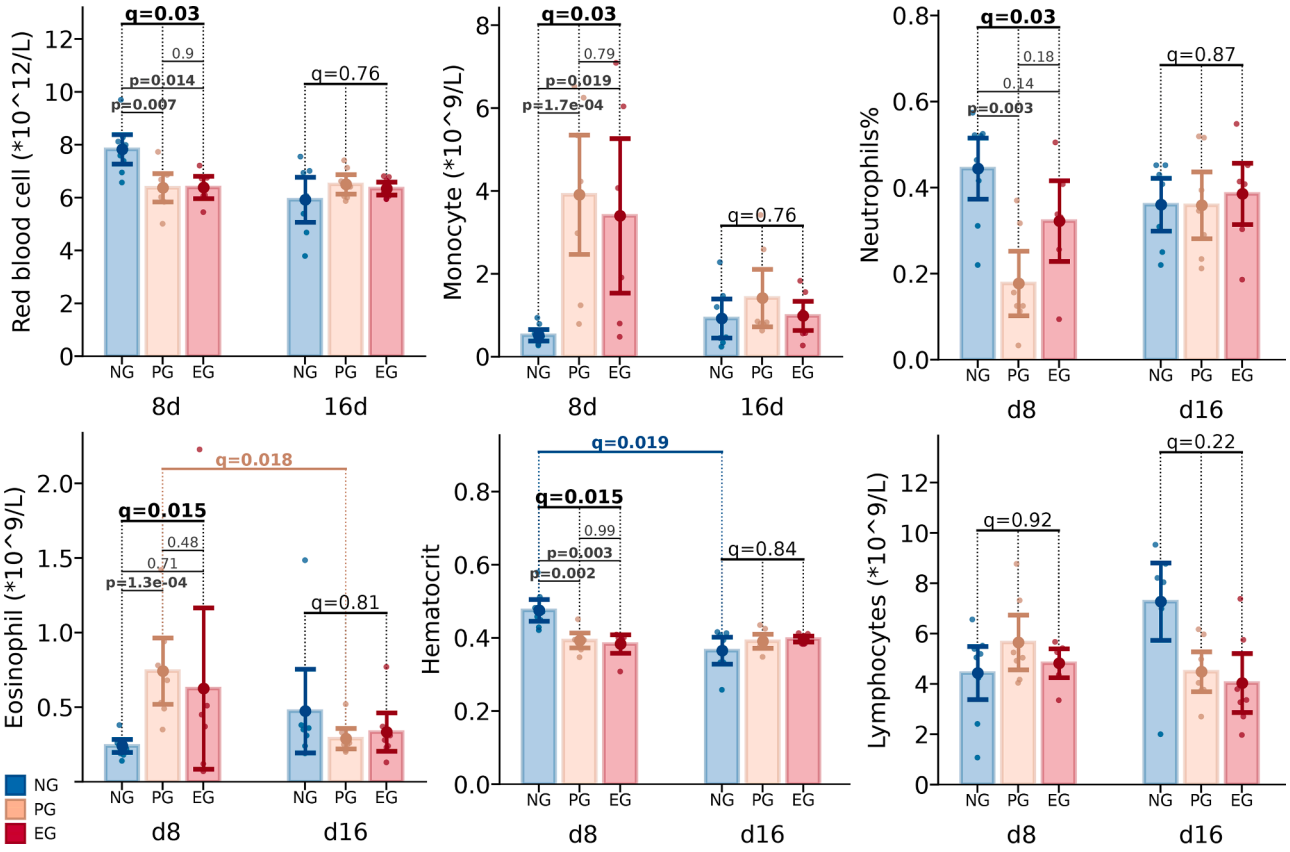

Fig. 1. Dynamics of haematological parameters in the rat experimental groups. NG - negative control group; PG - positive control group; EG - experimental group. 8d - day 8 of the experiment, 16d - day 16 of the experiment.

experimental material fixed in 2.5 % glutaraldehyde solution in phosphate buffer (pH 7.4). The samples were post-fixed in 2 % osmium tetroxide solution at 4 °C, washed in phosphate buffer, and dehydrated in a graded ethanol series (50 %, 70 %, 96 %, 100 %). All specimens were then embedded in an epon-araldite mixture.

Ultrathin sections (60 nm thickness) were obtained using a Leica UC7 ultramicrotome (Leica). Morphological assessment was conducted using a JEM 1400Plus transmission electron microscope (Jeol) equipped with a OneView CCD camera (Gatan, Germany). Images were acquired using an InLens detector at various magnifications up to 50,000×.

Quantification of MVs was performed using transmission electron microscopy (TEM) images. For each experimental group, a minimum of 25 random electron micrographs per animal were evaluated at 10,000× magnification (accelerating voltage of 80 kV). MVs were identified based on their characteristic spherical morphology and size range (typically 20–200 nm in diameter). The number of MVs per field was manually counted by two independent observers. The mean number of MVs per field was calculated for each group, and statistical analysis was conducted to compare MV production between groups.

2.8. Immunological assay

Multiplex analyses were conducted to evaluate the cytokine/chemokine profile in rat serum using the Luminex FLEXMAP 3D Instrument System (Thermo Fisher Scientific). The ProcartaPlex Rat Cytokine & Chemokine Panel 22plex (Thermo Fisher Scientific, Cat # EPX220-30122-901) was employed following the manufacturer's protocol. Data analysis was performed using the xPONENT software. Three histological sections were analyzed from each animal at all time points. Each sample was analyzed in triplicate.

2.9. Statistical analysis

Statistical analysis was performed in Python 3.9.16 using SciPy v1.13.1. Three group comparisons were performed using one-way Anova or Kruskal-Wallis tests where appropriate. Pairwise independent T-test with or without Welch correction or Mann-Whitney U tests were applied for post hoc comparison. Chi-square test was used to test differences in proportions. When comparing hematologic data, the FDR-BH multiple testing adjustment (denoted as q) was applied. A comparison of immunological data was performed without multiple comparison adjustments due to the low variability of these data (denoted as p). Network analysis was performed with NetworkX v3.2.1. Correlation was calculated using Spearman's r coefficient for each group independently. The significance level for all tests was set at 0.05 for raw and adjusted values. Principal coordinate analysis was performed using scikit-learn v1.5.1 on standard scaled data. Visualization was performed using Matplotlib v3.7.1 and seaborn v0.11.2.

3. Results

The experimental model investigating the effects of subinhibitory concentrations of meropenem on inflammatory response modulation and extracellular membrane vesicle production by the multidrug-resistant *B. fragilis* strain BFR KZ01 revealed characteristic changes in hematological parameters.

In typical B. fragilis infections, characteristic changes in hematological parameters often include an increase in white blood cell count, particularly neutrophils, as part of the acute inflammatory response. This is usually accompanied by a decrease in red blood cell count and hematocrit. Additionally, an elevation in monocyte and eosinophil counts is commonly observed, reflecting the ongoing immune response to the bacterial challenge.

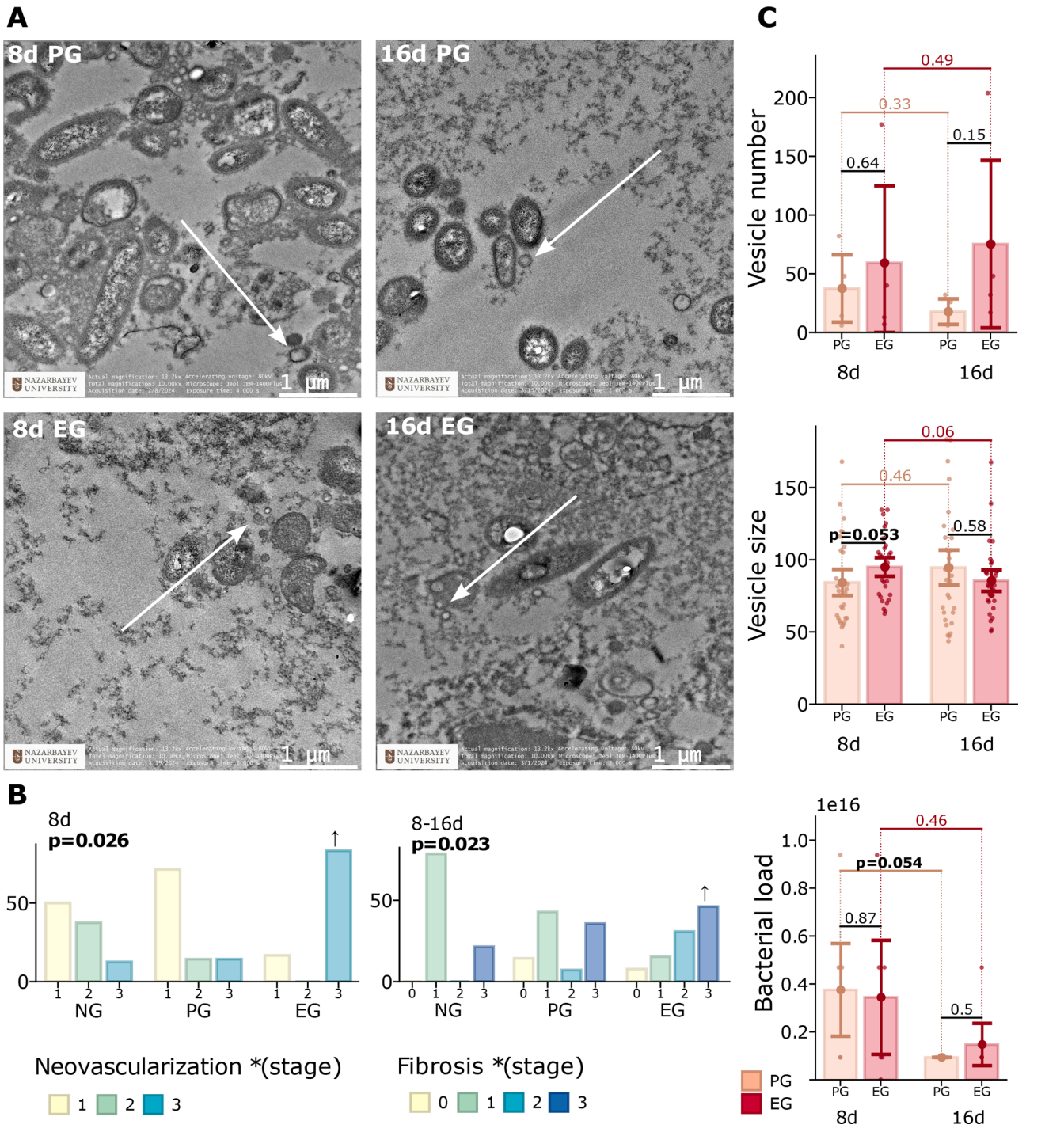

Fig. 2. Effects of subinhibitory concentrations of meropenem on MV production and *B. fragilis* growth in a rat model. A) MV production in PG and EG groups on days 8 and 16. Scale bars: 1 μm. MVs are indicated by red arrows and are characterized by their spherical shape and size range of approximately 20–200 nm in diameter. B) Changes in abscess model tissue, neovascularization, and fibrosis percentage by NG, PG, EG group. C) Bacterial load per 1 mL of exudate in PG and EG groups.

In our study, we observed on day eight post-infection, a decrease in erythrocyte count (PG, $p = 0.007$; EG, $p = 0.014$), neutrophil count (PG, $p = 0.003$; EG, $p = 0.14$), and hematocrit (PG, $p = 0.002$; EG, $p = 0.003$) was observed, accompanied by a significant increase in monocyte and eosinophil counts (Fig. 1). However, by day 16, most parameters had normalized, with the exception of an elevated lymphocyte count ($q = 0.22$, $p = 0.03$).

We observed increased MV production in the EG group compared to the PG group on both day 8 and day 16 of the experiment (Fig. 2), which may be attributed to the antibiotic effect. Furthermore, on day 8 post-infection, an increase in vesicle size was noted in the EG group (average size 95.00 nm vs 84.25 nm for PG, $p = 0.053$), whereas by day 16, their size decreased ($p = 0.06$). Bacterial load quantification per 1 mL of exudate revealed slightly higher *B. fragilis* colonization in the EG group compared to the PG group by day 16 (Fig. 2).

Histological examinations revealed the presence of fibrosis in the tissues of animals from all groups. The NG group predominantly exhibited first-degree fibrosis, while third-degree fibrosis was more common in the PG and EG groups ($p = 0.023$). Additionally, the EG group was characterized by a significant enhancement in

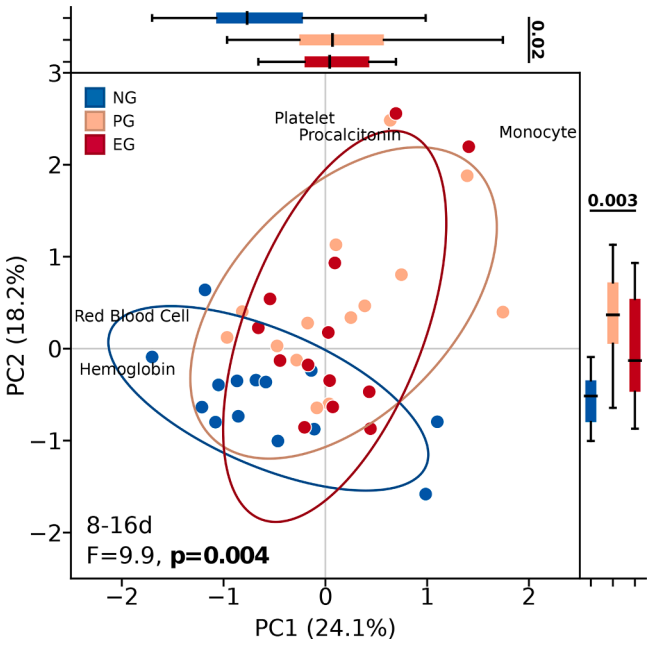

Fig. 3. Principal Component Analysis of hematological parameters across different groups in the experimental model of *B. fragilis*-induced abscess. The scatter plot illustrates the distribution of samples in the space defined by the first two principal components (PC1 and PC2), which account for 24.1 % and 18.2 % of the data variability, respectively. Box plots flanking the right and top margins of the graph depict the distribution of PC1 and PC2 values for each experimental group.

neovascularization ($p = 0.026$), a finding that could have potential implications for future research and treatment strategies.

Administration of subinhibitory concentrations of meropenem resulted in increased inflammatory response and fibrosis in the animal model, as evidenced by histological examination (Fig. 2 and Supplement figure 1) (Fig. 3).

Our use of Principal Component Analysis was instrumental in visualizing the differences in hematological profiles between groups and assessing the impact of *B. fragilis* infection and meropenem treatment on these parameters in the abscess model. The graph depicts vectors corresponding to various hematological parameters: erythrocytes, hemoglobin, platelets, procalcitonin, and monocytes. Their positioning indicates their contribution to group separation. Statistical analysis ($F = 9.9$, $p = 0.004$) shows significant differences between EG and PG groups on days 8–16 of the experiment and the negative control group (NG).

Examination of the overall immunological response using multiplex detection of chemokines and cytokines (ProcartaPlex Rat Cytokine & Chemokine Panel 22plex) in the serum of experimental animals revealed specific features of the immunological profile in response to *B. fragilis* inoculation and antibiotic induction. We observed substantial changes in the concentrations of four key inflammatory mediators (Fig. 4).

IL-6 concentration was significantly elevated in both PG and EG groups on day 8 of the experiment, with a more pronounced increase in the EG group. By day 16, IL-6 levels decreased in both groups but remained above control values.

IFN-γ concentration was elevated in PG and EG groups on day 8, with a more marked increase in the EG group. On day 16, IFN-γ levels decreased compared to the negative control group.

IL-5 levels showed a moderate increase in PG and EG groups on day 8 of the experiment. By day 16, IL-5 concentration decreased in both groups but was significantly lower than the control group level ($p = 0.024$).

G-CSF (CSF-3) demonstrated a notable concentration increase in the PG and EG groups on day 8 of the experiment compared to the NG control group. By day 16, G-CSF levels decreased in all groups but remained higher in PG and EG compared to NG.

All four cytokines - IL-6, IFN-γ, G-CSF (CSF-3), and IL-5 - are classified as proinflammatory cytokines. Their elevated levels in the experimental groups indicate activation of a proinflammatory immune response to *B. fragilis* inoculation and exposure to subinhibitory concentrations of the antibiotic.

These data demonstrate dynamic changes in crucial cytokine concentrations in response to *B. fragilis* introduction and exposure to subinhibitory antibiotic concentrations, with the most pronounced changes observed on day 8 of the experiment (Fig. 5).

Network analysis of correlation patterns among various immunological factors revealed complex systemic changes in the immune response. The experimental group receiving subinhibitory doses of meropenem (EG) demonstrated the most pronounced alterations in network complexity, particularly from day 8 to day 16 post-infection. Meanwhile, the positive control group (PG) showed an increase in network complexity over time, suggesting a natural progression of infection and immune response.

The EG group initially exhibited a highly complex network, followed by a significant reduction in its complexity. This pattern may indicate that subinhibitory antibiotic exposure induces more profound systemic changes than the infection alone. The persistent complexity in the composite network (d8-d16) within the EG group suggests the long-term effects of the antibiotic on host-pathogen interactions. In contrast, the negative control group also displayed a complex network of mutual associations at both time points.

4. Discussion

Our study reveals a novel adaptation mechanism of *B. fragilis* in response to subinhibitory concentrations of meropenem, characterized by enhanced MV production and modulation of the host inflammatory response. This discovery complements our previous genomic observations and complex host-pathogen interactions in vivo.

Our prior work, demonstrated radical changes in *B. fragilis* gene expression profiles in response to meropenem treatment, particularly activating seven genes associated with transmembrane transport and antimicrobial efflux mechanisms (Zholdybayeva et al., 2024). Based on these genetic analysis results, our current study utilized a rat model of *B. fragilis* BFR KZ01 infection to examine the effects of subinhibitory meropenem concentrations on MV production and inflammatory reactions.

We observed a notable difference in MV size in response to antibiotic administration, correlating with previously shown activation of transmembrane transport genes. Existing hypotheses on MV biogenesis suggest both vesicle formation resulting from bacterial lysis and the existence of a specific process for vesicle formation and packaging of specific cargo (Sartorio et al., 2023). This suggests a complex adaptive response of *B. fragilis* to subinhibitory meropenem concentrations. Although we did not demonstrate direct meropenem efflux through vesicles in this study, the observed MV alteration aligns with reports of similar phenomena in other bacteria exposed to subinhibitory antibiotic concentrations, such as metronidazole (Ribeiro de Freitas et al., 2022) and amoxicillin (Onorini et al., 2023). Studies conducted on other microorganisms have demonstrated that antibiotics can significantly increase the production of membrane vesicles (MVs) in various bacterial species, including Escherichia coli and Pseudomonas aeruginosa. Subinhibitory concentrations of several antibiotics, such as ciprofloxacin, meropenem, and polymyxin B, can enhance MV secretion and alter their characteristics, including size and zeta potential. Antibiotic-induced MVs may carry elevated levels of virulence factors and resistance genes, potentially exacerbating infections and contributing to antimicrobial resistance (Andreas et al., 2017; Li et al., 2024). Furthermore, MVs produced under antibiotic stress conditions may elicit stronger immune responses, including enhanced antibody production and

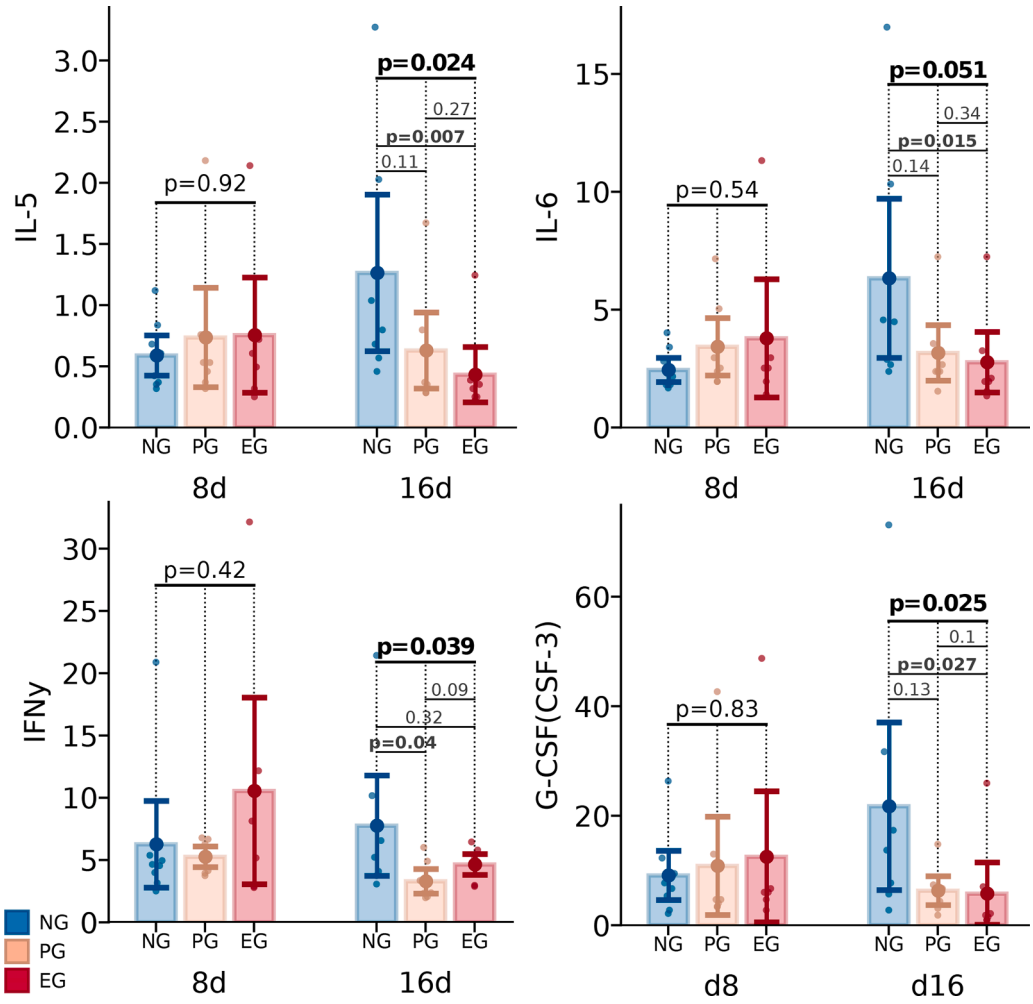

Fig. 4. Dynamics of IL-5, IL-6, IFN-γ and G-CSF (CSF-3) concentrations in the serum of experimental animals. NG - negative control group; PG - positive control group; EG - experimental group.

cytokine release (Hadadi-Fishani et al., 2021).

The enhanced production of MVs by B. fragilis under subinhibitory concentrations of meropenem aligns with recent advancements in the field of synthetic biology and MV engineering. Kelwick et al. (2023) have highlighted the potential of utilizing bacteria as delivery vectors for engineered MVs (Kelwick et al., 2023), which could have significant implications for therapeutic applications. The ability to modulate MV production through controlled antibiotic exposure may provide a valuable tool for optimizing MV-based delivery systems. Moreover, the altered characteristics of antibiotic-induced MVs, such as changes in size and potentially cargo composition, could be exploited to enhance their efficacy as carriers of therapeutic molecules. These findings open new avenues for developing targeted therapeutic strategies and improving our approaches to combating bacterial infections.

Notably, despite the absence of clear distinctions between the experimental group (EG) and positive control group (PG) in principal component analysis (PCA), we detected significant systemic shifts in the hematological profiles of *B. fragilis*-infected rats. Our observations are consistent with a growing body of literature demonstrating that individual hematological parameters may exhibit increased sensitivity to *B. fragilis*-induced changes compared to complex analytical approaches. For instance, Flores et al. (2014) revealed that individual variability in microbiome-associated changes might surpass the significance of group treatment effects, emphasizing the need for more detailed analysis at the individual subject level (Flores et al., 2014).

Wexler et al. (2007) demonstrated *B. fragilis'* ability to induce specific changes in certain immune cell populations (Wexler et al., 2007). These shifts in immune profile may not be adequately reflected in the generalized picture provided by PCA, indicating the need for more sensitive and specific analytical methods.

Our results provide new insights into *B. fragilis* adaptive strategies under antibiotic pressure and their potential impact on host-pathogen communication dynamics. These findings not only expand our understanding of bacterial adaptability mechanisms but also have practical implications for optimizing antibiotic dosing strategies in clinical practice.

The observed immunological shifts likely result from a cascade of events, including reduced neutrophil production, macrophage activation, and acute phase protein induction. By day 16, a slight decrease in lymphocyte production was noted, indicating the development of an adaptive immune response. This progression aligns with the classical model of immune response in bacterial infections and may explain the observed differences in fibrosis and neovascularization between EG and PG animal groups.

Importantly, while *B. fragilis* cell counts were approximately equal in both groups on day 8, by day 16, the EG group showed increased bacterial colonization and neovascularization compared to the positive control group without antibiotic treatment. This was accompanied by a sustained increase in membrane vesicle sizes, suggesting a potential bacterial adaptation mechanism.

The observed increase in bacterial colonization and enhanced neovascularization in the experimental group (EG) by day 16 of the study

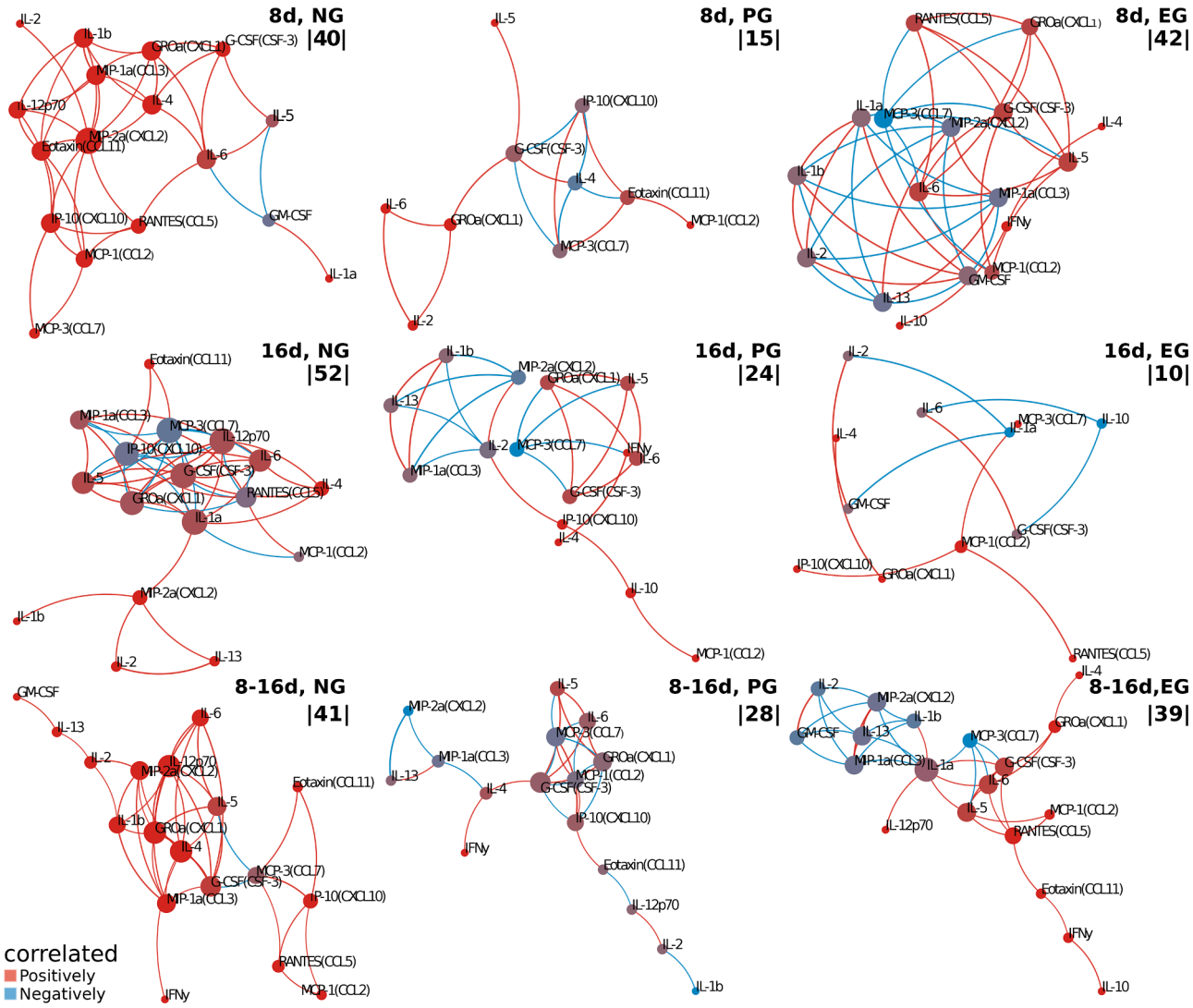

Fig. 5. Network analysis of correlations between various immunological factors across different experimental groups and time points. Animals Groups: NG - negative control group; PG - positive control group (infected); EG - experimental group (infected, treated with subinhibitory concentrations of meropenem). Time Points: d8 - day 8; d16 - day 16; d8–16 - combined analysis of days 8–16. Network structure: each node represents a different cytokine or chemokine measured in the experiment. Red lines indicate positive correlations. Blue lines indicate negative correlations. The thickness of the point represents the strength of the correlation.

presents an intriguing phenomenon that the complex interaction between *B. fragilis*, subinhibitory meropenem concentrations, and the host immune system can explain. We posit that several interrelated mechanisms mediate this effect. Primarily, subinhibitory meropenem concentrations may induce an adaptive response in *B. fragilis*, leading to increased expression of genes associated with colonization and survival. Choi et al. (2016) demonstrated that exposure to subinhibitory antibiotic concentrations can activate stress response mechanisms in bacteria, enhancing their ability to adhere and form biofilms (Choi et al., 2016). In our case, this may explain the enhanced *B. fragilis* colonization in the EG group.

Round and Mazmanian (2010) demonstrated *B. fragilis'* ability to induce Foxp3+ regulatory T-cell (Treg) development in the intestine (Round and Mazmanian, 2010). They showed that polysaccharide A (PSA), produced by *B. fragilis*, stimulates Treg development through TLR2 signaling, leading to suppression of Th17 inflammatory responses. In the context of our study, this may explain why subinhibitory meropenem concentrations result in an altered immune response. It is possible that in the presence of the antibiotic, *B. fragilis* enhances the production of PSA or other immunomodulating factors, leading to a more favorable environment for colonization. This immunomodulation may also contribute to the observed neovascularization, as the altered balance of pro- and anti-inflammatory factors can influence angiogenesis.

Furthermore, the enhanced neovascularization in the EG group may result from combined bacterial colonization and antibiotic exposure. *B. fragilis* is known for its ability to induce angiogenesis through various mechanisms, including vascular endothelial growth factor production (Vieira et al., 2016).

These interconnected mechanisms create a complex picture of *B. fragilis* adaptation to subinhibitory antibiotic concentrations, leading to paradoxical enhancement of colonization and stimulation of neovascularization.

Our results demonstrate that subinhibitory meropenem concentrations significantly influence the pathophysiological development of *B. fragilis* infection, modulating both the bacterial response and the host immune reaction. This leads to a more complex and potentially chronic disease course, underscoring the need to consider antibiotic dosing strategies in clinical practice carefully. The obtained results highlight the profound impact of subinhibitory antibiotic concentrations on *B. fragilis* infection dynamics, opening new avenues for research into host-pathogen interactions and personalized medical approaches to

treating infectious diseases.

CRediT authorship contribution statement

Saniya Kozhakhmetova: Investigation, Supervision, Resources, Formal analysis, Writing – original draft, Writing – review & editing. **Ayazhan Bekbayeva:** Methodology, Writing – original draft. **Elena Zholdybayeva:** Formal analysis, Writing – review & editing. **Tatyana Krivoruchko:** Formal analysis, Writing – review & editing. **Natalya Dashevskaya:** Writing – review & editing. **Zhanel Mukhanbetzhanova:** Methodology, Writing – original draft. **Elizaveta Vinogradova:** Writing – original draft, Data curation, Software, Visualization. **Almagul Kushugulova:** Writing – original draft, Writing – review & editing. **Samat Kozhakhmetov:** Writing – original draft, Writing – review & editing.

Funding sources

This research has been is funded by the Science Committee of the Ministry of Science and Higher Education of the Republic of Kazakhstan (Grant No. AP19679239).

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

During the preparation of this work the authors used Claude AI to improve language and readability. After using this service, the authors reviewed and edited the content as needed and takes full responsibility for the content of the publication.

Declaration of competing interest

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.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2024.100294.

Data availability

All data relevant to this study are fully presented in the manuscript. No additional datasets were generated or analyzed during the course of this work.

References

Ahmadi Badi, S., Khatami, S.H., Hiva Irani, S., Siadat, S.D., 2019. Induction effects of Bacteroides fragilis Derived outer membrane vesicles on toll like Receptor 2, toll like Receptor 4 genes expression and cytokines concentration in human intestinal epithelial cells. Cell J. 21 (1), 57–61. https://doi.org/10.22074/cellj.2019.5750.

Andreas, B., Lisa, K., Helge, K., Alexander, M., Martina, B., 2017. Antibiotic-mediated modulations of outer membrane vesicles in enterohemorrhagic Escherichia coli O104:H4 and O157:H7. Antimicrob. Agents Chemother. 61 (9). https://doi.org/10.1128/aac.00937-17.

Bos, J., Cisneros, L.H., Mazel, D., 2021. Real-time tracking of bacterial membrane vesicles reveals enhanced membrane traffic upon antibiotic exposure. Sci. Adv. 7 (4). https://doi.org/10.1126/sciadv.abd1033.

Choi, V.M., Herrou, J., Hecht, A.L., Teoh, W.P., Turner, J.R., Crosson, S., Wardenburg, J. B., 2016. Activation of Bacteroides fragilis toxin by a novel bacterial protease contributes to anaerobic sepsis in mice. Nat. Med. 22 (5), 563–567. https://doi.org/10.1038/nm.4077.

Flores, G.E., Gregory Caporaso, J., Henley, J.B., Rideout, J.R., Domogala, D., Chase, J., Leff, J.W., et al., 2014. Temporal variability is a personalized feature of the human microbiome. Genome Biol. 15 (12), 531. https://doi.org/10.1186/s13059-014-0531-y.

Gattuso, P., Reddy, V., David, O., Spitz, D., 2009. Differential Diagnosis in Surgical Pathology. Saunders/Elsevier, Philadelphia, PA. LK -. https://worldcat.org/title/460904305.

Gilmore, W.J., Johnston, E.L., Bitto, N.J., Zavan, L., O'Brien-Simpson, N., Hill, A.F., Kaparakis-Liaskos, M., 2022. Bacteroides fragilis outer membrane vesicles preferentially activate innate immune receptors compared to their parent bacteria. Front. Immunol. 13, 970725. https://doi.org/10.3389/fimmu.2022.970725.

Hadadi-Fishani, M., Najar-Peerayeh, S., Siadat, S.D., Sekhavati, M., Mobarez, A.M., 2021. Isolation and immunogenicity of extracted outer membrane vesicles from pseudomonas aeruginosa under antibiotics treatment conditions. Iran. J. Microbiol. 13 (6), 824–831. https://doi.org/10.18502/ijm.v13i6.8087.

Kelwick, R.J.R., Webb, A.J., Heliot, A., Segura, C.T., Freemont, P.S., 2023. Opportunities to accelerate extracellular vesicle research with cell-free synthetic biology. J. Extracell. Biol. 2 (5), e90. https://doi.org/10.1002/jex2.90.

Kozhakhmetova, S., Zholdybayeva, E., Tarlykov, P., Atavliyeva, S., Syzdykov, T., Daniyarov, A., Mukhtarova, K., Ramankulov, Y., 2021. Determinants of resistance in Bacteroides fragilis strain BFR_KZ01 isolated from a patient with peritonitis in Kazakhstan. J. Glob. Antimicrob. Resist. 25, 1–4. https://doi.org/10.1016/j.jgar.2021.02.022.

Li, Q., Li, J., He, T., Ji, X., Wei, R., Yu, M., Wang, R., 2024. Sub-MIC antibiotics modulate productions of outer membrane vesicles in tigecycline-resistant Escherichia Coli. Antibiotics. https://doi.org/10.3390/antibiotics13030276.

Onorini, D., Schoborg, R., Borel, N., Leonard, C., 2023. Beta lactamase-producing Neisseria gonorrhoeae alleviates Amoxicillin-induced chlamydial persistence in a novel in vitro co-infection model. Curr. Res. Microb. Sci. 4, 100188. https://doi.org/10.1016/j.crmicr.2023.100188.

Patrick, S., McKenna, J.P., O'Hagan, S., Dermott, E., 1996. A comparison of the haemagglutinating and enzymic activities of Bacteroides fragilis whole cells and outer membrane vesicles. Microb. Pathog. 20 (4), 191–202. https://doi.org/10.1006/mpat.1996.0018.

Ribeiro de, F., Michele, C., de Almeida, P.E., Vieira, W.V., Ferreira-Machado, A.B., Resende, J.A., da Silva, V.L., Diniz, C.G., 2022. Inflammatory modulation and outer membrane vesicles (OMV) production associated to Bacteroides fragilis response to subinhibitory concentrations of metronidazole during experimental infection. Anaerobe 73, 102504. https://doi.org/10.1016/j.anaerobe.2021.102504.

Round, J.L., Mazmanian, S.K., 2010. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proc. Natl. Acad. Sci. U.S.A. 107 (27), 12204–12209. https://doi.org/10.1073/pnas.0909122107.

Sartorio, M.G., Pardue, E.J., Scott, N.E., Feldman, M.F., 2023. Human gut bacteria tailor extracellular vesicle cargo for the breakdown of diet- and host-derived glycans. Proc. Natl. Acad. Sci. 120 (27), e2306314120. https://doi.org/10.1073/pnas.2306314120.

Stentz, R., Horn, N., Cross, K., Salt, L., Brearley, C., Livermore, D.M., Carding, S.R., 2015. Cephalosporinases associated with outer membrane vesicles released by Bacteroides Spp. protect gut pathogens and commensals against β-lactam antibiotics. J. Antimicrob. Chemother. 70 (3), 701–709. https://doi.org/10.1093/jac/dku466.

Toyofuku, M., Nomura, N., Eberl, L., 2019. Types and origins of bacterial membrane vesicles. Nat. Rev. Microbiol. 17 (1), 13–24. https://doi.org/10.1038/s41579-018-0112-2.

Toyofuku, M., Schild, S., Kaparakis-Liaskos, M., Eberl, L., 2023. Composition and functions of bacterial membrane vesicles. Nat. Rev. Microbiol. 21 (7), 415–430. https://doi.org/10.1038/s41579-023-00875-5.

Vieira, A.T., Fukumori, C., Ferreira, C.M., 2016. New insights into therapeutic strategies for gut microbiota modulation in inflammatory diseases. Clin. Transl. Immunology. 5 (6), e87. https://doi.org/10.1038/cti.2016.38.

Wexler, H.M., 2007. Bacteroides: the good, the bad, and the nitty-gritty. Clin. Microbiol. Rev. 20 (4), 593–621. https://doi.org/10.1128/CMR.00008-07.

Yekani, M., Rezaee, M.A., Beheshtirouy, S., Baghi, H.B., Bazmani, A., Farzinazar, A., Yousef Memar, M., Sóki, J., 2022. Carbapenem resistance in Bacteroides fragilis: a review of molecular mechanisms. Anaerobe 76, 102606. https://doi.org/10.1016/j.anaerobe.2022.102606.

Zakharzhevskaya, N.B., Vanyushkina, A.A., Altukhov, I.A., Shavarda, A.L., Butenko, I.O., Rakitina, D.V., Nikitina, A.S., et al., 2017. Outer membrane vesicles secreted by pathogenic and nonpathogenic Bacteroides fragilis represent different metabolic activities. Sci. Rep. 7 (1), 5008. https://doi.org/10.1038/s41598-017-05264-6.

Zholdybayeva, E., Kozhakhmetova, S., Bayanbek, D., Bekbayeva, A., Auganova, D., Kulmambetova, G., Tarlykov, P., 2024. Multi-omics approach for understanding the response of Bacteroides fragilis to carbapenems. Heliyon (17), 10. https://doi.org/10.1016/j.heliyon.2024.e37049.