Quarterly
Volume 63
Issue 4•2024
OCTOBER – DECEMBER

CODEN:
PMKMAV 63 (4)
2024

POLISH SOCIETY OF MICROBIOLOGISTS
POLSKIE TOWARZYSTWO MIKROBIOLOGÓW

Advancements of Microbiology

formerly Postępy Mikrobiologii

Impact Factor = 0,300 (2023)
MNiSW Score = 20,00 (2024)

http://am-online.org/

JOURNAL ADDRESS

Sławkowska 17, 31-016 Kraków, Polska
e-mail: editorial.office@am-online.org
phone: (+48) 885 191 121

ISBN 978 - 83 - 923731 - 3 - 1

Information about the cover photo

Adherence of *Neisseria gonorrhoeae* to surface of SiHa cells (human cervical carcinoma cell line)

Preparation and imaging:

Joanna Białecka PhD, Centre of Microbiological Research and Autovaccines, Cracow, Poland,
Kamil Drożdż MSc, Department of Molecular Medical Microbiology;
Chair of Microbiology, Faculty of Medicine Jagiellonian University Medical College in Krakow;
Monika Gołda-Cępa PhD, Materials and Surface Chemistry Group,
Department of Inorganic Chemistry, Faculty of Chemistry, Jagiellonian University

POLISH SOCIETY OF MICROBIOLOGISTS

ADVANCEMENTS OF MICROBIOLOGY
2024, 63, 4, 181–189
https://doi.org/10.2478/am-2024-0015

IMPORTANCE OF HUMAN FAECAL BIOBANKING: FROM COLLECTION TO STORAGE

Izabela Lewandowska[1]*, Katarzyna Grzech[1], Jolanta Krzysztoń-Russjan[2]*

[1] Pure Clinical Lab Network Ltd. Co., Słupsk, Poland
[2] Department of Medical Biology, National Institute of Cardiology, 04-628 Warsaw, Poland

Submitted in August 2024, accepted in October 2024

Abstract. The freezing and storage conditions of faecal samples in biobanks influence the preservation of the integrity and stability of genetic material and play a crucial role in scientific and clinical research quality. In recent years, there has been a particular increase in the number of studies related to the gut microbiome and the importance of its impact on the functioning human body. The review includes research on faecal microbiota transplantation (FMT), microbiome analysis, '-omics' research, cancer and parasites. The primary topic addressed in this research is the impact of storage conditions and freezing methods for faecal samples on the stability and diversity of the gut microbiome. Standardizing procedures for storing and analyzing faecal samples is essential to carry out this task. This standardization is not a goal but a necessity for the quality control of the storage of faecal samples to reach comparative results and to develop new diagnostic methods. Collected data presented here highlight a crucial step in microbiota research concerning optimizing biobanking conditions for faecal samples.

Keywords: accreditation, biobank, cryopreservatives, faecal samples, storage conditions

1. Introduction

The storage of biological material is an essential part of the study that influences the final result. Depending on the aim of the study, several factors of the research stage should be previously optimized and validated, including essential factors, such as temperature and period of storage, while considering the application of stabilizing components. The stool is a specific biological material when it is impossible to carry out tests within a short period from its collection or in clinical studies involving many subjects and biological samples collected for harmonization/searching for new diagnostic methods or therapies. The most common approach to increase the availability of such material is to lower the storage temperature, add stabilizing compounds and standardize this pre-laboratory stage.

The type of biological material, the storage period, and the study's purpose influence the biobanking process in faecal biobanking; CTQ (critical to quality) focuses on controlling shifts in microbial composition caused by stabilization agents, period of storage and extraction methods while ensuring optimal DNA yield. Repeatability and reproducibility are essential for consistent, high-quality data across experiments (Doukhaine *et al.* 2021). This optimization is crucial in ensuring the high quality of the biological material obtained after thawing, making the research successful. Due to the complexity, banking faecal samples is challenging. Optimization of biobanking conditions enables multidirectional research and epidemiological studies. Freezing samples should safeguard their composition for future analyses to ensure consistency of results over a more extended unit of time. Accurate biobanking allows the integrity

* Corresponding Authors: Izabela Lewandowska, Pure Clinical Lab Network Ltd. Co., Słupsk, Poland e-mail: i.lewandowska.edu@gmail.com, Jolanta Krzysztoń-Russjan, Department of Medical Biology, National Institute of Cardiology, 04-628 Warsaw, Poland; e-mail: jkrzyszton@ikard.pl

Cite as:
Importance of Human Faecal Biobanking: from collection to storage. Lewandowska I. *et al.,* ADV MICROBIOL-NY, 2024, 63, 4, 181–189, https://doi.org/10.2478/am-2024-0015

of the genetic material intended for preservation and performing optimal molecular analyses, '-omics', or epidemiological studies. For therapies related to intestinal microbiota transplantation, the choice of faecal freezing is crucial for storing samples from microbiota donors to ensure the quality and safety of the material intended for transplantation (Coppola *et al.* 2019).

Different freezing methods are used in biobanks storing faecal samples, depending on their purpose, the research scope, and the application. However, the insufficient standardization of biobanking methods can result in a lower level of stability of stored samples and a lack of reproducibility of test results. Implementing international requirements for standardization of biobanking methods can significantly improve the quality of stored biological materials and facilitate data sharing between resources, especially in rare diseases (Coppola *et al.* 2019).

This work aimed to analyze published biobanking modalities, the selection of compounds that prevent damage to the cell and tissue (known as cryoprotectants) before freezing faecal samples, and also review recommendations for the collection, freezing and storage of faeces to identify the impact of freezing modalities in microbiological studies, what are the challenges and limitations of faeces biobanking and future directions and emerging trends. This work also highlights the need for further research in this area to urgently address the identified challenges and explore the potential of emerging trends, offering a hopeful outlook for the future of biobanking.

2. Collection of faecal samples for testing and their transport

Sampling is the first stage of the study, which must consider several processes occurring immediately after the faecal passage. Oxidation, hydrolysis, or enzymatic degradation are just some of the reactions that can contribute variably to changes in the sought-after parameters of the test sample before analysis (O'Sullivan *et al.* 2018). The optimization of collection, transport, and storage methods is crucial as it directly impacts the quality of the research and the accuracy of the results, reducing the impact of degradation to negligible levels. Given the intricate interplay of factors and the diverse array of tests performed on faecal samples, it is challenging to establish a single protocol to optimize the steps mentioned above. Immediately after passing stool, the sample is affected by higher oxygen concentrations than those prevailing in the intestines. By conducting experiments under *in vivo* conditions ignoring the difference in atmospheric oxygen concentrations and the gut microenvironment, the final result may deviate critically from the initial content (Thomas *et al.* 2015; Widjaja *et al.* 2023). When testing anaerobic bacteria, the time between donation and testing is crucial and should be as short as possible. Widjaja *et al.* (2023) pointed to several media for faecal sample protection collected under anaerobic conditions until transported to the anaerobic chambers for the experiment. Of further interest is a 2021 patented self-sampling device that maintains anaerobic conditions and limits donor contact with the sample (Widjaja *et al.* 2023). In the absence of access to such devices, when a donor collects a sample outside the laboratory, it is recommended to use substrates that protect the integrity of the material from destabilizing agents. These substrates have a transport and a protective function against various temperatures. One of the most frequently mentioned transport media in recent papers was the OMNIgene-GUT commercial kit. Doukhaine *et al.* (2021) designated this medium as effective in maintaining the neutrality of the microbiome. This underscores the critical importance of selecting the appropriate stabilization medium to ensure sample integrity, which is essential in sample processing for biobanking.

3. Sample processing for biobanking

While not mandatory, validation of faecal sample processing is recommended to recognize and mitigate potential impacts on sample quality, ensuring adherence to biobanking standards as outlined by the ISO, CEN standards, and BioMolecular Resources Research Infrastructure (BBMRI) guidelines. Based on the published data, several factors, such as different collection tubes and storage conditions, affect the sample stability and method validation, emphasizing the importance of precisely documenting these factors by biobanks (Neuberger-Castillo *et al.* 2020). The most important is to maintain experimental conditions as close to *in vivo* conditions as possible and process the sample in anaerobic chambers to eliminate the influence of oxygen. Bellali *et al.* (2019) developed a protocol that limited sample exposure to oxygen to 2 minutes (by collecting samples into anaerobic jars and processing steps quickly), resulting in an 87% recovery of viable anaerobic bacteria. The protocol was compared with a modified protocol using antioxidants, in which the sample was exposed to oxygen for 120 minutes. For this protocol, the recovery of viable bacteria was up to 67%. In the absence of an anaerobic atmosphere, antioxidant enrichment of the biological samples is a reasonable alternative for this experiment, providing reassurance about the validity of the research. During processing, aliquoting of samples is required. This process eliminates the risk of thawing and freezing, which degrades

most of the faecal parameters. According to the Good Laboratory Practice recommendation, homogenizing samples before aliquoting should be performed (OECD 2004). It was found that the faecal sample as a whole is differentiated in terms of aerobic bacteria in the outer layers and anaerobic bacteria in the deeper layers, which can be further subdivided into a central fermentative and a reservoir (Swidsinski *et al*. 2010; Santiago *et al*. 2014). In his experiment, Santiago *et al*. (2014) compared the results of pyrosequencing the 16S rRNA gene analysis of samples from the outer layers, inner layers, and homogenized samples, indicating a similar abundance of measurements obtained. Performing this step in the laboratory according to the procedure minimizes the risk of inappropriate homogenization due to too much hardness of the sample (Bristol scale 1.2) or unpleasant sensations during the process.

4. Securing samples for storage

Preservation of biological material plays an essential role in biobanking. Comparisons of faecal freezing without and with any cryoprotectants were concerned with the composition of the gut microbiota of fresh and frozen faeces for faecal microbiota transplantation. Some papers in this area have studied the stability and survival of viruses causing gastrointestinal infections (Alghamdi *et al*. 2022, Yang *et al*. 2022). Available results from direct freezing of faeces at –80°C and storage for up to 48 h without cryoprotectants indicate this method as the 'gold standard' for preserving the qualitative and quantitative composition of the intestinal microbiota, compared with preservation with 10% glycerol (Deschamps *et al*. 2020). Yang's (2022) metagenomics study showed that the faecal sample can be successfully stored as a supernatant at –80°C. However, freezing without a cryoprotectant will not be advisable in every experiment. Preservatives can stabilize the number of faecal microorganism species, but storing samples without that type of compounds can lead to an increase in the number of specific taxa (e.g. operational taxonomic units- OTU *Enterobacteriaceae*) (Li *et al.* 2023). In a study where the use of fresh and frozen faeces for FMT was compared, Bilinski *et al*. (2022) showed that freezing whole faeces without any cryoprotectants has an impact on the biodiversity and survival of the bacterial intestinal microbiota – the number of viable cells decreased more than fourfold, from about 70% to 15%. Among the set of commercials facilitating the preservation of faecal samples, e.g. OMNIgene-GUT mentioned earlier, was independently recognized as the best alternative to the 'gold standard', established based on the outcomes from qualitative-quantitative composition analysis. Similar results were achieved in a protocol study for faecal samples detecting bile acids using OMNIgene-GUT, demonstrating its efficacy for metabolomics studies (Neuberger-Castillo *et al*. 2021). Of interest may be the preliminary results of an experiment by Young et al. (2020) on using a substrate based on guanidinium tthiocyanate – eNAT when stored at room temperature for about 30 days and then transferred into –80°C for longer time of the period storage. The most often self-prepared cryoprotectants applied before sample freezing included, among others, ethanol, DMSO-EDTA salt solution (DESS) and guanidine thiocyanate. In a Japanese study, the effects of DESS or guanidine thiocyanate at room temperature or 4°C showed the a- and b-diversities with no significant differences between *Bacteroides* and *Bifidobacterium* spp. profiles (Kawada *et al*. 2019). On the other hand, Hale *et al*. (2015) found no significant effect on the microbial community of the samples studied while storing faecal samples from spider monkeys in 100% ethanol at room temperature for eight weeks. Song *et al*. (2016) came to similar conclusions, where they demonstrated that 95% ethanol effectively preserves the diversity and composition of the gut microbiome stored at room temperature for at least eight weeks. However, this solution is highly flammable and expensive to transport. The effect of 10% glycerol concentrations (through its ability to permeate cell membranes) on metabolic and biological parameters has been described. As one of the more commonly chosen protectants due to its protection of bacterial viability after freezing, it also had another characteristic – after thawing, it offers an excellent environment for microbes to thrive, thereby causing changes in the microbiome community (Widjaja 2023). Deschamps (2020) also demonstrated the effect of glycerol in reducing metabolic activity, which may be related to cell damage. According to Biclot *et al*. (2022), glycerol contributes to the formation of ice crystals inside cells, ultimately leading to cell lysis. These disparate data point to the need for further research to better understand under which conditions the integrity of samples can best be maintained for different types of analysis. Other publications analyzed the effect of stabilizing agents used in microbiome studies, i.e. Tris-EDTA buffer and 70% ethanol. Unfortunately, buffers containing EDTA may not optimally preserve the microbiological composition of faecal samples. In Young *et al*. (2020) study, samples stored in Tris-EDTA buffer at ambient temperature had lower abundances of *Bifidobacterium* and *Anaerostipes* spp. and higher abundances of *Bacteroides* and *Proteobacteria* spp. than samples stored in Tris-EDTA buffer and immediately frozen to –80°C. In the case of 70% ethanol, using it as a protectant for faecal samples at room temperature yielded results similar to those without preservatives. Hence, 70% ethanol as a protectant for this type of storage is strongly discouraged (Song *et al*. 2016). Wu *et al*.

(2021) verified a self-prepared preservation buffer (PB) that could stabilize human saliva samples, thus demonstrating the suitability of this buffer for stabilizing faecal samples and intended for sequencing without freezing facilities or logistical constraints. The study results indicate that using a cryoprotectant has a beneficial effect on the microorganisms in the faeces, the stability of the sample, and the reproducibility of the results of various diagnostic methods. In addition, commercial media can be used in molecular biology studies due to their properties, i.e., the stabilization of microbial DNA in eNAT® medium or the lack of effect on DNA extraction efficiency in OMNIgene-GUT medium (Young *et al*. 2020; Doukhaine *et al*. 2021). Understanding that choosing preservation methods is not a one-size-fits-all solution is crucial. The efficacy of these methods varies across different groups of microorganisms, making it a significant and relevant area of research. The storage temperature and duration of storage also play a pivotal role in influencing microbial DNA yield (Wu *et al*. 2021).

5. Storage

When creating a sample storage protocol with specific assumptions for the experiment, storage time and temperature should be analyzed. The bacterial composition of faeces changes after 15 minutes of storage at room temperature, which can be an obstacle for, among other things, epidemiological studies; therefore, it is desirable to freeze samples immediately (Tamada *et al*. 2022). For metabolomic studies, temperature determines microbiological and enzymatic activity and, thus, the final results. O'Sullivan *et al*. (2018) highlighted the testing of previously frozen faecal samples for glutamate and branched-chain amino acid (BCAA) levels, where higher concentrations were found relative to measurements on fresh samples. For this type of study, storing samples at temperatures below 0 can significantly affect the final results. A thorough analysis of the modes and conditions of storage on the quality and quantity of the microbiota of stored human faeces is crucial in selecting optimal conditions depending on the intended use of the collected samples (Hickl *et al*. 2019). Preserving the integrity of the genetic material of the samples after collection and storage determines the correct interpretation of the data (Hickl *et al*. 2019; Tamada *et al*. 2022). Optimizing a faecal sample's freezing and storage conditions includes the choice of container, cryoprotectant, freezing, and storage method. Many containers suitable for deep-freezing are available, e.g. Eppendorf tubes, cryo-tubes without code and with 2D code and SBS (Society for Biomolecular Screening) tubes adapted for automatic biological material handling. Before biobanking, it is necessary to analyze the amount of frozen material, the available equipment, the number of personnel, and the choice of container. When selecting tubes with a stabilizing/transport medium, you must consider the stabilizer's possible influence on the parameter under study, e.g., the change in bacterial abundance in the buffer medium through chemical lysis or interference in metabolite studies (Chen *et al*. 2020; Neuberger-Castillo *et al*. 2021). Regardless of the container used, each Biobank needs to check the required quality parameters (depending on the purpose of the study) to ensure that the selected materials and equipment will meet the storage criteria under the prevailing conditions. Another critical factor in the correct storage process is the accurate temperature for the research. The literature data analyzed for his article of a study of the effect of the length of storage on the stability of stored faecal samples highlighted the dependence of quality parameters on different storage temperature ranges.

6. Stability studies of faecal samples during storage in the temperature range between 15–25°C

In routine diagnostic laboratories, faecal samples are usually stored at room temperature during the laboratory examination. After treatment, the remaining residue, until the result is stored at 4°C. For many research units without access to freezing equipment wishing to store faecal samples, the storage method at room temperature seems interesting. Due to several metabolic processes occurring in faecal samples at ambient temperature or fungal overgrowth (Thomas *et al*. 2015), using preservatives is crucial to maintain the sample's integrity. Their use eliminates the need for research to analyze the effect of humidity on fresh samples. In a study by Park *et al*. (2020), after evaluating the microbial profile, the authors confirmed the usefulness of transport media, i.e. NBgene-Gut and OMNIgene-GUT, for storing faecal samples at room temperature over two months. Thanks to the stabilizing media, transported faecal samples, e.g. with RNAlater® or OMNIgene-GUT, can be stored for 14–60 days (depending on the kit specification) (DNA Genotek 2010; Life Technologies 2011; Song *et al*. 2016; Wu *et al*. 2021).

In contrast, Wu *et al*. (2021) confirmed the efficacy of the 'Self-made PB buffer', which protected the microbiome in faecal samples for up to 4 weeks at room temperature. The authors suggest PB buffer as a cheaper alternative for storage at room temperature compared to its commercial counterparts. However, the limitations of this method over the long term indicate the need for further research into the long-term stability of samples. All the substrates mentioned are stored at lower temperatures (–20°C and –80°C, respectively) (DNA Genotek 2010; Life Technologies 2011; Wu *et al*.

2021). Other studies have noted the effect of room temperature on changing the ratio of Gram-negative and Gram-positive bacteria, probably due to the higher stability of bacterial DNA in Gram-negative bacteria relative to that of Gram-positive bacteria at room temperature (Hickl *et al.* 2019; Li *et al.* 2023).

7. Stability testing of faecal samples during storage at 4°C

In biobanks, temperatures of 4°C are not helpful for the long-term storage of biological samples. Amar et al. (2005) showed the advantage of molecular methods used for archived frozen fecal samples against conventional method immediately used for detection with the lowest percentage reconfirmation of target for *Giardia* spp, and *C. perfringens*. Results obtained by PCR indicated a 96% concordance for *Cryptosporidium* spp. after ten years of storage and a 68% concordance for *Giardia* after two years of storage, with positive results from a direct preparation. In most scientific studies on metabolites or the microbiome, a temperature of 4°C is not recommended for storage of media samples beyond 12 h because of the continuous proliferation and metabolism of some microorganisms, as well as the change in oxygen concentration in the samples, which significantly affects the anaerobic community (Thomas *et al.* 2015; Cunningham *et al.* 2020). In their study, Nogata *et al.* (2019) proved that using a medium such as Carry Blair during storage at 4°C can prevent changes in the microbiome community. For metabolomic testing of faecal water, according to O'Sullivan *et al.* (2018), storing faecal samples at this temperature for up to 24 hours shows a good alternative compared to testing on fresh samples. Wu *et al.* (2021), who studied the microbiome's composition in faecal samples subjected to different temperatures, including 4°C for up to 4h, proved the lack of effect of short-term storage on microbiome composition. Also, Cunningham *et al.* (2020) demonstrated that stool storage without cryoprotectants at room temperature and 4°C for up to 48 h has no significant effect on the microbiome in samples. The discrepancies in the period may be due to differences in the analytical methods used in the two studies, highlighting the need for standardization of procedures in this area. However, it is crucial to note that Cunningham's method should not be recommended in studies on determining the composition of faecal anaerobic microbiome by culture methods. Instead, the focus should be on pre-laboratory procedures that may cause errors affecting the microbiome's composition. If not carefully avoided, these errors can significantly alter it, underscoring the weight of a decision in maintaining its integrity.

8. Studies on the stability of faecal samples during storage in the temperature range –20 ÷ –30°C

The freezing method at –20°C has been repeatedly described as one of the most commonly used for long-term storage of samples (Biclot *et al.* 2022). Ice crystals may appear at this temperature, but the manufacturer of RNAlater® assures RNA stability in samples indefinitely (Life Technologies 2011). Despite using this medium in many experiments, the manufacturer's instructions do not provide information about applying it to faecal samples. An epidemiological study by Souza *et al.* (2021), conducted on archival (1998–2005) faecal samples frozen at –20°C, found genetic material in 6.8% of the samples analyzed for Adenovirus. Due to the lack of comparative results in fresh faecal samples, it is impossible to address the stability of faecal samples in the context of the presence of Adenovirus. Bilinski *et al.* (2022), in a study of faecal samples frozen at –30°C, found evidence in favour of a significant effect of low temperature on the composition of the microbiome, even though it is this way of freezing stool has more than 90% effectiveness in transplanting the microbiota in patients with *Clostridioides difficile* infection. The review did not analyze studies on the impact of the transplanted microbiota on the recipient's organism, as the main aim of this procedure is to eradicate *Clostridioides*. However, such studies may contribute to a better understanding of the transplant preparation process.

9. Stability testing of faecal samples during storage at –80°C

Biobanking using the rapid freezing method at –80°C has been recognized as the gold standard in microbiome research (Hickl *et al.* 2019; Guan *et al.* 2021; Wu *et al.* 2021; Li *et al.* 2023). This method seems more effective, especially for short-term storage of samples (up to 48 h) (Deschamps *et al.* 2020). However, some studies allow it to be used as a long-term storage method, especially in colorectal studies (Wirth *et al.* 2020). Compared to regular freezing at –80°C, the rapid drop in temperature reduces the ice crystal formation phenomenon while maintaining the integrity of the cells, further improving the isolation of Gram-positive bacterial DNA (Thomas *et al.* 2015; Li *et al.* 2023). In a study by Coryell *et al.* (2021) on the detectability of SARS-CoV-2 in faecal samples, a higher detection rate of viral RNA was demonstrated in samples stored in DNA/RNA shield stabilizing buffer, compared to samples without the addition of a stabilized stored at the same temperature. In the previously mentioned RNAlater® study results, confirmation was obtained for long-term storage of faecal samples without significant changes in microbiome

composition (Flores *et al*. 2015, Tap *et al*. 2019). In the case of the study by Liang *et al.* (2020) on OTU-level abundance in frozen samples, the RNAlater® substrate is not recommended due to its high diversity. Biclot *et al*. (2022) showed the effect of freezing faecal samples at –80°C on the composition of anaerobic bacterial species. They found that storing samples at this low temperature without additives, the so-called "dry condition", significantly showed the broader species richness. In addition to using –80°C for storage, this temperature is also used to transport faecal samples for essential analyses (Williams *et al*. 2019).

10. Stability studies of faecal samples during storage in liquid nitrogen

Due to relatively high storage costs, using liquid nitrogen for freezing faecal samples is not the first chosen method. Wu *et al.* (2021) compared the economic aspects of freezing using selected techniques in their publication. They found a financial alternative to the liquid nitrogen method (using low-cost buffers allowing samples to be kept for up to 4 weeks, based on the storage temperature. It is worth noting that liquid nitrogen, especially in combination with 10% glycerol, is an effective and reliable method for the long-term storing of faecal samples (Li *et al.* 2023). Relating to storing samples in liquid nitrogen, researchers also use nitrogen jet cooling to store samples at –80°C (Hickl *et al.* 2019; Guan *et al*. 2021).

This work's temperature division indicates various methods for preserving faecal samples, highlighting their effectiveness based on research. The purpose, planned costs, and future application of the results should all be considered.

Table I. presents various faecal microbiome preservation methods tested across different studies, providing an overview of their effectiveness based on storage duration and temperature. The best long-term preservation method remains freezing at –80°C. In a 2020 study, Young *et al.* demonstrated that the eNAT medium can

Table I
Summary of selected storage conditions and preserving faecal samples for a microbiome study.

Stabilization compounds/ solutions	Storage temperature	Storage period	Influence on stability of microbiota	Source
Stratec DNA stabilizer	Room temperature	7 days	Stable microbiota profiles, minimal changes in diversity.	(Chen *et al*., 2020)
OMNIgene-GUT DNA stabilizer	Room temperature	7 days	Microbiota minimal changes, high DNA stability.	(Chen *et al*., 2020)
DESS (DMSO-EDTA-salt)	Room temperature	1–3 weeks	Microbiome stability without significant changes in diversity (using targeted extraction methods).	(Kawada *et al*., 2019)
Homemade preservation buffer (EDTA, DSD*, SCTD*, ammonium sulfate)	Room temperature	4 weeks	Stability in room temperature (or in high temperatures up to 5 days), ideal when freezing isn't available.	(Wu & Chen *et al.,* 2021)
OMNIgene-GUT or NBgene-GUT	Room temperature	65 days	The best method for room temperature storage is stable microbiome profiles comparable to frozen samples	(Park *et al.,* 2020)
95% Ethanol, OMNIgene-GUT	Room temperature	8 weeks	Both methods effectively maintain microbiome stability	(Song *et al.,* 2016)
Ethanol (100%)	Room temperature	8 weeks	Stable microbiome, alternative to freezing, suitable for long-term storage (study on spider monkey's stool)	(Hale *et al*., 2015)
No stabilizer	–20°C	24 h	Minimal changes in taxonomic diversity, good DNA quality.	(Cardona *et al*., 2012)
No stabilizer	–20°C (after 48h at 4°C)	48–96 h	Minimal changes in microbiome and SCFA composition, indicating that 24h refrigeration preserves sample integrity.	(Cunningham *et al*., 2020)
No stabilizer	–80°C	48 h	Stable microbiota diversity, high DNA yield, minimal changes in bacterial composition	(Biclot *et al*., 2022)
eNAT® medium	–80°C	30 days at room temperature, then freezing	Stability of microbiome maintained after 30 days at room temperature, no significant changes in diversity and composition	(Young *et al*., 2020)
10% Glycerol	–80°C/liquid nitrogen	12 months	Microbiome stability maintained for 12 months, better diversity than ethanol-preserved samples at room temperature	(Li *et al*., 2023)

* DSD- disodium salt dihydrate, SCTD- sodium citrate trisodium salt dihydrate

store samples at room temperature for up to 30 days without compromising microbial diversity or composition. Similar results can be noticed in Park et al.'s study (2020) using OMNIgene-Gut and NBgene-Gut, where the microbiome was stable for up to 65 days. The choice of the optimal storage temperature and economic considerations is necessary to analyze the impact of additional factors affecting the sample during storage.

Considering all the beneficial and adverse factors in the biobanking of faecal samples mentioned above, it is evident that the crucial factors determine the specific aim of the study. Beneficial Factors play a key role in maintaining the quality, stability, and integrity of faecal samples, thereby supporting the accuracy of research outcomes. On the other hand, adverse factors can result in sample degradation, alterations in composition, or difficulties in maintaining the necessary biobanking conditions. Microbial growth in stored faecal samples below –20°C seems unlikely, however, and there are bacteria in the environment, i.e. *Planococcus halocryophilus*, which can grow at –15°C and retain metabolic activity at –20°C (Mykytczuk *et al.* 2013). Below –20°C metabolic processes leading to cell lysis occurring reduce the number of viable bacteria and expose genetic material, facilitating the extraction step (Hale *et al.* 2015; Ahrabi *et al.* 2016).

11. Applicable standards for the freezing and storage of faecal samples

The increased awareness of the need for high-quality samples and the desire to standardize collection and transport criteria has created an international document – ISO 20387:2018 Biotechnology – Biobanking – General Requirements for Biobanking (Dagher 2022). The document systematizes the quality control and management system requirements of Biobanking entities wishing to confirm the reliability of the high-quality activities performed. To describe the different analytical steps, the technical committee has successively published standards describing pre-analytical laboratory processes for pretreatment in molecular biology (e.g. for tissue, plasma, serum, urine), microbiology, and parasitology. No guidelines that relate only to faecal samples in a broad context have been produced. Looking through ISO resources, you can find documents that generally define Biobank's handling of biological material. One such guideline is ISO 21899- "Biotechnology – Biobanking – General requirements for the validation and verification of processing methods for biological material in biobanks" This standard covers a wide range of biological samples, so it could potentially apply to faecal material if the biobank is involved in such work, as long as the methods used meet the validation requirements outlined in the document. Another important: ISO/AWI TS 18701- "Molecular in vitro diagnostic examinations – Specifications for pre-examination processes for human specimens – Isolated microbiome DNA", is a forthcoming technical standard that will provide guidelines for the biobanking of faecal samples, including methods for their processing and storage. Its goal is to ensure consistency and quality in these procedures, enabling faecal samples to be effectively used in scientific and medical research. ISO/DIS 20070 "Biotechnology – Biobanking – Requirements for sample containers for storing biological materials in biobanks" focuses on the requirements for the management and quality of human biobanks, including the collection, processing, and storage of biological materials. In the context of faecal biobanking, this standard would ensure that proper protocols are followed to maintain the integrity and usability of faecal samples for future research, guaranteeing consistency in sample handling and data management.

At the same time, European technical specifications were being developed at the European Committee for Standardisation (CEN) to respond to the reproducibility crisis that emerged due to the lack of repeatability of experiments or errors in medical analysis. The main objective of the specifications was to define the requirements for the pre-analytical procedure with its complete documentation (Stumptner *et al.* 2022). In 2021, a standard describing the requirements for microbiome DNA isolation (CEN/TS 17626- Molecular in vitro diagnostic examinations – Specifications for pre-examination processes for human specimen – Isolated microbiome DNA) was developed, which, to date, is the only document directly referring to the handling of faecal samples. Access to CEN/TS 17626 is the start to achieving standardization in handling and treating faeces in all research fields (Stumptner *et al.* 2022), from assessing sample composition to clinical trials with biobanked biological samples. Researchers are only required to use standardized methods for faecal testing if the work leads to diagnostic procedures to which the IVDR (In Vitro Diagnostic Regulation) is applied. In many cases, using these standards benefits the accuracy and reliability of the assays obtained. In addition to being reproducible, the results of such studies can be used for accreditation and the creation or updating of standards (Stumptner *et al.* 2022).

12. Clinical applications of faecal biobanking

According to the comprehensive approach to the patient and a greater understanding of the gut-brain microbiome axis, scientists have increased the number of tests performed to find solutions to many diseases. As previously mentioned, the importance of biobanking

in scientific research and clinical and laboratory diagnostics has increased. From the publications analyzed over the last five years, eight main research directions emerge, i.e. FMT, microbiome studies,- omics (genomic, transcriptomic, proteomic, metabolomic), cancer, bacteria, viruses and parasites and others. These publications analyzed or studied faecal samples stored under different conditions and periods. Sample storage is just one of the many uses of a biobank. Transparent procedures for handling banked samples, broadly written informed consents, and precise quality valuation of samples determine the success of storing material in research and clinical experiments (Coppola *et al.* 2019), such as research on cancer, metabolic diseases, infectious diseases, research transplantation and also epidemiological and population studies.

13. Summary

Analysis of the available literature on the storage of faecal samples has shown the importance of the appropriate storage method and cryoprotectants for maintaining the integrity of genetic material and the stability of the biological composition. In microbiome studies, choosing a proper cryoprotectant and storage temperature is crucial, mainly if the microbiome composition is being analyzed.

Further research into optimal storage conditions for faecal samples and developing more precise guidelines may improve the quality and reliability of microbiological results. The publications analyzed were based on in-house protocols, often impossible to reproduce under other conditions. The following International Standards Operations (ISO) underline several aspects important in biobanking, i.e., ISO 21899 with the processing of biological materials using validated and/or verified methods fit for the purpose, both ISO 18701 and similar CEN/TS 17626 with requirements and recommendations for the pre-examination phase of several human specimens, such as stool, saliva, skin and urogenital specimens intended for microbiome DNA examination, and ISO 20070 which focuses on information security management systems in an organization The choice of freezing method depends on the goal and period of storage, as well as the resources of the Biobank. Faecal samples can be helpful in screening, cancer diagnosis, and research into gastrointestinal diseases, as well as bacterial and viral infections. Potential directions for further research should include continued research into optimal storage conditions for faecal samples, including the best cryoprotectant and storage temperature. It will undoubtedly contribute to increased efficacy in FMT therapy and the impact of microbiome composition on recipient health.

Research with faecal samples used in diagnostics of various diseases and developing more comprehensive standards for storing and analyzing faecal samples that enable reliable and reproducible results will interest future researchers.

ORCID
Izabela Lewandowska https://orcid.org/0009-0009-4629-7368
Jolanta Krzysztoń-Russjan https://orcid.org/0000-0001-7200-6178

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

Reference

1. Ahrabi A.F., Handa D., Codipilly C.N., Shah S., Williams J.E., McGuire M.A., Potak D., Aharon G.G., Schanler R.J.: Effects of extended freezer storage on the integrity of human milk. *J Pediatr*. **177**, 140–143 (2016)
2. Alghamdi S., Ashshi A., Kabrah A., Atwah B., Alkurbi M.O., Assas M.B.: The impact of gastrointestinal tract infection on acquiring dengue fever virus infection and haemorrhagic fever in Jeddah region, Saudi Arabia. *Cell Mol. Biol*. **68**(9), 45–50 (2022)
3. Amar C.F., East C.L., Grant K.A., Gray J., Iturriza-Gomara M., Maclure E.A., McLauchlin J.: Detection of viral, bacterial, and parasitological RNA or DNA of nine intestinal pathogens in fecal samples archived as part of the English infectious intestinal disease study: assessment of the stability of target nucleic acid. *Diagn. Mol. Pathol.* **14**(2), 90–96 (2005)
4. Bellali S., Lagier J.C., Raoult D., Bou Khalil J.: Among Live and Dead Bacteria, the Optimization of Sample Collection and Processing Remains Essential in Recovering Gut Microbiota Components. *Front Microbiol* **10**, 1606 (2019)
5. Biclot A., Huys G.R.B., Bacigalupe R., D'hoe K., Vandeputte D., Falony G., Tito R.Y., Raes J.: Effect of cryopreservation medium conditions on growth and isolation of gut anaerobes from human faecal samples. *Microbiome* **10**(1), 80 (2022)
6. Bilinski J. & Dziurzynski M. *et al.*: Fresh Versus Frozen Stool for Fecal Microbiota Transplantation-Assessment by Multimethod Approach Combining Culturing, Flow Cytometry, and Next-Generation Sequencing. *Front. Microbiol.* **13**, 872735 (2022)
7. Cardona S., Eck A., Cassellas M., Gallart M., Alastrue C., Dore J., Azpiroz F., Roca J., Guarner F., Manichanh C.: Storage conditions of intestinal microbiota matter in metagenomic analysis. *BMC Microbiol* **12**, 158 (2012)
8. Chen C.C., Wu W.K., Chang C.M., Panyod S., Lu T.P., Liou J.M., Fang Y.J., Chuang E.Y., Wu M.S.: Comparison of DNA stabilizers and storage conditions on preserving fecal microbiota profiles. *J. Formos. Med. Assoc.* **119**(12), 1791–1798 (2020)
9. Coppola L., Cianflone A., Grimaldi A.M., Incoronato M., Bevilacqua P., Messina F., Baselice S., Soricelli A., Mirabelli P., Salvatore M.: Biobanking in health care: evolution and future directions. *J Transl Med.* **17**(1), 172 (2019)
10. Coryell M.P. & Iakiviak M. *et al.*: A method for detection of SARS-CoV-2 RNA in healthy human stool: a validation study. *Lancet Microbe,* **2**(6), 259–266 (2021)
11. Cunningham J.L., Bramstång L., Singh A., Jayarathna S., Rasmusson A.J., Moazzami A., Müller B.: Impact of time and temperature on gut microbiota and SCFA composition in stool samples. *PLoS One*, **15**(8), 0236944 (2020)

12. Dagher G.: Quality matters: International standards for biobanking. *Cell Prolif* **55**(8) 13282 (2022)
13. Deschamps C. & Fournier E. *et al.*: Comparative methods for fecal sample storage to preserve gut microbial structure and function in an in vitro model of the human colon. *Appl Microbiol Biotechnol* **104**(23), 10233–10247 (2020)
14. DNA Genotek. PD-PR-01036. Storage recommendations for samples collected using Omnigene products. Available at: https://www.dnagenotek.com. (2010)
15. Doukhaine E., Gamwell L., Bouevitch A., Elgoff C., Lynch D., Smith C., Del Duca A., Odouli R., Ferber J., Li D., Merino C.: Critical To Quality pre-analytical factors and their impact on microbiome analysis. Available at: https://www.dnagenotek.com/us/pdf/Critical-To-Quality-pre-analytical-factors-and-their-impact-on-microbiome-analysis-MK-00629.pdf. (2021)
16. Flores R., Shi J., Yu G., Ma B., Ravel J., Goedert J.J., Sinha R.: Collection media and delayed freezing effects on microbial composition of human stool. *Microbiome* **3**, 33 (2015)
17. Guan H. & Pu Y. *et al.*: Comparison of Fecal Collection Methods on Variation in Gut Metagenomics and Untargeted Metabolomics. *mSphere*, **6**(5), e0063621 (2021)
18. Hale V.L., Tan C.L., Knight R., Amato K.R.: Effect of preservation method on spider monkey (Ateles geoffroyi) fecal microbiota over 8 weeks. *J Microbial Methods.* **113**, 16–26 (2015)
19. Hickl O., Heintz-Buschart A., Trautwein-Schult A., Hercog R., Bork P., Wilmes P., Becher D.: Sample Preservation and Storage Significantly Impact Taxonomic and Functional Profiles in Metaproteomics Studies of the Human Gut Microbiome. *Microorganisms*, **7**(9), 367 (2019)
20. Kawada Y., Naito Y., Andoh A., Ozeki M., Inoue R.: Effect of storage and DNA extraction method on 16S rRNA-profiled fecal microbiota in Japanese adults. *J Clin Biochem Nutr*. **64**(2), 106–111 (2019)
21. Li X.M., Shi X., Yao Y., Shen Y.C., Wu X.L., Cai T., Liang L.X., Wang F.: Effects of Stool Sample Preservation Methods on Gut Microbiota Biodiversity: New Original Data and Systematic Review with Meta-Analysis. *Microbiol Spectr* **11**(3), e0429722 (2023)
22. Liang Y. & Dong T. *et al.*: Systematic Analysis of Impact of Sampling Regions and Storage Methods on Fecal Gut Microbiome and Metabolome Profiles. *mSphere,* **5**(1), e00763-1 (2020)
23. Life Technologies. RNAlater® tissue collection: RNA stabilizationnsolution. Available from: https://tools.thermofisher.com/content/sfs/manuals/cms_056069.pdf (2011)
24. Mykytczuk N.C., Foote S.J., Omelon C.R., Southam G., Greer C.W., Whyte L.G.: Bacterial growth at –15 °C; molecular insights from the permafrost bacterium Planococcus halocryophilus Or1. *ISME J.* **7**(6), 1211–1226 (2013)
25. Neuberger-Castillo L., Ammerlaan W., Betsou F.: Fitness for purpose of stabilized stool samples for bile acid metabolite analyses. *Sci Rep.* **11**(1), 7904 (2021)
26. Neuberger-Castillo L., Hamot G., Marchese M., Sanchez I., Ammerlaan W., Betsou F.: Method Validation for Extraction of DNA from Human Stool Samples for Downstream Microbiome Analysis. *Biopreserv Biobank*, **18**(2), 102–116 (2020)
27. OECD, Organization for Economic Co-operation and Development (OECD): OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring Number 14: Advisory Document of the Working Group on Good Laboratory Practice – The Application of the Principles of GLP to in vitro Studies. OECD Publishing (2004)
28. O'Sullivan V., Madrid-Gambin F., Alegra T., Gibbons H., Brennan L.: Impact of Sample Storage on the NMR Fecal Water Metabolome. *ACS Omega*, **3**(12), 16585–16590 (2018)
29. Park C., Yun K.E., Chu J.M., Lee J.Y., Hong C.P., Nam Y.D., Jeong J., Han K., Ahn Y.J.: Performance comparison of fecal preservative and stock solutions for gut microbiome storage at room temperature. *J Microbiol.* **58**(8), 703–710 (2020)
30. Santiago A., Panda S., Mengels G., Martinez X., Azpiroz F., Dore J., Guarner F., Manichanh C.: Processing faecal samples: a step forward for standards in microbial community analysis. *BMC Microbiol.* **14**, 112 (2014)
31. Song S.J., Amir A., Metcalf J.L., Amato K.R., Xu Z.Z., Humphrey G., Knight R.: Preservation Methods Differ in Fecal Microbiome Stability, Affecting Suitability for Field Studies. *mSystems* **1**(3), e00021-16 (2016)
32. Souza Y.F.V.P., Souza E.V., Azevedo L.S., Medeiros R.S., Timenetsky M.D.C.S.T., Luchs A.: Enteric adenovirus epidemiology from historical fecal samples in Brazil (1998–2005): Pre-rotavirus vaccine era. *Infect Genet Evol***. 94**, 105007 (2021)
33. Stumptner C., Stadlbauer V., O'Neil D., Gessner A., Hiergeist A., Zatloukal K., Abuja P.M.: The Pre-Analytical CEN/TS Standard for Microbiome Diagnostics-How Can Research and Development Benefit? *Nutrients,* **14**(9), 1976 (2022)
34. Swidsinski A., Loening-Baucke V., Kirsch S., Doerffel Y.: Biostructure fonctionnelle du microbiote colique(zone de fermentation centrale, zone de réservegerminale et couche de mucus séparatrice) chez lessujets sains et chez les patients atteints de diarrhéetraités par Saccharomyces boulardii [Functional biostructure of colonic microbiota (central fermenting area, germinal stock area and separating mucus layer) in healthy subjects and patients with diarrhea treated with Saccharomyces boulardii]. *Gastroenterol Clin Biol* **34** Suppl **1**, 79–92 (2010)
35. Tamada H., Ito Y., Ebara T., Kato S., Kaneko K., Matsuki T., Sugiura-Ogasawara M., Saitoh S., Kamijima M.: Epidemiological Studies of Children's Gut Microbiota: Validation of Sample Collection and Storage Methods and Microbiota Analysis of Toddlers' Feces Collected from Diapers. *Nutrients*, **14**(16), 3315 (2022)
36. Tap J., Cools-Portier S., Pavan S., Druesne A., Öhman L., Törnblom H., Simren M., Derrien M.: Effects of the long-term storage of human fecal microbiota samples collected in RNAlater. *Sci Rep.* **9**(1), 601 (2019)
37. Thomas V., Clark J., Doré J.: Fecal microbiota analysis: an overview of sample collection methods and sequencing strategies. *Future Microbiol.* **10**(9), 1485–504 (2015)
38. Widjaja F., Rietjens I.M.C.M.: From-Toilet-to-Freezer: A Review on Requirements for an Automatic Protocol to Collect and Store Human Fecal Samples for Research Purposes. *Biomedicines,* **11**(10), 2658 (2023)
39. Williams G.M., Leary S.D., Ajami N.J., Chipper Keating S., Petrosin J.F., Hamilton-Shield J.P., Gillespie K.M.: Gut microbiome analysis by post: Evaluation of the optimal method to collect stool samples from infants within a national cohort study. *PLoS One* **14**(6), e0216557 (2019)
40. Wirth U. & Garzetti D. *et al.*: Microbiome Analysis from Paired Mucosal and Fecal Samples of a Colorectal Cancer Biobank. *Cancers (Basel)*, **12**(12), 3702 (2020)
41. Wu C. & Chen T. *et al.*: The maintenance of microbial community in human fecal samples by a cost effective preservation buffer. *Sci Rep*. **11**, 13453 (2021)
42. Yang S., He Y., Zhang J., Zhang D., Wang Y., Lu X., Wang X., Shen Q., Ji L., Lu H., Zhang W.: Viral metagenomics reveals diverse viruses in the fecal samples of children with diarrhea. *Virol Sin.* **37**(1), 82–93 (2022)
43. Young R.R., Jenkins K., Araujo-Perez F., Seed P.C., Kelly M.S.: Long-term stability of microbiome diversity and composition in fecal samples stored in eNAT medium. *Microbiologyopen*, **9**(7), e1046 (2020)

ADVANCEMENTS OF MICROBIOLOGY
2024, 63, 4, 191–198
https://doi.org/10.2478/am-2024-0016

CONTEMPLATING THE BIBLIOMETRIC DATA ONTO SEPSIS AND GUT MICROBIOME: MAPPING TRENDS, COLLABORATIONS, AND GLOBAL CONTRIBUTIONS

Li Zhang[1], Yi Wang[2], Ping Li[1], Xiang Li[2], Xiangyou Yu[1, 2]*

[1] Department of Nursing, Xinjiang Medical University, Xinjiang Uygur Autonomous Region, 830001, China
[2] Critical Medicine Center, The First Affiliated Hospital of Xinjiang Medical University, Urumqi 830054, Xinjiang Uygur Autonomous Region, China

Submitted in July 2024, accepted in November 2024

Abstract. Sepsis is a leading cause of hospital mortality, closely linked to gut dysfunction and dysbiosis. The gut microbiome's role in sepsis pathogenesis and progression necessitates a comprehensive bibliometric analysis to elucidate current research trends. Utilizing the Science Citation Index Expanded (SCI-E) database, literature was systematically retrieved using the terms: sepsis AND ("gut" OR "gastrointestinal") AND ("microbiome" OR "microbiota" OR "microflora" OR "bacillus"). After data refinement and duplicate removal, 2485 articles were included for statistical analysis using R software's bibliometric package, with Excel used to visualize publication trends. Findings demonstrate a progressive annual increase in published studies and citations. The United States and France emerged as primary contributors, exhibiting extensive international collaboration. Among leading institutions, the University of California ranked highest in research output, while Wiersinga WJ from the University of Amsterdam led in publication volume and collaborative networks. Research predominantly focuses on critical care medicine, immunology, and microbiology, with keywords such as sepsis, microbiome, microbiota, and microflora recurring. Current trends indicate a growing focus on the relationship between sepsis and gut microbiome dynamics, with a notable gap in evidence-based clinical applications. The prominence of the United States in the field underscores the need for well-designed clinical trials and prospective cohort studies to advance therapeutic strategies. Strengthening global collaboration, particularly through increased involvement of Chinese researchers, is crucial for a comprehensive understanding and future advancements in this complex and evolving field.

Keywords: bibliometrics study, gut microbiome, sepsis

1. Introduction

Sepsis, a life-threatening condition resulting from the body's dysregulated response to infection (Singer *et al*. 2016), is a significant contributor to mortality in severe patients, with a mortality rate of up to 29.0%. Septic shock is a severe form of sepsis and is by the means of serious, circulatory, cellular, and metabolic abnormality, because of the hypotension, which is not corrected by the fluid resuscitation, that accounts for the increase in the death rate (Liu *et al*. 2022). The pathogenesis of sepsis involves a complex interplay of infection, inflammation, immunity, coagulation, and, notably, intestinal dysfunction and flora imbalance (Haak and Wiersinga 2017, Adelman *et al*. 2020). Among critically ill patients, the gut microbiome has gained prominence in sepsis research, drawing increasing attention from intensive care unit (ICU) physicians seeking ways to enhance gut microbiome health for improved survival in patients with sepsis.

The gut microbiome comprises the interaction between intestinal flora and the host, encompassing the intestine, intestinal epithelial cells, secretions, ingested food, and nutrients (Cresci and Bawden 2015, Heintz-Buschart and Wilmes 2018). Of particular significance is the role of intestinal flora, which plays a crucial role

* Corresponding Author: Xiangyou Yu, Department of nursing, Xinjiang Medical University, Xinjiang Uygur Autonomous Region, 830001. China; Critical Medicine Center, The First Affiliated Hospital of Xinjiang Medical University, Urumqi 830054, Xinjiang Uygur Autonomous Region, China, email: yuxiangyou2023@sina.com

Cite as:
Contemplating the bibliometric data onto sepsis and gut microbiome: mapping trends, collaborations, and global contributions. Zhang L. *et al.,* ADV MICROBIOL-NY, 2024, 63, 4, 191–198, https://doi.org/10.2478/am-2024-0016

in nutrient metabolism, digestion, absorption, resistance to external bacterial colonization, anti-tumor activities, and maintenance of the intestinal barrier and immune function (Jandhyala *et al*. 2015, Hills *et al.* 2019). The pathogenesis of sepsis involves the interaction of infection, inflammation, immune dysfunction, coagulation defect, and the disrupted blood-gut barrier is the central part of it. In sepsis, gut permeability increases and the gut bacteria and endotoxins can migrate out of the gut into the bloodstream, which sparks the body's inflammatory reaction and organ dysfunction. The blood-gut barrier's impairment gives rise to the difficult immune system situation, moreover, the vicious cycle of inflammation – bacterial multiplication – and the further endotoxin that is added into the bloodstream. Thus, the restoration of the blood-gut barrier is being the key issue in treating sepsis as well as the gastrointestinal microbiome as a component that participates in this process providing the default barrier and the health of the mucosal wall (Dubin 2018). Consequently, investigating the relationship between sepsis and the gut microbiome has become a key focus of numerous studies.

Current research on sepsis and the gut microbiome takes various angles, with some studies examining microbiome composition (Miller *et al*. 2021), others exploring microbiota dynamics (Kullberg *et al.* 2021), and additional investigations considering broader aspects of flora involvement. While reviews provide comprehensive summaries and analyses of the interplay between sepsis and the gut microbiome (Tourelle *et al*. 2021, Zanza *et al*. 2022), a macroscopic analysis of the overall research landscape in this field is lacking.

To address this gap, our study utilizes bibliometric methods in literature research to systematically analyze published works. Through retrieval, statistical processing, and comprehensive analysis of relevant literature, we aim to unveil the current state of research on the gut microbiome of sepsis patients. Bibliometric enables exploration of key aspects such as geographic distribution of research, institutional affiliations, prominent researchers, favored journals, and prevalent keywords within the literature corpus (Brandt *et al*. 2019). This methodological approach aims to provide an in-depth understanding of the current focus and developmental trajectory in the expansive field of sepsis and gut microbiome research.

2. Material and Methods

2.1. Data Source

The Science Citation Index Expanded (SCI-E) database in the Web of Science Core Collection (WOSCC) was utilized as the primary data source for retrieval. SCI-E is a leading and the most common used database for bibliometric research (Wu *et al*. 2022, Wu *et al*. 2024), encompassing English literature and, to some extent, literature in other languages with English abstracts, providing a comprehensive representation of global scientific research.

2.2. Search Strategy

A "subject" retrieval method was employed for this study, using the retrieval formula: sepsis AND ("gut" OR "gastrointestinal") AND ("microbiome" OR "microbiota" OR "flora" OR "bacillus"). The search was restricted to publications from 2004 onwards. This search strategy included sepsis broadly without differentiating between bacterial and fungal origins. Therefore, the results reflect a general analysis of sepsis cases that may include studies on both bacterial and fungal sepsis, depending on the scope of individual articles.

2.3. Analysis

All records and references from the search results were exported in plain text format and analyzed using the bibliometric software package in R. The analysis included; number of documents published by each country in the field, cooperative relationships between countries, number of documents published by institutions, cooperative relationships between institutions, number of documents published by researchers, cooperative relationships between researchers, citation status of researchers, number of documents published in journals, utilization of keywords.

2.4. Statistical Analysis

Trend charts illustrating the annual number of published documents and the number of cited documents were generated using Excel software. Qualitative data were presented quantitatively and as percentages, facilitating a rigorous statistical exploration of the bibliometric landscape. This methodology provides insights onto the global distribution, collaborative networks, institutional contributions, researcher engagement, citation impact, and journal dissemination within the field of sepsis and gut microbiome research.

3. Results

3.1. General Information

A meticulous examination of relevant research literature resulted in the retrieval of 2558 records. Post-duplicate removal, 2485 articles were considered

Table I
Type of publications on sepsis and gut microbiome.

Type	Publications	Percentage (%)*
Original articles	1671	67.24
Reviews	279	11.23
Clinical trial	105	4.23
Meeting	228	9.18
Editorials	72	2.90
Abstract	63	2.54
Online first	27	1.09
Case Report	40	1.61

Note: *percentage of 2485

Table II
Research directions of publications on sepsis and gut microbiome.

Directions	Publications	Percentage (%)*
Infectious diseases	1657	66.68
Gastroenterology hepatology	1428	57.46
Biochemistry molecular biology	1074	43.22
Immunology	1039	41.81
Pathology	956	38.49
Microbiology	931	37.46
Nutrition dietetics	914	36.78
Pharmacology pharmacy	879	35.37
Food Science technology	708	28.49
Genetics heredity	703	28.29

Note: *percentage of 2485

for detailed analysis (Table I). This diverse dataset comprised 1671 original treatises, 279 review papers, 105 clinical studies (distinctly separated from the original treatises), 72 editorial materials, 228 conference proceedings papers, 63 conference abstracts, 27 advanced online publications, and 40 case reports. Analysis of the dataset revealed a discernible temporal pattern, illustrating a consistent year-on-year increase in the number of published documents (Fig. 1). Concurrently, the citation frequency of this literature exhibited a parallel upward trend, indicating a growing impact within the scientific community over time (Fig. 1). The cumulative citations for these documents reached an impressive 62247, with an average citation frequency of 25.05 times per document. A thorough examination of research directions, as depicted in Table II, illuminated the primary thematic concentrations within the dataset. Predominantly, the studies were centered on Infectious Diseases, Gastroenterology, Hepatology, Biochemistry and Molecular Biology. Additionally, substantial engagement was observed in the domains of Immunology, Pathology, and Microbiology, signifying the interdisciplinary nature of research exploring the intricate dynamics between sepsis and the gut microbiome. These results collectively contribute to a comprehensive understanding of the study landscape, delineating the composition of the literature corpus, temporal dynamics, and thematic concentrations. Such insights are instrumental in appreciating the evolving trends and scientific contributions within the realm of sepsis and gut microbiome research.

3.2. Analysis of Countries and Institutions

The examination of source countries within the literature corpus reveals substantial contributions from the United States, France, and the United Kingdom, displaying a marked prominence over other nations (Fig. 2). Notably, the United States exhibits a noteworthy lead, surpassing the combined literature output of the third to seventh-ranked countries. This underscores the significant influence wielded by these leading nations in shaping discourse on sepsis and the gut microbiome. An assessment of international collaboration scores, serving as an indicator of research cooperation enthusiasm, discloses the collaborative nature inherent in the field. The United States emerges as a key collaborator, boasting the highest cooperation score, followed by France, Germany, and other European

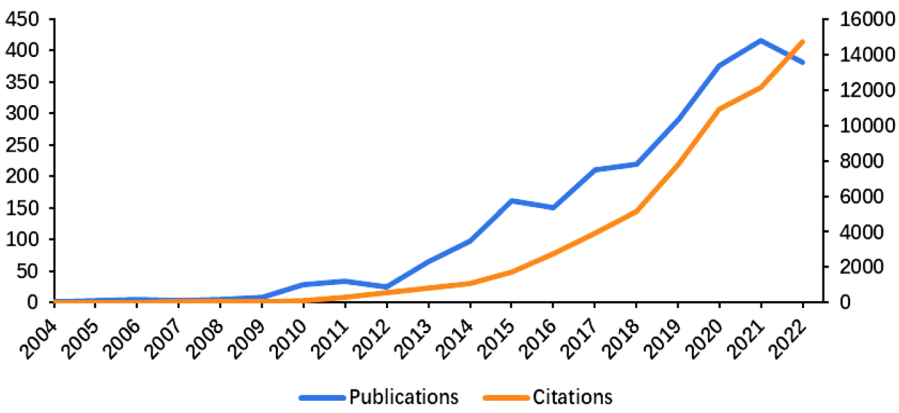


Fig. 1. Annual trends of publications and citations.

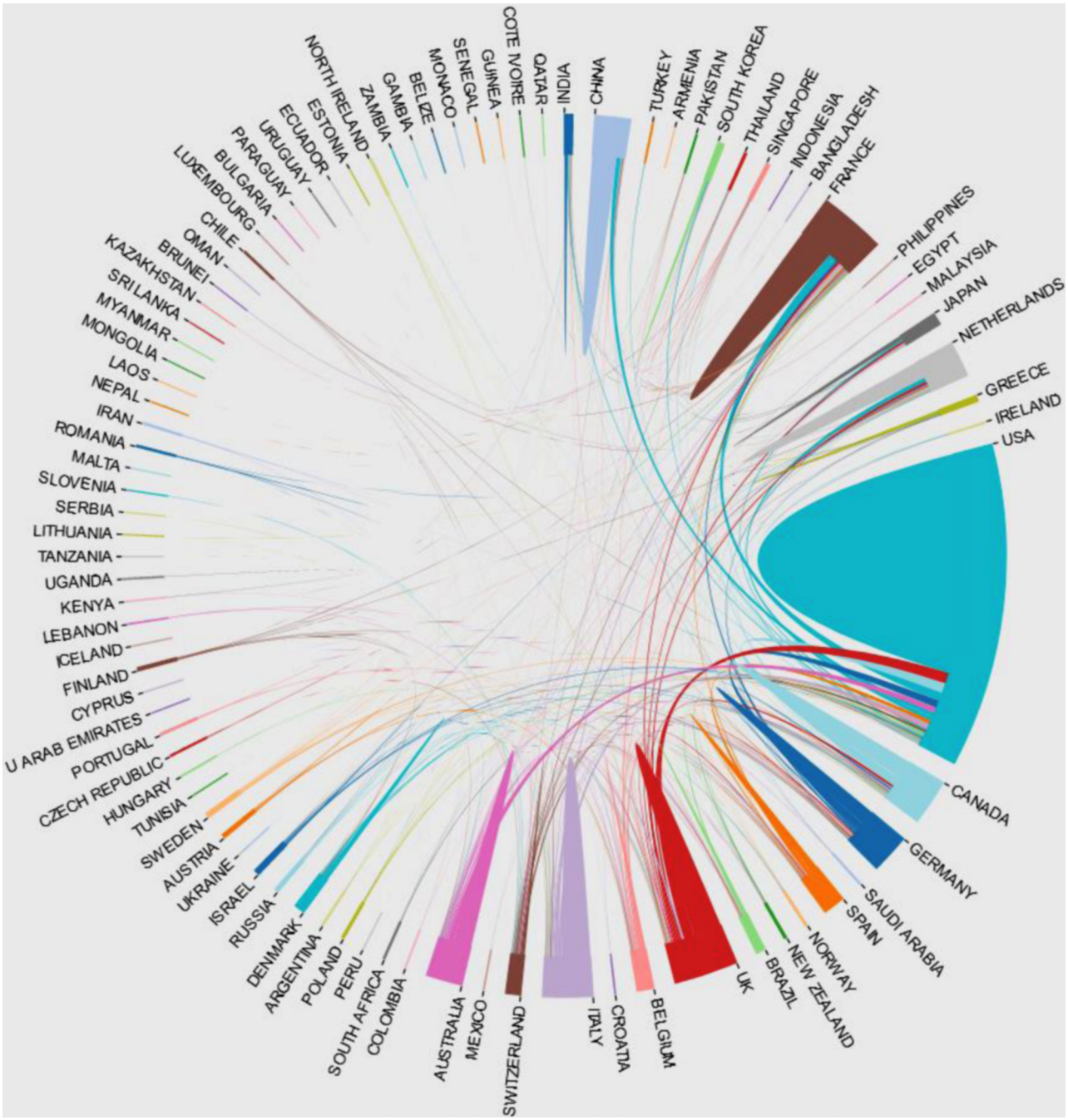


Fig. 2. Countries in publications and cooperations.

nations (Table III). Importantly, despite China holding the eighth position in total publications, its cooperation enthusiasm ranks tenth, indicative of a more reserved engagement in collaborative research initiatives within this scientific domain. Upon analyzing the institutions contributing to the published literature (Table IV), five of the top ten research institutions are situated in the United States, with the University of California leading the cohort. France is represented by two institutions in the top ten, while the Netherlands, the United Kingdom, and China each have one institution. The central cooperation score unveils the collaborative dynamics among these institutions, with the University of California demonstrating the highest enthusiasm for collaboration, followed by the University of Amsterdam and the University of Chicago. Noteworthy is the independent research stance of Southern Medical University China, ranking ninth in the number of published documents. This institution stands out for its self-reliant research endeavors, displaying limited collaboration with external entities, marking a distinctive scholarly approach to sepsis and gut microbiome research. These findings provide a grounded and professionally articulated insight into global contributions, international collaboration dynamics, and institutional engagements within the expansive field of sepsis and gut microbiome

Table III
Centrality of countries in the studies on sepsis and gut microbiome.

Ranks	Countries	Centrality
1	USA	0.36
2	France	0.24
3	Germany	0.21
4	England	0.20
5	Italy	0.17
6	Netherlands	0.16
7	Spain	0.15
8	Australia	0.13
9	Canada	0.12
10	China	0.11

Table IV
Top 10 institutes by number of publications on sepsis and gut microbiome.

Institutions	Publications	Percentage (%)*
University of California	88	3.54
University of Amsterdam	85	3.42
Udice French Research Universities	63	2.54
University of Chicago	60	2.41
Institute National De La Sante	50	2.01
University of Michigan	45	1.81
Harvard University	39	1.57
Newcastle University UK	35	1.41
Southern Medical University China	34	1.37
University of Minnesota	30	1.21

Note: *percentage of 2485

literature. Such insights enrich our understanding of the collaborative and independent scientific pursuits instrumental in advancing knowledge within this domain (Table V).

Table V
Top 10 institutes by centrality on sepsis and gut microbiome.

Ranks	Institutions	Centrality
1	University of California	0.24
2	University of Amsterdam	0.23
3	University of Chicago	0.18
4	Udice French Research Universities	0.17
5	Institut National De La Sante	0.15
6	University of Michigan	0.14
7	University of Florida	0.13
8	Chulalongkorn University	0.12
9	Northumbria University	0.11
10	Universidade De Sao Paulo	0.11

3.3. Author's Analysis

Wiersinga WJ from the University of Amsterdam in the Netherlands was identified as the most productive author in this field, as evident in the analysis results (Table VI). Professor Wiersinga also holds the highest centrality score (Table VII), highlighting the extensive impact of their contributions within the scholarly network. Further scrutiny reveals that Professor Wiersinga has authored a substantial number of articles on sepsis and gut microbiome, with over half published in prestigious journals such as JAMA, Lancet sub-journal, Gut, and other top-tier publications. Notably, collaborative associations among the top 10 authors include Wiersinga WJ and van der Poll T from the University of Amsterdam, and Berrington JE and Stewart CJ from Newcastle University, UK.

Table VI
Top 10 authors by number of publications on sepsis and gut microbiome.

Ranks	Authors	Publications	Percentage (%)*
1	Wiersinga WJ	44	1.77
2	Alverdy John C	17	0.68
3	Berrington JE	14	0.56
4	Leelahavanichkul A	15	0.60
5	Embleton ND	12	0.48
6	van der Poll T	11	0.44
7	Stewart CJ	10	0.40
8	Dickson RP	7	0.28
9	Wang W	6	0.24
10	Haak BW	6	0.2

Note: *percentage of 2485.

Table VII
Top 10 authors by centrality on sepsis and gut microbiom.

Ranks	Authors	Centrality
1	Wiersinga WJ	0.26
2	Alverdy John C	0.23
3	Leelahavanichkul A	0.19
4	Embleton ND	0.18
5	Wang YJ	0.17
6	Cao ZJ	0.17
7	Wang W	0.17
8	He ZY	0.15
9	Liu S	0.14
10	Li SL	0.13

3.4. Journals Analysis

The analysis of journals (Table VIII) reveals that Figshare, a prominent open data storage platform, leads in the number of publications on sepsis and gut

Table VIII
Top 10 journals by publication of literatures on sepsis and microbiome

Journals	Publications	Percentage (%)*	2022 IF
Figshare	166	6.67	Database
Nutrients	90	3.64	6.706
Critical Care	81	3.24	19.334
Plos One	78	3.13	3.752
Scientific Reports	73	2.93	4.996
European Nucleotide Archive	68	2.73	Database
Frontiers in Microbiology	60	2.42	6.064
Frontiers in Immunology	50	2.02	8.786
Pediatric Research	45	1.82	3.953
International Journal of Molecular Sciences	40	1.62	6.208

Note: *percentage of 2485.

microbiome research. Following closely are reputable academic journals, including Nutrients and Critical Care, among others. It is pertinent to note that Figshare, while a significant contributor, operates more as a data storage platform than a traditional academic journal. Among the academic journals publishing literature in this field, Gastroenterology (IF = 33.883) demonstrated the highest impact factor in 2022, with a total of 9 pertinent articles. This is followed by Critical Care, Frontiers in Immunology, and other journals, reflecting a diverse and impactful dissemination of research within the realm of sepsis and gut microbiome studies.

3.5. Keywords Analysis

A meticulous examination of all keywords utilized in the included literature underscores key themes prevalent in the field. The preeminent keyword is "sepsis," reflecting its centrality in scholarly discussions. Following closely are pivotal terms such as "microbiome," "microbiota," and "flora," highlighting the pronounced emphasis on microbial communities in this domain. Notably, six out of the top 10 keywords-specifically, "microbiome," "microbiota," "flora," "gut microbiota," "bacteria," and "gastrointestinal microbiome"-essentially revolve around or intricately relate to microecology. This prevalence of microecology-centric keywords signifies the integral role of microbial interactions and ecological dynamics within the broader context of sepsis and gut microbiome research (Table IX).

Table IX
Distribution of keywords.

Keywords	Frequency	Percentage (%)*
Sepsis	2174	87.48
Microbiome	991	39.88
Microbiota	765	30.78
Flora	382	15.37
Antibiotics	337	13.56
Gut microbiota	306	12.31
Immune system	218	8.77
Bacteria	184	7.40
Gut	163	6.56
Gastrointestinal microbiome	152	6.12

Note: *percentage of 2485.

4. Discussion

The outcomes of this study affirm the increasing research focus on the association between sepsis and the microbiome over the past decade. The upward trajectory in published literature reflects the growing acknowledgment of the clinical importance of investigating infectious diseases, particularly sepsis (Coopersmith *et al.* 2018). The observed dominance of the United States and China in the quantity of published literature underscores their significant contributions to the field. The United States not only exhibits a substantial publication volume but also a noteworthy centrality score, indicating a considerable influence within the scholarly network. Conversely, while China demonstrates prolific publication, its lower centrality score suggests potential for increased collaborative engagement on the international stage (Rhee *et al.* 2017).

Analysis of research institutions reveals the prevalence of European and American entities, with the University of California emerging as a central hub for collaborative research in this field. Noteworthy contributors include Fang Min and Wiersinga WJ from the University of Amsterdam, with Prof Wiersinga leading in both publication volume and collaborative endeavors. Journal analysis indicates that while research in this field is present in authoritative journals, there is a prevalent focus on severe diseases, microbiology, and immunology publications (Wiersinga and van der Poll 2022). This thematic alignment underscores the clinical and microbiological dimensions inherent in sepsis and gut microbiome studies. Examining the microecology, our study underscores the pivotal role of gut microbiome in human health. The intricate interplay of microbial entities, including Bacteroides, Bifidobacterium, and Lactobacillus, highlights their diverse contributions to nutrient metabolism, immune function, and intestinal barrier maintenance (Schmidt *et al.* 2018).

In the context of sepsis, our analysis reveals distinct alterations in the gut microbiome, characterized by reduced diversity, altered abundance of key microbial

groups, and overgrowth of pathogenic bacteria. This dysbiosis correlates positively with the mortality risk in patients exhibiting systemic inflammatory response syndrome and multiple organ dysfunction syndrome (Shimizu *et al.* 2011). The use of broad-spectrum antibiotics, a common therapeutic approach in sepsis, emerges as a significant factor contributing to gut microbiome imbalance (Condotta *et al.* 2013). This disruption is not only related to the duration of antibiotic use but also to specific antibiotic types and/or combinations of antibiotics, such as ceftriaxone and imipenem, with implications for the risk of opportunistic infections and antibiotic-associated complications (Haak and Wiersinga 2017). Clinical strategies for mitigating gut microbiome imbalance include judicious antibiotic use, supplementation with microecological agents, early enteral nutrition, and fecal flora transplantation (He *et al.* 2013, Wang *et al.* 2019; Ramirez *et al.* 2020, Johnstone *et al.* 2021, Cheema *et al.* 2022). These interventions aim to preserve microbial diversity, restore ecological balance, and improve patient outcomes.

Apart from the bacterial factors in the gut microbiota, the contribution of fungus in keeping the microorganism balance is a very important aspect that should be taken into consideration. Fungal species, primarily Candida, Aspergillus, and Zygomycetes, can considerably affect the intestinal microbiome. For example, Candida albicans is often related to dysbiosis, especially in people with weakened immune systems or those who are on the long-term use of broad-spectrum antibiotics. This non-optimal situation in the microbiota causes hypoproliferation, inflammation as well as negative changes in the intestinal barrier. Besides, the Aspergillus genus, although it isn't usually mentioned in gut health conversations, has been associated with dysbiosis in serious infections and it can disturb the microbiome community's normal activities in the gastrointestinal tract. Recognizing the role that fungi play in these processes is critically important if we are to develop successful strategies to fight against dysbiosis in sepsis patients (Chin *et al.* 2020).

In addition to macroscopic tendencies found in sepsis and gut microbiome research, the discovery of the etiological agents is very important in the differential diagnosis of sepsis microbe contributors. In our bibliometric style, we did not define independent causative pathogens, but prior studies often find that bacterial species, such as Escherichia coli, Klebsiella pneumoniae, and Staphylococcus aureus are common etiological factors in bacterial sepsis. These pathogens are frequently referred to disturbances in the gut microbiota, which are advantageous for the proliferation of pathogenic species and disadvantageous from the perspective of a high microbial diversity ratio. Bacteria, specifically Candida albicans, are also the predisposing factors for sepsis in cases of immunocompromised patients and they are associated with specific dysbiosis patterns in the gut microbiome (Jawhara 2022). The limitation in the bacterial and fungal etiologies can be the potential information on the dysbiosis and thus, the development of specific therapeutic strategies targeting the patient's microbes may prove to be the solution.

While this study provides a comprehensive macroscopic overview of the literature, its limitations include the lack of in-depth analysis of individual studies, interventions, and recent advancements. Additionally, the exclusion of non-English literature from the SCI-E database introduces a potential source of omission, emphasizing the need for a more inclusive approach to capture global contributions. The surge in research on the relationship between sepsis and gut microbiome signifies a burgeoning field with evolving clinical implications. The dominance of the United States in this research landscape underscores its leadership role. Future directions should prioritize evidence-based investigations, clinical trials, and prospective studies. Enhanced international collaboration, particularly involving Chinese researchers, holds promise for advancing the understanding and management of sepsis through the lens of gut microbiome dynamics.

5. Conclusion

Current study delves into the expanding landscape of sepsis and gut microbiome research, analyzing 2485 articles spanning diverse publication types and thematic concentrations. The temporal analysis reveals a consistent increase in publications, reflecting a burgeoning interest and impact within the scientific community. The dominance of the United States in both quantity and centrality underscores its pivotal role, with significant contributions from France, the United Kingdom, and others. Authorship analysis highlights Prof Wiersinga's prolific output and impact, while institutional dynamics and international collaboration further enrich the scholarly network. The examination of journals and keywords elucidates the thematic focus on microbiome-related concepts. The findings underscore altered gut microbiome dynamics in patients with sepsis, influenced by factors like antibiotic use, with clinical strategies proposed for mitigation. The study's limitations, including language exclusivity and the need for more detailed analyses, are acknowledged. Moving forward, the emphasis on evidence-based investigations, clinical trials, and international collaboration, particularly involving Chinese researchers, holds promise for advancing understanding and management in this evolving field. In essence, current study provides a nuanced and professionally articulated overview of

sepsis and gut microbiome research, paving the way for future exploration and advancements in evidence-based medicine.

ORCID
Xiangyou Yu: https://orcid.org/0009-0004-6909-142X
Li Zhang https://orcid.org/0000-0003-4131-6442
Yi Wang https://orcid.org/0000-0002-4985-2998
Ping Li https://orcid.org/0000-0001-7660-6082
Xiang Li https://orcid.org/0000-0002-2430-6798

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

Funding

National Natural Science Foundation of China (82160360).

References

1. Adelman M.W., Woodworth M.H., Langelier C., Busch L.M., Kempker J.A., Kraft C.S., & Martin G.S.: The gut microbiome's role in the development, maintenance, and outcomes of sepsis. *Crit. Care,* **24**, 1–10 (2020)
2. Brandt J.S., Hadaya O., Schuster M., Rosen T., Sauer M.V., & Ananth C.V.: A bibliometric analysis of top-cited journal articles in obstetrics and gynecology. *JAMA Netw. Open* **2**, e1918007 (2019)
3. Cheema H.A., Shahid A., Ayyan M., Mustafa B., Zahid A., Fatima M., Ehsan M., Athar F., Duric N., & Szakmany T.: Probiotics for the prevention of ventilator-associated pneumonia: An updated systematic review and meta-analysis of randomized controlled trials. *Nutrients* **14**, 8 (2022)
4. Chin V.K., Yong V.C., Chong P.P., Nordin S.A., Basir R., & Abdullah M.: Mycobiome in the gut: A multiperspective review. *Mediators Inflamm*. **2020**, 9560684 (2020)
5. Condotta S.A., Cabrera-Perez J., Badovinac V.P., & Griffith T.S.: T-cell-mediated immunity and the role of TRAIL in sepsis-induced immunosuppression. *Crit. Rev. Immunol.* **33**, 23–40 (2013)
6. Coopersmith C.M., et al.: Surviving sepsis campaign: research priorities for sepsis and septic shock. *Intensive Care Med*. **44**, 1400–1426 (2018)
7. Cresci G.A. & Bawden E.: Gut microbiome: What we do and don't know. *Nutr. Clin. Pract*. **30**, 734–746 (2015)
8. Dubin K.A.: Microbial dynamics during gut inflammation and infection. Weill Medical College of Cornell University (2018)
9. Haak B.W. & Wiersinga W.J.: The role of the gut microbiota in sepsis. *Lancet Gastroenterol. Hepatol*. **2**, 135–143 (2017)
10. He D., Wang H.Y., Feng J.Y., Zhang M.M., Zhou Y., & Wu X.T.: Use of pro-/synbiotics as prophylaxis in patients undergoing colorectal resection for cancer: A meta-analysis of randomized controlled trials. *Clin. Res. Hepatol. Gastroenterol*. **37**, 406–415 (2013)
11. Heintz-Buschart A., Wilmes P.: Human Gut Microbiome: Function Matters. *Trends Microbiol*. **26**(7), 563–574 (2018)
12. Hills R.D. Jr, Pontefract B.A., Mishcon H.R., Black C.A., Sutton S.C., Theberge C.R.: Gut Microbiome: Profound Implications for Diet and Disease. *Nutrients*. **11**(7), 1613 (2019)
13. Jandhyala S.M., Talukdar R., Subramanyam C., Vuyyuru H., Sasikala M., Nageshwar Reddy D.: Role of the normal gut microbiota. *World J Gastroenterol*. **21**(29) 8787–8803 (2015)
14. Jawhara S.: How gut bacterial dysbiosis can promote Candida albicans overgrowth during colonic inflammation. *Microorganisms* **10**, 1014 (2022)
15. Johnstone J. & Zytaruk N. et al.: Effect of probiotics on incident ventilator-associated pneumonia in critically ill patients: A randomized clinical trial. *JAMA*, **326**, 1024–1033 (2021)
16. Kullberg R.F., Wiersinga W.J., & Haak B.W.: Gut microbiota and sepsis: From pathogenesis to novel treatments. *Curr. Opin. Gastroenterol*. **37**, 578–585 (2021)
17. Liu Y.C., Yao Y., Yu M.M., Gao Y.L., Qi A.L., Jiang T.Y., Chen Z.S., Shou S.T., & Chai Y.F.: Frequency and mortality of sepsis and septic shock in China: A systematic review and meta-analysis. *BMC Infect. Dis*. **22**, 564 (2022)
18. Miller W.D., Keskey R., & Alverdy J.C.: Sepsis and the microbiome: A vicious cycle. *J. Infect. Dis*. **223**, S264–S269 (2021)
19. Ramirez J., Guarner F., Bustos Fernandez L., Maruy A., Sdepanian V.L., & Cohen H.: Antibiotics as major disruptors of gut microbiota. *Front. Cell Infect. Microbiol*. **10**, 572912 (2020)
20. Rhee C. & Clompas M.*et al*.: Incidence and trends of sepsis in US hospitals using clinical vs claims data, 2009–2014. *JAMA* **318**(13), 1241–1249 (2017)
21. Schmidt T.S.B., Raes J., & Bork P.: The human gut microbiome: From association to modulation. *Cell* **172**, 1198–1215 (2018)
22. Shimizu K. & Sugimoto H. et al.: Altered gut flora are associated with septic complications and death in critically ill patients with systemic inflammatory response syndrome. *Dig. Dis. Sci.* **56**, 1171–1177 (2011)
23. Singer M. & Deutschman C.S. et al.: The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA* **315**, 801–810 (2016)
20. Tourelle K.M., Boutin S., Weigand M.A., & Schmitt F.C.: Sepsis and the human microbiome: Just another kind of organ failure? A review. *J. Clin. Med*. **10**, 4831 (2021)
24. Wang J.W., Kuo C.H., Kuo F.C., Wang Y.K., Hsu W.H., Yu F.J., Hu H.M., Hsu P.I., Wang J.Y., & Wu D.C.: Fecal microbiota transplantation: Review and update. *J. Formos. Med. Assoc*. **118**, S23–S31 (2019)
25. Wiersinga W.J. & van der Poll T.: Immunopathophysiology of human sepsis. *E.Bio.Medicine*. **86**, 104363 (2022)
26. Wu F., Gao J., Kang J., Wang X., Niu Q., Liu J., & Zhang L.: Knowledge mapping of exosomes in autoimmune diseases: A bibliometric analysis (2002–2021). *Front. Immunol*. **13**, 939433 (2022)
27. Wu T., Wu Y., Nie K., Yan J., Chen Y., Wang S., & Zhang J.: Bibliometric analysis and global trends in uterus transplantation. *Int. J. Surg*. **110**, 4932–4946 (2024)
28. Zanza C., Romenskaya T., Thangathurai D., Ojetti V., Saviano A., Abenavoli L., Robba C., Cammarota G., Franceschi F., & Piccioni A.: Microbiome in critical care: An unconventional and unknown ally. *Curr. Med. Chem*. **29**, 3179–3188 (2022)

ADVANCEMENTS OF MICROBIOLOGY
2024, 63, 4, 199–222
https://doi.org/10.2478/am-2024-0017

DESCRIPTION OF METHODOLOGY FOR TESTING THE SYNERGISTIC AND ADDITIVE EFFECTS OF ANTIBIOTICS *IN VITRO*

Paweł Z. Kmiecikowski[1,*], Aniela Gabriel[1], Dagmara Depka[2], Tomasz Bogiel[2*]

[1] Microbiology Student Science Club, Ludwik Rydygier Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, 9 Maria Skłodowska-Curie Street, 85-094 Bydgoszcz, Poland.

[2] Microbiology Department Ludwik Rydygier, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, 9 Maria Skłodowska-Curie Street, 85-094 Bydgoszcz, Poland.

Submitted in August 2024, accepted in November 2024

Abstract. Given the dynamic growth of antibiotic resistance observed over the past few decades, new therapeutic methods for life-threatening infections are crucial. Effective treatment of infections caused by multidrug-resistant microorganisms increasingly requires the use of last resort drugs, and new antibiotics generate resistance in less time than it takes to bring them to the therapy. Therefore it is advisable to seek solutions using currently available antibiotics. Effective therapy of infections with multidrug-resistant microorganisms may be enabled by the use of additive and synergistic interactions, resulting from the combination of different groups of antibiotics with the same or different modes of action – including those to which a particular bacterial strain is resistant. This paper describes methods for determining the synergistic and additive effects of antibiotics *in vitro*, including the CombiANT method under testing. The purpose of this study is to present possible methods for determining the additive and synergistic effects between antibiotics *in vitro* and to propose appropriate laboratory procedures for their use. This paper reviews the latest literature on methods for determining the antimicrobials interactions. Clinical studies indicate significant benefits of using the phenomenon of antibiotic addition or synergy in clinical practice, not only improving the effectiveness of therapy, but also minimizing side effects and reducing the risk of developing *de novo* antibiotic resistance. Despite the significant problem of increasing antibiotic resistance level, clinical practice still lacks clearly standardized methods for determining synergism. Further research is needed to determine the most beneficial standard available for a wide range of microbiology diagnostic facilities.

Keywords: antibiotic resistance, antibiotic synergism, checkerboard test, time-kill test

1. Introduction

The epidemiological transformation in the early 20th century involved a shift from infectious diseases to non-infectious diseases as the leading causes of death worldwide. It became a source of hope for the ultimate victory of medicine over infections, facilitated by the discovery of many antibiotics in the 1940s and 1950s. Unfortunately, after only a few decades, due to the rapid growth of antibiotic resistance, infectious diseases have once again become one of the greatest threats to public health. Scientists such as Venkatasubramanian Ramasubramanian, president of the Clinical Infectious Disease Society of India, have been warning of a new post-antibiotic era for some time now (Sayburn 2023).

In addition to the significant rate of development of global antibiotic resistance, a critical aspect of the treatment of infections is the epidemiological situation in Poland, compared to Europe. For example, in 2021, fluoroquinolones-resistant *Escherichia coli* isolates accounted for 33.1% of *E. coli* isolates in Poland (population-weighted European average: 21.9%), carbapenem-resistant *Klebsiella pneumoniae* isolates for 19.5% (population-weighted European average: 11.7%), and vancomycin-resistant *Enterococcus faecium* isolates for 34.3% (population-weighted European average: 17.2%).

* Corresponding Authors: Paweł Z. Kmiecikowski, Studenckie Koło Naukowe Mikrobiologii przy Katedrze Mikrobiologii, Tomasz Bogiel Katedra Mikrobiologii, Wydział Farmaceutyczny, Collegium Medicum w Bydgoszczy Uniwersytet Mikołaja Kopernika w Toruniu, ul. M. Curie Skłodowskiej 9, 85-094 Bydgoszcz, e-mail: kmiecikowski.pawel@gmail.com, t.bogiel@cm.umk.pl

Cite as:
Description of methodology for testing the synergistic and additive effects of antibiotics *in vitro*. Kmiecikowski P.Z. *et al.*, ADV MICROBIOL-NY, 2024, 63, 4, 199–222, https://doi.org/10.2478/am-2024-0000

More than half of the isolated *K. pneumoniae* strains showed multidrug resistance (MDR), which in this case meant resistance simultaneously to aminoglycosides, fluoroquinolones and third-generation cephalosporins (Żabicka and Grzegorczyk 2022). A significant difference between the degree of antibiotic resistance in Poland and Western and Northern European countries is also of great concern. In 2022, carbapenem-resistant *Acinetobacter baumannii* strains accounted for 76.4% of isolates in Poland, compared with only 3.5% in Germany and France and 2.7% in Sweden (European Center for Disease Prevention and Control).

The stagnation of the pharmaceutical market further hampers the effective treatment of infections. From 2017 to 2022, only 12 new antibiotics have been approved, and only two of them – vaborbactam and lefamulin – are representatives of new drug groups. A significant constraint and challenge of new antimicrobials introduction is the cost of bringing new therapeutics to market and the need for some of the latest agents to be seen as drugs of last resort. In most cases, resistance to new drugs is already reported within 2–3 years of their initial application (WHO 2021).

Although research is ongoing on new antibacterial agents and strategies, such as monoclonal antibodies or bacteriophages (WHO 2021), in light of such a dynamic development of the phenomenon of antibiotic resistance, it is necessary to search for new forms of treatment, including also these using already available antibiotics. A promising infection treatment method combines antibiotics with different mechanisms of action in therapy, including antibiotics to which bacteria were resistant in monotherapy. Their additive or synergistic action may allow them to reduce the value of their minimal inhibitory concentrations (MICs) or even break the barrier of resistance of a strain to particular drugs. Additional advantages of achieving a synergistic effect of a combined antibiotics approach include maximizing treatment effects with a reduced risk of developing *de novo* antibiotic resistance and possibly using lower drug doses (Garbusińska and Szliszka 2017). It is worth recalling that combined antimicrobial therapy is almost as old as antibiotic therapy itself, as evident in the history of the treatment of infections caused by *Mycobacterium tuberculosis*, in which the association of streptomycin with other drugs began soon after its discovery. Indeed, it was found that after the several months course of tuberculosis pharmacotherapy, the probability of developing resistance to streptomycin used in monotherapy could be as high as 100% (Brennan-Krohn and Kirby 2019a).

Several methods are currently used to study the additive and synergistic effects of antibiotics: e.g. methods based on strips impregnated with antibiotics in a concentration gradient, antimicrobials disc diffusion methods, antimicrobials micro-dilutions in agar methods, the checkerboard method, "time-kill" test, *in vitro* dynamic pharmacokinetic and pharmacodynamic (PK/PD) models, semi-mechanistic PK/PD models, and even *in vivo* animal models (Karakonstantis *et al.* 2022). This article characterizes and describes the step-by-step methodology of several of the methods mentioned above, which, in the authors' opinion, have the most significant potential for use in daily laboratory and clinical practice as routine methods for testing antibiotic combinations, which in the future may contribute to improving the clinical condition of many patients suffering from infections caused by multi-drug-resistant bacterial strains.

2. Methodology of literature search and selection

PubMed and Google Scholar databases were used for the literature review, searching for the phrases: antibiotic resistance, antibiotic synergism, antibiotic interaction methodology, antibiotic FICI assessing, checkerboard assay, "time-kill" curves, synergism disc-based methods, synergism gradient-based methods, antibiotic gradient-based methods from the last 10 years. Among the results obtained, articles on antibiotic combinations were selected, including descriptions of the most commonly used methods for testing synergism and antibiotic adherence in substantive, clinical and/or procedural terms. Information on the epidemiology of antibiotic resistance was obtained from the Surveillance Atlas of Infectious Diseases of the European Centre for Disease Prevention and Control.

3. Method based on antibiotics microdilution series in broth medium

The microdilution method (so-called "checkerboard test") is the most popular method for testing the *in vitro* activity of antibiotic combinations, a modification of the standard method for determining MIC in broth, a technique routinely used, for example, in assessing the MIC value of colistin. Most often, a 96-well polystyrene plate is used. It is adapted to test the simultaneous activity of two antibiotics in each well – antibiotic concentrations are placed onto the plate, creating a gradient "checkerboard" of dilutions of the two antimicrobials so that every possible combination of the two concentrations of the test drugs is evaluated within the chosen and planned concentration ranges. This method has two severe limitations. First, it is a static method in which the effect of antibiotics is assessed at a specific point in time without the possibility of being evaluated for several hours of incubation. It provides information only on the bacteriostatic effect without the possibility

of testing the killing properties of drugs (in the methodology used, these two effects are indistinguishable) (Brennan-Krohn and Kirby 2019a). Limiting the number of antibiotics tested to two is also essential. With more than that, the method quickly becomes impractical, even assuming that at least one of the drugs will be tested in a relatively narrow range of concentrations (Brennan-Krohn and Kirby 2019a; Doern 2014). In addition, this method requires many reagents, and it is necessary to prepare many antibiotic dilutions.

In the described method, standardized bacterial inocula (of constant density and volume) in Mueller-Hinton broth and antibiotics (usually two from two different groups) in appropriate concentrations are placed in the wells of a polystyrene plate, the concentrations of which can be related to the concentrations achievable in the patient's body fluids and which were obtained in serial two-fold dilutions (Doern 2014; Garbusińska and Szliszka 2017; Brennan-Krohn and Kirby 2019a). After the incubation time, bacterial growth is assessed in all of the wells of the plate. For the wells in which the bacterial growth is inhibited, the fractional inhibitory concentration index (FICI) is calculated and based on this, the antibiotic interaction is classified as antagonistic ($FICI > 4$), neutral ($1 < FICI \leq 4$), additive ($0.5 < FICI \leq 1$) or synergistic ($FICI \leq 0.5$). Although authors of scientific investigation sometimes use other ranges for FICI values interpretation or do not distinguish between antibiotic addition/synergy effects, the cited classification method is the most common. The FICI for a given concentration combination at which inhibition is observed is the sum of the fractional inhibitory concentration (FIC) of both drugs, i.e. the ratio of the drug concentration in that well to its MIC. Thus, since synergism is evidenced by an $FICI \leq 0.5$, and the error range for MIC testing in a standard dilution in broth is ± 1 two-fold dilution (with the error range increasing when testing drugs in combination), the definition of synergism is met if each drug in a well has a concentration of at least half that of its MIC assessed individually in a separate assay (Garbusińska and Szliszka 2017; Brennan-Krohn and Kirby 2019a).

The broth microdilution method continues to be modified and improved, such as using a bio-printer, which allows for precise micro-volume measurements and speeds up the entire procedure (Brennan-Krohn and Kirby 2019b). The procedure described below includes reagents and equipment commonly available in microbiology laboratories.

Procedure

1. Preparation of bacterial inoculum.
   - 1.1. Calibrate the densitometer against a control sample with an optical density of 0.5 on the McFarland scale.
   - 1.2. Collect the tested strain with a sterile loop from the agar medium and place it in a solution appropriate for the method (usually saline).
   - 1.3. Vortex it.
   - 1.4. Measure of the optical density of the suspension in a densitometer.
   - 1.5. Optionally, add the bacterial mass or the control strain being tested using a loop to an optical density of 0.5 on the McFarland scale.
2. Determination of MICs of the tested antibiotics (method of choice).
3. Preparation of antibiotic solutions.
   - 3.1. Select the antibiotic to be diluted in rows 1–12, i.e. horizontal rows (antibiotic A), and another in wells A→H, i.e. vertical columns (antibiotic B); consider the appropriateness of testing one in a broader range of concentrations.
   - 3.2. Prepare approximately 400 µl of antibiotic A solution and approximately 350 µl of antibiotic B at a concentration four- (for A) and eight- (for B) times higher than the resistance cut-off value of a given drug for the species of the strain being tested (Brennan-Krohn *et al.* 2017; EUCAST 2024). Select the solvent according to the CLSI (Clinical and Laboratory Standards Institute) guidelines: "Solvents and Diluents for Preparing Stock Solutions of Antimicrobial Agents". If the guidelines do not contain information on the tested antibiotic, use a solvent that maintains more excellent drug stability (Bellio *et al.* 2021).
     - 3.2.1. It is possible to use the following pattern:

       $C_a - C_k = V_r$

       $C_k - C_r = V_a$

       Where:

       C_a – initial concentration of the antibiotic solution [mg/l],

       C_k – final concentration of antibiotic solution [mg/l],

       C_r – solvent concentration [mg/l],

       V_r – solvent volume [µl],

       V_a – initial volume of antibiotic solution [µl].

       Adding V_a of the initial antibiotic solution to V_r of the solvent will provide an antibiotic solution with a concentration of C_k. C_k should have a value four times higher than the breakpoint value for resistance to a given drug for the strain of the species tested.
4. Preparation of a concentration checkerboard. (Fig. 1. Broth microdilution method – preparation of a concentration pattern.)
   - 4.1. Add 50 µl of MHB (Mueller-Hinton broth) to all wells in rows 1st to 11th and two wells in row 12th by automatic adjustable pipette. (Fig. 1A. Add Mueller-Hinton broth.)

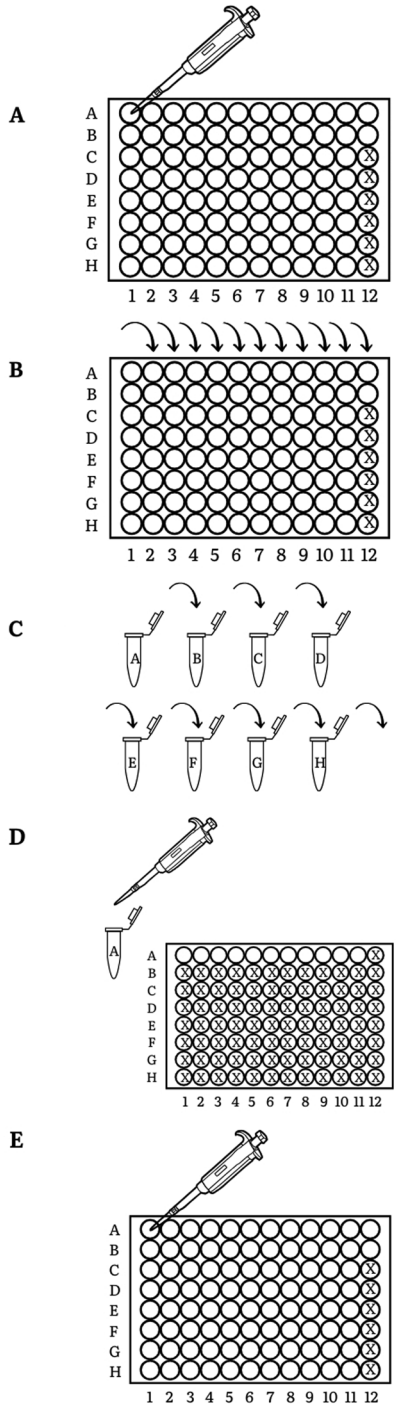


Fig. 1. Broth microdilution method – preparation of a concentration pattern.
A – add Mueller-Hinton broth.
B – prepare a series of microdilutions of the first antibiotic.
C – prepare a series of microdilutions of the second antibiotic.
D – add antibiotics together.
E – re-add of Mueller-Hinton broth.

4.2. Add 50 µl of antibiotic A to all wells (A-H) in row 1st.
4.3. With a multi-channel adjustable pipette, transfer 50 µl of solution from the wells of row 1st to the wells of row 2nd, from row 2nd to row 3rd, and so to row 11th. Mix the resulting solution each time with an automatic pipette (slowly aspirate and withdraw the solution several times). Transfer 50 µl of solution from wells 11A and 11B to 12A and 12B. Remove 50 µl of solution each from wells 11C-H. The well 12A will be a control for bacterial growth without antibiotics (positive control), the well 12B will serve as a procedure sterility control, and also for the remaining reagents and drugs solutions (negative control). (Fig. 1B. Prepare a series of microdilutions of the first antibiotic.)
4.4. Prepare 8 Eppendorf tubes (1,5 ml) and label them sequentially with the letters A-H.
4.5. Place 1 ml of MHB in all the labelled Eppendorf tubes using an automatic pipette.
4.6. Transfer 333 µl of antibiotic B solution (with a concentration of eight times the resistance breakpoint of the applied drug for the strains of the tested species) into the Eppendorf tube A. After placing antibiotic B in MHB at a ratio of 1:3, its solution with a concentration twice higher than the resistance breakpoint concentration of the drug will be obtained.
4.7. Transfer the volume of 333 µl from the Eppendorf tube A to the Eppendorf tube B and mix with an automatic pipette. Transfer 333 µl from the Eppendorf tube B to the Eppendorf tube C, mix and so on to the Eppendorf tube H. Remove 333 µl of solution from the Eppendorf tube H. (Fig. 1C. Prepare a series of microdilutions of the second antibiotic.)
4.8. Pipette 50 µl of solution from the Eppendorf tube A into wells of A row (A1-A11 – excluding well A12 – serving as a positive control), from the Eppendorf tube B into all wells in B row (B1-B12), from the Eppendorf tube C into wells C1-C11, and so on. (Fig. 1D. Add antibiotics together.)
4.9. Pipette (multi-channel pipette can be used) 50 µl of MHB into each well of the antimicrobials dilutions. Avoid adding MHB to the 12C-H wells when using the multi-channel pipette. (Fig. 1E. Re-add Mueller-Hinton broth.)
4.10. Add 50 µl of bacterial suspension to each well with antimicrobials solutions (except 12B – as a negative control).
5. Incubate for 16–24 hours at 37°C.
6. Bacterial growth assessment.
6.1. Assess turbidity in the wells after incubation. The absence of turbidity indicates that the antibiotics inhibit bacterial growth.
6.2. Record/capture in which antimicrobials concentration combinations growth inhibition is observed. For these combinations, FICI calculation is necessary, as follows:

$FICI = FIC_x + FIC_y$
$FIC_x = MIC_{xc} / MIC_x$
$FIC_y = MIC_{yc} / MIC_y$
Where:
MIC_x, MIC_y – MIC of drug X or Y used alone,
MIC_{xc}, MIC_{yc} – drug X or Y concentration in combination in a given well for which FICI is determined (Garbusińska i Szliszka 2017).

6.3. Interpret the FICI values according to the criteria given above and classify the particular antimicrobials concentrations combination as synergistic, additive, neutral or antagonistic.
6.4. If a so-called "skipped well" occurred in a row (e.g. no growth in well C8, while growth in C9, no growth in C10), the FICI for C10 was calculated to avoid misinterpretation, i.e. a false positive result (Brennan-Krohn *et al.* 2017).

4. "Time-kill" test

The "time-kill" test is a microbiological method which provides information on both the synergistic effect of antibiotics and the kinetics of bacterial growth and activity of the bactericidal preparation (Brennan--Krohn and Kirby 2019b). The test is based on the analysis of microbial survival in prepared concentrations of two antibiotics at selected time intervals. The interaction of the two drugs is determined by comparing the CFU/ml value (colony-forming unit per ml) between the tested combination of antibiotics and the best-performing single antibiotic. The obtained results are presented on the "time-kill" curves. Synergism is established when the difference between the two trials exceeds $\geq 2_{log10}$ (Brennan-Krohn and Kirby 2019b). A particular combination is considered bactericidal when the difference in CFU/ml between the combination of two antibiotics at the start of incubation and after 24 hours is $\geq 3_{log10}$. The "time-kill" test is an alternative to the checkerboard assay (Garbusińska and Szliszka 2017).

Its decisive advantage is the ability to determine both the bacteriostatic and bactericidal action (as opposed to the checkerboard assay, which can evaluate only the bacteriostatic action). In addition, the "time-kill" test makes it possible to determine the action of a combination of antibiotics at different time points (Brennan-Krohn and Kirby 2019b). The disadvantage of the "time-kill" test is that it is more labor-intensive and time-consuming compared to the checkerboard assay. In addition, the "time-kill" test is much more expensive.

Procedure

1. Preparation of antibiotic solutions.
   1.1. Determination of the antimicrobial agent concentration based on its solubility and the desired final concentration. The solvent selection should follow the CLSI (Clinical and Laboratory Standards Institute) guidelines.
2. Initiation of pre-culture.
   2.1. Prepare a 0.5 McFarland suspension in 0.9% NaCl from an overnight culture. Adjust the bacterial concentration with a densitometer to obtain a turbidity of 0.5 on a McFarland scale.
   2.2. Add 100 µl of the bacterial suspension to 5 ml of CAMBH (Mueller-Hinton Broth, cation adjusted). Transfer a drop of the diluted suspension via a sterile inoculating loop on a blood agar plate to confirm the purity of the inoculum. The incubation of the control culture is recommended at 35°C
   2.3. Incubate the remaining suspension at 35°C for at least 3 hours until logarithmic growth is achieved.
3. Antimicrobial solutions.
   3.1. Add 10 ml of CAMBH to 5 glass culture tubes.
   Tube 1: Add the first antibiotic in an amount corresponding to the target antibiotic concentration.
   Tube 2: Add the second antibiotic in an amount corresponding to the target antibiotic concentration.
   Tube 3: Add the same amount of the first and the second antibiotic as in tubes 1 and 2.
   Tube 4: Growth control – do not add antibiotics.
   Tube 5: Negative control – neither antibiotic nor microorganism should be added.
4. Perform a series of dilutions.
   (Fig. 2. The "time-kill" method – a series of dilutions.)
   4.1. Prepare six 96-well plates (labelled: t_0, t_1, t_2, t_4, t_6, t_{24}) with 2 ml wells which will be used for a series of dilutions. Place 900 µl of 0.9% NaCl in rows B-H, columns 1st-5th. (Fig. 2A Prepare six 96-well plates (t_0, t_1, t_2, t_4, t_6, t_{24}) with 0.9% NaCl.)
   4.2. Transfer the culture in the logarithmic growth phase of the initial inoculum. Transfer 1 ml of suspension to a culture glass tube. To obtain a density of 1.0 McFarland use CAMBH to adjust – dilute, or add more microbial cells to concentrate the suspension.
   4.3. Add 100 µl of the suspension to tubes 1st–4th and mix gently. (Fig. 2B. Add the bacterial suspension to test tubes 1st–4th.)
   4.4. Withdraw 150 µl from each tube at time t_0 (immediately after adding the bacterial suspension) and after 1, 2, 4, 6 and 24 hours. Add portions to wells in appropriately marked rows 1 (t_0, t_1, t_2, t_4, t_6, t_{24}) of the 96-well plates. (Fig. 2C. Transfer the suspension portion to the appropriate plate a predetermined times.)

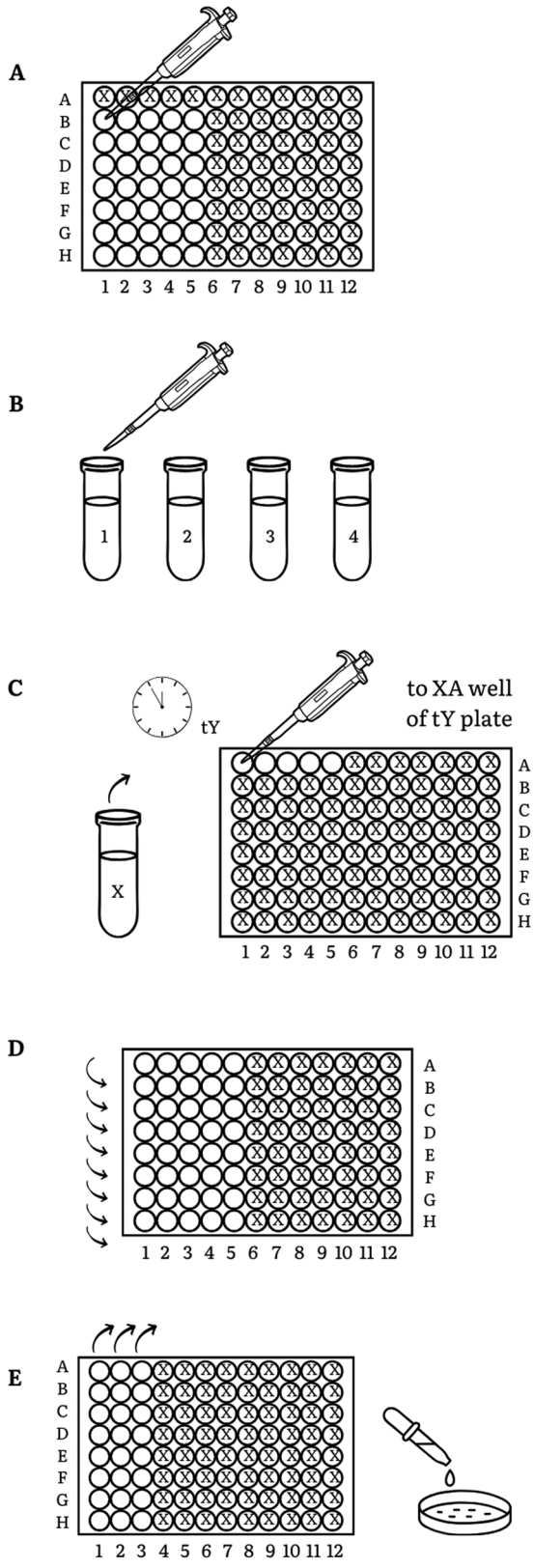


Fig. 2. The "time-kill" method – a series of dilutions.
A – prepare six 96-well plates (t_0, t_1, t_2, t_4, t_6, t_{24}) with 0.9% NaCl
B – add the bacterial suspension to test tubes 1st–4th.
C – transfer the suspension portion to the appropriate plate at predetermined times.
D – obtain a dilution of 1:10.
E – transfer a portion of the suspension to the plates using the "drop plate" method.

4.5. Using a multi-channel pipette, withdraw 100 µl volume from row A and transfer it to row B. Aspirate and withdraw the solution several times to mix evenly. This action will result in a dilution of 1:10. Repeat for rows B-H. Remove 100 µl from row H. (Fig. 2D. Obtain a dilution of 1:10.)
4.6. Using a multi-channel pipette, collect 10 µl from each well in columns 1st-3rd of all plates and transfer to appropriately labelled Mueller--Hinton agar plates for colony counting using the "drop plate" method. The labelling pattern should allow for identification of the antibiotics used, their concentrations, and the time after which a portion of the bacterial suspension was transferred to the plate (t_0-t_{24}). Allow the drop to dry completely. (Fig. 2E. Transfer a portion of the suspension to the plates using the "drop plate" method.)
4.7. Place a 10 µl drop from the negative control to the selected plate after 24 hours to confirm procedure sterility. Invert the plate and incubate overnight under recommended conditions.

5. Reading the results.
5.1. Negative control check. If any growth is observed, the results are unreliable.
5.2. The colonies obtained should have the uniform morphology expected for the strain used.
5.3. Identify drops with 3–30 colonies for each series of dilutions. Count the colonies in these drops and record them along with the dilution factor. If there are no drops with 3–30 colonies in the dilution series, count the last drop with more than 30 and the first with colonies.
5.4. Calculate the CFU/ml value in the sample for each dilution series based on the number of colonies in the drop using the formula:
CFU/ml = 100 n/d
Where:
n – number of colonies,
d – dilution factor (1 for undiluted sample – row A, while 0,1 for the first dilution – row B, 0,01 for the second dilution – row C, etc.).

6. Analysis of results.
6.1. Plot the corresponding growth curves from three cultures containing antibiotics and control growth, using time units on the x-axis and CFU/ml values on the y-axis.
6.2. Calculate the difference between growth in the tube with no antibiotics after 24 hours and the most active single factor simultaneously (in CFU/ml). The combination is considered syn-

ergistic if the difference is ≥ 2 $\log_{10}$. Calculate the difference in CFU/ml between the combination in the tube at 24 hours and at time 0 point. Consider the combination bactericidal if the difference is ≥ 3 $\log_{10}$.

5. CombiANT test

Another method for testing antibiotic combinations, CombiANT, is not yet available in research practice or microbiological diagnostics. It was first presented in 2020, and initial studies indicate identical results of the CombiANT technique compared to the checkerboard assay (Fatsis-Kavalopoulos *et al.* 2020). Despite the lack of equipment and algorithms for validated results interpretation for individual bacterial species, due to the many advantages of this method and perhaps a breakthrough in the facilitation of testing antibiotic combinations, the decision was made to describe this procedure in detail in this paper.

CombiANT is a diffusion-based assay providing quantitative information on the interactions of three pairs of antibiotics. The selected antibiotics are placed in three reservoirs and then in a standard agar plate. Two fields can be distinguished. On the outside of the insert is a field where each antibiotic acts individually. Inside the antibiotic plate is a triangular field where the interaction between the individual antibiotic pairs occurs. The results are generated by an algorithm developed by the creators, calibrated to the type and rate of diffusion of each antibiotic in the chosen substrate. The extent of antibiotic interaction is determined quantitatively based on points at the edge of the inhibition zone, according to the FICI formula. The FICI values were interpreted according to clinical thresholds, where ≤ 0.5 indicates antimicrobials synergy, FICI > 0.5 and ≤ 4 indicates addition, while > 4 indicates antagonism (Tang *et al.* 2024).

The CombiANT method can be used without determining the strain's susceptibility to a given antibiotic. This fact is a definitive advantage, as it reduces the waiting time for the antimicrobial sensitivity result of the tested strain. The CombiANT test has the same accuracy as the broth microdilution method but is more efficient and less complex (Fatsis-Kavalopoulos *et al.* 2020). An additional advantage of this method is the possibility of accurately determining the inhibitor concentration since a continuous range of antibiotic concentrations is used. In contrast, the checkerboard assay has only 2-fold antimicrobials dilutions. A significant disadvantage of this method is the need to purchase special inserts, which do not have laboratory applications for other studies.

Procedure (Fig. 3. CombiANT method – the insert and experimental protocol.)

1. Preparation of the antibiotic insert (Fig. 3A. The insert design: antibiotic reservoirs (a-c), interaction imaging area (d)).

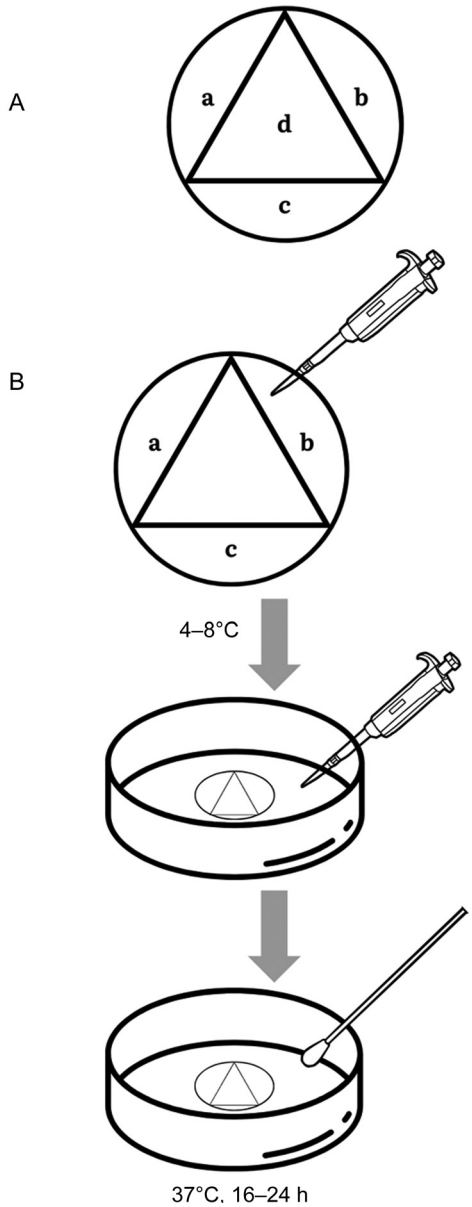


Fig. 3. CombiANT method – the insert and experimental protocol.
A – the insert design: antibiotic reservoirs (a-c), interaction imaging area (d).
B – the assay protocol: „The insert is loaded by adding 0.5 mL liquid agar (60°C) containing antibiotics into the reservoirs. The prepared inserts can be stored at 4–8°C. To activate an insert, add a second layer of 25 mL agar to enclose the insert and fill the plate, thereby permitting diffusion of the antibiotics to the agar surface and the reservoir periphery. After solidification, a bacterial cell suspension of 0.5 McFarland is inoculated on the agar surface using a sterile cotton swab and exposed to the antibiotic gradient landscape. The finished plates are incubated at 37°C, and stable zones of growth inhibition are established within 16–24 hours." (Fatsis-Kavalopoulos *et al.* 2020)

1.1. Add 0.5 ml of liquid agar medium to each of the reservoirs (at 60°C) containing previously added antibiotics (labelled A, B, and C, respectively). The antibiotic concentration is calculated based on the result of the calibration of the analytical algorithm (Fig. 3B). Preparation for digital imaging. Inactivated plates can be stored at 4–8°C for 7 days.
1.2. Applying the insert to the culture plate and adding a second agar layer activate the insert. This will allow antibiotics to diffuse.

2. Inoculation.
2.1. Prepare a bacterial cells suspension with a density of 0.5 on McFarland scale in compliance with the European Committee on Antimicrobial Susceptibility Testing (EUCAS) guidelines for the disk diffusion method.
2.2. Applicate the suspension to the solidified agar using a cotton swab, forming a bacterial lawn.

3. Incubation.
3.1. Incubate for 16–24 hours at 37°C.
3.2. After the appropriate incubation time, inhibition zones will form, consistent with the resulting diffusion gradient.
3.3. A triangular interaction imaging surface will be created inside the insert with active antibiotics. In each corner of this area, two adjacent antibiotics interact.

4. Quantitative measurements of drug interactions.
4.1. Perform plate imaging to measure antibiotic interaction, e.g. with a gel-doc camera or a handheld mobile device.
4.2. Perform the analysis with an appropriately calibrated algorithm that uses previously developed diffusion models.
4.2.1. Indicate which antibiotic has been placed in each tank of the test.
4.2.2. The algorithm developed by the method developers recalls stored diffusion maps corresponding to the antibiotics used in the assay and combines them into a test-specific model. It is then matched to the imaging performed on the test.
4.2.3. The algorithm determines IC (inhibitory concentration) and CP (combination inhibitory point) for all three antibiotics, individually and in combination. The extent of antibiotic interaction is determined quantitatively based on points at the edge of the inhibition zone, according to the FICI formula.

5. Calculation of FICI value.
$FICI_{AB} = C_A / IC_A + C_B / IC_B$
Where:
C_A, C_B – concentrations of antibiotic A and B, respectively,
IC_A, IC_B – inhibitory concentrations of antibiotic A and B, respectively.

6. Strips impregnated with antibiotic gradient used in methodology for determining antimicrobials synergistic effect

The synergistic effects of antimicrobial agents *in vitro* can also be evaluated using methods available in most microbiological laboratories, i.e., strips impregnated with antibiotic in the concentration gradient. The following methods have been developed so far:

- E-test fixed ratio method,
- cross method,
- MIC:MIC ratio evaluation,
- E-test agar method (Laishram *et al.* 2017; Guzek 2023)

Before performing the methods mentioned above, the MIC values for all antibiotics tested should be determined.

Methods based on antibiotic-impregnated strips with the concentration gradient have an advantage over other methods due to their simplicity and easy access to this procedure for all laboratories. However, synergism can be tested only between two antibiotics in this way. Furthermore, the limitation of these techniques is the lack of their use against resistant isolates whose MIC values exceed the maximum antimicrobial concentration placed on a strip scale since the exact MIC value must be known *a priori* to assess the synergy.

E-test fixed ratio method – procedure

1. Prepare suspensions of the tested bacteria (density 0.5 McFarland) and inoculate on Mueller-Hinton Agar medium (MHA).
2. Apply a strip containing antibiotic A on the MHA.
2.1. The area where the strip containing antibiotic A is located should be precisely marked.
3. Incubate a plate at room temperature for one hour to let the antibiotic diffuse from the strip into the MHA medium.
4. Remove the antibiotic A strip.
4.1. Clean the strip with alcohol and leave it as a template for reading the MIC value.
4.2. Apply the strip containing antibiotic B to the same spot.
5. Incubate the culture on the plate for 16–18 hours at 35°C ± 2°C (as recommended by the strip manufacturer).
6. Reading and interpretation of results: read the MIC values for the strips used and calculate the FICI value after the incubation (Fig. 4. Assessment of the synergistic effect of antibiotics by the method of constant coefficients.) (Laishram *et al.* 2017; Sreenivasan *et al.* 2022; Guzek 2023), which should be interpreted according to a standardized criteria.

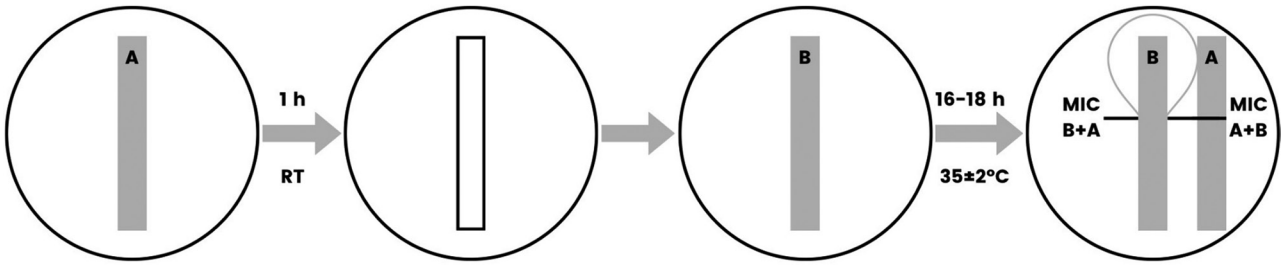


Fig. 4. Assessment of the synergistic effect of antibiotics by the method of constant coefficients.

Cross method – procedure

1. Inoculate the MHA plate with the tested bacterial suspension (density 0.5 McFarland).
2. Place two strips containing antibiotics A and B.
   2.1. Arrange the strips to intersect at an angle of 90° at the concentration marked as the previously determined MIC values.
3. Culture on a plate incubation for 16–20 hours at 35°C ± 2°C (as recommended by the strip manufacturer).
4. Reading and interpretation of results: read the MIC values for the strips used and calculate the FICI value after the incubation (Fig. 5. Assessment of the synergistic effect of antibiotics by cross-method.), which should be interpreted according to standardized criteria.

Due to its high availability and ease of performing, the cross method is eagerly chosen to evaluate the combination of two antibiotics. However, the results of this procedure can differ significantly from those obtained in the "time-kill" test, which is considered the gold standard for this purpose (Nasomsong *et al.* 2022).

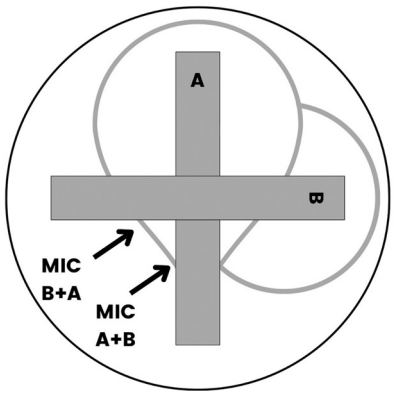


Fig. 5. Assessment of the synergistic effect of antibiotics by cross-method.

MIC:MIC ratio evaluation – procedure

1. Apply two strips with a concentration gradient of antibiotics A and B onto the MHA medium with a bacterial suspension plated initially.
   1.1. Mark previously obtained MIC value for each drug separately with a marker on the bottom of the plate.
2. Incubate the plate for one hour at room temperature to allow the antibiotics to diffuse from the strips.
3. Evaluation of antimicrobials synergism.
   3.1. Remove the first antibiotic strip from the culture after one hour.
   3.2. Place a new strip containing antibiotic A on the spot where the antibiotic B strip was located so that the MIC value from strip A corresponds to the MIC value for antibiotic B marked previously with the marker.
   3.3. Follow the same procedure for the second strip.
4. Culture on a plate incubation for 16–20 hours at 35°C ± 2°C (as recommended by the strip manufacturer).
5. Reading and interpretation of results: read the MIC values for the strips used and calculate the FICI value, which should be interpreted according to standardized criteria (Laishram *et al.* 2017; Guzek 2023). (Fig. 6. Evaluation of the synergistic effect of antibiotics by the MIC:MIC ratio evaluation.)

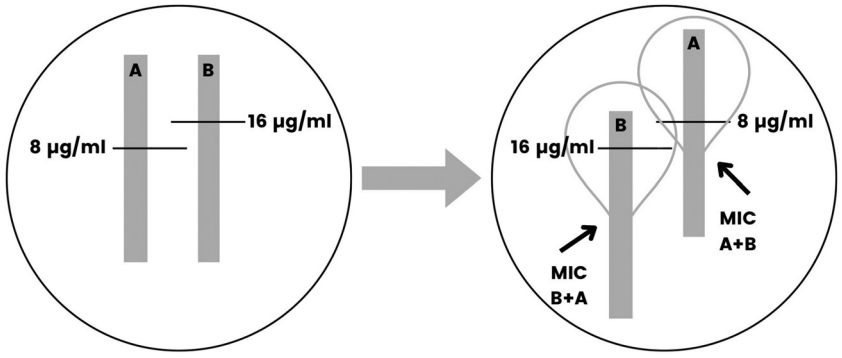


Fig. 6. Evaluation of the synergistic effect of antibiotics by the MIC:MIC ratio evaluation.

According to some previous results, in comparison with gradient diffusion methods used to assess the synergy of tobramycin and ceftazidime in multidrug-resistant *Pseudomonas aeruginosa*, the results obtained by Okoliegbe *et al.* suggest that the MIC:MIC ratio method should be considered as the preferred method for antibiotic interaction studies in diagnostic practice (Okoliegbe *et al.* 2021).

E-test agar method – procedure

1. Prepare the medium containing antibiotic A at a concentration of 0.5 × MIC or 0.25 × MIC of the given antimicrobial agent.
2. Place a strip with an antibiotic B on the medium plate.
3. Assess the MIC value of antibiotic B on the medium with antibiotic A.
4. Compare the obtained MIC value with the MIC value in the drug-free medium.
5. Interpretation of the results: Synergy is observed when there is more than a three-fold reduction in the MIC value on the medium containing the tested antibiotic (Laishram *et al.* 2017).

Marie *et al.* used this method to evaluate the combination of sulbactam and tazobactam with meropenem or colistin against the multidrug-resistant *A. baumannii* strain. However, the results obtained by this method did not coincide with the checkerboard assay, which demonstrated higher sensitivity in terms of synergism but identical in terms of addition (Marie *et al.* 2015).

7. Alternative methods for the determination of synergistic and additive antibiotic activity

Three of the many methods for determining antibiotic synergistic and additive activity not described in this article are worth mentioning. All of them are achievable in any basic medical diagnostic laboratory – double disc synergy test (a modification of the procedure typically used to assess the presence of drug resistance mechanisms in microorganisms), paper strip diffusion (which uses strips soaked in various antimicrobial solutions at concentrations equal to or higher than their MIC values) and the overlay inoculum susceptibility disc method.

Double-disc synergy test – procedure

1. Applicate discs soaked with the antibiotics tested to the medium with the bacterial suspension. Apply a distance of 20 mm or corresponding to the sum of the radii of the inhibition zones of each antibiotic separately.
2. Incubate the plate for 24 hours at 35°C.
3. Interpret the results by assessing the diameter of the growth inhibition zones around the discs.
   3.1. Antibiotic synergism is demonstrated by an increase ≥ 2 mm in the growth zone diameter compared to the size of the diameter of the single agent, the combination of the growth inhibition zone between the drug A and B, or the growth inhibition zone appearing between the diffusing agents.
   3.2. An increase of the inhibition zone by < 2 mm is classified as slight synergy.
   3.4. Antagonism is detected when the zone of inhibition flattens at the diffusion interface of two antimicrobials (Laishram *et al.* 2017).

This method is not commonly used due to the relatively subjective and only qualitative assessment of the synergistic effect of antibiotics.

Paper strip diffusion – procedure

1. Place the strips perpendicular to each other on an MHA medium with a previously applied inoculum of the tested microorganisms.
2. Leave the filter paper strips with the antibiotics for several hours to allow antibiotics to diffuse into the medium.
3. Incubate the plate for 18–24 hours at 37°C (Laishram *et al.* 2017).

   Apply antibiotic-soaked strips to a new MHA medium for 24 hours to allow antibiotics to diffuse into the medium, and then apply the microorganism using a membrane transfer technique as a possible alternative.
4. Interpretation of the results
   4.1. A neutral/additive effect is considered when two oval areas of growth inhibition zones, connected at a 90-degree angle, appear.
   4.2. Synergism is identified as enlargement or the presence of an inhibition zone around a 90-degree angle.
   4.3. Antagonism is noted at incision or narrowing around an angle between strips.

This method is cost- and labour-intensive, and the results obtained by this technique are only qualitative.

Overlay inoculum susceptibility disc method – procedure

1. Prepare an agar medium containing antibiotic A at a concentration equal to half the MIC value for this antibiotic (so-called base agar layer).
2. Apply an agar layer without antibiotic (so-called inoculum layer) containing 10^6 CFU/ml of the tested strain.
3. Place discs containing different concentrations of antibiotic B on the surface after solidification of the previous agar layers.

4. Incubate for 24 hours at 37°C.
5. Interpretation of the results
   5.1. Synergism occurs when the diameter of the inhibition zone around the disc with antibiotic B increases by at least 19%.
   5.2. An additive effect is assessed with an increase in the inhibition zone diameter around the disc with antibiotic B of less than 19%.
   5.3. No visible change in the inhibition zone indicates an indifferent effect of the combinations of antimicrobials used.

8. Conclusions

Due to the dynamic increase in antibiotic resistance of microorganisms, mainly bacteria, and the long time required to develop and approve a new antibiotic for clinical practice, it is necessary to introduce the evaluation of the effectiveness of the already-known antibiotic combinations into routine diagnostic practice. There are many methods for testing synergistic and additive antibiotic activity, which vary in the availability of the necessary reagents and equipment, the difficulty and time of performance, as well as the reliability of the results obtained and the unambiguity of their interpretation (Papoutsaki *et al.* 2020; Okoliegbe *et al.* 2021). Depending on the study, the level of compliance with individual methods varies significantly. Differences may be related to the strain of bacteria tested, antibiotics used, or even the presence of particular resistance mechanisms. In their study of methods for detecting synergism in multidrug-resistant Gram-negative rods, Gaudereto *et al.* obtained compliance with the MIC: MIC method and "time-kill" ratio method at the level of 35–71% (Gaudereto *et al.* 2020). Therefore, a clear comparison of individual methods in a universal way for antibiotics and bacterial strains is currently beyond the capabilities of researchers due to divergent results depending on the strains, antibiotics and their combinations (Doern 2014).

The "gold standard" test remains the "time-kill" method, which provides information on bactericidal and bacteriostatic action at different time points and is a reliable reference method in comparing different procedures. However, the "time-kill" test is a time-consuming and relatively difficult method, so using strips with antimicrobials concentration gradients seems to be an attractive alternative. It is necessary to validate the results to ensure they are as reliable as possible.

In addition to routine testing of synergistic and additive activity of antibiotics, it is also necessary to develop new methods to implement in diagnostic and clinical practice. The CombiANT method seems to be a promising procedure, which may become a method of great diagnostic importance soon, considering its ability to quickly test a combination of three drugs, easy interpretation of the results and accuracy comparable to the microdilution method (Fatsis-Kavalopoulos *et al.* 2020).

The checkerboard assay, the most popular method for synergism testing, as a modification of the MIC determination by antimicrobials broth microdilutions method, is an intuitive and easily accessible procedure due to the broad access to the required equipment and reagents. However, in contrast to the "time-kill" method, it only provides information on the bacteriostatic effect, which is the most significant limitation that may affect further clinical management.

The most accessible, easiest and cheapest methods are those using antibiotic concentration gradient strips or discs. Although validating the methodology based on their use and introducing uniform criteria for interpretation of the results obtained is difficult, in some situations, they may be the only possible procedure. Therefore, further studies are needed to introduce them into routine diagnostic and clinical practice while obtaining results comparable to other methods. It is essential to differentiate the reliability of the results of individual procedures with gradient strips, among which the MIC:MIC ratio method seems to be the most reliable one (Okoliegbe *et al.* 2021).

ORCID
Paweł Z. Kmiecikowski https://orcid.org/0009-0004-1315-7194
Aniela Gabriel https://orcid.org/0009-0006-6193-5610
Dagmara Depka https://orcid.org/0000-0003-2387-9778
Tomasz Bogiel https://orcid.org/0000-0001-6787-1378

Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

References

1. Bellio P., Fagnani L., Nazzicone L., Celenza G.: New and simplified method for drug combination studies by checkerboard assay. *MethodsX*, **8**, 101543 (2021)
2. Brennan-Krohn T., Kirby J.E.: When One Drug Is Not Enough: Context, Methodology, and Future Prospects in Antibacterial Synergy Testing. *Clin. Lab. Med.* **39**, 345–358 (2019a)
3. Brennan-Krohn T., Kirby J.E.: Antimicrobial Synergy Testing by the Inkjet Printer-assisted Automated Checkerboard Array and the Manual Time-kill Method. *J. Vis. Exp.* **146**, 10.3791/58636 (2019b)
4. Brennan-Krohn T., Truelson K.A., Smith K.P., Kirby J.E.: Screening for synergistic activity of antimicrobial combinations against carbapenem-resistant Enterobacteriaceae using inkjet printer-based technology. *J. Antimicrob. Chemother.* **72**, 2775–2781 (2017)
5. Doern C.D.: When does 2 plus 2 equal 5? A review of antimicrobial synergy testing. *J. Clin. Microbiol.* **52**, 4124–8 (2014)

6 https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Break point_tables/v_14.0_Breakpoint_Tables.pdf
EUCAST. European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 14.0, valid from 2024-01-01. 19.05.2024
7. https://atlas.ecdc.europa.eu/public/index.aspx. European Centre for Disease Prevention and Control. Surveillance Atlas of Infectious Diseases, 13.04.2024
8. Fatsis-Kavalopoulos N., Roemhild R., Tang P.-C., Kreuger J., Andersson D.I.: CombiANT: Antibiotic interaction testing made easy, *PLOS Biol.* **18**, e3000856 (2020)
9. Gaudereto J.J., Neto L.V.P., Leite G.C., Espinoza E.P.S., Martins R.C.R., Prado G.V.B., Rossi F., Guimarães T., Levin A.S., Costa S.F.: Comparison of methods for the detection of in vitro synergy in multidrug-resistant gram-negative bacteria. *BMC Microbiol*. **20**, 97 (2020)
10. Garbusińska A., Szliszka E.: Aktywność leków przeciwdrobnoustrojowych zastosowanych w kombinacjach wobec pałeczek Gram-ujemnych w badaniach *in vitro*. *Post. N. Med.*, **XXX(08)**, 427–433 (2017)
11. Guzek A.: Metodologia oznaczania synergizmu między antybiotykami z zastosowaniem pasków z gradientem stężeń antybiotyków w warunkach *in vitro*. *Forum Zakażeń*, **14**, 19–24 (2023)
12. Karakonstantis S., Ioannou P., Kofteridis D.D.: In search for a synergistic combination against pandrug-resistant *A. baumannii*; methodological considerations. *Infection*, **50**, 569–581 (2022)
13. Laishram S., Pragasam A.K., Bakthavatchalam Y.D., Veeraraghavan B.: An update on technical, interpretative and clinical relevance of antimicrobial synergy testing methodologies. *Indian J. Med. Microbiol.* **35**, 445–468 (2017)
14. Marie M.A.M., Krishnappa L.G., Alzahrani A.J., Mubaraki M.A., Alyousef A.A.: A prospective evaluation of synergistic effect of sulbactam and tazobactam combination with meropenem or colistin against multidrug resistant *Acinetobacter baumannii*. *Bosn. J. Basic Med. Sci.* **15**, 24–29 (2015)
15. Nasomsong W., Nulsopapon P., Changpradub D., Pungcharoenkijkul S., Hanyanunt P., Chatreewattanakul T., Santimaleeworagun W.: Optimizing Doses of Ceftolozane/Tazobactam as Monotherapy or in Combination with Amikacin to Treat Carbapenem-Resistant *Pseudomonas aeruginosa*. *Antibiotics* (*Basel*), **11**, 517 (2022)
16. Okoliegbe I.N., Hijazi K., Cooper K., Ironside C., Gould I.M.: Antimicrobial Synergy Testing: Comparing the Tobramycin and Ceftazidime Gradient Diffusion Methodology Used in Assessing Synergy in Cystic Fibrosis-Derived Multidrug-Resistant *Pseudomonas aeruginosa. Antibiotics* (*Basel*), **10**, 967 (2021)
17. Papoutsaki V., Galani I., Papadimitriou E., Karantani I., Karaiskos I., Giamarellou H.: Evaluation of in vitro methods for testing tigecycline combinations against carbapenemase-producing *Klebsiella pneumoniae* isolates. *J. Global Antimicrob. Resist.* **20**, 98–104 (2020)
18. https://pharmaceutical-journal.com/article/news/only-12-new-antibiotics-entered-the-market-in-five-years-who-warns-in-review
Sayburn A.: Only 12 new antibiotics entered the market in five years, WHO warns in review. *The Pharmaceutical Journal*, **310** (2023), 04.12.2024
19. Sreenivasan P., Sharma B., Kaur S., Rana S., Biswal M., Ray P., Angrup A.: In-vitro susceptibility testing methods for the combination of ceftazidime-avibactam with aztreonam in metallobeta-lactamase producing organisms: Role of combination drugs in antibiotic resistance era. *J. Antibiot*. (*Tokyo*), **75**, 454–462 (2022)
20. Tang P.-C., Sánchez-Hevia D.L., Westhoff S., Fatsis-Kavalopoulos N., Andersson D.I.: Within-species variability of antibiotic interactions in Gram-negative bacteria. *mBio*, **15**, e0019624 (2024)
21. https://iris.who.int/bitstream/handle/10665/354545/9789240047655-eng.pdf?sequence=1.
WHO. World Health Organization: 2021 Antibacterial agents in clinical and preclinical development: an overview and analysis, 13.04.2024
22. https://antybiotyki.edu.pl/wp-content/uploads/Biuletyn/Biuletyn-Informacyjny-2_2022.pdf
Żabicka D., Grzegorczyk M.: Oporność na antybiotyki w Polsce i Europie w 2021 roku – dane sieci EARS-Net. *Oporność na antybiotyki. Biuletyn informacyjny*, **2** (2022), 19.05.2024

Ze względu na praktyczne walory pracy dla szerszej dostępności artykułu dla diagnostów laboratoryjnych i innych zainteresowanych osób w Polsce publikujemy także wersję polskojęzyczną.

METODOLOGIA BADANIA DZIAŁANIA SYNERGISTYCZNEGO I ADDYCYJNEGO *IN VITRO* LEKÓW PRZECIWBAKTERYJNYCH

Paweł Z. Kmiecikowski[1,*], Aniela Gabriel[1], Dagmara Depka[2], Tomasz Bogiel[2*]

[1] Studenckie Koło Naukowe Mikrobiologii przy Katedrze Mikrobiologii Collegium Medicum w Bydgoszczy Uniwersytet Mikołaja Kopernika w Toruniu

[2] Katedra Mikrobiologii Collegium Medicum w Bydgoszczy Uniwersytet Mikołaja Kopernika w Toruniu

Streszczenie: W związku z dynamicznym wzrostem antybiotykooporności obserwowanym na przestrzeni ostatnich kilkudziesięciu lat, wskazane jest poszukiwanie nowych metod terapeutycznych w leczeniu zagrażających życiu infekcji. Efektywne leczenie zakażeń wielolekoopornymi drobnoustrojami coraz częściej wymaga korzystania z leków ostatniej szansy, a nowe antybiotyki generują oporność w krótszym czasie, niż zajmuje ich wprowadzenie do lecznictwa. Wskazane zatem jest poszukiwanie rozwiązań z wykorzystaniem aktualnie dostępnych antybiotyków. Skuteczną terapię zakażeń wielolekoopornymi drobnoustrojami umożliwiać może wykorzystanie oddziaływań addycyjnych oraz synergistycznych, wynikających z łączenia różnych grup antybiotyków, włączając te o odmiennych mechanizmach działania – również takich, na które z osobna dany szczep bakterii jest oporny. W niniejszej pracy zostały opisane dostępne metody oznaczania działania synergistycznego oraz addycyjnego antybiotyków w warunkach *in vitro* oraz metoda CombiANT, będąca aktualnie w fazie testów. Celem pracy jest przedstawienie możliwych metod, pozwalających na oznaczenie addycyjnego i synergistycznego działania między antybiotykami *in vitro* oraz zaproponowanie odpowiednich procedur laboratoryjnych do ich stosowania. W niniejszej pracy dokonano przeglądu najnowszego piśmiennictwa naukowego dotyczącego dostępnych metod oznaczania oddziaływania antybiotyków względem siebie. Badania kliniczne wskazują na znaczne korzyści wykorzystywania zjawiska addycji oraz synergii antybiotyków w praktyce klinicznej, nie tylko poprawiając skuteczność terapii, ale również minimalizując działanie niepożądane oraz zmniejszeniu ryzyka rozwoju antybiotykooporności *de novo*. Pomimo znaczącego problemu, jakim jest rosnący poziom antybiotykooporności, w praktyce klinicznej w dalszym ciągu brakuje jednoznacznej standaryzacji metod oznaczania synergizmu. Konieczne są dalsze badania, w celu określenia najkorzystniejszego standardu, dostępnego dla szerokiego zakresu zakładów diagnostyki mikrobiologicznej.

Słowa kluczowe: antybiotykooporność, synergizm antybiotykowy, test szachownicy, test „time-kill"

1. Wprowadzenie

W czasie transformacji epidemiologicznej, do której doszło na początku XX wieku, dokonało się przesunięcie głównej przyczyny zgonów na świecie z chorób infekcyjnych na te nieinfekcyjne. Pojawiła się nadzieja na ostateczne zwycięstwo medycyny nad chorobami powodowanymi przez drobnoustroje, czemu sprzyjało odkrycie w latach 40. i 50. ubiegłego stulecia wielu antybiotyków. Niestety po upływie zaledwie kilku dekad, ze względu na dynamiczny wzrost antybiotykooporności, choroby zakaźne ponownie stały się jednym z największych zagrożeń zdrowia publicznego. Naukowcy, w tym tacy jak Venkatasubramanian Ramasubramanian, prezes Klinicznego Towarzystwa Chorób Zakaźnych w Indiach, już od jakiegoś czasu przywołują ostrzeżenia o nowej erze postantybiotykowej (Sayburn 2023).

W sytuacji znaczącego tempa narastania antybiotykooporności na świecie, sytuacja epidemiologiczna w Polsce wydaje się szczególnie trudna, również na tle Europy. Dla przykładu w 2021 roku izolaty *Escherichia coli* oporne na fluorochinolony stanowiły w Polsce 33,1% izolowanych szczepów *E. coli* (populacyjnie ważona średnia europejska: 21,9%), izolaty *Klebsiella pneumoniae* oporne na karbapenemy – 19,5% (populacyjnie ważona średnia europejska: 11,7%), a izolaty

* Autor korespondencyjny: Paweł Z. Kmiecikowski, Studenckie Koło Naukowe Mikrobiologii przy Katedrze Mikrobiologii, Tomasz Bogiel Katedra Mikrobiologii, Wydział Farmaceutyczny, Collegium Medicum w Bydgoszczy Uniwersytet Mikołaja Kopernika w Toruniu, ul. M. Curie-Skłodowskiej 9, 85-094 Bydgoszcz e-mail: kmiecikowski.pawel@gmail.com , t.bogiel@cm.umk.pl

Cite as:
Metodologia badania działania synergistycznego i addycyjnego *in vitro* leków przeciwbakteryjnych. Kmiecikowski P.Z. *i wsp*., ADV MICROBIOL-NY, 2024, 63, 4, 199–222

Enterococcus faecium oporne na wankomycynę – 34,3% (populacyjnie ważona średnia europejska: 17,2%). Ponad połowa izolowanych szczepów *K. pneumoniae* wykazywała wielolekooporność (MDR, ang. multi--drug resistance), co oznaczało, w tym przypadku, oporność jednocześnie na aminoglikozydy, fluorochinolony i cefalosporyny III generacji (Żabicka i Grzegorczyk 2022). Niepokojąca jest duża różnica między stopniem antybiotykooporności w Polsce a w krajach Europy zachodniej i północnej. W 2022 roku szczepy *Acinetobater baumannii* oporne na karbapenemy stanowiły w Polsce 76,4% izolatów, podczas gdy w Niemczech i we Francji zaledwie 3,5%, a w Szwecji – 2,7% European Centre for Disease Prevention and Control (ECDC).

Skuteczne leczenie zakażeń utrudnia ponadto stagnacja na rynku farmaceutycznym. Od 2017 do 2022 roku zatwierdzono zaledwie 12 nowych antybiotyków, w tym tylko dwa – waborbaktam i lefamulina – należą do nowych grup leków. Dużym ograniczeniem i wyzwaniem są koszty wprowadzania nowych terapeutyków na rynek oraz konieczność zastrzeżenia niektórych najnowszych środków jako leków ostatniej szansy. Oporność na te nowe leki zgłaszana jest jednak już w ciągu 2–3 lat od ich wprowadzenia do stosowania (World Health Organization 2021).

Choć trwają badania nad nowymi preparatami i strategiami przeciwbakteryjnymi, takimi jak przeciwciała monoklonalne czy bakteriofagi (World Health Organization 2021), w świetle tak dynamicznego rozwoju antybiotykooporności konieczne jest poszukiwanie nowych sposobów leczenia zakażeń z wykorzystaniem dostępnych już antybiotyków. Bardzo obiecującą metodą jest łączenie w terapii antybiotyków o różnych mechanizmach działania – również takich, na które z osobna dany szczep bakterii jest oporny. Ich addycyjne lub synergistyczne działanie może pozwolić na zmniejszenie wartości ich minimalnego stężenia hamującego (MIC, ang. minimal inhibitory concentration), a nawet przełamać barierę oporności szczepu na leki. Dodatkowymi zaletami uzyskania synergizmu skojarzonych antybiotyków są: maksymalizacja efektów leczenia przy zmniejszonym ryzyku rozwoju antybiotykooporności *de novo* oraz możliwość korzystania z mniejszych dawek leków (Garbusińska i Szliszka 2017). Warto przypomnieć, że łączenie antybiotyków w terapii jest praktyką niemal tak starą jak sama antybiotykoterapia. Jest to widoczne w historii leczenia zakażeń wywoływanych przez prątki gruźlicy (*Mycobacterium tuberculosis*), w której szybko po odkryciu streptomycyny rozpoczęto kojarzenie jej z innymi lekami. Stwierdzono bowiem, że w ciągu kilkumiesięcznej farmakoterapii gruźlicy prawdopodobieństwo rozwoju oporności na streptomycynę stosowaną w monoterapii może wynosić nawet 100% (Brennan-Krohn i Kirby 2019a).

Obecnie stosuje się kilka metod badania działania addycyjnego i synergistycznego antybiotyków. Są to metody wykorzystujące paski nasycone gradientem stężeń antybiotyków, preparaty przeciwdrobnoustrojowe w krążkach, substancje przeciwbakteryjne rozcieńczane w agarze, metodę szachownicy, tzw. test "time-kill", dynamiczne modele farmakokinetyczne i farmakodynamiczne (PK/PD) *in vitro*, półmechaniczne modele PK/PD, a nawet modele zwierzęce *in vivo* (Karakonstantis *i wsp.* 2022). W artykule scharakteryzowano i opisano krok po kroku metodologię kilku wspomnianych metod, które w ocenie autorów mają największy potencjał na wykorzystywanie w codziennej praktyce laboratoryjnej i klinicznej jako rutynowe metody badania połączeń antybiotyków, co w przyszłości może przyczynić się do poprawy stanu klinicznego wielu pacjentów cierpiących z powodu zakażeń wywołanych wieloantybiotykoopornymi szczepami bakterii.

2. Metodyka wyszukiwania i doboru piśmiennictwa

Do przeglądu piśmiennictwa wykorzystano bazy PubMed i Google Scholar, wyszukując frazy: antibiotic resistance, antibiotic synergism, antibiotic interaction methodology, antibiotic FICI assessing, checkerboard array, „time-kill" curves, synergism disk-based methods, synergism gradient-based methods, antibiotic gradient-based methods z ostatnich 10 lat. Spośród uzyskanych wyników wyselekcjonowano artykuły badania połączeń antybiotyków, z uwzględnieniem opisów najczęściej stosowanych metod badania synergizmu i addycji antybiotyków w ujęciu merytorycznym, klinicznym i/lub proceduralnym. Informacje na temat epidemiologii antybiotykooporności uzyskano z bazy Surveillance Atlas of Infectious Diseases Europejskiego Centrum ds. Zapobiegania i Kontroli Chorób.

3. Metoda szeregów mikrorozcieńczeń dwóch antybiotyków w bulionie

Metoda mikrorozcieńczeniowa (tzw. test szachownicy) to najpopularniejsza metoda badania aktywności połączeń antybiotyków w warunkach *in vitro*, będąca modyfikacją standardowej metody oznaczania MIC w bulionie, techniki rutynowo wykorzystywanej np. przy określaniu wartości MIC kolistyny. Najczęściej wykorzystuje się 96-dołkową polistyrenową płytkę, którą dostosowuje się do badania aktywności dwóch antybiotyków. Tworzy się gradientową "szachownicę" rozcieńczeń dwóch antybiotyków, tak aby w poszczególnych dołkach oceniona została każda możliwa kombinacja stężeń badanych leków. Wiąże się to z dwoma

poważnymi ograniczeniami – jest to metoda statyczna, w której działanie antybiotyków oceniane jest w konkretnym punkcie czasowym, bez możliwości ich oceny na przestrzeni kilkunastu godzin inkubacji, oraz dostarcza informacji tylko o działaniu bakteriostatycznym, bez możliwości zbadania właściwości bójczych leków (w zastosowanej metodologii te dwa efekty są nie do odróżnienia) (Brennan-Krohn i Kirby 2019a). Istotne jest także rzeczywiste ograniczenie liczby badanych antybiotyków do dwóch. Przy większej ich liczbie metoda ta szybko staje się niepraktyczna, nawet przy założeniu, że co najmniej jeden z nich będzie badany w wąskim zakresie stężeń (Doern 2014; Brennan-Krohn i Kirby 2019a). Ponadto w metodzie tej potrzeba wielu odczynników oraz konieczne jest przygotowanie szeregu rozcieńczeń antybiotyków.

W opisywanej metodzie do dołków płytki polistyrenowej dodaje się wystandaryzowane inokula bakteryjne (o stałej gęstości i objętości) w bulionie Muellera-Hinton i antybiotyki w odpowiednich stężeniach (najczęściej dwa z dwóch różnych grup). Stężenia antybiotyków, które uzyskuje się w serii dwukrotnych rozcieńczeń, powinny odnieść się do stężeń możliwych do uzyskania w płynach ustrojowych pacjenta (Doern 2014; Garbusińska i Szliszka 2017; Brennan-Krohn i Kirby 2019a). Po okresie inkubacji ocenia się wzrost bakterii we wszystkich dołkach płytki. Dla stężeń w dołkach, w których wzrost został zahamowany, wylicza się wskaźnik FICI (ang. fractional inhibitory concentration index) i na jego podstawie klasyfikuje się interakcję antybiotyków jako działanie antagonistyczne (FICI > 4), obojętne ($1 < FICI \leq 4$), addytywne ($0{,}5 < FICI \leq 1$) lub synergistyczne ($FICI \leq 0{,}5$). Przytoczony sposób klasyfikacji jest najczęstszy, choć autorzy prac naukowych korzystają czasem z innych przedziałów dla FICI bądź nie wyróżniają addycji antybiotyków. Wskaźnik FICI dla danej kombinacji stężeń, przy których zaobserwowano zahamowanie wzrostu, jest sumą FIC (ang. fractional inhibitory concentration) obu leków, czyli stosunku stężenia leku w tym dołku do jego MIC. Zatem, skoro o synergizmie świadczy $FICI \leq 0{,}5$, a zakres błędu dla badania MIC w standardowym rozcieńczaniu w bulionie wynosi ± 1 dwukrotne rozcieńczenie (przy czym zakres błędu zwiększa się przy badaniu leków w skojarzeniu), to definicja synergizmu jest spełniona, jeśli każdy lek w dołku ma stężenie co najmniej o połowę niższą niż jego MIC oceniane indywidualnie w osobnym oznaczeniu (Garbusińska i Szliszka 2017; Brennan-Krohn i Kirby 2019a).

Metoda mikrorozcieńczeń w bulionie jest nadal modyfikowana i udoskonalana, np. poprzez wykorzystanie bio-drukarki, umożliwiającej precyzyjne odmierzanie mikroobjętości i przyspieszającej oznaczenie (Brennan-Krohn i Kirby 2019b). Opisana poniżej procedura uwzględnia odczynniki i aparaturę powszechnie dostępne w laboratoriach mikrobiologicznych.

Procedura

1. Przygotowanie inokulum bakteryjnego.
   - 1.1. Skalibruj densytometr względem próbki kontrolnej o gęstości optycznej 0.5 w skali McFarlanda.
   - 1.2. Zbierz badany szczep sterylną ezą z podłoża agarowego i umieść go w odpowiednim dla metody roztworze (najczęściej w roztworze fizjologicznym soli).
   - 1.3. Wymieszaj przez worteksowanie.
   - 1.4. Zmierz gęstość optyczną zawiesiny w densytometrze.
   - 1.5. W razie potrzeby dodaj masy bakterii lub szczepu kontrolnego za pomocą ezy w celu otrzymania zawiesiny o gęstości optycznej 0.5 w skali McFarlanda.
2. Oznaczenie MIC badanych antybiotyków (metoda do wyboru).
3. Przygotowanie roztworów antybiotyków.
   - 3.1. Wybierz antybiotyk, który będzie rozcieńczany w rzędach od 1. do 12., czyli rzędach poziomych (antybiotyk A), a który w dołkach A → H, czyli kolumnach pionowych (antybiotyk B), uwzględniając zasadność zbadania jednego z nich w szerszym zakresie stężeń.
   - 3.2. Przygotuj około 400 µl roztworu antybiotyku A i około 350 µl – antybiotyku B o stężeniu czterokrotnie (A) i ośmiokrotnie (B) wyższym niż graniczna wartość oporności danego leku dla gatunku badanego szczepu (Brennan-Krohn *i wsp.* 2017; European Committee on Antimicrobial Susceptibility Testing 2024). Wybierz rozpuszczalnik zgodnie z wytycznymi CLSI (Clinical and Laboratory Standards Institute) "Solvents and Diluents for Preparing Stock Solutions of Antimicrobial Agents". Jeśli wytyczne nie zawierają informacji o badanym antybiotyku, użyj rozpuszczalnika, który daje większą stabilność leku (Bellio *i wsp.* 2021).
     - 3.2.1. Można skorzystać ze wzoru:

       $C_a - C_k = V_r$

       $C_k - C_r = V_a$

       gdzie C_a – stężenie wyjściowego roztworu antybiotyku [mg/l],

       C_k – stężenie końcowe roztworu antybiotyku [mg/l]

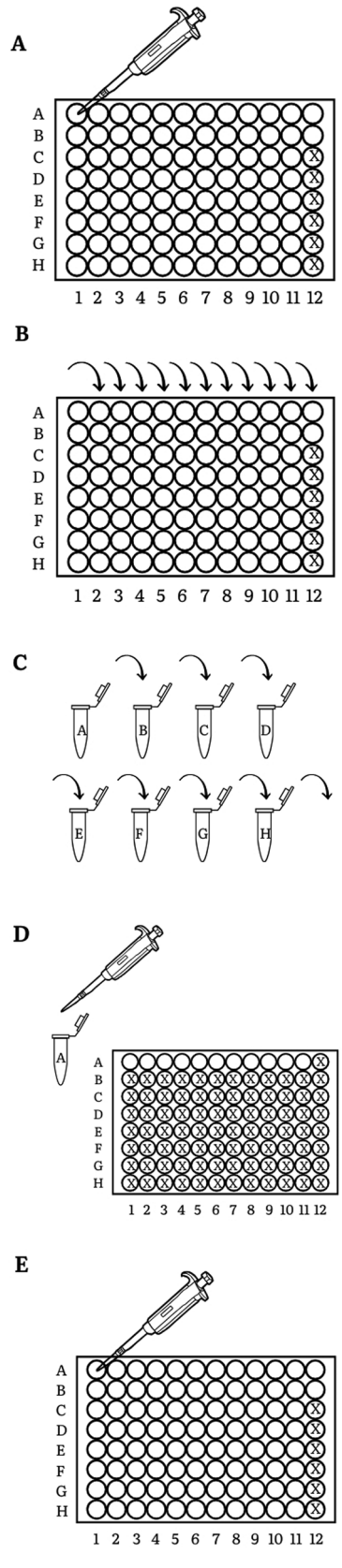
       C_r – stężenie rozpuszczalnika [mg/l]

       V_r – objętość rozpuszczalnika [µl]

       V_a – objętość wyjściowego roztworu antybiotyku [µl]

   Dodanie V_a wyjściowego roztworu antybiotyku do V_r rozpuszczalnika pozwoli uzyskać roztwór antybiotyku o stężeniu C_k. C_k powinno mieć wartość czterokrotnie większą niż graniczna wartość oporności danego leku dla gatunku badanego szczepu.

4. Przygotowanie szachownicy stężeń. (Ryc. 1. Metoda mikrorozcieńczeń w bulionie – przygotowanie szachownicy stężeń.)
   4.1. Pipetą automatyczną dodaj do wszystkich dołków w rzędach od 1. do 11. oraz do dwóch w rzędzie 12. po 50 µl MHB (bulion Muellera-Hinton). (Ryc. 1A. Dodanie bulionu Muellera-Hinton.)
   4.2. Nanieś do wszystkich dołków (A-H) w rzędzie 1. po 50 µl antybiotyku A.
   4.3. Przenieś pipetą wielokanałową 50 µl roztworu z dołków rzędu 1. do dołków z rzędu 2., z 2. do 3. i tak do rzędu 11. Każdorazowo wymieszaj uzyskany roztwór pipetą automatyczną (kilkukrotnie wolno naciągaj i wypuszczaj roztwór). Przenieś po 50 µl roztworu z dołków 11A i 11B do 12A i 12B. Usuń z dołków 11C-H po 50 µl roztworu. Dołek 12A będzie służyć jako kontrola wzrostu bakterii bez antybiotyków (kontrola dodatnia), a dołek 12B – kontrola jałowości odczynników i leków (kontrola ujemna). (Ryc. 1B. Przygotowanie szeregu mikrorozcieńczeń pierwszego antybiotyku.)
   4.4. Przygotuj 8 probówek typu Eppendorf i oznacz je kolejno literami A-H.
   4.5. Umieść za pomocą pipety automatycznej po 1 ml MHB we wszystkich probówkach Eppendorf.
   4.6. Przenieś 333 µl roztworu antybiotyku B (o stężeniu ośmiokrotnie większym niż graniczna wartość oporności danego leku dla gatunku badanego szczepu) do probówki A. Po umieszczeniu antybiotyku B w MHB w proporcji 1:3, otrzymany zostanie jego roztwór o stężeniu dwukrotnie wyższym niż graniczna wartość stężenia, które warunkuje oporność na dany lek.
   4.7. Przenieś 333 µl z probówki A do probówki B, wymieszaj pipetą automatyczną. Przenieś 333 µl z probówki B do probówki C, wymieszaj i tak dalej kolejno aż do probówki H. Z probówki H usuń 333 µl roztworu. (Ryc. 1C. Przygotowanie szeregu mikrorozcieńczeń drugiego antybiotyku.)
   4.8. Do dołków w rzędzie A (A1-A11 – bez dołka A12, tj. kontroli dodatniej) rozpipetuj po 50 µl roztworu z probówki A, z probówki B do wszystkich dołków w rzędzie B (B1-B12), z probówki C do dołków C1-C11 i tak dalej. (Ryc. 1D. Dodanie antybiotyków do siebie.)
   4.9. Do każdego dołka z roztworami dodaj pipetą (można użyć pipety wielokanałowej) po 50 µl MHB. Zachowaj ostrożność, aby przy korzystaniu z pipety wielokanałowej nie dodać MHB do dołków 12C-H. (Ryc. 1E. Ponowne dodanie bulionu Muellera-Hinton.)
   4.10. Dodaj 50 µl zawiesiny bakterii do wszystkich dołków z roztworami (oprócz 12B, tj. kontroli ujemnej).



Ryc. 1. Metoda mikrorozcieńczeń w bulionie – przygotowanie szachownicy stężeń.

A – dodanie bulionu Muellera-Hinton.
B – przygotowanie szeregu mikrorozcieńczeń pierwszego antybiotyku.
C – przygotowanie szeregu mikrorozcieńczeń drugiego antybiotyku.
D – dodanie antybiotyków do siebie.
E – ponowne dodanie bulionu Muellera-Hinton.

5. Inkubacja przez 16–24 godzin w temperaturze 37°C.
6. Ocena wzrostu.
   6.1. Oceń zmętnienie w dołkach po zakończeniu inkubacji. Brak zmętnienia oznacza zahamowanie wzrostu bakterii przez antybiotyki.
   6.2. Zapisz, w których połączeniach stężeń zaobserwowano zahamowanie wzrostu. Dla tych kombinacji oblicz wskaźnik FICI:
   $FICI = FIC_x + FIC_y$
   $FIC_x = MIC_{xc} / MIC_x$
   $FIC_y = MIC_{yc} / MIC_y$
   gdzie MIC_x, MIC_y – MIC leku X lub Y użytego samodzielnie,
   MIC_{xc}, MIC_{yc} – stężenie leku X lub Y w skojarzeniu w danym dołku, dla którego wyznaczane jest FICI (Garbusińska i Szliszka 2017).
   6.3. Oceń wartości FICI według kryteriów podanych powyżej i sklasyfikuj połączenia w różnych stężeniach jako synergistyczne, addytywne, obojętne bądź antagonistyczne.
   6.4. Jeśli w rzędzie nastąpiło tzw. "pominięcie dołka" (np. w dołku C8 brak wzrostu, w C9 wzrost, w C10 brak wzrostu), wylicz FICI dla C10, aby uniknąć błędnej interpretacji, tj. np. wyniku fałszywie dodatniego (Brennan-Krohn *i wsp.* 2017).

4. Test "time-kill"

Test "time-kill" jest metodą, której wynik dostarcza informacji zarówno na temat synergistycznego działania antybiotyków, jak i dynamiki wzrostu bakterii oraz aktywności bakteriobójczej preparatu (Brennan-Krohn i Kirby 2019b). Test opiera się na analizie przeżywalności drobnoustrojów w badanych stężeniach dwóch antybiotyków w wybranych odstępach czasowych. Wzajemne oddziaływanie dwóch leków ustala się, porównując liczbę przeżywających bakterii jako CFU/ml (liczba jednostek tworzących kolonie na ml) w badanej kombinacji antybiotyków i ich liczbę w najlepiej działającym pojedynczym chemioterapeutyku. Uzyskane wyniki prezentowane są na krzywych zabicia "time-kill". Synergizm stwierdza się, gdy różnica między dwoma próbami wynosi $\geq 2_{log10}$ (Brennan-Krohn i Kirby 2019b). W momencie, gdy różnica w liczbie CFU/ml między kombinacją dwóch antybiotyków w momencie rozpoczęcia inkubacji i po 24 h wynosi $\geq 3_{log10}$, uznaje się ją za bakteriobójczą. Test "time-kill" przedstawiany jest jako alternatywa dla metody szachownicy (Garbusińska i Szliszka 2017).

Zdecydowaną jego zaletą w przeciwieństwie do metody szachownicy, która jest w stanie określić jedynie działanie bakteriostatyczne, jest możliwość określenia zarówno działania bakteriostatycznego, jak i bakteriobójczego. Dodatkowo test "time-kill" umożliwia określenie działania kombinacji antybiotyków w różnych punktach czasowych (Brennan-Krohn i Kirby 2019b). Wadą testu "time-kill" jest natomiast większa pracochłonność i czasochłonność wykonania w porównaniu do metody szachownicy. Ponadto wykonanie testu "time-kill" jest bardziej kosztochłonne.

Procedura

1. Przygotowanie roztworów antybiotyków.
   1.1. Należy określić docelowe stężenie chemioterapeutyku w oparciu o jego rozpuszczalność oraz o pożądane stężenia końcowe. Wyboru rozpuszczalnika należy dokonać w oparciu o wytyczne CLSI.
2. Hodowla wstępna.
   2.1. Z jednodniowej hodowli bakteryjnej przygotuj w 0,9% NaCl zawiesinę o gęstości optycznej 0,5 w skali McFarlanda. Skontroluj zmętnienie za pomocą densytometru.
   2.2. Dodaj 100 µl zawiesiny bakteryjnej do 5 ml CAMBH (ang. Mueller-Hinton Broth, cation adjusted). Przenieś kroplę rozcieńczonej zawiesiny przy pomocy sterylnej pętli inokulacyjnej na płytkę z agarem z krwią w celu potwierdzenia czystości inokulum. Inkubuj tę hodowlę w 35°C.
   2.3. Inkubuj pozostałą część zawiesiny w temperaturze 35°C przez minimum 3 h, do czasu uzyskania wzrostu w fazie logarytmicznej.
3. Roztwory związków przeciwdrobnoustrojowych.
   3.1. Dodaj po 10 ml CAMBH do 5 szklanych probówek hodowlanych a następnie:
   Probówka nr 1: Dodaj pierwszy antybiotyk w ilości odpowiadającej jego docelowemu stężeniu.
   Probówka nr 2: Dodaj drugi antybiotyk w ilości odpowiadającej jego docelowemu stężeniu antybiotyku.
   Probówka nr 3: Dodaj tą samą ilości antybiotyku pierwszego i drugiego, co do probówek 1 i 2.
   Probówka nr 4: Kontrola wzrostu – nie należy dodawać antybiotyku.
   Probówka nr 5: Kontrola ujemna – nie należy dodawać ani antybiotyku, ani później drobnoustrojów.
4. Wykonanie serii rozcieńczeń. (Ryc. 2. Metoda „time-kill" – wykonanie serii rozcieńczeń.)
   4.1. Przygotuj sześć 96-dołkowych płytek (oznaczonych: t_0, t_1, t_2, t_4, t_6, t_{24}) ze studzienkami o pojemności 2 ml, które posłużą do serii rozcieńczeń. Umieść po 900 µl 0,9% roztworu NaCl w rzędach B-H, kolumny 1–5. (Ryc. 2A. Przygotowanie sześciu 96-dołkowych płytek (t0, t1, t2, t4, t6, t24) z 0,9% chlorkiem sodu.)
   4.2. Przygotuj początkowe inokulum. W tym celu przenieś 1 ml zawiesiny do szklanej probówki w momencie, gdy hodowla osiągnie fazę logarytmicznego wzrostu. Rozcieńcz za pomocą

CAMBH lub zagęść w celu uzyskania gęstości 1,0 w skali McFarlanda.

4.3. Dodaj 100 µl zawiesiny do probówek 1–4 i delikatnie wymieszaj. (Ryc. 2B. Dodanie zawiesiny bakteryjnej do probówek 1–4.)

4.4. Pobierz 150 µl z każdej probówki w czasie t_0 (tuż po podaniu zawiesiny bakteryjnej) oraz po 1, 2, 4, 6 i 24 godzinach. Dodawaj kolejne porcje do kolejnych dołków w rzędach o numerach 1 odpowiednio oznaczonych (t_1, t_2, t_4, t_6, t_{24}) płytek 96-dołkowych. (Ryc. 2C. Przeniesienie porcji zawiesiny do odpowiednich płytek w ustalonych wcześniej punktach czasowych.)

4.5. Pobierz za pomocą pipety wielokanałowej 100 µl z rzędu A i przenieś do rzędu B. Wymieszaj zawartość dołków (kilkukrotnie wolno naciągaj i wypuszczaj roztwór). Uzyskane zostanie w ten sposób rozcieńczenie 1:10. Powtórz czynności dla rzędów B-H. Usuń 100 µl z rzędu H. (Ryc. 2D. Uzyskanie rozcieńczenia 1:10.)

4.6. Pobierz za pomocą pipety wielokanałowej po 10 µl z każdego dołka w kolumnach 1–3 wszystkich płytek i przenieś na odpowiednio oznakowane płytki z agarem Muellera-Hinton w celu zliczania kolonii przy użyciu metody "drop plate". Odpowiednie oznakowanie powinno umożliwić identyfikację użytych antybiotyków, ich stężeń i czasu, po którym przeniesiono porcję zawiesiny bakteryjnej na płytkę (t_0-t_{24}). Pozostaw krople do całkowitego wyschnięcia. (Ryc. 2E. Przeniesienie porcji zawiesin na płytki metodą "drop plate".)

4.7. Po upływie doby umieść kroplę 10 µl pobraną z kontroli negatywnej na wybraną płytkę, w celu potwierdzenia sterylności. Wszystkie płytki po odwróceniu powinny być inkubowanie przez noc w warunkach zgodnych z zaleceniami.

5. Odczytanie wyników.

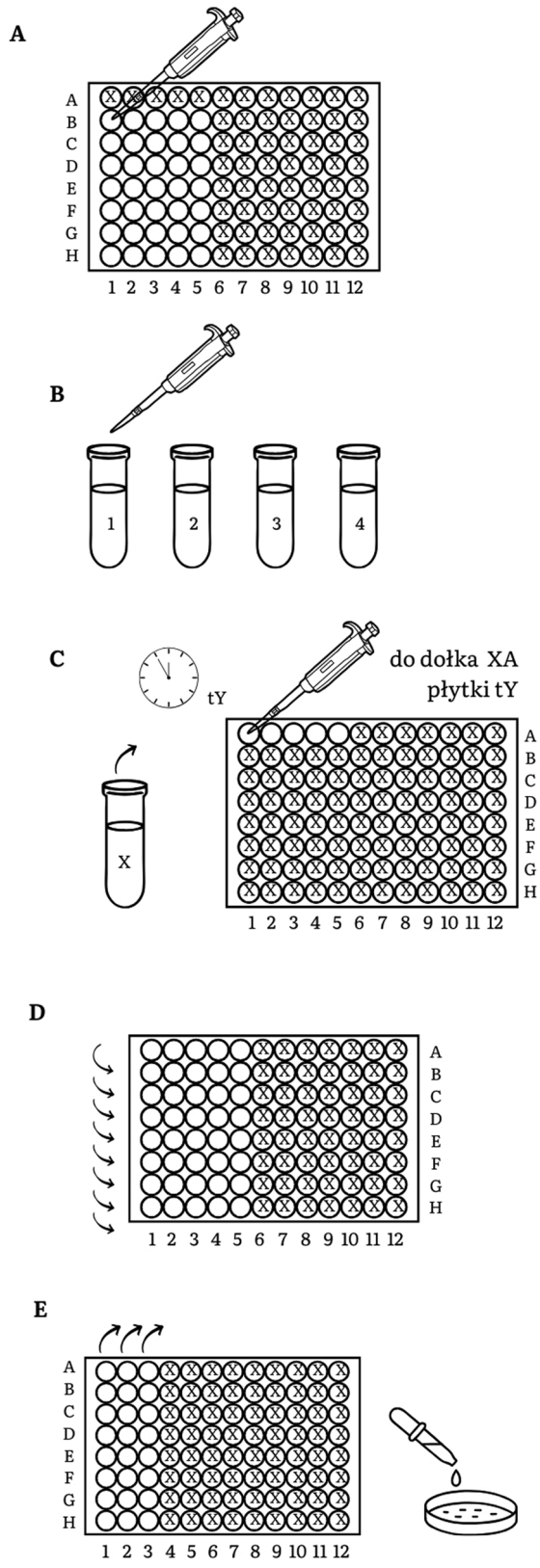
5.1. Sprawdź kontrolę ujemną. Jeśli zaobserwowano jakikolwiek wzrost, wyniki oznaczenia nie powinny być wykorzystywane.

5.2. Widoczne na płytkach kolonie powinny posiadać jednolitą morfologię, oczekiwaną dla danego szczepu.

5.3. Znajdź krople z 3–30 koloniami dla każdej serii rozcieńczeń. Zlicz kolonie w tych kroplach i zapisz wraz ze współczynnikiem rozcieńczenia. Jeśli w serii rozcieńczeń nie ma kropli z 3–30 koloniami, policz kolejno – kolonie w ostatniej kropli z ponad 30 koloniami i w pierwszej kropli z koloniami.

5.4. Oblicz dla każdej serii rozcieńczeń wartości CFU/ml w próbce na podstawie liczby kolonii w kropli, używając wzoru:

$$CFU/ml = 100\ n / d$$



Ryc. 2. Metoda „time-kill" – wykonanie serii rozcieńczeń.

A – przygotowanie sześciu 96-dołkowych płytek (t_0, t_1, t_2, t_4, t_6, t_{24}) z 0,9% chlorkiem sodu.

B – dodanie zawiesiny bakteryjnej do probówek 1–4.

C – przeniesienie porcji zawiesiny do odpowiednich płytek ustalonych wcześniej punktach czasowych.

D – uzyskanie rozcieńczenia 1:10.

E – przeniesienie porcji zawiesin na płytki metodą "drop plate".

gdzie:
n – liczba kolonii
d – współczynnik rozcieńczenia (1 dla nierozcieńczonej próbki – wiersz A, 0,1 dla pierwszego rozcieńczenia – wiersz B, 0,01 dla drugiego rozcieńczenia – wiersz C, itd.)

6. Analiza wyników.
   6.1. Wykreśl krzywe wzrostu z trzech hodowli zawierających antybiotyk oraz kontrolę wzrostu. Oznacz czas na osi *x*, natomiast wartości CFU/ml na osi *y*.
   6.2. Oblicz różnicę pomiędzy wzrostem w probówce z dwoma antybiotykami po upłynięciu 24 godzin a najbardziej aktywnym pojedynczym antybiotykiem w tym samym czasie (w CFU/ml). Kombinację antybiotyków należy uznać za synergistyczną, jeśli różnica wynosi $\geq 2_{\log 10}$. Oblicz różnicę w CFU/ml między kombinacją antybiotyków w probówce w 24. godzinie i w czasie 0. Uznać kombinację za bakteriobójczą należy, jeśli różnica wynosi $\geq 3_{\log 10}$.

5. Test CombiANT

Kolejna metoda badania połączeń antybiotyków, CombiANT, nie jest jeszcze dostępna w praktyce badawczej ani diagnostyce mikrobiologicznej, a jej efekty w stosowaniu klinicznym nie są jeszcze znane. Po raz pierwszy została zaprezentowana w 2020 roku, ale dotychczasowe badania wskazują, że wyniki oznaczeń techniką CombiANT są identyczne jak w metodzie szachownicy (Fatsis-Kavalopoulos *i wsp.* 2020). Mimo braku dostępności aparatury i algorytmów interpretacji wyników zwalidowanych dla poszczególnych gatunków bakterii, z powodu wielu zalet tej metody i być może przełomowego ułatwienia badania połączeń antybiotyków zdecydowano o opisie tej procedury w niniejszej pracy.

CombiANT to test oparty na dyfuzji dostarczający informacji ilościowych o interakcjach trzech par antybiotyków. Wybrane antybiotyki umieszczane są w trzech zbiorniczkach, a wkładka z nimi lokowana w standardowej płytce z agarem. Wyróżnić można dwa pola. Na zewnątrz wkładki znajduje się pole, w którym każdy z antybiotyków działa indywidualnie. Wewnątrz płytki z antybiotykami znajduje się trójkątne pole, w którym dochodzi do interakcji pomiędzy poszczególnymi parami antybiotyków. Wyniki są generowane przez algorytm opracowany przez twórców, który został skalibrowany pod względem rodzaju i szybkości dyfuzji poszczególnych antybiotyków w wybranym podłożu. Zakres interakcji antybiotyków jest określany ilościowo na podstawie punktów na krawędzi strefy zahamowania, zgodnie ze wzorem FICI. Wartość FICI zostały zinterpretowane zgodnie z progami klinicznymi, gdzie $\leq 0{,}5$ wskazuje na synergię, FICI $> 0{,}5$, a ≤ 4 wskazuje na addycję, natomiast > 4 – na antagonizm (Tang *i wsp.* 2024).

Metodę CombiANT można zastosować bez wcześniejszego oznaczenia wrażliwości szczepu na dany antybiotyk. Jest to zdecydowana zaleta, ponieważ pozwala to na skrócenie czasu oczekiwania na wynik lekowrażliwości szczepu badanego. Test CombiANT ma taką samą dokładność, jak metoda mikrorozcieńczeń w bulionie, jednak jest bardziej wydajny i mniej złożony (Fatsis-Kavalopoulos *i wsp.* 2020). Dodatkowym atutem tej metody jest możliwość dokładnego oznaczenia stężenia hamującego, ponieważ stosuje się ciągły zakres stężeń antybiotyków, natomiast testy szachownicy tylko dwukrotne rozcieńczenia. Istotną wadą tej metody jest konieczność wykorzystania specjalnie zaprojektowanych i wykonanych wkładek, które nie mają zastosowania w innych badaniach laboratoryjnych.

Procedura

1. Przygotowanie wkładki z antybiotykami (Ryc. 3. Metoda CombiANT – wkładka i protokół eksperymentalny.) zawierającej trzy rezerwuary (zbiorniczki) oznaczone jako a, b, c oraz trójkątne pole oddziaływania – oznaczone jako d. (Ryc. 3A. Konstrukcja wkładki: zbiorniki antybiotyków (a, b, c) i obszar obrazowania interakcji (d).)
   1.1. Dodaj 0,5 ml płynnego agaru (w temperaturze 60°C) do każdego ze zbiorniczków wkładki zawierających antybiotyki (oznaczone odpowiednio jako a, b, c). Stężenie antybiotyku jest obliczane na podstawie wyniku kalibracji algorytmu analitycznego, co jest przygotowaniem do obrazowania cyfrowego (Ryc. 3B).
   Przygotowane wkładki mogą być bezpiecznie przechowywane w temperaturze 4–8°C przez okres 7 dni.
   1.2. Umieść wkładkę w płytce hodowlanej i dodaj drugą warstwę agaru, w ten sposób ją aktywując. Umożliwiona zostanie dyfuzja antybiotyków.
2. Posiew badanego drobnoustroju.
   2.1. Przygotuj zawiesinę bakterii o gęstości 0,5 w skali McFarlanda, zgodnie z wytycznymi EUCAST (European Committee on Antimicrobial Susceptibility Testing), tak jak dla oznaczeń metodą dyfuzyjno-krążkową.
   2.2. Nanieś zawiesinę na zestalony agar za pomocą bawełnianej wymazówki, tworząc murawę bakteryjną.
3. Inkubacja.
   3.1. Inkubuj przez 16–24 godzin w temperaturze 37°C.
   3.2. Po odpowiednim czasie inkubacji wytworzą się strefy zahamowania wzrostu bakterii zgodne z powstałym gradientem stężeń.
   3.3. Wewnątrz wkładki z aktywnymi antybiotykami wytworzy się trójkątna powierzchnia obrazowania

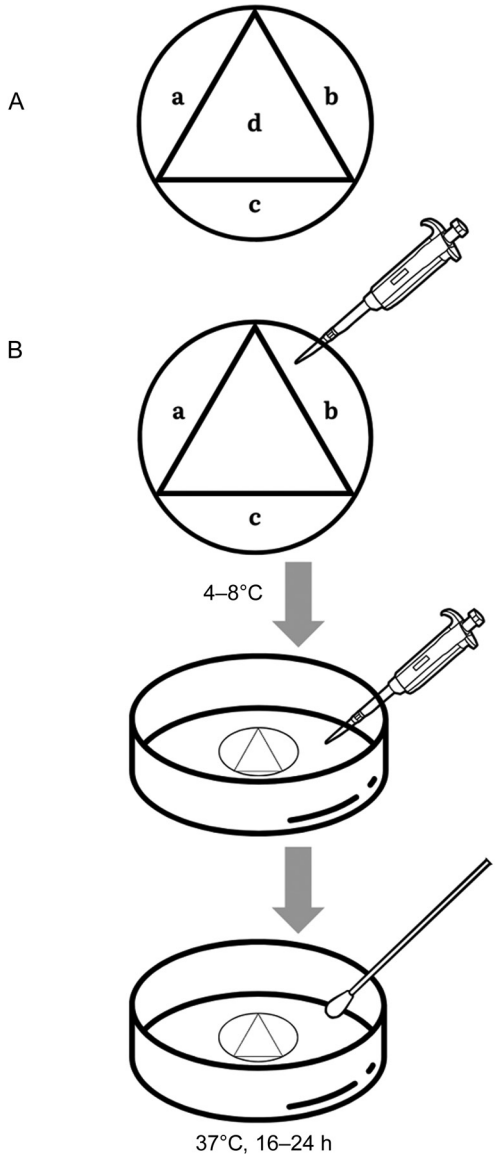


Fig. 3. Metoda CombiANT – wkładka i protokół eksperymentalny.

A – konstrukcja wkładki: zbiorniki antybiotyków (a, b, c) i obszar obrazowania interakcji (d).

B – protokół testu: Napełnienie wkładki poprzez dodanie do każdego ze zbiorników (a,b,c) 0,5 ml płynnego agaru (60°C) zawierającego odpowiedni antybiotyk (A,B,C). Tak przygotowane wkładki można przechowywać w temperaturze 4°C – 8°C do 7 dni. Aby aktywować wkładkę, należy umieścić ją na szalce i dodać 25 ml agaru, umożliwiając w ten sposób dyfuzję antybiotyków na powierzchnię agaru i obrzeża szalki. Po zestaleniu agaru nanieść zawiesinę bakterii o gęstości 0,5 McFarlanda na powierzchnię agaru za pomocą wymazówki. Inkubacja płytek w 37°C przez 16–24 godzin pozwala na uwidocznienie stref zahamowania wzrostu (Fatsis-Kavalopoulos *et al.* 2020).

interakcji. W każdym rogu obszaru dochodzi do interakcji dwóch sąsiadujących ze sobą antybiotyków.

4. Ilościowe pomiary interakcji leków.
   4.1. Wykonaj obrazowanie płytki w celu pomiaru interakcji antybiotyków, np. za pomocą kamery gel-doc lub ręcznego urządzenia mobilnego.
   4.2. Dokonaj analizy za pomocą odpowiednio skalibrowanego algorytmu, który wykorzystuje uprzednio opracowane modele dyfuzji.
      4.2.1. Określ, który antybiotyk został umieszczony w każdym zbiorniku testu.
      4.2.2. Algorytm opracowany przez twórców metody przywołuje zapisane mapy dyfuzji odpowiadające antybiotykom użytym w teście i łączy je w model specyficzny dla testu. Jest on następnie dopasowywany do wykonanego obrazowania testu.
      4.2.3. Algorytm wyznacza IC (ang. inhibitory concentration) oraz CP (ang. combination inhibitory point) dla trzech antybiotyków, zarówno indywidualnie, jak i w połączeniu. Zakres interakcji antybiotyków jest określany ilościowo na podstawie punktów na krawędzi strefy zahamowania, zgodnie ze wzorem FICI.
5. Obliczenie wartości FICI.
   $FICI_{AB} = C_A / IC_A + C_B / IC_B$
   gdzie:
   C_A, C_B – stężenie antybiotyku A i B
   IC_A, IC_B – stężenie hamujące antybiotyku A i B

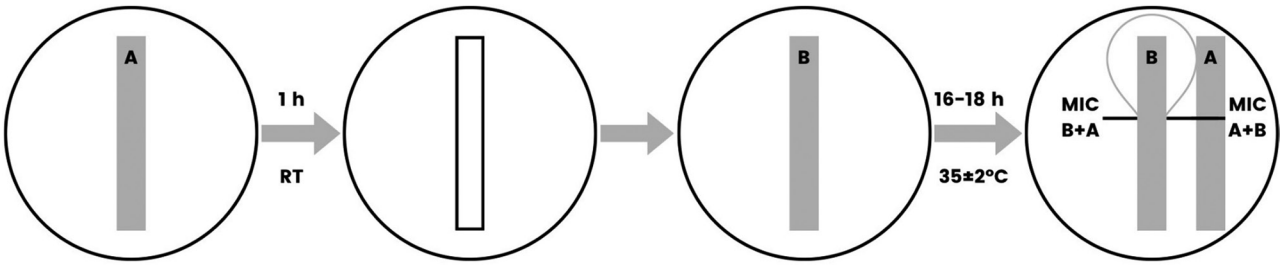
6. Paski z gradientami stężeń antybiotyków stosowane w metodach oznaczeń ich synergistycznego działania

Ocenę synergistycznego działania środków przeciwdrobnoustrojowych *in vitro* można przeprowadzić również, stosując metody dostępne w większości laboratoriów mikrobiologicznych, tj. wykorzystujące paski nasączone antybiotykiem w gradiencie stężeń. Dotychczas opracowano następujące metody:

- metoda stałych współczynników (ang. E-test fixed ratio method),
- metoda krzyżowa (metoda kąta 90°, ang. cross method),
- metoda proporcji – MIC:MIC,
- podłoży zawierających antybiotyk i pasków z gradientem stężeń (ang. E-test agar method) (Laishram *i wsp.* 2017; Guzek 2023).

Wykonanie badania każdą z wyżej wymienionych metod należy poprzedzić oznaczeniem wartości najmniejszego stężenia hamującego (MIC) wszystkich badanych antybiotyków.

Metody oparte na wykorzystaniu pasków nasączonych antybiotykiem w gradiencie stężeń mają przewagę nad innymi metodami ze względu na prostotę wykonania oraz łatwy dostęp do tej procedury dla wszystkich laboratoriów. Jednak w ten sposób można badać synergizm tylko pomiędzy dwoma antybiotykami. Ograniczeniem technik wykorzystujących paski z zawartością



Ryc. 4. Ocena synergistycznego działania antybiotyków metodą stałych współczynników.

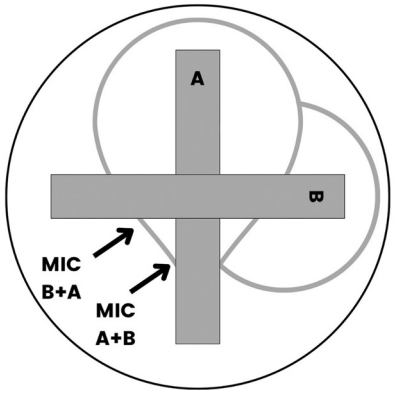
antybiotyku w gradiencie stężeń jest ponadto brak możliwości zastosowania ich wobec izolatów opornych, których wartości MIC wykraczają poza skalę paska, gdyż do oceny synergii należy znać dokładną wartość MIC.

Metoda stałych współczynników – procedura

1. Przygotuj badaną zawiesinę bakterii (gęstość 0,5 w skali McFarlanda) i nanieś ją na podłożu Mueller-Hinton Agar (MHA).
2. Nałóż na powierzchnię agaru pasek z gradientem stężeń badanego antybiotyku A.
   Dokładne zaznacz pole, na którym znajduje się pasek zawierający antybiotyk A.
3. Inkubuj hodowlę na płytce w temperaturze pokojowej przez godzinę w celu dyfuzji antybiotyku z paska do podłoża.
4. Usuń pasek z antybiotykiem A.
   4.1. Oczyść pasek alkoholem i pozostaw jako wzór do odczytu wartości MIC.
   4.2. Nałóż na to samo miejsce pasek z gradientem stężeń antybiotyku B.
5. Inkubuj płytkę przez 16–18 godzin w 35°C ± 2°C (według zaleceń producenta pasków).
6. Odczyt i interpretacja wyników: po inkubacji odczytaj wartości MIC dla zastosowanych pasków i oblicz wartości współczynnika FICI, który należy interpretować według standardowo przyjętych kryteriów (Laishram *i wsp.* 2017; Sreenivasan *i wsp.* 2022; Guzek 2023). (Ryc. 4. Ocena synergistycznego działania antybiotyków metodą stałych współczynników.)

Metoda krzyżowa – procedura

1. Badaną zawiesinę bakterii (gęstość 0,5 w skali McFarlanda) nanieś na płytkę z podłożem MHA.
2. Umieść dwa paski z gradientem stężeń badanych antybiotyków A i B w taki sposób, aby przecinały się pod kątem 90° na wysokości wcześniej oznaczonych wartości MIC.
3. Inkubuj płytkę przez 16–20 godzin w 35°C ± 2°C (według zaleceń producenta pasków).
4. Odczyt i interpretacja wyników:
   Po inkubacji odczytaj wartości MIC obu zastosowanych antybiotyków i oblicz wartość współczynnika FICI, który należy interpretować według standardowo przyjętych kryteriów. (Ryc. 5. Ocena synergistycznego działania antybiotyków metodą krzyżową.)

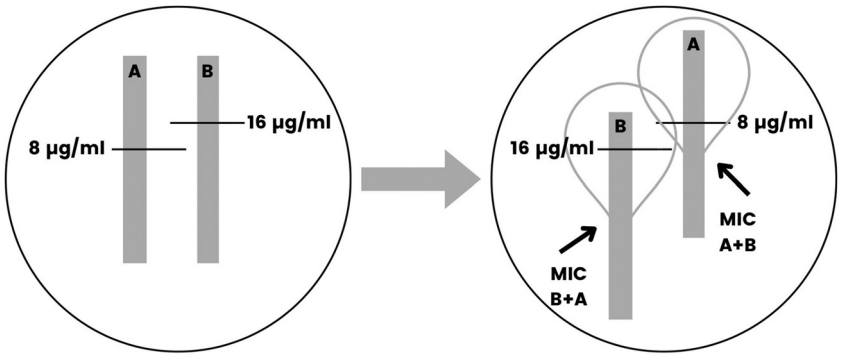


Ryc. 5. Ocena synergistycznego działania antybiotyków metodą krzyżową.

Ze względu na wysoką dostępność oraz łatwość wykonania, metoda krzyżowa jest chętnie wybierana do oceny skutków połączenia dwóch antybiotyków. Jednakże, wyniki tej procedury potrafią znacznie odbiegać od tych otrzymanych w testach "time-kill", będących złotym standardem (Nasomsong *i wsp.* 2022).

Metoda proporcji MIC:MIC – procedura

1. Nałóż równolegle dwa paski z gradientem stężeń badanych antybiotyków A i B na płytkę MHA z naniesioną zawiesiną bakterii. Zaznacz na spodzie płytki markerem w odpowiednim miejscu zbadane wcześniej wartość MIC dla każdego z antybiotyków.
2. Inkubuj płytki przez godzinę w temperaturze pokojowej celem dyfuzji antybiotyków.
3. Ocena synergizmu.
   3.1. Po upływie godziny usuń paski z antybiotykiem z płytki.
   3.2. Nałóż nowy pasek z gradientem stężeń antybiotyku B na miejsce, w którym znajdował się pasek z antybiotykiem A, w taki sposób, żeby wartość MIC z paska B korespondowała z oznaczoną markerem wartością MIC dla antybiotyku A.
   3.3. Postępuj analogicznie zastępując pasek B paskiem z antybiotykiem A.
4. Inkubuj płytkę przez 16–20 godzin w 35°C ± 2°C (według zaleceń producenta pasków).



Ryc. 6. Ocena synergistycznego działania antybiotyków metodą proporcji MIC:MIC.

5. Odczyt i interpretacja wyników:
   Dokonaj odczytu wartości MIC zastosowanych antybiotyków A i B. Oblicz wartość współczynnika FICI, który należy interpretować według standardowo przyjętych kryteriów (Laishram *i wsp.* 2017; Guzek 2023). (Ryc. 6. Ocena synergistycznego działania antybiotyków metodą proporcji MIC:MIC.)

W porównaniu metod dyfuzji gradientowej tobramycyny i ceftazydymu stosowanych w ocenie synergii w przypadkach zakażeń wielolekoopornymi *Pseudomonas aeruginosa*, wyniki uzyskane przez Okoliegbe i wsp. sugerują zasadność uznania metody MIC:MIC jako preferowanej w badaniach interakcji antybiotyków w praktyce diagnostycznej (Okoliegbe *i wsp.* 2021).

Metoda podłoży z zawartością antybiotyku i pasków z gradientem stężeń – procedura

1. Przygotuj podłoże zawierające jeden z badanych antybiotyków (A) w stężeniu 0,5 × MIC lub 0,25 × MIC.
2. Umieść pasek z gradientem stężeń antybiotyku B na tym podłożu.
3. Odczytaj wartość MIC antybiotyku B na podłożu z antybiotykiem A.
4. Porównaj uzyskaną wartość MIC z wartością MIC w podłożu wolnym od leku.
5. Interpretacja wyniku:
   Działanie synergistyczne stwierdza się, gdy występuje ponad trzykrotne zmniejszenie wartości MIC na podłożu zawierającym badany antybiotyk (Laishram *i wsp.* 2017).
   Marie i wsp. używali tej metody w celu oceny połączenia sulbaktamu i tazobaktamu z meropenemem lub kolistyną wobec wielolekoopornego szczepu *Acinetobacter baumannii*. Wykazali, że w przypadku tych połączeń wyniki uzyskane metodą podłoży z zawartością antybiotyku i pasków z gradientem stężeń nie pokrywały się z wynikami uzyskanymi metodą szachownicy, która wykazała większą czułość w oznaczaniu synergizmu. W przypadku oddziaływania addycyjnego obie metody wykazały podobną czułość (Marie *i wsp.* 2015).

7. Inne metody oznaczania synergizmu i addycji antybiotyków

Spośród wielu nieopisanych w artykule metod oznaczania synergizmu i addycji antybiotyków na uwagę zasługują trzy, których wykonanie jest osiągalne w każdym podstawowo wyposażonym medycznym laboratorium diagnostycznym. Są to: metoda podwójnych krążków (modyfikacja procedury standardowo wykorzystywanej w celu oceny występowania mechanizmów oporności drobnoustrojów na leki), metoda nasączania bibuły filtracyjnej (wykorzystuje ona paski bibuły filtracyjnej nasączone różnymi roztworami przeciwdrobnoustrojowymi o stężeniu równym lub wyższym od ich wartości MIC) oraz metoda warstwowa.

Metoda dwóch krążków – procedura

1. Nałóż krążki nasączone badanymi antybiotykami na podłoże z naniesioną zawiesiną bakterii w odległości 20 mm lub odpowiadającej sumie promieni stref zahamowania wzrostu dla każdego antybiotyku oddzielnie.
2. Inkubuj płytki przez 24 godziny w temperaturze 35°C.
3. Zinterpretuj wyniki poprzez ocenę średnicy strefy zahamowania wzrostu wokół krążków.
   3.1. Synergizm antybiotyków stwierdza się przy powiększeniu średnicy strefy zahamowania wzrostu o ≥ 2 mm w porównaniu ze średnicą pojedynczego środka, połączeniu strefy zahamowania wzrostu między lekiem A i B lub przy pojawieniu się strefy zahamowania wzrostu pomiędzy dyfundującymi lekami.
   3.2. Nieznaczne działanie synergistyczne wskazuje wzrost strefy zahamowania wzrostu o < 2 mm.
   3.3. Antagonizm stwierdza się przy spłaszczeniu strefy zahamowania wzrostu na styku dyfuzji dwóch środków przeciwdrobnoustrojowych (Laishram *i wsp.* 2017).

Metoda ta nie jest powszechnie stosowana ze względu na subiektywną i wyłącznie jakościową ocenę synergistycznego działania antybiotyków.

Metoda nasączania bibuły filtracyjnej – procedura

1. Na płytce MHA z naniesionym inokulum badanego drobnoustroju umieść wcześniej przygotowane paski bibuły nasączone antybiotykami pod kątem prostym względem siebie, stykające się.
2. Pozostaw paski bibuły z antybiotykami na kilka godzin w celu dyfuzji w podłożu.
3. Inkubuj płytki przez 18–24 godzin w temperaturze 37°C (Laishram *i wsp.* 2017).
   3.1. Możliwą alternatywą jest nałożenie pasków nasączonych antybiotykiem na czyste podłoże MHA na 24 godziny w celu dyfuzji, a następnie naniesienie badanego szczepu za pomocą techniki transferu membranowego.
4. Interpretacja wyników.
   4.1. Za efekt addycji uznaje się występowanie dwóch owalnych obszarów zahamowania wzrostu wokół pasków z antybiotykami łączących się pod kątem prostym.
   4.2. Synergizm stwierdza się przy poszerzeniu lub obecności strefy zahamowania wokół kąta prostego.
   4.3. Antagonizm stwierdza się przy wcięciu lub zwężeniu stref zahamowania wzrostu wokół pasków z antybiotykami od strony kąta prostego pomiędzy paskami (Laishram *i wsp.* 2017).

Metoda ta jest kosztochłonna i pracochłonna, a wyniki uzyskane tą techniką są jedynie jakościowe.

Metoda warstwowa – procedura

1. Przygotuj podłoże agarowe, zawierające antybiotyk A w stężeniu równym połowie jego wartości MIC dla badanego szczepu (tzw. warstwa agaru bazowego) oraz jako podłoże kontrolne agar bez antybiotyku.
2. Nanieś warstwę inokulum (agaru bez antybiotyku) zawierającą 10^6 CFU/ml badanego szczepu, na podłoże z antybiotykiem A oraz podłoże kontrolne.
3. Po zestaleniu agaru na płytce badanej i kontrolnej umieść na powierzchni krążki z antybiotykiem B w różnych stężeniach.
4. Inkubuj przez 24 godziny w temperaturze 37°C.
5. Interpretacja wyników:
   5.1. Synergizm stwierdza się przy wzroście średnicy strefy zahamowania wzrostu drobnoustrojów wokół krążka z antybiotykiem B o co najmniej 19% w stosunku do kontroli.
   5.2. Efekt addycji stwierdza się przy wzroście średnicy strefy zahamowania wzrostu wokół krążka z antybiotykiem B o mniej niż 19%.
   5.3. Brak zmian strefy zahamowania wzrostu wskazuje na brak działania połączeń stosowanych preparatów (Laishram *i wsp.* 2017).

8. Wnioski

Ze względu na dynamiczny wzrost poziomu antybiotykooporności drobnoustrojów i długi czas potrzebny do opracowania i dopuszczenia do praktyki klinicznej nowego antybiotyku konieczne jest wprowadzanie do rutynowej praktyki diagnostycznej oceny działania połączeń antybiotyków. Opisano wiele metod badania synergizmu i addycji antybiotyków, które różnią się dostępnością odczynników i aparatury, trudnością i czasem wykonania, a także uzyskiwanymi wynikami i jednoznacznością ich interpretacji (Papoutsaki *i wsp.* 2020; Okoliegbe *i wsp.* 2021). Opisywany w różnych badaniach poziom zgodności wymienionych tu metod jest znacząco różny, co może być związane z badanym szczepem bakterii, wykorzystywanymi antybiotykami czy nawet mechanizmami oporności. Gaudereto *i wsp.* w badaniu metod wykrywania synergizmu w przypadku wielolekoopornych pałeczek Gram-ujemnych uzyskali zgodność metody proporcji MIC:MIC i "time-kill" na poziomie 35–71% (Gaudereto *i wsp.* 2020). Wydaje się, że jednoznaczne porównanie poszczególnych metod w sposób uniwersalny wobec różnych antybiotyków oraz szczepów bakterii aktualnie pozostaje poza granicami możliwości badaczy (Doern 2014).

Obecnie "złotym standardem" badania połączeń antybiotyków pozostaje metoda "time-kill", która dostarcza dodatkowo informacji o działaniu bakteriobójczym i bakteriostatycznym w różnych punktach czasowych i stanowi wiarygodny punkt odniesienia dla różnych procedur. Test "time-kill" jest jednak metodą czasochłonną i relatywnie trudną metodologicznie, przez co atrakcyjną alternatywą wydają się przede wszystkim metody wykorzystujące paski z gradientami stężeń. Konieczna jest jednak walidacja uzyskiwanych dzięki nim wyników, tak aby były one jak najbardziej wiarygodne.

Oprócz rutynowego badania synergizmu i addycji w działaniu antybiotyków potrzebne jest jednak opracowywanie nowych metod możliwych do wdrożenia w praktyce diagnostycznej i, w efekcie, klinicznej. Obiecującą procedurą wydaje się metoda CombiANT, która dzięki możliwości szybkiego oznaczenia kombinacji trzech par leków, łatwej interpretacji wyników oraz dokładności porównywalnej do metody mikrorozcieńczeń w bulionie może być w niedalekiej przyszłości metodą o dużym znaczeniu diagnostycznym (Fatsis-Kavalopoulos *i wsp.* 2020).

Test szachownicy, najpopularniejsza metoda badania synergizmu, jako modyfikacja oznaczania MIC antybiotyku w bulionie jest procedurą intuicyjną i łatwo dostępną ze względu na szeroki dostęp do potrzebnej do wykonania badania aparatury i odczynników. W przeciwieństwie do metody "time-kill" dostarcza jednak informacji wyłącznie o efekcie bakteriostatycznym, co jest ograniczeniem mogącym mieć wpływ na dalsze postępowanie kliniczne.

Najbardziej dostępne, najłatwiejsze i najtańsze są metody wykorzystujące paski z gradientami stężeń antybiotyków. Pilnie potrzebna jest walidacja ich metodologii i konieczność wprowadzenia jednolitych kryteriów interpretacji uzyskanych wyników. W niektórych sytuacjach metody paskowe mogą być jedyną

możliwą do wykonania procedurą, dlatego tym bardziej potrzebne są dalsze badania porównawcze względem innych metod, które pozwolą wprowadzić je do rutynowej praktyki diagnostycznej, a w efekcie klinicznej. Ważne jest przedstawienie zróżnicowania wiarygodności wyników poszczególnych procedur z zastosowaniem pasków z gradientami stężeń, spośród których metoda proporcji MIC:MIC wydaje się być najbardziej rzetelna (Okoliegbe *i wsp.* 2021).

ORCID
Paweł Z. Kmiecikowski https://orcid.org/0009-0004-1315-7194
Aniela Gabriel https://orcid.org/0009-0006-6193-5610
Dagmara Depka https://orcid.org/0000-0003-2387-9778
Tomasz Bogiel https://orcid.org/0000-0001-6787-1378

Konflikt interesów

Autorzy nie zgłaszają żadnych powiązań finansowych ani osobistych z innymi osobami lub organizacjami, które mogłyby negatywnie wpłynąć na treść niniejszej publikacji i/lub rościć sobie prawa autorskie do tej publikacji.

Piśmiennictwo

1. Bellio P., Fagnani L., Nazzicone L., Celenza G.: New and simplified method for drug combination studies by checkerboard assay. *MethodsX*, **8**, 101543 (2021)
2. Brennan-Krohn T., Kirby J.E.: When One Drug Is Not Enough: Context, Methodology, and Future Prospects in Antibacterial Synergy Testing. *Clin. Lab. Med*. **39**, 345–358 (2019a)
3. Brennan-Krohn T., Kirby J.E.: Antimicrobial Synergy Testing by the Inkjet Printer-assisted Automated Checkerboard Array and the Manual Time-kill Method. *J.Vis. Exp.* **146**, 10.3791/58636 (2019b)
4. Brennan-Krohn T., Truelson K.A., Smith K.P., Kirby J.E.: Screening for synergistic activity of antimicrobial combinations against carbapenem-resistant Enterobacteriaceae using inkjet printer-based technology. *J. Antimicrob. Chemother*. **72**, 2775–2781 (2017)
5. Doern C.D.: When does 2 plus 2 equal 5? A review of antimicrobial synergy testing. *J. Clin. Microbiol*. **52**, 4124–8 (2014)
6 https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Break point_tables/v_14.0_Breakpoint_Tables.pdf
EUCAST. European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 14.0, valid from 2024-01-01. 19.05.2024
7. https://atlas.ecdc.europa.eu/public/index.aspx. European Centre for Disease Prevention and Control. Surveillance Atlas of Infectious Diseases, 13.04.2024
8. Fatsis-Kavalopoulos N., Roemhild R., Tang P.-C., Kreuger J., Andersson D.I.: CombiANT: Antibiotic interaction testing made easy, *PLOS Biol.* **18**, e3000856 (2020)
9. Gaudereto J.J., Neto L.V.P., Leite G.C., Espinoza E.P.S., Martins R.C.R., Prado G.V.B., Rossi F., Guimarães T., Levin A.S., Costa S.F.: Comparison of methods for the detection of in vitro synergy in multidrug-resistant gram-negative bacteria. *BMC Microbiol*. **20**, 97 (2020)
10. Garbusińska A., Szliszka E.: Aktywność leków przeciwdrobnoustrojowych zastosowanych w kombinacjach wobec pałeczek Gram-ujemnych w badaniach *in vitro*. *Post. N. Med.*, **XXX(08)**, 427–433 (2017)
11. Guzek A.: Metodologia oznaczania synergizmu między antybiotykami z zastosowaniem pasków z gradientem stężeń antybiotyków w warunkach *in vitro*. *Forum Zakażeń*, **14**, 19–24 (2023)
12. Karakonstantis S., Ioannou P., Kofteridis D.D.: In search for a synergistic combination against pandrug-resistant *A. baumannii*; methodological considerations. *Infection*, **50**, 569–581 (2022)
13. Laishram S., Pragasam A.K., Bakthavatchalam Y.D., Veeraraghavan B.: An update on technical, interpretative and clinical relevance of antimicrobial synergy testing methodologies. *Indian J. Med. Microbiol.* **35**, 445–468 (2017)
14. Marie M.A.M., Krishnappa L.G., Alzahrani A.J., Mubaraki M.A., Alyousef A.A.: A prospective evaluation of synergistic effect of sulbactam and tazobactam combination with meropenem or colistin against multidrug resistant *Acinetobacter baumannii*. *Bosn. J. Basic Med. Sci.* **15**, 24–29 (2015)
15. Nasomsong W., Nulsopapon P., Changpradub D., Pungcharoenkijkul S., Hanyanunt P., Chatreewattanakul T., Santimaleeworagun W.: Optimizing Doses of Ceftolozane/Tazobactam as Monotherapy or in Combination with Amikacin to Treat Carbapenem-Resistant *Pseudomonas aeruginosa*. *Antibiotics* (*Basel*), **11**, 517 (2022)
16. Okoliegbe I.N., Hijazi K., Cooper K., Ironside C., Gould I.M.: Antimicrobial Synergy Testing: Comparing the Tobramycin and Ceftazidime Gradient Diffusion Methodology Used in Assessing Synergy in Cystic Fibrosis-Derived Multidrug-Resistant *Pseudomonas aeruginosa. Antibiotics* (*Basel*), **10**, 967 (2021)
17. Papoutsaki V., Galani I., Papadimitriou E., Karantani I., Karaiskos I., Giamarellou H.: Evaluation of in vitro methods for testing tigecycline combinations against carbapenemase-producing *Klebsiella pneumoniae* isolates. *J. Global Antimicrob. Resist.* **20**, 98–104 (2020)
18. https://pharmaceutical-journal.com/article/news/only-12-new-antibiotics-entered-the-market-in-five-years-who-warns-in-review
Sayburn A.: Only 12 new antibiotics entered the market in five years, WHO warns in review. *The Pharmaceutical Journal*, **310** (2023), 04.12.2024
19. Sreenivasan P., Sharma B., Kaur S., Rana S., Biswal M., Ray P., Angrup A.: In-vitro susceptibility testing methods for the combination of ceftazidime-avibactam with aztreonam in metallobeta-lactamase producing organisms: Role of combination drugs in antibiotic resistance era. *J. Antibiot*. (*Tokyo*), **75**, 454–462 (2022)
20. Tang P.-C., Sánchez-Hevia D.L., Westhoff S., Fatsis-Kavalopoulos N., Andersson D.I.: Within-species variability of antibiotic interactions in Gram-negative bacteria. *mBio*, **15**, e0019624 (2024)
21. https://iris.who.int/bitstream/handle/10665/354545/9789240047655-eng.pdf?sequence=1.
WHO. World Health Organization: 2021 Antibacterial agents in clinical and preclinical development: an overview and analysis, 13.04.2024
22. https://antybiotyki.edu.pl/wp-content/uploads/Biuletyn/Biuletyn-Informacyjny-2_2022.pdf
Żabicka D., Grzegorczyk M.: Oporność na antybiotyki w Polsce i Europie w 2021 roku – dane sieci EARS-Net. *Oporność na antybiotyki. Biuletyn informacyjny*, **2** (2022), 19.05.2024

ADVANCEMENTS OF MICROBIOLOGY
2024, 63, 4, 223–238
https://doi.org/10.2478/am-2024-0018

POLYURETHANES AS BIOMATERIALS IN MEDICINE: ADVANCED APPLICATIONS, INFECTION CHALLENGES, AND INNOVATIVE SURFACE MODIFICATION METHODS

Kamil Drożdż[1], Monika Gołda-Cępa[2], Monika Brzychczy-Włoch[1*]

[1] Department of Molecular Medical Microbiology, Chair of Microbiology, Faculty of Medicine, Jagiellonian University Medical College, Krakow 31-121, Poland
[2] Faculty of Chemistry, Jagiellonian University, Krakow 31-007, Poland

Submitted in December 2024, accepted in December 2024

Abstract. Polyurethanes (PUs) are exceptionally versatile polymers widely utilized in medicine due to their outstanding mechanical properties, biocompatibility, and adaptability to various applications. This article explores advanced applications of polyurethane biomaterials in medicine, the challenges posed by infections associated with their use, and innovative surface modification techniques to improve their functionality. PUs are employed in a diverse array of medical devices, including non-implantable applications such as wound dressings, catheters, and infusion sets; short-term implants like bone stabilizers and tracheostomy tubes; and long-term implants such as tissue regeneration scaffolds, artificial blood vessels, and heart valves. Despite their many advantages, their use carries a significant risk of infections, including ventilator-associated pneumonia, infective endocarditis, and urinary tract infections. An important challenge lies in bacterial biofilms, which complicate treatment and enhance bacterial resistance to antibiotics. To address these issues, innovative PU surface modification methods are being developed, including laser texturing, nanoparticle deposition with antibacterial properties, ion implantation, cold metal spraying, the integration of biodegradable and biocompatible components, and plasma modifications. These advanced techniques aim to enhance polyurethane biomaterials' antibacterial properties and biocompatibility, thereby reducing infection risks and improving clinical outcomes. This article underscores the importance of ongoing research to effectively combat biomaterial-associated infections and broaden the medical applications of polyurethanes. The development of advanced surface modification methods holds great promise for improving patient quality of life and the efficacy of medical treatments.

Keywords: biomaterial-associated infections, biomaterials, innovative surface modification methods, medical applications of polyurethanes, polyurethanes

1. Introduction

Polyurethanes (PUs) are widely used in the production of medical devices. Due to their specific properties, some types of PUs can also be classified as biomaterials, depending on their application and compatibility with biological systems. A biomaterial can be described as "any substance or combination of substances, other than drugs, synthetic or natural, that can be used for any period of time and extends or replaces partially or completely any tissue, organ or function of the body" by the U.S. National Institutes of Health (Bergman and Stumpf 2013).

The first research on polyurethanes was carried out by Otto Bayer in 1937. He studied the polyaddition reaction between diisocyanate and polyester diol,

* Corresponding Authors: Monika Brzychczy-Włoch, Jagiellonian University Medical College, Faculty of Medicine, Chair of Microbiology, Department of Molecular Medical Microbiology, Czysta 18, 31-121 Krakow, Poland, e-mail: m.brzychczy-wloch@uj.edu.pl

Cite as:
Polyurethanes as Biomaterials in Medicine: Advanced Applications, Infection Challenges, and Innovative Surface Modification Methods. Drożdż K. *et al.,* ADV MICROBIOL-NY, 2024, 63, 4, 223–238, https://doi.org/10.2478/am-2024-0018

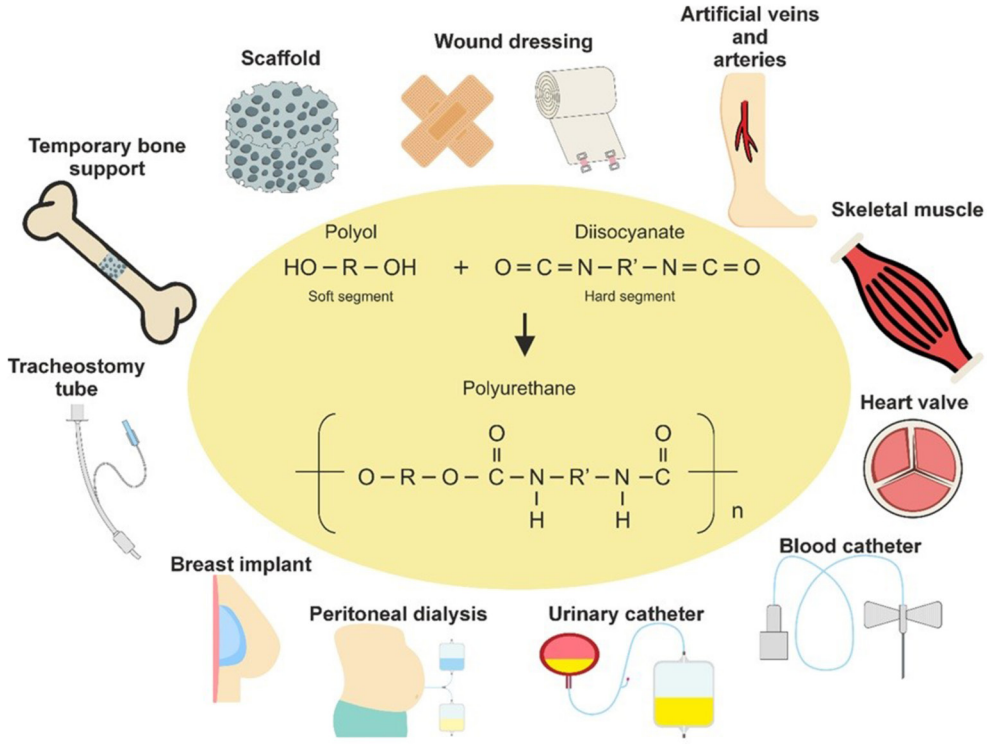


Fig. 1. Diagram of the synthesis of polyurethanes and their application in medical devices.

which enabled their use during the Second World War, mainly as adhesives and foams. Soon after the war, with the development of commercial production of flexible PU foams in the 1950s, PU industrial production began to include elastomers and coatings. During the same period, in response to the commercial success of Nylon 6.6, a condensation polymer developed by Du Pont, German teams led by Bayer investigated new methods of polymerization, which led to the creation of polyurethanes with properties similar to nylon. These new polyurethanes had lower water absorption and better mechanical and electrical stability than nylon. The evolution of PU technology continued, including developing more sustainable bio-based materials and improved production methods in an environmentally friendly direction (Ratner *et al.* 2013; Wendels and Avérous 2021).

Polyurethanes are synthesized through a complex process of urethane linkage. The process starts with a two-step synthesis strategy using three precursors: diisocyanates, diols and chain extenders, which together form the hard and soft segments of the polymer. The key process is the polyaddition reaction, in which the isocyanate NCO group reacts with the labile hydrogen of active compounds such as hydroxyls or amines to form permanent urethane bonds. These bonds a small but essential part of the chain, are formed by the reaction of isocyanates with alcohols (Fig. 1). Macroglycol, the main component, determines most of these bonds, which affect flexibility and fatigue resistance. Because the material is one large molecule, it cannot be dissolved or forced to flow by applying heat and pressure. This feature means that once a thermoset elastomer is formed, it cannot be further processed (Ratner *et al.* 2013). Therefore, PUs have simple architecture. Nowadays, commercially relevant PUs are block copolymers. The polymer molecules have alternating segments consisting of repeating „A" or „B" units. In addition, the materials are designed so that one segment – called the hard segment – is glassy or crystalline at the temperature of use, while the other segment – called the soft segment – is rubbery (Ratner *et al.* 2013).

PUs are a class of polymers that have, today, achieved industrial importance due to their tough and elastomeric properties and good fatigue resistance (Gunatilake *et al.* 2003; Rusu et al. 2020). In addition to their elastomeric properties, PUs have several beneficial interfacial characteristics. Most notably, PUs are abrasion and impact resistant, which makes them suitable for as coatings, and the materials also have good blood contact properties, making them useful in biomaterial applications (Ratner *et al.* 2013).

The main objective of this work is to provide a comprehensive overview of polyurethanes and their various applications in medicine and to outline the current challenges of biomaterial infections, especially those involving polyurethane-based devices. In addition, the paper discusses innovative surface modification techniques for polyurethanes to enhance their antimicrobial properties.

2. Characteristics and Applications of Polyurethane-Based Biomaterials

2.1. Segmented Structure of Polyurethanes

PUs stand out as one of the most versatile classes of polymer materials. Their versatility is primarily due to their segmented structure, including hard and soft segments (Rusu *et al.* 2020). The soft segments in polyurethanes are responsible for the material's elastomeric properties. Their chemical structure is based on polyols, such as polyethers and polyesters. The low glass transition temperature of the soft segments allows for flexibility and influences the material's hydrophilicity, which determines the degree of water diffusion into the polymer. As a result, the degradation rate of soft segments is related to hydrophilicity and the presence of labile groups. Polyethers are commonly used to impart flexibility and increase stability to the material. Another class of soft segments includes A-B-A tri-block polyols, which are used in producing resorbable polyurethanes due to their versatile properties (Mirhosseini *et al.* 2019; Zhang *et al.* 2019).

2.2. Mechanical Properties of Hard Segments

The hard segments in polyurethanes provide increased mechanical resistance. They consist of isocyanates and chain extenders. Their high transition temperature and pronounced crystallinity contribute to the material's mechanical strength. The content and chemistry of the hard segments have a direct impact on tensile strength and modulus. The amount of isocyanates and chain extenders determines the physical characteristics of polyurethane, and their type (aromatic or aliphatic) affects reactions with nucleophilic reagents and the material's toxicity. Aromatic isocyanates are highly reactive but can lead to side effects in carcinogenic degradation products, whereas aliphatic isocyanates have a lower toxicity potential (Somdee *et al.* 2019; Rogulska 2023).

2.3. General Characteristics

They are valued for their excellent biocompatibility, exceptional resistance to hydrolytic processes, high abrasion resistance and outstanding mechanical durability, including resistance to bending. Their diversity manifests in adhesives, coatings, sealants or foams, available in a wide range of Shore hardnesses (Shin *et al.* 2018). Thanks to such broad properties, it is possible to use PUs to create short- and long-term biomaterials.

2.4. Classification of Polyurethane Biomaterials

According to the U.S. Food and Drug Administration (FDA) and the European MDCG 2021–24, implants made of biomaterials are devices placed inside the body or on the body's surface (EC 2021; FDA 2023). A distinction can be made between long-term implants, which have a lifespan of 30 days or more in contact with a tissue before biodegradation or removal to promote healing to the tissue remodeling phase. In contrast, short-term implants have less than 30 days of lifespan in contact with living tissues. Finally, non-implantable devices are materials designed to be placed on the body's surface (EC 2021; FDA 2023). The properties mentioned above render polyurethanes ideal materials for various medical device components (Fig. 1).

2.4.1. Non-Implantation Applications of Polyurethanes

Polyurethanes are ideal materials for a wide range of medical devices due to their versatility. In non-implantation applications, they are valued for their excellent mechanical and chemical properties. For instance, polyurethane-based materials are used in wound dressings and membranes (Khodabakhshi *et al.* 2019; Yeoh *et al.* 2020; Li *et al.* 2022). In peritoneal dialysis catheters, polyurethanes provide flexibility and biocompatibility, enhancing patient comfort (Crabtree 2023; Peng *et al.* 2023). Due to their chemical resistance and durability, polyurethanes are also used to manufacture catheters and urine collection bags (Wang and Wang 2012).

2.4.2. Applications in Long-Term Implants

Polyurethanes are also utilized in intravenous line (IV) tubing due to their flexibility and kink resistance, ensuring continuous fluid delivery (Wang and Wang 2012, Tokhadzé *et al.* 2021). However, Tokhadzé *et al.* (2021) found that polyurethane catheters can cause significant drug loss, particularly with diazepam and insulin, which is clinically relevant for long-term infusions (Tokhadzé *et al.* 2021). Polyurethanes are also employed in artificial veins and arteries (Jia *et al.* 2020; Wendels and Avérous 2021; Zhang *et al.* 2021), aligning with blood flow dynamics and reducing thrombosis risk (Kim *et al.* 2016). Additionally, they aid in nerve regeneration, promoting the recovery of peripheral and sciatic nerves (Niu and Galluzzi 2020; Toichi Nasab *et al.* 2022; Zhang *et al.* 2023). In artificial heart valves, polyurethanes provide the necessary flexibility and strength to mimic natural valve function (Santerre *et al.* 2005; Shin *et al.* 2018; Kazerouni *et al.* 2021). Their biostability and low incidence of postoperative complications make them suitable for outer shells in breast implants, enhancing aesthetic results and patient satisfaction (Navas-Gómez and Valero 2020; Catanuto *et al.* 2023).

2.4.3. Applications in Short-Term Implants

Polyurethane tracheostomy tubes increase patient comfort and reduce tissue irritation due to their softness and flexibility (Björling 2009; Guo *et al.* 2020). In long-term implants, the durability and biocompatibility

of polyurethanes are crucial. They are used to create scaffolds supporting the regeneration of various tissues, including cartilage (Wendels and Avérous 2021) and muscle (Ergene *et al.* 2019; Jo *et al.* 2020; Wendels and Avérous 2021). Polyurethanes support healing by acting as bone stabilizers or adhesives in situations that require temporary reinforcement of bone structures (Oliveira *et al.* 2016; Rode *et al.* 2020; Li *et al.* 2023). Li *et al.* (2023) developed an injectable polyurethane adhesive with catechol groups and disulfide bonds that shows strong wet adhesion and biodegradability, making it ideal for complex bone injuries (Li *et al.* 2023).

These examples highlight polyurethanes' versatility and essential role in medical technology, from external devices to long-term implants, significantly contributing to improved patient outcomes and quality of life.

2.5. Trade Names of Polyurethane-Based Biomaterials

Medical-grade PU-based polymers can be found under several trade names, such as Carbothane™, Pellethane® or Tecoflex™ from Lubrizol (USA) and Carbosil® and Bionate® from DSM (Netherlands) (Wendels and Avérous 2021).

3. Instances of infections linked to the utilization of biomaterials

Advances in medical technology have made the use of various PU medical devices an integral part of patient care, significantly improving survival rates and quality of life. However, it should be explicitly stated that parent polyurethanes have no intrinsic antimicrobial activity, which poses additional challenges in preventing infections associated with their use. The colonization of medical devices by microorganisms can lead to severe infections, such as ventilator-associated pneumonia (Pen *et al.* 2020), infective endocarditis (Selton-Suty *et al.* 2012; Cahill *et al.* 2016), or vascular prosthesis infections (Gouveia e Melo *et al.* 2021). Understanding the microbiology of these infections is key to developing effective prevention and treatment strategies.

The available literature data is general and does not apply exclusively to polyurethane medical devices. This is because hospitals and clinical teams often do not consider the diversity of biomaterials used in medical devices, which significantly hinders detailed analyses of their impact on infection risk.

3.1. Infections involving biomaterials in the respiratory system

The insertion of respiratory biomaterials, such as tracheostomy tubes, is often essential in treating patients requiring long-term respiratory support. The tracheostomy procedure is an alternative to prolonged endotracheal intubation, offering better control of the patient's airway and reducing the risk of nosocomial infections. Nevertheless, using such devices may be associated with complications, such as granuloma formation, which can lead to bleeding, airway obstruction or scab formation. In addition, tracheostomy tubes may provide a site for colonization by microorganisms, promoting infection development (Cheung and Napolitano 2014).

These include different bacterial species such as *Klebsiella pneumoniae*, *Pseudomonas. aeruginosa*, *Staphylococcus aureus*, and *Acinetobacter baumannii* (Restrepo *et al.* 2013; Huang *et al.* 2018; Ścibik *et al.* 2020; Thirumurthi *et al.* 2021; Raveendra *et al.* 2022) and *Streptococcus pneumoniae* (Thirumurthi *et al.* 2021). Bacterial colonization on tracheostomy tubes can further lead to ventilator-associated pneumonia (VAP) (Restrepo *et al.* 2013; Ferro *et al.* 2021), which can be caused by a single pathogen or have a polymicrobial origin (Ścibik *et al.* 2020).

A study conducted in twenty-seven intensive care units across nine European countries reported a VAP rate of 18.3 cases per 1,000 days of mechanical ventilation. The predominant pathogens were *S. aureus*, *P. aeruginosa*, and *Acinetobacter* spp. (Koulenti *et al.* 2016). Additionally, it was shown that patients with SARS-CoV-2 infection had a higher risk of developing VAP (50.5%) compared to patients without viral infections (25.3%) (Rouzé *et al.* 2021). On the other hand, a meta-analysis that included 8,282 cases from twenty provinces in China revealed that the cumulative incidence of VAP in mainland China was 23.8% between 2006 and 2014 (Ding *et al.* 2017).

In addition to colonizing the tracheostomy tube, the natural microbiome colonizing patients is also essential. It is estimated that approximately 20% of healthy individuals are chronically colonized by *S. aureus* (Pickens and Wunderink 2022). Studies indicate that between 15.2% and 22.1% of VAP cases are caused by MRSA strains (Feeney *et al.* 2018; Pasha *et al.* 2020). Furthermore, a systematic review and meta-analysis suggest that MRSA colonization status may be helpful as an indicator of the risk of developing a VAP infection, which may support decision-making about the use of empiric therapy (Butler-Laporte *et al.* 2018).

In addition, patients colonized by MRSA tend to have more prolonged mechanical ventilation and poorer clinical outcomes, highlighting the need for strict infection control and targeted therapies (Feeney *et al.* 2018)

As a result, it can lead to patient death. Scientific sources report that VAP mortality rates range from 20–30% (Restrepo *et al.* 2013), with some studies indicating that the mortality rate can be as high as 49.2% (Tamaya *et al.* 2012). In patients who have undergone heart surgery, VAP was identified as the most significant independent risk factor for in-hospital mortality.

The mortality rate in patients with VAP was 49.2%, compared to 2% among those without VAP (Tamaya *et al.* 2012).

3.2. Cardiovascular infections with the use of biomaterials

The use of medical devices, including pacemakers, defibrillators, stents, and heart valves, has increased dramatically over the past fifty years. Today, more than 1.7 million cardiovascular devices and over one million other medical devices are implanted worldwide annually (Scialla *et al.* 2021).

Infective endocarditis (IE) associated with implantable devices, such as artificial valves, is referred to as prosthetic valve endocarditis (PVE). PVE is a severe complication of heart valve replacement surgery, with a reported mortality rate ranging from 20% to 80% (Angelina *et al.* 2016). The pathophysiology of PVE varies depending on the time elapsed since the operation, allowing for a distinction between two types of PVE: early and late. Early PVE is diagnosed within the first year after the implantation of an artificial valve, while late PVE is diagnosed after this period (Ramos-Martínez *et al.* 2023).

In early PVE, the lack of endothelialization of the suture ring and adjacent tissue plays a key role. When combined with the presence of adhesion proteins such as fibrinogen and fibronectin, it facilitates the development of infection. Additionally, early PVE often results from accidental contamination during surgery or hematogenous spread occurring postoperatively within the first hours to months. In contrast, during late PVE, the heart valve structures are fully covered by endothelium, and the pathogenesis of the disease more closely resembles that of native valve endocarditis (NVE) (Galar *et al.* 2019). A large cohort study involving 1,354 cases of PVE demonstrated that early PVE is more commonly associated with nosocomial pathogens, whereas late PVE is characterized by greater microbiological diversity. The hospital mortality rate for PVE was reported to be 32.6% (Ramos-Martínez *et al.* 2023; Ivanovic *et al.* 2019). In contrast, other studies suggest that the most common microorganisms responsible for early PVE (within two months of implantation) are *S. aureus* (36%), coagulase-negative staphylococci (17%), and fungi. For PVE occurring later, the incidence of *S. aureus* and coagulase-negative staphylococci decreases to 18–20%, with a corresponding increase in infections caused by enterococci and streptococci (10–13%) (Ivanovic et al. 2019). Patients with *S. aureus*-induced PVE represent a unique subgroup characterized by an increased risk of complications and a higher mortality rate (Tan *et al.* 2015). The causative factors in late PVE are similar to those in native valve endocarditis. It is usually caused by bacteria, such as α-haemolytic streptococci and CoNS (Coagulase Negative Staphylococci), which colonize various human body surfaces (Ivanovic et al. 2019; Berisha *et al.* 2022). Patients with *S. aureus*-induced PVE represent a distinct subgroup characterized by an increased risk of complications and a higher mortality rate (Rivoisy *et al.* 2018).

Other examples of biomaterial-related infections include infections of synthetic blood vessels, which typically result from direct contamination during surgery (Hasse *et al.* 2013). These infections affect approximately 1–6% of patients, with a higher risk (around 6%) for prostheses placed in the groin region (Wilson *et al.* 2016). Treatment usually involves antibiotic therapy and surgical prosthesis removal, a particularly challenging process due to the biofilm's presence and the infection's depth (Leroy *et al.* 2012). Gram-positive bacteria, such as *S. aureus* and coagulase-negative staphylococci, and less commonly Gram-negative bacteria like *Escherichia coli*, are most frequently responsible for these infections. The biofilms produced by these bacteria significantly complicate the elimination of infections (Gharamti and Kanafan 2018). However, a recent retrospective study revealed that vascular prosthesis infections have evolved, with a notable increase in infections caused by Gram-negative bacteria exhibiting high antibiotic resistance, particularly in early infections (Gouveia e Melo *et al.* 2021).

Another group of biomaterials, some made from polyurethanes, includes implantable cardiac electronic devices (ICEDs). This group comprises implantable cardiac defibrillators (ICDs), cardiac resynchronization therapy devices (CRTDs), and permanent pacemakers (PPMs) (Armaganijan and Healey 2011). In 2010, approximately 40,000 ICEDs were implanted in the UK (Sandoe *et al.* 2014).

Studies indicate that the infection rate associated with ICEDs is relatively low, ranging from 0.5% to 2.2%. However, the mortality rate of these infections is as high as 35% (Sandoe *et al.* 2014; Sławiński *et al.* 2019). In Poland, the infection rate is reported to be 1.2% (Sławiński *et al.* 2019). More than eighteen scientific reports, including at least 100 patients each, were analyzed in recent guidelines on diagnosing, preventing, and treating infections associated with implantable cardiac electronic devices (ICEDs). The etiological agents were found to be highly recurrent. Gram-positive bacteria were the predominant group, isolated in 67.5% to 92.5% of cases, with CoNS and *S. aureus* being the most common pathogens. Gram-negative bacilli accounted for 6% to 10.6% of all isolates. Polymicrobial infections were reported in seven studies, ranging from 2% to 24.5%. Fungal infections, in contrast, were rare, with a prevalence of less than 2% (Sandoe *et al.* 2014).

Central venous catheters (CVCs) are essential in managing critically ill patients, particularly those in intensive care units (ICUs). They facilitate intravenous

access, enabling the administration of drugs and fluids, as well as the collection of blood for laboratory analysis (Gomes Resende de Souza da Silva *et al.* 2021). However, their use requires proper preparation and precise insertion techniques to minimize the risk of procedural complications. Central line-associated bloodstream infections (CLABSIs) are among the most common infections in ICUs. In developing countries, incidence rates range from 1.7 to 44.6 cases per 1,000 catheter days (Fontela *et al.* 2012). These infections are caused by various species, with CoNS (e.g., *Staphylococcus epidermidis*) being predominant, particularly in patients using long-term central catheters (Özalp Gerçeker *et al.* 2019; Akaishi *et al.* 2023). Other common pathogens include *S. aureus* (Sellamuthu *et al.* 2023), *Enterococcus faecium* and *Enterococcus faecalis* (Belloni *et al.* 2022). Gram-negative bacteria, such as *Klebsiella* spp. and *Pseudomonas* spp., are responsible for many infections, particularly in pediatric and oncology wards (Tomar *et al.* 2015). This group also includes Gram-negative bacteria, such as *Klebsiella* spp. and *Pseudomonas* spp., responsible for many infections, particularly in pediatric and oncology wards (Tomar *et al.* 2015). Furthermore, the emerging phenomenon of antimicrobial resistance among clinically significant Gram-positive and Gram-negative bacteria, particularly those in the ESKAPE group (*E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa,* and *Enterobacter* spp.), poses a significant challenge for the prevention and treatment of infections (De Oliveira et al. 2020).

3.3. Urinary Tract Infections Associated with Biomaterial Usage

Another infection associated with biomaterials is catheter-associated urinary tract infection (CAUTI). CAUTI occurs when bacteria enter the urinary tract due to using a urinary catheter, a tube inserted into the bladder through the urethra to drain urine when a patient cannot urinate independently.

Catheter-associated urinary tract infections are significant due to their high prevalence, as they are one of the most common healthcare-acquired infections, accounting for up to 40% of hospital-acquired infections (Rubi *et al.* 2022). Moreover, 15–25% of hospitalized patients use urinary catheters, and approximately 75% of UTIs developing in hospitals are associated with catheter use (CDC 2024). Some studies indicate that the costs per patient with CAUTI range from $876 when a patient requires hospitalization, additional diagnostic tests, and medications to as much as $10,197 when a patient is hospitalized in the ICU (Hollenbreak and Schilling 2018). Other data suggest that reducing the incidence of CAUTIs can lower healthcare costs by $4,501 for every 1,000 catheterized patient days (Sutherland *et al.* 2015).

The most commonly identified microorganisms in the biofilms of long-term catheterized patients include *Proteus mirabilis* (Melo *et al.* 2016; Yuan *et al.* 2021; Herout *et al.* 2023), *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *Proteus stuartii*, *Morganella morganii*, and *E. faecalis* (Lassek *et al.* 2015; Puspitasari *et al.* 2021). Less frequently, *S. aureus* has been found to be responsible for 0.5% to 2% of all CAUTIs (Walkera *et al.* 2017).

4. Biofilm formation on polyurethanes

Bacterial biofilm represents a significant challenge in each of the infections mentioned above. A biofilm is a structured community of microorganisms growing within a self-produced matrix of polymeric materials (Das *et al.* 2019). It is estimated that 40% to 80% of all bacterial and archaeal cells exist within biofilm structures (Flemming and Wuertz 2019). An example of such biofilm formation on polyurethane surfaces, derived from the author's research, is shown in Fig. 2.

The formation of a biofilm occurs in four stages: initial attachment, biofilm accumulation, maturation, and dispersion (Fig. 3).

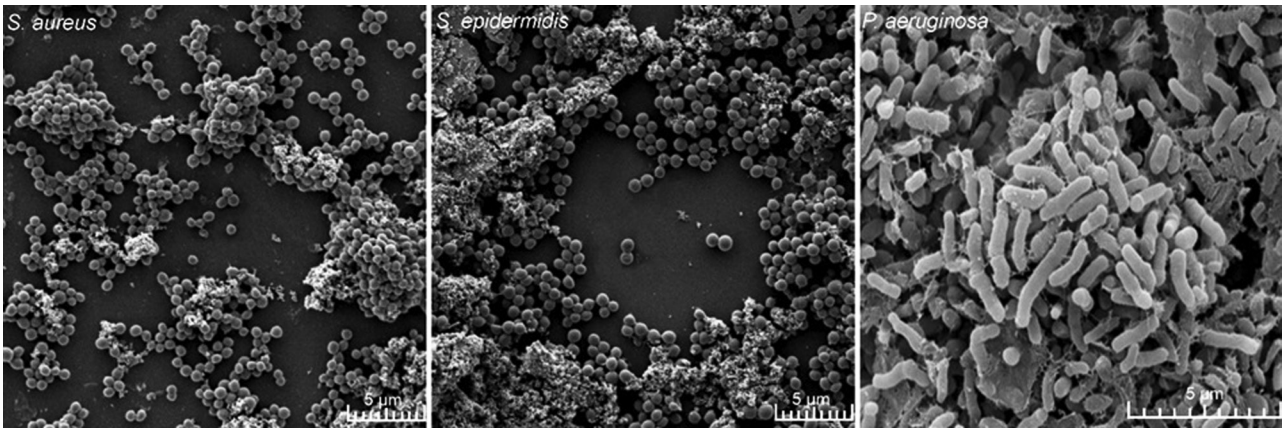


Fig. 2. Biofilm formation by *S. aureus*, *S. epidermidis* and *P. aeruginosa* on polyurethane surfaces. Images were obtained by this paper authors using a conventional scanning electron microscope (SEM) protocol at 10,000× magnification.

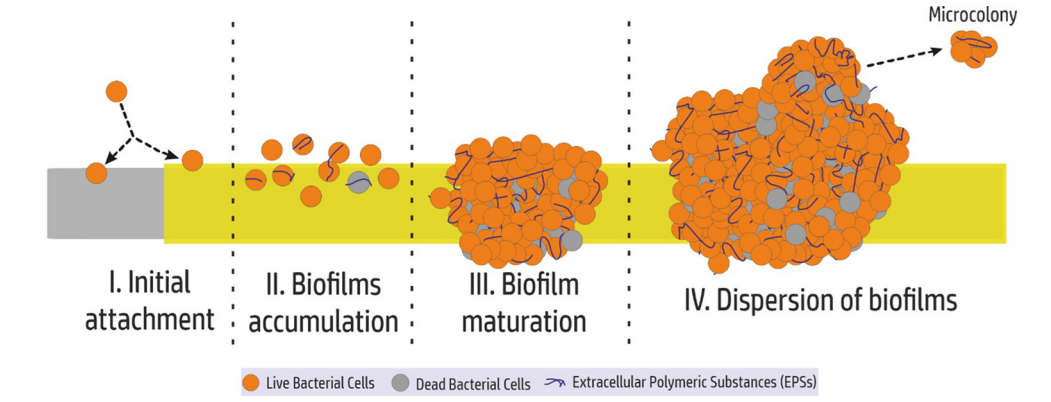


Fig. 3. Stages of bacterial biofilm formation on polyurethane medical devices: stage I – Initial attachment, stage II – Biofilm accumulation, stage III Biofilm maturation, stage IV – Dispersion of biofilms.

The first and most critical stage of biofilm formation by bacteria is the reversible adhesion of planktonic bacteria. This process occurs due to the ability of bacteria and surfaces to form covalent or ionic bonds. Additionally, weaker forces, such as polar bonds, hydrogen bonds, or Lifshitz-van der Waals interactions, can enhance or lead to strong interactions when many contact points are involved (Carniello *et al.* 2018; Joshi *et al.* 2020). Bacteria can also be modeled as smooth, inert colloidal microparticles. Many researchers utilize the Derjaguin-Landau-Verwey-Overbeek (DLVO) model to describe this process (Carniello *et al.* 2018). However, this model is a generalization due to the high variability in bacterial surface properties, which depend on species, strain, population heterogeneity, and even the cell cycle phase. Bacterial cell protrusions, such as nanofibers, pili, or fimbriae, act as adhesins, further facilitating attachment (Arciola *et al.* 2018; Vissers *et al.* 2018). The roughness of bacterial surfaces, caused by protrusions of varying lengths and widths, complicates the classical approach to adhesion, as the concept of distance between surfaces becomes ambiguous in these cases (Carniello *et al.* 2018).

In the second stage, bacterial adhesion transitions from a reversible to a more permanent state through physicochemical mechanisms that do not yet involve changes in gene expression. This process occurs on a timescale of minutes and depends on surface characteristics (e.g., hydrophobicity, charge) and environmental conditions (Carniello *et al.* 2018). The contact time between bacteria and the surface influences the binding strength amplification. This process is described by models incorporating changes in adhesion forces, browning motion, and temporal desorption (Fang *et al.* 2014; Carniello *et al.* 2018).

Mechanisms of bond strengthening and the transition to irreversible adhesion include:

Removal of interfacial water: This allows closer contact between bacteria and the surface, enabling the formation of acid-base interactions (Olsson *et al.* 2010).

Nanoscopic deformation of the cell wall: Deformation under adhesion forces increases the interactions between the bacterium and the surface, thereby strengthening adhesion (Chen *et al.* 2014).

Multi-molecule binding: Over time, additional adhesion structures (e.g., pili) become involved in binding to the surface, reducing the likelihood of simultaneous detachment (Sjollema *et al.* 2017).

Subsequently, extracellular polymeric substances (EPS) production begins, marking the transition to a strictly biological process. Studies indicate that pili-mediated adhesions in *P. aeruginosa* stimulate gene expression changes, initiating EPS production within 1–2 hours after contact with the surface (Crouzet *et al.* 2017). In *S. aureus*, EPS production reinforces binding in response to environmental conditions that weaken adhesion forces. Notably, in 3–24 hours of biofilms, the production of eDNA and poly-N-acetylglucosamine (PNAG) and the expression of their associated genes decreased as adhesion forces strengthened (Harapanahalli *et al.* 2015).

The third stage is biofilm maturation, during which bacteria proliferate to form a heterogeneous three-dimensional structure. Within this structure, zones of variable cell growth and gene expression emerge. At this stage, a high density of bacterial cells is established, enabling communication through quorum sensing. This process facilitates biofilm growth and supports a coordinated response to environmental changes, such as fluctuations in nutrient availability and stress conditions (Mukherjee *et al.* 2017).

The final stage of biofilm development is dispersion, during which individual bacterial cells or microcolonies are released, potentially producing new biofilms (Arciola *et al.* 2018). A combination of biological mechanisms and environmental factors regulates biofilm dispersion. Key processes include the activity of enzymes that degrade the extracellular polymeric matrix (EPS) and quorum sensing systems that synchronize the release of bacteria from the biofilm (Le *et al.* 2019).

Additionally, changes in nutrient and oxygen availability can trigger dispersal, enabling bacteria to adapt to fluctuating environmental conditions (Wang *et al.* 2020). This process is further associated with activating motility-related genes, facilitating the colonization of new surfaces following release from the biofilm (Rumbaugh and Sauer 2020).

Biofilms on biomaterials present a serious challenge in medicine and engineering, causing infection-related complications, device durability, and treatment efficacy. Antibiotic resistance, recognized as one of the most significant global health threats, is increasingly associated with biofilms, which exhibit adaptive resistance depending on their growth stage. As a community of microorganisms embedded in an EPS, biofilms enable bacteria to survive environmental stresses, enhancing their resistance to antibiotics and host defense mechanisms (Dostert *et al.* 2021). Biofilms are multiresistant to antibiotics and play a critical role in chronic infections, accounting for over 65% of all human infections (Dostert *et al.* 2021). The National Institutes of Health (NIH) also estimates that approximately 80% of surgical site infections in the U.S. are linked to biofilm formation (Hrynyshyn *et al.* 2022). No specific countermeasures targeting biofilms have been developed (Dostert *et al.* 2021). Furthermore, the lack of reliable and standardized clinical methods for detecting biofilms makes diagnosis and treatment challenging (Xu *et al.* 2020).

When biofilms develop on medical devices made of various biomaterials, long-term therapy is often required, and in many cases, implant removal becomes necessary (Li *et al.* 2023).

The economic burden of treating biofilm-related infections is substantial. For example, treating *P. aeruginosa* biofilm infections in hospital settings costs between $20,000 and $80,000 per patient (Gupta and Ayan 2017). Additionally, biofilm-associated infections on medical devices, such as joint prostheses and implants, cost $1 billion annually in the U.S. (Rogers and Hudson 2013).

Given these challenges, there is an urgent need to develop alternatives to traditional antibiotics that can directly inhibit or eliminate biofilms.

5. Surface Modification Techniques for Polyurethanes to Enhance Antimicrobial Properties

Modern materials engineering and biomedicine increasingly utilize advanced surface modification techniques to meet the demands placed on contemporary biomaterials. It is essential to tailor the mechanical properties and optimize polymeric materials' chemical and topographical characteristics to enhance their functionality in various medical and technological applications. Developing innovative modification methods opens up new opportunities in designing materials with highly specific properties. This progress can drive advancements in tissue engineering, implant manufacturing, and drug delivery technologies (Fig. 3).

5.1. Laser Surface Texturing

One technique for processing polyurethanes is laser surface texturing (LST). This method modifies the surfaces of polymer biomaterials using a laser beam. LST offers numerous advantages, such as altering surface roughness and chemistry in a single step without using toxic substances. Laser surface texturing can also alter polymer surfaces on the macro-, micro, and nanoscale with high spatial and temporal resolution (Lippert 2004) (Fig. 4a).

Laser processing technology enables the creation of submicrometer structures on polyurethane surfaces, first documented for components around 250 nm in size. Studies have shown that periodic line-like patterns with spatial periods of up to 500 nm can be achieved using this technique. The depth of these structures ranges from 0.88 to 1.25 μm for periods larger than 2.0 μm. It reaches up to 270 nm for periods between 500 nm and 1.0 μm, demonstrating precise control over the parameters of the textures obtained (Estevam-Alves *et al.* 2016).

In comparison, the average size of most bacteria falls within the 1–2 μm range. As a result, surfaces with submicron topographies have been found to exhibit antimicrobial properties (Siddiquie *et al.* 2020). These properties are particularly relevant in the context of biomaterial-associated infections (BAI), where bacterial adhesion to the biomaterial surface is a critical first step in biofilm formation (Arciola *et al.* 2018). Submicron surface structures reduce bacterial adhesion by inducing stress on bacterial cell walls when the spacing between protrusions is less than 1.5 μm and by trapping air to decrease the apparent solid surface area available for bacterial attachment. While this does not equate to bacteriostatic or bactericidal activity, limiting bacterial adhesion is an important indicator of antimicrobial efficacy in preventing biofilm formation and subsequent infections (Siddiquie *et al.* 2020).

5.2. Nanoparticle Grafting

Nanoparticle grafting involves attaching nanoparticles to the surface of a polymer, often through covalent bonds, to enhance surface properties. This process allows for the modification of both the physical and chemical properties of the polymer and the nanoparticles, thereby expanding their application potential across various technological fields. Grafting nanoparticles onto polymers is typically achieved using chemical methods such as 'grafting to' and 'grafting from.'

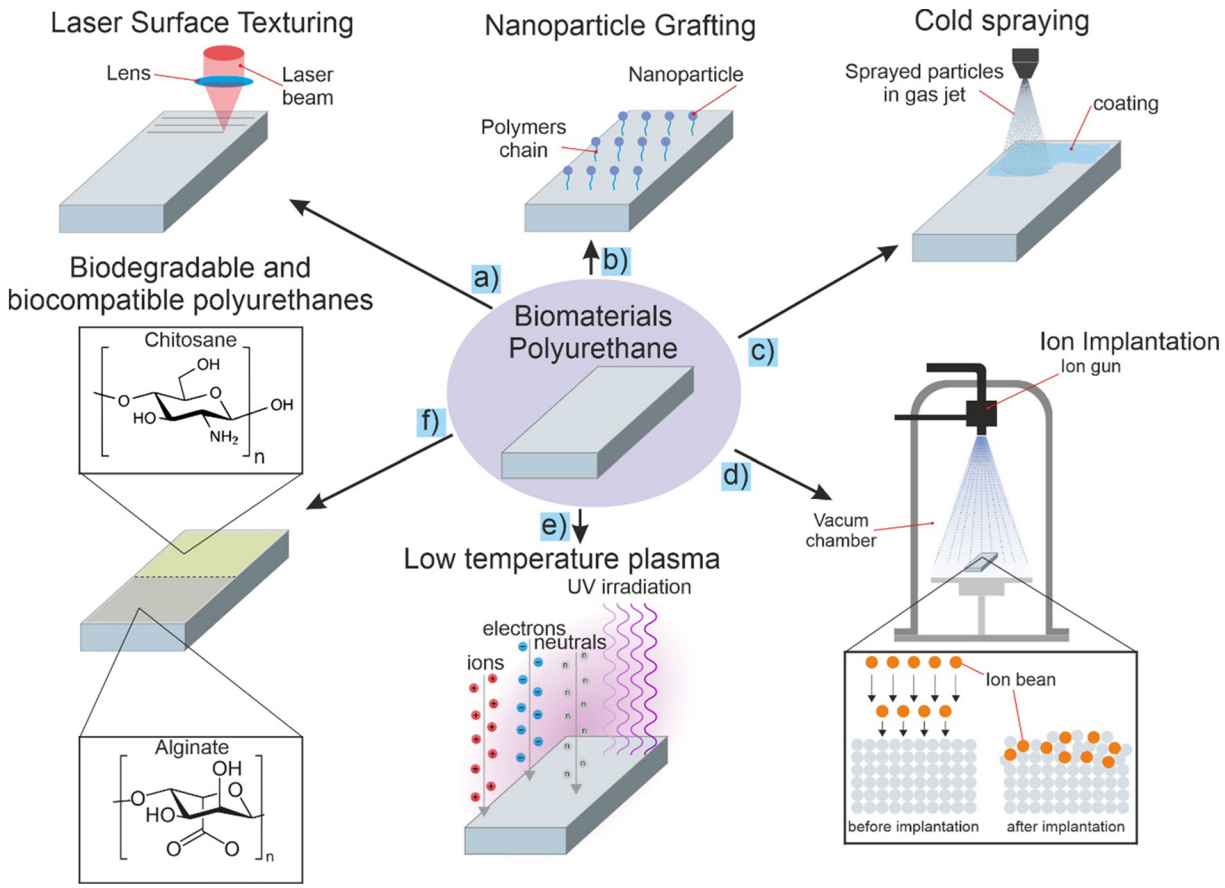


Fig. 4. Methods applied for the surface treatment of polyurethane polymers: a) Laser Surface Texturing, b) Nanoparticle Grafting, c) Cold spraying metallization, d) Ion Implantation, e) Low-temperature plasma, f) Biodegradable and biocompatible polyurethanes.

This technique is gaining popularity due to its ability to improve nanoparticles' dispersion, chemical reactivity, and stability within polymer matrices, making them more effective in advanced material applications (Kango *et al.* 2013) (Fig. 4b).

Various chemical techniques, including silane coupling agents, atom transfer radical polymerization (ATRP), and reversible addition-fragmentation chain transfer (RAFT) polymerization, are employed to graft polymers onto nanoparticles. These techniques allow fine-tuning of the surface properties of nanoparticles and their compatibility with polymer matrices, making them more versatile and practical in a wide range of applications (Kango *et al.* 2013).

One example of nanoparticle grafting is the creation of polyurethane nanofibers with incorporated ZnAg composite nanoparticles for use in antibacterial wound dressings. The grafting process involved synthesizing ZnAg composite nanoparticles (silver and zinc oxide) and incorporating them into polyurethane nanofibers (PUZnAg) through electrospinning. The resulting PUZnAg nanofibers demonstrated excellent antibacterial properties. Specifically, the $PUZnAg_2$ sample, containing 8% ZnAg by weight, exhibited 100% antibacterial efficacy against *E. coli*, *S. aureus*, and *Bacillus subtilis*, completely inhibiting bacterial growth. Furthermore, the nanofibers effectively prevented bacterial growth in the medium for up to 72 hours (Jatoi *et al.* 2020).

A similar technique was used for the single-phase synthesis of silver (Ag) nanoparticles embedded in polyurethane (PU) nanofibers. These PU/Ag nanofibers showed antibacterial efficacy against *E. coli* and *S. aureus*, forming inhibition zones of 11.4 mm and 10.8 mm in diameter, respectively. Additionally, the PU/Ag nanofibers were shown to be biocompatible, promoting fibroblast proliferation making them promising materials for medical applications, such as wound dressings (Pant *et al.* 2019).

Another potential application of polyurethanes is in flexible nanocomposite foams based on biocompatible thermoplastic polyurethane (TPU) and ZnO nanoparticles, which have potential uses as wound dressings. The TPU/ZnO foams were produced using the thermally induced phase separation (TIPS) method. This resulted in a highly porous structure with pore sizes ranging from 10 to 60 µm, allowing water vapor transport up to 8.9 mg/cm^2-h. The TPU/ZnO foams exhibited strong antibacterial activity against Gram-positive bacteria, such as *E. faecalis* and *S. aureus*, and Gram-negative bacteria, such as *E. coli* and *P. aeruginosa*. The highest

reduction in bacterial numbers, by up to 10^3 Colony Forming Units (CFU), was observed in foams containing 10% ZnO (Bužarovskaa *et al.* 2019).

5.3. Ion Implantation

Ion implantation is a process in which ions of a specific element are accelerated and introduced into the surface of a polymer, leading to the formation of metallic nanoparticles within the polymer matrix. This technique allows for the modification of both the chemical and physical properties of the surface without altering the bulk material's properties. Ion implantation enables the creation of advanced metal-polymer nanocomposites with enhanced mechanical properties, such as increased strength, wear resistance, and improved electrical conductivity. This method is widely applied in medical implants, biomaterials, and electronics (Popok *et al*. 2014). Ion implantation also provides precise control over the depth and distribution of nanoparticles within the polymer, influencing properties such as surface conductivity and roughness. This level of control is crucial for applications like surface plasmon resonance, which plays a key role in photonics and sensor technology (Salvadori *et al*. 2014) (Fig. 4d). PUs are being modified to enhance their antimicrobial properties, which are essential for reducing the risk of infections associated with medical implants.

Recent studies have demonstrated significant microbiological effects of modifying polyurethane (PU) surfaces using nitrogen ion implantation (N_2^+). This treatment substantially reduced the viability of *S. epidermidis* colonies, decreasing their survival by 3 to 5 times compared to unmodified PU. Additionally, a significant reduction in the total number of bacteria adhering to the surface was observed. These effects remained stable over time, with antibacterial properties persisting for up to 11 months post-treatment. The reduction in bacterial adhesion and viability was closely associated with structural changes in the surface, including increased roughness, the development of an undulating morphology, and enhanced surface hydrophilicity (Morozov *et al*. 2019).

The plasma ion implantation (PIII) method also shows great promise in improving the antimicrobial properties of polymeric biomaterials, such as polyurethane (PU) and polyethylene terephthalate (PET). This technique significantly reduces bacterial adhesion on modified materials, crucial for preventing infections related to medical implants. For instance, PIII-modified PET surfaces treated with acetylene (C_2H_2) exhibited a marked decrease in the adhesion of bacteria, including *S. aureus*, *S. epidermidis*, *E. coli*, and *P. aeruginosa*. Structural changes induced by the modification, such as increased roughness and enhanced hydrophilicity, were key factors contributing to the reduction in bacterial growth. These findings highlight the potential of the PIII method as an effective tool for creating antimicrobial biomaterials that reduce infection risks and enhance the safety of medical implants (Huang *et al*. 2004).

5.4. Cold spraying

Cold spraying, also known as cold dynamic gas spray (CGDS) technology, is an advanced method of applying coatings and depositing powder materials to various surfaces. The process involves accelerating powder particles to supersonic speeds (300–1200 m/s) using a gas jet (e.g., nitrogen, helium or air) passed through a special nozzle. After hitting the substrate, the particles experience intense plastic deformation phenomena, which enables effective adhesion without melting the material (Dai *et al*. 2024).

Cold spraying is emerging as a promising technique for biomedical applications, particularly for improving the properties of PUs. The process involves the deposition of various materials, including metals, ceramics, and composites, onto the surface of polymers without the risk of thermal degradation, which is essential for medical materials. One of the key application areas for this technique is to improve biomaterials' biocompatibility and antimicrobial properties, which is particularly important in medical devices such as prostheses and internal fixation systems. With cold spraying coatings, these biomaterials can achieve improved cell adhesion, reduced cytotoxicity, and improved mechanical properties, making them ideal for implant applications (Vilardell *et al*. 2015; Dosta *et al*. 2018) (Fig. 4c).

Another example of using cold spray metallization techniques is the production of copper coatings on carbon steel. Silva *et al.* (2019) demonstrated that these coatings, characterized by a dense microstructure and low porosity, exhibit strong antibacterial properties against *S. aureus*, achieving complete bacterial mortality (100%) within 10 minutes of direct contact under controlled experimental conditions. The antibacterial mechanism involves the release of copper ions, which damage bacterial cell membranes and disrupt protein structures. The high copper content on the surface is essential for antibacterial efficacy. Notably, the coating produced by this method is nearly oxide-free, ensuring direct contact between the copper and bacteria, which enhances its antimicrobial performance. These findings suggest that copper cold spraying coatings could be effective antibacterial surfaces, with potential applications in hospital equipment and touch surfaces in public spaces (Silva *et al.* 2019).

5.5. Polyurethane-biopolymer composites for antimicrobial and biofilm-preventive applications

The integration of natural biopolymers into polyurethane (PU) matrices is a promising approach to enhance their antimicrobial properties and prevent

biofilm formation. Modifying PUs with components like chitosan and alginate during synthesis makes it possible to create materials with improved biocompatibility, biodegradability, and antimicrobial properties (Uscátegui *et al*. 2019) (Fig. 4f). Composites are materials composed of two or more components with different physical, chemical or mechanical properties that remain separate but work together to produce unique properties (Biermann 2019)

Chitosan is a natural polysaccharide derived from crustaceans, making it a dedicated and renewable resource. Studies have shown that it is biocompatible, biodegradable, bioadhesive, non-toxic, and possesses antimicrobial properties (Confederat *et al*. 2021). Due to these attributes, chitosan is widely used in applications such as wound dressings, surgical sutures, and scaffolds in tissue engineering, among others (Wu *et al*. 2018). Similarly, alginate, a polysaccharide derived from brown algae, is valued for its biocompatibility and capacity to support tissue regeneration. Alginate-modified PUs form hydrogels that can trap bacteria, limiting their mobility and reducing biofilm growth. These composites have demonstrated antimicrobial activity by acting as physical barriers and delivering antimicrobial agents directly to the infection site (Zafar *et al*. 2022).

Building on this foundation, the research team led by Villani *et al*. developed a polyurethane composite incorporating chitosan (Chit) as a functional filler within a thermoplastic polyurethane (TPU) matrix. With its well-documented antimicrobial properties, chitosan was integrated into the TPU matrix to enhance biological performance. The resulting TPU-Chit composite exhibited significant antimicrobial activity, particularly against *S. aureus*. This antimicrobial effect was primarily attributed to the anti-adhesion properties of chitosan, which reduced bacterial attachment to the composite surface and inhibited biofilm formation. The effectiveness of the composite was selective, with *S. aureus* growth reduced by 20% to over 50%, depending on bacterial concentration, while no significant effect was observed against *E. coli*. Additionally, scanning electron microscopy (SEM) confirmed structural damage to *S. aureus* bacteria adhering to the composite surface, including cell wall deformation, indicating a reduced capacity for growth and adhesion (Villani *et al*. 2020).

The research conducted by Khan *et al*. focused on the development of polyurethane (PUR) membranes modified with sodium alginate (SAg) to enhance their antimicrobial properties. By incorporating sodium alginate as a filler, the team explored its impact on the structural and functional characteristics of the membranes. Antimicrobial testing of the PUR-alginate membranes was performed using Gram-negative (*E. coli*) and Gram-positive (*Bacillus cereus*) bacteria. The study revealed that the membranes effectively inhibited bacterial growth, with *B. cereus* demonstrating a higher sensitivity to the alginate content. Membranes with increased alginate concentrations produced larger bacterial growth inhibition zones, indicating a clear link between alginate levels and antimicrobial efficacy. In the case of *E. coli*, a gradual reduction in bacterial proliferation was observed, likely due to the enhanced hydrophilicity of the alginate-modified membranes. Khan *et al.* proposed that the antimicrobial mechanism arises from the interaction of alginate's carboxyl groups (-COOH) with bacterial cells. The dissociation of protons from these groups lowers the local pH, compromising bacterial cell walls. Additionally, carboxylate ions can bind to positively charged components of bacterial cells, disrupting essential cellular processes. Overall, incorporating alginate into polyurethane membranes improved their hydrophilicity and significantly enhanced their antimicrobial performance, particularly against *B. cereus* (Khan *et al*. 2021).

In summary, incorporating natural biopolymers like chitosan and alginate into polyurethane matrices enhances their antimicrobial efficacy by disrupting bacterial adhesion and biofilm formation. These modifications address critical challenges in biomaterials engineering, particularly for medical devices prone to infection.

5.6. Plasma modification

Plasma, often referred to as the fourth state of matter, is a powerful tool for surface modification of polymers, including polyurethanes. Plasma treatment involves the exposure of polyurethane surfaces to a gaseous environment containing ions, radicals, and photons (visible and near-UV), leading to physical and chemical surface modifications (Friedrich *et al*. 2012; Thakur and Vasudevan 2021). The concept of polymer functionalization is based on exposing the modified surface to plasma, which results in the attachment of atoms or fragments of dissociated plasma gas as functional groups through H-substitution in the polymer chain. Since many different fragments and atoms are present in plasma, a wide range of functional groups can be produced (Friedrich *et al*. 2012; Thakur and Vasudevan 2021) (Fig. 4e).

The interaction of plasma with polymer materials can be divided into three stages:

Surface cleaning: In this stage, organic impurities are mainly removed.

Chemical modification: In this stage, C-C and C=C bonds are broken, and fragments of dissociated plasma gas are incorporated as functional groups through H-substitution in the polymer chain.

Surface etching and nanotopography creation: For longer exposure times, plasma etches the surface and creates nanotopography.

However, a significant challenge lies in introducing plasma energy into polyurethanes. While this energy is necessary to sustain the plasma state, it also poses a risk of degrading the polymer material. Excessive energy delivery can break C-H and C-C chemical bonds, which are crucial for the structural stability of polymers (Friedrich *et al*. 2012; Thakur and Vasudevan 2021).

In biomaterials engineering, there are various surface treatment methods in which plasma plays a key role. Low-temperature plasma is one of the most effective techniques for surface modification of biomaterials, such as polyurethanes. Due to its unique properties and wide range of applications, this method allows selective surface modification in inert (e.g., argon) or reactive (e.g., oxygen) atmospheres, providing precise control over the chemical changes introduced. For polyurethanes, the modification must be tailored to the specific material, considering chemical composition, crystallinity, and thickness factors. It is essential to optimize parameters like the type of feed gas (oxidizing, reducing, inert), gas partial pressure, plasma generator power, and exposure time. Thanks to its flexibility, low-temperature plasma is particularly effective in enhancing the surface properties of polyurethanes without altering their bulk characteristics (Alves *et al.* 2011; Cvrček *et al*. 2019; Drożdż *et al*. 2024).

6. Summary

Polyurethanes (PUs) have emerged as highly versatile materials in modern medicine, offering unparalleled opportunities for innovation in medical devices ranging from wound dressings to artificial heart valves. Their exceptional mechanical properties, adaptability, and biocompatibility make them indispensable in diverse medical applications. However, parent PUs lack intrinsic antimicrobial properties, posing significant challenges in preventing biofilm formation and associated infections.

Innovative surface modification techniques, such as laser texturing, nanoparticle deposition, ion implantation, and plasma treatments, show great promise in addressing these challenges. These methods enhance the antimicrobial properties of PUs and improve their biocompatibility, paving the way for safer and more effective medical devices. Furthermore, integrating natural polymers and biodegradable components into PU matrices offers an additional avenue for enhancing their functionality while aligning with sustainability goals.

Looking forward, further research should focus on translating these laboratory advancements into clinical settings, optimizing the long-term performance of PU-based biomaterials, and exploring their applications in regenerative medicine and personalized therapies. Overcoming these challenges will require multidisciplinary collaboration, combining materials science, microbiology, and clinical expertise to unlock the full potential of polyurethanes in healthcare.

By advancing the design and functionality of polyurethane biomaterials, the scientific community can contribute to groundbreaking innovations that will enhance patient outcomes and inspire new directions in biomaterials science. This review underscores the need for continued focus on addressing infection risks while maximizing the unique advantages of polyurethanes in modern medicine.

ORCID
Kamil Drożdż https://orcid.org/0000-0003-2508-7934
Monika Brzychczy-Włoch https://orcid.org/0000-0002-7415-0154
Monika Gołda-Cępa https://orcid.org/0000-0001-9054-4682

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents

References:

1. Akaishi T., Tokuda K., Katsumi M. et al.: Blood Culture Result Profile in Patients With Central Line-Associated Bloodstream Infection (CLABSI): A Single-Center Experience. *Cureus* **15**(6), e40202 (2023)
2. Alves P., Pinto S., de Sousa H.C., Gil M.H.: Surface modification of a thermoplastic polyurethane by low-pressure plasma treatment to improve hydrophilicity. *J. Appl. Polym. Sci*. **122**, 2302–2308 (2011)
3. Angelina J.T., Ganesan S., Panicker T.M.R., Narayani R., Korath M.P., Jagadeesan K.: Pulsed laser deposition of silver nanoparticles on prosthetic heart valve material to prevent bacterial infection. *Mater. Technol*. **32**(3), 148–155 (2017)
4. Arciola C.R., Campoccia D., Montanaro L.: Implant infections: adhesion, biofilm formation and immune evasion. *Nat Rev Microbiol* **16**(7), 397–409 (2018)
5. Armaganijan L., Healey J.S.: Pacemakers and Implantable Cardioverter Defibrillators in the Elderly. In: Katlic M. (ed) *Cardiothoracic Surgery in the Elderly*. *Springer*, *New York* (2011)
6. Belloni S., Caruso R., Cattani D. et al.: Occurrence rate and risk factors for long-term central line-associated bloodstream infections in patients with cancer: A systematic review. *Worldviews Evid. Based Nurs*. **19**(2), 100–111 (2022)
7. Bergmann, C.P., Stumpf, A.: Biomaterials. In: Dental Ceramics. Topics in Mining, Metallurgy and Materials Engineering. *Springer, Berlin, Heidelberg* (2013)
8. Berisha B., Ragnarsson S., Olaison L., Rasmussen M.: Microbiological etiology in prosthetic valve endocarditis: A nationwide registry study. *J. Intern. Med*. **292**(3), 428–437 (2022)
9. Biermann D.: Composite materials. In: CIRP Encyclopedia of Production Engineering. *Springer Berlin Heidelberg, Berlin, Heidelberg*, 1–5 (2018)
10. Björling G., Axelsson S., Johansson U.-B., Lysdahl M., Markström A., Schedin U., Karlsson S.: Clinical use and material wear of polymeric tracheostomy tubes. *Laryngoscope* **117**(9), 1552–1559 (2007)
11. Butler-Laporte G., De L'Étoile-Morel S., Cheng M.P., McDonald E.G., Lee T.C.: MRSA colonization status as a predic-

tor of clinical infection: A systematic review and meta-analysis. *J. Infect*. **77**(6), 489–495 (2018)
12. Bužarovska A., Dinescu S., Lazar A.D., Serban M., Pircalabioru G.G., Costache M., Gualandi C., Avérous L.: Nanocomposite foams based on flexible biobased thermoplastic polyurethane and ZnO nanoparticles as potential wound dressing materials. *Mater. Sci. Eng. C.* **104**, 109893 (2019)
13. Cahill T.J., Baddour L.M., Habib G., Hoen B., Salaun E., Pettersson G.B., Schäfers H.J., Prendergast B.D.: Challenges in infective endocarditis. *J. Am. Coll. Cardiol.* **69**(3), 325–344 (2017)
14. Carniello V., Peterson B.W., van der Mei H.C., Busscher H.J.: Physico-chemistry from initial bacterial adhesion to surface-programmed biofilm growth. *Adv Colloid Interface Sci* **261**, 1–14 (2018)
15. Catanuto G., Virzì D., Latino M., Musmeci N., Fichera D., Balafa K., Cannata I., Rocco N., Marino M., Castiglione G.: One-stage implant-based breast reconstruction with polyurethane-coated device: standardized assessment of outcomes. *Aesthet. Surg. J.* **44**(5), 491–498 (2024)
16. Centers for Disease Control and Prevention: Catheter-associated Urinary Tract Infection Basics, (15.04.2024), https://www.cdc.gov/uti/about/cauti-basics.html (27.11.2024)
17. Chen Y., Harapanahalli A.K., Busscher H.J., Norde W., van der Mei H.C.: Nanoscale cell wall deformation impacts long-range bacterial adhesion forces on surfaces. *Appl. Environ. Microbiol.* **80**(2), 637–643 (2014)
18. Cheung N.H., Napolitano L.M.: Tracheostomy: epidemiology, indications, timing, technique, and outcomes. *Respir. Care* **59**(6), 895–915 (2014)
19. Confederat L.G., Tuchilus C.G., Dragan M., Sha'at M., Dragostin O.M.: Preparation and antimicrobial activity of chitosan and its derivatives: a concise review. *Molecules*, **26**, 3694 (2021)
20. Crabtree J.H.: Clinical biodurability of aliphatic polyether-based polyurethanes as peritoneal dialysis catheters. *ASAIO J*. **49**(3), 290–294 (2003)
21. Crouzet M., Claverol S., Lomenech A.M., Le Sénéchal C., Costaglioli P., Barthe C., Garbay B., Bonneu M., Vilain S.: *Pseudomonas aeruginosa* cells attached to a surface display a typical proteome early as 20 minutes of incubation. *PLoS ONE* **12**(7):e0180341 (2017)
22. Cvrček L., Horáková M.: Plasma modified polymeric materials for implant applications (in) Non-Thermal Plasma Technology for Polymeric Materials, Ed. Thomas S., Mozetič M., Cvelbar U., Špatenka P., Praveen K.M., *Elsevier*, pp. 367–407 (2019)
23. Dai S., Cui M., Li J., Zhang M.: Cold Spray Technology and Its Application in the Manufacturing of Metal Matrix Composite Materials with Carbon-Based Reinforcements. *Coatings* **14**(7), 882 (2024)
24. Das S., Singh S., Matchado M.S., Srivastava A., Bajpai A.: Biofilms in Human Health. In: Kumar S., Chandra N., Singh L., Hashmi M., Varma A. (eds) Biofilms in Human Diseases: Treatment and Control. *Springer, Cham*, p. 27–42 (2019)
25. De Oliveira D.M.P., Forde B.M., Kidd T.J., Harris P.N.A., Schembri M.A., Beatson S.A., Paterson D.L., Walker M.J.: Antimicrobial resistance in ESKAPE pathogens. *Clin. Microbiol. Rev*. **33**(3), (2020)
26. Ding C., Zhang Y., Yang Z., Wang J., Jin A., Wang W., Chen R., Zhan S.: Incidence, temporal trend and factors associated with ventilator-associated pneumonia in mainland China: a systematic review and meta-analysis. *BMC Infect. Dis*. **17**, 468 (2017)
27. Dosta S., Cinca N., Vilardell A.M., Cano I.G., Guilemany J.M.: Cold spray coatings for biomedical applications (in) Cold-Spray Coatings, Ed. Cavaliere P., *Springer, Cham*, pp. 533–557 (2017)
28. Dostert M, Trimble M.J., Hancock R.E.W.: Antibiofilm peptides: overcoming biofilm-related treatment failure. *RSC Adv*. **11**, 2718–2728 (2021)
29. Drożdż K., Gołda-Cępa M., Chytrosz-Wróbel P., Kotarba A., Brzychczy-Włoch M.: Improving biocompatibility of polyurethanes applied in medicine using oxygen plasma and its negative effect on increased bacterial adhesion. *Int. J. Biomater.*, Article ID 5102603 (2024)
30. Ergene E., Yagci B.S., Gokyer S., Eyidogan A., Aksoy E.A., Huri P.Y.: A novel polyurethane-based biodegradable elastomer as a promising material for skeletal muscle tissue engineering. *Biomed. Mater*. **14**(2), 025014 (2019)
31. Estevam-Alves R., Günther D., Dani S., Eckhardt S., Roch T., Mendonca C.R., Cestari I.N., Lasagni A.F.: UV Direct Laser Interference Patterning of polyurethane substrates as tool for tuning its surface wettability. *Appl. Surf. Sci*. **374**, 222–228 (2016)
32. European Commission, Medical Device Coordination Group: Guidance on classification of medical devices. MDCG 2021-24, październik 2021, https://health.ec.europa.eu/system/files/2021-10/mdcg_2021-24_en_0.pdf (27.11.2024)
33. Fang B., Jiang Y., Rotello V.M., Nüsslein K., Santore M.M.: Easy come easy go: surfaces containing immobilized nanoparticles or isolated polycation chains facilitate removal of captured *Staphylococcus aureus* by retarding bacterial bond maturation. *ACS Nano*, **8**(2), 1180–1190 (2014).
34. Feeney M., Lindsey D., Vazquez D., Porter K., Murphy C.: Identifying risk factors for MRSA pneumonia in SICU patients with ventilator-associated pneumonia. *Crit. Care Med.* **46**(1), 330 (2018)
35. Ferro A., Kotecha S., Auzinger G., Yeung E., Fan K.: Systematic review and meta-analysis of tracheostomy outcomes in COVID-19 patients. *Br. J. Oral Maxillofac. Surg*. **59**(9), 1013–1023 (2021)
36. Flemming H-C., Wuertz S.: Bacteria and archaea on Earth and their abundance in biofilms. *Nat Rev Microbiol* **17**(4), 247–260 (2019)
37. Fontela P.S., Platt R.W., Rocher I., Frenette C., Moore D., Fortin É., Buckeridge D., Pai M., Quach C.: Epidemiology of central line-associated bloodstream infections in Quebec intensive care units: A 6-year review. *Am. J. Infect. Control* **40**(3), 221–226 (2012)
38. Friedrich J.: Interaction between plasma and polymers (in) The Plasma Chemistry of Polymer Surfaces: Advanced Techniques for Surface Design, Wiley-VCH, pp. 11–33 (2012)
39. Galar A., Weil A.A., Dudzinski D.M., Muñoz P., Siedner M.J.: Methicillin-resistant *Staphylococcus aureus* prosthetic valve endocarditis: Pathophysiology, epidemiology, clinical presentation, diagnosis, and management. *Clin. Microbiol. Rev*. **32**(2) (2019)
40. Gharamti A., Kanafani Z.A.: Vascular graft infections: An update. *Infect. Dis. Clin. North Am*. **32**(4), 789–809 (2018)
41. Gomes Resende de Souza da Silva A., Bisinoto Alves S., Eurípedes Resende Guimarães E., Rodrigues Braga J., Carneiro Cunha Neves H., de Lima Vieira dos Santos S., Carmo Moreira M.A.: Central line-associated bloodstream infection trend in Brazilian adult intensive care units: an ecological study. *J. Infect. Dev. Ctries* **15**(11), 1744–1749 (2021)
42. Gouveia e Melo R., Martins B., Mendes Pedro D., Mimoso Santos C., Duarte A., Fernandes e Fernandes R., Garrido P., Mendes Pedro L.: Microbial evolution of vascular graft infections in a tertiary hospital based on positive graft cultures. *J. Vasc. Surg*. **74**(1), 276–284.e4 (2021)
43. Gunatillake P.A., Martin D.J., Meijs G.F., McCarthy S.J., Adhikari R.: Designing biostable polyurethane elastomers for biomedical implants. *Aust. J. Chem*. **56**(6), 545–557 (2003)
44. Guo Y., Zhang H., Duan S., Ding X., Hu Y., Ding X., Xu F.-J.: Bulk modification of thermoplastic polyurethanes for self-sterilization of trachea intubation. *Macromol. Biosci*. **21**(2), 2000318 (2021)

45. Gupta T.T., Ayan H.: Non-Thermal Plasma In Conjunction With Chlorhexidine (Chx) Digluconate Sterilize The Biofilm Contaminated Titanium Surface. 2017 IEEE *Int. Conf. Plasma Sci.* (ICOPS), 1–1 (2017)
46. Harapanahalli A.K., Chen Y., Li J., Busscher H.J., van der Mei H.C.: Influence of adhesion force on icaA and cidA gene expression and production of matrix components in *Staphylococcus aureus* biofilms. *Appl. Environ. Microbiol*. **81**(10), 3414–3421 (2015)
47. Hasse B., Husmann L., Zinkernagel A., Weber R., Lachat M., Mayer D.: Vascular graft infections. *Swiss Med. Wkly*. **143**, w13754 (2013)
48. Herout R., Khoddami S., Moskalev I., Reicherz A., Chew B.H., Armbruster C.E., Lange D.: Role of bacterial surface components in the pathogenicity of *Proteus mirabilis* in a murine model of catheter-associated urinary tract infection. *Pathogens* **12**(4), 509 (2023)
49. Hollenbeak C.S., Schilling A.L.: The attributable cost of catheter-associated urinary tract infections in the United States: A systematic review. *American Journal of Infection Control* **46**(7), 751–757 (2018).
50. Hrynyshyn A, Simões M, Borges A.: Biofilms in Surgical Site Infections: Recent Advances and Novel Prevention and Eradication Strategies. *Antibiotics* **11**, 69 (2022)
51. Huang Y., Jiao Y., Zhang J., Xu J., Cheng Q., Li Y., Liang S., Li H., Gong J., Zhu Y., et al.: Microbial etiology and prognostic factors of ventilator-associated pneumonia: A multicenter retrospective study in Shanghai. *Clin. Infect. Dis*. **67**(1), S146–S152 (2018)
52. Ivanovic B., Trifunovic D., Matic S., Petrovic J., Sacic D., Tadic M.: Prosthetic valve endocarditis – A trouble or a challenge? *J. Cardiol*. **73**(2), 126–133 (2019)
53. Jatoi A.W.: Polyurethane nanofibers incorporated with ZnAg composite nanoparticles for antibacterial wound dressing applications. *Compos. Commun*. **19**, 103–107 (2020)
54. Jia W., Li M., Weng H., Gu G., Chen Z.: Design and comprehensive assessment of a biomimetic tri-layer tubular scaffold via biodegradable polymers for vascular tissue engineering applications. *Mater. Sci. Eng.* C **110**, 110717 (2020)
55. Jo S.B., Erdenebileg U., Dashnyam K., Jin G.Z., Cha J.R., El-Fiqi A., Knowles J.C., Patel K.D., Lee H.H., Lee J.H., Kim H.W.: Nano-graphene oxide/polyurethane nanofibers: mechanically flexible and myogenic stimulating matrix for skeletal tissue engineering. *J. Tissue Eng.* **11**, (2020)
56. Joshi A.S., Singh P., Mijakovic I.: Interactions of Gold and Silver Nanoparticles with Bacterial Biofilms: Molecular Interactions behind Inhibition and Resistance. *Int J Mol Sci.* **21**(20), 7658 (2020)
57. Kango S., Kalia S., Celli A., Njuguna J., Habibi Y., Kumar R.: Surface modification of inorganic nanoparticles for development of organic-inorganic nanocomposites – A review. *Prog. Polym. Sci.* **38**(8), 1232–1261 (2013)
58. Kazerouni Z.S., Telloo M., Farazin A., Saber-Samandari S., Sheikhbahaei E., Kamyab-Moghadas B., Joneidi-Yekta H., Esmaeili S., Khandan A.: A mitral heart valve prototype using sustainable polyurethane polymer: fabricated by 3D bioprinter, tested by molecular dynamics simulation. *AUT J. Mech. Eng*. **5**(1), 109–120 (2021)
59. Khan S.U., Sultan M., Islam A., Sabir A., Hafeez S., Bibi I., Ahmed M.N., Khan S.M., Khan R.U., Iqbal M.: Sodium alginate blended membrane with polyurethane: Desalination performance and antimicrobial activity evaluation. *Int. J. Biol. Macromol*. **182**, 72–81 (2021)
60. Khodabakhshi D., Eskandarinia A., Kefayat A., Rafienia M., Navid S., Karbasi S., Moshtaghian J.: *In vitro* and *in vivo* performance of a propolis-coated polyurethane wound dressing with high porosity and antibacterial efficacy. *Colloids Surf. B Biointerfaces*, **178**, 177–184 (2019)
61. Kim G.B., Guo J., Hu J., Shan D., Yang J.: Novel applications of urethane/urea chemistry in the field of biomaterials (in) Advances in Polyurethane Biomaterials, Ed. S.L. Cooper, J. Guan, *Woodhead Publishing*, p. 115–147 (2016)
62. Koulenti D., Tsigou E., Rello J.: Nosocomial pneumonia in 27 ICUs in Europe: perspectives from the EU-VAP/CAP study. *Eur. J. Clin. Microbiol. Infect. Dis*. **36**, 1999–2006 (2017)
63. Lassek C., Burghartz M., Chaves-Moreno D., Otto A., Hentschker C., Fuchs S., Bernhardt J., Jauregui R., Neubauer R., Becher D., Pieper D.H., Jahn M., Jahn D., Riedel K.: A metaproteomics approach to elucidate host and pathogen protein expression during catheter-associated urinary tract infections (CAUTIs). *Mol. Cell. Proteomics* **14**(4), 989–1008 (2015)
64. Le KY, Villaruz AE, Zheng Y, He L, Fisher EL, Nguyen TH, Ho TV, Yeh AJ, Joo H-S, Cheung GYC, Otto M: Role of phenol-soluble modulins in *Staphylococcus epidermidis* biofilm formation and infection of indwelling medical devices. *J Mol Biol* **431**(16), 3015–3027 (2019)
65. Leroy O., Meybeck A., Sarraz-Bournet B., d'Elia P., Legout L.: Vascular graft infections. *Curr. Opin. Infect. Dis*. **25**(2), 154–158 (2012)
66. Li Q., Tang B., Liu X., Chen B., Wang X., Xiao H., Zheng Z.: Overcoming the dilemma of *in vivo* stable adhesion and sustained degradation by the molecular design of polyurethane adhesives for bone fracture repair. *Adv. Mater*. **13**(5), 2301870 (2023)
67. Li S., Zhang Y., Ma X., Qiu S., Chen J., Lu G., Jia Z., Zhu J., Yang Q., Chen J., Wei Y.: Antimicrobial lignin-based polyurethane/Ag composite foams for improving wound healing. *Biomacromolecules*, **23**(4), 1622–1632 (2022)
68. Lippert T.: Laser Application of Polymers. In: Lippert T. (eds) Polymers and Light. Advances in Polymer Science, vol 168. *Springer, Berlin, Heidelberg*, pp. 51–246 (2021)
69. Melo L.D.R., Veiga P., Cerca N., Kropinski A.M., Almeida C., Azeredo J., Sillankorva S.: Development of a phage cocktail to control *Proteus mirabilis* catheter-associated urinary tract infections. *Front. Microbiol*. **7**, 1024 (2016)
70. Mirhosseini M.M., Haddadi-Asl V., Jouibari I.S.: A simple and versatile method to tailor physicochemical properties of thermoplastic polyurethane elastomers by using novel mixed soft segments. *Mater. Res. Express*, **6**(6), 065314 (2019)
71. Morozov I.A., Kamenetskikh A.S., Beliaev A.Yu., Scherban M.G., Lemkina L.M., Eroshenko D.V., Korobov V.P.: The Effect of Damage of a Plasma-Treated Polyurethane Surface on Bacterial Adhesion. *Cell Biophys*. **64**, 410–415 (2019)
72. Mukherjee S., Moustafa D., Smith C.D., Goldberg J.B., Bassler B.L.: The RhlR quorum-sensing receptor controls *Pseudomonas aeruginosa* pathogenesis and biofilm development independently of its canonical homoserine lactone autoinducer. Lee V.T., editor. *PLOS Pathog*. **13**(7), e1006504 (2017)
73. Huang N., Yang P., Leng Y.X., Wang J., Sun H., Chen J.Y., Wan G.J.: Surface modification of biomaterials by plasma immersion ion implantation. *Surf. Coat. Technol*. **186**(1–2), 218–226 (2004)
74. Navas-Gómez K., Valero M.F.: Why polyurethanes have been used in the manufacture and design of cardiovascular devices: A systematic review. *Materials* **13**(15), 3250 (2020)
75. Niu Y., Galluzzi M.: A biodegradable block polyurethane nerve-guidance scaffold enhancing rapid vascularization and promoting reconstruction of transected sciatic nerve in Sprague-Dawley rats. *J. Mater. Chem. B* **8**, 11063–11073 (2020)
76. Oliveira G.J.P.L., Barros-Filho L.A.B., Barros L.A.B., Queiroz T.P., Marcantonio E. Jr.: *In vitro* evaluation of the primary stability of short and conventional implants. *J. Oral Implantol*. **42**(6), 458–463 (2016)
77. Olsson A.L.J., van der Mei H.C., Busscher H.J., Sharma P.K.: Novel analysis of bacterium–substratum bond maturation

measured using a quartz crystal microbalance. *Langmuir* **26**(13), 11113–11117 (2010)
78. Özalp Gerçeker G., Yardımcı F., Aydınok Y.: Central Line-Associated Bloodstream Infections in Children With Hematologic and Oncologic Diseases: First Prevalence Results From a University Hospital. *J. Pediatr. Oncol. Nurs*. **36**(5), 327–336 (2019)
79. Pant B., Park M., Park S.-J.: One-Step Synthesis of Silver Nanoparticles Embedded Polyurethane Nanofiber/Net Structured Membrane as an Effective Antibacterial Medium. *Polymers* **11**(7), 1185 (2019)
80. Pasha S.A.R., Naseer A.A., Hajira S.N., Bhat P., Yoganand R.: Microbiological profile of ventilator-associated pneumonia (VAP) in geriatric patients and their antibiotic susceptibility pattern with detection of MRSA, ESBLs, and MBLs in intensive care unit of a tertiary care centre from South India. *Int. J. Curr. Microbiol. App. Sci*. **9**(2), 551–563 (2020)
81. Pen D.-L., Yan G.-F., He L.-Y., Yan W.-L., Chen W.-M., Liu J., Ying J.-Y., Wang C.-Q., Lu G.-P.: The role of bacterial colonization of ventilator circuit in development of ventilator-associated pneumonia: a prospective observational cohort study. *Clin. Microbiol. Infect*. **27**(3), 467.e1–467.e7 (2021)
82. Peng J., Li K., Du Y., Yi F., Wu L., Liu G.: A robust mixed-charge zwitterionic polyurethane coating integrated with antibacterial and anticoagulant functions for interventional blood-contacting devices. *J. Mater. Chem. B* **11**, 8020–8032 (2023)
83. Pickens C.I., Wunderink R.G.: Methicillin-resistant *Staphylococcus aureus* hospital-acquired pneumonia/ventilator-associated pneumonia. *Semin. Respir. Crit. Care Med*. **43**(2), 304–309 (2022)
84. Popok V.N., Nuzhdin V.I., Valeev V.F., Stepanov A.L.: Copper nanoparticles synthesized in polymers by ion implantation: Surface morphology and optical properties of the nanocomposites. *J. Mater. Res*. **30**(1), 86–92 (2014)
85. Puspitasari D., Rusli E.A., Husada D., Kartina L.: *Escherichia coli* and *Klebsiella pneumonia* as the most common bacteria causing catheter-associated urinary tract infection. *Mol. Cell. Biomed. Sci*. **5**(3), 121 (2021)
86. Ramos-Martínez A. et al.: Clinical presentation, microbiology, and prognostic factors of prosthetic valve endocarditis. Lessons learned from a large prospective registry. *PLoS ONE* **18**(9), e0290998 (2023)
87. Ratner B.D., Hoffman A.S., Schoen F.J., Lemons J.E.: Biomaterials Science: An Introduction to Materials in Medicine. *Academic Press, San Diego*, (2013)
88. Raveendra N., Rathnakara S.H., Haswani N., Subramaniam V.: Bacterial biofilms on tracheostomy tubes. *Indian J. Otolaryngol. Head Neck Surg*. **74**, 4995–4999 (2022)
89. Restrepo M.I., Peterson J., Fernandez J.F., Qin Z., Fisher A.C., Nicholson S.C.: Comparison of the bacterial etiology of early-onset and late-onset ventilator-associated pneumonia in subjects enrolled in 2 large clinical studies. *Respir. Care* **58**(7), 1220–1225 (2013)
90. Rivoisy C. & Lefort A. et al.: Prosthetic valve *Candida* spp. endocarditis: New insights into long-term prognosis – The ESCAPE study. *Clin. Infect. Dis*. **66**(6), 825–832 (2018)
91. Rode C., Wyrwa R., Weisser J., Schnabelrauch M., Vučak M., Grom S., Reinauer F., Stetter A., Schlegel K.A., Lutz R.: A novel resorbable composite material containing poly(ester-co-urethane) and precipitated calcium carbonate spherulites for bone augmentation—development and preclinical pilot trials. *Molecules* **26**(1), 102 (2021)
92. Rogers A.D., Hudson D.A.: Ten principles to prevent biofilm-related infections in surgical practice. *S. Afr. Med. J*. **103**, 701 (2013)
93. Rogulska M.: The influence of diisocyanate structure on thermal stability of thermoplastic polyurethane elastomers based on diphenylmethane-derivative chain extender with sulfur atoms. *Materials*, **16**(7), 2618 (2023)
94. Rouzé A. & Nseir S. et al.: Relationship between SARS-CoV-2 infection and the incidence of ventilator-associated lower respiratory tract infections: a European multicenter cohort study. *Intensive Care Med*. **47**, 188–198 (2021)
95. Rubi H., Mudey G., Kunjalwar R.: Catheter-associated urinary tract infection (CAUTI). *Cureus* **14**(10), e30385 (2022)
96. Rumbaugh K.P., Sauer K.: Biofilm dispersion. *Nat. Rev. Microbiol*. **18**, 571–586 (2020)
97. Rusu L.-C., Ardelean L.C., Jitariu A.-A., Miu C.A., Streian C.G.: An insight into the structural diversity and clinical applicability of polyurethanes in biomedicine. *Polymers*, **12**(5), 1197 (2020)
98. Salvadori M.C., Teixeira F.S., Sgubin L.G., Cattani M., Brown I.G.: Surface modification by metal ion implantation forming metallic nanoparticles in an insulating matrix. *Appl. Surf. Sci*. **310**, 158–163 (2014)
99. Sandoe J.A.T., Barlow G., Chambers J.B., Gammage M., Guleri A., Howard P., Olson E., Perry J.D., Prendergast B.D., Spry M.J., Steeds R.P., Tayebjee M.H., Watkin R. et al.: Guidelines for the diagnosis, prevention and management of implantable cardiac electronic device infection. Report of a joint Working Party project on behalf of the British Society for Antimicrobial Chemotherapy (BSAC, host organization), British Heart Rhythm Society (BHRS), British Cardiovascular Society (BCS), British Heart Valve Society (BHVS) and British Society for Echocardiography (BSE). *J. Antimicrob. Chemother*. **70**(2), 325–359 (2015)
100. Santerre J.P., Woodhouse K., Laroche G., Labow R.S.: Understanding the biodegradation of polyurethanes: From classical implants to tissue engineering materials. *Biomaterials* **26**(35), 7457–7470 (2005)
101. Scialla S. & Raucci M.G. et al.: Trends in managing cardiac and orthopaedic device-associated infections by using therapeutic biomaterials. *Polymers* **13**(10), 1556 (2021)
102. Ścibik Ł., Ochońska D., Gołda-Cępa M., Brzychczy-Włoch M., Kotarba A.: Microbiological analysis of tracheostomy tube biofilms and antibiotic resistance profiles of potentially pathogenic microorganisms. *Otolaryngol. Pol*. **76**(5) (2022)
103. Sellamuthu R., Nair S., Chandrasekar J. et al.: Risk Factors of Central Line-Associated Bloodstream Infection (CLABSI): A Prospective Study From a Paediatric Intensive Care Unit in South India. *Cureus* **15**(8), e43349 (2023)
104. Selton-Suty C., Célard M., Le Moing V., Doco-Lecompte T., Chirouze C., Iung B., Strady C., Revest M., Vandenesch F., Bouvet A.: AEPEI Study Group: Preeminence of *Staphylococcus aureus* in infective endocarditis: A 1-year population-based survey. *Clin. Infect. Dis*. **54**(9), 1230–1239 (2012)
105. Shin E.J., Choi S.M.: Advances in Waterborne Polyurethane-Based Biomaterials for Biomedical Applications (in) Novel Biomaterials for Regenerative Medicine, Ed. H. Chun, K. Park, C.H. Kim, G. Khang, *Springer, Singapore*, p. 229–241 (2018)
106. Siddiquie R.Y., Gaddam A., Agrawal A., Dimov S.S., Joshi S.S.: Anti-Biofouling Properties of Femtosecond Laser-Induced Submicron Topographies on Elastomeric Surfaces. *Langmuir* **36**(19), (2020)
107. Silva F.S., Cinca N., Dosta S., Cano I.G., Guilemany J.M., Caires C.S.A., Lima A.R., Silva C.M., Oliveira S.L., Caires A.R.L., Benedetti A.V.: Corrosion resistance and antibacterial properties of copper coating deposited by cold gas spray. *Surf. Coat. Technol*., **361**, 292–301 (2019)
108. Sjollema J., van der Mei H.C., Hall C.L., Peterson B.W., de Vries J., Song L., de Jong E.D., Busscher H.J., Swartjes J.J.T.M.: Detachment and successive re-attachment of multiple, reversibly-binding tethers result in irreversible bacterial adhesion to surfaces. *Sci. Rep*. **7**:4369 (2017)

109. Sławiński G., Lewicka E., Kempa M., Budrejko S., Raczak G.: Infections of cardiac implantable electronic devices: Epidemiology, classification, treatment, and prognosis. *Adv. Clin. Exp. Med*. **28**(2), 263–270 (2019)
110. Somdee P., Lassú-Kuknyó T., Kónya C., Szabó T., Marossy K.: Thermal analysis of polyurethane elastomers matrix with different chain extender contents for thermal conductive application. *J. Therm. Anal. Calorim*., **138**, 1003–1010 (2019)
111. Sutherland T., Beloff J., McGrath C., Liu X., Pimentel M.T., Kachalia A., Bates D., Urman R.D.: A single-center multidisciplinary initiative to reduce catheter-associated urinary tract infection rates: Quality and financial implications. *The Health Care Manager* **34**(3), 218–224 (2015).
112. Tamayo E. & Bustamante J. et al.: Ventilator-associated pneumonia is an important risk factor for mortality after major cardiac surgery. *J. Crit. Care* **27**(1), 18–25 (2012)
113. Tan H.-L., Chai L.Y.A., Yeo T.-C., Chia B.-L., Tambyah P.A., Poh K.-K.: Predictors of in-hospital adverse events in patients with prosthetic valve infective endocarditis. *Heart Lung Circ*. **24**(7), 705–709 (2015)
114. Thakur N., Vasudevan S.N.: Plasma treatment and seed quality advancement: a review. *Agric. Rev*. **42**, 197–202 (2021)
115. Thirumurthi S., Kanagamuthu P., Srinivasan R., Dhanasekaran B.: Predominance of bacterial flora causing lower respiratory tract infections following tracheostomy in patients of Chettinad Hospital & Research Institute, Chennai, India. *J. Evol. Med. Dent. Sci.* **10**(35), 2964–2968 (2021)
116. Tofighi Nasab S., Hayati Roodbari N., Goodarzi V., Ali Khonakdar H., Mansoori K., Nourani M.R.: Novel electrospun conduit based on polyurethane/collagen enhanced by nanobioglass for peripheral nerve tissue engineering. *J. Biomater. Sci. Polym. Ed.* **33**, 801–822 (2022)
117. Tokhadzé N., Chennell P., Pereira B., Mailhot-Jensen B., Sautou V.: Critical drug loss induced by silicone and polyurethane implantable catheters in a simulated infusion setup with three model drugs. *Pharmaceutics* **13**(10), 1709 (2021)
118. Tomar S., Lodha R., Das B. et al.: Central line-associated bloodstream infections (CLABSI): Microbiology and antimicrobial resistance pattern of isolates from the Pediatric ICU of a tertiary care Indian hospital. *Clin. Epidemiol. Global Health* **3**(1), S16–S19 (2015)
119. U.S. Food and Drug Administration: Evidentiary Expectations for 510(k) Implant Devices, (07.08.2023), https://www.fda.gov/regulatory-information/search-fda-guidance-documents/evidentiary-expectations-510k-implant-devices (27.11.2024)
120. Uscátegui Y.L., Díaz L.E., Gómez-Tejedor J.A., Vallés-Lluch A., Vilariño-Feltrer G., Serrano M.A.. Valero M.F.: Candidate polyurethanes based on castor oil (Ricinus communis), with polycaprolactone diol and chitosan additions, for use in biomedical applications. *Molecules*, **24**, 237 (2019)
121. Vilardell A.M., Cinca N., Concustell A., Dosta S., Cano I.G., Guilemany J.M.: Cold spray as an emerging technology for biocompatible and antibacterial coatings: state of art. *J. Mater. Sci.,* **50**, 4441–4462 (2015)
122. Villani M., Consonni R., Canetti M., Bertoglio F., Iervese S., Bruni G., Visai L., Iannace S., Bertini F.: Polyurethane-based composites: Effects of antibacterial fillers on the physical-mechanical behavior of thermoplastic polyurethanes. *Polymers* **12**(2), 362 (2020)
123. Vissers T., Brown A.T., Koumakis N., Dawson A., Hermes M., Schwarz-Linek J., Schofield A.B., French J.M., Koutsos V., Poon W.C.K.: Bacteria as living patchy colloids: Phenotypic heterogeneity in surface adhesion. *Sci Adv.* **4**(4) (2018)
124. Walker J.N., Flores-Mireles A.L., Pinkner C.L., et al.: Catheterization alters bladder ecology to potentiate *Staphylococcus aureus* infection of the urinary tract. *Proc. Natl. Acad. Sci. U.S.A.* **114**(41), E8721–E8730 (2017)
125. Wang J., Liu Q., Dong D., Hu H., Wu B., Ren H.: In-situ monitoring of the unstable bacterial adhesion process during wastewater biofilm formation: A comprehensive study. *Environ. Int.* **140**, 105722 (2020)
126. Wang W., Wang C.: Polyurethane for biomedical applications: a review of recent developments (in) The Design and Manufacture of Medical Devices, Ed. J. Paulo Davim, *Woodhead Publishing*, p. 115–151 (2012)
127. Wendels S., Avérous L.: Biobased polyurethanes for biomedical applications. *Bioactive Materials*. **6**(4), 1083–1106 (2021)
128. Wilson W.R. & Baddour L.M. et al.: Vascular graft infections, mycotic aneurysms, and endovascular infections: A scientific statement from the American Heart Association. *Circulation* **134**(20), e412–e460 (2016)
129. Wu H., Williams G.R., Wu J., Wu J., Niu S., Li H., Wang H., Zhu L.: Regenerated chitin fibers reinforced with bacterial cellulose nanocrystals as suture biomaterials. *Carbohydr. Polym*. **180**, 304–313 (2018)
130. Xu Y, Dhaouadi Y, Stoodley P, Ren D.: Sensing the unreachable: challenges and opportunities in biofilm detection. Curr. Opin. Biotechnol. 64, 79–84 (2020)
131. Li P, Yin R, Cheng J, Lin J.: Bacterial Biofilm Formation on Biomaterials and Approaches to Its Treatment and Prevention. *Int. J. Mol. Sci.* **24**, 11680 (2023)
132. Yeoh F.H., Lee C.S., Kang Y.B., Wong S.F., Cheng S.F., Ng W.S.: Production of biodegradable palm oil-based polyurethane as potential biomaterial for biomedical applications. *Polymers*, **12**(8), 1842 (2020)
133. Yuan F., Huang Z., Yang T., Wang G., Li P., Yang B., Li J.: Pathogenesis of *Proteus mirabilis* in catheter-associated urinary tract infections. *Urol. Int*. **105**(5–6), 354–361 (2021)
134. Zafar K., Zia K.M., Alzhrani R.M., Almalki A.H., Alshehri S.: Biocompatibility and hemolytic activity studies of synthesized alginate-based polyurethanes. *Polymers*, **14**, 2091 (2022)
135. Zhang B., Xu Y., Ma S., Wang L., Liu C., Xu W., Shi J., Qiao W., Yang H.: Small-diameter polyurethane vascular graft with high strength and excellent compliance. *J. Mech. Behav. Biomed. Mater*. **121**, 104614 (2021)
136. Zhang L., Zhang C., Zhang W., Zhang H., Hou Z.: Synthesis and properties of biodegradable poly(ester-urethane)s based on poly (ε-caprolactone) and aliphatic diurethane diisocyanate for long-term implant application: effect of uniform-size hard segment content. *J. Biomater. Sci. Polym. Ed*. **30**(13), 1212–1226 (2019)
137. Zhang Q., Chen J., Feng Y., Lin J., Li J., Wang Y., Tan H.: Electroactive scaffolds of biodegradable polyurethane/polydopamine-functionalized graphene oxide regulating the inflammatory response and revitalizing the axonal growth cone for peripheral nerve regeneration. *J. Mater. Chem. B* **11**, 6308–6318 (2023)

CONTENTS