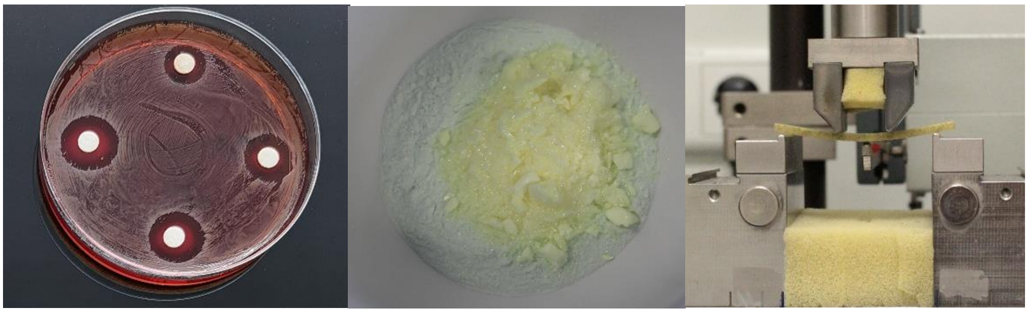
JUSTUS-LIEBIG-UNIVERSITÄT GIESSEN

Microbiological and physical properties of biofilm-active PMMA bone cements in arthroplasty



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Martina Humez

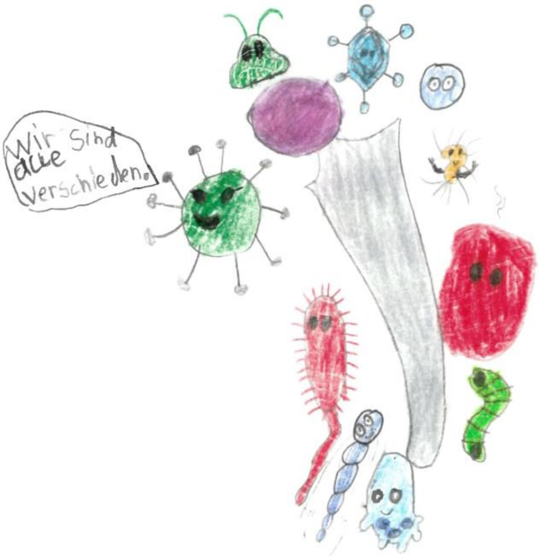
Für Marie-Florence und Maxime.

Ihr habt mich gelehrt,

dass echtes Wissen mit Staunen beginnt und niemals endet.

Diese Arbeit ist euch gewidmet –

als Erinnerung daran, niemals aufzuhören, Fragen zu stellen.



„Once the emotions have been aroused

– a sense of the beautiful, the excitement of the new and the unknown –

then we wish for knowledge."

| Rachel Carson, 1956

Contents

I. Abstract & Zusammenfassung

1. Abstract

Periprosthetic joint infection (PJI) remains one of the most serious complications in arthroplasty, associated with high morbidity, mortality, and substantial socioeconomic burden. Antibiotic-loaded bone cements (ALBCs) play a central role in both the prevention and treatment of PJI. In primary arthroplasty, they enable high local antibiotic concentrations at the implant-tissue interface, reducing early bacterial adhesion and lowering infection risk. In revision surgery, especially for established infection, ALBCs facilitate targeted antimicrobial delivery as cement spacers, while simultaneously providing mechanical stability for temporary or definitive fixation. Despite decades of clinical experience with ALBCs, fundamental questions remain incompletely understood: the selection of the most appropriate antimicrobial drugs for admixing to acrylic bone cements, pharmacokinetic carrier elution aspects, cement matrix interactions, antibiotic admixing methodology, and antibiotic dose limitations. Furthermore, it is not clear to which extent laboratory experiences can be transferred to the clinical setting. These knowledge gaps are particularly relevant in the context of rising antimicrobial resistances, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE), for which therapeutic options are increasingly restricted.

This cumulative dissertation integrates material science, microbiology, and translational in-vivo modelling to advance the evidence-based use of ALBCs in arthroplasty and revision surgery. Across four studies, the work systematically examines (i) the feasibility of formulating novel daptomycin-loaded poly-methyl-methacrylate (PMMA) cements for PJIs caused by vancomycin-resistant bacteria, (ii) the mechanical, chemical, and kinetic consequences of manual antibiotic admixing, (iii) the impact of the cement matrix on antibiotic release and long-term antimicrobial activity, and (iv) the translational antimicrobial efficacy of commercial single antibiotic-loaded bone cements (SALBCs) and dual-antibiotic loaded bone cements (DALBCs) in a validated *Galleria mellonella* implant infection model. A fifth investigation, presented at the EBJIS (European Bone & Joint Infection Society) congress and published as abstract, proposes an optimal daptomycin dosage and suitable PMMA cement matrix for spacer applications using the *Galleria mellonella* biofilm model.

The results demonstrate that acrylic bone cements are not interchangeable materials: the polymer composition and additives, hydrophilicity, viscosity, sterilisation method and manufacturing process collectively determine the antibiotic elution, antimicrobial efficacy and mechanical stability. DALBCs consistently outperformed SALBCs in infection prevention, achieving superior antibiofilm effects, and providing markedly increased survival in larvae infected with multi-drug-resistant *Staphylococcus aureus* and *Enterococcus faecalis*. High local antibiotic concentrations generated by antibiotic elution from acrylic bone cements were able to overcome resistance phenotypes, emphasising the unique pharmacodynamic environment of ALBC.

Systematic evaluation of manual admixing revealed that dry mixing of the powder in cartridge mixing devices did neither improve mechanical stability nor antibiotic release. It generated abrasive plastic debris capable of embedding into the cement highlighting previously unrecognised risks. Fractionated bowl mixing was identified as the only safe and reproducible preparation method when manual admixing is unavoidable. Cement brand was found to be a dominant predictor of antibiotic release, with the polymer composition in Palacos® cements consistently outperforming the one in Simplex® cements across all antibiotics tested.

Finally, integrating in-vitro release kinetics with the *Galleria mellonella* biofilm model enabled the identification of 1.5 g daptomycin per 40 g PMMA (Palacos® R+G and Simplex® T) as the

optimal risk-benefit balance between antimicrobial efficacy and mechanical integrity for cement spacers in VRE caused infections.

In summary, this thesis provides a comprehensive evidence base for the rational selection, mixing, and clinical application of ALBCs. It clarifies the material dependent performance and establishes best-practice standards for manual admixing, validates an efficient in-vivo screening platform for cement performance, and supports the development of future daptomycin-loaded DALBC formulations capable of addressing the growing challenge of multi-drug-resistant PJI. These results are of high clinical relevance for surgeons who deal with these difficult-to-treat orthopaedic infections.

2. Zusammenfassung

Die periprothetische Gelenkinfektion zählt zu den schwerwiegendsten Komplikationen in der Endoprothetik und ist mit einer hohen Morbidität, Mortalität, sowie einer erheblichen sozioökonomischen Belastung verbunden. Antibiotikabeladene Knochenzemente (ALBC) spielen sowohl bei der Prävention als auch bei der Behandlung von Gelenkinfektionen eine zentrale Rolle. In der Primärendoprothetik ermöglichen sie hohe lokale Wirkspiegel an der Schnittstelle zwischen Implantat und Gewebe, wodurch eine frühe bakterielle Besiedlung des Implantates reduziert und das Infektionsrisiko gesenkt wird. In der Revisionschirurgie, insbesondere bei etablierten Infektionen, ermöglichen ALBCs als Zementspacer eine gezielte Abgabe von Antibiotika und bieten gleichzeitig mechanische Stabilität für eine temporäre oder definitive Fixation. Trotz jahrzehntelanger klinischer Erfahrung mit antibiotikabeladenen Knochenzementen sind grundlegende Fragen noch nicht vollständig geklärt: die Auswahl der am besten geeigneten Antibiotika für die Beimischung zu Knochenzementen auf Acrylbasis, pharmakokinetische Aspekte der Elution, Wechselwirkungen mit der Zementmatrix, Methoden zur Beimischung von Antibiotika und Dosierungsbeschränkungen für Antibiotika. Darüber hinaus ist unklar, inwieweit Laborergebnisse auf die klinische Praxis übertragen werden können. Diese Unklarheiten sind besonders relevant im Zusammenhang mit zunehmenden Antibiotikaresistenzen, darunter Methicillin-resistente *Staphylococcus aureus* (MRSA) und Vancomycin-resistente Enterokokken (VRE), für die die Behandlungsmöglichkeiten zunehmend eingeschränkt sind.

Diese kumulative Dissertation integriert Materialwissenschaft, Mikrobiologie und in-vivo Biofilmmodelle, um den evidenzbasierten Einsatz von ALBCs in der Endoprothetik und Revisionschirurgie weiterzuentwickeln. In vier Studien untersucht die Arbeit systematisch (i) die Machbarkeit der Formulierung neuartiger mit Daptomycin beladener Knochenzemente zur Behandlung von Infektionen durch Vancomycin-resistente Erreger, (ii) die mechanischen, chemischen und pharmakokinetischen Auswirkungen der manuellen Beimischung von Antibiotika, (iii) den Einfluss der Zementmatrix auf die Antibiotikafreisetzung und die langfristige antimikrobielle Aktivität und (iv) die Effektivität kommerziell verfügbarer ein- und zweifach antibiotikabeladener Knochenzemente im validierten *Galleria mellonella* Biofilmmodell. Eine fünfte Untersuchung, die auf dem Kongress der EBJIS (European Bone & Joint Infection Society) vorgestellt wurde und als Abstract verfügbar ist, schlägt eine optimale Daptomycin Zumischung, sowie die geeignet Zementmatrix zur Herstellung von Spacern unter Verwendung des *Galleria mellonella* Biofilmmodells vor.

Die Ergebnisse zeigen, dass Knochenzemente keine austauschbaren Materialien sind: Die Polymerzusammensetzung und Additive, Hydrophilie, Viskosität, Sterilisationsmethode und Herstellungsverfahren bestimmen gemeinsam die Antibiotikafreisetzung, antimikrobielle Wirksamkeit und mechanische Stabilität. Zweifach antibiotikabeladene Zemente übertrafen einfach beladene Zemente durchweg bei der Infektionsprävention, erzielten überlegene Effekte gegen Biofilm und führten zu deutlich höheren Überlebensraten in den *Galleria* Larven, welche mit multiresistentem *Staphylococcus aureus* und *Enterococcus faecalis* infiziert waren. Die hohen lokalen Wirkspiegel, die durch die Antibiotikafreisetzung aus Knochenzement erreicht werden, konnten Resistenzphänotypen überwinden und verdeutlichen die besondere Wirkweise lokaler Antibiotikaträger.

Die systematische Bewertung der manuellen Antibiotikazumischung zeigt, dass das Trockenmischen des Pulvers in einer Kartusche weder die mechanische Stabilität noch die Antibiotikafreisetzung verbesserte. Zudem entstanden abrasive Kunststoffpartikel, die im Zementteig verbleiben können und ein bisher unterschätztes Risiko darstellen. Das fraktionierte Beimischen in einer Schale wurde als einzige sichere und reproduzierbare Methode identifiziert, wenn eine manuelle Antibiotikazugabe unvermeidlich ist. Darüber hinaus erwies sich die Wahl der Zementmarke als entscheidender Faktor für die Antibiotikafreisetzung, wobei Palacos®-Zemente mit ihrer Polymerzusammensetzung bei allen getesteten Antibiotika durchweg besser abschnitten als Simplex®-Zemente.

Schließlich ermöglichte die Kombination aus in-vitro Freisetzungsdaten und dem *Galleria mellonella* Biofilmmodell die Identifizierung von 1,5 g Daptomycin pro 40 g Knochenzementpulver (Palacos® R+G und Simplex® T) als optimaler Kompromiss zwischen antimikrobieller Wirksamkeit und mechanischer Stabilität für die Herstellung von Spacern bei Infektionen mit Vancomycin-resistenten Enterokokken.

Zusammenfassend liefert diese Dissertation eine umfassende wissenschaftliche Grundlage für die rationale Auswahl, Zumischung und klinische Anwendung von antibiotikabeladenen Knochenzementen. Sie verdeutlicht die materialabhängigen Determinanten der Antibiotikafreisetzung, etabliert evidenzbasierte Standards für die manuelle Zumischung, validiert ein effizientes in-vivo Screening Modell zur Bewertung unterschiedlicher Antibiotikakombinationen im Knochenzement und unterstützt die Entwicklung zukünftiger Daptomycin-beladener Formulierungen zur Bewältigung der wachsenden Herausforderung periprothetischer Infektionen, die von multi-resistenten Erregern verursacht werden. Diese Ergebnisse sind von hoher klinischer Relevanz für Chirurgen, die sich mit diesen schwer zu behandelnden orthopädischen Infektionen befassen.

3. Glossary

Expression	Definition
ALBC	Antibiotic-Loaded Bone Cement
AJRR	American Joint Replacement Registry
AOANJRR	Australian Orthopaedic Association National Joint Replacement Registry
ASA	American Society of Anaesthesiologists
BPO	Benzoyl Peroxide
CDP-1	Crystalline Degradation Product-1
CFU	Colony Forming Units
CoNS	Coagulase Negative Staphylococci
CRP	C-Reactive Protein
DAIR	Debridement, Antibiotics and Implant Retention
DALBC	Dual Antibiotic-Loaded Bone Cement
DIN	Deutsche Industrie Norm
DmpT	N,N-Dimethyl-p-Toluidine
EBJIS	European Bone & Joint Infection Society
ECOFF	Epidemiological Cut-off Value
EO	Ethylene Oxide
EPRD	Endoprothesenregister Deutschland
ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp*.
EPS	Extracellular Polymeric Substance
EQ-5D	EuroQol-5 Dimensions
EUCAST	European Annual Antimicrobial Susceptibility Testing
FDA	U.S. Food and Drug Administration
FNOF	Fractured Neck of Femur
GISA	Glycopeptide-Intermediate-Resistant *Staphylococcus aureus*
GRE	Glycopeptide-Resistant Enterococci
HA	Hemiarthroplasty
HPLC	High-Performance Liquid Chromatography
IDSA	Infectious Diseases Society of America
IL	Interleukin
ISO	International Organization for Standardisation
IUPAC	International Union of Pure and Applied Chemistry
IV	Intravenously Administered Antibiotics
IZT	Inhibition Zone Testing
LROI	Landeliijke Registratie Orthopedische Interventies
NJR	National Joint Registry
MBEC	Minimum Biofilm Eradication Concentration

MBIC	Minimum Biofilm Inhibitory Concentration
MDR	Multi-Drug-Resistant Bacteria
MIC	Minimum Inhibitory Concentration
MMA	Methyl-Methacrylate
MSSA	Methicillin-Sensitive *Staphylococcus aureus*
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MRSE	Methicillin-Resistant *Staphylococcus epidermidis*
MS	Mass Spectrometry
MSIS	Musculoskeletal Infection Society
THA	Total Hip Arthroplasty
TKA	Total Knee Arthroplasty
TNF	Tumour Necrosis Factor
Tris-HCl	Tris-Hydrochloride
OD	Optical Density
OECD	Organisation for Economic Co-operation and Development
OR	Operating Room
PBS	Phosphate-Buffered Saline
PG	Phosphatidylglycerol
PIF	Pro-Implant Foundation
PJI	Periprosthetic Joint Infection
PMMA	Poly-Methyl-Methacrylate
PO	Perioperative Administered Antibiotics
PRISS	Prosthesis Related Infections Shall be Stopped
RANKL	Receptor Activator of Nuclear Factor B Ligand
RCT	Randomized Controlled Trial
RNA	Ribonucleic Acid
SALBC	Single Antibiotic-Loaded Bone Cement
SAR	Swedish Arthroplasty Register
TSB	Tryptic Soy Broth
UK	United Kingdom
U.S.	United States
VISA	Vancomycin-Intermediate-Resistant *Staphylococcus aureus*
VRE	Vancomycin-Resistant Enterococci
WHO	World Health Organisation

II. Introduction

1. Background and clinical relevance

Osteoarthritis is the leading disease for which procedures of arthroplasty are performed, particularly affecting the knee, hip, ankle, and finger joints in patients over 60 years of age (Hunter & Bierma-Zeinstra, 2019). Fractured neck of femur (FNOF) constitutes the second most frequent cause of arthroplasty, especially in patients aged 80 years and above (Rupp et al., 2021b). Joint replacement surgery aims to reduce pain, improve mobility and quality of life. This intervention has become increasingly common, as demonstrated by data from the Organisation for Economic Co-operation and Development (OECD): the median incidence of hip arthroplasty across 35 OECD countries is 174 implants per 100,000 inhabitants, while the median for knee arthroplasty across 33 countries is 137 implants per 100,000 inhabitants (OECD, 2021).

Acrylic-based bone cement, commonly referred to as poly-methyl-methacrylate cement (PMMA), is used to fixate implants in these procedures. Such interventions are classified as cemented total hip arthroplasty (THA), cemented hemiarthroplasty (HA) in FNOF cases, and total knee arthroplasty (TKA). In Germany, cemented TKA predominates, with 98.6% of primary TKAs being either fully cemented or hybrid when cement is only partially applied for usually the tibial implant (Humez et al., 2024). Internationally, the Netherlands, Sweden, and the United Kingdom (UK) report comparable cementation rates exceeding 90% for TKA. However, a slight trend toward cementless techniques has emerged in recent years, particularly in Australia and the United States (U.S.). For THA, cementation rates vary considerably between countries, with Nordic nations, such as Sweden, reporting proportions of approximately 60%, compared to less than 25% in Germany (Humez et al., 2024).

1.1. Infection as cause for revision surgery

Given the high frequency of hip and knee replacements, minimizing revision risks is a primary objective, as the growing number of primary procedures inevitably increases the absolute number of revisions. Revision surgeries are associated with substantial hospital and insurance costs, increased patient morbidity and mortality, and psychological stress for surgeons and patients. Among revision causes, periprosthetic joint infections (PJI) remains one of the most severe complications following arthroplasty. Despite advances in surgical techniques and infection prevention strategies, PJI persists in approximately 0.5%–2.3% of primary THA and TKA cases (Aftab et al., 2025; Patel, 2023). The absolute number of PJI cases continues to rise globally, driven by increasing arthroplasty volumes and a growing proportion of patients with compromised health status. Notably, the prevalence of patients with ASA (American Society of Anaesthesiologists) scores ≥3 is increasing, suggesting a further rise in PJI incidence in the future.

Registry data confirm this trend, as infection has become one of the most frequent reasons for revision in THA and TKA (**Figure 1**). The German Arthroplasty Register (EPRD) and the UK National Joint Registry (NJR) report aseptic loosening followed by infection as the leading revision causes (EPRD, 2025; NJR, 2025). In contrast, the Australian (AOANJRR), Dutch (LROI), Swedish (SAR), and U.S. (AJRR) registries identify infection as the most common reason for revision, followed by loosening (AAOS, 2025; Lewis et al., 2025; LROI, 2025; SAR, 2024). Recent analyses suggest these figures may even underestimate the true incidence of infection as the LROI captures only one-third of PJI cases in THA and TKA (van Veghel et al., 2024).

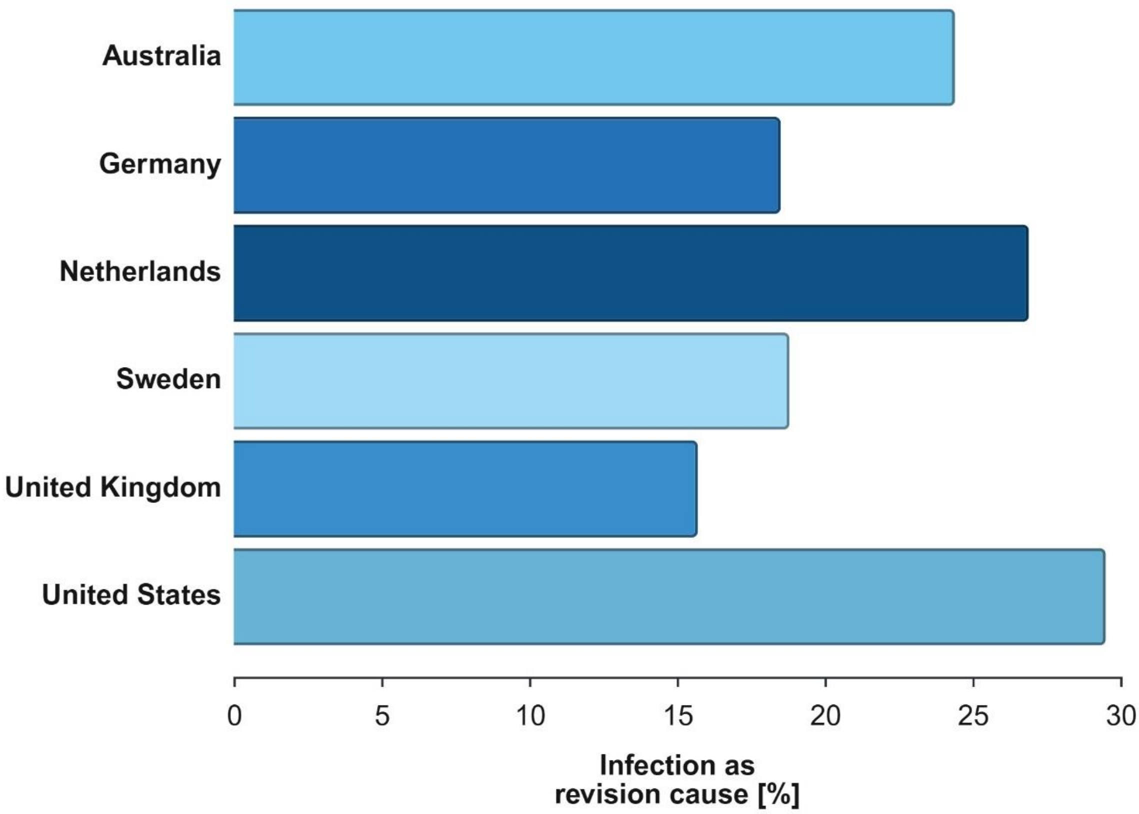


Figure 1 | Proportion of infection as leading revision cause in primary THA based on international arthroplasty register reports from Australia - AOANJRR (Lewis et al., 2025), Germany - EPRD (EPRD, 2025), the Netherlands - LROI (LROI, 2025), Sweden - SAR (SAR, 2024), United Kingdom - NJR (NJR, 2025) and the United States - AJRR (AAOS, 2025)).

The clinical impact of PJI is profound, with morbidity and mortality rates reaching 24–26% for THA, alongside impaired joint function and diminished quality of life (Natsuhara et al., 2019). Patients with PJI often face prolonged hospitalization, multiple reoperations, and psychological distress. Health-related quality of life scores (EQ-5D) are consistently lower in this population (Aftab et al., 2025; Walter et al., 2021; Xu et al., 2023). Mortality following PJI is significantly higher compared to aseptic revision arthroplasty, with a fourfold increase in 90-day mortality, double the five-year mortality, and a 1.5-fold increase in 10-year all-cause mortality (**Figure 2**) (Lum et al., 2018; Xu et al., 2023; Zmistowski et al., 2013). This renders PJI more lethal than the most common cancers, such as breast cancer in women (~10% 5-year mortality) and prostate cancer in men (~15% 5-year mortality) (Coleman et al., 2025; Reinhard et al., 2024).

From a surgical perspective, PJI management is highly complex. On basis of the important differentiation between acute and chronic infection cases, the surgical strategies go from implant maintaining to one- or two-stage prosthesis exchange philosophies. DAIR (debridement, antibiotics, and implant retention) aims to preserve the implant while reducing bacterial load through radical debridement combined with systemic and local antibiotics. This approach is generally reserved for selected patients with early-detected infections and immature biofilms (Sigmund et al., 2025). One-stage revision seeks to minimise patient burden by performing a single surgery in which the infected implant is removed and replaced, enabling shorter hospitalization and faster mobilization. However, careful patient selection is essential to mitigate the re-revision risk that comes with an increased perioperative mortality; particularly in cases of polymicrobial infection (Resl et al., 2025).

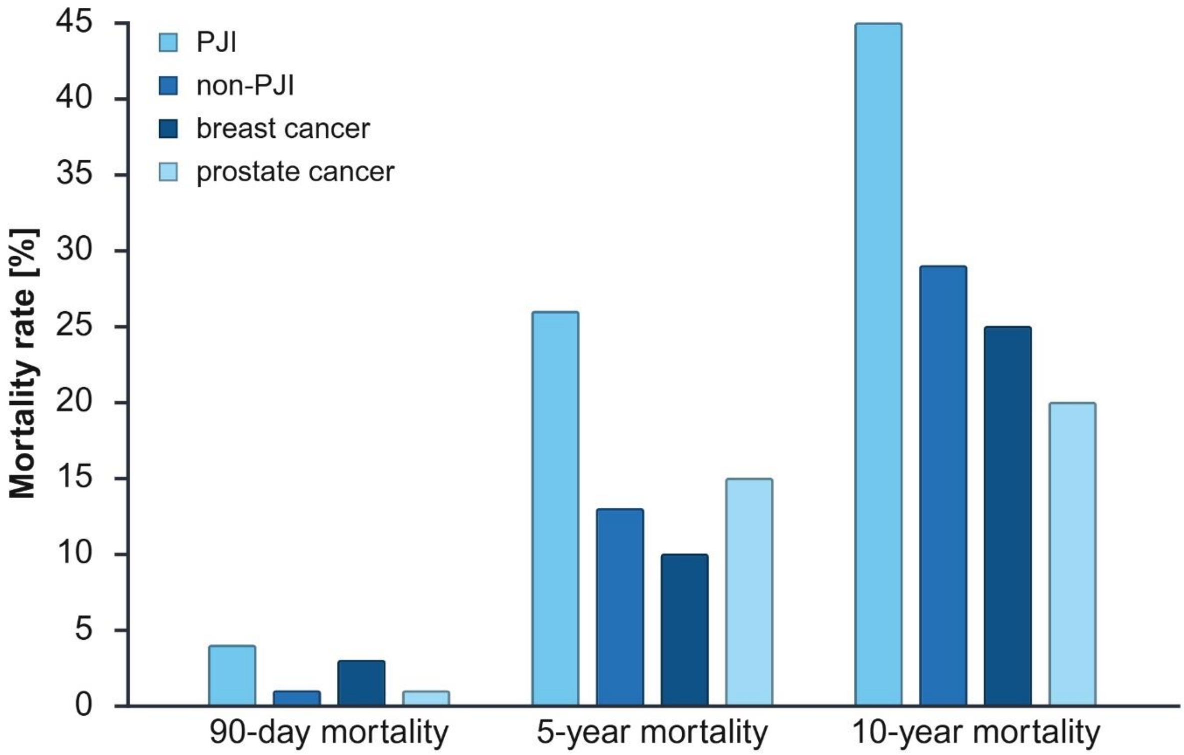


Figure 2 | Mortality rates following periprosthetic joint infection compared to the most common cancers: breast cancer in women and prostate cancer in men (Björklund et al., 2022; Coleman et al., 2025; Uyar et al., 2025; Xu et al., 2023).

Two-stage revision remains the gold standard for septic cases, involving implant removal, an interim phase (6–8 weeks) with an antibiotic-loaded PMMA cement spacer, and subsequent reimplantation (Lehner et al., 2020; Romanò et al., 2012; Samuel, 2012). In complex infections, multiple antibiotic-loaded PMMA spacers support infection eradication (Anagnostakos & Fink, 2018; Kelm et al., 2006; Prats-Peinado et al., 2024; Tsung et al., 2014). Notably, the likelihood of re-revision due to pathogen persistence or superinfections during treatment is higher following septic revisions than other causes of failure, emphasising the need to prevent infection recurrence by the most powerful prophylactic measures (Kirschbaum et al., 2022).

Finally, the economic burden is substantial. In the United States, PJI treatment costs are 76% higher than those for primary arthroplasty (Sadoghi et al., 2025). In Europe, direct reimbursement for PJI treatment reached €346 million in 2019 for 20,414 revisions (Alt et al., 2025). Alt et al. (2025) have noted that these figures likely underestimate the true financial impact. Indirect costs, including prolonged recovery, reduced quality of life, and lost productivity among working-age patients, remain unaccounted for (Sadoghi et al., 2025).

2. Definition and pathogenesis of periprosthetic joint infections

PJI is characterised by microbial colonisation of tissues around an implant, predominantly through biofilm formation on the implant surface. Clinically, patients typically present with pain at the implant site accompanied by local signs of inflammation such as erythema and increased temperature. In chronic cases, progressive complications may include implant loosening, wound dehiscence, fistula formation, and purulent discharge. Advanced infections may manifest with a sinus tract, which constitutes a definitive diagnostic criterion for PJI (Patel, 2023). The onset of infection can occur within weeks or even years following arthroplasty. It is classified as either an early postoperative infection, usually resulting from intraoperative contamination or wound healing problems, or a hematogenous infection originating from a remote infection site. Both variants frequently present as acute infections caused by highly virulent organisms such as *Staphylococcus aureus*, with clear clinical symptoms including pain, fever, and swelling. Conversely, low-grade infections evolve over months or years and are primarily associated with low-virulence pathogens such as *Cutibacterium acnes* or *Staphylococcus epidermidis*. These cases often exhibit non-specific symptoms, and conventional inflammatory markers may remain inconclusive (McNally et al., 2021). The likelihood of infection, or the transition from contamination to clinically relevant infection, can be conceptualized as a function of the total bacterial load multiplied by pathogen virulence, divided by the host’s immune competence. Thus, all three parameters collectively determine the probability of developing a PJI:

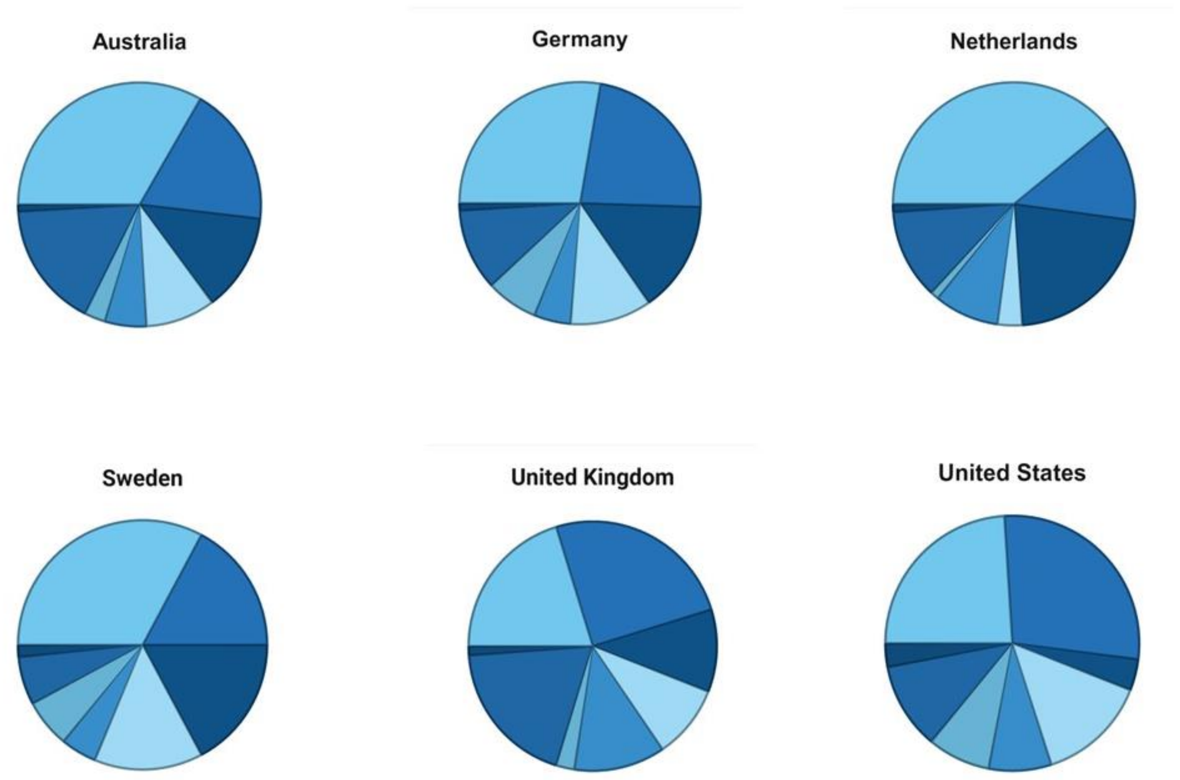
$$\boldsymbol{Infection} = \frac{\boldsymbol{degree\ of\ contamination\ x\ virulence\ of\ germs}}{\boldsymbol{immune\ competence}}$$

2.1. Degree of contamination

The first parameter in the calculation is the surgery-related factor of bacterial load. Strict perioperative hygiene protocols are implemented to reduce bacterial load and thereby lower the probability of developing a PJI. Sweden initiated a national initiative, Prosthesis Related Infections Shall be Stopped (PRISS), that explicitly targets operative-environment determinants of infection risk (Wildeman et al., 2024). The measures include a standardised hygiene protocol, locking operating rooms (OR) during joint replacement procedures, limiting personnel traffic and headcount, and improved disposable attire for the OR team. Laminar airflow is widely installed, and double-gloving with regular glove changes is recommended. In addition to operating room and team hygiene, patient skin is prepared by removing hair and disinfection, and continuous lavage is used as an adjunctive measure (Chan & Partington, 2018; Jones et al., 2018; Malhotra et al., 2018).

2.2. Virulence of germs

The second factor comprises the causative pathogens and their virulence. In the U.S., PJIs are most attributed to staphylococci (~56%), followed by streptococci (~14%), enterococci (~8%), and Gram-negative bacteria (~11%) (**Figure 3**) (Patel, 2023). A similar distribution has been reported for Germany, with staphylococci (~66%) predominating, followed by streptococci (~11%), enterococci (~5%), and Gram-negative organisms (~11%) (**Figure 3**) (Ergin et al., 2024; Rupp et al., 2021a). In the UK, enterococci appear slightly more frequently (~10%) compared with the other selected countries (**Figure 3**) (Dedeogullari et al., 2025). Within the staphylococci, skin-associated *Staphylococcus aureus* and *Staphylococcus* *epidermidis* are the predominant species. Notably, coagulase-negative *Staphylococcus* (CoNS) readily form biofilms on implant surfaces and are often methicillin-resistant, making them inherently less susceptible to the systemic perioperative antibiotic prophylaxis (usually cefazolin or cefuroxime) and more difficult to treat in established infections; as their name implies, they do not produce the coagulase enzyme.



	Frequency [%]					
Microorganism	**Australia**	**Germany**	**Netherlands**	**Sweden**	**United Kingdom**	**United States**
Staphylococcus aureus	36	28	36	21	17	24
Staphylococcus epidermidis	20	23	12	11	21	28
other *Staphylococcus* species	14	15	20	11	9	4
***Streptococcus* species**	10	11	3	9	8	14
***Enterococcus* species**	6	5	8	3	10	8
***Cutibacterium* species**	3	7	1	4	2	8
Gram-negative bacteria	18	11	11	4	16	11
Fungi	<1	1	<1	<1	1	3

Figure 3 | PJI causing microorganisms and their frequency for Australia (Manning et al., 2020), Germany (Rupp et al., 2021a), the Netherlands (van Veghel et al., 2025), Sweden (Sebastian et al., 2021), UK (Dedeogullari et al., 2025) and the U.S. (Patel, 2023).

Streptococci implicated in PJI frequently originate from mucocutaneous reservoirs, including the urogenital tract (*Streptococcus dysgalactiae*, *Streptococcus agalactiae*). For enterococci, *Enterococcus faecium* is commonly encountered. At lower frequencies, Gram-negative pathogens such as *Pseudomonas aeruginosa* and *Escherichia coli* are causative, and low-grade infections are mainly associated with *Cutibacterium acnes*. Fungal PJIs are rare overall, but *Candida* species have been reported more frequently in the U.S. than in Germany (Patel, 2023; Rupp et al., 2021a). The increasing prevalence of multi-drug-resistant organisms (MDR), including MRSA, methicillin-resistant *Staphylococcus epidermidis* (MRSE), vancomycin-resistant enterococci (VRE) and vancomycin-intermediate-resistant *Staphylococcus aureus* (VISA), further complicates antimicrobial strategies (Almeida-Santos et al., 2025; Rupp et al., 2021a).

These pathogens give rise to surgical site-related infections in the context of the implant. Planktonic bacteria may then adhere to the implant surface and progressively form a biofilm whose extracellular polymeric substance (EPS) confers protection from host immunity and impedes antibiotic penetration (**Figure 4**). This process is more intricate in implant-associated infections than previously assumed (Sauer et al., 2022). Despite rigorous protocols, neither the patient nor the OR can be rendered completely sterile. Consequently, every arthroplasty inherently carries a residual risk of PJI, and the objective is to minimise this probability as a complete elimination would therefore never be possible. Depending on the pathogen, its resistance profile, and host immune competence, as few as 100–1,000 bacteria may suffice to convert contamination into infection. Intraoperative contamination may occur even with strict protocol adherence, and an infection can occur when the patient's immune system fails to eradicate all present bacteria. In the presence of easily colonised, non-vascularized foreign bodies, like metal or polyethylene implants, a small number of bacteria are sufficient to establish an infection. Biofilm formation represents an efficient bacterial survival strategy: organisms become embedded in a matrix of extracellular polysaccharides, proteins, lipids, and DNA, and transition metabolically from planktonic to sessile states with reduced activity and heightened tolerance (**Figure 4**). They may persist in this state for prolonged periods and subsequently revert to a metabolically active planktonic phenotype. In arthroplasty, it is therefore crucial to prevent biofilm formation as early and effectively as possible (Sauer et al., 2022; Zimmerli et al., 2004).

As mentioned before, bacteria embedded within a biofilm exhibit significantly enhanced protection against antimicrobial agents compared to their planktonic counterparts. This increased tolerance is primarily due to the EPS matrix, which acts as a physical and chemical barrier. From a treatment point of view, biofilm-related infections are notoriously difficult to eradicate and often require substantially higher antimicrobial concentrations. To evaluate antimicrobial efficacy, two parameters are commonly used: the minimum inhibitory concentration (MIC), which represents the lowest concentration needed to inhibit planktonic bacterial growth, and the minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC), which indicates the concentrations required to prevent biofilm formation or disrupt established biofilms. The distinction between MIC and MBIC/MBEC is clinically critical because MBIC/MBEC values are typically much higher than MIC values, reflecting the resilience of biofilm communities. Therefore, eliminating bacteria before they transition from the planktonic phase to a biofilm phenotype is essential for effective infection control, as early intervention reduces the risk of persistent, device-related infections and improves therapeutic outcomes.

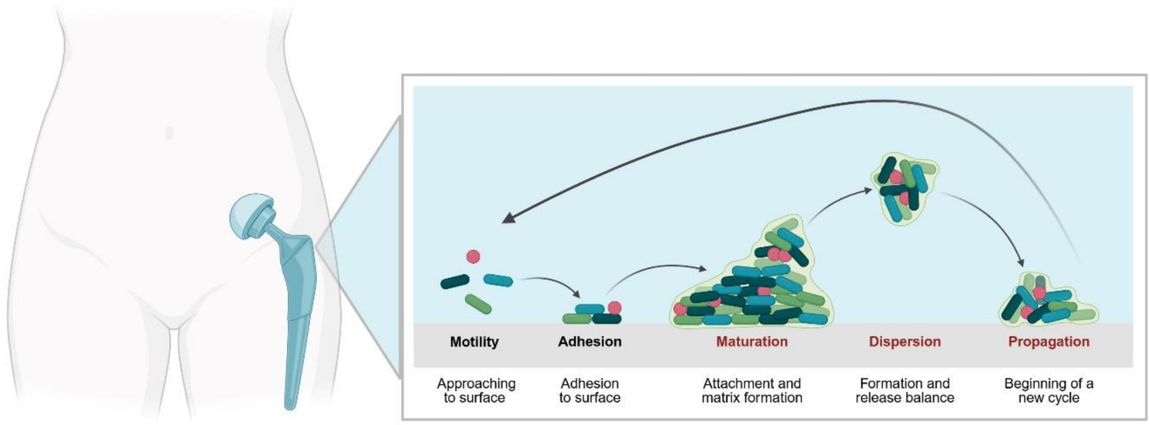


Figure 4 | Biofilm development on a hip implant illustrating the classical five-step model: initial planktonic phase, bacterial adhesion to the surface, biofilm maturation, subsequent dispersion, and propagation of sessile communities. Own illustration based on Sauer et al., (2022).

2.3. Immune competence of patient

The third factor is the patient's immune competence. Comorbidities such as obesity, diabetes mellitus, and cardiovascular disease increase the PJI risk; some determinants are modifiable (e.g., smoking), although outcomes depend critically on patient compliance (Berberich et al., 2022). The ASA Physical Status classification is widely used to index preoperative health and perioperative risk; patients with ASA ≥ 3 (multiple comorbidities) exhibit a significantly increased risk of PJI. Registry data indicate the prevalence of higher ASA scores among primary THA populations: Australia ~43% (Lewis et al., 2025), Germany ~31% (EPRD, 2025), Netherlands ~26% (LROI, 2025), Sweden ~20% (SAR, 2024), UK ~18% (NJR, 2025), U.S. ~34% (Silman et al., 2021) (**Figure 5**). Prior surgery, revision procedures, and prolonged operative time further elevate the risk (Berberich et al., 2022).

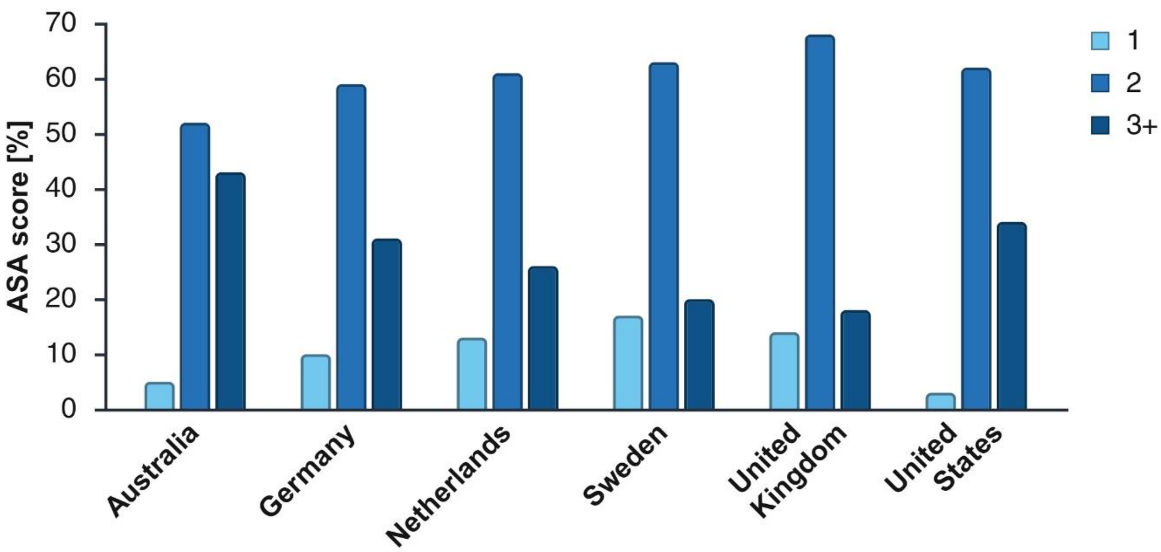


Figure 5 | Distribution of ASA Physical Status scores among patients undergoing primary total hip arthroplasty, based on data from national register reports: Australia - AOAJRR (Lewis et al., 2025), Germany - EPRD (EPRD, 2025), the Netherlands - LROI (LROI, 2025), Sweden - SAR (SAR, 2024), United Kingdom - NJR (NJR, 2025), and the United-States - Kaiser Permanente (Silman et al., 2021).

Patients with uncontrolled diabetes mellitus are predisposed to postoperative wound infections due to the inherent wound healing delays. Diabetes comes also in conjunction with severe obesity (BMI > 35) and/or cardiovascular disease (Lenguerrand et al., 2019). Severe kidney dysfunction, chronic immunosuppression, malnutrition, or anaemia are likewise associated with higher PJI rates (Kunutsor et al., 2016; Sanz-Ruiz et al., 2020).

2.4. Diagnostics

Diagnostic criteria for PJI differ across organizations, notably the European Bone and Joint Infection Society (EBJIS) and the Musculoskeletal Infection Society (MSIS), and there is ongoing discussion regarding the optimal diagnostic algorithm for confirmation or exclusion of a PJI case (McNally et al., 2021; Mühlhofer et al., 2021; Parvizi et al., 2013). Microbiological criteria (culture of tissue samples or synovial fluid), serological parameters (e.g., CRP-level, leukocyte count), and histological findings are differentially weighted and integrated with clinical symptoms. Common pitfalls include too short bacteria culture times that fail to detect low-grade infections (e.g., *Cutibacterium acnes*) and low specimen numbers (Li et al., 2020). Subsequent PJI management is inherently complex and requires multidisciplinary collaboration, including infectious-disease specialists, microbiologists, orthopaedic surgeons and other disciplines to tailor the most appropriate treatment pathway for the patient.

3. Acrylic bone cements for arthroplasty

3.1. History

The development of PMMA as an acrylic bone cement began in 1901 when Otto Röhm laid the foundation for acrylic polymer chemistry with his dissertation entitled "Über Polymerisationsprodukte der Acrylsäure" ("Polymerisation products from acrylic acid"). He referred to the resulting material as "organic glass," which later became widely known as Plexiglas®. This discovery marked the starting point for the synthesis of PMMA and its subsequent applications. By 1928, Röhm and Otto Haas had advanced the technology to enable industrial-scale production of methyl-methacrylate (MMA), the monomer essential for PMMA synthesis. This achievement facilitated the transition from laboratory research to large-scale manufacturing, opening the door to practical applications. In 1935, Walter Bauer, head of the Röhm & Haas research laboratory, patented a process for producing dental prostheses using MMA. This represented the first medical application of this material. Shortly thereafter, in 1936, Heraeus (Kulzer) introduced a heat-curing technique by combining PMMA powder with liquid monomer and benzoyl peroxide (BPO), which polymerised at approximately 100 °C. This innovation allowed the fabrication of durable components for dental and medical use. The versatility of PMMA was further demonstrated in 1941 when pre-cured PMMA plates (Paladon® 65) were employed for the first time in plastic surgery to repair cranial defects. Two years later, in 1943, Heraeus (Kulzer) and Degussa patented a process for cold-curing PMMA cement at room temperature, a breakthrough that established the chemical principles still applied in modern acrylic bone cement formulations. The first orthopaedic application of cold-curing PMMA occurred in 1951 when surgeon Sven Kiaer used the material as an anchoring agent for acrylic glass caps on the femoral head following cartilage removal. This milestone marked the beginning of PMMA's role in orthopaedic surgery, paving the way for its widespread use in joint arthroplasty and other implant fixation procedures (Bistolfi et al., 2019; Kühn, 2014). The late 1950s marked a turning point in orthopaedic surgery with the pioneering work of Sir John Charnley, who in 1958 successfully anchored a total hip implant using cold-curing PMMA cement. Charnley referred to this material as "acrylic-based bone cement," and early clinical studies demonstrated excellent biocompatibility and long-term implant survival up to three decades (Charnley, 1960, 1970; Charnley et al., 1968). In 1959, Heraeus (Kulzer) introduced Palacos®, the first PMMA-based bone cement manufactured in Germany according to an in-house formulation. A major innovation occurred in 1969 when Professor Hans-Wilhelm Buchholz proposed the incorporation of antibiotics into PMMA cement to reduce the at this time high postoperative infection rates (Buchholz & Engelbrecht, 1970). His correspondence with Heraeus (Kulzer) emphasized the potential clinical significance of antibiotic-loaded bone cement and raised the question of whether active substances could be released from the polymer matrix. Although initially met with scepticism, famously expressed by Charnley in his remark, "My dear Buchholz, nothing leaks out of a stone", the addition of gentamicin sulphate to Palacos® R yielded promising results and, infection rates were subsequently reduced following joint replacement surgery. Still today, PMMA cements are among the most widely used medical devices worldwide (Leta et al., 2023). Their primary function is to provide mechanical fixation of implants within bone, ensuring rapid primary stability, uniform load distribution, and, when loaded with antibiotics, local antimicrobial activity (Kühn, 2014; Kühn et al., 2016).

3.2. Chemical properties

Modern acrylic bone cements are polymers based on acrylic acid that set without the application of external heat. They are made up of two basic components, the polymer (poly-methyl-methacrylate, PMMA for short) in powder form and the monomer (methyl-methacrylate, MMA for short) in liquid form (**Table 1**). Upon mixing of these components, chemically an exothermic polymerisation reaction commences. On continuation, the cement viscosity increases with time until complete curing in a firm and stable matrix (Kühn, 2022; Paul & Kühn, 2023). The powder component consists of polymers and/or copolymers, X-ray contrast agents (barium sulphate or zirconium dioxide), an activator (benzoyl peroxide), active ingredients (antibiotics) and, optionally, colouring agents (**Table 1**). The liquid component consists of pure MMA, usually stabilised with small amounts of hydroquinone, an initiator N,N-dimethyl-p-toluidine (DmpT) and, optionally, also a colourant. The MMA molecules in the liquid component are therefore the basic building blocks of PMMA in its non-polymerised form (Frommelt & Kühn, 2005; Kühn, 2001, 2014, 2022).

Powder = Polymer (PMMA/Copolymer)		Liquid = Monomer (MMA)	
Component	**Function**	**Component**	**Function**
Benzoyl peroxide (BPO)	Initiator	N,N-dimethyl-p-toluidine (DmpT)	Activator
Zirconium dioxide or barium sulphate	Radio pacifier	Hydroquinone	Stabiliser
Green pigment (chlorophyllin = E141) or blue pigment (E313)	Colouring agent	Green pigment (chlorophyllin = E141) or not stained	Colouring agent
Antibiotics (e.g. gentamicin, clindamycin, vancomycin, tobramycin)	Infection prophylaxis or treatment		

Table 1 | Overview of the primary ingredients in PMMA cement, categorised by powder and liquid components, and their chemical or clinical functions.

The MMA molecule, an ester of methacrylic acid, contains a reactive C=C bond that enables chain growth (Kühn, 2001, 2014). The redox initiator system, benzoyl peroxide (BPO) in the powder and DmpT in the liquid, generates free radicals that open the C=C bond, initiating polymerisation. This process transforms the initially fluid mixture into a mouldable dough and ultimately into a solid matrix. Heat release peaks in the curing phase, but in-vivo temperatures remain below protein coagulation thresholds due to prosthesis heat absorption and tissue perfusion (Biehl et al., 1974; Breusch & Malchau, 2005; Toksvig-Larsen et al., 1991).

Although PMMA cements may appear uniform at first glance, they differ significantly in composition and production processes. The powder component can consist of pure polymers, copolymers, or blends, and this composition critically influences key properties such as elasticity and hydrophilicity (Lewis, 2015; Meeker et al., 2019). Sterilisation method is another determinant of material performance: gamma irradiation enables rapid, cost-effective sterilisation but can compromise the mechanical integrity of polymer beads, whereas ethylene oxide (EO) sterilisation preserves powder quality and mechanical stability (Kühn, 2014; Weisman et al., 2000). To facilitate postoperative radiographic assessment, radiopaque agents such as barium sulphate or zirconium dioxide are incorporated into the powder. Zirconium dioxide offers superior radiopacity, smaller and more homogeneous particle size, and additionally functions as a thermal conductor during polymerisation (Kühn, 2014; Kühn et al., 2016). Furthermore, acrylic bone cements may be antibiotic loaded to provide local antimicrobial activity; however, only specific antibiotics are suitable for incorporation (Kühn et al., 2017).

The polymerisation of PMMA cement is an exothermic process, releasing energy that manifests as a measurable temperature increase over time. Under standardised laboratory conditions (ISO 5833:2002), peak temperatures can exceed 80 °C, raising theoretical concerns about thermal necrosis in-vivo. However, clinical measurements demonstrate substantially lower temperatures, typically ranging between 40 °C and 46 °C (Biehl et al., 1974; Frommelt & Kühn, 2005; Spierings, 2005; Toksvig-Larsen et al., 1991). This reduction is primarily attributed to the high thermal conductivity of metallic implants, which dissipate heat effectively, and to physiological cooling mechanisms provided by blood circulation and surrounding tissues. Consequently, no active cooling measures are required during implantation (Bishop et al., 1996; Eitenmüller et al., 1981). As the in-vivo temperatures remain below the threshold for protein coagulation. Additionally, cement volume influences thermal behaviour: thin layers, such as those forming the prosthesis fixation mantle, exhibit significantly lower peak temperatures compared to large cement masses.

3.3. Physical properties

PMMA cements form a dual interface: interdigitating with cancellous bone and creating a form-fit with the prosthesis surface. During daily functional loading, acrylic bone cement must meet stringent mechanical requirements due to its critical role as an interface between bone and prosthesis (Lee, 2005). The cement mantle is responsible for securely anchoring the implant, transmitting patient body weight to the bone, and accommodating peak loads such as those occurring during stumbling or falls (Breusch, 2005). In addition, it must exhibit sufficient elasticity to mitigate stress concentrations. Insufficient load distribution can lead to bone resorption and implant loosening (Breusch & Malchau, 2005; Spierings, 2005, 2007). To evaluate these properties, standardised mechanical tests according to ISO 5833:2002 are performed under both static and dynamic loading conditions. These tests determine key parameters such as compressive strength, flexural strength, elasticity, and shear resistance (Kühn, 2014; Lewis, 2003). The resulting data provide essential insights into the long-term survival of cemented implants and serve as indicators of cement quality. In-vivo, the initially brittle cement matrix absorbs fluids, becoming softer and more elastic, which improves load transfer (Daniels et al., 2005; Kühn, 2014; Nottrott et al., 2008). Softening affects creep and glass transition behaviour (Kühn, 2014; Lee et al., 2002). Porosity and density influence structural stability (Wang et al., 1993). The globally recognised benchmark for PMMA cements is ISO 5833:2002. In addition to defining general specifications, the standard outlines a series of mandatory tests (**Table 2**).

ISO 5833:2002	
Chapter	**Testing method**
Appendix A	Shelf life of liquid component
Appendix B	Sticky or phase of the liquid-powder mixture for use of the cement dough
Appendix C	Maximum temperature and curing time of the liquid-powder mixture
Appendix D	Intrusion of the liquid-powder cement mixture for use in the dough
Appendix E	Compressive strength of polymerised cement
Appendix F	Bending modulus and bending strength of polymerised cement

Table 2 | Overview of the mandatory tests for PMMA cement in accordance with ISO 5833:2002 including mechanical performance evaluations as specified in Appendices E and F.

Annex A addresses monomer stability and its impact on shelf life. Appendix B specifies the determination of tack-free time under controlled laboratory conditions (23 °C ± 1 °C, > 40% relative humidity), defined as the point at which the cement no longer adheres to a gloved latex finger. In clinical practice, this parameter may vary due to environmental and procedural factors. Appendix C evaluates cement penetration depth into cancellous bone, requiring a minimum of 2 mm under standard conditions. Appendix D determines maximum curing temperature and setting time. Appendices E and F assess mechanical performance through static tests: Appendix E measures compressive strength, while Appendix F determines bending strength and bending modulus (**Figure 6**). These parameters provide critical insight into the viscoelastic behaviour of bone cements. Compliance with all ISO-defined criteria is mandatory for product approval (ISO 5833, 2002).

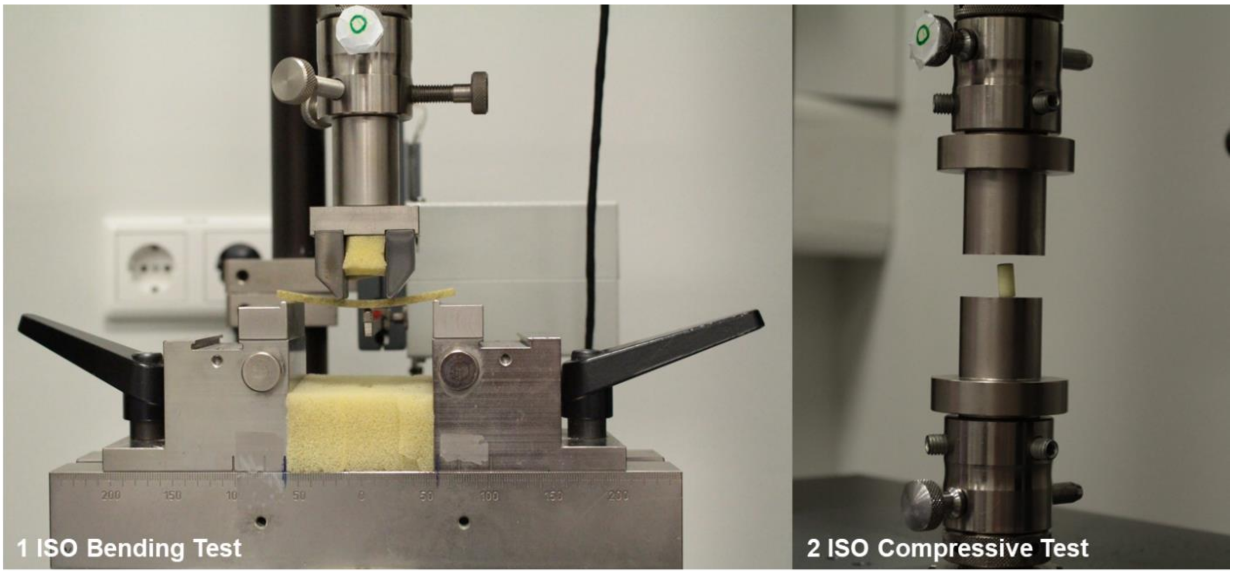


Figure 6 | Test equipment for determining the bending modulus and strength (1) and compressive strength (2), in accordance with the procedures outlined in Appendices E and F of ISO 5833:2002.

Impact strength can be assessed according to DIN 53435:2018, as this parameter provides a more accurate representation of the behaviour of PMMA cements under high-rate, dynamic loading conditions (**Figure 7**). It quantifies the energy required to initiate fracture, which is clinically relevant in scenarios such as accidental trauma where bones and implants may be subjected to sudden forces. In contrast, ISO 5833:2002 primarily addresses static mechanical properties, such as compressive and bending strength (**Figure 6**).

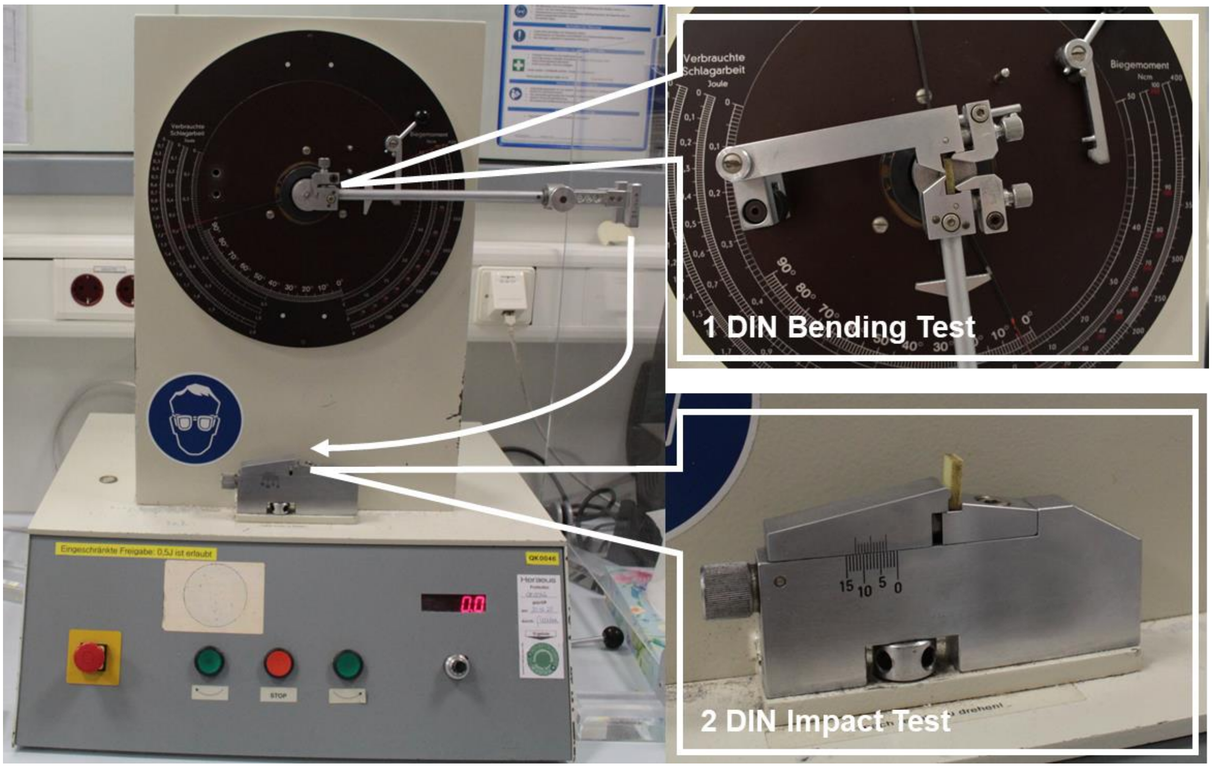


Figure 7 | Test equipment for determining the bending strength (1) and impact resistance/strength (2), in accordance with DIN 53435:2018.

3.4. Preparation and handling

The preparation of PMMA cement is performed intraoperatively by operating room (OR) staff using different mixing systems, including open bowl mixing, vacuum mixing in cartridges, or fully closed vacuum mixing systems. Cement handling comprises four phases: mixing, waiting (sticky phase), working, and setting (**Figure 8**). Polymerisation begins immediately upon combining the powder and liquid components (Kodama, 2022). The MMA monomer reacts with the PMMA powder, initiating an exothermic polymerisation process. As the powder absorbs the liquid, monomers start to form the first polymer chains, and the mixture transitions from a low-viscosity liquid to a mouldable dough, eventually hardening into a solid matrix (**Figure 8**). The initiator system, BPO in the powder and DmpT in the liquid, is consumed during this reaction; without these components, the cement would not cure within the required timeframe for orthopaedic surgery (Kühn, 2014, 2022; Paul & Kühn, 2023). Directly after mixing, the cement exhibits a sticky, stringy consistency known as the waiting phase or doughing time (defined by ISO standards). The cement must become tack-free before application (Kodama, 2022; Kühn, 2005). This transition typically occurs within 20 seconds to 5 minutes, depending on cement type and ambient temperature (cooler conditions prolong the phase). As polymerisation progresses, viscosity increases and stickiness decreases, marking the end of the waiting phase. Once tack-free, the cement enters the working phase, during which it can be applied to bone and the prosthesis inserted. This phase offers a limited time window that varies with cement formulation and environmental conditions. As viscosity continues to rise, the cement loses mouldability and can no longer penetrate cancellous bone, making further manipulation impossible. During the setting phase, polymerisation completes, releasing heat in an exothermic reaction. The prosthesis must remain fixed until the cement fully hardens and cools down. The setting time, defined by ISO standards, corresponds to the point at which the cement reaches its maximum curing temperature and achieves secure fixation (Kühn, 2014; Kühn et al., 2005; Paul et al., 2023).

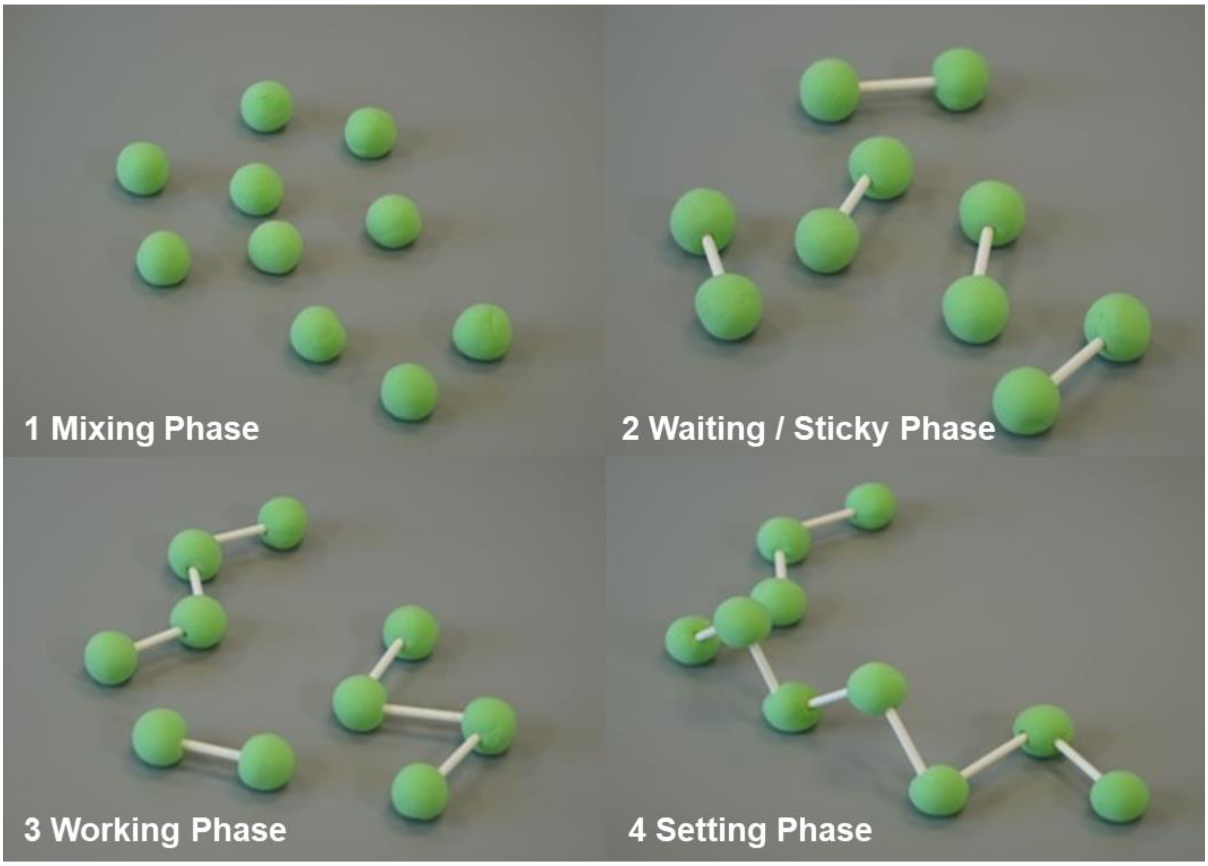


Figure 8 | The handling of PMMA bone cement progresses through four phases. The mixing phase, during which the polymer powder and liquid monomer are combined (1). The waiting (or sticky) phase is characterised by the formation of a viscous dough that remains sticky (2). The working phase is initiated once the cement becomes tack-free and suitable for application (3). The setting phase commences when the material is no longer mouldable and continuing until complete polymerisation and curing are achieved (4). Own illustration published in Paul et al. (2023).

4. Antibiotic strategies for infection prevention

4.1. Combination of systemic and local antibiotics

To reduce the risk of PJI, antibiotic prophylaxis is employed alongside strict hygiene measures. Antibiotics support host defences by targeting potential pathogens and preventing contaminations developing into infections. In established infections, antibiotics aim to significantly reduce bacterial load following surgical debridement and, if necessary, implant removal (Hanssen & Rand, 1999; Hanssen & Spangehl, 2004; Izakovicova et al., 2019). Prophylactic regimens are typically empirical, based on the most likely causative organisms, whereas therapeutic regimens are based on the confirmed pathogens and are guided by antibiograms (Izakovicova et al., 2019; Otto-Lambertz et al., 2017; Zimmerli et al., 2004).

Antibiotic administration can be categorized as systemic or local. Systemic delivery, via intravenous (IV) or oral administration, results in absorption and distribution through the circulatory (blood) and organ systems, achieving peak concentrations in blood plasma and organs such as the kidneys. However, due to the limited vascularisation of bone tissue, systemic therapy often yields suboptimal bone concentrations (Cartau et al., 2025; Frommelt, 2006). Furthermore, obesity, common among arthroplasty patients, alters the pharmacokinetics of antibiotic distribution and reduces the concentrations of those antibiotics in adipose tissue which are rather hydrophile (Märtson et al., 2025).

To support the action of systemic antibiotics and to achieve a high local antibiotic concentration directly at the implant site, the local application mode of antibiotics is often chosen as supplement (Engesaeter et al., 2003). Antibiotics are released directly from carrier systems like bone cement or bone substitute material into the bone or the joint compartment (Carli et al., 2018). In this way, high antibiotic concentrations are produced in site where a possible contamination by bacteria has occurred or where an infection has already developed. At the same time, the systemic burden is relatively low with locally administered antibiotics because the active substance remains largely on site because of the limited vascularisation of the bone and joint tissue (Kendoff et al., 2016). The systemic and local antibiotic application can optimally supplement one another: two independent antibacterial front lines can be established. This is of more clinical relevance if, as in most cases, different antibiotic classes are used systemically and locally. As a proof of principle, a cephalosporin (e.g. cefuroxim) is typically administered for the perioperative systemic prophylaxis and an aminoglycoside (e.g. gentamicin) is used for local prophylaxis with bone cement or a bone substitute carrier (Wahlig & Dingeldein, 1980; Wahlig et al., 1978; Wahlig et al., 1984).

Local antibiotic application methods include different modalities which range from direct intraosseous injection, topical antibiotic powdering of the implant before wound closure until antibiotic delivery via implanted carrier materials (**Figure 9**) (Berberich, 2025). Intraosseous infusion via bone or joint catheters can achieve high local concentrations, but these systems remain experimental (Berberich, 2025; Zou et al., 2024). Topical powdering, commonly with vancomycin, is widely practiced in some regions (e.g., the U.S.), but evidence for its efficacy is limited (Doxey et al., 2024). Although simple and cost-effective, this technique lacks sustained release and may result in rapid drug absorption (Abuzaiter et al., 2023). Current studies remain inconclusive, and routine use in arthroplasty cannot yet be recommended (Saka et al., 2024; Xie et al., 2024). Antibiotic carriers are classified as resorbable or non-resorbable. Resorbable carriers include autografts, allografts, calcium phosphate, calcium sulphate, hydroxyapatite, bio glass, and hydrogels (Berberich, 2025), many of which are still under evaluation and lack randomized controlled trials (RCTs). Among these, antibiotic-loaded calcium sulphate is the most widely used in joint replacement surgery, demonstrating inhibition and even eradication of biofilm (Howlin et al., 2015; Wahl et al., 2017). Limitations include lack of initial mechanical stability and the potential of delaying wound healing due to increased seroma formation and fluid secretion (Shi et al., 2022).

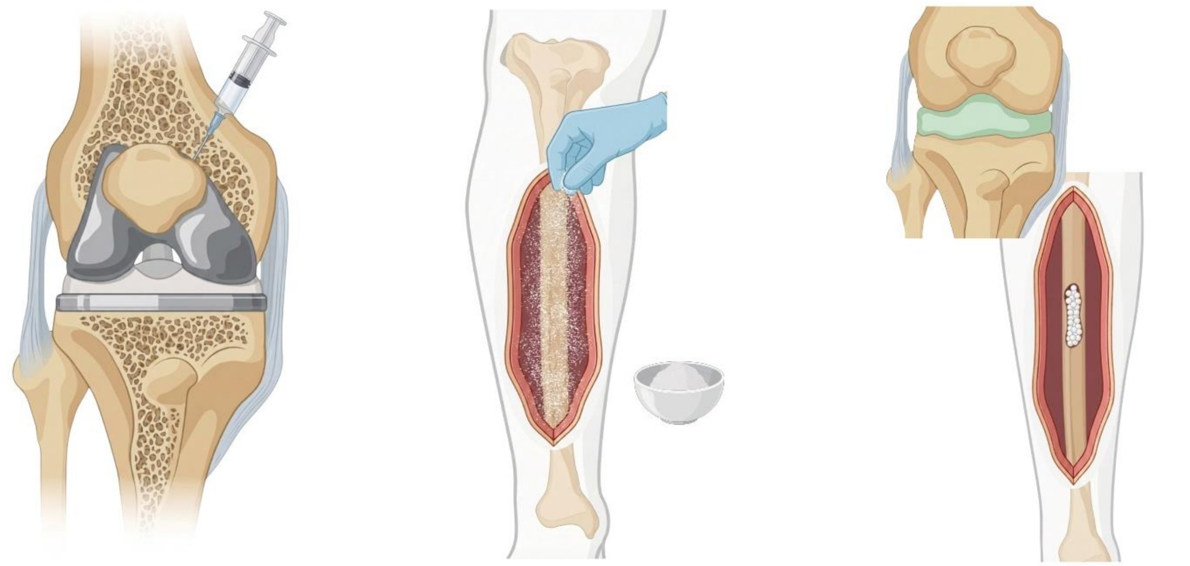


Figure 9 | Principal methods for local antibiotic delivery. Intraosseous or intra-articular administration of liquid antibiotics via syringes or catheter systems (A). Topical application of antibiotic powder directly on the prosthesis or surrounding soft tissue prior to wound closure (B). Implantation of antibiotic-loaded materials into bone defects or joints spaces (e.g., PMMA cement spacers or calcium sulphate beads) (C). Own illustration based on Berberich (2025).

PMMA cement, a non-resorbable carrier, has been established for decades and validated through large-scale studies and registry-based meta-analyses (Sprowson et al., 2016) (Gil-Gonzalez et al., 2024; Leong et al., 2020; Sabater-Martos et al., 2023). Its huge advantage of the other carriers is that PMMA cement serves at the same time as a fixation material for implants and prevents biofilm formation or, as spacer cement in two-stage revision protocols, supports treatment of already established infections via local antibiotic delivery. Another key factor is its sustained antibiotic release, which has been found to be critical for biofilm prevention (Cara et al., 2020).

4.2. Role of antibiotic-loaded bone cement

ALBCs were introduced in the 1970s to prevent bacterial colonisation of cured bone cement and adjacent tissues by organisms susceptible to the incorporated antibiotic (Sabater-Martos et al., 2023). This preventive function is primarily associated with single-antibiotic loaded bone cement (SALBC) (**Table 3**) (Gil-Gonzalez et al., 2024; Leong et al., 2020; Parvizi et al., 2008). Among the first antibiotics used was the aminoglycoside gentamicin, incorporated into Palacos® R+G (Heraeus Medical GmbH), which has demonstrated excellent clinical outcomes for over five decades (Gil-Gonzalez et al., 2024; Sanz-Ruiz et al., 2017; SAR, 2024).

In revision surgeries, ALBC plays a critical role. Although surgical debridement removes most microorganisms from the infected site, complete sterilisation cannot be achieved by debridement alone. Therefore, the local release of high antibiotic concentrations from bone cement, combined with systemic antibiotic therapy, enhances infection control. This therapeutic function, and in some cases infection eradication, is best achieved by combinations of antibiotics in bone cement (e.g. dual antibiotic-loaded bone cements (DALBC)), which contain two different antibiotics (**Table 3**). Their superior antimicrobial action reflects the synergistic antibiotic elution from the carrier substance, the broader microbial spectrum and the lower risk of resistance development. DALBC are recommended for both septic and aseptic revisions to mitigate the inherently higher infection risk of any revision procedures and the risk of an erroneous or incomplete microbiological diagnosis (Sanz-Ruiz et al., 2017; Sanz-Ruiz et al., 2020). Furthermore, DALBCs are frequently used for arthroplasty procedures in high-risk patients with a combination of comorbidities and in cemented hemiarthroplasties in the particularly frail FNOF patients (Agni et al., 2023; Berberich et al., 2021; Sprowson et al., 2016; Szymski et al., 2023). When the local antibiotic concentration released from ALBC exceeds the MIC, it can effectively eradicate bacteria before biofilm formation. ALBC formulations are classified by antibiotic dose: high-dose (≥2 g antibiotic per 40 g cement, typically DALBC) and low-dose (≤2 g antibiotic per 40 g cement, typically SALBC) (Malhotra et al., 2018). For primary arthroplasty, low-dose cement containing a broad-spectrum antibiotic is used for prophylaxis, whereas high-dose cement is employed to treat established infections (**Table 3**). This is the case in two-stage revision procedures where DALBC is used for spacer production. Temporary PMMA spacers maintain joint space, minimise soft tissue contracture, provide stability, and can allow limited mobility. These spacers are impregnated with antibiotics to prevent recolonisation during the 6 to 8 weeks implantation period, making sustained antibiotic release above the MIC essential (Fink et al., 2011).

	SALBC	**DALBC**
Description	Single-antibiotic-loaded bone cement	Dual-antibiotic-loaded bone cement
Antibiotic combinations	Gentamicin / tobramycin	Gentamicin + clindamycin / Gentamicin + vancomycin
Antibiotic dosage	Low-concentration / low-dose > 5% antibiotic powder in cement powder > 2g of antibiotic powder per 40g cement powder	High-concentration / high-dose < 5% antibiotic powder in cement powder < 2g antibiotic powder per 40 g cement powder
Indication	i. Infection prevention in primary joint replacement procedures	i. Prevention re-colonisation in septic / aseptic revision procedures. ii. Support infection eradication in septic revisions as spacer iii. Infection prevention in primary joint replacement producers for high-risk patients
Commercial products	Gentamicin: Palacos® R+G (Heraeus Medical) Refobacin® Bone Cement R (Zimmer Biomet) SmartSet® GHV (DePuy Synthes) Cemex® Genta (Tecres) Tobramycin: Antibiotic Simplex® with Tobramycin (Stryker)	Gentamicin + clindamycin: Copal® G+C (Heraeus Medical) Gentamicin + vancomycin: Copal® G+V (Heraeus Medical) VancoGenx® (Tecres) Subiton G+V (Subiton) Spectrum™ GV Bone Cement (OsteoRemedies)

Table 3 | Comparative overview of SALBC and DALBC: antibiotic composition, dosage, and indication-specific differences. SALBC formulations incorporate one antibiotic, typically gentamicin, whereas DALBC combines two antibiotics (e.g., gentamicin with clindamycin or vancomycin) to broaden antimicrobial coverage and address resistant pathogens. The table also lists the most used commercially available ALBC products.

4.3. Requirements and frequently used antibiotics

For antibiotic selection in ALBC, the active substance should ideally exhibit broad-spectrum activity against pathogens commonly associated with PJI. A bactericidal mode of action with low intrinsic resistance is preferred to ensure rapid bacterial eradication. Under certain conditions, even antibiotics typically classified as bacteriostatic (e.g., clindamycin) may show bactericidal effects when applied locally at high concentrations. Although the antibiotics used in SALBC and particularly in DALBC formulations provide broad-spectrum coverage, their efficacy remains concentration-dependent and must be considered in relation to the MIC distribution of PJI-relevant pathogens (Berberich, 2025).

Several prerequisites must be fulfilled for an antibiotic to be suitable for incorporation into PMMA cement. The antibiotic must be water-soluble to allow effective elution from the cement matrix and thermostable to withstand the exothermic polymerisation process, which could otherwise degrade heat-sensitive compounds (Kühn, 2014; Levack et al., 2021). Sterility is essential to prevent contamination during preparation, and the antibiotic should be available in powdered form for mixing with the PMMA powder component. Furthermore, it must be chemically stable, not interfere with the radical polymerisation reaction, and be capable of sterilisation and storage in manufactured formulations. Ideally, the antibiotic should exhibit good bone penetration as well as low risk of allergic reactions (Breusch & Malchau, 2005; Kühn, 2014). Its impact on the mechanical properties of the cement should be minimal, as alterations can compromise implant stability. The physical state of the antibiotic also influences PMMA curing; liquid formulations generally reduce mechanical strength, making crystalline antibiotics preferable (Hetzmannseder et al., 2021). However, exceptions exist, rifampicin, despite being crystalline, can inhibit PMMA polymerisation (Funk et al., 2019).

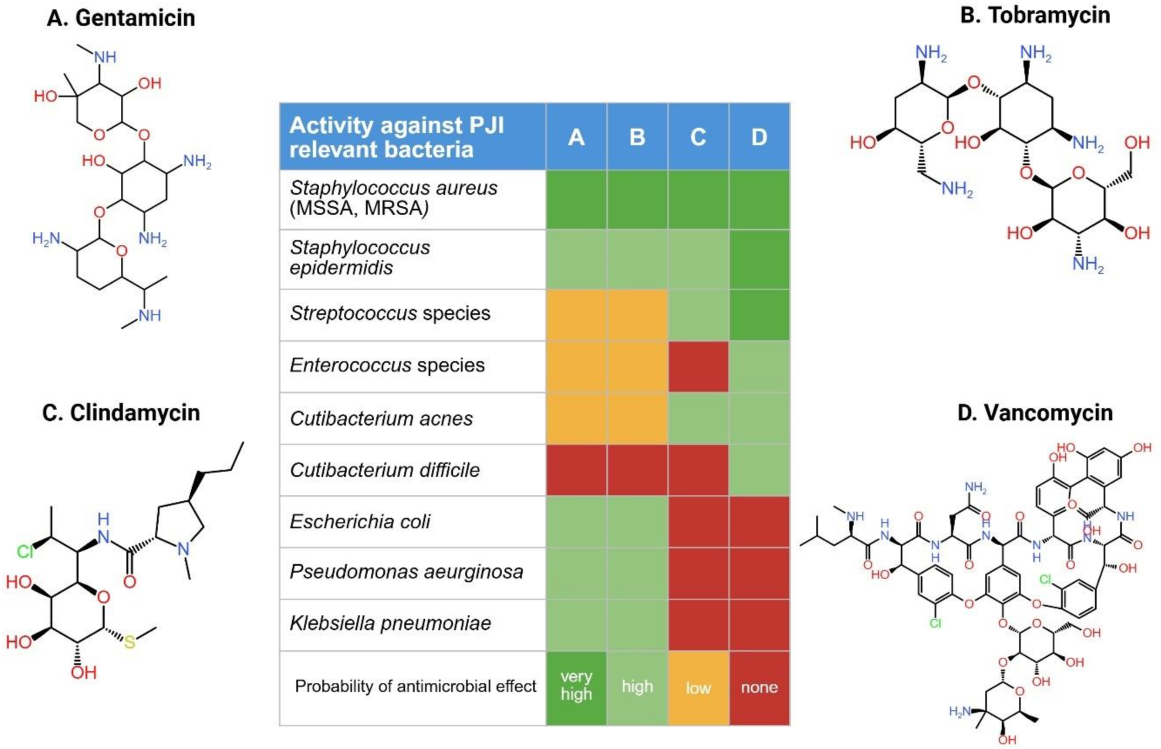
Historically, the development of ALBC began with the addition of penicillin and erythromycin, which were later abandoned due to insufficient efficacy (Hinarejos et al., 2013; Pardo-Pol et al., 2024). Gentamicin emerged as a safe and effective option and remains widely used. Today, commercially available ALBC formulations typically include gentamicin, tobramycin, clindamycin, or vancomycin (**Figure 10**) (Leta et al., 2023).

Gentamicin is a bactericidal aminoglycoside antibiotic with broad-spectrum activity, primarily against Gram-negative bacteria such as *Escherichia*, *Enterobacter*, *Klebsiella*, *Salmonella*, *Serratia*, *Shigella*, and certain species of *Proteus* and *Pseudomonas aeruginosa* (**Figure 10**). It also exhibits efficacy against some Gram-positive organisms, including staphylococci and enterococci. Gentamicin has been clinically and therapeutically established for decades and meets key requirements for incorporation into acrylic bone cement, including thermal stability and broad antimicrobial activity, as documented in numerous in-vivo and clinical studies (Salvati et al., 1986). Aminoglycosides such as gentamicin inhibit the protein synthesis by binding to the 16S ribosomal RNA of the 30S ribosome (**Figure 11**). As a result, gentamicin promotes mistranslation resulting in error prone protein synthesis. This leads to the incorporation of mis- or nonfunctional proteins which damage the bacterial cell membrane and other compartments, ultimately leading to cell death (Krause et al., 2016).

Potential systemic risks of gentamicin, such as nephrotoxicity and ototoxicity, have long been recognised for aminoglycosides but the toxicity is concentration-dependent (Federspil et al., 1976). However, elution profiles from PMMA cement demonstrate very low systemic concentrations, well below these thresholds (Salvati et al., 1986; Wahlig et al., 1984).

Gentamicin remains the antibiotic of choice in many commercially available PMMA cements due to its broad spectrum, bactericidal and strict concentration-dependant activity, and proven clinical efficacy (Gao et al., 2023). Examples for gentamicin containing commercially available ALBCs are Palacos® R+G (Heraeus Medical GmbH, Wehrheim, Germany), Refobacin® Bone

Cement R (Zimmer Biomet, Valence, France), SmartSet® GHV (DePuy Synthes, Warsaw, Indiana, USA) and Cemex® Genta (Tecres S.p.A, Sommacampagna, Italy).



Activity against PJI relevant bacteria	A	B	C	D
Staphylococcus aureus (MSSA, MRSA)	very high	very high	very high	very high
Staphylococcus epidermidis	high	high	high	very high
Streptococcus species	low	low	high	very high
Enterococcus species	low	low	none	high
Cutibacterium acnes	low	low	high	high
Cutibacterium difficile	none	none	none	high
Escherichia coli	high	high	none	none
Pseudomonas aeurginosa	high	high	none	none
Klebsiella pneumoniae	high	high	none	none
Probability of antimicrobial effect	very high	high	low	none

Figure 10 | Chemical structures of the most used antibiotics in ALBC: gentamicin (A), tobramycin (B), clindamycin (C) and vancomycin (D). Their predicted antimicrobial activity against clinically relevant pathogens in PJI, based on susceptibility data from the European Annual Antimicrobial Susceptibility Testing (EUCAST) breakpoints (EUCAST, 2025b). Colour coding indicates the probability of effective antimicrobial activity: Dark green = very high probability. Light green = high probability. Orange = low probability. Red = no probability. Own illustration based on Berberich (2025).

Tobramycin is a bactericidal aminoglycoside antibiotic, highly effective against a broad spectrum of Gram-negative bacteria, including *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella*, *Enterobacter*, *Proteus*, and *Serratia species*, and some Gram-positive organisms such as *Staphylococcus aureus*, especially MRSA (**Figure 10**). It is commonly used in both systemic and local treatments for bone and joint infections, fulfilling essential criteria for incorporation into acrylic bone cement: thermal stability, proven efficacy, and pharmacokinetic profiles evidenced in both in-vivo and clinical studies (Brogden et al., 1976; Federspil et al., 1976; Fiel & Roesch, 2022).

Tobramycin binds to the 16S ribosomal RNA of the 30S ribosome, disrupting the initiation complex and causing mistranslation during protein synthesis (**Figure 11**). This leads to the production of defective proteins that compromise bacterial cell membranes and ultimately result in bacterial death (Brogden et al., 1976). Systemic administration is associated with nephrotoxicity and ototoxicity, which are concentration dependent. However, when tobramycin is incorporated into PMMA cement or other antibiotic carriers like calcium sulphate, elution studies indicate that systemic absorption is very low (Pargas et al., 2022).

Tobramycin, similarly, to gentamicin meets all key requirements for incorporation into PMMA cements and usage in PJI prevention. One example for commercially available PMMA cement containing tobramycin is Antibiotic Simplex® with Tobramycin (Stryker Howmedica Osteonics, Mahwah, New York, USA).

Clindamycin is a lincosamide antibiotic, primarily bacteriostatic (and bactericidal at higher concentrations), targeting Gram-positive cocci (*Staphylococcus aureus* including some MRSA strains, *Streptococcus spp.*) and anaerobic bacteria such as *Bacteroides fragilis* and *Clostridium spp.* (**Figure 10**). It lacks activity against most Gram-negative aerobes due to intrinsic resistance. Widely used for decades in treating bone and joint infections due to its high bone penetration, including osteomyelitis, clindamycin is also well-established for local delivery in orthopaedic settings, satisfying critical criteria for inclusion in acrylic bone cement: thermal stability, satisfactory bone penetration, and efficacy supported by preclinical and clinical studies (Abdelaziz et al., 2019).

Clindamycin binds to the 50S ribosome, interaction at the peptidyl transferase centre of 23S ribosomal RNA, inhibiting peptide bond formation and blocking elongation interrupting protein synthesis and bacterial proliferation (**Figure 11**) (Ali et al., 2025; Dhawan & Thadepalli, 1982).

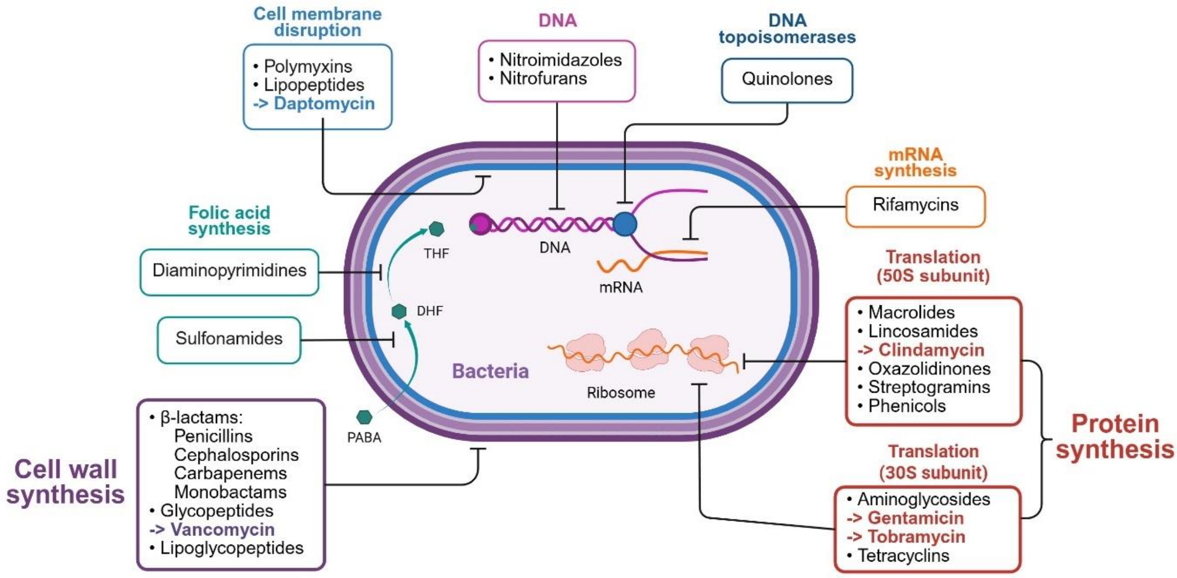


Figure 11 | Antibiotic classes and their mechanisms of action on bacterial metabolism. Antibiotics most frequently incorporated into ALBC (gentamicin, tobramycin, clindamycin, vancomycin) are emphasised in bold and highlighted in colour. Own illustration based on Bbosa et al. (2014).

Systemic administration carries risks of gastrointestinal adverse events, notably *Clostridioides difficile*-associated colitis, as well as occasional hepatotoxicity, neutropenia, and rare severe dermatologic reactions. Unlike aminoglycosides, clindamycin does not cause nephro- or ototoxicity. When used locally in PMMA cement, elution studies show minimal systemic absorption and plasma levels etc., drastically lower than those linked to adverse effects, supporting a favourable safety profile for orthopaedic applications (Ali et al., 2025). Clindamycin is mainly used for ALBC formulations in combination with gentamicin achieving a high local antibiotic release due to the synergistic elution effect. The only commercially available ALBC formulation is Copal® G+C (Heraeus Medical GmbH, Wehrheim, Germany) and Refobacin® Revision (Zimmer Biomet, Valence, France).

Vancomycin is a bactericidal glycopeptide antibiotic primarily active against Gram-positive bacteria, including MRSA, coagulase-negative staphylococci, *Enterococcus spp.* (e.g., *E.*

faecalis, *E. faecium*), *Streptococcus pneumoniae*, *Clostridioides difficile*, *Listeria*, *Bacillus*, and other Gram-positive anaerobes (**Figure 10**) (Levine, 2006; McHenry & Gavan, 1983).

Vancomycin binds to the D-alanine–D-alanine terminus of the peptidoglycan precursors in the bacterial cell wall, thereby inhibiting trans glycosylation and transpeptidation reactions needed for cell wall cross-linking (**Figure 11**). This disruption of cell wall synthesis results in osmotic instability and cell lysis. Due to its bulky, hydrophilic structure, vancomycin does not penetrate Gram-negative bacterial outer membranes and thus lacks significant activity against those organisms (Rybak, 2006).

In orthopaedic applications, vancomycin is thermally stable and has been widely incorporated into PMMA cement for local delivery. Elution studies demonstrate sustained antibiotic release: high initial burst followed by days to weeks of effective concentrations at the site, while systemic absorption remains low, avoiding nephro- or ototoxic thresholds (Kuechle et al., 1991; Penner et al., 1996). While vancomycin can cause nephrotoxicity and ototoxicity when administered systemically, typically associated with high peak levels, these risks are negligible with local delivery via PMMA cement, due to the low serum level achieved.

Vancomycin is mainly used in combination with gentamicin; commercially available DALBC formulations are Copal® G+V (Heraeus Medical GmbH, Wehrheim, Germany), VancoGenx® (Tecres S.p.A. Sommacampagna, Italy), Subiton G+V (Subiton, Buenos Aires, Argentina), Spectrum™ GV Bone Cement (OsteoRemedies LCC, Memphis, Tennessee, USA).

4.4. Principle of antibiotic elution from acrylic bone cements

Antibiotic release from PMMA cement occurs through water uptake and diffusion within the cement matrix (Paul & Kühn, 2023). This water-based diffusion process is essential for achieving high local antibiotic concentrations at the implant site to prevent bacterial colonisation and biofilm formation (**Figure 12**). Diffusion explains the net release of molecules from a region of higher concentration to one of lower concentration. Anti-infective release is proportional to the cement's ability to absorb water, both over time and across surface area. This is more relevant than the absolute amount of antibiotic incorporated. Porosity plays a critical role in antibiotic elution from PMMA cement because it directly affects water penetration and diffusion pathways within the cement matrix. A higher porosity increases water uptake, which accelerates the dissolution of antibiotic particles embedded in the cement. This creates more channels for diffusion, allowing antibiotics to migrate from the interior of the cement to its surface. Greater surface area due to pores enhances the contact between the cement and surrounding fluids, promoting sustained release over time (Coraça-Huber et al., 2025; Meeker et al., 2019; van de Belt et al., 2000).

Hydrophilicity is a critical factor influencing antibiotic elution from PMMA cement because it determines the extent of water absorption, which drives the diffusion process. A hydrophilic cement matrix absorbs more water from surrounding tissue fluids, allowing antibiotic particles embedded in the cement to dissolve and migrate through the matrix. This property supports a sustained release of antibiotics, maintaining concentrations above the MIC for longer periods, which is essential for preventing bacterial colonisation and biofilm formation. The chemical composition of the cement strongly affects hydrophilicity: PMMA-MMA copolymers exhibit higher water affinity and therefore enable prolonged antibiotic release, whereas PMMA-styrene copolymers are less hydrophilic and typically cause an initial burst release followed by a rapid decline in antibiotic concentration (Coraça-Huber et al., 2025; Karpiński et al., 2024; Levack et al., 2021; Meeker et al., 2019). Clinically, this difference is significant because maintaining effective antibiotic levels for several days or weeks is crucial in revision arthroplasty and spacer applications, where infection control depends on sustained local antimicrobial activity.

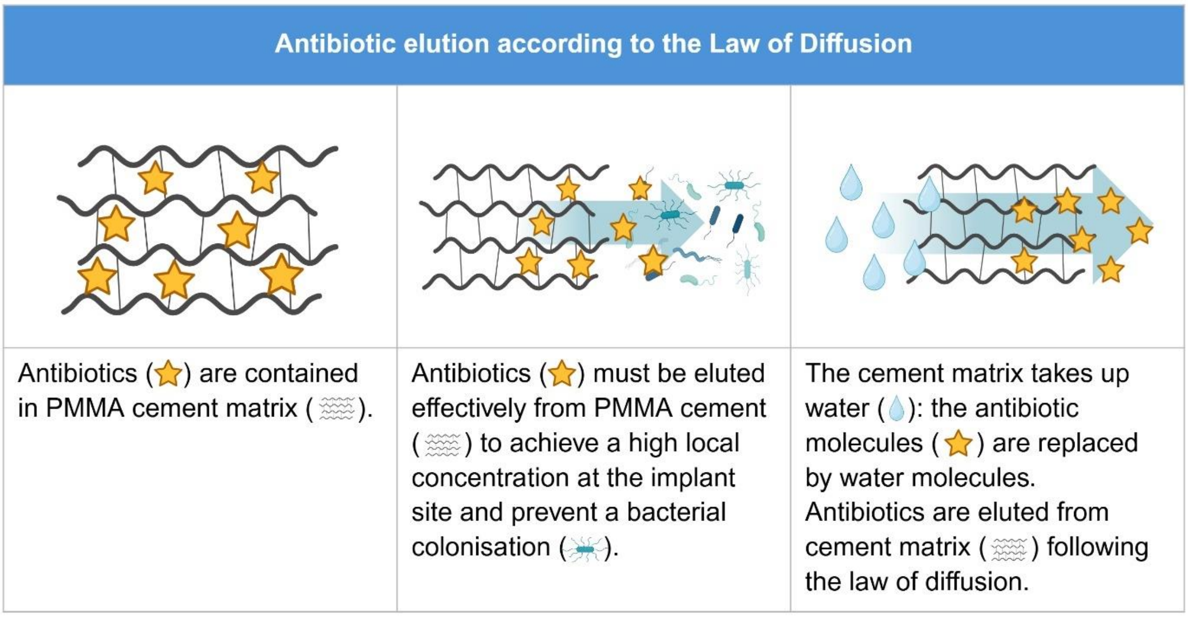


Figure 12 | Schematic representation of antibiotic (or other anti-infective substance) release from PMMA cement according to the law of diffusion. Own illustration based on Kühn, 2014.

An important phenomenon influencing antibiotic release is the synergistic elution effect. When two antibiotics are combined in PMMA cement, the overall elution can significantly exceed the sum of their individual release rates (Kühn, 2014; Kühn et al., 2016, 2017; Neut et al., 2003). This occurs because the presence of multiple antibiotic particles increases matrix porosity and water penetration, facilitating diffusion. However, this effect only applies when the antibiotics act synergistically and not antagonistically. Clinically, this principle is exploited in DALBC formulations, which often combine the hydrophilic gentamicin with vancomycin or clindamycin to broaden antimicrobial coverage and enhance elution.

Additional factors such as the physical form of the antibiotic and even its manufacturer can influence release kinetics. For example, Lee et al. demonstrated variability in vancomycin hydrochloride elution among different brands, with sterile formulations showing superior performance. Importantly, the relationship between antibiotic concentration in PMMA and elution rate is non-linear; increasing the incorporated amount does not necessarily improve release (Warwick et al., 2024).

4.5. Evaluation of antibiotic release and antimicrobial efficacy

The measurement of antibiotic elution and antimicrobial activity is a critical step in the development and quality control of ALBCs, as these parameters directly influence infection prevention and clinical performance. Ideally, elution kinetics and antimicrobial efficacy must both ensure that the ALBC formulations achieve therapeutic concentrations above MIC of PJI relevant pathogens for a clinically relevant time. PMMA cement samples are typically prepared as standardised discs with a diameter of approximately 25 mm to ensure reproducibility across all test methods (**Figure 13**). These discs are incubated in a dissolution medium, most commonly phosphate-buffered saline (PBS), within sterile storage tubes (e.g., Falcon® tubes) under controlled conditions. At defined time intervals, the medium is completely replaced to maintain consistent elution dynamics, and the collected eluates are transferred into separate sterile tubes for subsequent analysis. These eluates serve as the basis for various testing protocols, including antibiotic release profiling and antimicrobial efficacy assessments.

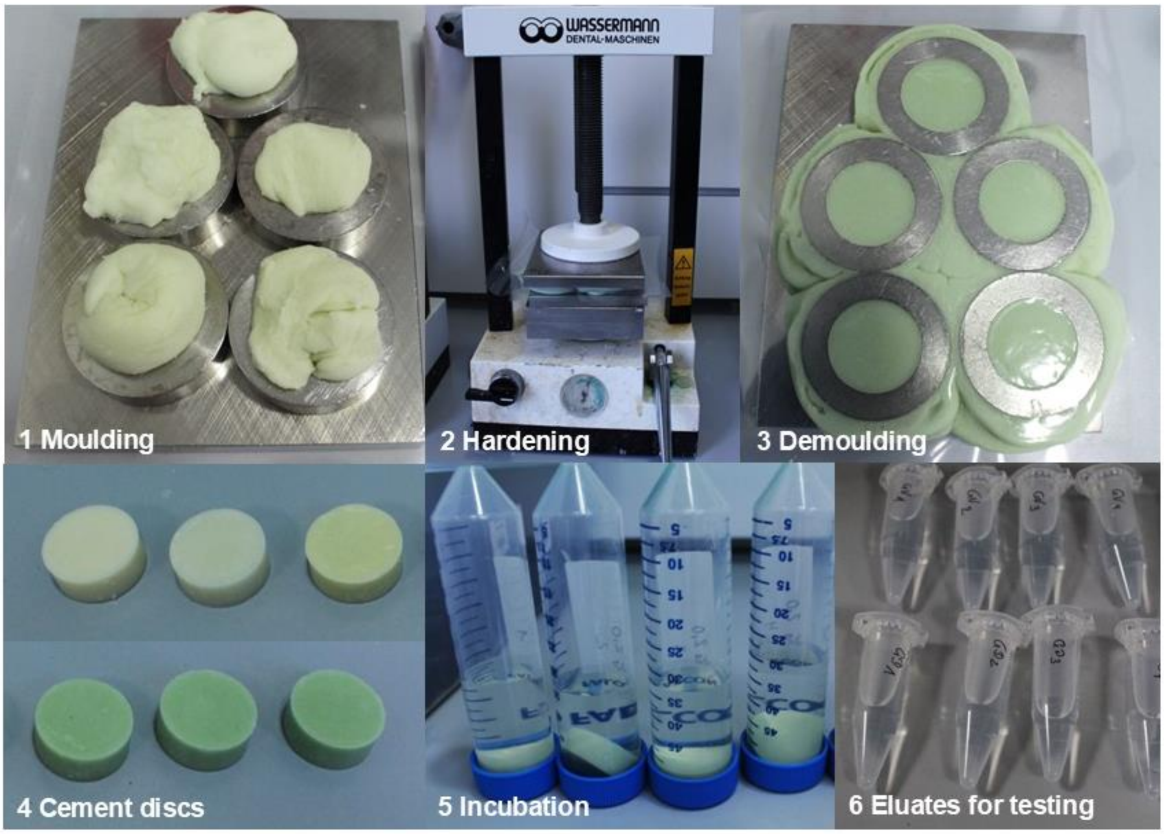


Figure 13 | Preparation of PMMA cement discs for antibiotic elution and antimicrobial efficacy testing. PMMA cement was mixed and introduced into cylindrical moulds (1). Moulds were placed in a dental press to ensure hardening under pressure (2). Hardened cement samples were removed from the press (3). Samples were demoulded to obtain standardised discs (4). Discs were incubated in PBS within Falcon tubes, with medium exchanged at predefined intervals during eluate collection (5). Collected eluates were stored in sterile tubes for subsequent analyses, including HPLC, inhibition zone assays, and proliferation tests (6).

High-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) is among the most widely used techniques for quantifying antibiotic release from PMMA cement. In a typical protocol, cement samples are incubated in a dissolution medium (e.g., 0.1 M Tris-HCl buffer) and eluates are collected over defined intervals (e.g., 5 days). Some antibiotics (e.g., gentamicin) are quantified by summing the signals of its major components detected during LC-MS/MS, whereas others (e.g., daptomycin) are measured directly. This approach provides precise release profiles and allows comparison of different formulations under standardised conditions (Aiken et al., 2015; Amin et al., 2012).

To evaluate antimicrobial efficacy, a microplate proliferation assay is employed to determine the ability of cement surfaces to inhibit bacterial growth. Cement discs are incubated in PBS and eluates are collected at multiple time points over a longer period (e.g., 42 days). After exposure to test strains, bacterial proliferation is quantified by optical density (OD) measurements following amplification in a selected medium (e.g., tryptic soy broth (TSB)). A material is classified as antimicrobial if ≥99.9% inhibition of daughter cell formation is achieved compared to a blank PMMA control without any antibiotics (Alt et al., 2004a, 2004b; Bechert et al., 2000). The inhibition zone test provides a qualitative and semi-quantitative measure of antimicrobial activity. Agar plates (e.g., Müller-Hinton) inoculated with test strains are either treated with eluates from cement samples or cement discs are placed directly onto the agar surface. Antibiotic diffusion into the agar creates a clear zone where bacterial growth is inhibited; the diameter of this zone correlates with antimicrobial efficacy. The zones of inhibition are measured and compared to non-antibiotic loaded blank PMMA samples (Ensing et al., 2008; Fuchs et al., 2001; Huys et al., 2002).

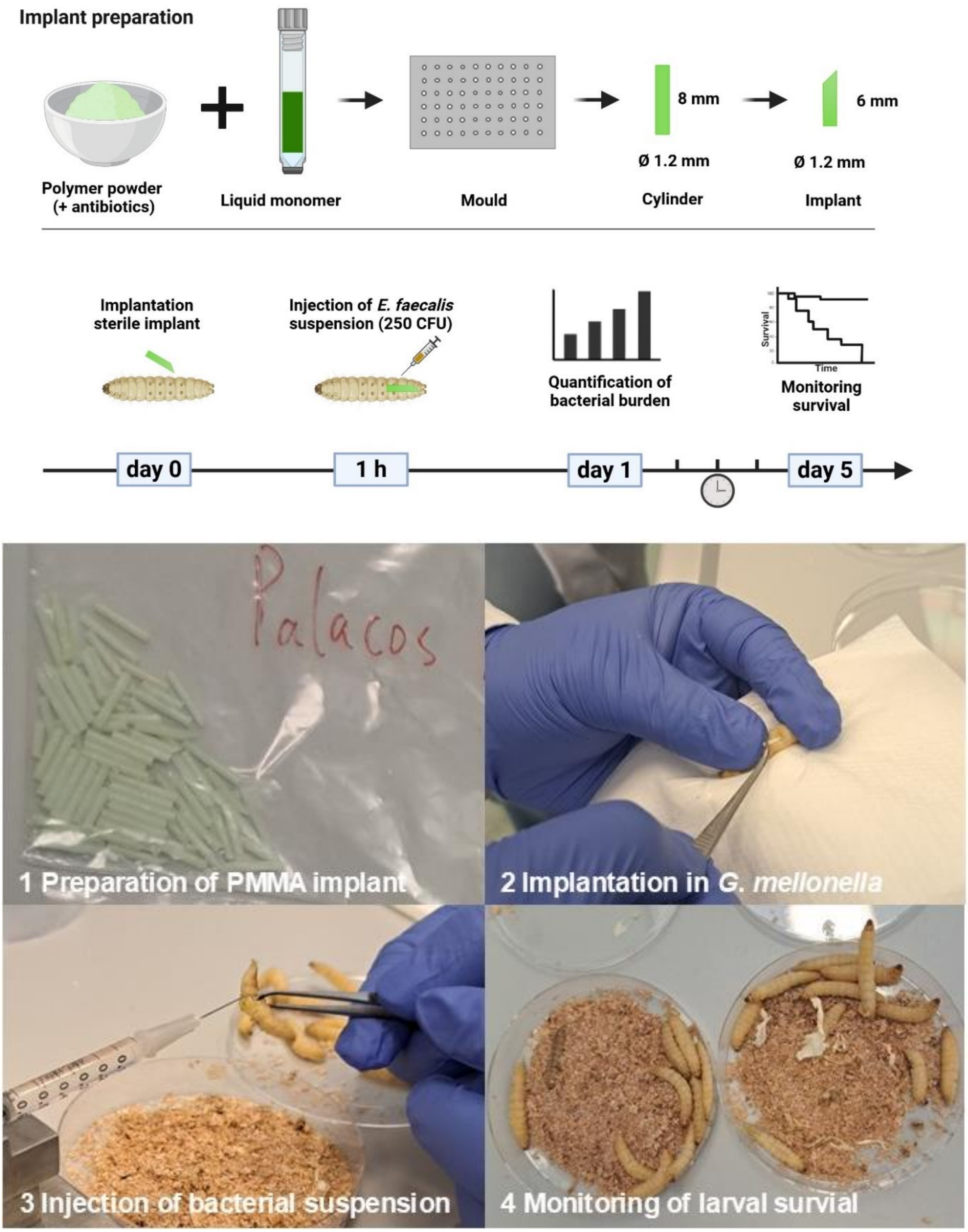


Figure 14 | Preparation and experimental use of PMMA cement implants in the *Galleria mellonella* infection model. Cylindrical PMMA implants are fabricated using a Teflon mould and subsequently shaped to facilitate implantation into the larval body (1). The sterile implants are directly implanted in the larvae of *G. mellonella* (2). Larvae are inoculated with a bacterial suspension (e.g., *Enterococcus faecalis*) (3). Bacterial burden is quantified by homogenizing larvae and determining colony-forming units (CFU). Larval survival is monitored over a 5-day observation period to assess the infection-preventive efficacy of antibiotic-loaded implants (4). Own illustration based on Zhao et al. (2024).

An advanced method for evaluating the infection-preventive properties of ALBC is the in-vivo biofilm model using larvae of the greater wax moth (*Galleria mellonella*) (Glavis-Bloom et al., 2012; Wilson-Sanders, 2011; Zhao et al., 2024). In this approach, sterile PMMA cement implants are inserted into the larval body, followed by inoculation with clinically relevant bacterial strains (**Figure 14**). This setup mimics the early postoperative scenario where an implant is at risk of colonisation. Antibiotic-loaded implants are then assessed for their ability to inhibit bacterial adhesion and subsequent biofilm formation on the implant surface. Key endpoints include larval survival, reduction in bacterial burden, and prevention of biofilm development compared to antibiotic-free controls (**Figure 14**). The model provides a dynamic environment with host immune interactions, offering a more realistic representation of how ALBC can prevent implant-associated infections rather than merely treating established biofilms (Mannala et al., 2021; Tsai et al., 2016; Zhao et al., 2025; Zhao et al., 2024).

5. Rationale for antibiotic admixing in acrylic bone cements

5.1. Limitations of commercially available ALBC

As described in the Chapter 4.3. (II. Introduction, pp. 24-28), commercially available ALBC formulations typically contain gentamicin, tobramycin, clindamycin, or vancomycin. These antibiotics do not provide sufficient coverage against all clinically relevant pathogens, including MDR bacterial and fungal species. Although fungal PJIs are relatively rare, their incidence appears to be increasing, particularly in immunocompromised patients or those with multiple prior revisions (Sznajder et al., 2025). Candida species, especially *C. albicans* and *C. parapsilosis*, are the most implicated pathogens, and their management is complex due to limited systemic treatment options, prolonged therapy requirements, and the lack of any commercially available PMMA cement containing antifungal agents (Sambri et al., 2022). As a result, antifungal agents such as amphotericin B or voriconazole have been incorporated into PMMA spacers in selected cases, although data on their elution behaviour and long-term stability remain limited (Czuban et al., 2019; Frank et al., 2025; Krampitz et al., 2023).

This lack of coverage for certain pathogens becomes particularly relevant during septic revision surgery, especially within the interim phase of a two-stage revision. In such cases, PMMA spacers are frequently loaded with at least two antibiotics, and occasionally additional agents, to adequately address the microorganisms identified through microbiological diagnostics (Prats-Peinado et al., 2024). Consequently, especially in specialised revision centres, surgeons routinely supplement PMMA cements with additional anti-infective substances when commercially available formulations do not provide the required antimicrobial spectrum. These clinical practices are well documented in the literature, and established guidelines exist for the diagnosis and treatment of PJI (Hertzberg-Boelch et al., 2022; Lüdemann et al., 2023; Izakovicova et al., 2019; Mühlhofer et al., 2021; PIF, 2023). These guidelines provide detailed recommendations on the most appropriate antimicrobial substances for specific clinical scenarios and indicate which agents can be added to PMMA cement. The Pro-Implant Foundation (PIF) has published a pocket guide with detailed recommendations for revision protocols and corresponding systemic and local antibiotic regimens (PIF, 2023).

Enterococci are responsible for approximately 3–10 % of PJIs, and the management of these infections remains challenging due to high failure rates and frequent relapses (Almeida-Santos et al., 2025; Patel, 2023; Rupp et al., 2021a). The underlying reasons for treatment failure in enterococcal implant-associated infections are not yet fully understood. But contributing factors are thought to include the limited bactericidal activity of β-lactam and glycopeptide antibiotics against enterococci, antimicrobial tolerance, intracellular persistence, and the absence of potent antibiofilm-active agents effective against these organisms. Treatment complexity is further increased by the ability of enterococci to acquire resistance determinants against β-lactam, glycopeptide, aminoglycoside, and oxazolidinone antibiotics (Tafin et al., 2011). As a result, surgeons often employ non-standardised therapeutic strategies because the optimal combination of surgical and antimicrobial management for enterococcal PJI remains uncertain. Nevertheless, susceptibility to vancomycin and daptomycin among enterococcal isolates in PJI remains high. Renz et al. (2019) investigated 75 episodes of enterococcal PJI: 41 in hip prostheses, 30 in knee prostheses, two in elbow prostheses, and two in shoulder prostheses. *Enterococcus faecalis* was identified in 64 episodes, *E. faecium* in 10 episodes, and *E. casseliflavus* in one case. Approximately half of these infections were polymicrobial. In total the overall treatment success rate was high (84%). Taken together, these findings indicate that although enterococcal PJI presents notable clinical challenges, consistent surgical and antimicrobial strategies can achieve favourable outcomes, with failures occurring primarily within the first three years and only a minority of patients developing subsequent infections caused by different pathogens.

5.2. Challenges of anti-infective admixing

Commercially available ALBCs do not provide comprehensive antimicrobial coverage against all possible PJI germs, particularly against MDR bacteria, or against fungal pathogens. Consequently, the manual admixing of additional anti-infective agents is often required to achieve adequate pathogen-specific coverage. However, surgeons are not always aware of which anti-infective substances can be safely incorporated into PMMA cement, which dosages are appropriate for fixation versus spacer cement, or which material-related specifications must be considered during admixing.

Although the incorporation of additional antimicrobials has become routine practice in many revision centres, the process itself is more complex than often assumed, and there is no universally standardised protocol or structured training. Institutions such as the Pro-Implant Foundation (PIF, 2023, 2025) provide valuable instruction, but access is limited, resulting in inconsistent dissemination of practical knowledge and persistent uncertainty among surgeons performing manual admixing (**Figure 15**).

Several critical factors must be addressed when modifying PMMA cements. From a regulatory standpoint, once anti-infective agents are manually added, the surgeon becomes the legal “manufacturer” of the modified cement and assumes responsibility for its performance (Heraeus Medical, 2025; Malhotra et al., 2018). Mechanically, PMMA cement stability is negatively influenced by the addition of powdery antimicrobial agents, to the extent of weakening depending on both the total amount and the specific physicochemical properties of the substance (Egger et al., 2024; Kühn, 2014; Persson et al., 2006). For instance, the incorporation of antifungal agents such as voriconazole can significantly compromise mechanical strength (Krampitz et al., 2023). To maintain acceptable mechanical properties, it is generally recommended that the total amount of admixed anti-infective powder does not exceed approximately 10% of a 40 g cement powder portion, including the antibiotics already premixed by the manufacturer (Kühn, 2014). Thus, careful calculation of the total additive load is essential.

In addition to mechanical considerations, the elution behaviour of antimicrobial agents is highly relevant. Commercially manufactured ALBCs typically show more predictable and often more efficient elution profiles because their formulations are engineered for optimal porosity, powder dispersion, and polymer–monomer interactions. In contrast, elution from manually admixed cements is more variable and strongly dependent on the homogeneity of powder distribution, the solubility of the chosen anti-infective, and the degree of porosity introduced during mixing (Kittinger et al., 2024a; Kwong et al., 2024). Inadequate homogenisation can reduce the release surface area, leading to subtherapeutic elution, whereas excessive additive loading may increase porosity and cause an initial burst release followed by insufficient sustained concentrations. Additionally, the antibiotic elution is not directly proportional to the amount of antibiotics added (Warwick et al., 2024). Understanding these pharmacokinetic differences is crucial to ensure that local antibiotic levels exceed the MIC of the targeted pathogens throughout the required treatment window.

Anti-infective agents selected for incorporation should ideally be sterile and water-soluble to enable effective diffusion from the PMMA matrix. Surgeons must also be aware that certain antibiotics remain unsuitable for admixing despite microbiological susceptibility, as some, including rifampicin, interfere with PMMA polymerisation, making their incorporation more complex than other antibiotics (Sanz-Ruiz et al., 2018).

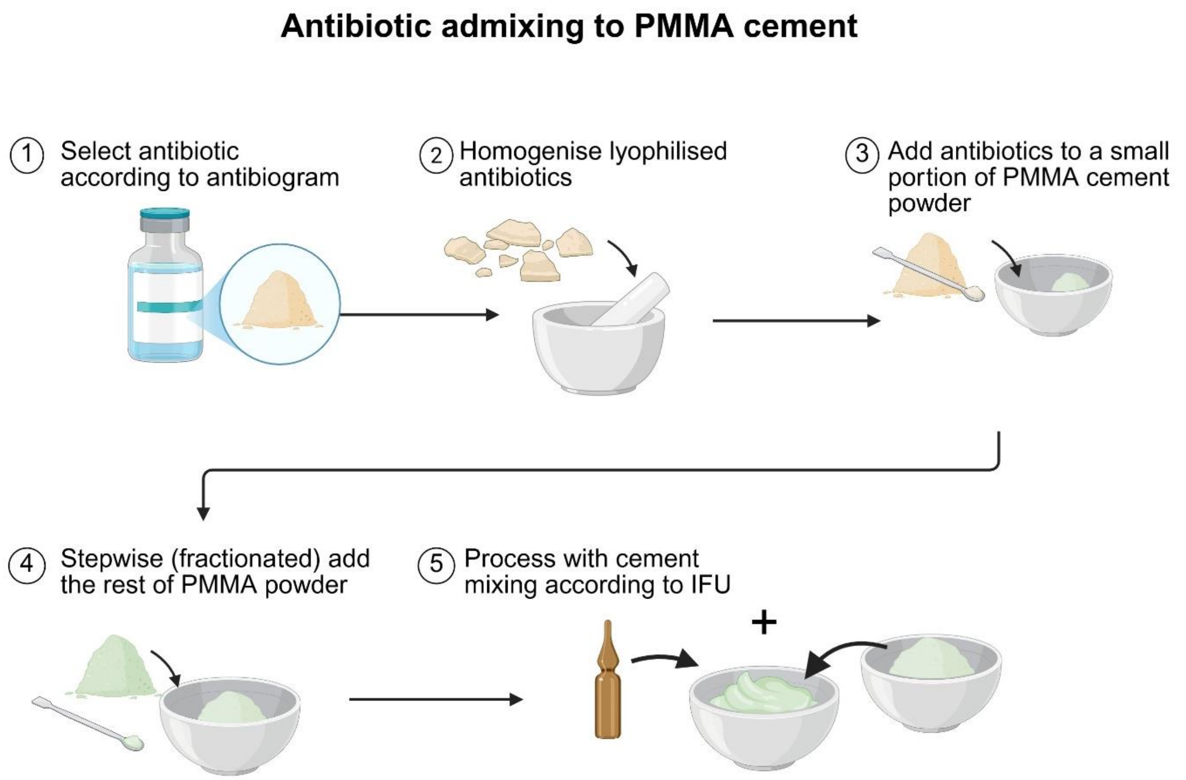


Figure 15 | Fractionated admixing of an anti-infective agent into PMMA cement. Select a sterile antibiotic in powder form based on the antibiogram (1). If the antibiotic is supplied as a lyophilised agent, pulverise it to obtain a fine, homogenous powder (2). Combine the antibiotic with an equal portion of PMMA cement powder and mix thoroughly to create a uniform pre-mixture (3). Gradually add the remaining PMMA powder in small portions to the pre-mixture, ensuring continuous homogenisation until all powder is incorporated (4). Proceed with cement preparation following the manufacturer's instruction for use (IFU) (5). Own illustration based on Kühn (2014).

To achieve optimal distribution of anti-infective agents, fractionated admixing is strongly recommended (**Figure 15**) (Kühn, 2014; PIF, 2025). In this technique, the selected antibiotic quantity is first mixed thoroughly with a very small portion of the PMMA powder (e.g., 1 g antibiotic with 1 g cement powder) to create a homogeneous pre-mix. Prior to this the granulated or lyophilised antibiotic is grounded first. This pre-mix is then incrementally combined with additional small portions of cement powder until the entire powder component has been evenly homogenised. Only after this step the pre-mix is added to the liquid monomer. Although fractionated admixing is time-consuming, particularly in the intraoperative setting, it ensures uniform distribution of the antimicrobial agent. In contrast, directly mixing the full antibiotic dose into the entire powder portion often results in inhomogeneity, reduced mechanical stability, and unpredictable elution profiles, including undesirable burst release or insufficient long-term release.

5.3. Challenges of resistant bacteria

A wide range of microorganisms can cause PJI; particularly difficult to treat are the so-called ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp*.) which are collectively recognised for their capacity to "escape" the effects of conventional antimicrobial therapy and for their association with multi-drug resistance (Anemüller et al., 2019; Miller & Arias, 2024). Within the context of PJI, staphylococci and enterococci remain the most prevalent and clinically most significant organisms. Their increasing share of resistant strains is causing the biggest headaches with clinicians (II. Introduction, Chapter 2.2., pp. 10-12).

Biofilm formation adds a further layer of complexity to antimicrobial resistance in PJI. Both MRSA and CoNS form highly structured, polysaccharide-rich biofilms on implant surfaces with formation of biofilm-tolerant small colony variants and persister cells (Chapman et al., 2024; Conlon, 2014; Sauer et al., 2022).

Staphylococcus aureus exhibits an extensive repertoire of virulence factors, including adhesins, cytolytic toxins, exoenzymes, immune-evasion mechanisms, and superantigens, all of which facilitate adherence, invasion, and survival within host tissues (Urish & Cassat, 2020). MRSA is one of the most frequent causes of antibiotic-resistant healthcare-associated infections worldwide. First identified in the 1960s, MRSA spread rapidly through the 1990s and is now endemic in hospitals globally, with additional emergence of community-associated strains across many regions, including Europe (ECDC, 2024). *S. aureus* is a major cause of bacteraemia, endocarditis, skin and soft-tissue infections as well as PJI. It remains the most common pathogen responsible for postoperative wound infections. Transmission frequently occurs via fomites, including clothing and personal devices, and colonisation may persist for prolonged periods. Despite declining incidence in certain regions, e.g. Germany (current prevalence of 4%), MRSA continues to pose a major clinical threat due to its persistent morbidity and mortality (**Figure 16**). WHO still tracks its prevalence globally, which highlights its clinical importance in hospitals and communities (WHO, 2023). Some regions continue to suffer under the MRSA burden with a high prevalence, e.g. India (56%), and the U.S. (38%) (**Figure 16**). Even within Europe the geographic variability in MRSA prevalence is high with 11 of 44 reporting countries showing MRSA rates below 5% (e.g. the Netherlands, Sweden, Germany), but 13 reporting rates ≥25% (e.g. Italy, Greece) (ECDC, 2024; Turner et al., 2019).

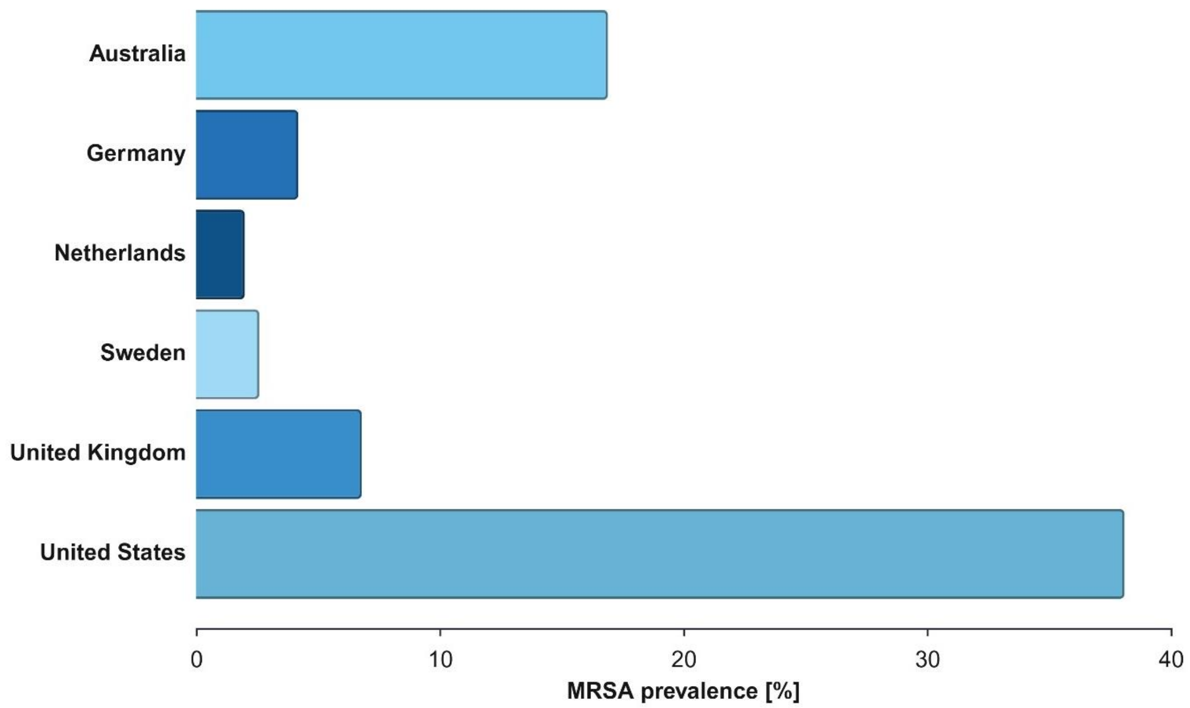


Figure 16 | Prevalence of methicillin resistant *S. aureus* (MRSA) reported by the World Health Organization (WHO): Australia (17%), Germany (4%), the Netherlands (2%), Sweden (3%), United Kingdom (7%) and the United States (38%) (WHO, 2023).

In Australia, the MRSA prevalence with 16% is higher than in the UK (7%) (WHO, 2023). Countries with a massive decline in MRSA prevalence over the last decade have successfully implemented aggressive “search-and-destroy” screening and containment strategies (Turner et al., 2019).

Methicillin resistance in *S. aureus* is mediated by the mecA gene carried on the SCCmec element, encoding the penicillin-binding protein PBP2a, which exhibits low affinity for β-lactam antibiotics and thereby confers resistance to the entire class. Additional resistance determinants, acquired through pathogenicity islands, phages, plasmids, and transposons, confer resistance to penicillin (blaZ), trimethoprim (dfrA, dfrK), macrolides and lincosamides (constitutive ermC), and tetracyclines (tetK, tetL) (Turner et al., 2019). Emerging vancomycin resistance is of particular concern. VISA typically arise after prolonged exposure, whereas VRSA results from acquisition of the vanA operon from *Enterococcus faecalis* (Hiramatsu, 2001). Fortunately, VRSA remains rare, with an estimated global prevalence of 1.5% among 5,855 *S. aureus* isolates in a recent meta-analysis (Shariati et al., 2020; Turner et al., 2019).

In studies of bone and joint infections, including PJI, MRSA and CoNS accounted for 45% of isolates in an Australian multicentre analysis. They are also frequent in polymicrobial infections (36%), especially in those involving Gram-negative bacilli and enterococci (Peel et al., 2012). Evidence from randomized trials or large cohort studies guiding optimal antimicrobial therapy for MRSA and CoNS in PJI remains limited. Current international guidelines recommend vancomycin as first-line therapy, with daptomycin or linezolid as alternatives (Anemüller et al., 2019; Le Vavasseur & Zeller, 2022; Osmon et al., 2013). For MRSA implant-associated osteomyelitis, the Infectious Diseases Society of America (IDSA) recommends intravenous vancomycin or daptomycin combined with rifampicin for the initial two weeks, followed by rifampicin plus an oral companion agent, fluoroquinolone, trimethoprim–sulfamethoxazole, tetracycline, or clindamycin, for a total of 3–6 months in early-onset infections (Urish & Cassat, 2020). In contrast, MSSA remains susceptible to methicillin, oxacillin, and cefoxitin (Turner et al., 2019). The implication of MRSA in bone and joint infections has demonstrated a lower treatment success (Urish & Cassat, 2020).

5.4. Growing prevalence of vancomycin-resistant enterococci

Next to staphylococci, enterococci represent one of the most clinically significant groups of nosocomial MDR organisms. Like MRSA, infections caused by enterococci predominantly affect vulnerable patient populations with multiple comorbidities, prolonged hospital stays, and frequent exposure to invasive procedures. Their relevance is particularly pronounced in immunocompromised individuals, critically ill patients, and those with intravascular or orthopaedic implants. Unlike staphylococci and streptococci, enterococci do not produce potent pro-inflammatory toxins; instead, their pathogenicity is largely mediated by a diverse range of adhesion proteins and biofilm-associated properties that facilitate adherence, colonisation, and persistence on host tissues and medical devices. Within this genus, *Enterococcus faecium* has emerged as the most clinically challenging species (Almeida-Santos et al., 2025; Arias & Murray, 2012; Radford-Smith & Anthony, 2025).

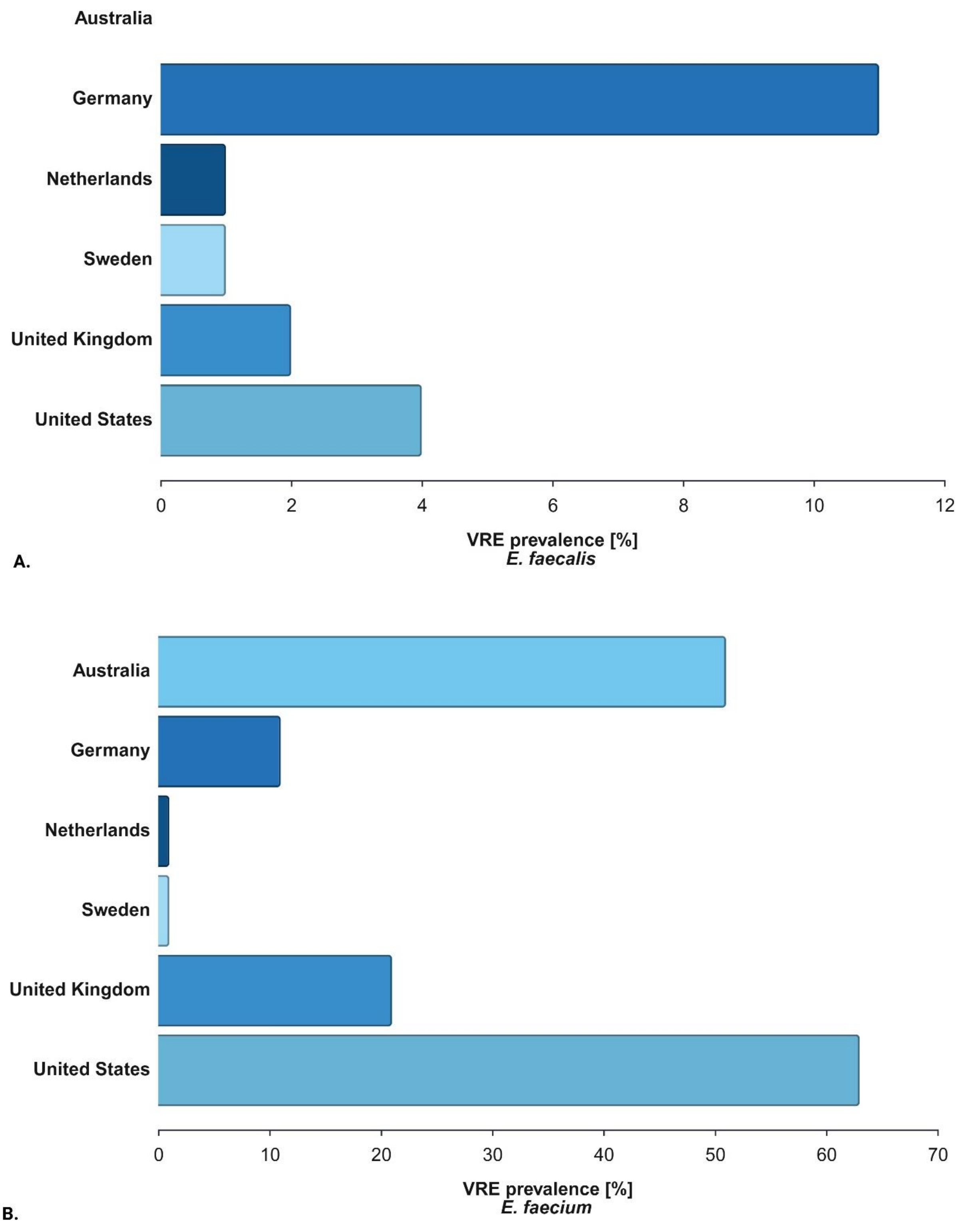


Figure 17 | Prevalence of vancomycin-resistant *E. faecalis* (VRE) (A) reported by the World Health Organization (WHO): Australia (0%), Germany (11%), the Netherlands (1%), Sweden (1%), United Kingdom (2%) and the United States (4%). Prevalence of vancomycin-resistant *E. faecium* (VRE) (B) reported by the World Health Organization (WHO): Australia (51%), Germany (11%), the Netherlands (1%), Sweden (1%), United Kingdom (21%) and the United States (63%) (WHO, 2023)

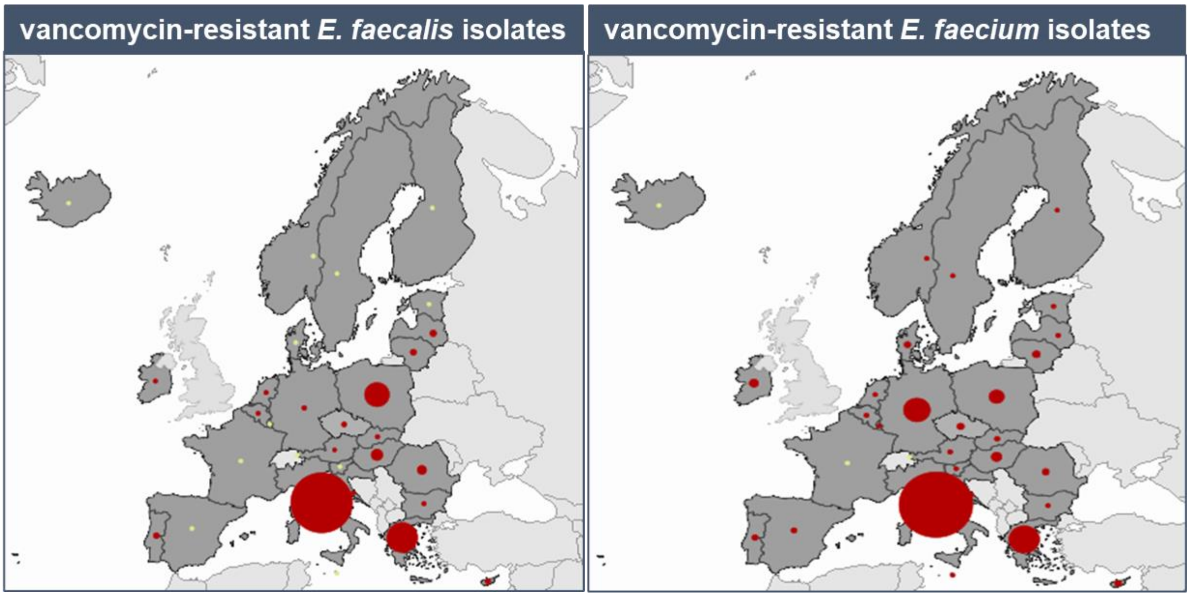


Figure 18 | Number of vancomycin-resistant *Enterococcus faecalis* and *Enterococcus faecium* isolates across Europe, based on surveillance data reported by the European Centre for Disease Prevention and Control (ECDC) (ECDC, 2024).

E. faecium is a commensal organism residing in the human gastrointestinal tract. Although typically of low pathogenic potential, it can cause severe infections such as bloodstream infections, endocarditis, and peritonitis. Vancomycin resistance in *E. faecium* varies widely across Europe, ranging from <1% (Finland, France, Luxembourg, the Netherlands, Sweden) to ≥50% (Croatia, Cyprus, Greece, Lithuania) (**Figure 17, 18**) (ECDC, 2024). Intermediate levels are observed in Germany (~11%) (ECDC, 2024; Markwart et al., 2019). In contrast, resistance rates are markedly higher in many non-European regions, including Australia (~51%) and the United States (65%) (**Figure 17**) (CDC, 2023b; NSQHS, 2023).

E. faecalis, another common inhabitant of the gastrointestinal tract, generally exhibits lower vancomycin resistance rates than *E. faecium*, but still poses major clinical challenges due to its intrinsic antimicrobial tolerance and strong capacity for biofilm formation. *E. faecalis* is a well-known pathogen in urinary tract infections, endocarditis, dental infections, and healthcare-associated infections. Increasing rates of vancomycin-resistant *E. faecalis* have been reported in several southern European countries, including Italy and Greece (**Figure 18**) (Ayobami et al., 2020). Conversely, no vancomycin-resistant *E. faecalis* was detected in recent surveillance data from Australia (NSQHS, 2023), while lower rates were reported in the UK (2%) (UK Health Security Agency, 2021) and the U.S. (4%) (**Figure 17**) (CDC, 2023a).

Enterococci have demonstrated the capacity to acquire resistance to nearly every antimicrobial agent used against them. Vancomycin was widely employed from the mid-20th century for ampicillin-resistant enterococci and β-lactam–allergic patients without notable resistance until the mid-1980s. High-level resistance is believed to originate from horizontal gene transfer from environmental *Paenibacillus species*. Resistance is mediated by amino acid substitutions in the D-Ala-D-Ala termini of peptidoglycan precursors, reducing vancomycin binding affinity (**Figure 19, 20**) (Arias & Murray, 2012). The two principal operons are VanA, conferring high-level resistance to vancomycin and teicoplanin, and VanB, causing variable vancomycin resistance without affecting teicoplanin susceptibility (Dubin & Pamer, 2014).

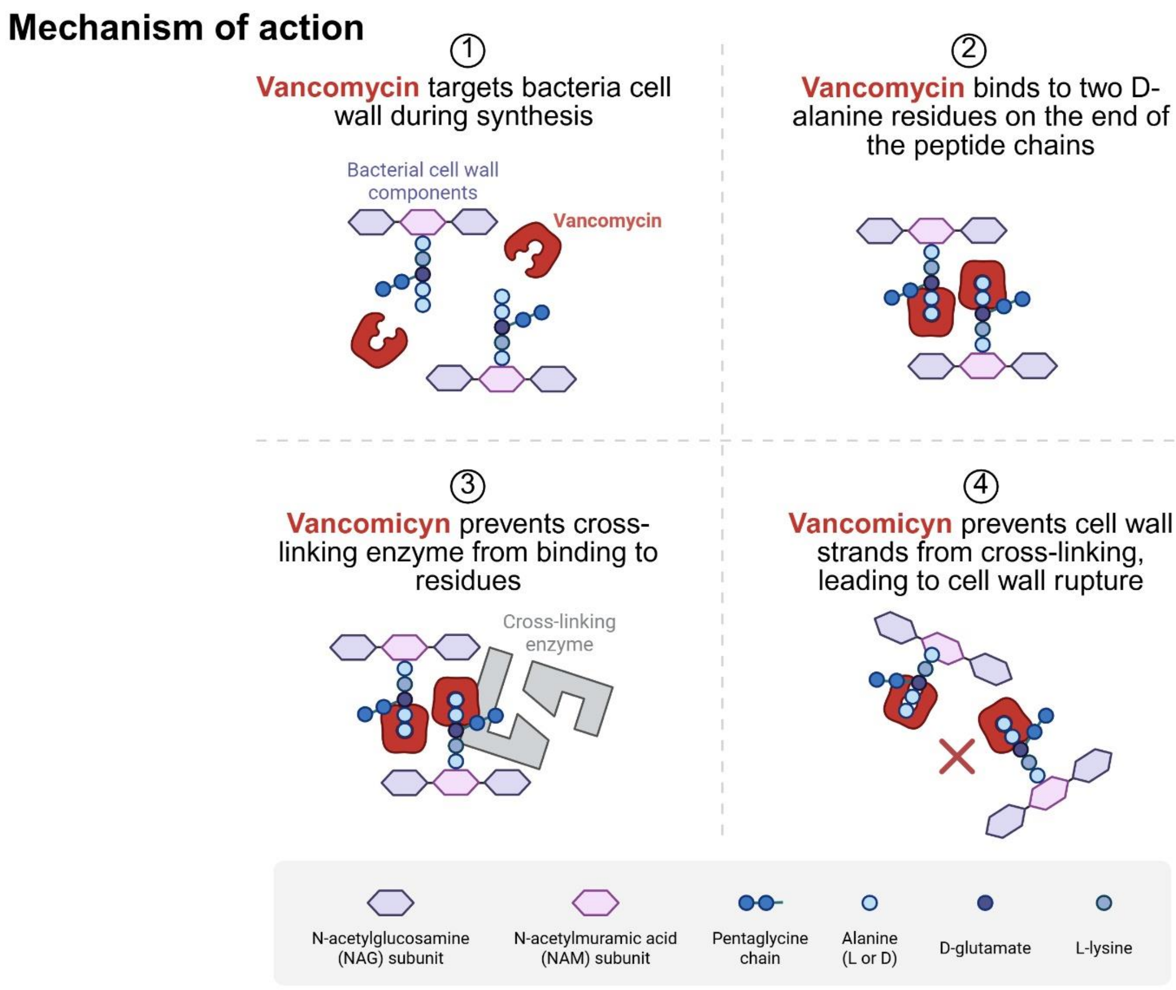


Figure 19 | Mechanism of action of vancomycin. Vancomycin binds to the D-Ala-D-Ala termini of peptidoglycan precursor chains, preventing the cross-linking enzymes from accessing their substrates. This inhibition ultimately disrupts cell-wall synthesis and leads to bacterial cell-wall rupture. Illustration from BioRender Science Templates based on Kwun et al. (2013).

Importantly, VRE also exhibit pronounced biofilm-associated tolerance, which is distinct from genetically encoded resistance. Biofilm formation reduces metabolic activity, limits antibiotic penetration, and supports the survival of persister cells (Le Pont et al., 2024). This tolerance significantly contributes to chronicity and treatment failure in PJI, even when isolates appear susceptible in-vitro, and underscores the need for combined surgical and antimicrobial strategies.

Therapeutic options for VRE are considerably more limited than for vancomycin-susceptible strains, and high-quality evidence from RCTs or large cohorts is lacking. International guidelines consistently identify linezolid and high-dose daptomycin as the principal systemic agents for invasive VRE infections (Boulekbache et al., 2024; Chang et al., 2017; Wilcox, 2003). Linezolid remains the only FDA (U.S. Food and Drug Administration) approved drug for VRE; however, its bacteriostatic activity and risk of time-dependent bone marrow suppression limit its utility for the prolonged treatment durations required in osteomyelitis and PJI (Bender et al., 2018). Daptomycin provides rapid bactericidal activity, but VRE typically display higher MIC values than other Gram-positive pathogens, requiring doses of 10–12 mg/kg/day. Combination therapy with β-lactam antibiotics may further enhance bactericidal activity by reducing the bacterial surface charge and improving daptomycin binding (La & Kim, 2022). Broader reviews highlight ongoing challenges, including emerging resistance to last-line agents and the need for therapeutic drug monitoring for linezolid, daptomycin, and teicoplanin to optimize dosing and minimise toxicity (Cairns et al., 2023). As with all forms of PJI, effective antimicrobial therapy must be combined with appropriate surgical management, including debridement, implant retention or exchange strategies, tailored to infection duration, microbial factors, and patient comorbidities. Medical therapy alone is insufficient to eradicate biofilm-associated VRE.

The rise in VRE also impacts decisions regarding local antibiotic therapy in revision surgery. In clinical practice, surgeons sometimes increase vancomycin concentrations in PMMA spacers up to 4 g per 40 g of polymer powder; however, such dosages significantly compromise mechanical stability and heighten the risk of systemic nephrotoxicity. According to the Pocket Guide to Diagnosis & Treatment of PJI from the Pro-Implant Foundation (PIF, 2023), up to 2 g daptomycin may be added to fixation cement and up to 3 g to spacer cement, while higher concentrations (>2 g) lead to non-ISO compliant mechanical properties.

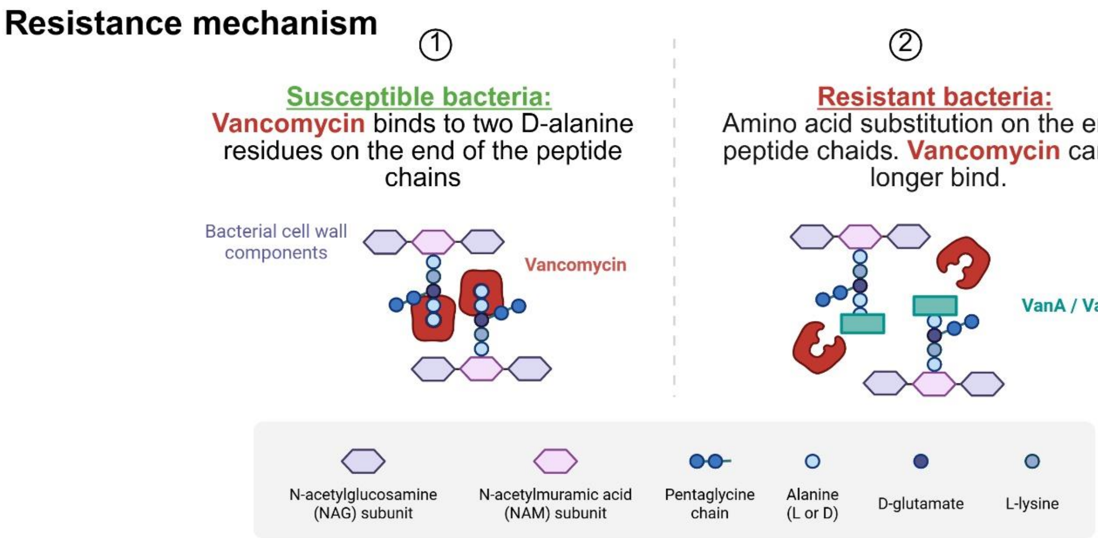


Figure 20 | Vancomycin resistance mechanism. Vancomycin resistance is mediated by an amino-acid substitution at the terminus of the peptidoglycan precursor, which markedly reduces vancomycin's binding affinity and prevents effective target engagement. Own illustration based on Radford-Smith & Anthony (2025).

6. Daptomycin as an antibiotic option in acrylic bone cements

6.1. Chemistry and pharmacology

Daptomycin is considered a last resort antibiotic for the treatment of infections caused by Gram-positive pathogens, most notably *Staphylococcus aureus* (including MRSA) and VRE (Gray & Wenzel, 2020; Grein et al., 2020). Clinically, it is primarily indicated for the management of complicated skin and soft-tissue infections, bacteraemia, and right-sided infective endocarditis (Gray & Wenzel, 2020; Wang et al., 2014).

Structurally, daptomycin is a cyclic lipopeptide composed of 13 amino acids, featuring a hydrophilic, water-soluble core and a lipophilic acyl side chain (**Figure 21**). The N-terminus consists of a 13-amino-acid peptide linked to a decanoyl fatty acid chain, whereas the C-terminal amino acid is connected through an ester bond to the hydroxyl group of the side chain. Its International Union of Pure and Applied Chemistry (IUPAC) name is N-decanoyl-L-tryptophyl-D-asparaginyl-L-aspartyl-L-threonylglycyl-L-ornithyl-L-aspartyl-D-alanyl-L-aspartylglycyl-D-seryl-threo-3-methyl-L-glutamyl-3-anthraniloyl-L-alanine-lactone, and its empirical formula is $C_{72}H_{101}N_{17}O_{26}$, corresponding to a molecular mass of approximately 1,620 Dalton (Sauermann et al., 2008; Tedesco & Rybak, 2004).

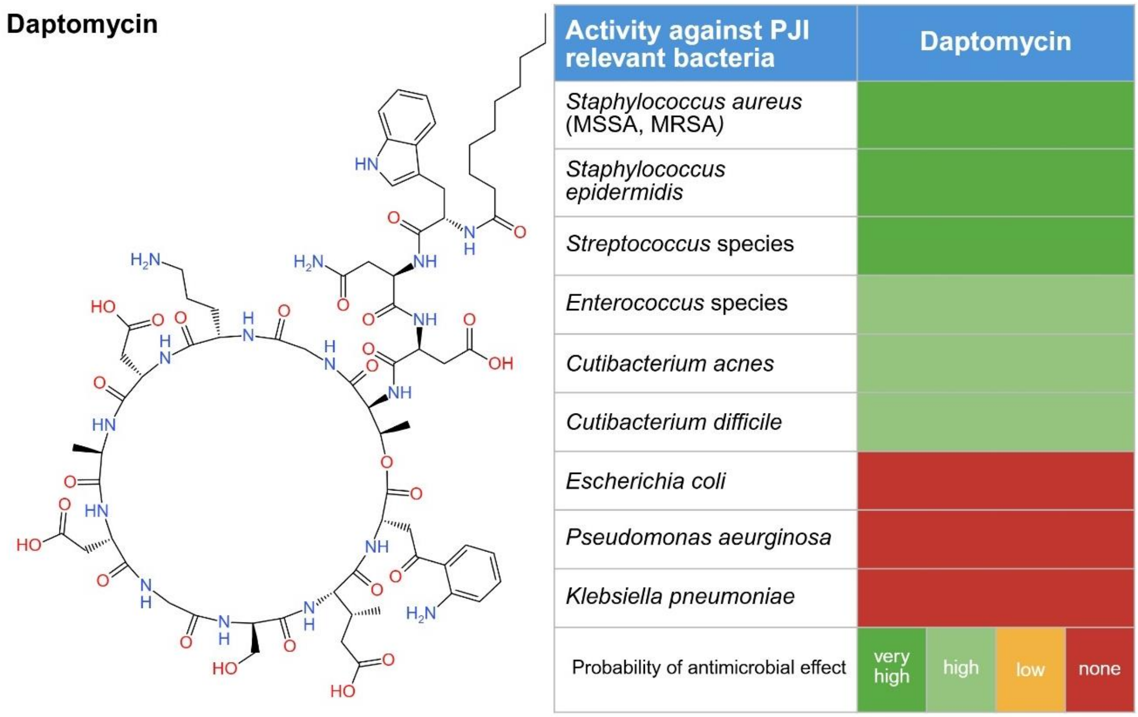


Figure 21 | Chemical structure of daptomycin. Their predicted antimicrobial activity against clinically relevant pathogens in PJI, based on susceptibility data from EUCAST breakpoints (EUCAST, 2025b). Colour coding indicates the probability of effective antimicrobial activity: Dark green = very high probability. Light green = high probability. Orange = low probability. Red = no probability.

The development of this calcium-dependent, membrane-binding cyclic lipopeptide began in the 1980s, when daptomycin was isolated from *Streptomyces roseosporus*, a Gram-positive soil actinomycete (D'amato et al., 1975; EMA, 2022; Gray & Wenzel, 2020). After promising early studies, progress was interrupted because high-dose administration in animal and initial clinical trials resulted in adverse effects, particularly myopathy, which temporarily stopped further development. With the increasing prevalence of bacterial resistance in the late 1990s, including MRSA, glycopeptide-intermediate *S. aureus* (GISA), and glycopeptide-resistant enterococci (GRE), especially *E. faecium*, the need for alternative treatment options prompted renewed interest in daptomycin. Other Gram-positive species, such as CoNS and *Corynebacterium spp*., had also become problematic due to the acquisition of multiple resistance determinants. Following its re-evaluation, daptomycin received approval from the FDA and was introduced to the market in 2003 as the first agent of a new class of cyclic lipopeptides, representing the last time when a new antibiotic class reached the market (Gray & Wenzel, 2020). In Europe, the first authorization was granted in 2006 (EMA, 2022; Salzberger & Heinzl, 2007).

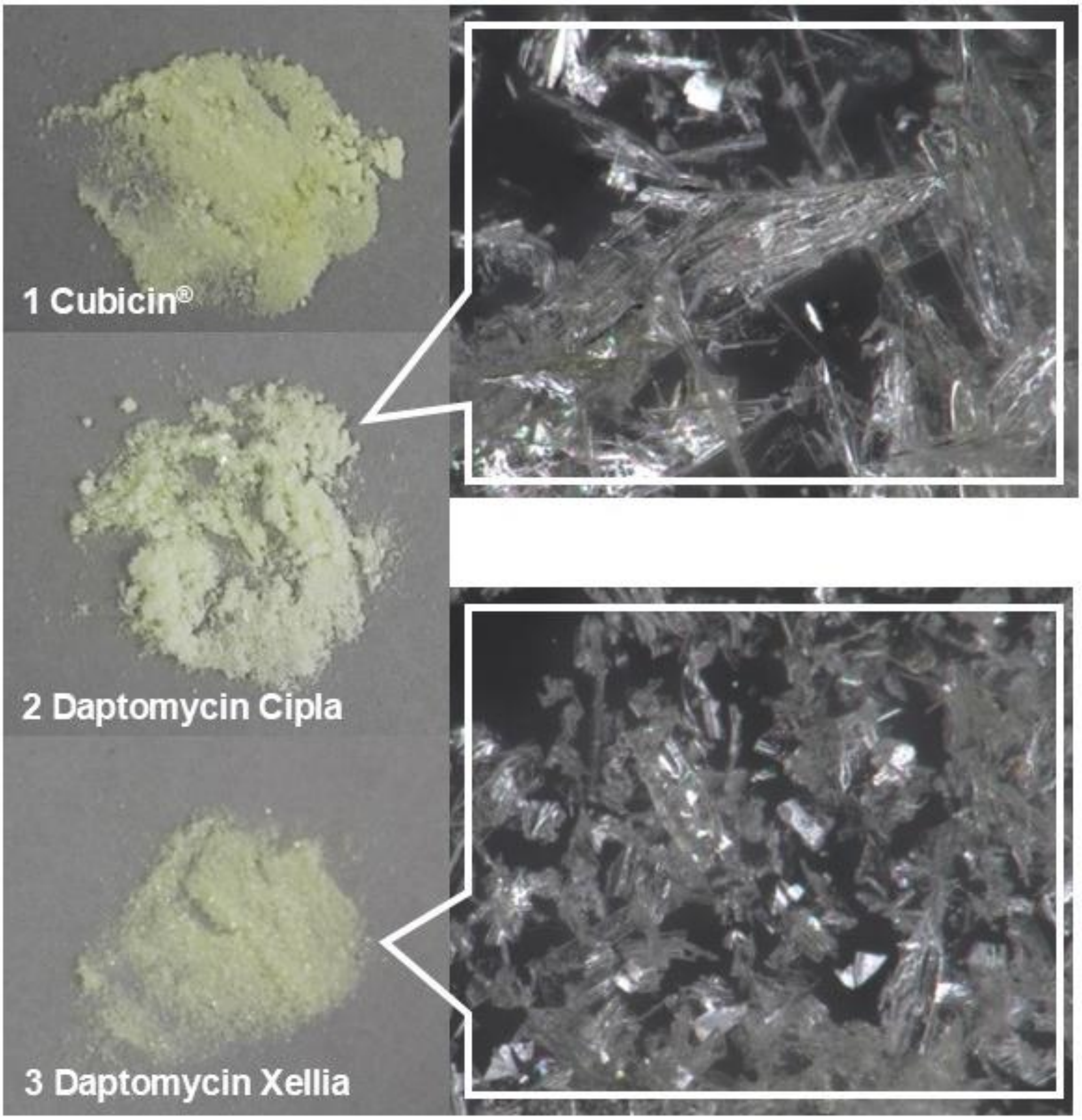


Figure 22 | Macroscopic appearance of daptomycin powders obtained from three different manufacturers: (1) Cubicin® (MSD Sharp & Dohme GmbH, Munich, Germany), (2) Daptomycin Cipla (Cipla Europe NV, Antwerp, Belgium), and (3) Daptomycin Xellia (Xellia Pharmaceuticals Ltd., Budapest, Hungary). Manufacturer-dependent differences in powder morphology are also evident upon microscopic examination (Keyence digital microscope model series VHX-6000; magnification ×200).

Daptomycin demonstrates clinically relevant penetration into bone tissue (Grillon et al., 2019; Montange et al., 2014; Rosslenbroich et al., 2012). This aspect is particularly critical when antibiotics are applied locally for PJI prevention or treatment. Healthy cortical bone is inherently poorly vascularised, and infected bone tissue exhibits even further reduced perfusion due to inflammatory processes. Consequently, if an antibiotic does not achieve therapeutic concentrations within the bone, it cannot effectively eradicate the resident bacterial populations. Daptomycin is administered intravenously and dosed according to body weight. The currently approved regimen is 6 mg/kg for *S. aureus* bacteraemia, including right-sided endocarditis, and 4 mg/kg for complicated skin and skin-structure infections (MSD, 2022).

Daptomycin is currently available from several pharmaceutical manufacturers (**Figure 22**). The formulations most used for manual admixing are those originally intended for the preparation of intravenous injection solutions. These include the lyophilised reference product Cubicin® (MSD Sharp & Dohme GmbH, Munich, Germany) (MSD, 2022), as well as generic lyophilised formulations such as Daptomycin Cipla, which contains sodium hydroxide as an excipient for pH adjustment (Cipla Europe NV, Antwerp, Belgium) (Cipla, 2021), and Daptomycin-ratiopharm®, which also uses sodium hydroxide for pH regulation (ratiopharm GmbH, Ulm, Germany) (ratiopharm, 2022). Another daptomycin variant is produced by Xellia Pharmaceuticals (Xellia Pharmaceuticals Ltd., Budapest, Hungary) (Xellia, 2024). This formulation is likewise supplied as a lyophilised powder but is primarily intended for large-scale industrial manufacturing processes or chemical laboratory applications rather than routine clinical use (**Figure 22**).

6.2. Antimicrobial spectrum and activity

Daptomycin exhibits potent activity against a broad range of Gram-positive pathogens, including MRSA, MRSE, VISA, and VRE (La Plante & Rybak, 2004). It exerts rapid bactericidal effects by disrupting the integrity of the bacterial cytoplasmic membrane, leading to membrane depolarisation and subsequent inhibition of essential cellular processes. This distinct mechanism of action makes daptomycin an attractive candidate for incorporation into PMMA cement. In addition, it possesses antimicrobial activity against several anaerobic Gram-positive species, although it shows no activity against Gram-negative bacteria (Grein et al., 2020; La Plante & Rybak, 2004; Luther et al., 2014). The intrinsic resistance of Gram-negative bacteria is attributed to their outer membrane, which acts as a permeability barrier that daptomycin is unable to penetrate (Tedesco & Rybak, 2004).

Daptomycin demonstrates high efficacy against clinically relevant planktonic and biofilm-embedded Gram-positive pathogens, including *Staphylococcus spp*. (both methicillin- and vancomycin-resistant isolates), *Enterococcus spp*. (including vancomycin-resistant strains), and *Streptococcus spp*. (including penicillin-resistant isolates) (Anemüller et al., 2019; EMA, 2022; Tedesco & Rybak, 2004). Furthermore, several Gram-positive anaerobic bacteria, such as *Clostridium difficile*, *Clostridium perfringens*, *Propionibacterium ssp.*, have been shown to be susceptible to daptomycin (Tedesco & Rybak, 2004).

As introduced in Chapter 2.2. (II. Introduction, pp. 10-13), biofilm formation significantly complicates antimicrobial therapy because bacterial eradication often requires antibiotic concentrations 100- to 1,000-fold higher than the MIC. Biofilms also impair host immune defences, as their matrix restricts the penetration of antibodies and phagocytic cells. So, antibiotics with the ability to penetrate biofilms are of special interest: rifampicin, vancomycin, and daptomycin exhibit superior penetration into *Staphylococcus* biofilms (Boudjemaa et al., 2016; Domínguez-Herrera et al., 2012). Daptomycin is of particular interest as it is among the few antibiotics that demonstrate sufficient inhibitory activity against *S. aureus* biofilms derived from PJI isolates and can technically be admixed to PMMA cements (Boyer & Cazorla, 2021; Mandell et al., 2019).

The wild-type MICs for daptomycin are 0.5 µg/mL for *E. faecalis* and 2.0 µg/mL for *E. faecium* (Hindler et al., 2015). According to recent EUCAST data, the MIC distribution for *E. faecalis* ranges primarily from 0.25 µg/mL to 2 µg/mL, with most isolates exhibiting an MIC of 1 µg/mL and an ECOFF of 4 µg/mL (**Figure 23**). For *E. faecium*, MICs generally range from 0.5 µg/mL to 4 µg/mL, peaking at 2 µg/mL, with an ECOFF of 8 µg/mL. For MRSA, MICs fall between 0.25 µg/mL and 1 µg/mL, with the majority at 0.5 µg/mL and an ECOFF of 1 µg/mL (EUCAST, 2025b).

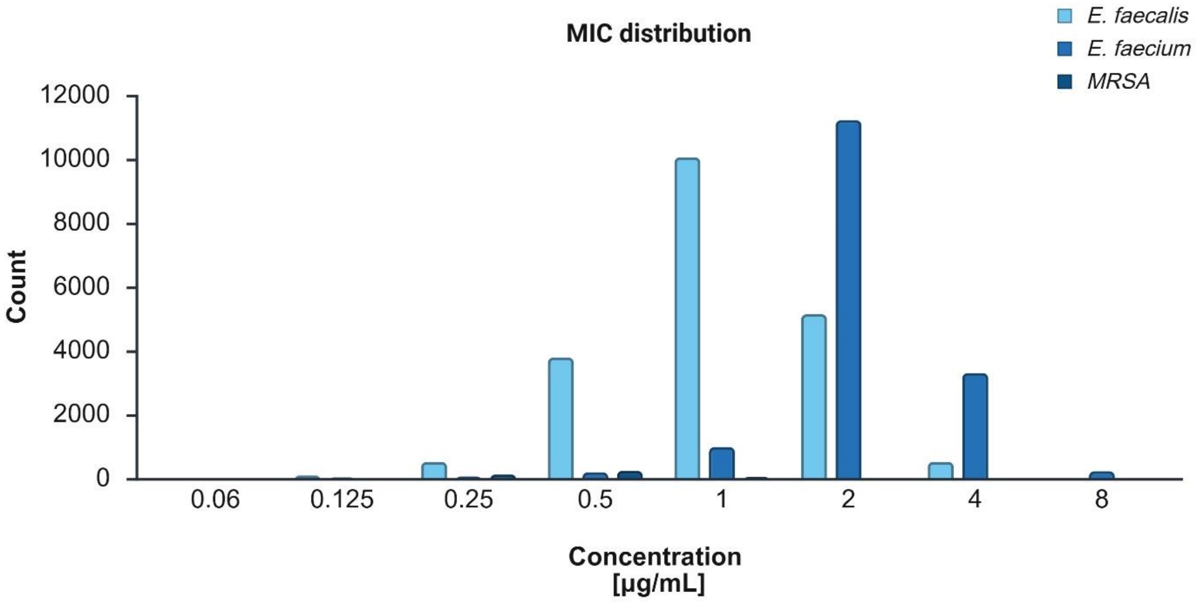


Figure 23 | Distribution of minimum inhibitory concentrations (MICs) of daptomycin against *Enterococcus faecalis*, *Enterococcus faecium*, and methicillin-resistant *Staphylococcus aureus* (MRSA), based on EUCAST MIC distribution data (EUCAST, 2025b).

6.3. Current understanding of the mode of action

Although daptomycin was discovered in the 1980s, its precise mechanism of action has long remained a matter of debate (Gray & Wenzel, 2020; Grein et al., 2020). Overall, daptomycin exerts rapid bactericidal activity primarily through disruption of multiple functional aspects of the bacterial cytoplasmic membrane.

As summarized by Grein and colleagues, early studies proposed that daptomycin interferes with peptidoglycan biosynthesis, accompanied by potassium efflux from *Staphylococcus aureus* cells. Subsequent investigations suggested effects on cell division as well as on the synthesis of secondary cell wall polymers. In parallel, several membrane-perturbing mechanisms were proposed, including induction of altered membrane curvature, membrane depolarisation, pore formation, and reorganization of local membrane architecture (Grein et al., 2020). While substantial evidence supports the bacterial cell membrane as the primary target of daptomycin, effects on the cell wall have also been reported (Pogliano et al., 2012). Notably, discrepancies between findings obtained in-vivo and in-vitro contributed to the ongoing controversy surrounding its mode of action (Gray & Wenzel, 2020).

The presence of physiological concentrations of free calcium ions (Ca^{2+}) is generally regarded as essential for the antibacterial activity of daptomycin (EMA, 2022; Pogliano et al., 2012). In contrast to most other lipopeptide antibiotics, daptomycin carries a net negative charge of −3 at neutral pH. Binding of Ca^{2+} partially neutralizes this charge and promotes oligomerisation of the peptide. A daptomycin-Ca^{2+} complex with an overall neutral charge is formed at a stoichiometry of 2:3 (daptomycin/ Ca^{2+}), which exhibits increased affinity for negatively charged phospholipids, particularly phosphatidylglycerol. It represents a major phospholipid component of Gram-positive bacterial membranes, and its presence was considered a prerequisite for daptomycin activity (Gray & Wenzel, 2020).

Based on these observations, two major mechanistic models have dominated the scientific discussion. The first, derived primarily from structural studies, proposes that Ca^{2+}-daptomycin complexes assemble into oligomeric aggregates that, upon interaction with phosphatidylglycerol containing membranes, rearrange into pore-like structures, leading to ion leakage and dissipation of membrane potential (**Figure 24**). The second model suggests that daptomycin inserts into specific phosphatidylglycerol (PG) enriched membrane domains, thereby disrupting essential processes such as cell wall biosynthesis and cell division.

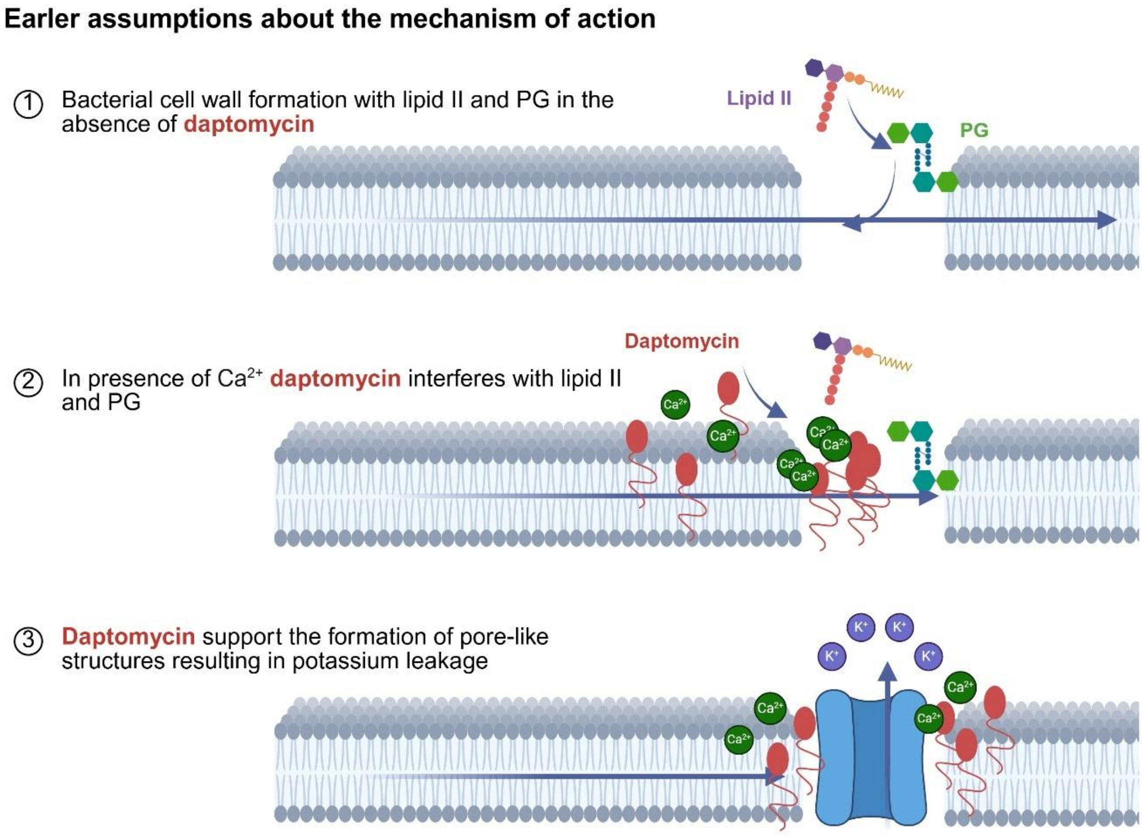


Figure 24 | Lipid II and the membrane phospholipid phosphatidylglycerol (PG) represent essential precursors in the bacterial cell wall biosynthesis pathway (1). Daptomycin exploits this vulnerability by binding to these key components, thereby interrupting cell wall assembly (2). This disruption compromises membrane integrity, leading to structural defects and subsequent ion efflux, including the release of potassium (3). Own illustration based on Pogliano et al. (2012).

In 2020, these previously competing hypotheses were reconciled by the work of Grein et al. Using microbiological and biochemical assays in combination with fluorescence microscopy and optical sectioning of intact staphylococcal cells and model membrane systems, they identified carrier-bound cell wall precursors as specific molecular targets of daptomycin (**Figure 25**). Ca^{2+}-daptomycin oligomers preferentially localize to the division septum, where they interact with undecaprenyl-linked cell envelope precursors in a PG-dependent manner. The formation of a tripartite complex consisting of Ca^{2+}-daptomycin, PG, and bactoprenylcoupled lipid intermediates results in inhibition of cell wall synthesis, and, upon prolonged exposure, extensive membrane rearrangements. Ultimately, daptomycin disperses throughout the cytoplasmic membrane, leading to disintegration of the membrane bilayer, membrane leakage, and cell death (Grein et al., 2020).

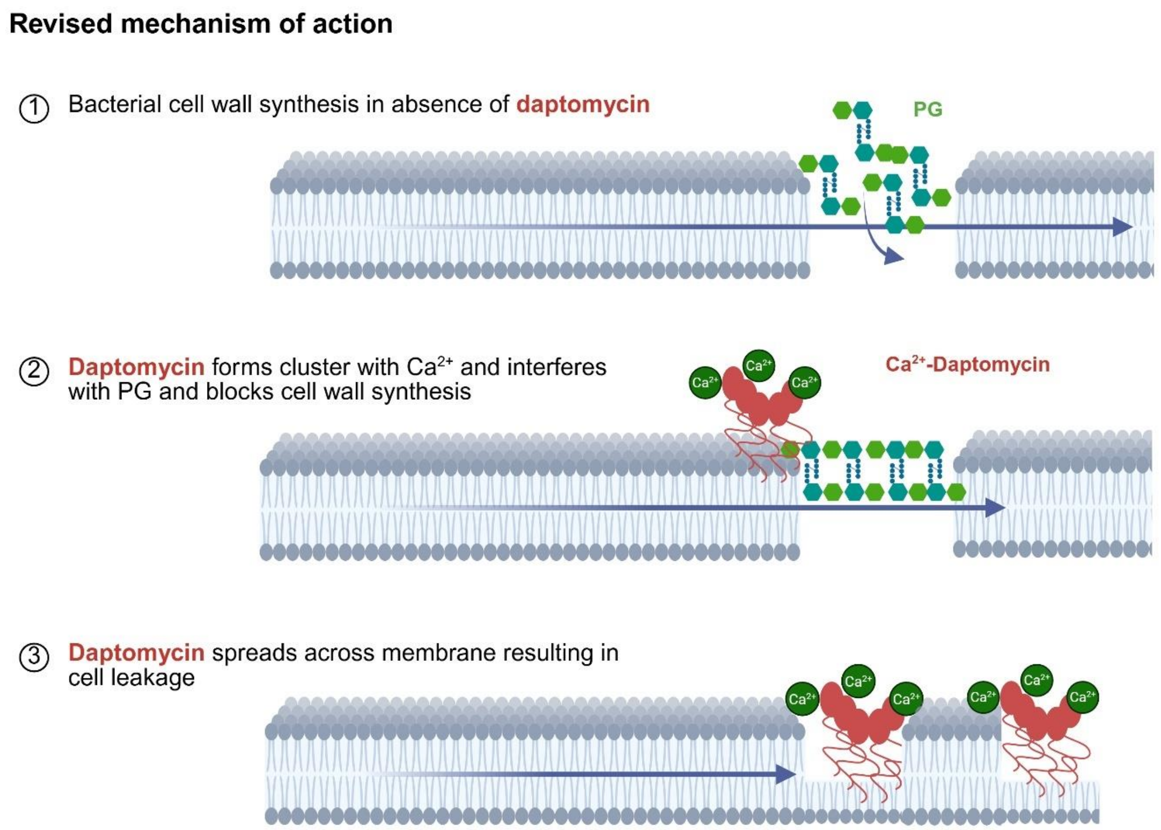


Figure 25 | In the absence of daptomycin, the cell wall synthesis is normal and new cell wall material is formed (1). When daptomycin binds calcium (forming Ca^{2+}-DAP), it forms clusters and accumulates at the division septum. This area is rich in negatively charged lipids, mainly phosphatidylglycerol (PG), and in cell wall precursors attached to bactoprenol. Ca^{2+}-daptomycin forms a three-component complex with PG and these bactoprenol-linked intermediates (2). These complex blocks cell wall synthesis. With continued daptomycin exposure, the Ca^{2+}-daptomycin spreads across the entire cell membrane and the membrane begins to break apart, leading to leakage of cell contents and ultimately cell death (3). Own illustration based on Grein et al. (2020) and Gray & Wenzel (2020).

Owing to its distinct mechanism of action, daptomycin retains activity against bacterial strains resistant to other major classes of antibiotics, including β-lactams (such as methicillin), glycopeptides (e.g., vancomycin), quinupristin/dalfopristin, linezolid, and other agents commonly used against Gram-positive pathogens (EMA, 2022). Daptomycin a comparatively low tendency to induce bacterial resistance. Recent findings show that, although daptomycin can interact with the membrane-associated transport machinery that many Gram-positive bacteria use to protect themselves from antimicrobial peptides, this system neither provides protection against the drug nor becomes activated in its presence. This indicates that daptomycin's mechanism of disrupting the bacterial membrane enables it to circumvent a major resistance pathway (Faure et al., 2024).

6.4. Comparative aspects of vancomycin and daptomycin

Both vancomycin and daptomycin are restricted to parenteral administration owing to negligible gastrointestinal absorption. However, the two agents differ substantially in their pharmacokinetic properties, which has important implications for systemic therapy as well as for local antibiotic delivery strategies. Daptomycin is characterised by a very small apparent volume of distribution, reported to range from approximately 0.06 to 0.16 L/kg, indicating limited tissue penetration and confinement predominantly to the intravascular and extracellular fluid compartments. This pharmacokinetic behaviour is largely attributable to its high plasma protein binding, which is approximately 90% under physiological conditions. Consequently, only a small unbound fraction of daptomycin is microbiologically active, and this must be considered when interpreting plasma concentrations relative to MICs (Estes & Derendorf, 2010; Gregoire et al., 2021).

In contrast, vancomycin exhibits a considerably larger volume of distribution, approximating total body water (0.4–1 L/kg), reflecting more extensive tissue distribution. Plasma protein binding of vancomycin is moderate, typically ranging between 30% and 55%. Both antibiotics are predominantly eliminated via renal excretion, necessitating dose adjustment in patients with impaired renal function (Estes & Derendorf, 2010). Vancomycin comes with several disadvantages for local antibiotic application. First, vancomycin exhibits unfavourable elution kinetics when incorporated into PMMA cements or biodegradable carriers. Elution is typically characterised by an initial burst release during the first 24–48 hours, followed by a rapid decline to subtherapeutic concentrations. Importantly, a substantial proportion of vancomycin remains permanently trapped within PMMA matrices, resulting in incomplete release and limited sustained antimicrobial activity at the implantation site (Anagnostakos et al., 2009; Smolle et al., 2023). Consequently, increasing the total vancomycin content does not proportionally enhance long-term antimicrobial exposure and may instead compromise the mechanical integrity of the cement. Second, vancomycin activity is primarily time-dependent and relies on maintaining drug concentrations continuously above the MIC. This pharmacodynamic profile is suboptimal for local delivery systems with declining elution profiles, where concentrations frequently fall below effective levels after the early postoperative phase. Sub-inhibitory vancomycin concentrations may promote bacterial persistence, biofilm formation, and adaptive resistance mechanisms, including cell wall thickening (Pogliano et al., 2012; Rybak et al., 2009). Third, vancomycin shows limited bactericidal activity against established biofilms, particularly on implant surfaces in contrast to daptomycin (Boudjemaa et al., 2016; Domínguez-Herrera et al., 2012; Siala et al., 2014; Stewart et al., 2009). In addition, its molecular size and hydrophilicity restrict penetration into dense biofilm matrices, further reducing its effectiveness in the context of implant-associated infections and infection prevention (Anagnostakos et al., 2009). Fourth, massively increasing vancomycin loading in local carriers generates potential safety concerns. Although systemic absorption from local delivery systems is typically limited, clinically relevant serum levels have been detected, particularly when high doses are used or when renal function is a priori impaired. This raises concerns regarding nephrotoxicity and underscores that a massive increase in vancomycin dose does not represent a safe or effective strategy to overcome pharmacokinetic limitations (Colding-Rasmussen et al., 2018).

In contrast, daptomycin displays concentration-dependent bactericidal activity, making it better suited for local delivery systems that generate high initial antibiotic concentrations. First studies have demonstrated a potential usage of daptomycin in PJI revision surgeries (Carli et al., 2020). Moreover, daptomycin's distinct mechanism of action reduces the risk of cross-resistance with glycopeptides and supports its use in both infection treatment and prevention strategies where vancomycin coverage is insufficient.

7. Research gap and aims of this thesis

7.1 Research gap

Although ALBCs have been used in arthroplasty for infection prophylaxis and treatment for more than five decades, fundamental aspects of their use in clinical practice remain insufficiently understood. Their use has largely been driven by early in-vitro data and empirical clinical success, rather than by a comprehensive understanding of the underlying material properties and pharmacokinetic aspects. In practice, PMMA cement formulations are often regarded as interchangeable, despite existing evidence that antibiotic release characteristics vary considerably between different cement matrices. These differences may influence the ability to achieve sufficient local antibiotic concentrations above the MIC or MBIC/MBEC for those pathogens which are responsible for PJI. A better understanding of these parameters will presumably translate into better clinical results with respect to infection prevention and/or infection treatment.

Furthermore, in cases where pathogens display resistance to the antibiotics available in commercial acrylic bone cements, surgeons frequently rely on empirical or hands-on solutions without good evidence basis. Standardised guidelines are lacking, and the potential consequences, such as altered polymerisation kinetics, impaired mechanical performance, or subtherapeutic elution, are often insufficiently recognised. Knowledge gaps are even greater with respect to antifungal agents, for which validated protocols and systematic elution data are even more scarce. Mixing procedures themselves are commonly guided by a „trial-and-error "-mentality rather than by a controlled, reproducible methodology, and dosing recommendations are frequently based on the assumption that higher drug loads automatically translate into improved elution. For pathogens such as vancomycin-resistant enterococci, therapeutic options are particularly limited due to constraints on maximum antibiotic loading that still preserves cement handling and mechanical properties. Additional controversy surrounds the clinical utility of DALBCs compared to SALBCs.

Overall, the pharmacokinetics of antibiotic release, the interaction between antimicrobials, cement matrices, host factors, and bacterial biofilms, as well as the clinical implications of these interactions, remain insufficiently elucidated, highlighting a substantial and ongoing research gap.

7.2. Aims of this thesis

The identified research gaps are multifaceted and broad in scope, rendering it unfeasible to address all of them within the framework of a single thesis. Nevertheless, this work aims to elucidate selected aspects that may support orthopaedic surgeons in evidence-based decision-making regarding the use of ALBCs. It aims to address these gaps through an integrated series of in-vitro and in-vivo investigations focusing on antibiotic choice, cement matrix properties, mixing methodology, and translational efficacy.

By integrating material science, microbiology, and translational infection modelling, this thesis aims to:

- Provide evidence-based guidance for selecting and optimising ALBC formulations in clinical practice.
- Clarify how cement matrix composition, antibiotic choice, and mixing technique jointly determine elution, efficacy, and mechanical performance.
- Establish standardised testing methods for comparing ALBCs.

- Demonstrate the relevance of in-vitro findings by validating them in an in-vivo model.
- Support development of a new daptomycin-based DALBC against vancomycin-resistant bacteria.

7.2.1. Evaluate the feasibility of a daptomycin-loaded acrylic bone cement

The first aim of this thesis is to determine the feasibility of formulating a DALBC combining gentamicin with daptomycin for the management of PJIs caused by vancomycin-resistant pathogens. To achieve this, the thesis investigates:

- The minimum effective daptomycin concentration required to achieve antimicrobial activity against VRSA, VRE, and VISA, assessed via standardised proliferation assays and inhibition zone testing (IZT).
- The mechanical stability of PMMA cement containing varying daptomycin concentrations, evaluated according to ISO 5833:2002 and DIN 53435:2018 to ensure clinically acceptable bending strength, bending modulus, compressive strength, and impact resistance.
- The elution behaviour of daptomycin in combination with gentamicin and the potential for synergistic release patterns.
- The influence of sterilisation methods (gamma irradiation vs ethylene oxide) on daptomycin activity, a critical parameter for future industrial production.

This aim represents a foundational step toward establishing whether a gentamicin and daptomycin loaded DALBC could serve as a viable alternative to currently available vancomycin-containing cements for the prevention or eradication of PJIs caused by vancomycin-resistant organisms.

7.2.2. Develop evidence-based recommendations for manual admixing

The second aim of this thesis is to address the largely undocumented challenges associated with manual admixing of antimicrobial agents into PMMA cement, an off-label yet common clinical practice when commercially available cements do not match pathogen susceptibilities. The goal is to establish evidence-based recommendations that support safe, reproducible use of manually admixed ALBCs in clinical scenarios involving resistant organisms. To this end, the thesis investigates:

- The impact of different mixing procedures, including additional homogenisation through dry mixing, on the uniform distribution of antimicrobial powders within the polymer matrix.
- The mechanical consequences of manual admixing, including effects on bending strength, modulus, and impact resistance relative to industrially premixed cements.
- Microstructural alterations, such as abrasion and particle shedding from mixing cartridges during dry mixing, assessed by microscopic analysis.
- Elution characteristics of manually admixed antibiotic–cement formulations and their implications for achieving therapeutic local concentrations.

Building upon these findings, this aim further seeks to compile a practical, surgeon-oriented overview summarising (i) which antimicrobial agents can reasonably be admixed into PMMA cement, (ii) in what quantities, (iii) how such additions affect handling and mechanical properties, and (iv) whether synergistic elution effects may be expected. Overall, these results should contribute to a safer and more reproducible use of customised ALBCs in the management of PJI cases with resistant pathogens.

7.2.3. Determine whether antibiotic release differs between cement brands

The third aim of this thesis is to investigate how differences in PMMA cement matrices influence antibiotic release kinetics and antimicrobial performance, given that commercially available ALBCs vary substantially in polymer composition, viscosity profile, hydrophilicity, and radio pacifier content. The goal is to establish whether such matrix-dependent factors result in clinically relevant differences in antibiotic availability over time and thereby inform evidence-based cement selection. To this end, the thesis investigates:

- Short- and long-term antibiotic elution profiles of Palacos® R+G and Simplex® T under standardised conditions using defined specimen geometry and harmonised HPLC protocols, enabling direct comparability between matrices.
- Functional antimicrobial activity over extended time periods, assessed through inhibition zone testing against *S. aureus*, *S. epidermidis*, and *E. coli* to determine whether quantitative elution differences translate into biological efficacy.
- The influence of polymer characteristics, including viscosity and hydrophilicity, on the amount and duration of antibiotic release.
- The feasibility and necessity of standardised elution methodology, addressing the substantial methodological variability in the literature and validating consistent protocols as a prerequisite for meaningful comparison between cement brands.

By clarifying how cement matrix properties influence antibiotic release and antimicrobial activity, this aim contributes to more informed, evidence-based selection of PMMA cements for both infection prophylaxis and PJI treatment.

7.2.4. Translate in-vitro findings into an in-vivo biofilm model

The fourth aim of this thesis is to evaluate whether antibiotic load and antibiotic combination-specific differences in antimicrobial inhibition are transferrable from the bench to the in-vivo situation by using the *Galleria mellonella* model for early implant-associated infection. Of highest relevance is here to determine whether antibiotic concentrations eluted from different ALBC formulations are sufficient to inhibit or eradicate biofilm-embedded MDR pathogens under physiologically more realistic conditions. To this end, the thesis investigates:

- The in-vivo efficacy of distinct commercial ALBCs (Palacos® R+G, Copal® G+V, Copal® G+C) in preventing or reducing implant-associated infections caused by MDR *S. aureus* and *E. faecalis* in both biofilm-coated implant and haematogenous infection models.
- The antimicrobial effect across different stages, including planktonic bacteria, biofilm on surrounding tissues, and biofilm on cemented implant surfaces, capturing a more complete picture of PJI.
- The capacity of high local antibiotic concentrations to overcome resistance observed in-vitro, particularly in DALBC formulations with a synergistic release behaviour.

By integrating in-vitro release characteristics with in-vivo infection dynamics, this aim contributes to a better understanding of how ALBC formulations perform under clinically relevant biological conditions.

7.2.5. Identify the optimal daptomycin dosage for spacers in an in-vivo model

The fifth aim of this thesis is to identify the optimal daptomycin dosage for incorporation into PMMA spacers intended for treating vancomycin-resistant infections under the premises that antibiotic loading must achieve a balance between antimicrobial efficacy and mechanical stability. The goal is to define dose-response relationships for daptomycin in clinically used cement carriers and to validate these findings in an in-vivo model of VRE implant infection. To this end, the thesis investigates:

- The antimicrobial efficacy of PMMA spacers loaded with 1 g vs. 2 g daptomycin in the *Galleria mellonella* implant infection model, measuring survival, bacterial burden, and implant colonisation outcomes.
- Matrix-dependent performance differences between Palacos® R+G and Simplex® T when supplemented with daptomycin, reflecting the influence of the underlying cement chemistry on elution and efficacy.
- The consequences of increased daptomycin concentration in PMMA cement for the mechanical stability.
- The correlation between in-vitro and in-vivo antimicrobial activity, establishing whether optimised dosing regimens maintain both biological activity and material safety.

By defining a safe and effective daptomycin dosage for PMMA cement spacers, this aim contributes to the development of evidence-based, mechanically robust ALBC formulations capable of managing PJIs associated with VRE.

III. Publications contributing to this thesis

1. Daptomycin-impregnated PMMA cement against vancomycin-resistant germs: dosage, handling, elution, mechanical stability, and effectiveness

1.1. Summary

In this study, we investigated how the manual admixture of different concentrations of daptomycin into gentamicin-loaded PMMA cement influences its antimicrobial activity, antibiotic release characteristics, mechanical stability, and handling properties. The motivation for this work is based on the increasing prevalence of PJIs caused by vancomycin-resistant pathogens, VRE, VRSA and VISA, that are a challenge in revision arthroplasty (Markwart et al., 2019; Shariati et al., 2020). Since no commercial PMMA cement containing daptomycin exits, it was essential to determine whether daptomycin could be integrated into PMMA cement without compromising its mechanical stability (Leta et al., 2023).

Our primary objective was to identify a daptomycin concentration that provides reliable antimicrobial efficacy against vancomycin-resistant pathogens while still fulfilling the mechanical and handling requirements defined in ISO 5833:2002 and DIN 53435:2018. Additionally, we aimed to characterize how daptomycin affects antibiotic elution kinetics, especially in combination with gentamicin, since synergistic elution effects are known to increase local antibiotic concentrations (Berberich et al., 2022; Kühn, 2014). We also investigated on the effect of different sterilisation methods, gassing with ethylene oxide and gamma irradiation, on a daptomycin-loaded PMMA cement.

To achieve these objectives, I prepared PMMA cement samples by adding 0.5 g, 1.0 g, or 1.5 g of daptomycin to Palacos® R+G, which already contains 0.5 g gentamicin per 40 g of polymer powder. I evaluated the antimicrobial activity using the Certika® proliferation assay and inhibition zone testing against a spectrum of clinically relevant strains, including MRSE, MRSA, VRSA, and VRE (Alt et al., 2004a, 2004b; Bechert et al., 2000). Antibiotic elution profiles for daptomycin and gentamicin were quantified over five days using HPLC–MS/MS, enabling me to determine cumulative release and dose-dependent effects (Aiken et al., 2015; Amin et al., 2012). I further assessed mechanical properties, including bending strength, bending modulus, and compressive strength and examined handling characteristics such as doughing and setting times (DIN 53435, 2018; ISO 5833, 2002).

Our analyses demonstrated that the admixture of 0.5 g daptomycin was insufficient, as these samples showed no antimicrobial activity in either the proliferation assay or inhibition zone tests. In contrast, cements containing 1.0 g and 1.5 g daptomycin inhibited all tested resistant strains. The strongest antimicrobial effect consistently occurred at the 1.5 g concentration, confirming this as the most effective formulation in-vitro. Daptomycin release occurred in a dose-dependent manner, with the cumulative amount increasing from 263.8 µg (0.5 g) to 611.4 µg (1.0 g) and 1039.7 µg (1.5 g) over five days. Gentamicin release was also enhanced at higher daptomycin concentrations, indicating a synergistic elution effect. Antibiotic release peaked within the first 24 hours for all formulations, followed by a gradual decline. When compared to Copal® G+V, vancomycin exhibited a much higher initial release but dropped sharply (approximately 85% reduction) by day 2. All formulations fulfilled the mechanical thresholds. The cement loaded with 1.0 g daptomycin showed the highest bending strength, but the 1.5 g formulation remained well above required limits. Increasing daptomycin concentrations slightly decreased bending strength and impact resistance;

however, none of these reductions compromised compliance with mechanical standards. We found that the cement containing 1.5 g of daptomycin exhibited a prolonged doughing time and a faster setting time compared to both Palacos® R+G and Copal® G+V. Despite these differences, the handling characteristics remained within standards.

Based on my investigations, we can conclude that the combination of 1.5 g daptomycin and 0.5 g gentamicin per 40 g PMMA cement represents a good formulation as it provides antimicrobial activity against vancomycin-resistant bacteria, shows a synergistic elution effect, fulfils ISO and DIN mechanical standards and handling properties. Given the increasing prevalence of vancomycin-resistant bacteria in PJIs and the limitations of vancomycin-containing ALBCs, this formulation offers a promising alternative for PMMA cement spacers in two-stage revision surgery.

1.2. Contribution

The tests on antimicrobial activity (proliferation assay, inhibition zone testing), antibiotic elution (HPLC) as well as mechanical stability (ISO 5833:2002, DIN 53435:2018) were performed by me. The influence of the sterilisation method on antimicrobial activity was handed over to an external certified lab (INNOVENT e.V., Jena). Rainer Strathausen, Sebastian Vogt, Klaus-Dieter Kühn and I designed the research and analysed the data. I wrote the manuscript and reviewed it with all authors.

1.3. Reference

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Article

Daptomycin-Impregnated PMMA Cement against Vancomycin-Resistant Germs: Dosage, Handling, Elution, Mechanical Stability, and Effectiveness

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Abstract: Background: The number of periprosthetic joint infections caused by vancomycin-resistant pathogens is increasing. Currently, no PMMA cement is commercially available to cover VRE. Daptomycin shows promising results in treating infection, offering a good safety profile and a reduced risk of developing resistance. The purpose of this in vitro study was to investigate the mechanical stability, handling properties, elution behavior, and antimicrobial effectiveness of PMMA cement loaded with three different daptomycin concentrations in comparison to commercially available antibiotic-loaded bone cement (ALBC). Methods: Mechanical properties and handling characteristics (ISO 5833, DIN 53435), HPLC elution, antimicrobial effectiveness with proliferation assay (DIN 17025), and inhibition zone testing were investigated. Results: All tested daptomycin concentrations met the ISO and DIN standards for mechanical strength. Loading of 40 g of PMMA cement with 0.5 g of daptomycin did not show any antimicrobial effectiveness, in contrast to 1.0 g and 1.5 g. PMMA cement with 1.5 g of daptomycin was the best in terms of elution and effectiveness, and it showed good ISO mechanical strength; ISO doughing was sticky for a little longer and setting was faster compared to the vancomycin-containing reference cement. Conclusion: PMMA cement containing 0.5 g of gentamicin and 1.5 g of daptomycin could be a good alternative to the already established COPAL® (Wehrheim, Germany) G+V for the treatment of PJIs caused by VRE.

Keywords: daptomycin; PMMA cement; mechanical properties; antimicrobial effectiveness; vancomycin-resistant germs; PMMA spacer

1. Introduction

Periprosthetic joint infections (PJIs) are a challenging complication in joint replacement surgery that often results in worse outcomes for the patients, especially when the causative pathogen is a multidrug-resistant germ [1] and/or the patient is at high risk [2,3]. Poly(methyl methacrylate) (PMMA) bone cements loaded with one (i.e., single-antibiotic-loaded bone cement (SALBC)) or two antibiotics (i.e., dual-antibiotic-loaded bone cement

(DALBC)) are used for the prevention or treatment of PJIs, e.g., for a spacer in the interim period of a two-stage exchange procedure [4–7]. DALBC supports reducing PJIs especially well compared to SALBC [8]. Commercially available antibiotic-loaded bone cements (ALBCs) mainly contain the antibiotics gentamicin or tobramycin (aminoglycosides), vancomycin (a glycopeptide), and clindamycin (a lincosamide) [9]. The number of PJIs caused by resistant germs, including vancomycin-resistant *Staphylococcus aureus* (VRSA), vancomycin-resistant *Enterococci* (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), and methicillin-resistant *Staphylococcus epidermidis* (MRSE), is rising [10,11], and the manual addition of vancomycin to commercially available ALBCs is insufficient to cover these germs. The aforementioned bacterial species have mainly developed resistance against vancomycin and methicillin; therefore, the antibiotics used for their treatment must be adapted to the resistance pattern of the causative bacteria [12]. One antibiotic that has been found to be effective against Vancomycin-intermediate-resistant *S. aureus* (VISA), VRSA, and VRE is daptomycin; it also shows antimicrobial activity against anaerobic bacteria, but none against Gram-negative bacteria [10,13]. Daptomycin has a unique mode of action and disrupts the cell membrane integrity of bacteria [14].

There is no daptomycin ALBC commercially available, and the manual admixture of daptomycin is highly expensive (Cubicin, ~780 EUR/2 g). In clinical practice, surgeons increase the vancomycin concentration up to 4 g per 40 g of polymer powder to treat vancomycin-resistant germs. But a massively increased antibiotic concentration in PMMA spacers negatively influences their mechanical stability [15], and the risk of local and systemic kidney toxicity is highly increased. According to the "Pocket Guide to Diagnosis & Treatment of PJI" from the PRO-IMPLANT Foundation (PIF), 2 g of daptomycin can be added to a fixation cement, and 3 g of daptomycin can be added to a spacer cement [16]. The addition of more than 2 g of daptomycin results in non-ISO-compliant mechanical properties.

With this investigation, we wanted to figure out what concentration of daptomycin can be added to PMMA cement to efficiently inhibit bacterial growth and, at the same time, deliver mechanical stability according to ISO standards.

2. Results

2.1. Antimicrobial Effectiveness Determined by Proliferation Assay

For methicillin-resistant *S. epidermidis* (MRSE), the blank sample and PMMA cement loaded with 0.5 g of gentamicin and daptomycin (GD0.5) did not show any antimicrobial activity (Figure 1a). GD1.0 (1.0 g of daptomycin + 0.5 g of gentamicin) and GD1.5 (1.5 g of daptomycin + 0.5 g of gentamicin) inhibited bacterial growth, and GD1.5 showed better antimicrobial properties compared to GD1.0. For vancomycin-resistant *S. aureus* (VRSA), the blank sample and GD0.5 did not show antimicrobial activity (Figure 1b), whereas GD1.0 and GD1.5 were able to inhibit bacterial growth. The same observation was made for vancomycin-resistant *E. faecium* (VRE), where only GD1.0 and GD1.5 showed antimicrobial activity (Figure 1c). For methicillin-resistant *S. aureus* (MRSA), the blank sample and GD0.5 did not inhibit bacterial growth, in contrast to GD1.0 and GD1.5. Overall, the antimicrobial efficacy of GD1.5 and GD1.0 did not differ widely, with GD1.5 showing the greatest effect on bacterial growth.

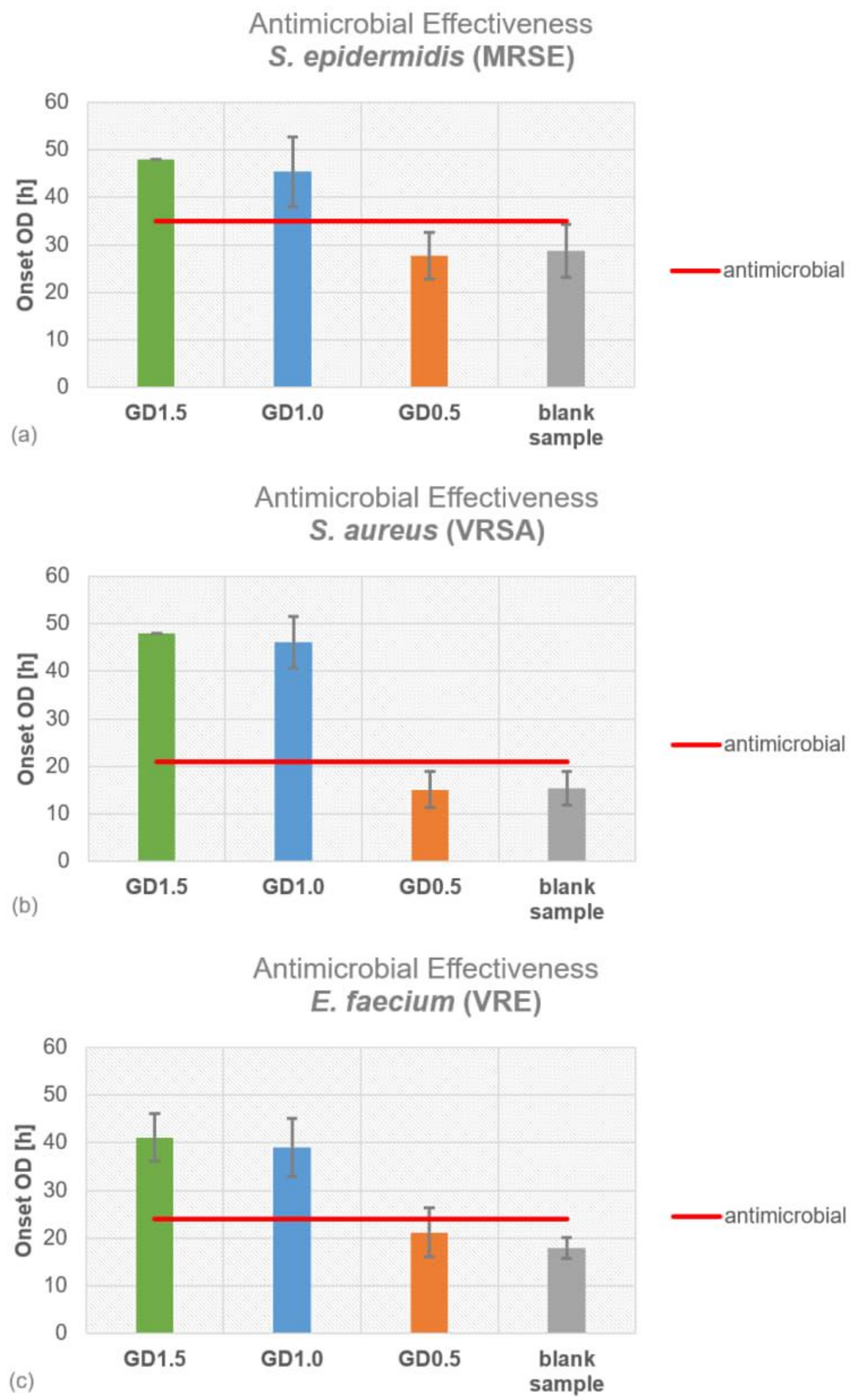


Figure 1. *Cont.*

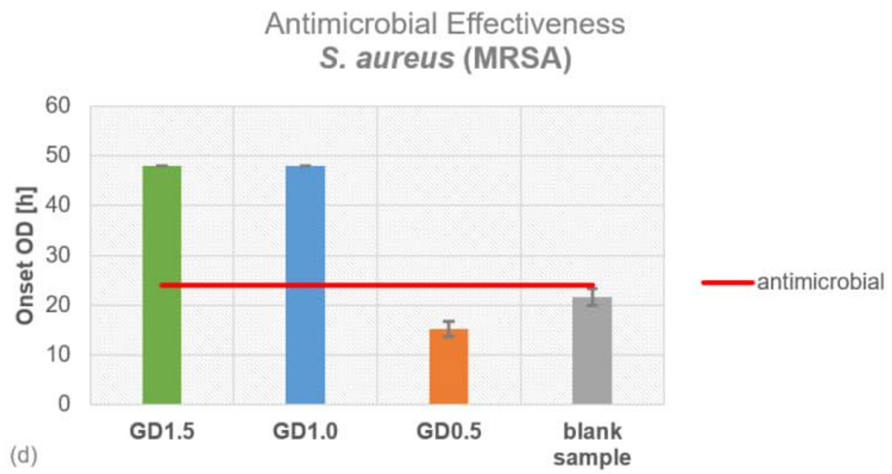


Figure 1. Antimicrobial effectiveness of different daptomycin concentrations (0.5 g, 1.0 g, of 1.5 g) in PMMA bone cement samples, as determined by Certika® (Barranquilla, Colombia) proliferation assay for (**a**) methicillin-resistant *S. epidermidis*, (**b**) vancomycin-resistant *S. aureus*, (**c**) vancomycin-resistant *E. faecium*, and (**d**) methicillin-resistant *S. aureus*.

2.2. Antimicrobial Effectiveness Based on Inhibition Zone Tests

The inhibition zones of daptomycin-containing PMMA cements showed the best effectiveness with a concentration of 1.5 g of daptomycin. Figure 2 shows the antimicrobial effectiveness of the tested samples relative to the amount of daptomycin added: 1.5 g led to an average inhibition zone of 2269 $\pm$ 171 mm^2, 1.0 g led to an inhibition zone of 2219 $\pm$ 346 mm^2, and 0.5 g led to an inhibition zone of 2134 $\pm$ 198 mm^2. GD1.5, GD1.0, and GD0.5 showed antimicrobial effectiveness against *B. subtilis* (Figure 2).

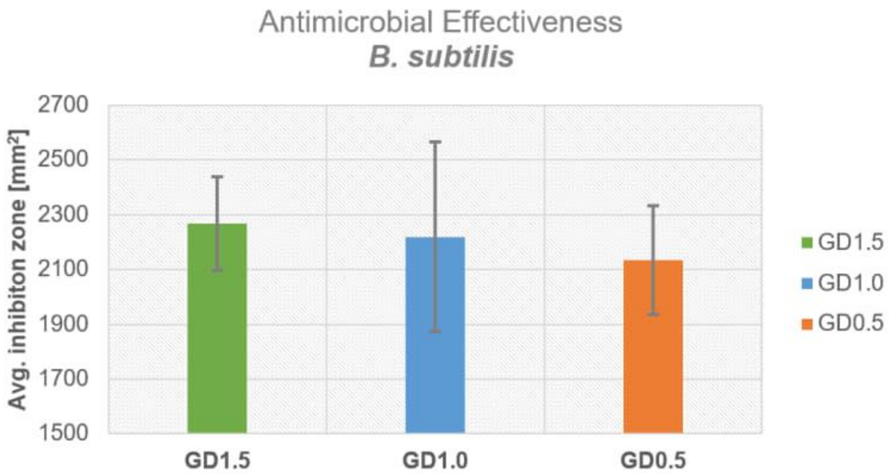


Figure 2. Antimicrobial effectiveness as determined by inhibition zone tests for all three different daptomycin concentrations.

2.3. Influence of the Sterilization Method

Non-sterilized and gamma-irradiated pure daptomycin powder showed comparable effectiveness against *B. subtilis* (Figure 3). The effectiveness of daptomycin sterilized with ethylene oxide was significantly reduced in comparison to the unsterile and gamma-irradiated powders.

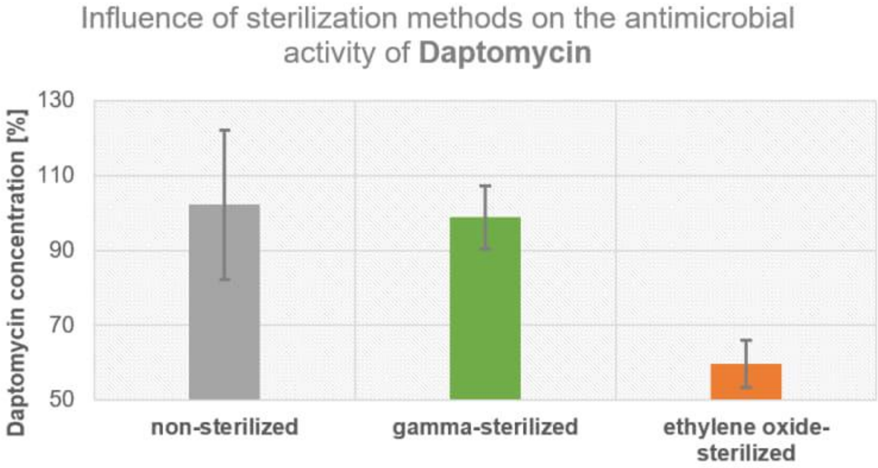


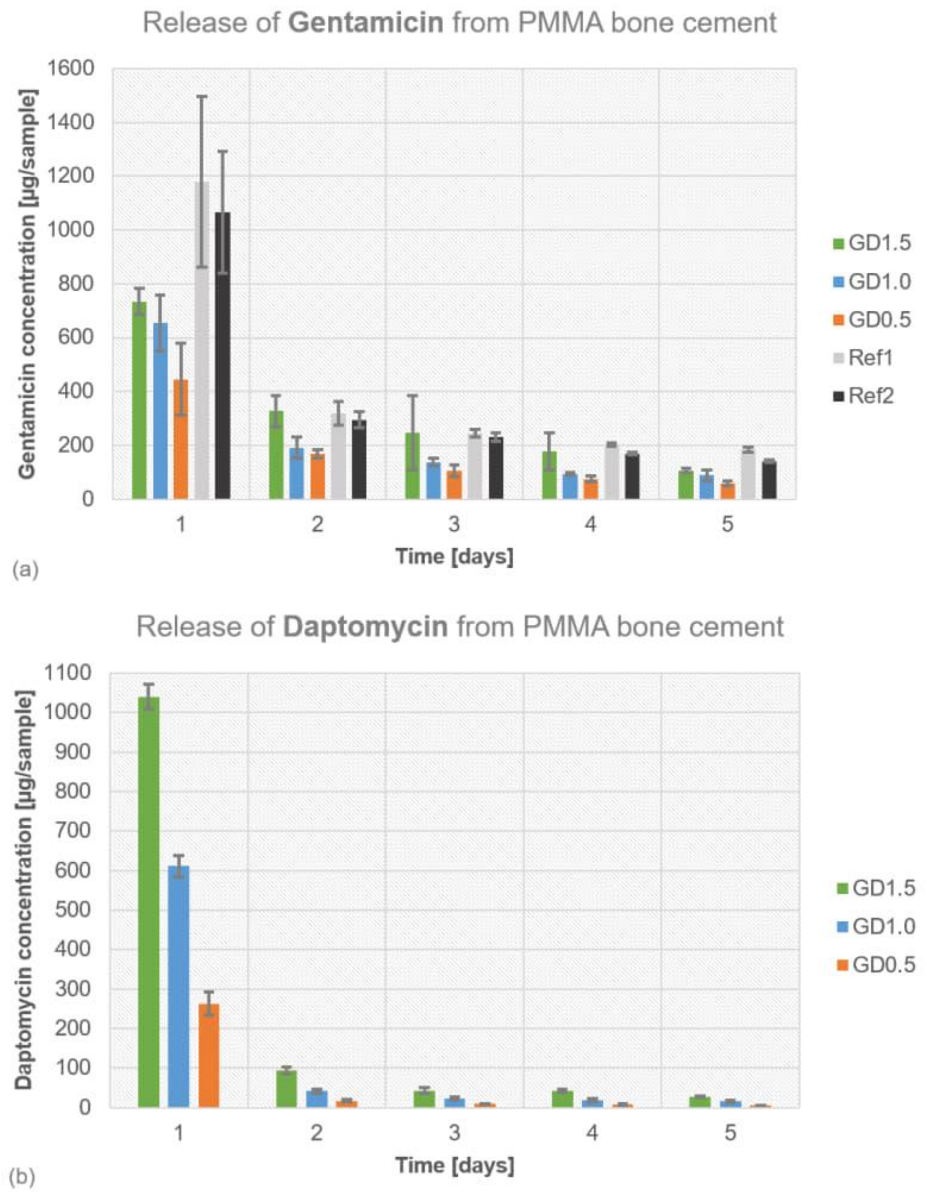
Figure 3. Influence of gamma and ethylene oxide sterilization on the antimicrobial effectiveness of daptomycin against *B. subtilis*, in contrast to non-sterilized daptomycin.

2.4. Antibiotic Release Profile of Gentamicin, Daptomycin, and Vancomycin

The highest volume of gentamicin released from daptomycin + gentamicin-loaded PMMA cement samples was observed on day 1, followed by a continuous decrease in antibiotic release (Figure 4a). The gentamicin release was higher for those samples with a higher daptomycin concentration: GD1.5 showed the highest gentamicin release over five days compared to GD1.0 and GD0.5, indicating a synergistic elution effect. PALACOS® R+G and COPAL® G+V showed the highest antibiotic release on day 1 compared to the test samples with manually admixed daptomycin. The total release of daptomycin was higher (1039.7 ± 31 µg) compared to gentamicin (734.1 ± 48 µg) for GD1.5 (Figure 4b,c). Adding 0.5 g of daptomycin resulted in a twofold higher antibiotic release rate: GD1.5 with 1039.7 ± 31 µg, GD1.0 with 611.4 ± 27 µg, and GD0.5 with 263.8 ± 28 µg. Ref2 (COPAL® G+V) was assessed for vancomycin elution (Figure 4d), showing the highest initial release of all tested samples on day 1 (1460.2 ± 70 µg), followed by a massive decrease to 221.4 ± 19 µg on day 2. Compared to the GD samples and Ref1 (PALACOS® (Wehrheim, Germany) R+G), Ref2 (COPAL® G+V) showed the highest total amount of antibiotics eluted. Compared to the vancomycin release (Figure 4d) from Ref2 (COPAL® G+V), the total amount of daptomycin released was lower, indicating an overall better elution from Ref2 (COPAL® G+V) compared to the GD samples.

2.5. Mechanical Stability of Daptomycin-Loaded Bone Cement

All tested daptomycin-containing PMMA cement samples fulfilled the ISO and DIN requirements (Figure 5a). PALACOS® R+G (Ref1) showed a bending strength of 71 ± 1 MPa, surpassing the threshold of 50 MPa. COPAL® G+V (Ref2) was close to the threshold, with a bending strength of 58 ± 3 MPa. The ISO bending strength was highest for GD1.0 (72 ± 1 MPa), followed by GD0.5 (70 ± 2 MPa) and GD1.5 (67 ± 2 MPa), which therefore come with a higher bending strength than COPAL® G+V (Ref2). The bending modulus of Ref1 (2922 ± 66 MPa) was comparable to that of Ref2 (2900 ± 30 MPa), fulfilling the minimum threshold of 1800 MPa (Figure 5b). GD1.0 had the highest bending modulus, with 3342 ± 113 MPa, followed by GD1.5 (3148 ± 168 MPa) and GD0.5 (3120 ± 95 MPa); all of the GD samples surpassed the references. Ref2 fulfilled the minimum requirement (70 MPa) for ISO compressive strength, with 78 ± 0 MPa, whereas Ref1 exceeded it (87 ± 1 MPa) (Figure 5c). The compressive strength was highest for GD1.5 (93 ± 3 MPa), followed by GD0.5 (92 ± 1 MPa) and GD1.0 (90 ± 2 MPa).



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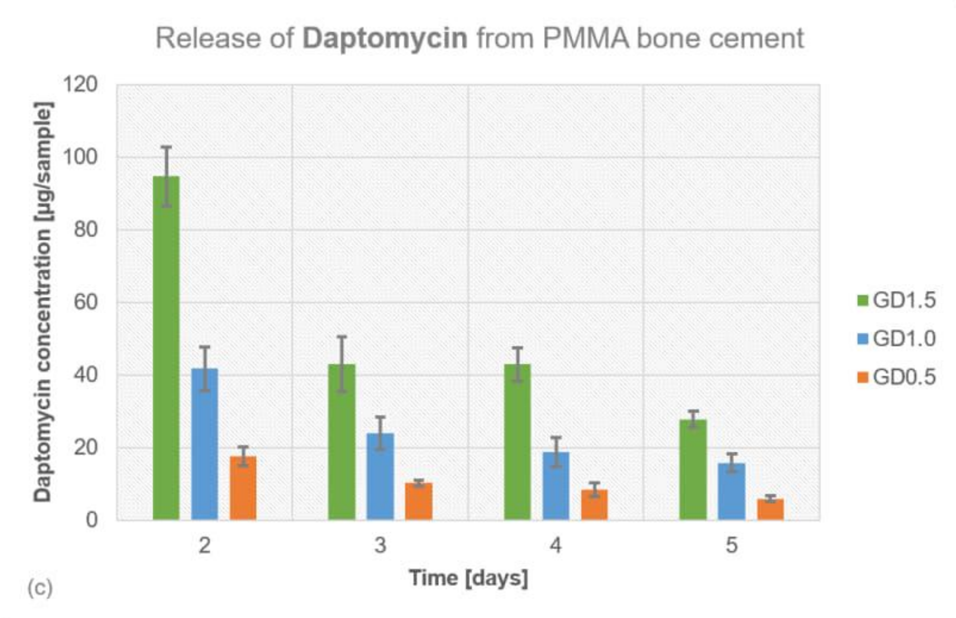

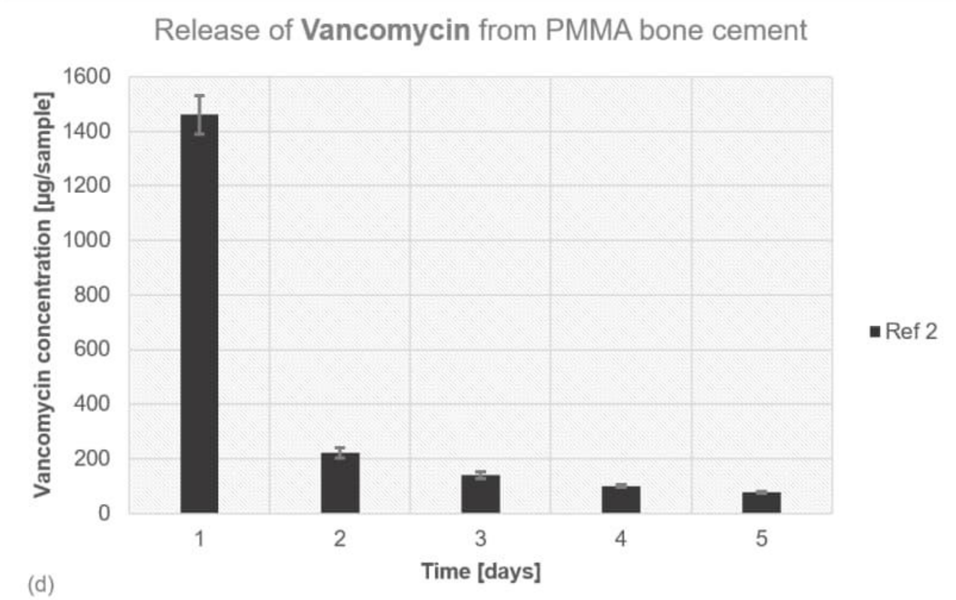

Figure 4. Release profiles from PMMA bone cement were determined with HPLC for (**a**) gentamicin days 1–5, (**b**) daptomycin days 1–5, (**c**) daptomycin days 2–5, and (**d**) vancomycin days 1–5. Ref1 (PALACOS® R+G), Ref2 (COPAL® G+V).

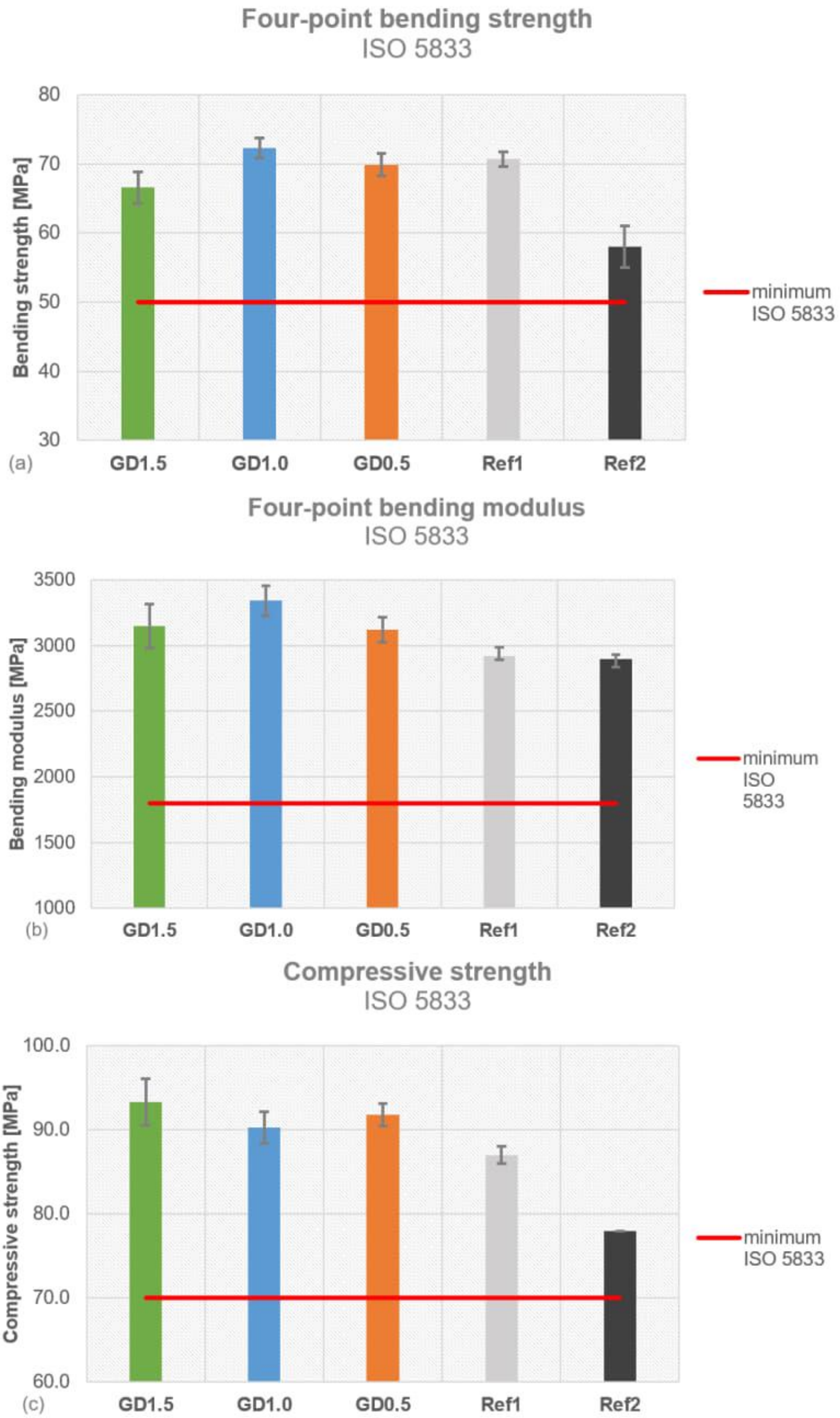

Figure 5. Mechanical stability tested for all daptomycin concentrations compared to PALACOS® R+G and COPAL® G+V: (**a**) four-point bending strength; (**b**) four-point bending modulus; (**c**) compressive strength.

All GD cement samples fulfilled the requirements for mechanical stability according to DIN 53435. Ref2 exceeded the minimum threshold (50 N/mm^2) of DIN bending strength, with 69 ± 3 N/mm^2, as did Ref1 with 81 ± 1 N/mm^2 (Figure 6a). The DIN bending strength decreased with the increase in the daptomycin concentration, from 74 ± 3 N/mm^2 (GD0.5) to 70 ± 3 N/mm^2 (GD1.0) and 64 ± 4 N/mm^2 (GD1.5). GD1.0 showed a comparable DIN bending strength to Ref2, whereas GD1.5's was slightly below that of Ref2. The DIN impact resistance was measured for COPAL® G+V (Ref2) as 3.0 ± 0.3 kJ/m^2, which was set as a reference. The DIN impact resistance is shown as the difference compared to COPAL® G+V (Ref2) (Figure 6b). Ref1 (3.5 ± 0.3 kJ/m^2), GD1.0 (3.2 ± 0.4 kJ/m^2), and GD0.5 (3.1 ± 0.5 kJ/m^2) exceeded the impact resistance of Ref2, while GD1.5 (2.6 ± 0.6 kJ/m^2) showed the highest difference in DIN impact resistance. The higher the daptomycin concentration, the lower the measurements for DIN bending strength and impact resistance, indicating that a high daptomycin concentration in PMMA bone cement reduces its mechanical properties. The ISO bending strength for GD1.5 was lower compared to all other concentrations and the references.

2.6. Handling Properties of Daptomycin-Loaded Bone Cement

GD1.5 was slightly faster-setting compared to COPAL® G+V (Ref2), and even more so compared to PALACOS® R+G (Ref1) (Table 1), resulting in a faster setting behavior. The ISO doughing time was lowest for Ref2 and highest for GD1.5, indicating a slower doughing process for GD1.5. The density of all tested cement samples was similar.

Table 1. Handling properties of PMMA bone cement containing 0.5 g of gentamicin + 1.5 g of daptomycin compared to commercially available bone cements Ref1 and Ref2.

Handling Properties	**GD1.5**	**PALACOS® R+G Ref1**	**COPAL® G+V Ref2**
ISO Setting Time (min:s)	06:45 ± 00:00	09:30 ± 00:10	08:15 ± 00:18
ISO Doughing Time (min:s)	01:30 ± 00:00	00:55 ± 00:05	01:00 ± 00:05
Density (g/cm^3)	1.13 ± 0.01	1.15 ± 0.00	1.12 ± 0.01

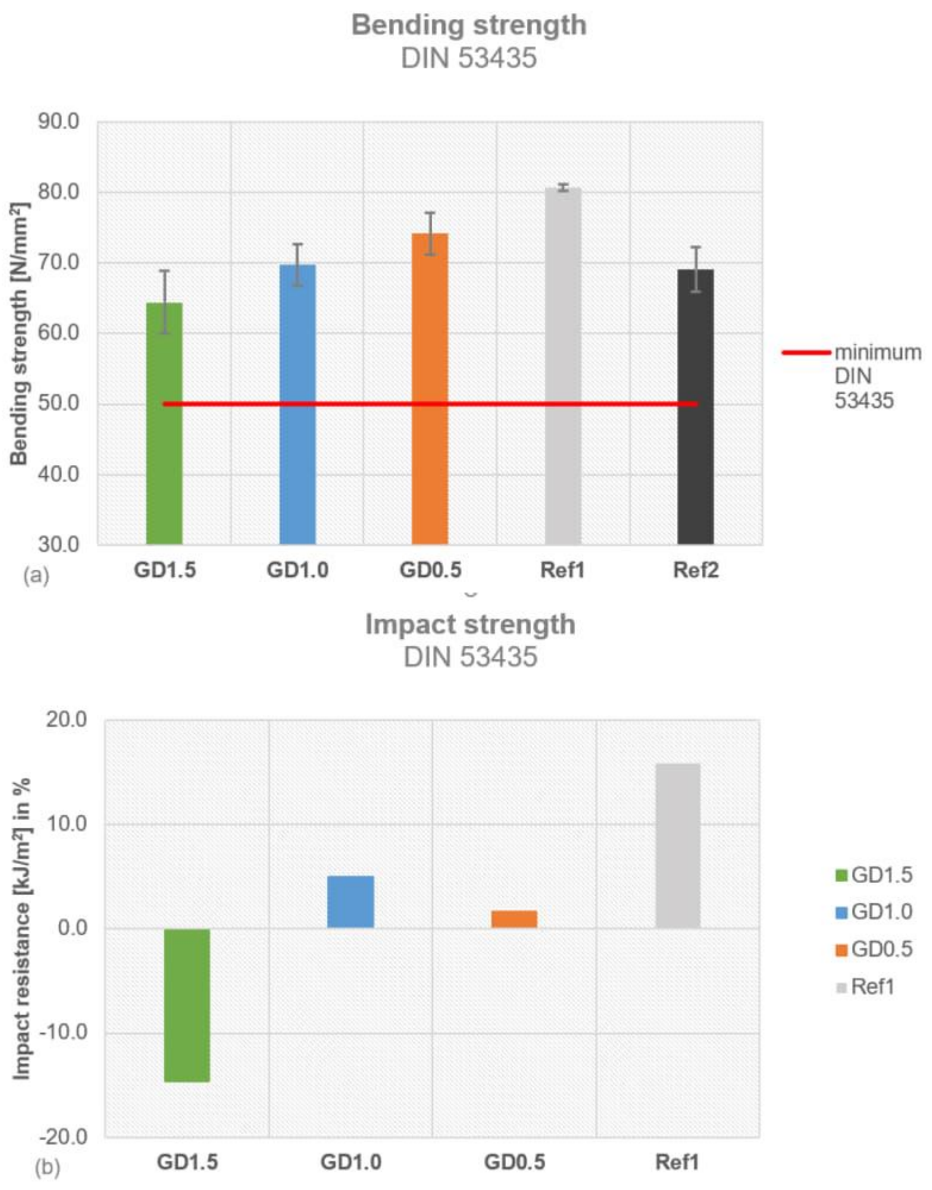

Figure 6. Mechanical stability tested for all daptomycin concentrations (GD1.5, GD1.0, and GD0.5) compared to PALACOS® R+G (Ref1) and COPAL® G+V (Ref2): (**a**) DIN bending strength; (**b**) DIN impact strength shown as the difference compared to Ref2.

3. Discussion

As PJIs still pose a threat to patients and the healthcare system, especially when caused by resistant germs, all kinds of support are needed to prevent PJIs or treat them in a two-stage revision protocol [17]. Daptomycin-containing PMMA cement is recommended for PJIs when combatting vancomycin-resistant germs, e.g., VRE.

In clinical practice, spacers for two-stage revision procedures are created by using industrially manufactured cement already containing vancomycin, or by manually admixing vancomycin with PMMA cement [6,18,19]. The use of the commercially available cement COPAL® G+V is already established for the prevention of PJIs, especially against the most frequent PJI germs *S. aureus* and *S. epidermidis* [8,18], but does not cover VRSA, VISA, or VRSE. In general, it's recommended to use a fixation or spacer cement containing two complementary antibiotics to best cover the spectrum of PJI pathogens [12]. The spectrum of vancomycin is predominantly limited to Gram-positive bacteria; therefore, a broad-spectrum antibiotic is needed to also cover Gram-negative bacteria, e.g., gentamicin [13]. Additionally, the combination of two antibiotics offers a synergistic elution effect that leads to an overall increase in antibiotic elution from PMMA bone cement, resulting in a stronger antimicrobial effect [2]. Antibiotic-loaded bone cement with gentamicin and daptomycin can make a difference in the prevention and treatment of PJIs caused by vancomycin-resistant pathogens [20]. Daptomycin offers a good safety profile and has a unique mode of action that may also be effective against bacteria in biofilms [20], coming with a low resistance profile [21]. According to Gray and Wenzel [14], knowledge on the exact mode of action is still missing, but they observed that the development of resistance to daptomycin was slower compared to other antibiotics with single protein targets. Rouse et al. [22] figured out with a rat model that a PMMA cement with daptomycin may be an option for the local treatment of resistant bacteria causing osteomyelitis. The manual admixture of daptomycin with PMMA bone cement is suggested for PJI cases caused by VRSA, VRE, MRSA, and MRSE with a vancomycin MIC of greater than 2 μg/mL [10]. A first case study in 2013 already showed the ability of PMMA spacers with daptomycin to eradicate an infection in a two-stage revision hip surgery [23]. In our study, we found antimicrobial effectiveness for GD1.5 and GD1.0, whereas a concentration of 0.5 g of daptomycin was not sufficient. This is in line with findings from Eick et al. [24], who concluded from their inhibition zone testing that 1.5 g of daptomycin showed an antimicrobial effect, in contrast to 0.5 g. This may also be caused by the combination of two antibiotics (gentamicin and daptomycin) and the resulting synergistic elution effect. The combination of 0.5 g of gentamicin and 1.5 g of daptomycin showed the best antibiotic elution profile, as well as a synergistic elution effect supporting the prevention of infection [15]. The overall elution profile of daptomycin was comparable to the findings of Meeker et al. [25], showing peak elution for the first 24 h. Our observed synergistic elution effect contrasts with the results reported by Antonello et al. [20], who concluded a rather antagonistic interaction of daptomycin and gentamicin from their study review. But studies on a *Galleria melonella* larvae biofilm model also showed a synergistic effect of combining gentamicin with daptomycin for the treatment of vancomycin resistant *E. faecium* [26]. We observed an effect on the bacterial growth comparable to that reported by Webb et al. [27], indicating an inhibitory effect of daptomycin on the growth of resistant strains of Gram-positive bacteria. Overall, the synergistic elution effect and the high daptomycin release suggested a positive effect of combining 0.5 g of gentamycin and 1.5 g of daptomycin in PMMA bone cement. We also investigated the vancomycin elution, with COPAL® G+V as a reference. Despite finding the highest vancomycin elution for day 1, the elution had already decreased by ~85% on day 2. We doubt that this vancomycin concentration, in a clinical setting, would be sufficient to meet the MIC of VRSA (MIC $\geq$ 16 μg/mL) and VRE (MIC $\geq$ 32 μg/mL) [10], but we would also suggest further investigations in a biofilm model.

To ensure patient safety, daptomycin must be sterilized with either ethylene oxide or gamma radiation. Our findings suggested that daptomycin-loaded bone cement should be sterilized using gamma radiation to maintain its antimicrobial effectiveness, because sterilization with ethylene oxide reduced the efficacy of daptomycin significantly.

To best treat MRSE, MRSA, or enterococci, the *PIF Pocket Guide* [16] recommends increasing the vancomycin concentration in commercially available COPAL® G+V (40 g) by another 2 g. But an increase in the addition of antibiotic powder beyond a total concentration of 10% results in a spacer cement that no longer fulfills the mechanical ISO requirements for bone cement [15,19,28]. Despite Lunz et al. [29] pointing out that an antibiotic concentration exceeding 10% of the powder volume significantly reduces the mechanical strength of PMMA spacers, they recommend manually admixing 4 g of vancomycin with 40 g of PALACOS® R+G instead of using the commercially available COPAL® G+V. These spacers do not comply with the ISO requirements, as the bending strength for PALACOS® R+G + 4 g of vancomycin is below the minimum threshold of 50 MPa and comes with the potential risk of bone cement or spacer fracture [15,19,21,29]. Therefore, we assessed the mechanical stability of a daptomycin-containing bone cement. The ISO bending modulus of the GD samples was increased compared to the reference samples, as admixing antibiotics increases the hydrophilic characteristics of PMMA cements, which results in increased elasticity of the cement [15]. The mechanical properties of GD1.5, GD1.0, and GD0.5 were all above the corresponding minimum thresholds, as the concentration of the added daptomycin was below 10% of the total PMMA cement powder volume [15,22,30]. Considering the mechanical properties, a concentration of 1.0 g of daptomycin would be ideal, but this does not offer a sufficient antibiotic release needed for preventing infection. As the bone cement GD1.5 showed antimicrobial effectiveness and promising mechanical properties, handling characteristics were only assessed for this bone cement sample. The ISO setting time, assessed according to ISO 5833:2002 [31], determines the timepoint when the bone cement is completely set and cannot be handled any longer. The ISO doughing time describes the time until the PMMA cement reaches the dough state. The ISO setting time and ISO doughing time were comparable to those of PALACOS® R+G and COPAL® R+V, indicating a slightly faster setting time for GD1.5, which means a shorter application time window.

Our investigations indicated that the performance of a PMMA bone cement containing 0.5 g of gentamicin and 1.5 g of daptomycin is the optimal choice considering its antibiotic effectiveness, antibiotic release, and mechanical stability (Figure 7). According to the recommendations from the PRO-IMPLANT Foundation, 3.0 g of daptomycin can be added to a PMMA spacer cement made from 40 g of powder [16], but Kühn [15] suggested not adding more than 2 g of daptomycin, which is in line with our findings. PMMA spacers with manually added antibiotics must also fulfill the legal requirements for medical devices and comply with the ISO standards [15]. This is rather important from a legal perspective, as the surgeon becomes the legal manufacturer of the product by admixing antibiotics. We assumed that the concentration of 3 g of daptomycin was recommended because the antimicrobial effect was perceived as insufficient, so this indicates that the antibiotic release is too weak. It is also described in the literature that the "Daptomycin dose in ALBC for spacer should be 3.3-times the original dose to double the release" [21]. As the mode of action of daptomycin is dependent on calcium ions, solely adding more daptomycin does not necessarily improve the antimicrobial effectiveness [14]. The mode of action is dependent on a sufficient concentration of calcium ions [14] in the surrounding tissue; to increase the inhibitory effect of daptomycin eluted from PMMA bone cement, calcium ions could potentially be added [30]. We want to investigate this in a further study. As manually admixed ALBC is mainly used for spacers, we recommend investigating a longer period of more than 14 days to better simulate the spacer interim period of a two-stage revision protocol [19]. Despite this, we also recommend further investigations with daptomycin-containing PMMA cement in biofilm models.

We want to highlight that a surgeon ordering daptomycin from the pharmacy will receive a different product than the industrially used daptomycin. The clinical available "Cubicin" [32], in addition to daptomycin, also contains sodium hydroxide, the potential influence of which on the mechanical properties and antibiotic elution of PMMA bone cement is not yet clarified. Following the recommendations of the PRO-IMPLANT foundation [16], the addition of Cubicin is costly: for a fixation cement it costs ~780 EUR (2 g), and even more for a spacer cement (~1500 EUR (3 g)), with including the price for a gentamicin-loaded PMMA bone cement as basis for admixing. From a financial perspective, a commercially available PMMA bone cement with gentamicin and daptomycin could be of interest.

Tests // Samples		GD1.5 Test cement 0.5 g Gentamicin + **1.5 g Daptomycin**	GD1.0 Test cement 0.5 g Gentamicin + **1.0 g Daptomycin**	GD0.5 Test cement 0.5 g Gentamicin + **0.5 g Daptomycin**	Ref1 PALACOS® R+G 0.5 g Gentamicin	Ref2 COPAL® G+V 0.5 g Gentamicin + 2.0 g Vancomycin
Antimicrobial Effectiveness	Certika® Proliferation Assay *Enterococcus faecium (EDCC 5271)* *Staphylococcus epidermidis (EDCC 5130)* *Staphylococcus aureus (CCUG 45315)* *Staphylococcus aureus (EDCC 5274)*	+	+	−		
	Inhibition Zone Testing (48 h) *Bacillus spizizenii (ATC 6633)*	+	+	−		
Sterilization Method	Inhibition Zone Testing (48 h) *Bacillus spizizenii (ATC 6633)*					
Antibiotic Release	HPLC Daptomycin (5 days)	+	−	−	−	−
Mechanical Stability	ISO Bending Test (> 50 MPa)	+	+	+	+	+
	ISO Bending Modulus (> 1800 MPa)	+	+	+	+	+
	ISO Compressive Strength (> 70 MPa)	+	+	+	+	+
	DIN Bending Strength (> 50 N/mm²)	+	+	+	+	+
	DIN Impact Strength [kJ/m²] in %	+	+	+	+	+
Handling Properties	ISO Setting Time [min]	+			+	+
	ISO Doughing Time [min]	+			+	+
	Density [g/cm³]	+			+	+

Figure 7. Overview of the tests performed to evaluate the best concentration of daptomycin that can be added to PMMA bone cement (color coding: white = not evaluated, green/+ = effective or requirements fulfilled; red/− = ineffective or requirements not fulfilled).

4. Materials and Methods

4.1. PMMA Cements and Bacteria

PALACOS® R, PALACOS® R+G, and COPAL® G+V (Heraeus Medical GmbH, Wehrheim, Germany) were used. PALACOS® R is a plain PMMA cement without antibiotics, PALACOS® R+G contains 0.5 g of gentamicin, and COPAL® G+V contains 0.5 g of gentamicin combined with 2 g of vancomycin. PALACOS® R+G was loaded with 1.5 g, 1.0 g, and 0.5 g of daptomycin powder (Xellia Pharmaceuticals ApS, Copenhagen, Denmark). PALACOS® R, PALACOS® R+G, and COPAL® G+V were used as references (Table 1). Daptomycin was manually admixed at three different concentrations of 1.5 g (GD1.5), 1.0 g (GD1.0), and 0.5 g (GD0.5). Test strains derived from clinical isolates from the Eugen Domann Culture Collection (EDCC) and Culture Collection University of Gothenburg (CCUG), with different resistance patterns against gentamicin, methicillin, and vancomycin, were used to test the antimicrobial properties of the bone cement samples in vitro (Figure 8).

Tests // Samples		GD1.5 Test cement 0.5 g Gentamicin + **1.5 g Daptomycin**	GD1.0 Test cement 0.5 g Gentamicin + **1.0 g Daptomycin**	GD0.5 Test cement 0.5 g Gentamicin + **0.5 g Daptomycin**	Ref1 PALACOS® R+G 0.5 g Gentamicin	Ref2 COPAL® G+V 0.5 g Gentamicin + 2.0 g Vancomycin	blank sample PALACOS® R without antibiotics	Daptomycin Daptomycin powder
Antimicrobial Effectiveness	Certika® Proliferation Assay *Enterococcus faecium (EDCC 5271)* *Staphylococcus epidermidis (EDCC 5130)* *Staphylococcus aureus (CCUG 45315)* *Staphylococcus aureus (EDCC 5274)*	x	x	x	/	/	x	/
	Inhibition Zone Testing (48 h) *Bacillus spizizenii (ATC 6633)*	x	x	x	/	/	x	/
Sterilization Method	Inhibition Zone Testing (48 h) *Bacillus spizizenii (ATC 6633)*	/	/	/	/	/	/	x
Antibiotic Release	HPLC (5 days)	x (Gentamicin, Daptomycin)	x (Gentamicin, Daptomycin)	x (Gentamicin, Daptomycin)	x (Gentamicin)	x (Vancomycin)	/	/
Mechanical Stability	ISO Bending Test (> 50 MPa)	x	x	x	x	x	/	/
	ISO Bending Modulus (> 1800 MPa)	x	x	x	x	x	/	/
	ISO Compressive Strength (> 70 MPa)	x	x	x	x	x	/	/
	DIN Bending Strength (> 50 N/mm²)	x	x	x	x	x	/	/
	DIN Impact Strength [kJ/m²] in %	x	x	x	x	x	/	/
Handling Properties	ISO Setting Time [min]	x	/	/	x	x	/	/
	ISO Doughing Time [min]	x	/	/	x	x	/	/
	Density [g/cm³]	x	/	/	x	x	/	/

Figure 8. PMMA bone cement test samples and the tests performed to determine their antimicrobial effectiveness, antibiotic release, mechanical stability, and handling properties (x = tested; / = not tested).

4.2. Certika® Proliferation Assay

The Certika® microplate proliferation assay was used to determine the antimicrobial efficacy of material surfaces by measuring their ability to prevent the multiplication of microorganisms and germs on a surface. The testing was conducted according to a method developed by QualityLabs BT GmbH, published by Bechert et al. in 2000 [33–35]. Bone cement samples (seven replicates each) with a diameter of 6 mm were prepared using molds. The tested material was defined as antimicrobial if the formation of at least 99.9% of the daughter cells during the observation time was prevented in comparison to the blank sample. Statistical analysis was performed in line with DIN EN ISO/EC 17025 [36].

4.3. Inhibition Zone Testing

To detect the antimicrobial effectiveness, inhibition zone tests were performed. Agar plates with Bacto agar (2% agarose) and Tris-buffered minimum medium (Ca 10 µM, phosphate 130 µM) were prepared and incubated with *Bacillus subtilis* ssp. *Bacillus spizizenzii* ATCC 6633 [37]. Two bone cement samples with a diameter of 6.0 mm were placed on one plate, executing four repetitions per cement sample. The agar plates were incubated for 48 h at 36 °C. IMAGE pro scanning software was used to determine the sizes of the inhibition zones, as well as for statistical analysis. Afterwards, the average values and standard deviations were calculated for all of the test samples. If daptomycin was effective against *B. subtilis*, it would diffuse in the agar, creating a clear area, known as the zone of inhibition. In this clear zone, the growth of the bacteria is inhibited. The size of this zone was measured and used to interpret the effectiveness of PMMA bone cement containing daptomycin as an antimicrobial agent.

To determine the influence of sterilization on the antimicrobial effectiveness of non-sterilized, ethylene-oxide-sterilized, and gamma-sterilized daptomycin against *B. subtilis*, inhibition zone tests were performed by an external certified lab (INNOVENT e.V. Jena). Data collection and statistical analysis were performed using IMPAGE pro V2 scanning software.

4.4. High-Performance Liquid Chromatography (HPLC)

To determine the release profile of gentamicin and daptomycin dissolved from bone cement samples over 5 days (60 dissolution samples each), HPLC with MS/MS detection was performed [38,39]. As the medium for dissolution, 0.1 M Tris-hydrochloride buffer (pH 7.4) was used. Gentamicin was determined by analyzing its major components separately (resulting in three signals/peaks during LC-MSMS) and summing these three signals to a total concentration. Daptomycin was directly determined using LC-MSMS. The method was validated online using one set of matrix calibration standards and two sets of quality control samples. The calibration samples were used to calculate the results, and the quality control samples were used to monitor the quality of the analytical run. The mean values and standard deviations were calculated for all of the test samples.

4.5. Mechanical Stability Testing According to ISO 5833 and DIN 53435

To ensure that the added daptomycin did not negatively influence the mechanical stability of the PMMA cement, mechanical tests for bending strength, bending modulus, and compressive strength were performed according to ISO 5833:2002 [31]. To determine the compressive strength, the cement rods (12 mm height, 6 mm diameter) were loaded with a constant crosshead speed of 19.8–25.4 mm/min. The tests were run at 23 ± 1 °C with dry specimens prepared 24 h before testing. For the bending strength and bending modulus, rectangular specimens (3.3 mm × 75.0 mm × 10.0 mm) were used; they were loaded with a constant crosshead speed of 5 mm/min. The tests were run at 23 ± 1 °C with dry specimens prepared 24 h before testing. The four-point bending test rig had 60 mm between the outer loading points and 20 mm between the inner loading points. The tests were continued until failure, and the maximum force was used to calculate the bending strength. Value calculations and statistical analysis were performed as described in the ISO standard [31].

The DIN impact strength and bending strength were also determined according to DIN 53435 [40]. The rectangular specimens (3.0 mm × 15.0 mm × 10.0 mm) were stored for at least 12 h under standard climatic conditions using the appropriate impact direction (i.e., consumption of at least 10%, and at most 80%, of the maximum impact by the test specimens). The bone cement samples were placed vertically in the test device, and the pendulum was adjusted to 90° and the height of the drop. According to DIN 53435 [40], the average and standard deviation were calculated in kJ/m^2. For the DIN bending strength, a bending force of 400 Ncm was applied to the bone cement samples until they broke. The bending strength was measured and calculated, and statistical analysis was performed according to DIN 53435 [40].

4.6. Handling Properties of PMMA Cement

The ISO setting time and ISO doughing time were assessed, and statistical analysis was performed [31].

5. Conclusions

Our results suggest adding 1.5 g of daptomycin to PMMA cement combined with 0.5 g of gentamicin for PJI cases caused by vancomycin-resistant germs. All mechanical and handling properties, along with the elution profile and effectivity of 1.5 g of daptomycin added to 40 g of PMMA, fulfilled all clinical requirements. Due to its antimicrobial spectrum against vancomycin-resistant germs (e.g., VRE), a PMMA cement containing 0.5 g of gentamicin and 1.5 g of daptomycin instead of vancomycin could be a good option for the treatment of PJIs. Further investigations of its performance in biofilm models and against clinical isolates are recommended.

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2. Enhancing PMMA cements with manually added antimicrobial agents

2.1. Summary

In this study, we investigated the impact of manual admixing and additional dry homogenisation of antimicrobial agents into PMMA cements, with a specific focus on the antibiotic vancomycin. Our aim was to evaluate whether additional dry mixing of the powder and anti-infective substance blend improves the cement's mechanical properties, antibiotic release behaviour, and mixture homogeneity, or whether it introduces problems such as abrasion and particle detachment from mixing system components. This question is clinically relevant because in many PJI cases, anti-infective agents must be manually added to PMMA cement when no suitable industrially premixed cement exists (Berberich et al., 2023; Krampitz et al., 2023; Tsung et al., 2014).

The main objectives of our work were to determine whether additional dry mixing enhances the homogeneity of vancomycin-loaded PMMA cement and whether this step improves or worsens antibiotic elution, mechanical stability, or microbiological efficacy (Gergely et al., 2016; Kühn et al., 2017; McLaren et al., 2009). Additionally, we wanted to identify potential risks introduced during dry mixing, such as plastic abrasion from mixing cartridges and paddles. An important aspect was to provide practical recommendations for surgeons regarding safe and effective manual admixing procedures.

I prepared four sets of PMMA cement samples using Palacos® R+G as the base cement, manually admixing 2.2 g vancomycin to the polymer powder. Two sample sets were mixed conventionally without dry mixing, and two underwent additional dry mixing inside two different mixing cartridges (Palamix® 2 and Optivac®). To benchmark performance, I used Copal® G+V (microbiological reference) and Copal® G+C pro (mechanical reference). I assessed the mechanical properties (ISO bending strength, bending modulus (ISO 5833, 2002), DIN impact strength (DIN 53435, 2018)). Antibiotic elution was measured on day 1, 3 and 7. Mixing cartridges and paddles were examined under a microscope to determine a potential abrasion.

We found no statistically significant differences between manually mixed samples and reference cements in ISO bending strength or bending modulus. Bending strengths among test samples ranged narrowly (62.2–63.2 MPa), closely aligning with reference values. DIN impact strength was more variable, with samples produced in the Optivac® system showing significantly lower values, suggesting that mixing-device design influences sensitivity to powder abrasion and localised weaknesses. Overall, additional dry mixing did not improve mechanical properties. Across all samples, vancomycin exhibited a burst release on day 1 (~maximum 647 µg/sample for the reference), followed by decreasing concentrations on days 3 and 7. Additional dry mixing did not lead to higher or more consistent vancomycin elution. On day 7, samples with dry mixing showed significantly lower release than the reference cement. Elution patterns between samples with and without dry mixing were statistically comparable ($p > 0.05$). No homogenisation related improvement could be demonstrated. Microscopy revealed that the inner surfaces of mixing cartridges showed scratching and abrasion for dry mixing. Small plastic particles detached during dry mixing and were embedded into the polymer powder. The mixing paddles, especially in the Optivac® system, showed surface roughening and visible material loss. Industrially premixed systems such as Copal® G+C pro did not show any abrasion.

We can conclude that manual dry mixing of cement powder with additional antimicrobial agents should be avoided. Additional dry homogenisation does not improve antibiotic release, mechanical properties and microbiological performance. Instead, it introduces the risk of abrasion, plastic particle contamination, and potential inflammatory complications (Gelb et al.,

1994; Jiang et al., 2016). For safe and effective manual preparation of antimicrobial PMMA bone cements, we recommend avoiding dry mixing in closed cartridges. Ideally, the orthopaedic surgeon should prefer industrially premixed cements whenever available, particularly for routine clinical use. We recommend in case a manual addition of an anti-infective substance is needed to follow our recommendations depicted in both tables (Table 2 and Table 3) contained in the publication. These findings contribute directly to improving the safety and reproducibility of manually prepared anti-infective-loaded PMMA cements in PJI revision surgery.

2.2. Contribution

The tests on mechanical stability (ISO 5833:2002, DIN 53435:2018) were performed by me. The test on antibiotic elution (HPLC) was handed over to an external certified lab (Analytisches Zentrum Biopharm GmbH, Berlin). The microscopical examination was done with support from Tim Schnieber and Betina Hinkelmann. The literature review was performed by Klaus-Dieter Kühn and me. All authors designed the research. Klaus-Dieter Kühn and I analysed the data. I wrote the manuscript and reviewed it with all authors.

2.3. Reference

Humez, M., Citak, M., Luck, S., Linke, P., Gehrke, T., Paul, C., & Kühn, K.-D. (2025). Enhancing PMMA Cements With Manually Added Antimicrobial Agents. *APMIS: acta pathologica, microbiologica, et immunologica Scandinavica*, *133*(5), e70029. https://doi.org/10.1111/apm.70029

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ORIGINAL ARTICLE

Enhancing PMMA Cements With Manually Added Antimicrobial Agents

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ABSTRACT

Periprosthetic joint infection (PJI) is an infrequent yet severe complication. A fundamental aspect of PJI treatment involves the use of polymethylmethacrylate (PMMA) cement augmented with antibiotics. For therapeutic application, it is often necessary to manually mix antibiotics with commercially produced PMMA cements. However, the potential issues arising from this manual admixing have not been thoroughly documented. This study aims to elucidate the impact of additional homogenization through dry mixing of a polymer-active substance blend on the quality of manually mixed PMMA cement. In this laboratory-based investigation, four cement samples were prepared using various methods for the manual incorporation and homogenization of the antibiotic vancomycin. The reference controls were Copal G+V and Copal G+C pro (Heraeus Medical GmbH, Wehrheim, Germany), representing commercially available PMMA cements and a closed mixing system. The samples were analyzed for mechanical, microbiological, and microscopic properties. The mechanical and microbiological analyses revealed no statistically significant differences between the manually mixed samples and the references. However, microscopic examination of the mixing cartridges' inner surfaces indicated scratching and signs of abrasion during the mixing process. The manual incorporation of antibiotics into PMMA cement should be limited to specific indications, with a preference for using commercially available mixtures whenever possible.

1 | Introduction

Periprosthetic joint infection (PJI) following hip and knee arthroplasty is an infrequent complication, yet it carries a high mortality rate of approximately 21% within 5 years [1, 2]. Upon accurate diagnosis of PJI [3], surgical revision supported by either locally or systemically administered antibiotics or antimycotics is the primary treatment approach [4–10]. During septic revision surgery in arthroplasty, the active substances used further support the treatment and ensure effective surgical debridement by reducing the bacterial load after the removal of all alloplastic (foreign) materials [4, 9, 11, 12]. Acrylic-based bone cements are utilized to fix prostheses in arthroplasty procedures [13] as well as for temporary spacers in two-stage revisions, serving as interim prostheses [8, 9, 14]. As a local drug carrier [15], PMMA cement can be specifically augmented with anti-infective agents [10, 16–19], which achieve high local drug concentrations post-implantation [20, 21].

When additional active substances are manually incorporated into industrially produced PMMA cement powder, the original medical device undergoes permanent alteration [22]. This modification is essential in cases of periprosthetic infections where fungi are identified as pathogens, as no industrially produced

TABLE 1 | (a) Overview of tested cement mixtures with added vancomycin and their homogenization (dry mixing) and corresponding references. (b) Overview of tests performed: + test was performed and – was not tested.

	Sample 1	Sample 2	Sample 3	Sample 4	Reference [1]	Reference [2]
(a)						
PMMA cement	Palacos *R*+G	Palacos *R*+G	Palacos *R*+G	Palacos *R*+G	Copal G+V	Copal G+C
Mixing system	Palamix 2	Optivac	Palamix 2	Optivac	Palamix 2	Pro-system
Vancomycin addition	+2.2 g	+2.2 g	+2.2 g	+2.2 g	—	—
± Dry mixing (DM)	–DM	–DM	+DM	+DM	–DM	–DM
(b)						
Microbiology						
Antibiotic release	±	±	±	±	±	—
Microscopy						
Abrasion	±	±	±	±	±	±
Mechanics						
ISO bending strength	±	±	±	±	±	±
ISO bending modulus						
DIN impact strength						

mixtures are available for cases [19]. Consequently, the orthopedic surgeon assumes the role of the manufacturer of the PMMA cement mixture under the Medical Device Regulation (MDR). Beyond the legal implications, it is critical that the admixed ingredient demonstrates sufficient and reproducible release from polymethylmethacrylate (PMMA) cement, for example, the manual addition of antibiotics can result in a lower release compared to commercially available antibiotic-loaded cements [23]. Furthermore, the resulting PMMA cement must comply with applicable standards (e.g., ISO 5844 [24]) taking into consideration that additional substances may interfere with the mechanical properties [25]. This requirement also applies when a spacer is reinforced with a metal inlay, ensuring mechanical integrity is maintained. Adding powder components, for example, antibiotics, also alters the powder-liquid ratio influencing the mechanical properties [26]; this also applies for the liquids present in an arthroplasty surgery (human blood, irrigation solutions) [27, 28]. Even the mixing method could alter the bending strength of the PMMA cement [29].

Ensuring a high-quality product necessitates the homogeneous distribution of manually added active substances [7, 30, 31]. To achieve this, the active ingredients are incorporated into the polymer powder within a sterile bowl using a mortar if needed to break down larger crystals [7]. This polymer/active ingredient mixture is then transferred into a cement cartridge and sealed. The mixture is further homogenized within the cartridge [32] using a stirring spatula at relatively high speeds. Subsequently, the cement dough is formed by adding the liquid monomer.

The process of additional dry mixing, intended to enhance the homogenization of the polymer/active ingredient mixture within the cartridge, raises several critical considerations. Abrasive components of the polymer powder may promote the detachment of plastic particles from the inner surface of the mixing cartridge and the mixing paddle. Furthermore, the active ingredient particles could be pulverized, potentially impairing both the release profile and the mechanical properties of the cement. In this study, we hypothesize that additional homogenization does not improve elution and mechanical properties of the PMMA cement.

2 | Materials and Methods

In this laboratory-based study, four cement samples were prepared using various methods for the manual addition and homogenization of the antibiotic vancomycin (Table 1). Copal G+V (Heraeus Medical GmbH, Wehrheim, Germany), which includes industrially admixed vancomycin, served as the reference for microbiological investigations. For mechanical property assessments, Copal G+C pro (Heraeus Medical GmbH, Wehrheim, Germany), provided in a closed mixing system and containing two antibiotics, was used as the reference.

2.1 | Material

To Palacos *R*+G (Heraeus Medical GmbH, Wehrheim, Germany), which already contains 0.5 g of gentamicin, 2.2 g of vancomycin (Table 1) was manually added. The references used were Copal G+V (containing 0.5 g gentamicin +2.0 g vancomycin) and Copal G+C pro (containing 1.0 g gentamicin +1.0 g clindamycin, Table 1).

2.2 | Cement Mixing

The four cement samples were mixed in a bowl [8]. Initially, approximately one-third of the polymer was combined with one-third of the vancomycin. The coarse vancomycin particles were crushed using a mortar and homogenized with the polymer [33]. This procedure was repeated twice, incorporating the remaining polymer and vancomycin. The homogenized polymer/active ingredient mixture was then combined with the monomer liquid to form a cement dough in the Palamix 2 (Heraeus Medical GmbH, Wehrheim, Germany) (sample 1) and in the Optivac (Zimmer-Biomet, Warsaw, USA) (sample 2) without vacuum. The dough was placed in a mold in accordance with ISO 5833 [24] and DIN 53435 [34]. After setting, the molded PMMA cement samples were removed from the mold and tested for ISO bending strength, ISO bending modulus, and DIN impact strength.

To assess the influence of additional dry mixing of the polymer compound, the manually prepared mixture was transferred from the bowl to the mixing systems Palamix 2 (sample 3) and Optivac (sample 4). In these systems, it was mixed without vacuum for 30 s at a mixing frequency of one stroke per second, without the monomer liquid. The inner surfaces and mixing paddles were then examined microscopically. Following this dry mixing procedure, the PMMA powder was mixed with the corresponding monomer liquid. The mixed powder was removed from the cement cartridge and transferred to a new cartridge already containing the monomer liquid. The reference samples, Copal G + V and Copal G + C pro, were both prepared according to the manufacturer's instructions for use.

2.3 | Mechanical Testing

The PMMA cement dough was placed in the mold in order to prepare test bodies according to ISO 5833 [24] or DIN 53435 [34] and the respective mechanical tests were performed. The following mechanical parameters relevant to PMMA cements were analyzed: ISO bending strength (Instron, Schenck, Darmstadt, Germany), rectangular molded bodies: $3 \times 75 \times 10$ mm; ISO bending modulus (Instron), rectangular molded bodies: $3 \times 75 \times 10$ mm; and DIN impact strength (Zwick, Ulm, Germany), rectangular molded bodies: $3 \times 10 \times 15$ mm. ISO bending modulus and strength, as well as DIN impact strength, were assessed as those are mechanical parameters most influenced by manual addition of antimicrobial agents.

2.4 | Microbiological Testing

Standardized cylindrical molded bodies (25×10 mm) were prepared for antibiotic release testing. Antibiotic release was determined in an external laboratory (Analytisches Zentrum Biopharm GmbH 12,681 Berlin) with a triple determination on days 1, 3, and 7. Potential deviations and differences in the release behavior are caused by inhomogeneities [25, 26].

2.5 | Microscopic Examination

The mixing cartridges and mixing paddles were examined under a microscope (Keyence digital microscope model series VHX-6000; Keyence Deutschland GmbH, Neu-Isenburg, Germany) for damage caused by abrasion after the cement mixture. Copal G + C pro was set as the reference system as well as Copal G + V prepared in Palamix.

2.6 | Statistics

Statistical analysis is specified by ISO 5833 [24] and DIN standard 53435 [34] (6-fold individual determination, limit values, acceptance criteria). For the elution data, the mean value was calculated as well as the standard deviation. The statistical significance was determined using a one-way ANOVA with Dunnett multiple comparisons test. A p value of 0.05 was defined as statistically significant. The Prism 5 software (GraphPad Software, San Diego, CA, USA) was used for the calculation.

2.7 | Literature

There is a wide range of experience with the manual addition of anti-infective substances to PMMA cement, which is listed in various publications. In the discussion section, current recommendations are summarized and presented as a take home message.

3 | Results

3.1 | Mechanical Test Results

Regarding mechanical stability, no statistically significant differences were observed between the manually admixed samples and the references (Figure 1), except for DIN impact strength. The references exhibited the highest values for ISO bending strength (Copal G + V: 63.5 ± 0.5 MPa and Copal G + C pro: 64.1 ± 2.6 MPa) and DIN impact strength (Copal G + V: 2.85 ± 0.21 kJ/m^2 and Copal G + C pro: 3.03 ± 0.32 kJ/m^2). Within test groups 1–4, the bending strengths ranged from 62.2 ± 2.1 MPa (sample 4) to 63.2 ± 1.0 MPa (sample 2) without any significant differences and the bending modulus ranged from 2789 ± 92 MPa (sample 3) to 2919 ± 41 MPa (ref. [1]). For ISO bending modulus, there was a significant difference between sample 3 ($p = 0.032$) and ref. [1] as well as in between both references ($p < 0.001$). DIN impact strength values were 2.36 ± 0.22 kJ/m^2 (sample 4) and 2.76 ± 0.23 kJ/m^2 (sample 1). Notably, the DIN impact strengths for some cement samples were significantly lower for sample 2 ($p = 0.04$) and sample 4 ($p < 0.01$).

3.2 | Microbiological Test Results

The antibiotic release profiles from the manually produced cement samples did not exhibit statistically significant differences when compared to those subjected to additional dry mixing (Figure 2: Reference to sample 1: $p = 0.1082$; reference to sample 2: $p = 0.1238$; reference to sample 3: $p = 0.1861$; reference to sample 4: $p = 0.1412$; one-way ANOVA: $p = 0.2698$). For all samples we observed an antibiotic burst on day 1 (Figure 2a) with a maximum vancomycin concentration of 647 µg/sample for the reference Copal G + V. On day 3 the vancomycin concentration dropped to a maximum of 398 µg/sample for the reference (Figure 2b). No statistically significant differences ($p > 0.05$)

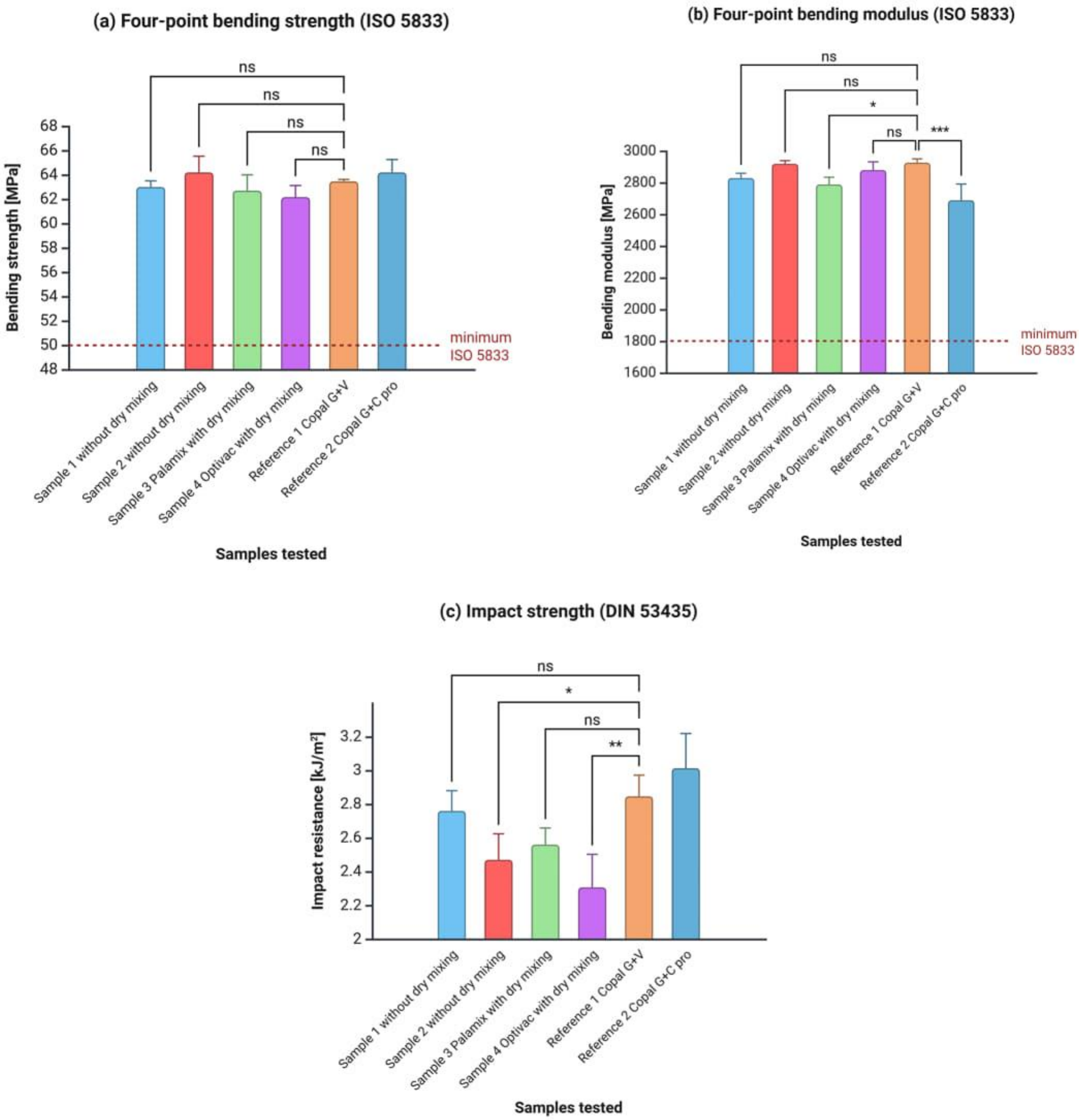

FIGURE 1 | Testing for mechanical stability according to (a) + (b) ISO 5833 and (c) DIN 5345 for all tested samples. Created in https://BioRender.com. ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

were observed in the antibiotic release between the two samples (samples 1 and 2) without additional dry mixing. Similarly, the elution of vancomycin from the samples (samples 3 and 4) with additional dry mixing did not show any statistically significant improvement in release ($p > 0.05$). The release of vancomycin from the reference sample was consistently compared to the manually prepared samples and showed on day 1 (Figure 2a) a significantly higher release compared to all manually prepared samples ($p < 0.01$) No significant difference to reference was observed on day 3 (Figure 2b). For day 7, the vancomycin elution from sample 1 and 4 was significantly lower compared to the reference ($p < 0.01$) (Figure 2c).

3.3 | Microscopic Results

The inner surface of the mixing cartridges used showed scratch marks after the dry mixing of the polymer powder. Small plastic particles were detached from the roughened surface and were released into the polymer powder. In addition, plastic particles were clearly visible on the front part of the mixing paddle. In the two mixing systems analyzed, it was also noticeable that individual plastic particles had broken out of the Optivac, while the surface of the Palamix 2 was rather scratched. Scratches were particularly visible on the front of the mixing paddle (Figure 3). For the reference mixing system Copal G + C pro, no scratches

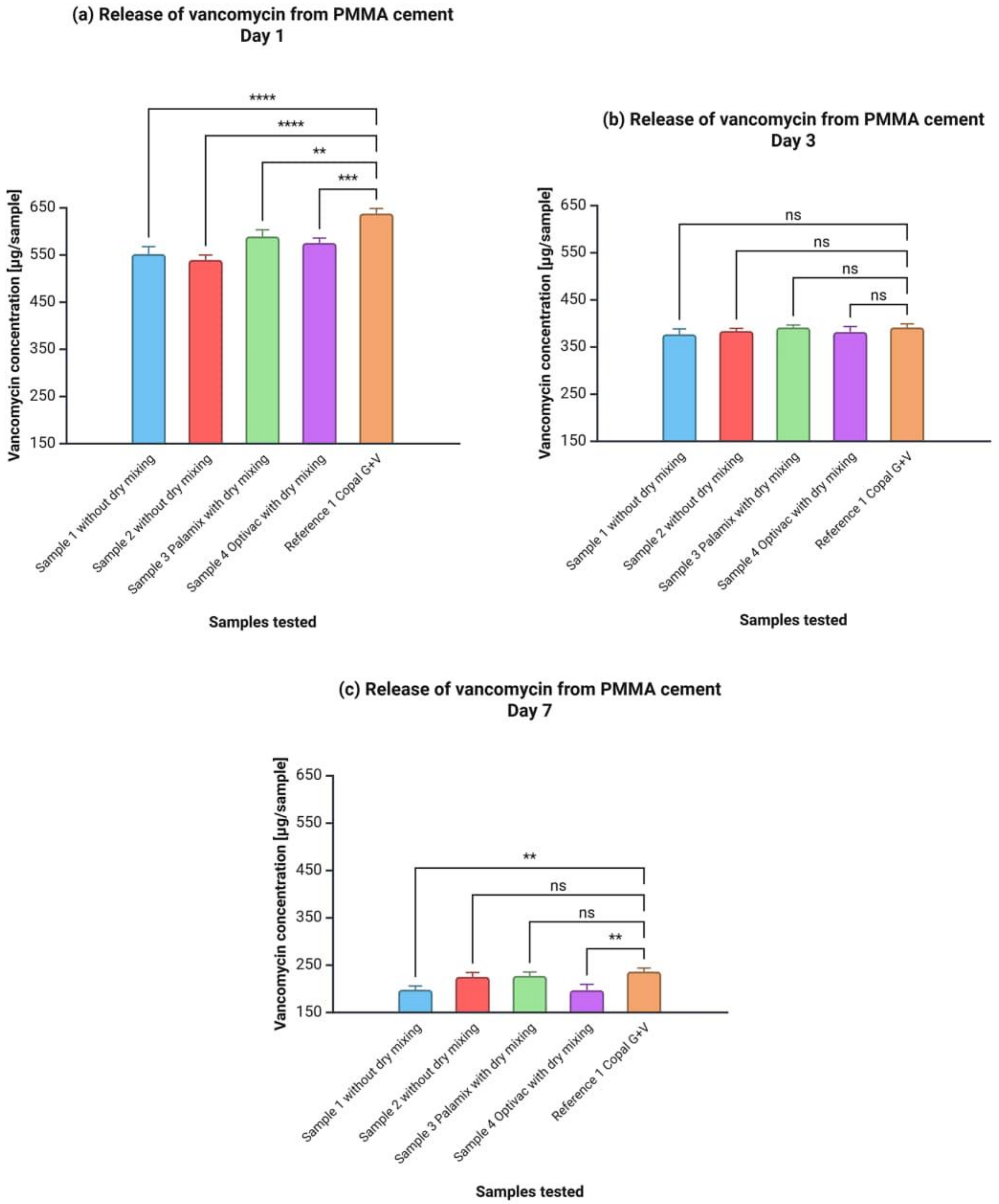

FIGURE 2 | Vancomycin release in µg/test specimen from the various manually prepared cement samples compared to reference Copal G + V on (a) day 1, (b) day 3, and (c) day 7. Created in https://BioRender.com. ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

could be found in the inner surface of the cartridge after cement extrusion. This was also applicable for the Copal G + V prepared in the Palamix system.

4 | Discussion

By definition, a revision PMMA cement contains more than

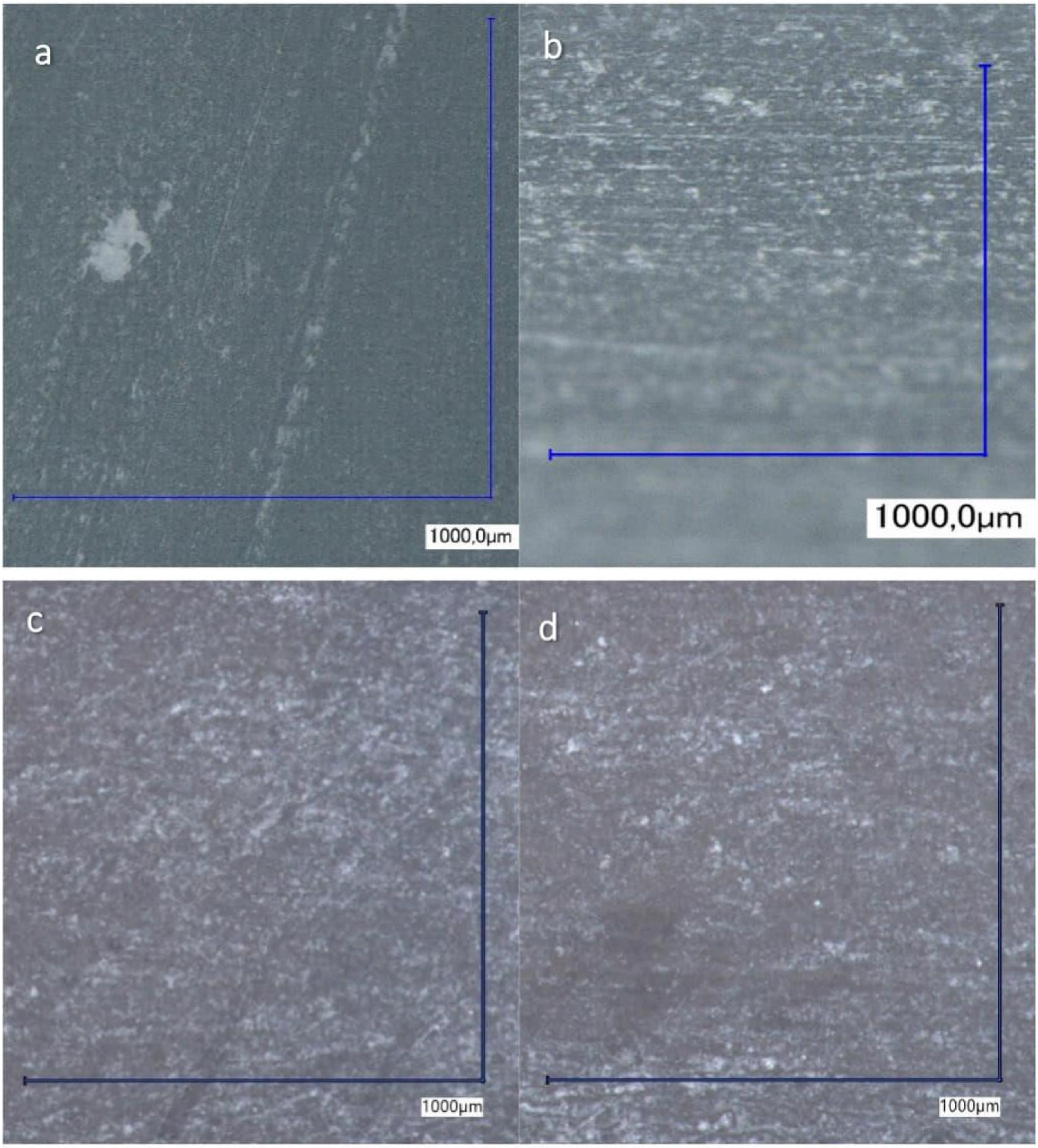

FIGURE 3 | Roughened surface of the stirrers of the Optivac and Palamix mixing systems tested after dry mixing of a polymer/active ingredient mixture in the mixing system. (a) Optivac, (b) Palamix, (c) COPAL G + C pro, (d) Copal G + V. (Keyence digital microscope model series VHX-6000). Pictures T. Schnieber, B. Hinkelmann.

one anti-infective substance at a concentration exceeding 2.5%. Antibiotic combinations, such as gentamicin and vancomycin or gentamicin and clindamycin, offer the advantage of synergistic antibiotic elution [15, 35, 36]. Manual admixing of anti-infective agents to PMMA powder is only indicated when no industrially produced cement is available that meets the perioperative microbiological requirements [18, 19, 30, 35]. To achieve a homogeneous distribution of manually added active ingredients, the powder mixtures are often dry-mixed in the mixing cartridge before combining with the monomer liquid. This additional homogenization step ensures that the cement dough is well-mixed [7]. In one-stage septic revisions, this manually prepared PMMA cement is used to fix a prosthesis [13]. In two-stage or multiple-stage revisions, it is used to create interim prostheses (spacers), which are removed after a retention period of 2–6 weeks [17].

According to our investigations, additional dry mixing of the PMMA powder did not improve homogenization. The mechanical properties were comparable with or without dry mixing. Omitting the additional dry mixing step offers advantages, such as saving time during surgery, as the manually prepared antibiotic-cement mixture can be immediately transferred to a mixing cartridge containing the monomer liquid. Consistent use of fractionated admixture and production of the dough in a suitable mixing system can eliminate the need for dry mixing. Microbiological characteristics indicate that the release of the added antibiotic is comparable even without dry mixing. PMMA cement mixing systems are not designed for homogenizing powder, especially abrasive powder. A few careful movements of the mixing paddle in the polymer, such as collecting vacuum or positioning the paddle, are not critical. Abrasion of the plastic occurs due to rapid mixing movements combined with the simultaneous rotation of the paddle within the polymer. This action causes abrasive components of the powder to be pressed and rubbed against the inner wall of the cartridge, leading to surface scratches and particle detachment. These particles can remain in or on the PMMA cement. The presence of such debris may induce an inflammatory response, which is also influenced by the particle size [37]. Particles within the joint tissue enhance macrophage activity, triggering an inflammatory reaction that leads to the destruction of adjacent cells, ultimately resulting in osteolysis [38]. While crunching noises may occur, their intensity depends on the plastic composition. Although the noise is a secondary phenomenon, the abrasion itself is significant. The clinical implications, such as potential inflammation or other bodily reactions, have not yet been clarified. These findings suggest that cement mixing processes should ideally specify the polymer first, with the liquid added subsequently. However, some cements on the market have manufacturer-specified component order. For instance, with Simplex P (Stryker Howmedica Osteonics, Kalamazoo, MI, USA), the polymer powder is specified first, followed by the addition of the monomer liquid according to the manufacturer's instruction for use (www.stryker.com) Initially, the powder is minimally dissolved by the monomer due to its composition. The polymers or copolymers used only allow wetting by the liquid monomer after a short period (10–20 s) following component combination. Therefore, the dry powder scrubs the inner surface of the cartridge before the powder soaks the liquid, resulting in potential abrasion due to intensive strokes of the stirring spatula.

The vancomycin elution over 7 days starting with an antibiotic burst on day 1 is in line with recent findings from Kwong et al. [39] indicating the highest antibiotic release for the commercially available Copal G + V.

Industrially produced mixtures containing two antibiotics are easy to use in the operating theater under sterile conditions, especially when using pre-packed systems (e.g., Copal G + C pro). These pre-packed PMMA cement systems consist of a mixing cartridge prefilled with cement containing a combination of two antibiotics (e.g., gentamicin and clindamycin), enabling homogeneous cement mixing. Such systems have been shown to reduce the risk of revision due to infection [40]. The combination of two antibiotics is not only established for revision surgery [41] but also used in patients with a fractured neck of the femur to reduce the revision risk [42, 43]. Other industrially manufactured PMMA cements are Copal G + C and Copal G + V (Heraeus Medical GmbH, Wehrheim, Germany), Refobacin Revision (Zimmer Biomet, Warsaw, USA), VancoGenx (Merete GmbH, Berlin, Germany), and Antibiotic Simplex with Erythromycin and Colistin (Stryker Howmedica Osteonics, Mahwah, USA) [31]. Both Copal G + C and Refobacin Revision contain 1.0 g gentamicin and 1.0 g clindamycin per 40 g PMMA powder. Although their polymer matrices differ, their cement properties are not significantly different (Table 2). In contrast, Copal G + V and VancoGenx have notable compositional differences (Table 2). Copal G + V contains 0.5 g gentamicin and 2.0 g vancomycin, whereas VancoGenx contains 1.0 g gentamicin and 1.0 g vancomycin. Over time, Copal G + V exhibits significantly better antibiotic elution than VancoGenx [15, 59].

If manual admixture is necessary, it is strongly recommended to use antimicrobial agents in powder form only, as liquids can significantly adversely affect the elution and mechanical properties of bone cement [60] (Table 3). The powder must be sterile, and its active ingredient content should be verified due to potential variability. Furthermore, the quality of anti-infective substances varied by different manufacturers [44]. The homogeneity of the antimicrobial admixture is crucial; for instance, crystalline powders may be more challenging to mix uniformly compared to fine powders. This difficulty arises because antimicrobial powders are typically intended for injection solution preparation. For manual mixing of larger amounts of active substances with PMMA cement, it is recommended to use a low-viscosity cement brand with a prolonged low-viscosity phase [19]. The addition of antibiotics or antimycotics can alter the color of the cement dough. For example, tigecycline and amphotericin B are orange-brown powders that color the cement accordingly, which does not indicate a polymerization failure [19, 50]. The PRO-IMPLANT Foundation pocket guide [58] and the ICM consensus on orthopedic infections [61] offer recommendations for the manual addition of antimicrobial agents. Notably, the ICM consensus recommendations [61] are not based on in vitro testing. To ensure the production of a spacer with adequate release of the anti-infective agent, refer to Table 2.

Combining two or more antibiotics can not only increase antibiotic release but in some cases also limit antibiotic release (e.g., gentamicin and fosfomycin) [47]. Certain antibiotics, such as rifampicin, should not be used for admixture as they interfere with the polymerization reaction [47]. Additionally, the PMMA cement brand and the quality of the antibiotic significantly influence the antimicrobial efficacy; for example, different vancomycin products can yield varying outcomes [44]. Hsun-Lee et al. [44] tested three different vancomycin brands with the highest elution for sterile vancomycin (Hospira Inc., Lake Forest, Illinois, USA) and the lowest elution for Lyo-Vancin (China Chemical & Pharmaceutical Co. Ltd., Taichung, Taiwan) which differs by 5 fold. Insufficient antibiotic concentrations can lead to the development of resistance if they fail to reach the minimum inhibitory concentration [21]. This issue can arise as antibiotics used for admixture do not solely consist of active components. The activity coefficient indicates the actual proportion of active substance present [47]. Additionally, some active substances available on the market were originally intended for other applications, such as infusions (e.g., amphotericin B or voriconazole) [19]. These substances may have low active

TABLE 2 | In clinical practice antimicrobials are added manually to PMMA cement.

Clinical situation/ pathogen	Antimicrobials	Commercially available/ manual admixing	Dosage fixation cement	Dosage spacer cement	Admixing procedure	Mechanical properties	Synergistic elution effect	Remarks	Literature
Susceptible pathogen unknown	Gentamicin + Clindamycin	COPAL G+C, Refobacin revision	1g+1g	1g+1g				Clindamycin is not available as sterile powder	[41]
	Gentamicin + Clindamycin + Vancomycin			1g+1g+2g				Source and quality of vancomycin is decisive	[15, 31, 44–46]
Methicillin-resistant: *Staphylococcus epidermidis* (MRSE) *Staphylococcus aureus* (MRSA) Oxacillin-resistant: *Staphylococcus aureus* (ORSA) *Enterococci*	Gentamicin + Vancomyin	COPAL G+V, VancoGenx	0.5g+2g	0.5g+2g				Source and quality of vancomycin is decisive	[39, 45–47]
	Gentamicin + Daptomycin	Admixing	0.5g+2g	0.5g+3g				Easy admixing of daptomycin	[18]
Vancomycin-resistant: *Enterococci* (VRE) *Staphylcoccus aureus* (VRSA/VISA)	Gentamicin + Linezolid	Admixing	0.5g+1g	0.5–1g+2g				Easy admixing of linzelolid Linezolid is costly	[14]
	Gentamicin + Daptomycin	Admixing	0.5g+2g	0.5–1g+3g				Easy admixing of daptomycin	[18]
	Gentamicin + Fosfomycin sodium	Admixing	0.5g+2g	0.5–1g+2–4g				Dosage form important, prefer sodium	[48, 49]
	Gentamicin + Tigecycline	Admixing	0.5g+0.5g	0.5–1g+1g				Tigecycline stains PMMA powder orange-brown	[50]

(Continues)

TABLE 2 | (Continued)

Clinical situation/ pathogen	Antimicrobials	Commercially available/ manual admixing	Dosage fixation cement	Dosage spacer cement	Admixing procedure	Mechanical properties	Synergistic elution effect	Remarks	Literature
Resistant gram-negative bacteria for example: *Escherichia coli* *Klebsiella* *Enterobacter* *Pseudomonas* spp.	Gentamicin + Colistin	Antibiotic simplex E+C	0.5+5–10 Mio IU	0.5–1+10–20 Mio IU				Antibiotic Simplex E+C no longer available, use colistin-sodium or colistin-sulfate for admixing, small ampoules with powder	[51, 52]
	Gentamicin + Fosfomycin sodium	Admixing	0.5g+2g	0.5g+2–4g				Formulation is important; recommendation to prefer fosfomycin sodium	[53]
	Gentamicin + Meropenem	Admixing	0.5g+2g	0.5–1g+3g				Easy admixing of meropenem	[54]
	Gentamicin + Ciprofloxacin	Admixing	0.5g+2g	0.5–1g+3g				Recommendation to prefer ciprofloxacin salt over pure ciprofloxacin	[55]
Fungi for example: *Candida* spp. *Aspergillus* spp.	Gentamicin + Amphotericin B	Admixing	0.5g+200mg	0.5–1g+400mg				PMMA cement is stained orange-brown To achieve a concentration of 200mg use 4×1.3g of amphotericin B	[56, 57]
	Gentamicin + Voriconazole	Admixing	0.5g+200mg	0.5–1g+400mg				To achieve a concentration of 200mg use 3.5g of voriconazole	[19]

Note: This table offers on overview on the most common germs and corresponding anti-infectives as well as recommendations for admixing. Clinical situation and anti-infective dosages according to PIF Pocket Guide [58]. Green, Easy admixing/good mechanical properties/synergistic elution effect; yellow, Careful admixing/reduced mechanical properties/reduced synergistic elution effect; red, Difficult admixing/limited mechancial properties/limited synergistic elution effect.

TABLE 3 | Recommendations for manual admixing of anti-infective substances to PMMA powder.

Never homogenize antibiotic powder dry in the mixing system	As this could potentially increase the abrasion risk
Prepare PMMA cement for spacer without vacuum	As vacuum mixing would reduce antibiotic elution
Not add liquid antibiotics to the powder	As this could interfere with the cement matrix and potentially weaken it
Pay attention to the quality of the antibiotic powder	As the antibiotic/antimycotic substance could come with potential filling substances reducing the total amount of active substance
Use sterile antibiotic powder	To reduce the risk for contamination

ingredient content, necessitating the use of large quantities to achieve sufficient local concentrations in arthroplasty.

Inhomogeneities can permanently weaken the cement matrix, rendering the cement body unable to withstand the mechanical demands as an anchoring material or spacer [16, 62]. Regarding mechanical properties, most in vitro studies focus on compression strength, which is not significantly affected by the addition of antimicrobial agents. It is advisable to consider ISO bending strength and DIN impact strength as indicators of the impact on mechanical properties as they are sensitive to weak points in the cement matrix (Table 2) [47, 63]. Potentially, these inhomogeneities can lead to cement fractures, necessitating further revisions [16]. The ISO bending modulus is of high relevance as large amounts of added ingredients can increase the bending modulus. This increase is primarily due to improved fluid absorption by the cement matrix, making the cement appear slightly more elastic [22]. Additionally, the active ingredients may not be reproducibly released from the cement [4].

The use of erythromycin and colistin-loaded Antibiotic Simplex in total knee arthroplasty did not reduce infection rates in long-term follow-up, suggesting it is not suitable for general use. The combination of erythromycin and colistin may be problematic due to erythromycin's weak mode of action and relatively high resistance levels [52, 64, 65]. Colistin is readily available as a sterile powder, typically in the form of colistin sulfate or colistin sodium. It is highly effective against most problematic gram-negative bacteria when combined with gentamicin, clindamycin, vancomycin, and meropenem [51, 66]. The pure colistin content is usually expressed in international units (IU), so the dosage for admixture in grams must be calculated in advance. Sterile meropenem powder has minimal impact on mechanical properties [54, 67] and is quite resistant to high polymerization temperatures, making it suitable for admixture with PMMA [68]. Both pure ciprofloxacin and its salts can be used, with ciprofloxacin salt potentially offering better release. However, higher concentrations (up to 5%) can decrease ISO mechanical strength [47, 55].

Cephalosporins such as cefuroxime and cefazolin, along with other related compounds, are available as sterile powders. These antibiotics are often heat-sensitive. When combined with other antibiotics like gentamicin and vancomycin, they exhibit a synergistic effect [31, 47, 62]. Linezolid, although effective as a sterile powder, remains quite expensive and elutes well from PMMA at concentrations above 1 g [14]. Fosfomycin is frequently unavailable as a sterile powder, necessitating the use of pharmaceutical formulations intended for other indications. When combined with gentamicin, both antibiotics demonstrate good efficacy [47, 49, 53], but fosfomycin can exhibit inhibitory effects when combined with vancomycin against *Staphylococcus aureus* [48].

While vancomycin is available in sterile powder form, the quality varies among manufacturers. The addition of vancomycin to PMMA significantly impacts mechanical properties, particularly ISO bending and DIN impact strength. There is a synergistic effect between gentamicin and vancomycin, both physically and pharmaceutically. Combining vancomycin, gentamicin, and clindamycin using Copal G + C and Copal G + V yields excellent mechanical and elution properties [31]. In some cases, vancomycin can be applied to the cement surface before it fully hardens in the body, resulting in a high vancomycin concentration immediately after implantation [45]. For stable long-term elution of vancomycin, Labmayr et al. [46] recommend adding superficial vancomycin to Copal G + V. Due to its small molecules, clindamycin is effectively released from bone cements along with gentamicin and is used by specialists to prevent infection in high-risk patients undergoing primary surgery [36, 41].

Antimycotic agents are not available as pure sterile powders. Voriconazole and amphotericin B are typically used as powders for infusion preparation, resulting in low concentrations of the active ingredient. For instance, 200 mg of pure voriconazole translates to 3.5 g of voriconazole powder. Concentrations exceeding 400 mg are challenging to mix homogeneously, leading to compromised mechanical properties. Using low-viscosity bone cement can help achieve a more homogeneous mixture [19]. Voriconazole is effective even at low concentrations, with its elution from PMMA enhanced by hydrophilic excipients in the powder formulation [19]. Amphotericin B in PMMA (AmBisome, Gilead Sciences, Ireland) weakens mechanical properties due to its formulation, which contains 1.33 g of powder for infusion preparation but only 50 mg of pure amphotericin B. Despite this, the efficacy at low concentrations is adequate [56].

This study has certain limitations, primarily due to its focus on products currently accessible to orthopedic surgeons. Potential admixtures, such as ceramics or carbons, were excluded as they remain under investigation and are not yet commercially available [69]. The emphasis was placed on antibiotics and antimycotics, which are predominantly utilized by orthopedic surgeons and have a well-established history of use spanning several decades.

5 | Conclusions

When manually preparing homogeneous antibiotic-bone cement mixtures, it is advisable to avoid dry mixing the powder before adding the liquid, as this can generate abrasion particles that may lead to inflammatory reactions. To ensure reliable mechanical properties, adequate antibiotic elution, and a synergistic elution effect, careful attention must be given to the optimal method of admixing anti-infective agents into PMMA bone cements.

Ethics Statement

This article does not include studies on humans or animals.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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3. A comparative study of extended gentamicin and tobramycin release and antibacterial efficacy from Palacos and Simplex acrylic cements

3.1. Summary

In this study, we investigated the comparative elution behaviour and long-term antibacterial efficacy of two commercially available aminoglycoside-loaded PMMA cements: Palacos® R+G (with gentamicin) and Simplex® T (with tobramycin). Our intention was to determine how differences in cement formulation influence antibiotic release and antimicrobial efficacy. This question is clinically relevant because ALBCs are widely used for both prevention and treatment of PJI, yet significant variability exists between cement types. Additionally, standardised protocols for the determination of antimicrobial efficacy are missing and results can be influenced by the methodology itself (Huys et al., 2002; Kok et al., 2025; Levack et al., 2021).

The main objectives of our work were to assess whether gentamicin-loaded Palacos® R+G exhibits superior short- and long-term antibiotic elution compared to tobramycin-loaded Simplex® T, and how these quantitative release profiles translate into sustained antibacterial activity over 42 days. We aimed to clarify whether the cement matrix composition, rather than the antibiotic type or concentration, primarily determines elution behaviour (Egger et al., 2024; Hertzberg-Boelch et al., 2022; Kühn, 2014).

To investigate these questions, we prepared standardised cylindrical cement samples (25 mm × 10 mm, surface area 8 cm²) from Palacos® R+G and Simplex® T according to their manufacturer instructions and without vacuum mixing (Heraeus Medical, 2025; Stryker, 2025). We performed quantitative elution analysis using HPLC/LC-MS/MS, collecting eluates over defined short-term (6 h, 12 h, 24 h) and long-term (1–42 days) intervals. In parallel, we evaluated biological activity using inhibition zone testing against *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Escherichia coli* following EUCAST 2025 guidelines (EUCAST, 2025a).

We found that Palacos® R+G exhibited markedly higher antibiotic release than Simplex® T across all time points. In the first 6 hours, Palacos® R+G released 96 µg/cm² of gentamicin, compared to only 4.83 µg/cm² of tobramycin from Simplex® T. Cumulative 24-hour release was 183.6 µg/cm² for Palacos® R+G versus 7.3 µg/cm² for Simplex® T. Over the full 42-day period, Palacos® R+G maintained a sustained elution profile (30.5 µg/cm² at day 42), while Simplex® T showed a clear decline, reaching only 7.5 µg/cm² by day 42. These differences were statistically significant. In line with these quantitative findings, Palacos® R+G demonstrated significantly greater and longer-lasting antibacterial activity. Inhibition zones remained detectable against all tested bacteria for the full 42-day period. Simplex® T, however, showed measurable activity only through day 14 for *S. aureus*, through day 28 for *S. epidermidis*, and only through day 7 for *E. coli*. At intermediate and late time points, Palacos® R+G consistently produced significantly larger inhibition zones ($p < 0.05$ or $p \leq 0.01$). No early-time-point advantage was observed for Simplex® T despite its higher antibiotic concentration.

Our findings show that the superior performance of Palacos® R+G cannot be explained by antibiotic type, since gentamicin and tobramycin exhibit comparable activity against the organisms tested. Instead, the differences reflect the underlying cement matrix properties (Kühn, 2014). Palacos® R+G likely permits greater water uptake and enhanced diffusion pathways. In contrast, the PMMA-styrene copolymer matrix of Simplex® T, together with its lower viscosity and variability during the doughing phase, appears to restrict water uptake and antibiotic elution (Dietz et al., 2024; Meeker et al., 2019). We can conclude that cement

composition plays a decisive role in determining antibiotic availability and long-term antimicrobial efficacy. This means that premixed PMMA formulations cannot be considered interchangeable even when loaded with antibiotics of similar spectrum.

3.2. Contribution

The tests on antimicrobial activity (inhibition zone testing) and antibiotic elution (HPLC) were performed by Débora Coraça-Huber. Débora Coraça-Huber, Klaus-Dieter Kühn and I designed the research and analysed the data. I wrote the manuscript and reviewed it with all authors.

3.3. Reference

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microorganisms

Article

A Comparative Study of Extended Gentamicin and Tobramycin Release and Antibacterial Efficacy from Palacos and Simplex Acrylic Cements

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Abstract

Antibiotic-loaded bone cements (ALBCs) are used to prevent and treat periprosthetic joint infections (PJI). This study compares the in vitro release and antibacterial effectiveness of gentamicin from Palacos® R+G and tobramycin from Simplex® T. Standardized cylindrical specimens of Palacos® R+G and Simplex® T were incubated in phosphate-buffered saline. Antibiotic release was quantified using high-performance liquid chromatography (HPLC) over 14 and 42 days. Antibacterial efficacy was assessed using inhibition zone tests (IZT) against *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Escherichia coli* over 42 days. Palacos® R+G exhibited a significantly higher and more sustained antibiotic release of gentamicin compared to tobramycin from Simplex® T. The cumulative release of gentamicin from Palacos® R+G was 1.148 μg/cm^2, while Simplex® T released 198.87 μg/cm^2 tobramycin over 14 days. Inhibition zone tests showed that Palacos® R+G maintained antibacterial activity for 42 days, while Simplex® T's activity diminished after 14 days. Statistical analysis confirmed significant differences in antibacterial efficacy between the two cements. Palacos® R+G demonstrated superior gentamicin release and sustained antibacterial activity compared to tobramycin from Simplex® T. These findings suggest that Palacos® R+G may offer better clinical outcomes in preventing and treating PJIs.

Keywords: antibiotic loaded bone cements (ALBCs); Simplex T; Palacos R+G; aminoglycoside; antimicrobial efficacy; HPLC elution; inhibition zone test

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1. Introduction

Polymethylmethacrylate (PMMA) bone cements are extensively utilized to anchor artificial joints. However, periprosthetic joint infections (PJI) remain a significant and devastating complication [1]. The treatment of joint infections poses a major and demanding challenge for both patients and surgeons [2]. Antibiotic-loaded bone cements (ALBCs) release antibiotics locally, thereby protecting the surgical field. In primary surgical procedures, ALBCs prevent biofilm formation on prostheses and significantly reduce the risk of infection within the joint [3]. In septic revisions, ALBCs serve as an adjuvant therapy to support bacterial eradication [4], effectively securing surgical debridement [5–7]. In two-stage revisions, antibiotic-loaded PMMA spacers are typically used in the interim phase. These spacers maintain a certain amount of joint stability and mobility and ensure high intra-articular antibiotic concentrations [8]. It is often recommended to use a combination of at least two antibiotics for spacer preparation: a broad-spectrum antibiotic (e.g., gentamicin), in combination with another antibiotic targeting the relevant pathogens [9,10]. According to the patient's antibiogram, antibiotics can be manually added to the PMMA cement spacer if needed [11]. Admixing antibiotics to commercially available cements requires the consideration of factors such as the influence of the antibiotic amount on both antibiotic elution and mechanical stability [12,13].

The determination of antibiotic elution from PMMA is crucial for evaluating the efficacy of these cement spacers. Various methods, including enzyme-linked immunosorbent assay (ELISA), inhibition zone test (IZT), agar diffusion test, high-performance liquid chromatography (HPLC), and proliferation assays, are employed to measure antibiotic release [14–21]. The literature reveals notable differences in results depending on the method used. Even the decision on a medium influences bacterial growth and therefore also the antimicrobial efficacy of the test samples [22,23]. For instance, a recent publication using ELISA [17] demonstrated significantly higher release rates for the Cemex® spacer (Tecres, Sommacampagna, Italy) to Refobacin® Bone Cement spacers (Zimmer Biomet, Warsaw, IN, USA). Conversely, studies utilizing the IZT method indicated that the Cemex® spacer had a lower release kinetic than the Copal® Exchange spacer [13]. Furthermore, HPLC-based analyses presented yet another set of differing results. Unfortunately, there is no uniform, standardized procedure for determining the efficacy and especially the elution of antibiotics from ALBCs [24], making it difficult to compare most publications in this field.

In addition to HPLC/LC-MS/MS and inhibition zone bioassays (IZT/agar diffusion), other methods for quantifying antibiotic elution from PMMA have been described in the literature: (i) UV-Vis spectrophotometry (particularly for tobramycin, $\lambda \approx 269$ nm) as a rapid, low-cost screening method, (ii) colorimetric ninhydrin assays or fluorescence polarization immunoassays for gentamicin, (iii) isothermal microcalorimetry to assess functional activity against (biofilm) bacteria on or near the PMMA surface, (iv) broth microdilution or time-kill assays using eluted samples, and (v) in vivo/intra-articular micro dialysis to characterize early elution kinetics after spacer implantation. Each method has specific strengths and limitations in terms of sensitivity, matrix interference, and clinical applicability [25].

These discrepancies highlight the importance of not only the methods themselves but also factors such as sample preparation and the concentration and quality of active substances [10,26,27]. Other factors include the thermostability of the incorporated antibiotics [26], the cement surface area [2,28] and the ability of the PMMA cement to absorb water, which correlates with the polymer base and the hydrophilicity of the PMMA cement [2,29]. The more water the cement matrix can absorb, the more antibiotic can be eluted. PMMA cements containing hydrophobic styrene result in lower hydrophilicity compared to pure PMMA-MMA copolymer cements [30]. Therefore, the antibiotic elution varies by cement type, with Simplex® P exhibiting the lowest release compared to Palacos® and Copal® formulations [31,32]. Even the mixing procedure influences antibiotic elution, depending on the cement porosity. A decrease in porosity due to vacuum mixing decreases antibiotic elution [29], whereas an increase in cement porosity by adding pore-induced particles increases the elution but decreases the mechanical properties significantly. The variability in these factors makes direct comparisons between studies challenging.

We aim to prove that the standardized application of HPLC and IZT for determining antibiotic elution from various ALBC formulations enables the consistent and comparable assessment of their antimicrobial efficacy, regardless of cement type and antibiotic concentration. We hypothesize that the temporal profile and total amount of antibiotic release differ significantly between commercially available bone cement formulations.

2. Materials and Methods

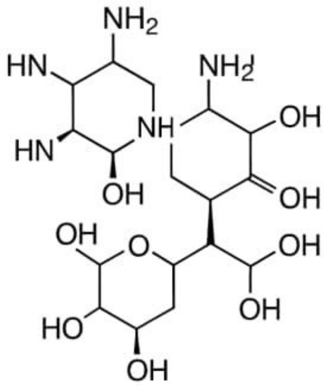
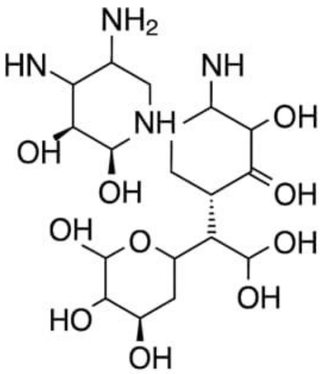
2.1. Bone Cements and Bacterial Strains

Two commercially available aminoglycoside-containing bone cements were used in this study; Palacos® R+G, containing 0.5 g gentamicin (Heraeus Medical GmbH, Wehrheim, Germany), and Simplex® T (also branded as Simplex® P with Tobramycin) with 1.0 g tobramycin (Stryker, Kalamazoo, MI, USA) (Table 1). Both cements were prepared according to the manufacturers' instructions for use [33,34] using the Palamix® Mixing Device (Heraeus Medical GmbH, Wehrheim, Germany) without vacuum, and shaped into standardized cylindrical specimens with a diameter of 25 mm and a height of 10 mm. Phosphate-buffered saline (PBS, pH 7.4) was used as the incubation medium for the antibiotic release and inhibition zone tests. Mueller–Hinton agar (Oxoid, ThermoFisher Scientific, Darmstadt, Germany) was used for antimicrobial susceptibility testing [35]. Reference bacterial strains from the DSMZ collection were employed; *Staphylococcus aureus* DSM 799 (ATCC 6538), *Staphylococcus epidermidis* DSM 1798, and *Escherichia coli* DSM 1576 (ATCC 8739) (Table 2), all susceptible against aminoglycoside antibiotics gentamicin and tobramycin. Both antibiotics are comparable in their antimicrobial spectrum as well as mode of action. All agar-based experiments followed the EUCAST 2025 [36] guidelines for antimicrobial testing.

Table 1. Commercially available PMMA bone cements used for testing. MMA = methyl methacrylate, DmpT = N,N-Dimethyl-p-Toluidine, HQ = hydroquinone, E141 = sodium–copper–chlorophyllin, PMMA = polymethylmethacrylate, MMA/MA-S = methyl methacrylate–styrol–copolymer, BPO = benzoyl peroxide, MA-MMA = methyl acrylate – methyl methacrylate–copolymer.

Bone Cement	Simplex® T	Palacos® R+G
Cement Viscosity	Low Viscosity	High viscosity
Polymer powder [g]/Liquid monomer [mL]	40:20	40:20
Monomer Components	MMA, DmpT, HQ	MMA, DmpT, HQ, E141
Polymer Combination	PMMA, MA-S, BPO	PMMA, MA-MMA, E141
Radiopacifier Contained	Barium sulfate	Zirconium dioxide
Antibiotic Contained	Tobramycin	Gentamicin
Antibiotic Amount [g] per 40 g	1.0	0.5
Antibiotic Concentration [%]	2.5	1.25

Table 2. Testing of minimum inhibitory concentration (MIC) for PJI-relevant germs against aminoglycoside antibiotics with a comparable spectrum of activity (+ = testing).

Antibiotics	Structure	*Staphylococcus aureus* (DSM 799)	*Staphylococcus epidermidis* (DSM 1798)	*Escherichia coli* (DSM 1576)
Gentamicin		+	+	+
Tobramycin		+	+	+

According to EUCAST 2025 [37], aminoglycoside breakpoints relevant to our study are as follows: Enterobacterales (including *E. coli*) are generally considered susceptible at MIC ≤ 2 mg/L for both gentamicin and tobramycin [38]; for staphylococci, gentamicin breakpoints are reported mainly for synergistic therapy. Thus, both gentamicin and tobramycin are in principle active against the reference strains used. Our E-test results confirmed this for *S. aureus* (MIC gentamicin 0.25 µg/mL) and *S. epidermidis* (0.125 µg/mL); the IZT data further showed inhibition zones against *E. coli*, confirming the biological activity of the antibiotics eluted from the cement.

2.2. Short- and Long-Term Release Rate of Gentamicin and Tobramycin

Standardized cylindrical bone cement specimens (25 mm diameter, 10 mm height, surface area: 8.0 cm^2) were incubated at 37 °C in 10.5 mL of dissolution medium (0.1 M phosphate buffer, pH 7.4). At pre-defined time points (for short term release 6 h, 12 h, 24 h, and for long term release 1 d, 3 d, 7 d, 14 d, 28 d, 42 d), aliquots were collected and the medium was fully renewed. All samples were stored at −20 °C until analysis. Calibration standards were prepared in the following concentration ranges: 100–7500 ng/mL for gentamicin, 50–2500 ng/mL for tobramycin. Each set included a zero sample (without internal standard) and a blank sample (with internal standard). To prepare the standards, 200 µL of working solution was spiked with 18 µL of the corresponding internal standard solution (gentamicin and tobramycin). These solutions were used for LC-MS/MS analysis (AZ Biopharm, Berlin, Germany). Study samples were diluted 20-fold for gentamicin and 5-fold for tobramycin and processed using the same protocol as for the calibration standards. Internal standard working solutions were added accordingly. All samples were analyzed in triplicate. Chromatographic separation was performed using a modular HPLC 1200 Series system (Agilent Technologies, Waldbronn, Germany) equipped with a Luna C18(2) column (150 × 2 mm) and two C18 guard columns (4 × 2 mm; Phenomenex, Aschaffenburg, Germany). Column temperature was maintained at 25 °C. The injection volume was 2 µL. Mobile phase A consisted of 0.11 M trifluoroacetic acid/methanol (50:50), and mobile phase B was acetonitrile. Isocratic separation was achieved at an A:B ratio of 95:5, with a flow rate of 0.25 mL/min. The run time was 2.5 min, and the total cycle time was less than 3 min. Under these conditions, the four gentamicin components (C1, C2, C2a, and C1a) were co-eluted. This HPLC method was previously described by Heller et al. [39] for quantifying gentamicin in biopsy samples. Detection was performed on an API 4000 QTrap mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with an electrospray ionization source operating in positive polarity and multiple reaction monitoring (MRM) mode. The following MRM transitions were monitored: 478.4 → 322.3 *m/z* (gentamicin C1), 464.4 → 322.3 *m/z* (gentamicin C2 and C2a), 450.3 → 322.3 *m/z* (gentamicin C1a), and 468.4 → 163.1 *m/z* (internal standard). The summed peak areas of the gentamicin components were analyzed using Analyst software version 1.4.2 (Applied Biosystems, ThermoFisher Scientific, Darmstadt, Germany), and data were processed with Microsoft Excel 365 (Microsoft Deutschland GmbH, Unterschleißheim, Germany). Tobramycin and its internal standard were analyzed under the same LC-MS/MS conditions. The MRM transitions monitored were 163.1 *m/z* for tobramycin and 478.4 → 322.3 *m/z* for its internal standard. Chromatograms were evaluated using Analyst 1.4.2 (AB Sciex LLC., Framingham, MA, USA) and processed with Microsoft Excel 365.

2.3. Comparative Long-Term Efficacy of Gentamicin and Tobramycin

The antibacterial efficacy of gentamicin and tobramycin released from the two bone cements tested was evaluated using the IZT method. The following reference strains from the DSMZ collection were used: *Staphylococcus aureus* DSM 799, *Staphylococcus epidermidis* DSM 1798 and *Escherichia coli* DSM 1576. The bacterial strains were initially tested for their susceptibility to gentamicin and tobramycin using E-tests (BioMérieux GmbH, Vienna, Austria). Each E-test was performed in triplicate. The minimum inhibitory concentration (MIC) values for the tested antibiotics were determined to be 0.25 µg/mL for *S. aureus*, 0.125 µg/mL for *S. epidermidis* and 0.125 µg/mL for *E. coli*. All antimicrobial susceptibility tests were performed on Mueller–Hinton agar according to EUCAST 2025 guidelines [36], using standardized agar volumes. Plates were incubated at 36 ± 1 °C for 24 h. To assess the release efficacy of gentamicin from Palacos® R+G and tobramycin from Simplex® T cements, standardized cylindrical specimens (25 mm diameter × 10 mm height) were used. One cement body per group was incubated in phosphate-buffered saline (PBS) at room temperature. Sampling was performed at the following time points: 1 h, 24 h, 7 d, 14 d, 21 d, 35 d, 42 d. At each time point, 60 µL of the incubation solution was withdrawn and applied directly onto Mueller–Hinton agar plates previously inoculated with one of the bacterial strains and pre-incubated for 24 h at 37 °C. For each time point and cement type, three independent cement specimens were tested (n = 3). The diameter of the inhibition zones was measured (in mm) for each of the tested bacterial strains (*S. aureus*, *S. epidermidis*, and *E. coli*). Mean values and standard deviations were calculated for each group. For all elution and efficacy tests, we used cement samples that were prepared in a standardized way, ensuring a defined surface.

2.4. Statistical Analysis

To evaluate the differences in antimicrobial activity between Palacos® R+G and Simplex® T, statistical analysis was performed on the inhibition zone diameters obtained for *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Escherichia coli*. Data were collected at seven time points over a 42-day period. The mean inhibition zones were compared using an unpaired two-tailed Student's *t*-test assuming unequal variances. Additionally, two-way ANOVA (factors: cement type and time), followed by Bonferroni-corrected post hoc pairwise comparison, was performed to confirm the statistical significance of the observed differences between groups. A significance threshold of $p < 0.05$ was adopted for all comparisons. Statistical analysis was conducted using Python version 3.12.10 and SciPy 1.16.0 with the SciPy library (NumFOCUS, Austin, TX, USA).

3. Results

3.1. Comparative Short-Term Release Rate of Gentamicin and Tobramycin

Tobramycin release from Simplex® T: The cumulative release of tobramycin from the Simplex® T bone cement was evaluated over a 24 h period (Figure 1). The initial burst release observed at 6 h reached a mean concentration of 4.83 μg/cm^2 (SD: 0.09), representing the highest value within the sampling period. This was followed by a sharp decrease in concentration at 12 h and 24 h, reaching 0.74 μg/cm^2 (SD: 0.02) and 0.73 μg/cm^2 (SD: 0.01), respectively. Overall, the cumulative release across all time points for Simplex® T resulted in a total mean tobramycin concentration of 7.30 μg/cm^2 (SD: 0.52).

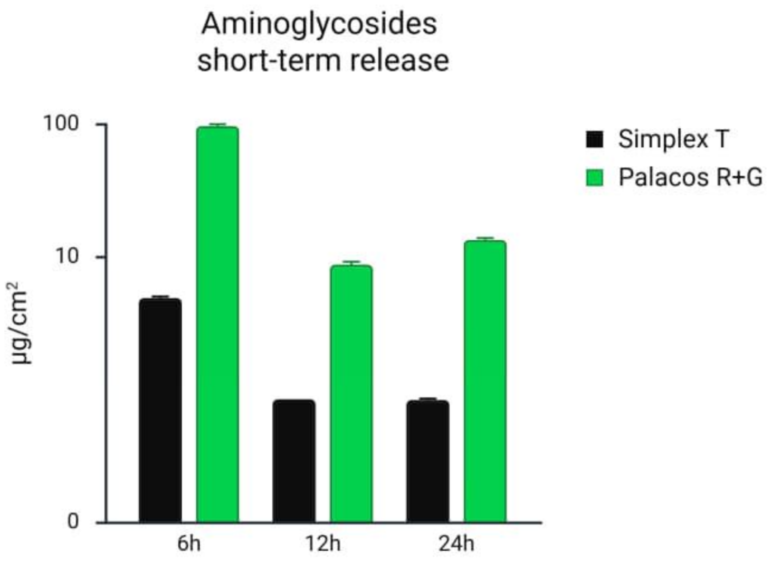


Figure 1. Short-term release of gentamicin from Palacos® R+G (1.25% gentamicin) and tobramycin from Simplex® T (2.5% tobramycin) bone cements over the first 24 h, quantified by HPLC and expressed in μg/cm^2. Palacos® R+G exhibited substantially higher and more sustained antibiotic release across all time points compared to Simplex® T. Statistical analysis showed a significant difference in release profiles between the two materials over time (one-way ANOVA, $p = 0.044$) and a trend toward significance in direct group comparison (unpaired *t*-test, $p = 0.059$).

Gentamicin release from Palacos® R+G: In contrast, Palacos® R+G demonstrated a significantly higher gentamicin release profile (Figure 1). At 6 h, the release peaked at a mean of 96.00 μg/cm^2 (SD: 2.3), nearly 20-fold higher than tobramycin from Simplex® T at the same time point. Although there was a notable decrease at 12 h (8.70 μg/cm^2, SD: 0.43), a secondary peak was observed at 24 h, reaching 13.30 μg/cm^2 (SD: 0.33). Gentamicin concentrations remained consistently higher than those observed for tobramycin from Simplex® T throughout the 24 h. The total cumulative gentamicin release from Palacos® R+G over the entire period was 183.61 μg/cm^2 (SD: 2.21), more than 25 times higher than that observed for tobramycin of Simplex® T.

The quantification of gentamicin release via HPLC over the first 24 h revealed that Palacos® R+G consistently released higher concentrations of antibiotic compared to tobramycin of Simplex® T (Figure 1). At all measured time points, the gentamicin release from Palacos® R+G remained substantially elevated, particularly in the early phase (e.g., 96.00 μg/cm^2 at 6 h vs. 4.83 μg/cm^2 for tobramycin of Simplex® T). Statistical analysis using a one-way ANOVA confirmed a significant difference between the two cements ($p = 0.044$), indicating that the overall gentamicin release profile was higher in the Palacos® R+G group with 1.25% gentamicin compared to Simplex® T with 2.5% tobramycin. The unpaired t-test showed a trend toward significance ($p = 0.059$), suggesting variability but supporting the same directional outcome. These findings reinforce the enhanced early and intermediate-term antibiotic elution performance of Palacos® R+G.

3.2. Comparative Long-Term Release Rate of Gentamicin and Tobramycin

The following data presents a direct comparison of gentamicin release profiles from Palacos® R+G and Simplex® T bone cements, measured in μg/cm^2, over a 42-day period (Figure 2). Palacos® R+G exhibited significantly higher antibiotic release across all time points. On day 1, Palacos® R+G showed a peak release of 82.2 μg/cm^2, more than double that of Simplex® T, which released 35.6 μg/cm^2. Although the release from both cements decreased over time, Palacos® R+G maintained a more sustained release pattern. For instance, by day 14, the release values were 37.2 μg/cm^2 for Palacos® R+G and only 10.3 μg/cm^2 for Simplex® T. At the end of the study period (day 42), Palacos® R+G continued to release gentamicin at 30.5 μg/cm^2, while Simplex® T showed a markedly lower release of 7.5 μg/cm^2. These results emphasize the superior long-term elution characteristics of Palacos® R+G, which may contribute to improved local antibiotic availability in clinical applications requiring extended prophylaxis or infection control.

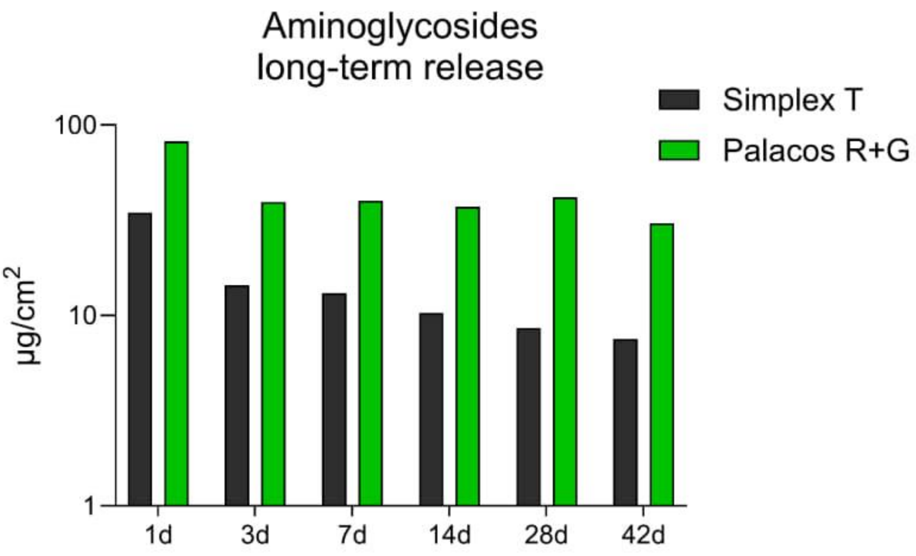


Figure 2. Long-term release of gentamicin from Palacos® R+G (1.25% gentamicin) and tobramycin from Simplex® T (2.5% tobramycin) bone cements, quantified by HPLC expressed in $\mu g/cm^2$. Palacos® R+G exhibited substantially higher and more sustained antibiotic release across all time points compared to Simplex® T over a 42-day period; Palacos® R+G demonstrated significantly higher and more sustained antibiotic release across all time points compared to Simplex® T ($p < 0.01$, unpaired *t*-test and ANOVA).

3.3. Antibiotic Susceptibility Tests Carried Out During Comparative Long-Term Tests on Gentamicin and Tobramycin from Palacos® R+G and Simplex® T

The antimicrobial activity of the ALBCs was assessed by measuring the diameter of inhibition zones against *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Escherichia coli* over a period of 42 days (Supplementary Data S1). Measurements were taken after applying eluates from cement samples incubated in PBS to pre-inoculated Mueller–Hinton agar plates. Figure 3A shows the inhibition zones against *S. aureus*. Palacos® R+G exhibited strong antibacterial activity at early time points, with a mean inhibition zone of approximately 24 mm at 1 h. This activity gradually decreased over time but remained detectable up to day 42. In contrast, Simplex® T showed significantly smaller zones, with measurable activity only until day 14, and no inhibition observed thereafter. Figure 3B illustrates the results for *S. epidermidis*. A similar trend was observed: Palacos® R+G showed robust antibacterial activity with inhibition zones above 20 mm in the first 24 h, which declined gradually but persisted throughout the 42-day period. Simplex® T demonstrated limited activity, with inhibition zones significantly smaller than those of Palacos® R+G and becoming undetectable by day 28. Figure 3C presents the inhibition data for *E. coli*. The activity of Palacos® R+G was again superior, showing measurable inhibition up to 28 days, whereas Simplex® T displayed minimal efficacy, with small inhibition zones only detectable up to 7 days. In all three bacterial models, Palacos® R+G demonstrated superior and more sustained antibacterial activity compared to Simplex® T. Palacos® R+G showed significantly larger inhibition zones than Simplex® T at later time points for *S. aureus* (days 14, 21, 35; $p < 0.05$), *S. epidermidis* (days 14, 21, 35, 42; $p \leq 0.01$), and *E. coli* (days 14, 35, 42; $p < 0.05$). Early time points (1 h, 24 h, 7 d) did not show significant differences between cements. These results confirm that Palacos® R+G exhibited superior and prolonged antibacterial efficacy compared to Simplex® T, particularly at intermediate and late time points.

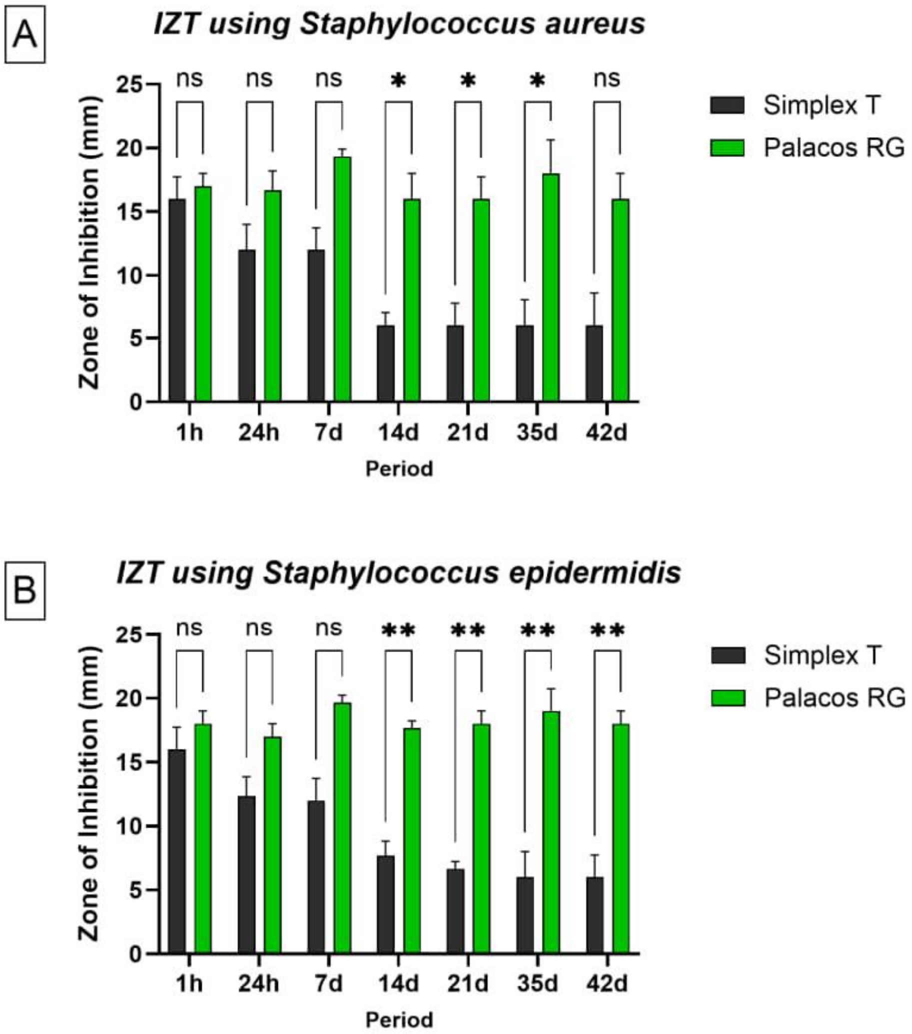


Figure 3. *Cont*.

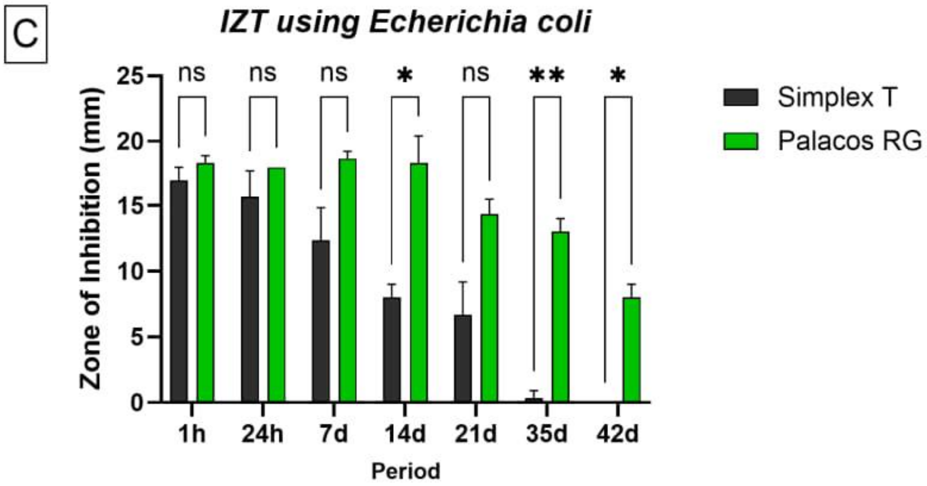


Figure 3. Inhibition zones (in mm) produced by eluates from Palacos® R+G (1.25% gentamicin; green bars) and Simplex® T (2.5% tobramycin; black bars) against three bacterial strains: (**A**) *Staphylococcus aureus*, (**B**) *Staphylococcus epidermidis*, and (**C**) *Escherichia coli*, measured at multiple time points (1 h to 42 days). Each bar represents the mean ± standard deviation (n = 3). Statistical analysis was performed using a two-way ANOVA (factors: cement type and time), followed by Bonferroni-corrected post hoc pairwise comparisons. Palacos® R+G showed significantly larger inhibition zones than Simplex® T at later time points for *S. aureus* (days 14, 32, 35; $p < 0.05$), *S. epidermidis* (days 14, 21, 35, 42 $p \leq 0.01$), and *E. coli* (days 14, 35, 42; $p < 0.05$). ns = not significant; * = $p < 0.05$; ** = $p \leq 0.01$.

4. Discussion

The methodology used in this study, including HPLC and IZT, provides a comprehensive evaluation of antibiotic elution and efficacy. HPLC is a highly sensitive and specific method that allows for precise quantification of antibiotic concentrations over time [39]. This method is particularly useful for detecting subtle differences in elution profiles and ensuring accurate measurements of antibiotic release [39]. Kittinger et al., 2024 [15] stated that HPLC showed the best differences in release kinetics and helped to prove that the total amount of antibiotic added is not directly proportional to the antibiotic elution. In contrast, IZT assesses the antibacterial activity of the eluted antibiotics by measuring the diameter of inhibition zones against specific bacterial strains [19]. Methodologically, the parallel use of quantitative (HPLC/LC-MS/MS) and functional (IZT) assays provided complementary information: HPLC/LC-MS/MS yielded quantitative elution kinetics, whereas IZT reflected the biological activity of the antibiotic fractions that diffused into the agar. However, the combination of HPLC and IZT in this study offers a robust assessment of both the quantitative and qualitative aspects of antibiotic elution and efficacy [15]. Differences between the two methods arise from matrix binding, diffusion radii, and medium conditions (moisture/nutrients), and are therefore expected [15]. Elution data should thus always be interpreted in conjunction with activity assays. Pharmacologically, gentamicin and tobramycin share similar activity against Enterobacterales; for *Pseudomonas aeruginosa*, tobramycin has been reported to show slightly higher in vitro activity, while for Staphylococci, gentamicin is the more established aminoglycoside (usually in combination for synergistic effect) [40]. In our model (*E. coli*), both materials showed only limited activity over time, likely reflecting the free drug concentration achievable in the cement matrix rather than a fundamental class-spectrum difference.

Other methods frequently used to determine the antimicrobial efficacy of antibiotic-loaded bone cements include ELISA, agar diffusion test, and proliferation assays. Each method has its advantages and limitations. ELISA is highly sensitive and specific for detecting and quantifying proteins, including antibiotics [17]. It is particularly useful for measuring low concentrations of antibiotics in complex biological samples [19]. However, ELISA requires specific antibodies that are not available for all antibiotics. The agar diffusion test is like IZT, and it assesses the antibacterial activity of antibiotics by measuring the zones of inhibition on agar plates [19]. It is relatively simple and cost-effective but lacks the precision of HPLC in quantifying antibiotic concentrations. Also, it is not suitable for the determination of antibiotic elution from PMMA cement samples as it underestimates the actual antibiotic elution [15]. Additionally, EUCAST [36] does not recommend agar diffusion tests like Kirby–Bauer disk diffusion for routine antimicrobial susceptibility testing. Instead, EUCAST primarily endorses broth microdilution for MIC determination and standardized disk diffusion tests. The “pour plate” or “incorporation” method, which involves mixing bacteria into molten agar, is not recommended due to its lack of standardization and reproducibility. This method is more suitable for testing bacteriophage or bacteriocin activity, some biocompatibility assays, and specific microbiology applications. Proliferation assays measure the effect of antibiotics on bacterial growth and proliferation. They provide valuable information on the biological efficacy of antibiotics, especially when combined with other testing methods, e.g., IZT or an in vivo biofilm model [41].

The composition of nutrient media and environmental conditions such as humidity significantly influence antibiotic activity and the outcomes of antimicrobial susceptibility testing. Variations in media components can affect bacterial growth, biofilm formation, and the expression of resistance genes, leading to differences in observed antibiotic efficacy [22,23,35]. Excess surface moisture can cause the irregular diffusion of antibiotics, resulting in larger and inconsistent inhibition zones. Conversely, a dry agar surface can limit antibiotic diffusion, leading to smaller inhibition zones and potentially false resistance results. High incubation humidity helps maintain standard diffusion dynamics by preventing the agar from drying out [22,23,35].

Given the variability in these factors, the objective of this study is to provide a comprehensive recommendation for the determination of antibiotic elution using HPLC and IZT. By standardizing these methods, we aim to facilitate more consistent and comparable evaluations of ALBC efficacy. The cements used in this study were different in composition and drug concentration, and the cement sample preparation involved standardized molded bodies with a defined surface. The same eluates were used for all efficacy tests to ensure uniformity.

The results of this study demonstrate a significant difference in the antibiotic elution profiles between Palacos® R+G and Simplex® T bone cements. Over a 14-day period, Palacos® R+G demonstrated significantly enhanced and prolonged aminoglycoside release compared to Simplex® T, despite containing only 0.5 g of gentamicin, whereas Simplex® T incorporates 1 g of tobramycin. This difference was particularly pronounced in the early phase, with Palacos® R+G showing a peak release nearly 20-fold higher than Simplex® T at the same time point. The IZT further corroborated these findings, showing that Palacos® R+G maintained antibacterial activity for up to 42 days, while Simplex® T’s activity diminished after 14 days. The superior elution profile of Palacos® R+G can be attributed to its formulation, which likely facilitates better antibiotic release and sustained antibacterial efficacy [30,42]. These results suggest that Palacos® R+G may offer better clinical outcomes in preventing and treating PJI due to its enhanced antibiotic elution and prolonged antibacterial activity. This finding is in line with the in vitro testing from Meeker et al., 2019 [31] that found that the elution profiles of vancomycin, daptomycin and tobramycin manually added to Simplex® and Palacos® bone cements were higher for the latter one. Despite Dietz et al., 2024 [43]’s finding of a high antibiotic release rate for Simplex® T on day 1, the decrease on day 2 was massive compared to Palacos® R+G. After day 1, Simplex® T did not even reach the MIC of the tested microorganisms, in contrast to Palacos® R+G cements. Although gentamicin and tobramycin exhibit minor structural differences, both aminoglycoside antibiotics share a comparable mode of action and display a similar antimicrobial spectrum [44,45]. While tobramycin is often considered more effective against Gram-negative bacilli, the existing literature also highlights that a substantial proportion of these pathogens remain susceptible to gentamicin, underscoring its continued clinical relevance [45,46]. The primary distinction lies in tobramycin's enhanced activity against *Pseudomonas aeruginosa* [45,47], a pathogen that, however, accounts for only 3–5% of all PJIs [48]. Tobramycin exhibited reduced antimicrobial activity against *Escherichia coli* in comparison to gentamicin. According to EUCAST 2025 [37], the clinical breakpoints for Enterobacterales are S $\leq$ 2 mg/L (gentamicin/tobramycin) [38]. Whether elution levels transiently exceed these thresholds depends heavily on cement matrix properties, porosity, water affinity, and assay conditions—factors which plausibly explain the observed differences between Palacos® R+G and Simplex® T.

These findings suggest that neither the choice of antibiotic nor the testing methodology alone can account for the observed differences in antimicrobial efficacy, thereby strongly implicating the PMMA cement formulation as the determining factor (Table 1). Notably, the observed variations in antibiotic elution and antimicrobial activity persisted across different testing methods. This interpretation is further supported by the fact that Simplex® T, despite containing twice the antibiotic load, exhibited lower elution levels compared to Palacos® R+G. Although Simplex® is known to exhibit the lowest antibiotic release profile among bone cements [31], it remains widely utilized in spacer fabrication [49–51] according to its worldwide availability. The pronounced decline in antibiotic elution over time from such spacers may compromise therapeutic efficacy, potentially leading to the incomplete eradication of residual pathogens and increasing the risk of antimicrobial resistance development [50]. Tseng et al. [51] attempted to enhance vancomycin release from Simplex® P cement to improve its suitability for spacer fabrication. However, our findings suggest that selecting an alternative commercially available PMMA may offer a more straightforward and effective approach to achieving sustained antibiotic release.

The observed differences in antibiotic elution between Simplex® T and Palacos® R+G may be partially attributed to their viscosity characteristics. Low-viscosity (LV) cements like Simplex® T (Table 1) typically require higher antibiotic concentrations (2.5% for Simplex® T) to achieve comparable release profiles to high-viscosity (HV) cements like Palacos® R+G. This relationship could serve as an additional explanatory factor for the inferior elution and antimicrobial efficacy observed with Simplex® bone cements, beyond previously discussed aspects. Simplex® features a complex chemical composition, including a PMMA-styrene copolymer matrix, and undergoes gamma irradiation for sterilization—both of which may influence its hydrophilicity and hence elution behavior. According to the Simplex® marketing material, the cement offers convenient application during both the LV and MV phases [52]. However, data from Fölsch et al. [53] demonstrated that Simplex® P can be safely applied at viscosities comparable to Palacos® R+G during the working phase. Notably, the sticky phase of Simplex® P is highly variable, ranging from approximately 2.5 to 3.0 min, whereas Palacos® R+G exhibits a more consistent sticky phase of 60–75 s. This variability may affect the application timing and the degree of cement intrusion. This is supported by further observations, highlighting differences in the doughing phase and intrusion behavior between the two cements [11]. Additionally, the manufacturer suggests an earlier loss of stickiness for Simplex® T, occurring around 1.3–2.0 min, which may influence clinical handling and application timing. This results in a potential risk of a too-early application when Simplex ® T is still in the sticky phase and not yet ready for application.

Antibiotic release from bone cement is known to be highest during the doughing phase (in which some cements can still be sticky), which represents a critical window for drug elution [30]. Premature application of the cement—such as is possible with Simplex® T—may lead to excessive early antibiotic loss, thereby reducing the amount available for sustained release. This phenomenon has important clinical implications, as it may compromise the infection prophylaxis during primary fixation. Optimizing the timing of cement application is therefore essential to maximize antibiotic retention and ensure effective local antimicrobial activity.

Overall, these results are consistent with the higher and prolonged release of gentamicin from Palacos® R+G observed in the elution studies, reinforcing its potential clinical advantage in infection prevention and spacer usage [54].

5. Conclusions

Standardizing antibiotic elution testing methods is crucial for generating reliable and comparable data which will enhance our understanding of the efficacy of antibiotic-loaded bone cements and improve clinical outcomes in managing PJIs. Palacos® R+G bone cement exhibits more efficient antibiotic release kinetics compared to Simplex® T, despite containing only 50% of the antibiotic load. Additionally, Palacos® R+G offers an extended antibacterial activity, lasting up to 42 days, indicating its potential for better clinical outcomes in preventing and treating PJIs. Antibiotic release corresponds with the optimal application time point: this time point varies significantly for Simplex® bone cements. These differences in viscosity, composition, and doughing time likely contribute to the inferior antibiotic elution and variable application behavior of Simplex® T compared to Palacos® R+G.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/microorganisms13092174/s1. Supplementary Data S1: Inhibition zone testing.

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Abbreviations

The following abbreviations are used in this manuscript:

ALBC	Antibiotic-loaded bone cement
ELISA	Enzyme-linked immunosorbent assay
EUCAST	European Society of Clinical Microbiology and Infectious Diseases

HPLC	High-performance liquid chromatography
HV	High-viscosity bone cement
IZT	Inhibition zone test
LV	Low-viscosity bone cement
MV	Medium-viscosity bone cement
PJI	Periprosthetic joint infection
PMMA	Polymethylmethacrylate

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IZT using Staphylococcus aureus

Time point	Mean (mm) Simplex T®	Mean (mm) Palacos® R+G	Adjusted p-value (Bonferroni)	Summary
1 h	16.00	17.00	>0.9999	ns
24 h	12.00	16.67	0.2505	ns
7 d	12.00	19.33	0.0808	ns
14 d	6.000	16.00	0.0336	*
21 d	6.000	16.00	0.0148	*
35 d	6.000	18.00	0.0293	*
42 d	6.000	16.00	0.0546	ns

IZT using Staphylococcus epidermidis

Time point	Mean (mm) Simplex T®	Mean (mm) Palacos® R+G	Adjusted p-value (Bonferroni)	Summary
1 h	16.00	18.00	>0.9999	ns
24 h	12.33	17.00	0.1113	ns
7 d	12.00	19.67	0.0728	ns
14 d	7.667	17.67	0.0069	**
21 d	6.667	18.00	0.0021	**
35 d	6.000	19.00	0.0080	**
42 d	6.000	18.00	0.0100	**

IZT using Echericha coli

Time point	Mean (mm) Simplex T®	Mean (mm) Palacos® R+G	Adjusted p-value (Bonferroni)	Summary
1 h	17.00	18.33	0.9351	ns
24 h	15.67	18.00	>0.9999	ns
7 d	12.33	18.67	0.2998	ns
14 d	8.000	18.33	0.0362	*
21 d	6.667	14.33	0.1404	ns
35 d	0.3333	13.00	0.0015	**
42 d	0.000	8.000	0.0362	*

4. Efficacy of dual-antibiotic-loaded bone cement against multi-drug-resistant *Staphylococcus aureus* and *Enterococcus faecalis* in a *Galleria mellonella* model of periprosthetic joint infection

4.1. Summary

In this study, we investigated the antimicrobial performance of three commercially available antibiotic-loaded PMMA cements, Palacos® R+G (with gentamicin), Copal® G+C (with gentamicin and clindamycin), and Copal® G+V (with gentamicin and vancomycin), using a *Galleria mellonella* model of implant-associated infection. Our primary intention was to determine how SALBC versus DALBC formulations differ in their ability to prevent or reduce infections caused by MDR *Staphylococcus aureus* and *Enterococcus faecalis*. This question is clinically relevant because DALBCs are increasingly used in high-risk arthroplasty and revision surgery, yet comparative in-vivo evidence, particularly against MDR pathogens, remains limited (Ahmed et al., 2024; Cara et al., 2022). Furthermore, current preclinical evaluations often rely on vertebrate models that are costly and restricted from an ethical standpoint, highlighting the need for validated invertebrate alternatives (Kiani et al., 2022).

The main objective of our work was to assess whether DALBC formulations provide increased local antimicrobial activity, biofilm inhibition, and infection protection compared to the widely used SALBC. A second objective was to validate the *G. mellonella* implant infection model as a high-throughput platform capable of capturing meaningful differences between cement formulations. We aimed to clarify whether the combination of two antibiotics improves performance against MDR bacteria that are resistant to gentamicin and/or clindamycin.

To investigate these questions, we prepared standardised cemented Kirschner wires incorporating each cement formulation and evaluated their antimicrobial activity through a combined in-vitro and in-vivo experimental design. In-vitro analyses included MIC/MBC testing of the MDR strains, agar diffusion assays using intact cemented implants or their eluates (day 1–5), antibiotic release kinetics measured by HPLC-MS/MS, and quantitative antibiofilm assays assessing bacterial burden in planktonic fractions, well-surface biofilms, and implant-associated biofilms. In parallel, we employed two *G. mellonella* models: a biofilm-implant model and a hematogenous implant seeding model (Mannala et al., 2021). In these assays, larvae received cemented implants that were either pre-incubated with bacterial suspensions (biofilm model) or implanted prior to bacterial injection (hematogenous model), and outcomes were measured as survival over five days and bacterial burden in tissue and on implants after 24 hours.

We found that Copal® G+C and Copal® G+V provided markedly increased antimicrobial efficacy compared to Palacos® R+G across nearly all assays. Despite high-level resistance of both MDR strains to gentamicin, and for *E. faecalis*, also to clindamycin, Palacos® R+G produced at least modest inhibition zones and limited antibiofilm effects. In contrast, Copal® G+C and Copal® G+V generated significantly larger inhibition zones against both pathogens. Copal® G+C produced the largest in-vitro inhibition against *S. aureus*, while Copal® G+V was most effective against *E. faecalis*. Antibiotic release analysis confirmed a characteristic burst on day 1 followed by a sustained low-level release through day 5, with Copal® G+C releasing nearly twice as much gentamicin as the other formulations. These quantitative release profiles translated into differences in biofilm inhibition. In *S. aureus* biofilm assays, Copal® G+C and Copal® G+V reduced bacterial burden by more than 1.5 log across planktonic, well-surface, and implant-associated compartments, whereas Palacos® R+G achieved only a modest reduction. Against *E. faecalis*, Copal® G+V produced the strongest antibiofilm effect, significantly reducing bacterial counts across all compartments, while Copal® G+C showed

partial activity. Palacos® R+G showed no meaningful antibiofilm efficacy against *E. faecalis*. In the in-vivo model *G. mellonella*, DALBC formulations dramatically improved larval survival and reduced bacterial colonisation. In the biofilm implant model, survival after *S. aureus* infection reached 70% for Copal® G+C and 90% for Copal® G+V, compared to only 36.7% with Palacos® R+G and 3.3% in the unloaded control. Comparable patterns were observed for *E. faecalis*. Bacterial burden analyses confirmed substantial log-scale reductions with DALBCs for both implant surfaces and surrounding tissues, up to complete bacterial clearance with Copal® G+V, whereas Palacos® R+G showed little or no effect. Similar outcomes were observed in the hematogenous infection model.

In total, our findings demonstrate that DALBCs Copal® G+C and Copal® G+V exhibit significantly greater antimicrobial, antibiofilm, and anti-infective efficacy than SALBC Palacos® R+G, even against MDR pathogens classified as resistant to the individual antibiotics. These results indicate that high local antibiotic concentrations achieved by synergistic antibiotic elution can overcome resistance phenotypes at the implant site (Cara et al., 2022). They also underscore that antibiotic choice and formulation composition decisively influence antimicrobial performance, meaning that PMMA cements cannot be considered interchangeable. Finally, our data establish the *G. mellonella* implant infection model as a relevant, scalable preclinical platform for evaluating PMMA-based antimicrobial strategies against implant-associated infections.

4.2. Contribution

The tests on antimicrobial activity (MIC determination, agar diffusion assay, antibiofilm assay, in-vivo biofilm model) were performed and analysed by You Zhao, Gopala Krishna Mannala, Raphaëlle Youf and Ruth Schewior. The test on antibiotic elution (HPLC) was performed and analysed by me. Martijn Riool, Gopala Krishna Mannala and I designed the research. Gopala Krishna Mannala wrote the original draft of the manuscript with the support of all authors and reviewed it with all authors.

4.3. Reference

Zhao, Y., Mannala, G. K., Youf, R., Humez, M., Schewior, R., Kühn, K.-D., Alt, V., & Riool, M. (2025). Efficacy of Dual-Antibiotic-Loaded Bone Cement Against Multi-Drug-Resistant *Staphylococcus aureus* and *Enterococcus faecalis* in a *Galleria mellonella* Model of Periprosthetic Joint Infection. *Antibiotics*, *14*(12), 1280. https://doi.org/10.3390/antibiotics14121280

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Article

Efficacy of Dual-Antibiotic-Loaded Bone Cement Against Multi-Drug-Resistant *Staphylococcus aureus* and *Enterococcus faecalis* in a *Galleria mellonella* Model of Periprosthetic Joint Infection

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Abstract

Background: Antibiotic-loaded bone cement (ALBC) is widely used for local antibiotic delivery in joint arthroplasty to prevent and treat prosthetic joint infections (PJIs). In this study, we evaluated the efficacy of cemented Kirschner (K)-wires coated with various ALBC formulations using a *Galleria mellonella* infection model against multidrug-resistant (MDR) *Staphylococcus aureus* and *Enterococcus faecalis*. **Methods:** We tested commercially available bone cements, including gentamicin-only formulations (PALACOS R+G) and dual-antibiotic formulations, combining gentamicin with either clindamycin (COPAL G+C) or vancomycin (COPAL G+V), alongside an antibiotic-free control (PALACOS R). In vitro assays—including minimum inhibitory/bactericidal concentration (MIC/MBC) determination, antibiotic release kinetics, agar diffusion, and antibiofilm evaluations—demonstrated effective antibiotic release and significant antimicrobial activity against both planktonic and biofilm-associated bacteria. **Results:** In vivo, ALBC-coated K-wires were well tolerated in *G. mellonella* and significantly protected the larvae from *S. aureus* infection compared to controls. Notably, dual-antibiotic formulations provided superior protection, correlating with substantial reductions in bacterial colonisation on implant surfaces and in surrounding tissues. **Conclusions:** These findings support the utility of the *G. mellonella* model as a high-throughput, cost-effective platform for the preclinical evaluation of antimicrobial strategies to prevent and treat PJIs and further demonstrate the effectiveness of dual-loaded ALBC against multidrug-resistant bacteria.

Keywords: antibiotic-loaded bone cement (ALBC); polymethylmethacrylate (PMMA); *Galleria mellonella*; prosthetic joint infection (PJI); multidrug-resistant bacteria

1. Introduction

Orthopaedic implants have transformed fracture fixation and joint replacement, greatly improving patient outcomes. In the USA, the annual number of procedures is projected to reach 1.22 million total knee arthroplasties (TKA) and 719,000 total hip arthroplasties (THA) by 2040 [1]. Despite their high success rates, these procedures frequently require revision surgeries, primarily due to aseptic loosening and infection. Prosthetic joint infections (PJIs) account for approximately 15% and 25% of THA and TKA revision surgeries, respectively [2]. A recent report by the European Bone and Joint Infection Society (EBJIS) indicated that, out of approximately two million joint replacements performed in Europe in 2019, over 20,000 resulted in PJIs, imposing a substantial economic burden on healthcare systems [3].

PJIs are predominantly caused by *Staphylococcus aureus*, coagulase-negative staphylococci, *Streptococcus* spp. and *Enterococcus* spp. These pathogens form biofilms on implant surfaces, facilitating bacterial persistence and resistance to antimicrobial therapies [4,5]. Furthermore, recent evidence shows that bacteria can evade host immune defences by colonising implant surfaces and forming biofilms [6]. The involvement of multidrug-resistant (MDR) pathogens in PJIs further complicates management, often requiring multiple cement spacer exchanges, increasing the risk of infection recurrence, and substantially adding to healthcare costs [7].

Cemented implants play a crucial role in managing PJIs, particularly in two-stage revision procedures and cases involving limb shortening, disuse osteopenia, and extensive bone and soft tissue loss [8]. Strategies such as antimicrobial coatings (e.g., silver, antibiotics) and antibiotic-loaded bone cements (ALBCs) have been widely adopted to mitigate these complications [9]. Poly(methyl methacrylate) (PMMA) bone cement, especially formulations loaded with single (e.g., gentamicin, tobramycin) or dual antibiotics (e.g., gentamicin combined with clindamycin or vancomycin), enables localised antibiotic delivery and is integral to two-stage exchange protocols [10]. Clinical studies suggest that dual-antibiotic-loaded formulations yield superior outcomes compared to single-antibiotic alternatives [11], which is supported by in vitro evidence demonstrating enhanced antibiotic release kinetics and biofilm inhibition [12].

Preclinical models are crucial for understanding PJI pathogenesis and developing therapeutic strategies. However, research on animal models of cemented implant infections remains limited. For example, Lin et al. demonstrated the effectiveness of vancomycin-loaded PMMA bone cement against *S. aureus* in a rabbit model [13]. Due to ethical and regulatory constraints, vertebrate models must adhere to the 3R principles (Replacement, Reduction, Refinement) and guidelines such as PREPARE [14,15]. Consequently, invertebrate models, including *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Galleria mellonella*, have emerged as robust alternatives for infection studies and high-throughput antimicrobial screening [16,17]. Compared with the other two models, *G. mellonella* offers several advantages for infection studies, including a body size large enough for device implantation, tolerance to 37 °C, closely matching human body temperature, and a complex innate immune system with haemocytes functionally analogous to mammalian phagocytes. Importantly, a *G. mellonella* as a model for implant-associated infections has been established, incorporating Kirschner (K)-wires and ALBC to evaluate biofilm formation and antimicrobial efficiency [18,19]. Additionally, Büssemaker et al. demonstrated the feasibility of using a *G. mellonella* implant infection model to evaluate silver coatings against bacterial infections [20].

In the current study, we build upon this model to investigate the antimicrobial properties of cemented K-wires against MDR pathogens, specifically *S. aureus* and *Enterococcus* *faecalis*. Using survival assays and bacterial burden analyses, we aim to assess the therapeutic efficacy of various antibiotic combinations against these challenging infections.

2. Results

2.1. *S. aureus* and *E. faecalis* Exhibit Resistance to Gentamicin and Clindamycin

The *S. aureus* EDCC 5055 and *E. faecalis* EUCC2 strains were susceptible to vancomycin (MIC: 1 and 1–2 µg/mL, respectively) but resistant to gentamicin and clindamycin (Table 1). The *S. aureus* strain had a gentamicin MIC of 4–8 µg/mL and an MBC of 8–16 µg/mL, whereas its clindamycin MIC was 32 µg/mL with an MBC of 64–128 µg/mL. *E. faecalis* exhibited high-level resistance to gentamicin and clindamycin, with MIC and MBC values above 128 µg/mL. Of note, the gentamicin and clindamycin used for MIC/MBC determination were obtained as standard infusion solutions, whereas the vancomycin tested was derived from a pharmaceutical-grade powder similar to formulations used in industrially manufactured PMMA cements.

Table 1. MIC and MBC values of *S. aureus* and *E. faecalis*.

Bacterial Strain	Antibiotic	MIC (µg/mL)	ECOFF [2]	Interpretation [1]	MBC (µg/mL)
S. aureus EDCC 5055	Gentamicin	4–8	2	R	8–16
	Clindamycin	32	0.25	R	64–128
	Vancomycin	1	2	S	1
E. faecalis EUCC2	Gentamicin	>128	128	R	>128
	Clindamycin	>128	-	R	>128
	Vancomycin	1–2	4	S	32

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of antibiotics against *S. aureus* EDCC 5055 and *E. faecalis* EUCC2 (in µg/mL). [1] Categorised as susceptible (S) or resistant (R) according to EUCAST, [2] including the corresponding epidemiological cut-off (ECOFF) values [21].

Despite their resistance to gentamicin and clindamycin, these MDR strains were selected intentionally to assess ALBC formulations containing gentamicin alone or in combination with clindamycin or vancomycin, to evaluate their efficacy against resistant pathogens.

2.2. Cemented K-Wires Suppress Bacterial Growth In Vitro

2.2.1. Antibiotic-Loaded Cemented K-Wires Inhibit *S. aureus* and *E. faecalis* Despite Resistance

The agar diffusion assay showed that antibiotic-loaded cemented K-wires inhibited bacterial growth, despite resistance to gentamicin. Control cemented K-wires (PALACOS R, no antibiotics) did not inhibit *S. aureus* growth, as expected (Figure 1A). However, gentamicin-loaded cemented K-wires (PALACOS R+G) produced an inhibition zone of 10.3 ± 5.6 mm. The gentamicin-vancomycin combination (COPAL G+V) further increased inhibition (13.3 ± 2.9 mm). The gentamicin-clindamycin combination (COPAL G+C) produced the largest inhibition zone (29.1 ± 2.4 mm), which likely reflects its higher gentamicin content (1.0 g per 40 g cement powder) and the resulting increased local antibiotic release compared with PALACOS R+G and COPAL G+V (both containing 0.5 g gentamicin). This effect may additionally be influenced by a potential synergistic interaction between antibiotics with different mechanisms of action.

For *E. faecalis*, PALACOS R did not produce an inhibition zone, as expected, whereas PALACOS R+G generated a small zone (6.0 ± 4.9 mm), suggesting localised activity despite resistance (Figure 1B). COPAL G+V displayed a significant inhibition zone (11.8 ± 1.0 mm), while COPAL G+C showed the highest inhibition (14.9 ± 1.2 mm), indicating superior efficacy.

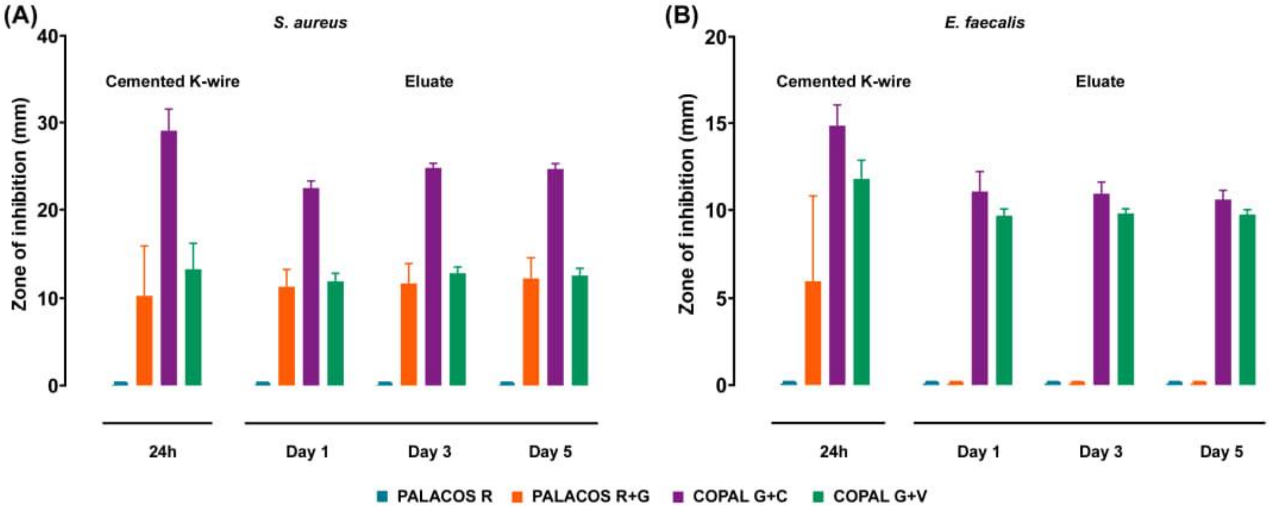

Figure 1. Cemented K-wires inhibit bacterial growth in vitro. Quantification of the mean inhibition zone diameters (in mm) against (**A**) *S. aureus* EDCC 5055 and (**B**) *E. faecalis* EUCC2 using cemented K-wires ($n = 8$ per group). Data are presented as mean ± standard deviation.

2.2.2. Antibiotic Eluates Maintain Antimicrobial Activity over Time

To evaluate sustained antimicrobial activity, an agar diffusion assay was performed using eluates from cemented K-wires incubated in PBS. For *S. aureus*, all antibiotic-loaded eluates inhibited bacterial growth after 1 day, with inhibition zones of PALACOS R+G, 11.3 ± 1.9 mm; COPAL G+V, 11.9 ± 0.9 mm; and COPAL G+C, 22.6 ± 0.8 mm (Figure 1A). A slight increase in inhibition zones was observed over 3 and 5 days.

For *E. faecalis*, eluates from COPAL G+V (9.7 ± 0.4 mm) and COPAL G+C (11.1 ± 1.1 mm) produced significant inhibition after 1 day (Figure 1B). No inhibition was detected with PALACOS R or PALACOS R+G, and no further increase was observed at later time points.

2.3. Antibiotic Release from Cemented K-Wires Shows a Burst on Day 1 Followed by a Slower Sustained Phase

To evaluate antibiotic release kinetics, cemented K-wires were incubated in PBS, and eluates were analysed on days 1, 3, and 5 using HPLC (Figure 2).

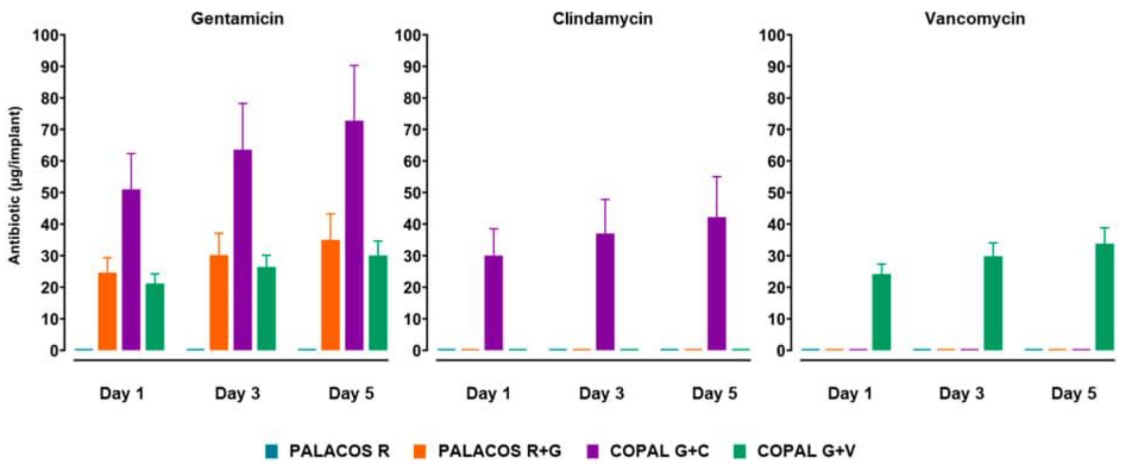

Figure 2. Antibiotic release kinetics from cemented K-wires. Quantitative analysis of cumulative antibiotic release for gentamicin, clindamycin, and vancomycin over five days, as determined by HPLC-MS/MS ($n = 5$ per group). A pronounced burst release was observed on day 1, followed by a slower, sustained phase. Data are presented as mean ± standard deviation.

A pronounced burst release was observed on day 1, after which the rate of release declined but continued at a lower, sustained level. PALACOS R+G released 24.6 ± 4.2 μg of gentamicin on day 1, with cumulative amounts increasing to 30.2 ± 6.9 μg on day 3 and 35.0 ± 8.3 μg on day 5 (Figure 2). COPAL G+C showed the highest overall release, with 51.0 ± 10.2 μg of gentamicin and 30.0 ± 7.6 μg of clindamycin on day 1, followed by a gradual increase over time. COPAL G+V exhibited a similar burst-sustained profile for gentamicin and vancomycin. Overall, COPAL G+C released nearly twice as much gentamicin as PALACOS R+G and COPAL G+V, reflecting its higher antibiotic loading.

2.4. Cemented K-Wires Exhibit Antibiofilm Activity In Vitro

2.4.1. Dual-Antibiotic-Loaded K-Wires Effectively Disrupt *S. aureus* Biofilms

To evaluate the impact of cemented K-wires on pre-formed biofilms, *S. aureus* and *E. faecalis* biofilms were established in 96-well plates, followed by the placement of cemented K-wires (Figure 3A). The antimicrobial efficacy of released antibiotics was assessed by quantifying the bacterial load in three compartments: (i) planktonic bacteria, (ii) biofilm on the well surface, and (iii) bacterial colonisation on the cemented K-wire surface.

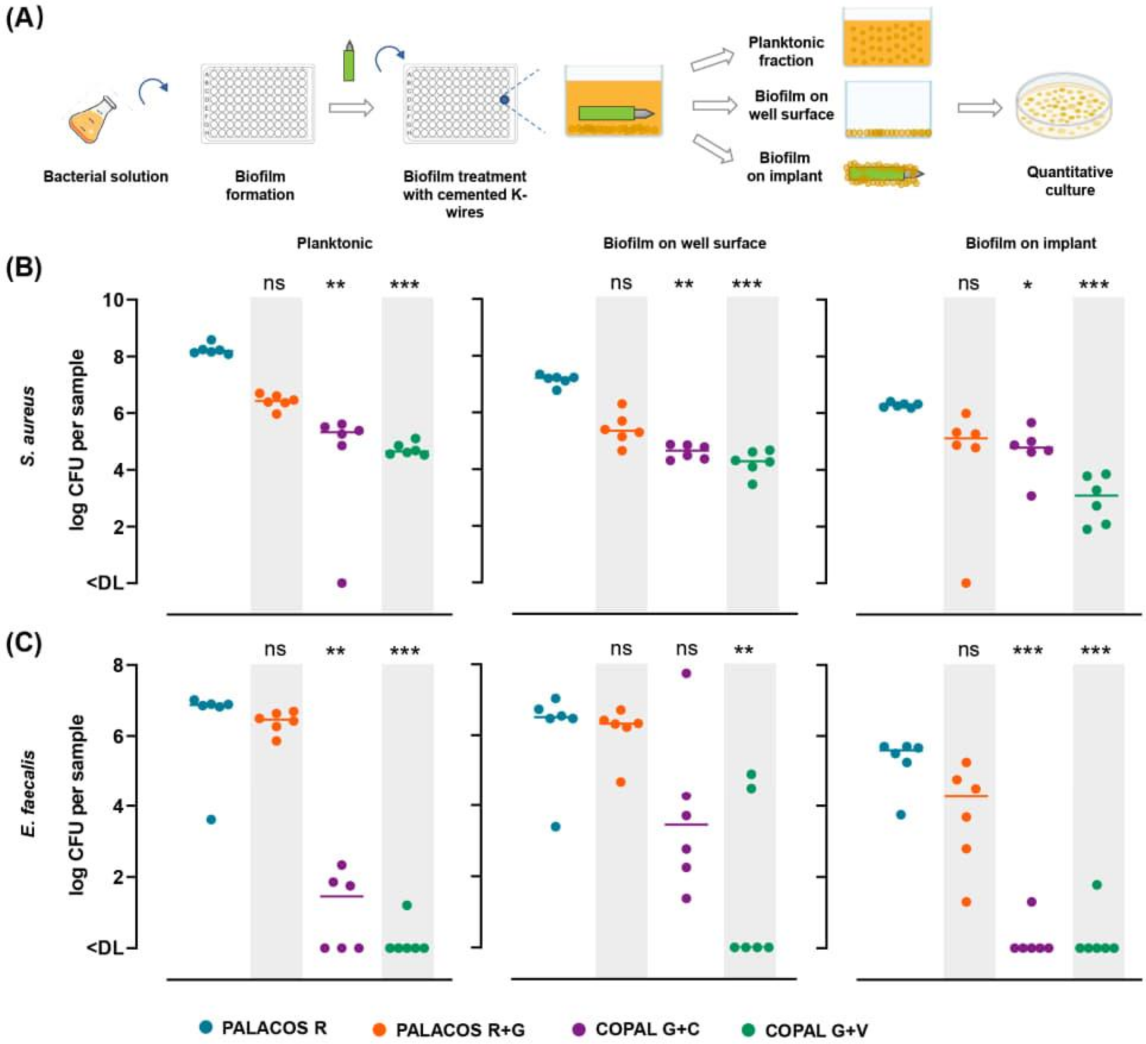

Figure 3. Cemented K-wires exhibit antibiofilm activity in vitro. (**A**) Schematic representation of the biofilm treatment protocol, in which pre-formed *S. aureus* and *E. faecalis* biofilms were treated with

cemented K-wires. (**B**) Quantification of viable *S. aureus* bacteria (log CFU) retrieved from the planktonic fraction (left), the biofilm on the well surface (middle), and the cemented K-wire surface (right). (**C**) Quantification of viable *E. faecalis* bacteria (log CFU) in the same compartments as in panel (**B**). Horizontal lines indicate median values (n = 6 for each compartment). Data were analysed using the Kruskal–Wallis rank-sum test, with statistical significance denoted as: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$), ns = non-significant.

For *S. aureus* biofilms, K-wires containing dual-antibiotic-loaded bone cement (COPAL G+C and COPAL G+V) significantly reduced the pre-formed biofilms on the well surfaces, decreased planktonic bacterial counts, and inhibited bacterial colonisation on the implant surface (Figure 3B). Compared to the PALACOS R control, COPAL G+C and COPAL G+V achieved a more than 1.5-log reduction in bacterial burden across all three compartments. In contrast, mono-antibiotic-loaded K-wires (PALACOS R+G) caused a modest 1.2-log reduction on the surface of the implant, indicating partial biofilm inhibition.

2.4.2. Vancomycin-Loaded Cemented K-Wires Reduce *E. faecalis* Biofilms

For *E. faecalis* biofilms, COPAL G+C and COPAL G+V cemented K-wires significantly reduced bacterial burden in the planktonic fraction and bacterial colonisation of the implant surface (at least $p < 0.01$ in all cases) (Figure 3C). The greatest reduction in biofilm on the well surface was observed with COPAL G+V ($p < 0.01$). However, COPAL G+C did not significantly reduce biofilm on the well surface ($p = 0.135$), possibly due to limited efficacy of clindamycin against established *E. faecalis* biofilms. PALACOS R+G showed no significant impact on *E. faecalis* biofilms, planktonic bacteria, or bacterial colonisation of the K-wire surface.

2.5. Antibiotic-Loaded Cemented K-Wires Prevent Biofilm Infections In Vivo

2.5.1. Dual-Antibiotic-Loaded Cemented K-Wires Protect Against Biofilm-Associated Pathogenicity

The ability of cemented K-wires to prevent biofilm formation was evaluated in a *G. mellonella* biofilm infection model (Figure 4A). For *S. aureus* biofilm infections, after 5 days, survival was significantly higher with PALACOS R+G (36.7 ± 8.8%; $p < 0.001$), COPAL G+C (70.0 ± 8.4%; $p < 0.001$), and COPAL G+V (90.0 ± 5.5%; $p < 0.001$), compared to non-loaded controls (PALACOS R; 3.3% survival) (Figure 4B).

A similar finding was observed for *E. faecalis* biofilm infections: PALACOS R+G (23.3 ± 7.7%; $p < 0.05$), COPAL G+C (70.0 ± 8.4%; $p < 0.001$), and COPAL G+V (80.0 ± 7.3%; $p < 0.001$), compared to the control (PALACOS R; 6.7 ± 4.6% survival) (Figure 4D).

2.5.2. Dual-Antibiotic-Loaded Cemented K-Wires Reduce Bacterial Load on Implants and in Tissue

To assess bacterial dissemination from biofilms, bacterial burden was quantified in larval tissue and on implant surfaces 24 h post-implantation.

For *S. aureus*, COPAL G+C and COPAL G+V significantly reduced bacterial burden by at least 6.6-log in both implant and tissue samples ($p < 0.01$ and $p < 0.05$, respectively), leading to bacterial clearance in nearly all samples (Figure 4C). PALACOS R+G showed no substantial reduction (log 7.4 CFU/tissue, log 6.5 CFU/implant), confirming limited efficacy against biofilms. The control group (PALACOS R) exhibited log 7.0 CFU/tissue and log 6.6 CFU/implant, confirming extensive bacterial colonisation.

For *E. faecalis*, COPAL G+V significantly reduced bacterial counts by over 2.4-log on implants and in tissue ($p < 0.001$ for both; Figure 4E), whereas COPAL G+C resulted in a smaller yet statistically significant ≥1.3-log reduction ($p < 0.05$ for both) compared to the control (PALACOS R: log 8.7 CFU/tissue, log 6.0 CFU/implant). PALACOS R+G did not significantly affect bacterial burden (log 8.4 CFU/tissue, log 5.8 CFU/implant).

These results demonstrate that dual-antibiotic-loaded cemented K-wires offer superior protection against biofilm infections by significantly reducing bacterial load and preventing systemic infection.

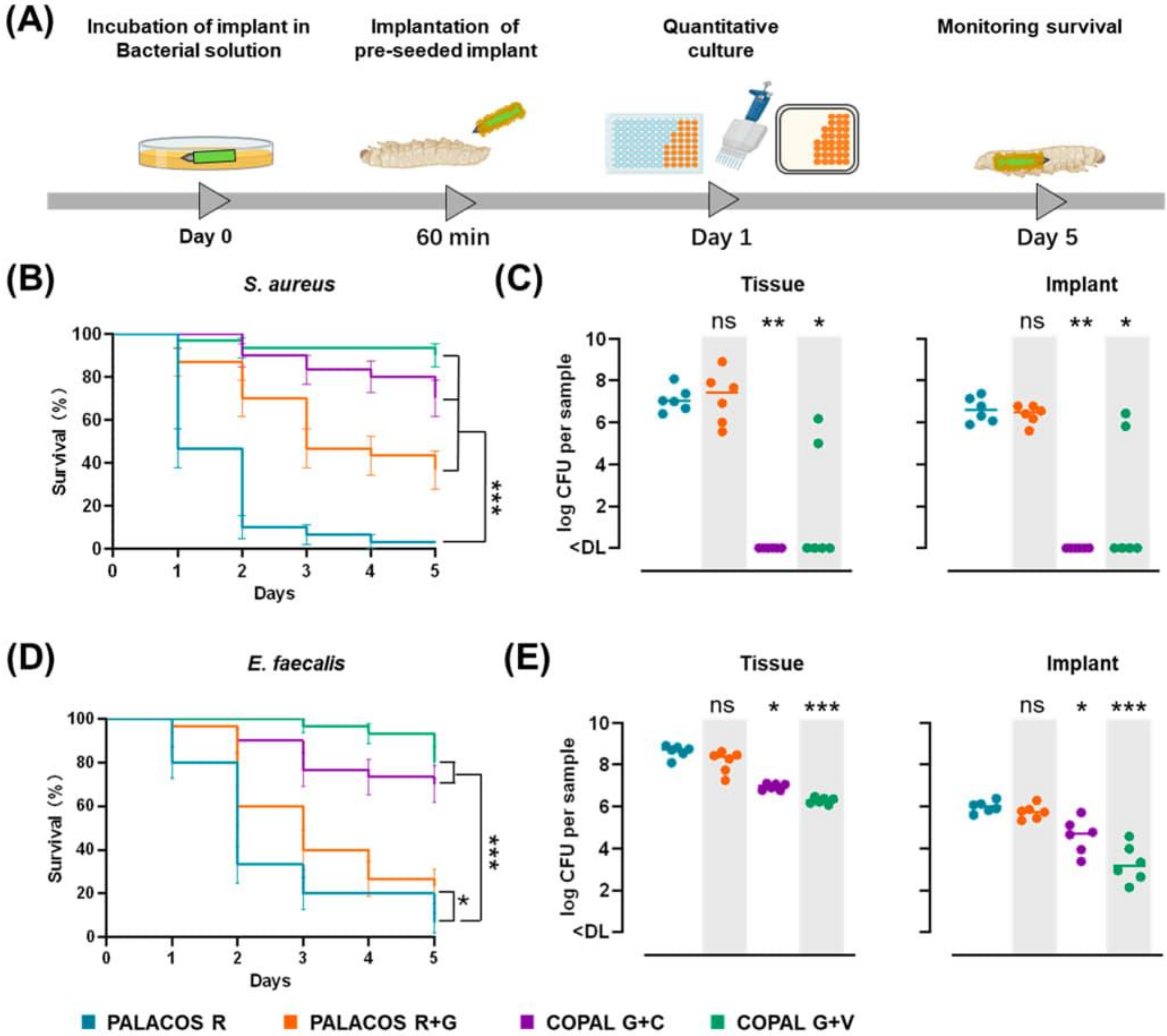

Figure 4. Antibiotic-loaded cemented K-wires prevent biofilm implant infections in *G. mellonella*. (**A**) Schematic representation of the biofilm infection model, in which cemented implants were pre-incubated for 60 min in *S. aureus* or *E. faecalis* suspensions (0.5–1 $\times$ 10^7 CFU/mL) before implantation. The mean number of *S. aureus* adhered to the surface before implantation was log 4.7 CFU (PALACOS R), log 2.8 CFU (PALACOS R+G) and log 3.5 CFU (COPAL G+V) per implant, whereas no bacteria could be observed on COPAL G+C (<DL; n = 3 per group). The mean number of *E. faecalis* adhered to the surface before implantation was log 5.9 CFU (PALACOS R), log 5.5 CFU (PALACOS R+G), log 3.9 CFU (COPAL G+C) and log 1.3 CFU (COPAL G+V) per implant (n = 3 per group). (**B**,**D**) Kaplan–Meier curves showing percent survival ($\pm$SEM) over 5 days for larvae infected with *S. aureus* (**B**) or *E. faecalis* (**D**) after implantation of pre-incubated cemented K-wires. (**C**,**E**) Bacterial burden (log CFU) in larval tissue and on the cemented K-wire surface after 24 h for *S. aureus* (**C**) and *E. faecalis* (**E**). Horizontal lines represent median values. Data were analysed from three independent experiments (n = 10 larvae per experiment) using the log-rank test for survival and the Kruskal–Wallis rank-sum test for bacterial burden. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = non-significant). The detection limits (DL) were 5 CFU/tissue and 3 CFU/implant.

2.6. Antibiotic-Loaded K-Wires Prevent Haematogenous Implant Infection in G. mellonella

2.6.1. Antibiotic-Loaded Cemented K-Wires Increase Survival in *S. aureus* and *E. faecalis* Infected Larvae

The in vivo antimicrobial efficacy of cemented K-wires was investigated using a *G. mellonella* haematogenous infection model, in which antibiotic-loaded K-wires were implanted before infection with *S. aureus* or *E. faecalis* (Figure 5A).

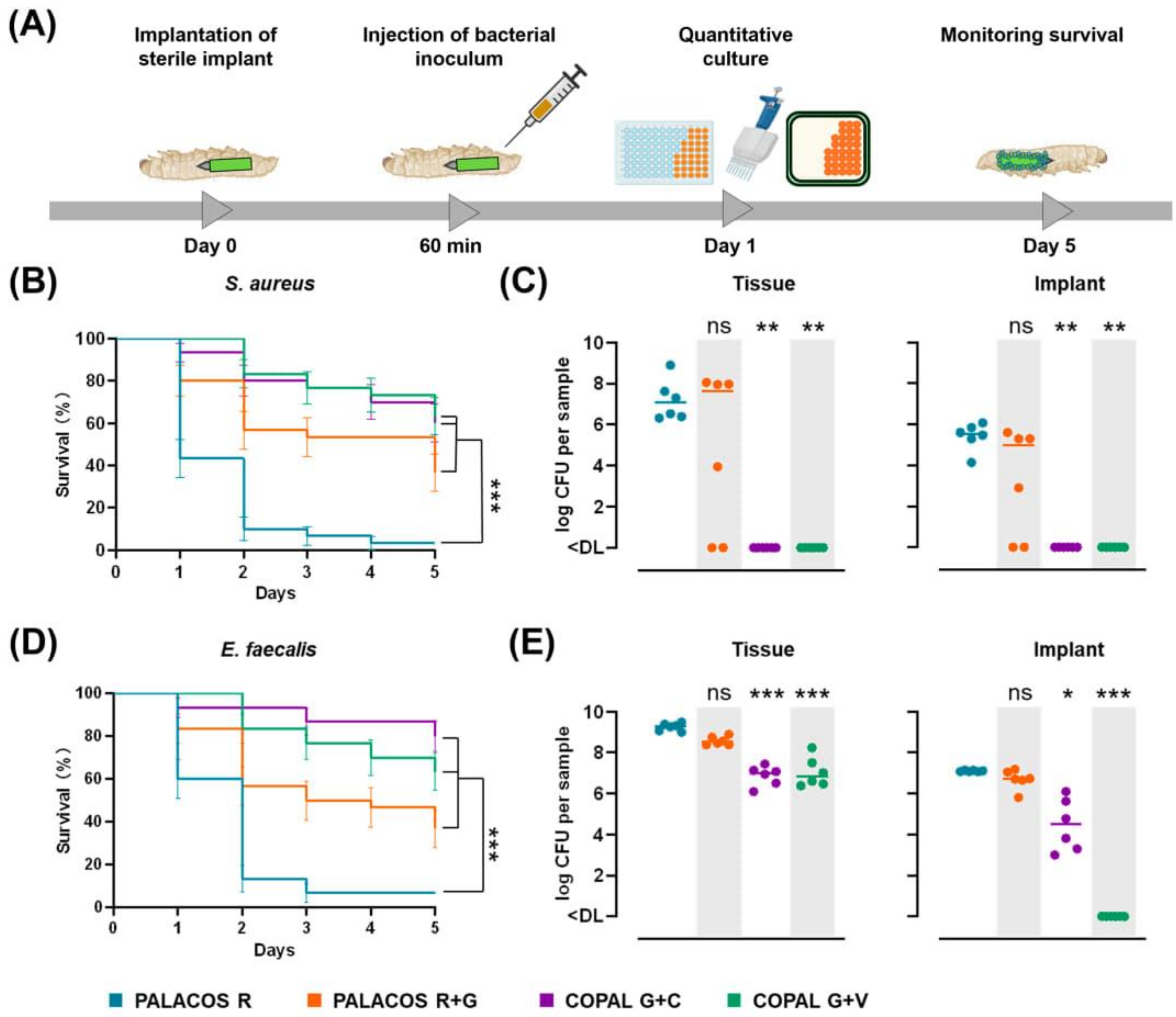

Figure 5. Antibiotic-loaded cemented K-wires prevent haematogenous implant infection in *G. mellonella*. (**A**) Schematic representation of the haematogenous infection model, where larvae received cemented K-wire implantation 60 min prior to infection with *S. aureus* or *E. faecalis* (5×10^4 CFU/larva). (**B**,**D**) Kaplan–Meier survival curves showing the percent survival (±SEM) over 5 days for larvae infected with *S. aureus* (**B**) or *E. faecalis* (**D**) after implantation of cemented K-wires loaded with gentamicin alone (PALACOS R+G) or in combination with clindamycin (COPAL G+C) or vancomycin (COPAL G+V). Non-loaded implants (PALACOS R) served as controls. (**C**,**E**) Bacterial burden (log CFU) in larval tissue and on the cemented implant K-wire surface after 24 h of incubation for *S. aureus* (**C**) and *E. faecalis* (**E**). Horizontal lines represent median values. Data were analysed from three independent experiments ($n = 10$ larvae per experiment) using the log-rank test for survival and the Kruskal–Wallis rank-sum test for bacterial burden. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = non-significant). The detection limits (DL) were 5 CFU/tissue and 3 CFU/implant.

For *S. aureus* infections, larvae implanted with antibiotic-loaded cemented K-wires demonstrated significantly improved survival rates compared to the non-loaded control (PALACOS R, 3.3% survival): COPAL G+V (63.3 ± 8.8%; $p < 0.001$), COPAL G+C (60.0 ± 8.9%; $p < 0.001$) and PALACOS R+G (36.7 ± 8.8%; $p < 0.001$) (Figure 5B).

For *E. faecalis* infections, antibiotic-loaded cemented K-wires also significantly improved survival, with COPAL G+V showing the highest protection (80.0 ± 7.3%; $p < 0.001$), followed by COPAL G+C (63.3 ± 8.8%; $p < 0.001$) and PALACOS R+G (36.7 ± 8.8%; $p < 0.001$), compared to PALACOS R (6.7% survival; Figure 5D).

Notably, PALACOS R+G significantly increased survival in larvae infected with *S. aureus* and *E. faecalis*, despite resistance to gentamicin, suggesting that high local antibiotic concentrations may overcome resistance locally and hinder infection progression. Importantly, sterile cemented implants had no impact on larval survival, confirming that implantation alone did not affect viability (Figure A1).

2.6.2. Dual-Antibiotic-Loaded Cemented K-Wires Eliminate Bacteria from Implants and Surrounding Tissue

To further evaluate the antimicrobial efficacy, bacterial burden was quantified in larval tissue and on the cemented K-wire surface after 24 h. For *S. aureus* infections, COPAL G+C and COPAL G+V reduced bacterial counts in tissue by approximately 7.1-log and on the implant surface by 5.5-log ($p < 0.01$ in all cases), leading to complete bacterial clearance in all cases (Figure 5C). In contrast, PALACOS R+G did not significantly reduce bacterial burden (log 7.6 CFU/tissue, log 5.0 CFU/implant) compared to the control (PALACOS R; log 7.1 CFU/tissue, log 5.5 CFU/implant).

For *E. faecalis* infections, COPAL G+C reduced bacterial counts by more than 2.3-log in tissue ($p < 0.001$) and on the implant surface ($p < 0.05$), while COPAL G+V led to a more than 2.4-log reduction in tissue ($p < 0.001$) and to elimination on the implant surface ($p < 0.001$) (Figure 5E). PALACOS R+G failed to significantly reduce bacterial counts, with similar bacterial loads (log 8.5 CFU/tissue, log 6.7 CFU/implant) compared to the control (PALACOS R; log 9.3 CFU/tissue, log 7.1 CFU/implant).

These findings highlight the effectiveness of dual-antibiotic-loaded cemented K-wires in preventing haematogenous *S. aureus* and *E. faecalis* infections, improving survival, and significantly reducing bacterial colonisation on implant surfaces and adjacent tissues.

3. Discussion

Commercially available PMMA bone cements play a crucial role in prosthesis fixation, fracture management, bone tumour treatment, and revision surgeries. Their primary functions include securing artificial joints, delivering local antibiotics, and enhancing mechanical stability. In this study, we evaluated the efficacy of antibiotic-loaded cemented implants against MDR *S. aureus* and *E. faecalis* using previously developed *G. mellonella* infection models [19] to mimic PJI. In vitro and in vivo analyses demonstrated that dual-antibiotic-loaded bone cements (COPAL G+C and COPAL G+V) were significantly more effective in biofilm elimination and infection prevention than PALACOS R+G, which contained only gentamicin. These findings are consistent with clinical studies reporting improved infection control with dual-antibiotic ALBC formulations [22,23]. By utilising clinically relevant, commercially available bone cements, we effectively mimicked real-world conditions and strengthened the relevance of the model.

Cemented implants, including the cemented K-wires used in this study, typically exhibit a pronounced burst release of antibiotics during the initial days, followed by a slower, sustained elution phase. Our efficacy assessments therefore primarily reflect this early burst-release period, and the infection control observed in our model is most appropriately interpreted in the context of high local antibiotic concentrations during the acute phase. Although sustained low-level antibiotic release from PMMA can contribute for extended periods [24], its contribution to long-term infection prevention was not directly evaluated here and remains to be further characterised, particularly in relation to late-onset infections.

However, such elevated antibiotic levels also raise concerns about local cytotoxicity. In vitro studies have shown that while gentamicin- or vancomycin-loaded PMMA cements exhibit only mild cytotoxic effects, combinations such as gentamicin + clindamycin can markedly impair cell viability and osteogenic activity [25]. Moreover, the exothermic polymerisation of PMMA generates free radicals that may induce local oxidative stress and tissue damage [26]. In our in vivo compatibility assay (Figure A1), the antibiotic dose in the cemented K-wires caused no observable harm to *G. mellonella* larvae, but caution is warranted when extrapolating these findings to mammalian systems. Promisingly, formulation strategies are being developed to mitigate toxicity without compromising antimicrobial efficacy. For example, co-loading vancomycin with the antioxidant N-acetylcysteine (NAC) has been shown to enhance the antibiotic elution profile and substantially reduce cytotoxicity, maintaining antibacterial activity for more than 35 days at lower vancomycin doses [26]. These findings emphasise the importance of balancing high local antimicrobial potency with host tissue compatibility in the design of ALBC formulations.

Interestingly, PALACOS R+G (containing 0.5 g gentamicin) demonstrated measurable antimicrobial activity against gentamicin-resistant *S. aureus* in the agar diffusion assay. Moreover, COPAL G+C (containing 1 g gentamicin and 1 g clindamycin) exhibited pronounced antimicrobial and anti-biofilm activity against *S. aureus* and *E. faecalis* strains classified as resistant to both gentamicin and clindamycin. Our findings suggest that both PALACOS R+G and COPAL G+C achieve high local antibiotic release, with dual-antibiotic-loaded bone cements providing a higher total amount of released antibiotic, potentially contributing to concentration-dependent bacterial killing and/or synergistic interactions between antibiotics [22,27]. However, true pharmacodynamic synergy was not formally assessed in this study and therefore cannot be concluded from our data. These findings support the view that antibiotics deemed ineffective systemically due to resistance may still exert local antimicrobial effects at high concentrations. Similarly, Metsemaker et al. reported that a doxycycline-coated titanium intramedullary nail provided complete protection against osteomyelitis caused by a doxycycline-resistant *S. aureus* strain, further supporting the importance of high local antibiotic concentrations in infection control [28].

Bacterial biofilms play a crucial role in antibiotic resistance, and our study demonstrates that COPAL G+V exhibited the highest biofilm disruption activity against both bacterial species, aligning with clinical reports of its growing use in PJI treatment [29]. The varying antimicrobial effects observed over time across different ALBC implants, as demonstrated in agar diffusion assays, are likely attributed to differences in antibiotic loading: COPAL G+C (1 + 1 g), COPAL G+V (0.5 + 2 g), and PALACOS R+G (0.5 g). For future in vivo studies, equalising antibiotic concentrations could allow for direct comparison of additive effects between antibiotics.

Notably, our study successfully adapted the *G. mellonella* model for evaluating cemented implants, an important step considering the widespread clinical use of cemented fixation. This model offers a cost-effective, high-throughput approach for investigating PJI prevention and treatment. Our findings, consistent with clinical observations and previous in vivo studies [13] and clinical settings [30], highlight that dual-antibiotic-loaded ALBC (COPAL G+C and COPAL G+V) is significantly more effective than mono-antibiotic ALBC (PALACOS R+G) during the early phase of infection. This is supported by reduced bacterial burden and early biofilm formation at 24 h post-implantation, as well as significantly improved larval survival over the 5-day observation period in both haematogenous and biofilm-associated infection models with MDR *S. aureus* and *E. faecalis*. Nevertheless, we acknowledge that the absence of bacterial quantification at later post-infection stages represents a limitation and may lead to a partial overestimation of efficacy.

A key advantage of the *G. mellonella* model is its low cost, high-throughput capacity, exemption from ethical approval requirements, and its ability to provide preliminary insights that often align with clinical trends [31]. As such, it serves as a preclinical screening tool that helps reduce reliance on vertebrate models and thereby minimises overall animal use. However, important limitations exist: *G. mellonella* lacks adaptive immunity, including antibody production, which plays a critical role in human PJI responses [32,33]. Moreover, the larvae do not possess a musculoskeletal system or bone-implant interface, limiting their ability to model orthopaedic implant environments. Therefore, while the model is valuable for rapid, cost-effective preliminary assessment of antimicrobial efficacy, results must be validated in more advanced systems, such as vertebrate models, before any clinical translation.

Nevertheless, qualitative parallels can be drawn between our findings and results from established vertebrate models of bone and implant-associated infection reported in the literature. Several studies using rat or rabbit models have demonstrated that antibiotic-loaded PMMA cements, particularly those containing vancomycin or broad-spectrum or dual-antibiotic formulations, can significantly reduce *S. aureus* infection, bacterial burden, and inflammatory responses in vivo, while providing sustained local antibiotic release around the implant [34–36]. Comparative investigations have also shown that differences in cement formulation and antibiotic composition lead to distinct biological and antimicrobial outcomes, underscoring the critical role of antibiotic-loaded PMMA in infection control [37]. These observations from vertebrate models are consistent with our results, in which the dual-antibiotic-loaded cements COPAL G+V and COPAL G+C outperformed the mono-antibiotic formulation PALACOS R+G in controlling implant-associated infection in the *G. mellonella* model, providing useful biological context for the superior early anti-infective performance of dual-antibiotic PMMA formulations.

Despite these limitations, the *G. mellonella* model remains a valuable tool for investigating implant-associated infections caused by MDR bacterial strains, particularly those belonging to the ESKAPE group (*Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) [38]. These pathogens pose a global health threat due to their ability to evade standard antibiotic treatments and cause persistent implant-associated infections. Future studies could utilise *G. mellonella* infection models to assess the efficacy of ALBC implants with diverse antibiotic combinations against ESKAPE pathogens and polymicrobial infections.

4. Materials and Methods

4.1. Bacterial Cultures

The MDR bacterial strains *S. aureus* EDCC 5055 and *E. faecalis* EUCC2 were used for all experiments. The *S. aureus* EDCC 5055 strain, characterised by its biofilm-forming capacity, was originally isolated from a wound infection [39]. The *E. faecalis* EUCC2 strain was isolated from a fracture-related infection at University Hospital Regensburg, Regensburg, Germany.

Prior to each experiment, bacteria were revived from frozen stocks (−80 °C) and grown overnight at 37 °C on LB agar plates (Carl Roth, Karlsruhe, Germany). A single colony was used to inoculate an overnight culture in tryptic soy broth (TSB; Merck, Darmstadt, Germany), incubated at 37 °C with shaking at 180 rpm. The overnight culture was diluted 1:100 in fresh TSB and incubated under the same conditions until it reached the mid-logarithmic growth phase. The bacteria were pelleted by centrifugation, washed once with phosphate-buffered saline (PBS; 140 mM NaCl, pH 7.4; Gibco, Life technologies, Paisley, UK), and resuspended in PBS. The bacterial concentration was adjusted for in vitro and in vivo experiments based on optical density measurements at 600 nm.

The inoculum suspension's final concentration was confirmed through quantitative culture. Briefly, 10-fold serial dilutions of the bacterial suspension were prepared in PBS and duplicate 5 µL aliquots were plated onto LB agar. Colony-forming units (CFU) were enumerated after overnight incubation at 37 °C, and CFU/mL was calculated.

4.2. Antibiotics and Bone Cement Formulations

A selection of antibiotics from different classes was used in this study, including the protein synthesis inhibitors gentamicin (aminoglycoside; infusion solution; 50 mg/mL; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and clindamycin (lincosamide; infusion solution; 50 mg/mL; 1A Pharma, Holzkirchen, Germany), as well as vancomycin (glycopeptide; pharmaceutical-grade powder, 1000 mg per vial; Dr. Friedrich Eberth Arzneimittel GmbH, Ursensollen, Germany), which inhibits bacterial cell wall synthesis. Vancomycin powder was reconstituted according to the manufacturer's instructions to obtain a stock solution of 50 mg/mL, matching the nominal concentration of the gentamicin and clindamycin infusion solutions. Working solutions of all antibiotics were subsequently prepared in Milli-Q water at 2.56 mg/mL and stored at 4 °C, protected from light, until use.

Bone cements used in this study were obtained from Heraeus Medical GmbH (Wehrheim, Germany) and included the following formulations: PALACOS R, an unloaded bone cement containing no antibiotics; PALACOS R+G, a bone cement containing 0.5 g gentamicin; COPAL G+C, a bone cement containing 1 g gentamicin and 1 g clindamycin; and COPAL G+V, a bone cement containing 0.5 g gentamicin and 2 g vancomycin. These bone cements were prepared and analysed as described in subsequent sections.

4.3. Antimicrobial Activity of Antibiotics in Solution (MIC/MBC)

To determine the antimicrobial activity of antibiotics, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were assessed according to EUCAST guidelines. For each bacterial strain, a 10 µL aliquot of the inoculum suspension (1×10^7 CFU/mL, resulting in a final concentration of 1×10^6 CFU/mL) was added to 90 µL of two-fold serially diluted antibiotic solutions in TSB (final concentrations ranging from 128 to 0.125 µg/mL) in a 96-well polystyrene flat-bottom microtiter plate (Sarstedt AG, Nümbrecht, Germany). A non-treated control was included, in which bacteria were incubated in TSB without antibiotics. Plates were incubated overnight at 37 °C and 180 rpm in a humidified environment.

After incubation, the wells were visually inspected for bacterial growth to determine the MIC, defined as the lowest antibiotic concentration that completely inhibited visible growth. To determine the MBC, 5 µL aliquots from each well were plated onto LB agar plates to quantify the number of viable bacteria. After overnight incubation at 37 °C, the MBC was identified as the lowest antibiotic concentration that killed $\geq$99.9% of bacteria within 24 h. Experiments were performed in duplicate, with $n = 3$ for all conditions, and antimicrobial susceptibility was interpreted using EUCAST clinical breakpoints.

4.4. Preparation of Cemented Implants

The radiopaque polymer powder (40–43 g, depending on the type of bone cement) was rapidly and thoroughly mixed with 20 mL of monomer liquid in a mixing bowl to form a homogeneous paste. Mixing lasted approximately 20 s. The paste was then transferred into Teflon moulds (Karl Lettenbauer, Erlangen, Germany) using a spatula.

To create cemented implants, 4 mm long K-wire segments with a 0.8 mm diameter ($TiAl_6V_4$; DePuy Synthes, Oberdorf, Switzerland) were rapidly inserted into the bone cement paste within the Teflon mould before the onset of cement polymerization, resulting in implants measuring 8 mm in length and 1.2 mm in diameter (Figure 6). The distal 1.5–2 mm of the K-wire tip was left uncemented to mimic the human situation, in which the articulating part of the prosthesis cannot be cemented. After 30 min, once polymerisation was complete, the samples were carefully removed from the mould using a metal pin. The implants were sharpened with an electric combination tool (Georg Roth GmbH, Fürth, Germany) and sterilised under ultraviolet (UV) light for 30 min before implantation into *G. mellonella* larvae.

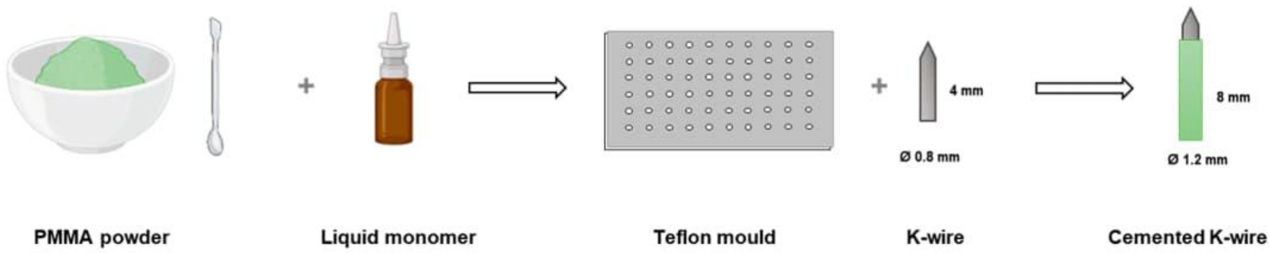

Figure 6. Schematic representation of the sample preparation.

4.5. Release Kinetics of Antibiotics from Cemented K-Wires

To assess the antibiotic release kinetics from cemented K-wires, samples (n = 5 per group) were incubated in 1.2 mL of PBS at 37 °C for up to 5 days. Eluate samples were collected after 1, 3, and 5 days for high-performance liquid chromatography (HPLC) analysis. At each time point, fresh PBS was added to maintain consistent incubation conditions. The concentrations of gentamicin, clindamycin, and vancomycin in the eluates were quantified using HPLC with MS/MS detection. The method was validated according to standard protocols, using one set of matrix calibration standards and two quality control samples. Calibration samples were used to calculate the results, while quality control samples monitored analytical accuracy. Antibiotic release data are presented as cumulative amounts released over time, expressed as mean and standard deviation for all test groups [40].

4.6. In Vitro Antimicrobial Activity of Cemented K-Wires

4.6.1. Agar Diffusion Assay

A modified Kirby-Bauer agar diffusion assay [41] was performed to evaluate the inhibition zone of cemented K-wires against *S. aureus* and *E. faecalis*. A bacterial suspension was prepared by suspending five bacterial colonies in 5 mL of TSB. The suspension was evenly spread onto the surface of Mueller Hinton Agar (MHA) II plates, and excess liquid was removed to ensure uniform distribution. After a brief drying period, the cemented K-wires were placed on the inoculated surface, and the plates were incubated for 24 h at 37 °C (n = 8 cemented K-wires per group).

The inhibition zones were measured at four positions around each cemented K-wire, and the mean diameter (mm) with standard deviation was calculated. Additionally, cemented K-wires were incubated in 500 µL PBS for up to 5 days. At 1, 3 and 5 days, 5 µL of the eluate was spotted on pre-inoculated MHA plates to assess the antimicrobial activity of the released antibiotics. Plates were incubated for 24 h at 37 °C, and the inhibition zone diameter was measured.

4.6.2. Antibiofilm Assay of Cemented K-Wires

To assess the antibiofilm activity of cemented K-wires (n = 6 per group), overnight cultures of *S. aureus* and *E. faecalis* were diluted 1:100 in fresh TSB, and 200 µL of the suspension was added to each well of a 96-well plate (approximately 2×10^5 CFU/well). The plates were incubated at 37 °C and 100 rpm for 24 h to allow biofilm formation.

After incubation, the wells were gently washed with PBS, followed by the addition of 200 µL of fresh TSB and placement of a cemented K-wire in each well. Plates were incubated for another 24 h at 37 °C and 100 rpm. After incubation, three measures of bacterial growth were quantified (Figure 3A): (i) planktonic growth in the medium, (ii) biofilm formation in the well, and (iii) biofilm formation on the implant surface. The medium was collected, and both the implants and wells were rinsed separately with PBS. Subsequently, the implants were then sonicated separately in 500 µL PBS and the wells in 200 µL PBS for 5 min at 45 kHz in a water bath sonicator (Ultrasonic Cleaner USC-T; VWR, Ismaning, Germany) and vortexed for 30 s to detach and disperse adherent biofilm cells. This procedure does not affect bacterial viability [42].

The medium and sonicates were serially diluted ten-fold, and 5 µL droplets of each dilution were plated onto LB agar plates. To enhance the detection limit, an additional 200 µL was directly plated onto LB agar. Plates were incubated overnight at 37 °C, and CFU were enumerated.

4.7. *G. mellonella Implant Infection Models*

4.7.1. Animals

G. mellonella larvae were obtained from Evergreen GmbH (Augsburg, Germany) and Fauna Topics GmbH (Marbach am Neckar, Germany) and maintained on wheat germ (Tropic Shop GmbH, Nordhorn, Germany) at room temperature prior to experiments and were incubated at 37 °C following implantation. For each survival experiment, 10 larvae in the last instar stage (weighing approximately 450–550 mg) were used per group, and each experiment was performed in triplicate (n = 30 larvae per group). Larvae randomly allocated to treatment or control groups before inoculation. For bacterial quantification on implant surfaces and in larval tissue, an additional set of six larvae per group was used. To assess the in vivo biocompatibility, the larvae received a sterile cemented K-wire, and their survival rate was monitored for 5 days (n = 10 larvae per group). All experiments adhered to the ARRIVE guidelines [43].

4.7.2. Biofilm Implant Infection Model

For a biofilm infection, cemented K-wires were pre-incubated with *S. aureus* or *E. faecalis*. The implants were immersed in TSB containing 0.5–1 $\times 10^7$ CFU/mL of the bacterial inoculum suspension and incubated at 180 rpm for 1 h, following established procedures [19,44]. After incubation, the K-wires were rinsed with PBS and implanted into the posterior segment of each larva by piercing the cuticle with the sharpened end of the implant. The larvae were maintained at 37 °C, and survival was monitored for 5 days. Before implantation, bacterial numbers on additional implants (n = 3) were determined using the quantitative culture method described below.

4.7.3. Haematogenous Implant Infection Model

To model haematogenous infection, cemented K-wires were implanted into the posterior segment of the larvae by piercing the cuticle with the sharpened end of the implant. The larvae were then incubated at 37 °C. After 1 h, each larva received an injection of 10 µL of *S. aureus* or *E. faecalis* inoculum suspension (5×10^6 CFU/mL in PBS), corresponding to 5×10^4 CFU/larva, directly at the site of the implanted cemented K-wire. The larvae were maintained at 37 °C, and survival was monitored for 5 days [19,44].

4.7.4. Quantitative Culture

To assess the antimicrobial activity of cemented K-wires, bacterial counts were quantified from the implant surface and larval tissue. At 1-day post-implantation, implants were carefully separated from larval tissue for bacterial quantification. Implants were rinsed with PBS, sonicated in 0.5 mL PBS for 5 min at 45 kHz in a water bath sonicator, and vortexed for 30 s to dislodge adherent bacteria.

Larval tissue samples were homogenised using a Precellys system (VWR). Larvae were surface-disinfected with 70% ethanol and subsequently mechanically disrupted in 1 mL PBS using six large (Ø 2.8–3.2 mm) and ~10 small (Ø 1.4–1.6 mm) yttrium-stabilised zirconium oxide grinding beads (Cerdur, Vechta, Germany). Homogenisation was carried out over six cycles of 30 s at 8000 rpm, with 30 s rest intervals between cycles, under continuous cooling at 4 °C.

The sonicates and homogenates were serially diluted ten-fold, and 5 µL of each dilution was plated onto mannitol salt agar (Sigma-Aldrich) or LB agar plates containing 15 µg/mL gentamicin to suppress the growth of larval bacterial flora. Plates were incubated overnight at 37 °C. To improve detection sensitivity, an additional 200 µL of the undiluted sample was plated. The lower detection limits were 3 CFU per implant and 5 CFU per tissue sample. For visualisation on a logarithmic scale, a value of 1 CFU was assigned when no bacterial growth was detected.

4.8. Statistics

Statistical analyses were performed using GraphPad Prism 9.5 (GraphPad Software, San Diego, CA, USA). Bacterial counts were analysed using the Kruskal–Wallis rank-sum test, represented as $\log_{10}$ CFU with the median value for each group. Differences in *G. mellonella* survival curves were analysed using the log-rank test. Survival data were represented as mean ± standard error of the mean (SEM) from three independent experiments, each with 10 larvae per group. A p-value of <0.05 was considered statistically significant.

5. Conclusions

This study shows that dual-antibiotic-loaded cemented implants—combining gentamicin with either clindamycin or vancomycin—effectively reduce bacterial colonisation by MDR *S. aureus* and *E. faecalis* in both haematogenous and biofilm infections. Significant bacterial burden reductions on implant surfaces and surrounding tissues were associated with improved survival outcomes in our infection models. These findings provide strong experimental data supporting the clinical use of dual-antibiotic ALBC formulations as a strategy to reduce revision surgeries and associated morbidity in orthopaedic practice.

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Institutional Review Board Statement: Ethical review and approval were not applicable for this study, because of the use of the non-vertebrate *Galleria mellonella* animal model.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

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Conflicts of Interest: M.H. is an employee of Heraeus Medical GmbH. K.D.K. and V.A. serve as advisors for Heraeus Medical GmbH. The company had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors declare no conflicts of interest.

Appendix A

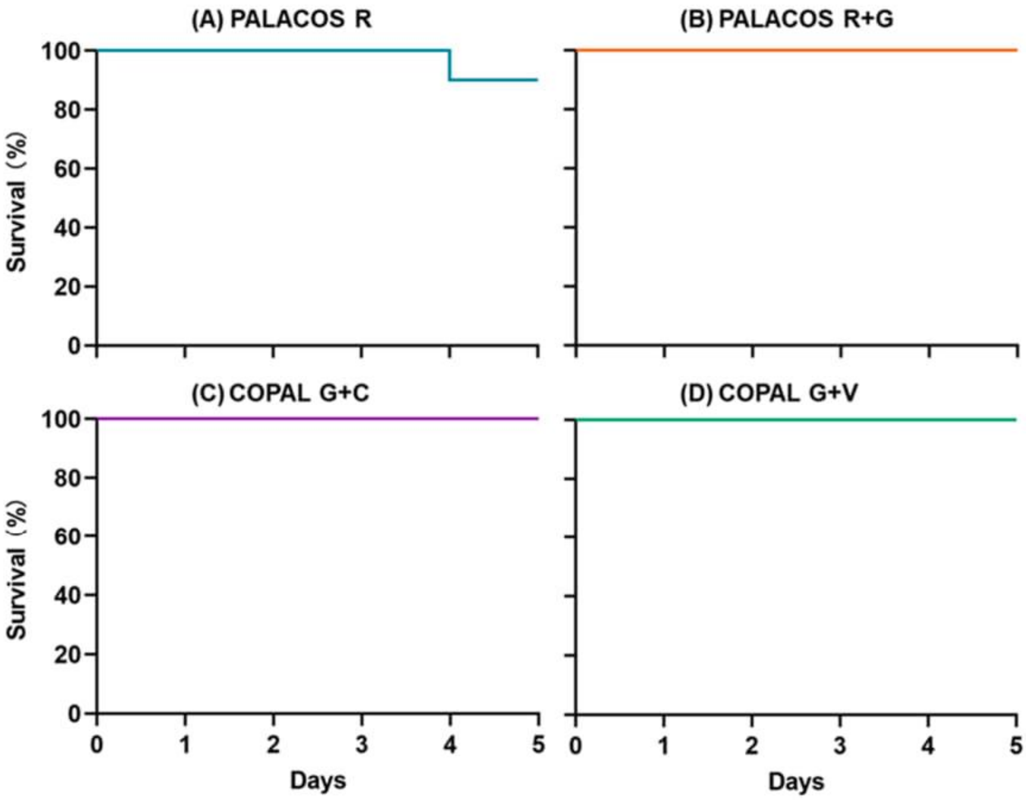

Figure A1. Biocompatibility assay of cemented K-wires in *G. mellonella*. Kaplan–Meier survival curves showing the percentage of larvae surviving over 5 days following implantation with cemented K-wires composed of: (**A**) non-loaded control bone cement (PALACOS R), (**B**) ALBC containing gentamicin alone (PALACOS R+G), (**C**) dual-antibiotic bone cement with gentamicin and clindamycin (COPAL G+C), and (**D**) dual-antibiotic bone cement with gentamicin and vancomycin (COPAL G+V). No significant differences in survival were observed among the groups (n = 10 larvae per group).

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5. Evaluation of daptomycin-supplemented antibiotic-loaded bone cement for treating vancomycin-resistant *Enterococcus faecalis* in the *Galleria mellonella* implant infection model

5.1. Summary

In this study, we investigated the mechanical performance, antimicrobial activity, and in-vivo efficacy of manually daptomycin-loaded PMMA cements using two widely used commercial formulations: Palacos® R+G and Simplex® T (Leta et al., 2023). Our intention was to determine how differences in polymer matrix composition and antibiotic loading influence both mechanical stability and antibacterial effectiveness, particularly against vancomycin-resistant *Enterococcus faecalis*. This question is clinically relevant because manually admixed ALBCs are commonly used in two-stage revision arthroplasty, yet their mechanical integrity and antimicrobial activity vary substantially by cement brand, antibiotic type, and amount added (Coraça-Huber et al., 2025; Kwong et al., 2024). Furthermore, increasing rates of VRE and other MDR pathogens highlight the need for alternative antibiotics such as daptomycin that remain effective against resistant strains (Ayobami et al., 2020).

The main objectives of our work were to determine how different concentrations of daptomycin (1 g, 1.5 g, 2 g) affect the mechanical properties of two chemically distinct PMMA matrices and how these dosing levels translate into short- and long-term antimicrobial activity over 42 days. Additionally, we wanted to prove that the in-vitro findings can be reflected in an in-vivo *Galleria mellonella* biofilm infection model that better mimics real life (Zhao et al., 2024). We aimed to clarify whether cement composition or antibiotic dose is the primary determinant of antimicrobial performance, and to establish thresholds at which mechanical standards defined by ISO 5833:2002 are no longer maintained.

To address these questions, we manually incorporated daptomycin powder into Palacos® R+G and Simplex® T at concentrations of 1.0 g, 1.5 g, and 2.0 g. We performed standardised mechanical testing, including ISO four-point bending strength, bending modulus, compressive strength (ISO 5833, 2002), and DIN impact resistance (DIN 53435, 2018), to assess the influence of daptomycin on material stability. In parallel, we performed microbiological analyses using inhibition zone testing and proliferation assays against VRE over a 42-day elution period. Additionally, to reflect PJI conditions more closely, we evaluated antimicrobial efficacy in-vivo using the *Galleria mellonella* larvae model, testing both infection prevention and infection treatment strategies with PMMA implants shaped from the corresponding cement formulations.

We found that adding daptomycin reduced mechanical strength in a dose- and cement-dependent manner. Although all samples remained above the ISO threshold for bending modulus, both four-point bending strength and compressive strength declined with antibiotic addition. Palacos® R+G showed a smaller reduction and remained within ISO limits at 1.5 g loading, whereas Simplex® T exhibited substantially greater variability and dropped below ISO requirements at the same dosage, indicating that Simplex® T is more susceptible to mechanical compromise when manually admixed with antibiotics. Similar patterns were observed in DIN bending and impact resistance tests, where daptomycin addition consistently reduced resistance to bending and impact, with Palacos® R+G again demonstrating more stable performance than Simplex® T. Antimicrobial activity depended strongly on the amount of daptomycin added and declined over prolonged elution. At early time points, 2 g daptomycin produced the largest inhibition zones for both cements, followed by 1.5 g. Samples containing only 1 g showed minimal or no inhibition by day 7. After 42 days antimicrobial activity persisted only for cements containing 1.5 g or 2 g, with Palacos® R+G consistently yielding larger

residual inhibition zones than Simplex® T. This suggests that both the antibiotic dose and the cement matrix influence long-term release behaviour. Proliferation assay results at early time points also reflected clear dose-dependent suppression of VRE, confirming that daptomycin at higher concentrations effectively inhibits bacterial replication. These in-vitro findings translated consistently into in-vivo outcomes. In the infection prevention model, non-loaded and single-antibiotic reference cements showed no protective effect, whereas daptomycin-loaded samples markedly improved larval survival. The highest survival rates were observed for Palacos® R+G with 2 g daptomycin, followed by lower daptomycin doses. Simplex® T also benefitted from daptomycin addition but showed lower survival than Palacos® R+G for corresponding doses. CFU analyses confirmed these trends, with higher daptomycin concentrations leading to substantially reduced bacterial burden in both larval tissue and on implant surfaces. Similarly, in the infection-treatment model, only daptomycin-loaded samples improved survival or reduced CFU counts, with Palacos® R+G with 2 g daptomycin again achieving the strongest antimicrobial effect.

Taken together, our results demonstrate that daptomycin can be effectively incorporated into PMMA cement to achieve substantial antimicrobial activity against VRE, provided that sufficiently high loading (≥1.5 g) is used. However, this manual admixture impacts mechanical properties in a cement-specific manner. Palacos® R+G retains mechanical stability more reliably across all tests, whereas Simplex® T shows significant reductions, occasionally dropping below ISO thresholds. These findings underscore that PMMA cements are not interchangeable, that manual antibiotic addition requires careful evaluation of mechanical limits, and that higher daptomycin doses offer superior antimicrobial protection in both in-vitro and in-vivo models. Overall, the combination of Palacos® R+G with 2 g daptomycin demonstrated the most favourable balance between mechanical performance and antimicrobial efficacy, supporting its potential use in high-risk two-stage revision procedures involving VRE.

5.2. Contribution

The tests on antimicrobial activity (proliferation assay, inhibition zone testing) and mechanical stability (ISO 5833:2002, DIN 53435:2018) were performed and analysed by me. The testing in the in-vivo biofilm model *G. mellonella* were performed by me with the support of You Zhao and Gopala Krishna Mannala. You Zhao analysed the data derived from the biofilm model. Martijn Riool, Klaus-Dieter Kühn and I designed the research. I wrote the manuscript and reviewed it with all authors.

5.3. Reference

Humez, M., Zhao, Y., Mannala, G. K., Alt, V., Riool, M. & Kühn, K.-D. (2025). Evaluation of Daptomycin-Supplemented Antibiotic-Loaded Bone Cement for Treating Vancomycin-Resistant *Enterococcus faecalis* in the *Galleria mellonella* implant infection model. Orthopaedic Proceedings, 107-B (SUPP_12), 13-13.
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Orthopaedic Proceedings

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FULL ACCESS

EVALUATION OF DAPTOMYCIN-SUPPLEMENTED ANTIBIOTIC-LOADED BONE CEMENT FOR TREATING VANCOMYCIN-RESISTANT ENTEROCOCCUS FAECALIS IN THE GALLERIA MELLONELLA IMPLANT INFECTION MODEL

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Cite this

Abstract

Aim

Periprosthetic infections caused by vancomycin-resistant pathogens, such as vancomycin-resistant *Enterococcus faecalis* (VRE), represent a major clinical challenge. Daptomycin can be incorporated into polymethyl methacrylate (PMMA) cement spacers. This study aimed to utilize the *in vivo Galleria mellonella* larvae implant infection model to determine the optimal dosage of daptomycin in different PMMA cements and evaluate its efficacy.

Method

Daptomycin (1 g or 2 g) was added to different PMMA cements (Cement 1 containing 1 g tobramycin; Cement 2 containing 0.5 g gentamicin), to produce bone cement implants. Control groups included non-loaded Cement 1 and Cement 2, as well as Reference 1 and Reference 2, without antibiotics. The test specimens were implanted into larvae of the greater wax moth (*G. mellonella*), followed by infection with 250 CFU of VRE DSM13591 after 1 hour. The survival of the larvae was monitored over time, and bacterial numbers in the larval tissue and on the implant surface were determined after 24h. These experiments were completed with *in vitro* proliferation assay and inhibition zone testing. Mechanical stability was measured according to ISO 5833 and DIN 53435.

Results

Survival and bacterial burden analysis demonstrated that the addition of 1 g or 2 g of daptomycin was effective in preventing VRE infections. There were significantly improved larval survival and reduced bacterial numbers in both the larval tissue and on the implant surface. In contrast, Cement 1 and Cement 2 alone did not enhance larval survival and showed outcomes comparable to the antibiotic-free control groups. The addition of daptomycin was found to be effective in inhibiting bacterial growth when compared to the references, with the largest inhibition observed for Cement 2 (2 g daptomycin). On day 42, sufficient bacterial growth inhibition was observed only with the addition of 2 g daptomycin. These results correlate with those from the proliferation assay. Adding more than 1.5 g daptomycin resulted in a reduction of mechanical strength, with Cement 1 no longer meeting the ISO standard for four-point bending strength.

Conclusions

The incorporation of daptomycin into ALBC successfully prevented VRE infections in an *in vivo* model. These findings emphasize the potential of the *G. mellonella* implant infection model as a valuable tool for evaluating the efficacy of ALBC against multidrug-resistant bacteria, thereby accelerating pre-clinical research and advancing strategies to combat PJI.

We observed differences in mechanical stability and antimicrobial efficacy between daptomycin-loaded Simplex Tobramycin and Palacos R+G with increased stability and bacterial growth reduction for the latter one.

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IV. Discussion

1. Feasibility confirmed for a daptomycin-loaded acrylic bone cement

In view of the rising incidence of vancomycin-resistant pathogens in PJI, the clinical demand for a PMMA cement incorporating daptomycin is increasing (Markwart et al., 2019; Shariati et al., 2020). At present, no commercially available acrylic bone cement contains daptomycin, requiring orthopaedic surgeons to manually prepare such formulations. This practice places the surgeon in the position of a de facto legal manufacturer and necessitates explicit patient consent due to the off-label use. Moreover, antibiotic release kinetics from these admixed preparations are inherently non-standardised. Currently, clinicians lack an ISO-compliant and biomechanically validated cement option capable of reliably targeting MDR Gram-positive bacteria.

In our investigations, only formulations containing 1.0 g or 1.5 g daptomycin combined with 0.5 g gentamicin achieved complete inhibition of MRSE, MRSA, VRSA, and VRE strains, whereas 0.5 g daptomycin alone was insufficient, consistent with previous findings reported by Eick et al. (2017). These results indicate that the threshold concentration required for dependable antimicrobial coverage against vancomycin-resistant bacteria exceeds 1 g per 40 g of cement powder. Additionally, we identified a synergistic antibiotic elution effect when daptomycin was combined with gentamicin, particularly at higher daptomycin concentrations. The early burst release observed in our elution profiles aligns with established release characteristics described by Kühn (2014) and is comparable to the behaviour documented for the reference cement Copal® G+V.

These findings underscore a broader principle in DALBCs: the strategic combination of a broad-spectrum aminoglycoside antibiotic, such as gentamicin, with a second antibiotic possessing a complementary mechanism of action. This can substantially enhance antimicrobial performance. While aminoglycosides provide solid base coverage, their efficacy is increasingly limited against resistant staphylococci. Incorporating a second agent, in our case daptomycin, not only broadens the antimicrobial spectrum but also enables synergistic or additive effects that elevate local antibiotic concentrations beyond levels achievable SALBCs (Malhotra et al., 2018). Such enhanced elution is particularly relevant for overcoming the high MBEC/MBIC typical for PJI. Moreover, combining two antibiotics reduces the likelihood of selecting for resistant subpopulations, an important consideration when treating MDR staphylococci. Together, our microbiological and elution data support the concept that DALBCs can provide more reliable and robust local antimicrobial activity, especially when targeting resistant or biofilm-forming bacteria.

1.1. Antimicrobial efficacy and elution dynamics

The use of DALBC is well established within the orthopaedic community for special arthroplasty procedures which are associated with higher infection risks. This reflects the previously demonstrated benefits of combining two antimicrobial agents in the cement (Abdel et al., 2019). Due to the broader antibacterial spectrum, the synergistic antibiotic elution effects, and the reduced likelihood of resistance development (Sanz-Ruiz et al., 2020; Tyas et al., 2018). Gentamicin, with its broad activity also against Gram-negative pathogens, represents an appropriate partner for daptomycin, which exhibits potent activity primarily against Gram-positive cocci. Non-appropriate antibiotic drug combinations and wrong dosages may increase the risk of treatment failure during revision procedures. Given the increasing prevalence of VRE and VRSA in PJIs, an effective local antibiotic delivery strategy offers substantial therapeutic benefit in addition to the systemic antibiotic use. Systemic therapies are limited by toxicity (e.g., linezolid) and high local MIC requirements (e.g., daptomycin dosing of 8–12 mg/kg in VRE bacteraemia), whereas local antibiotic delivery enables high antimicrobial concentrations with minimal systemic exposure. Clinical case evidence also supports successful use of daptomycin for VRE bone and joint infections, including patients with linezolid intolerance. Comparisons in PJI management further suggest that daptomycin may be associated with fewer late relapses than vancomycin in certain cohorts (La & Kim, 2022).

Antonello et al. (2022) conducted a systematic literature review summarising current insights into the synergistic elution characteristics of daptomycin. The combination of daptomycin and gentamicin has been evaluated in 16 studies, predominantly focusing on systemic administration. Only one study reported an antagonistic elution pattern, noting a delayed bactericidal effect of daptomycin against MRSA when combined with gentamicin; this observation was not reproducible in our experiments (La Plante & Woodmansee, 2009). In contrast, our in-vitro findings demonstrate that this antibiotic combination reliably reduces bacterial activity and overall bacterial burden. It must be acknowledged, however, that local application via PMMA cement results in substantially higher antibiotic concentrations than those achieved via systemic use. Numerous other investigations also support the notion of a synergistic effect of gentamicin and daptomycin: Credito et al. (2007) reported enhanced antimicrobial activity against MRSA and VRSA at concentrations below the respective MICs, and Tafin et al. (2011) demonstrated that combining both agents increased the cure rate of infections in a foreign-body infection model to 55%, compared with 25% for daptomycin alone and 50% for gentamicin alone. The setting here with guinea pigs reflected much better the clinical reality than pure in-vitro tests in the laboratory. In our in-vivo *Galleria mellonella* infection model, we could replicate these experiences by showing that only the combination of gentamicin and daptomycin resulted in a further and measurable reduction of bacterial burden in the larvae. This dual-antibiotic regimen was more effective than the combinations of linezolid with gentamicin or linezolid with rifampicin, and the most pronounced decrease in bacterial load for *E. faecalis* and VRE was observed with the daptomycin and gentamicin combination (Luther et al., 2014). Our data similarly support that PMMA cement containing 1.0 g or 1.5 g daptomycin combined with 0.5 g gentamicin consistently inhibits clinically relevant resistant pathogens, including MRSA, MRSE, VRSA, and VRE. In contrast, cement containing only 0.5 g daptomycin was insufficient to inhibit bacterial growth, indicating that this concentration does not reliably achieve MIC thresholds for highly resistant Gram-positive species. Pharmacokinetic properties may further explain discrepancies between systemic and local application. Daptomycin demonstrates limited tissue distribution, largely due to its high plasma protein binding capacity of approximately 92% (Dvorchik & Damphousse, 2005; Estes & Derendorf, 2010). Despite its long half-life of approximately eight hours, systemic administration may not achieve sufficiently high local concentrations around implants to eradicate biofilm-embedded bacteria (Dvorchik & Damphousse, 2005). Additionally, daptomycin's activity is dependent on the presence of calcium ions, suggesting that local calcium availability in peri-implant tissue could further potentiate the antimicrobial effect of daptomycin-loaded PMMA cement (Gray & Wenzel, 2020).

The first experiences with the local application mode of daptomycin reported by Rouse et al. (2006) showing its elution from self-made PMMA beads in a rat osteomyelitis model. In the clinical setting, Cortes et al. (2013) presented a case report in which they showed the successful eradication of MRSA in their two-stage PJI hip treatment approach using a daptomycin- and gentamicin-loaded PMMA spacer. Considering daptomycin's concentration-dependent bactericidal activity, our findings indicate that a loading of 1.0–1.5 g per 40 g PMMA represents a clinically meaningful and effective range for local delivery. This aligns with the results of Eick et al. (2017), who showed highest antimicrobial activity of 1.5 g daptomycin plus 0.5 g gentamicin in inhibition zone testing against *S. aureus* and *E. faecalis*.

Daptomycin is not the only antibiotic that can be considered for the management of MRSA and VRE associated PJIs. Another agent that has recently gained attention is tigecycline, a last-line antibiotic with broad activity against MDR pathogens. As demonstrated by Abramowicz et al. (2024) tigecycline can be incorporated into PMMA bone cement at concentrations of 0.5 g and 1.0 g without compromising its mechanical stability. The authors also reported that combining tigecycline (0.5–1.0 g) with 0.5 g gentamicin enhances antibiotic elution, suggesting a synergistic release profile. However, only the formulation containing 1.0 g tigecycline in combination with 0.5 g gentamicin produced sustained antimicrobial activity for up to 28 days. In contrast, our own, yet unpublished, data (III. Publications, Chapter 5., pp. 125-128) indicate that the combination of daptomycin and gentamicin results in sustained and clinically relevant antibiotic elution for at least 42 days. This prolonged release profile suggests that daptomycin-loaded PMMA cement may be better suited for use in temporary spacers during two-stage revision procedures.

For spacer applications, the enhanced early elution of daptomycin is clinically advantageous, ensuring therapeutic concentrations during the critical early postoperative period. In contrast, the initial burst release followed by a decline in vancomycin elution from vancomycin-loaded PMMA cement (e.g., Copal® G+V) may fail to maintain local vancomycin levels above MIC for VRE and VRSA, which often exhibit highly elevated MIC thresholds (MIC VRSA ≥ 16 µg/mL; MIC VRE ≥ 32 µg/mL) (Shariati et al., 2020). The elution characteristics of an antibiotic load of 0.5 g gentamicin combined with 1.5 g daptomycin provide a sustained advantage in maintaining local antibiotic concentrations above the required MIC. These in-vitro findings confirm exactly the results from the investigations in the in-vivo biofilm model *Galleria mellonella* where this combination and this antibiotic load in the cement showed the highest antimicrobial efficacy (IV. Discussion, Chapter 5., pp. 151-152). Our in-vitro and in-vivo experiments reinforce the hypothesis of synergistic antimicrobial effects of daptomycin with gentamicin and provide a valuable guidance for optimal antibiotic dosing. Daptomycin elutes more effectively at a dose of 1.5 g in PMMA and shows mutually enhanced antibiotic release from the carrier substance when it is combined with gentamicin. Similar conclusions were drawn by Luther et al. (2014) in the *Galleria mellonella* model, although their design assessed the systemic and not the local antibiotic application delivery mode.

1.2. Mechanical stability and handling properties

An important prerequisite for clinical applicability is the fulfilment of the mechanical parameters defined by ISO 5833:2002. All daptomycin-loaded cement formulations (0.5 g, 1.0 g, and 1.5 g) remained fully compliant with the required thresholds for bending strength, bending modulus, and compressive strength. This outcome is expected, as the total antibiotic load in these formulations (≤2 g per 40 g PMMA) remains well below the widely accepted upper limit of approximately 10% wt.%, above which the mechanical properties of ALBCs are known to deteriorate (Kühn, 2014). While DIN 53435:2018 bending and impact strength tests are not required for PMMA bone cements, unlike for dental acrylics, they nonetheless offer useful information on the mechanical characteristics of various ALBC formulations and help build a more complete picture of their performance.

Mechanical stability is particularly relevant in the context of such a clinical practice, where ≥4 g vancomycin is admixed into PMMA spacers for resistant staphylococcal or enterococcal infections exceeding the recommendation of max 10% wt.% (Lunz et al., 2022; Lunz et al., 2023). Once a spacer no longer meets ISO 5833:2002 mechanical stability requirements, particularly bending strength, its risk of fracture during motion increases significantly, posing a direct hazard to the patient (Lunz et al., 2022; Malhotra et al., 2018). Furthermore, when surgeons manually admix antibiotics, they assume the legal role of the manufacturer and become responsible in the event of mechanical failure or fractures. Thus, non-compliant spacers present dual risks: legal implications for the surgeon and substantial health risks for the patient. The present findings align with earlier analyses demonstrating that PMMA maintains adequate mechanical integrity when moderately loaded with antibiotics, whereas higher drug concentrations lead to measurable weakening (Kühn, 2014). To emphasise the clinical importance of this issue, an easy to capture "traffic-light labelling" was used in another publication with the purpose to raise awareness among surgeons as well as to illustrate which antibiotic combinations or quantities may be considered "risky" (Humez et al., 2025).

Notably, the mechanical performance of the daptomycin-loaded cements was comparable to, or in some cases superior to, established clinical reference products such as Palacos® R+G and Copal® G+V, with the 1.0 g daptomycin addition exhibiting the highest bending modulus. Based on an exclusively mechanical point of view one may argue that the combination of 1.0 g daptomycin with 0.5 g gentamicin would be the ideal choice. However, since clinical decision-making requires balancing optimal mechanical stability with the need for robust antimicrobial efficacy, we would recommend a dose of 1.5 g daptomycin combined with 0.5 g gentamicin as guidance for the clinician. In clinical practice, the primary objective of PMMA spacer application is the reliable eradication of infection. Therefore, the surgeon must ultimately determine which antibiotic combination and dosage are most appropriate for the individual case, ideally in close collaboration with microbiologists and hospital pharmacists. This interdisciplinary approach ensures that both mechanical considerations and antimicrobial requirements are adequately addressed, supporting the selection of a formulation that delivers the highest therapeutic benefit.

The addition of 1.5 g daptomycin resulted in a slightly accelerated setting time and moderately prolonged doughing time. However, both parameters remained within clinically acceptable ranges and should not negatively affect intraoperative usability of such a cement. Overall handling performance was comparable to that of established reference cements such as Copal® G+V and Palacos® R+G. This is highly relevant because substantial variations in handling characteristics can interfere with the critical timing of cement application when cement is applied for fixation (Kühn, 2014). However, when the cement is used to prepare a PMMA spacer, handling behaviour becomes considerably less important.

For implant fixation, the waiting or sticky phase marks the critical period in which PMMA changes from a sticky to a mouldable consistency, making it highly important for proper handling. Adequate waiting time prevents unintended cement migration into undesired anatomical compartments (e.g., spinal canal, soft tissues) and facilitates controlled application. The working time is the period during which the cement can be manipulated. A sufficiently long working phase ensures that surgeons can achieve accurate component positioning without premature curing, which is essential for forming a stable and homogeneous cement–bone interface, particularly in total hip and knee arthroplasty. The setting time defines the point at which the cement has hardened sufficiently to tolerate mechanical loading. Predictable setting behaviour allows surgeons to resume manipulation of the limb or spacer without risking micromotion and helps to avoid thermally induced bone damage due to the exothermic polymerisation of PMMA (Paul & Kühn, 2023).

1.3. Impact of sterilisation method on daptomycin activity

Sterilisation of PMMA cement powder as well as antibiotics can influence significantly the quality and properties of both, PMMA and antibiotics (Lewis & Mladsi, 1998; Paul & Kühn, 2023; Paul et al., 2023). Our investigations demonstrate that gamma irradiation preserves the antimicrobial activity of daptomycin, whereas sterilisation with ethylene oxide significantly diminishes its effectiveness. Daptomycin-containing PMMA cement sterilised with ethylene oxide exhibited a markedly reduced ability to inhibit bacterial growth compared with non-sterilised or gamma-sterilised cement. This finding has direct practical relevance: industrial production of daptomycin-loaded ALBCs would be expected to rely on gamma irradiation to maintain reliable bioactivity.

Ethylene oxide sterilisation exerts its microbicidal effect through alkylation of nucleophilic functional groups, including amino, hydroxyl, thiol, and carboxyl groups, present on biological molecules. The formation of alkylated adducts can impair the biological function of susceptible compounds (CDC, 2023c). Because daptomycin contains multiple nucleophilic amino acid residues and a structurally complex cyclic lipopeptide core, it is highly plausible that ethylene oxide modifies its chemical structure, thereby reducing its antimicrobial potency (Shintani, 2017). In contrast, gamma irradiation sterilises via ionising radiation that generates free radicals, which primarily damage microbial DNA and proteins rather than the antibiotic itself. Although gamma irradiation is known to degrade certain pharmaceutical compounds and can reduce the molecular weight of PMMA (approximately by half), leading to diminished mechanical stability (Kühn, 2014; Lewis & Mladsi, 1998) it remains the more suitable option in this context compared with ethylene oxide. Gamma irradiation may influence certain handling properties of PMMA cement, which is disadvantageous for surgical use, but it is still preferable when preservation of antibiotic activity is a priority. For these reasons, commercially available PMMA cements such as Copal® G+V are sterilised using gamma irradiation rather than ethylene oxide accepting a slight change in handling properties.

1.4. Limitations and opportunities for further research

Despite these encouraging findings, several important limitations must be acknowledged. More advanced biofilm models and extended (>14 days) elution studies are needed to better approximate the prolonged implantation intervals characteristic of clinical spacer use. Furthermore, clinical evidence remains limited and relies primarily on case reports, retrospective clinical series, and in-vitro investigations. This underscores the need for further research. The potential influence of pharmaceutical excipients, such as the sodium hydroxide present in Cubicin®, on polymerisation behaviour and drug release has not yet been quantified and warrants systematic evaluation. It should also be noted that the incorporation of daptomycin was evaluated in only a single PMMA cement formulation (Palacos®); therefore, its elution profile and antimicrobial activity may vary substantially when combined with other PMMA cement matrices (e.g. Simplex®). Additionally, given the calcium-dependent mechanism of action of daptomycin, future work may explore whether targeted modification of PMMA formulations with calcium ions could enhance local antimicrobial efficacy. Finally, validation of these in-vitro results using in-vivo biofilm models would be a critical next step in confirming the clinical relevance of this antibiotic combination.

Another important consideration is the variability in elution and mechanical behaviour among commercially available PMMA cements. Palacos® R+G is widely regarded as a cement with a high antibiotic elution profile, and therefore the release characteristics observed in this study cannot be directly extrapolated to other cement formulations with lower elution capacity. The same applies to mechanical performance: increasing amounts of daptomycin progressively reduce mechanical stability in Palacos® cements, but the magnitude of this effect may differ in other cement matrices due to variations in composition and polymerisation behaviour. Consequently, direct translation of our findings to other PMMA cements should be approached with caution, and future studies should systematically compare multiple cement types to determine how matrix-specific factors influence both elution efficiency and mechanical integrity

2. Evidence-based recommendations for manual admixing of anti-infective agents

In clinical scenarios where commercially available ALBCs do not provide adequate antimicrobial coverage for the identified pathogen, orthopaedic surgeons are required to manually admix antimicrobial agents into PMMA powder. This practice, although widely adopted, introduces substantial variability in the resulting cement's mechanical, chemical, and microbiological properties. As previously noted, this approach also constitutes an off-label use, meaning that surgeons assume full legal liability, as they effectively become the manufacturer of the final product. Guidance on antibiotic selection, dosing, and pathogen coverage for fixation and spacer cement is provided by the Pro-Implant Foundation in its established pocket guide (PIF, 2023). However, surgeons also require clearer evidence whether clinically relevant differences exist between pre-loaded and manually loaded ALBCs, how the admixing procedure should be performed (e.g. is dry-mixing necessary or not), and how reliably the manually prepared cement elutes antibiotics. To address these uncertainties, Table 2 of my publication, Humez et al. (2025), uses a traffic-light classification system to highlight critical considerations during admixing, accompanied by explanatory remarks and primary references for readers seeking additional detail. In this system, green denotes an uncomplicated admixing process, mechanical properties that remain within ISO limits, and evidence of sufficient and synergistic antibiotic elution. Yellow indicates that admixing is more demanding, for example, requiring careful antibiotic selection (e.g. fosfomycin sodium instead of fosfomycin – that is not available in sterile powder form), and that a reduction in mechanical properties approaching the ISO threshold as well as diminished synergistic elution must be expected. Red denotes a complex and often impractical admixing process, typically due to the large powder volumes required when agents contain substantial filler material (e.g. voriconazole, amphotericin B), resulting in mechanical properties that fall below ISO standards and significantly impaired antibiotic elution compared with commercially available DALBCs.

2.1. Effects of dry mixing on mechanical stability

The manual incorporation of vancomycin into Palacos® R + G resulted in cement formulations whose ISO compliant bending strength and bending modulus remained comparable to those of the corresponding industrially preloaded references, irrespective of whether an additional dry-mixing step was performed. Only the DIN impact strength exhibited reductions, most prominently in specimens prepared using the Optivac® mixing system. These observations support previous evidence suggesting that the admixing of antibiotic powder does not inherently diminish mechanical stability when the antibiotic is properly fractionated and uniformly mixed within the polymer matrix. They also underscore the critical role of meticulous powder incorporation, as structural inhomogeneities, such as large crystalline aggregates or incomplete distribution, may introduce mechanical weak points within the PMMA network (Kühn et al., 2017). However, the fact that dry mixing did not produce any measurable mechanical benefit challenges the belief that additional homogenisation inside the mixing cartridge improves the quality of the final cement (Zahar & Hannah, 2016). The present findings clearly indicate that this assumption is not supported by experimental data. One practical motivation for mixing the antibiotic directly in the PMMA powder contained in the cartridge was, of course, to reduce preparation time by avoiding the additional step of premixing the powder in a separate bowl and subsequently transferring it into the mixing cartridge.

2.2. Impact of dry mixing on antibiotic elution

Across all tested specimens, additional dry mixing of manually added vancomycin powder into the PMMA cement powder did not result in any measurable improvement in vancomycin elution. In fact, the reference cement Copal® G + V demonstrated a consistently higher burst release (647 µg/specimen) on day 1 than any manually admixed formulation, including those subjected to supplementary dry mixing. These findings support two clinically relevant conclusions: first, that dry mixing does not improve antibiotic availability or alter the pharmacokinetic release profile in a meaningful way; and second, that industrially manufactured ALBCs remain superior, particularly when an early high concentration release is required for effective biofilm disruption. Notably, the elution differences of manually added vancomycin were most pronounced on day 1 and day 7, underscoring that manual preparation rarely achieves the consistency and reproducibility characteristic of industrially optimised ALBCs.

2.3. Dry mixing as potential cause of particle abrasion

Perhaps the most consequential finding of this investigation was the identification of abrasive wear occurring inside plastic mixing cartridges during dry mixing. Microscopic examination revealed clear evidence of damage, including scratch marks on the inner cartridge walls, plastic particles embedded within or intermixed with the PMMA powder, and material detachment from the mixing paddles. These effects that were particularly pronounced when using the Optivac® mixing system. This phenomenon is mechanistically plausible, as PMMA cement mixing devices were not originally engineered for the homogenisation of dry powders, especially powders with abrasive or crystalline morphology. Their intended purpose is the controlled mixing of polymer powder with monomer liquid to produce a homogeneous cement dough (Kühn, 2014). Under normal operating conditions, a liquid phase is always present in the mixing chamber, and minor powder movement is not considered mechanically critical. Abrasion arises only when vigorous mechanical agitation is applied in the absence of a liquid, combined with paddle rotation and the presence of antibiotics with a more crystalline or abrasive structure. Over time, this motion results in measurable removal of plastic material from the inner cartridge surface, and the resulting debris can remain entrapped within the final cement dough.

This observation has important biological implications. Plastic debris introduced into the joint space may provoke macrophage activation and subsequent osteolysis, a pathway well-documented in the context of polyethylene wear particles (Jiang et al., 2016). Osteolysis around joint implants represents a biologically mediated response to particulate debris generated at articulating or interfacial surfaces (Ollivere et al., 2012). Even modern, wear resistant materials, including polyethylene, PMMA cement, metal alloys, and ceramics, produce micro abrasive particles during normal joint motion. These particles, often present in the submicron range, are highly biologically active and cannot be enzymatically degraded by host tissues. Instead, macrophages attempt to phagocytose the particles, and when phagocytic capacity is exceeded, a macrophage response occurs, characterised by the release of inflammatory mediators such as tumour necrosis factor-alpha (TNF-α), interleukin (IL-1β), IL-6, prostaglandin E2, and receptor activator of nuclear factor b ligand (RANKL) (Jiang et al., 2016). This cytokine environment promotes osteoclast differentiation and activation, leading to progressive periprosthetic bone resorption. As osteolysis advances, implants may lose fixation, and the resulting micromotion accelerates further particle generation, ultimately creating a self-reinforcing cycle that culminates in aseptic loosening, that is most common cause of late revision arthroplasty (Ollivere et al., 2012).

We hypothesise that plastic debris generated by dry mixing could elicit a similar inflammatory response, with biological activity likely influenced by particle size (Gelb et al., 1994). Early studies already demonstrated that PMMA cement wear particles can trigger macrophage activation and osteolysis. This concern is particularly relevant for articulating PMMA spacers, such as those used in two-stage knee revisions, where the femoral and tibial components articulate directly against one another. Gelb et al. (1994) established in a rat model that PMMA particle exposure induces a robust inflammatory response, and importantly, that the magnitude of this response depends on particle size distribution.

Taken together, these findings indicate that additional dry mixing does not enhance homogeneity, mechanical integrity, or antibiotic release, while potentially introducing clinically meaningful risks through the generation of biologically active plastic debris. It should be noted, however, that such debris becomes clinically relevant predominantly in scenarios where the cement-implant interface has already loosened. Although abrasion particles may theoretically adhere to the cement surface during late implant failure, this is expected to occur only infrequently. Nonetheless, even this low probability further underscores the importance of avoiding unnecessary manipulation steps that could increase the risk of particle formation without providing any therapeutic benefit.

2.4. Evidence-based recommendations for manual admixing

2.4.1. Do not dry mix antibiotic powder in the cartridge mixing system

Available evidence shows that dry mixing offers no mechanical or microbiological benefit and instead introduces a measurable risk of plastic abrasion within the mixing cartridge as explained in Chapter 2.3. (IV. Discussion, p. 137. Therefore, dry homogenisation of antibiotic powder in cement mixing cartridges should be avoided.

The fractionated mixing technique in an open bowl, combining polymer powder and anti-infective substance in small portions and homogenising larger crystals with a sterile mortar, produces a uniform admixture without causing cartridge abrasion. This method represents the safest and most controlled approach when manual antibiotic incorporation is required. It should therefore be considered the standard procedure for preparing manually loaded ALBCs. Once the mixture is homogenised in the bowl, it can be transferred to the cartridge system, and cement preparation can proceed according to the manufacturer's instructions.

2.4.2. Use antimicrobial agents in sterile powder form

The sterility, particle size distribution, and brand specific characteristics of antimicrobial powders have substantial influence on both elution kinetics and mixture homogeneity. For this reason, only sterile powders – if available - intended for parenteral use should be employed, and surgeons must also consider known differences in antibiotic quality, elution performance, and the true amount of active substance contained within various commercial powders. The addition of sterile liquid antibiotics is contraindicated because aqueous solutions interfere with PMMA polymerisation and compromise cement integrity. As demonstrated by Hetzmannseder et al. (2021) incorporating liquid antibiotics weakens both the mechanical properties and antimicrobial performance of PMMA cement. Antimicrobial liquids are chemically incompatible with the liquid monomer cement phase and therefore fail to integrate homogeneously; the result is poor mixing, formation of pores and structural defects, and a consequential reduction in compressive strength, bending strength, and impact resistance, especially when liquid antibiotics are added to the cement powder or dough. Simultaneously, antimicrobial efficacy becomes unreliable because the antibiotic is distributed unevenly and may undergo thermal degradation during polymerisation. In contrast, powder formulations maintain consistent mechanical performance and predictable release profiles, making them the better option (Hetzmannseder et al., 2021).

Furthermore, powder quality varies widely among manufacturers. Lee et al. (2016) showed that different vancomycin brands exhibit markedly different release profiles from PMMA, sometimes differing by a factor of five, where sterile pharmaceutical vancomycin achieved substantially higher elution than lyophilised formulations. This variability has direct clinical relevance, particularly regarding the ability to maintain antibiotic concentrations above MIC thresholds. In the most unfavourable scenario, a poorly eluting antibiotic is combined with a cement matrix that inherently restricts diffusion, resulting in subtherapeutic release toward the end of the spacer interval. Such insufficient elution could facilitate recolonisation of the spacer, prevent the complete eradication of infection, or even aid the selection of a-priori resistant organisms. This difference in elution patterns in varying vancomycin brands may arise as generic formulations contain variable levels of impurities and degradation products, such as crystalline degradation product-1 (CDP-1) (Lee et al., 2016). These substances may accumulate within the cement during mixing, altering internal porosity and microstructure, including pore number and pore geometry, thereby affecting the mobility and solubility of vancomycin within PMMA. Analogous to DALBCs where additional soluble components increase void formation and enhance elution, impurities in certain vancomycin brands may unintentionally modify diffusion pathways. Finally, when selecting antimicrobial powders for manual admixing, fine powders should be preferred over crystalline or coarse formulations, as the latter are more difficult to incorporate homogeneously and may further compromise mixture quality.

2.4.3. Prefer commercially available ALBCs

Industrial and approved ALBCs provide consistently more reliable performance compared with manually admixed formulations, offering high material homogeneity, validated sterility, optimised release kinetics, and omit potential mixing failures. For these reasons, commercially available ALBCs should be considered as primary option whenever their antimicrobial spectrum aligns with clinical requirements. This has previously been demonstrated by Ferraris et al. (2010) who directly compared the commercially premixed Palacos® R+G with manually admixed gentamicin-containing cements. Their in-vitro analysis showed that Palacos® R+G produced slightly larger and more homogeneous inhibition zones, indicating a more consistent and reliable antibacterial effect than manually prepared cements.

Despite these findings, the manual incorporation of antibiotics is repeatedly discussed as a potentially more cost-efficient alternative under economic pressure. Schwarz et al. (2021) highlighted several core reasons why many clinicians remain hesitant to rely on commercially ALBCs among them is the fear that commercially available ALBC quickly fall below MIC thresholds or that DALBC are not approved by the FDA for the routine use in e.g. high-risk patients in the U.S. In the U.S. this resulted in a decline in the usage of commercially ALBC from 90% in 2006 to 34.5% in 2017 (Schwarz et al., 2021). However, decisions regarding the choice of ALBC should be guided primarily by clinical efficacy and patient outcomes, rather than cost considerations alone. When provided in pre-filled cartridge mixing systems such as Copal® G+C pro, these products also reduce handling steps and thereby lower the risk of intraoperative contamination resulting in a reduced PJI risk (SAR, 2023).

The use of DALBCs can enhance release through synergistic antibiotic elution, however, not all combinations behave synergistically. In certain pairings, contrasting physicochemical interactions result in reduced release (Kühn, 2014). Of particular concern is the antagonistic interaction between gentamicin and fosfomycin in PMMA cement. Multiple studies demonstrate that the concurrent incorporation of these agents significantly decreases gentamicin elution, especially when fosfomycin is added as a non-sterile pharmaceutical formulation rather than as a pure sterile powder (Cara et al., 2022; Pruekprasert & Tunyapanit, 2005; Yuenyongviwat et al., 2017). Fosfomycin appears to alter the physicochemical environment within PMMA cement, disrupting hydrophilic diffusion channels and limiting gentamicin mobility. This antagonistic effect has been observed specifically in *Staphylococcus aureus* models, where combined gentamicin and fosfomycin demonstrate lower antimicrobial activity than cements containing only gentamicin (Cara et al., 2022). Because these alterations may lead to subtherapeutic local concentrations and risk insufficient pathogen suppression, current recommendations advise against this combination in routine PMMA admixture.

The amount of active ingredient and its true bioactive fraction, described by the activity coefficient, are also critical considerations (Kühn, 2014). This is especially relevant for antimycotics such as amphotericin B and voriconazole, where the actual active fraction is low, requiring large powder quantities for clinically relevant dosing. Both antimycotics are provided as sterile powders intended for infusion solutions, and their formulations contain a relatively high proportion of excipients. These high powder loads markedly reduce mechanical stability. For example, manual addition of voriconazole to Palacos® R+G decreases ISO compressive and bending strength toward or below the ISO threshold, particularly at higher dosages (Krampitz et al., 2023). Similar reductions in bending strength were observed for amphotericin B (Frank et al., 2025). Importantly, these findings extend beyond antimycotics: unevenly homogenised antibiotic particles weaken PMMA cements structurally, and certain antibiotic choices negatively influence mechanical stability (Paz et al., 2015).

In cases where it is necessary to admix high volumes of antimicrobial powder, for example when antimycotics must be incorporated, low-viscosity PMMA cements are generally the preferred choice. Their lower viscosity provides superior wetting properties and facilitates more uniform dispersion of crystalline or voluminous powders, reducing the likelihood of particle agglomeration and heterogeneity (Krampitz et al., 2023). Antimicrobial formulations containing excipients or fillers can alter the cement microstructure, thereby affecting key mechanical parameters such as bending modulus and impact strength. Consequently, when high-dose admixture cannot be avoided, low-viscosity cements with extended handling times should be selected to optimize powder distribution, whereas cement brands known to exhibit mechanical sensitivity to large antibiotic additions should be avoided.

Surgeons often add high amounts of vancomycin to PMMA spacers to ensure that local antibiotic concentrations exceed the MIC (Lunz et al., 2023). However, this practice is frequently driven more by perceived safety than by evidence-based guidelines (Warwick et al., 2024). Excessive vancomycin loading can compromise the mechanical integrity of the cement, an effect also supported by our own findings, and may expose patients to an increased risk of renal impairment due to systemic absorption of the antibiotic. To overcome these negative side effects, alternative strategies have been explored to enhance local antibiotic delivery without negatively affecting cement stability. Amerstorfer et al. (2017) investigated the concept of a superficial vancomycin coating on PMMA spacers. In their approach, PMMA cement containing vancomycin was moulded into a spacer, implanted before complete polymerisation, and subsequently coated with additional vancomycin powder manually pressed onto the surface. This method aims to achieve high initial local antibiotic concentrations while avoiding the mechanical weakening associated with excessive vancomycin doses. Labmayr et al. (2021) demonstrated that superficial vancomycin coating significantly enhanced antibiotic elution during the first 24 hours, without compromising mechanical stability, while simultaneously maintaining the long-term release characteristics typical of ALBC spacers.

In their comparative evaluation of ciprofloxacin- and vancomycin-loaded PMMA cement, Gandomkarzadeh et al. (2020) demonstrated that increasing antibiotic content leads to a dose-dependent deterioration of mechanical performance, primarily due to higher porosity within the cured cement matrix. These findings are consistent with our recommendation not to exceed 10% antibiotic content relative to cement powder. Even at low antibiotic loads, mechanical properties declined, although ISO requirements were still met for formulations containing 2.5 wt.% antibiotic. Higher concentrations (5–10 wt.%) produced substantial reductions in both compressive and bending strength, with vancomycin, likely due to its larger particle size and higher molecular weight, causing more pronounced mechanical weakening than ciprofloxacin at comparable loads. Porosity measurements confirmed a significant increase in void fraction for both ALBCs, correlating with reduced mechanical stability. While Gandomkarzadeh et al. (2020) attributed part of the mechanical decline to increased water uptake, this interpretation remains debated, as water absorption can, depending on the cement system, also increase ductility and thereby partially improve certain mechanical responses. Nevertheless, aging studies consistently demonstrate a characteristic decline in mechanical properties during the first 14 days followed by partial recovery, identifying the second week as the most representative time point for mechanical assessment (Dunne et al., 2008; Gandomkarzadeh et al., 2020; Lewis et al., 2010).

A recurring limitation in existing studies is the predominant focus on ISO compressive strength, which is relatively insensitive to clinically relevant failure mechanisms. Compressive strength often remains unchanged despite significant microstructural compromise. In contrast, ISO bending strength is a far more sensitive and clinically meaningful parameter (Egger et al., 2024; Kühn, 2014). Bending strength reflects the actual mechanical demands placed on PMMA spacers, which experience multidirectional bending loads, tensile stresses, shear forces, and cyclic fatigue rather than pure axial compression. PMMA is intrinsically strong under compression but comparatively weak in tension, making bending a more realistic predictor of fracture (Kühn, 2014). Variations in porosity, viscosity, and antibiotic distribution primarily influence bending properties, as demonstrated in Egger et al. (2024) where gentamicin loaded and unloaded cements maintained compressive strength but differed in porosity and elasticity. Therefore, ISO bending strength should be the primary metric for assessing spacer integrity.

In contrast to bending strength, bending modulus tends to increase upon admixture of certain antimicrobial agents, reflecting enhanced fluid uptake and associated increases in cement elasticity (Krampitz et al., 2023; Kühn et al., 2017). When considering mechanical performance over the typical 6–8-week spacer interval, higher antibiotic loads correlate with progressive reductions in bending strength, underscoring the need for conservative dosing (Krampitz et al., 2023; Lunz et al., 2022). In addition to mechanical risks, unevenly distributed active ingredients may also facilitate particle breakout. So, inhomogeneous distribution alters both elution kinetics and structural integrity, contributing to uncontrolled release patterns and increased risk for mechanical failure (Kühn et al., 2017).

Taken together, these considerations emphasize the importance of using commercially available ALBCs whenever possible. When manual admixing is necessary, strictly adhere to evidence-based recommendations such as those outlined by the PIF Pocket Guide, that is planned to be updated including our recommendations considering admixing procedure, mechanical properties and synergistic elution effect as shown in **Table 2** in our publication (III. Publications, Chapter 2., pp. 71-85).

2.4.4. Prepare acrylic bone cement spacers without vacuum

When preparing a temporary PMMA spacer, cement preparation without vacuum is recommended to increase the antibiotic elution (Egger et al., 2024). In contrast to fixation cement, where vacuum mixing is intentionally used to reduce entrapped air and porosity and thereby enhance long-term mechanical stability by preventing microcrack initiation, spacers are not intended to function as permanent load-bearing constructs (Gergely et al., 2016; Lewis, 2003). Their primary clinical function is local delivery of high antibiotic concentrations for biofilm eradication, not long-term mechanical fixation. A cement prepared without vacuum intentionally increases internal porosity and enlarges the surface area, which is essential because PMMA releases antibiotics mainly from its surface (van de Belt et al., 2000; Wang et al., 1993). Greater porosity therefore leads directly to increased elution capacity. Moreover, a more porous structure allows greater water uptake during the spacer interval, which enhances solute diffusion and further increases total antibiotic release. A scientifically important distinction is that mechanical requirements for spacers differ fundamentally from those for implant fixation. Vacuum mixed cement produces a stronger, denser matrix suitable for long-term stability, but this benefit is unnecessary for spacers, which typically remain in situ for only 6–12 weeks. Spacers are subjected primarily to short-term loading and are designed to tolerate only temporary functional stresses. As such, the transient reduction in mechanical strength resulting from cement mixing without vacuum is largely clinically acceptable and is outweighed by the substantial improvement in antimicrobial elution (Ajit et al., 2019).

Additionally, the elution profile of PMMA is logarithmic, with a desirable initial burst followed by a lower release phase. Non vacuum mixing supports this pattern by increasing surface-connected porosity, reducing diffusion path length, and enabling the formation of microchannels once the cement absorbs synovial fluid (Egger et al., 2024; Neut et al., 2003). In articulating spacer designs, extremely low porosity may also impede fluid penetration, reducing the reservoir effect that supports sustained elution.

2.5. Limitations and opportunities for further research

Although the discussion highlights the potential for abrasion derived plastic debris to trigger inflammatory reactions, the study did not include any biological assays; consequently, conclusions regarding the clinical relevance of these particles remain speculative in the absence of experimental validation. In addition, while microscopy provided visually compelling evidence of cartridge abrasion, no quantitative characterisation of the released debris, such as particle size distribution, particle count, or chemical composition, was performed. Quantitative particle analysis would be essential for understanding exposure levels. The elution experiments were conducted only over a period of seven days, whereas PMMA spacers typically remain in situ for more than 6 weeks, indicating that long-term elution kinetics should be evaluated. Furthermore, the investigation focused primarily on two commonly used mixing systems (Palamix® and Optivac®), even though additional cartridge mixing systems and cement matrices are available on the market. Notably, no biological assessment of the abrasion particles or their potential to induce macrophage activation, osteolysis, or other inflammatory pathways was undertaken.

Future studies should therefore include in-vitro macrophage stimulation assays, osteoblast/osteoclast culture models, and in-vivo studies to determine whether these particles elicit inflammatory cascades. In addition, extending mechanical and elution testing to a broader range of mixing systems and cement brands could be of interest.

3. Acrylic bone cement matrix influences antibiotic release profile

In clinical practice, PMMA cements are often regarded as interchangeable or as identical acrylic materials, an assumption reflected in the widespread perception that “cement is cement.” Consequently, even during spacer fabrication, limited attention is given to the underlying cement matrix, while the primary focus tends to be placed on the choice and quantity of antibiotics incorporated. However, antibiotic elution differs substantially between cement brands, and these differences are predominantly determined by the physicochemical composition of the respective polymer matrices. Despite the likelihood that such variations may influence clinical outcomes, particularly in situations where antibiotic concentrations released from spacers fall below the MIC for relevant pathogens, the number of studies systematically examining these effects remains limited. Moreover, although standardised testing approaches exist, they are not yet consistently established or uniformly applied. For this reason, we investigated the release kinetics and antibacterial efficacy of gentamicin from Palacos® R+G and tobramycin from Antibiotic Simplex® with tobramycin (Simplex® T), two of the most widely used cements globally (Leta et al., 2023). While these materials contain different aminoglycosides, gentamicin and tobramycin are structurally related and share a comparable mechanism of action as well as overlapping antimicrobial spectra, particularly against Enterobacterales and staphylococci (Brogden et al., 1976; Federspil et al., 1976; Fleischmann et al., 2020). Tobramycin is generally regarded as more potent against *Pseudomonas aeruginosa*, whereas gentamicin demonstrates stronger activity against staphylococcal species (Lode, 1998). Since *P. aeruginosa* accounts for only 3–5% of pathogens in PJIs, while staphylococci represent the predominant causative organisms, the clinical implications of this divergence are context dependent (Patel, 2023). By selecting *Escherichia coli* as one of the test organisms, both cements demonstrated limited antimicrobial activity over time, thereby minimizing the influence of spectrum-related class differences. The choice of antibiotics also reflects geographical preferences, as tobramycin-containing cements are more commonly used in the U.S. and Australia, whereas European practice more frequently relies on gentamicin-loaded cement formulations.

3.1. Standardised methodology for elution testing

HPLC remains the gold standard for quantifying antibiotic elution from ALBCs, as it enables highly sensitive detection of even subtle differences in release kinetics between cement formulations (Heller et al., 2005). Complementing HPLC with inhibition zone testing (IZT) provides an important functional perspective by assessing the biological activity of the eluted antibiotic, allowing both methodological approaches to yield a more comprehensive antimicrobial release profile (Balouiri et al., 2016; Kittinger et al., 2024b). Some variation between HPLC and IZT results is expected due to factors inherent to microbiological assays, such as agar composition, diffusion characteristics, and incubation conditions, but these differences are interpretable when the methodologies are applied together. In addition to variability dependent on the method, sample preparation constitutes a major determinant of elution behaviour. To ensure reproducibility and clinical relevance, cement specimens should be prepared according to a standardised protocol that reflects surgical practice. Because clinical spacers are typically mixed manually under atmospheric conditions rather than under vacuum, using a non-vacuum mixing approach preserves the porosity and fluid uptake characteristics that drive in-vivo antibiotic release (IV. Discussion, Chapter 2.4.4., p. 141). Employing Teflon moulds and predefined sample geometries further ensure consistent ratio surface to volume, which are critical parameters influencing diffusion-based elution (Janssen et al., 2023; Kittinger et al., 2024a, b). Following preparation, PMMA specimens should be incubated in PBS with daily replacement of the elution medium to avoid artificial accumulation of antibiotic, which would otherwise inflate measured concentrations and obscure differences between cements. The collected eluates can subsequently be evaluated by both HPLC and IZT. In IZT assays, eluates may be introduced into punched agar wells or applied to sterile Whatman paper discs. Direct placement of cement discs onto agar plates should be avoided, as this method has been demonstrated to produce misleading inhibition zone diameters and poor discrimination between cement types. As this method could lead to uncontrolled antibiotic diffusion into the agar matrix and increased moisture uptake at the interface between cement and agar (Kittinger et al., 2024b). Consequently, inhibition zones do not correlate with actual drug content, and meaningful differentiation between cement brands becomes impossible. Furthermore, this setup does not adequately reflect in-vivo conditions, where antibiotic elution is governed by continuous fluid exchange within the joint space and subsequent distribution into periprosthetic tissues. Methods relying on eluates therefore model more accurately the clinical environment.

Additionally, differences in sample preparation, e.g. choice of nutrient media, and methodological inconsistencies across the literature make comparisons across studies challenging (Steixner et al., 2021). Many historical elution studies rely on vacuum mixing, non-standardised sample geometries, static elution media, or directly tested cement disc on agar plates; all approaches that limit interpretability and comparability (Pithankuakul et al., 2015). Thus, the implementation of harmonised testing protocols is essential. In addition, current EUCAST recommendations explicitly advise against pour-plate or incorporation methods, because these approaches lack standardisation and reproducibility; instead, agar plates should first be poured, allowed to solidify, and then inoculated prior to application of antibiotic-containing eluates. Although EUCAST provides guidance for microbiological diffusion assays, there is currently no universally accepted standard specifically tailored to ALBC elution testing (EUCAST, 2025a). Standardisation of cement preparation, elution protocols, sampling intervals, and biological assays would substantially improve reproducibility and enable more accurate assessment of brand-specific differences in antibiotic release.

3.2. Cement brand characteristics impact antibiotic elution

Antibiotic elution from PMMA cements varies markedly between commercial formulations. The amount of antibiotic incorporated into the cement powder is not proportional to the amount ultimately released. Palacos® R+G exhibited substantially higher gentamicin release than Simplex® T released tobramycin, despite Simplex® T containing approximately twice the antibiotic amount per 40 g of powder. This disparity was evident in both the early and prolonged antibiotic elution phase. Most antibiotic release from PMMA occurs within the first hours to days, during which early burst kinetics dominate. In this critical phase, elution characteristics differ substantially between cements. This was also observed by Kittinger et al. (2024b) where Palacos® R+G demonstrated higher initial gentamicin release than the cement brand CMW® 1G although it contains less antibiotics. These early-phase differences are particularly important because methodological choices strongly influence whether such variations can be detected; only techniques that accurately quantify early release, HPLC and IZT, can capture these clinically relevant disparities (Kittinger et al., 2024b).

Within the first 24 hours, Palacos® R+G displayed a pronounced burst release, reaching 96 µg/cm² at 6 hours - nearly twenty-fold higher than the 4.83 µg/cm² released by Simplex® T at the same timepoint. Although concentrations declined thereafter, Palacos® R+G consistently maintained superior release, achieving a cumulative 24-hour elution of 183.61 µg/cm² compared with only 7.30 µg/cm² for Simplex® T. Over 42 days, this trend persisted: Palacos® R+G maintained levels of 30.5 µg/cm², whereas Simplex® T declined to 7.5 µg/cm², confirming that Palacos® R+G provides both a greater magnitude and longer duration of antibiotic availability.

These kinetic differences translated directly into functional antimicrobial activity. IZT revealed that Palacos® R+G maintained activity against *Staphylococcus aureus*, *Staphylococcus epidermidis,* and *Escherichia coli* throughout the entire period of 42 days, whereas Simplex® T retained measurable activity only until day 14, and in the case of *E. coli*, activity was lost after 7 days. This underlines that the bioavailability of the eluted antibiotic, rather than the nominal antibiotic load, determines antibacterial efficacy.

The observation that antibiotic content does not predict elution behaviour is consistent with earlier findings. Meeker et al. (2019) reported that, across several antibiotic and cement combinations, Palacos® consistently demonstrated higher release than Simplex®, independent of the antimicrobial agent used. Also, Squire et al. (2008) demonstrated that Palacos® R+G showed markedly increased antibiotic elution, especially, when mixed with vacuum, while Simplex® T achieved relevant concentrations only on day 1. Similarly, Dietz et al. (2024) showed that although Simplex® exhibits a steep burst release on day 1, exceeding that of Palacos®, this is followed by a rapid decline by day 2, whereas Palacos® maintains more sustained release. Thus, relying on one single timepoint increases the risk of overestimating the antimicrobial performance of certain cements. In contrast, the persistence of antibiotic concentrations above the MIC over time is essential for effective spacer function. Prolonged antibiotic concentrations below MIC during the later stages of PMMA spacer implantation may have clinical consequences. When antibiotic release falls below the MIC, bacterial populations, particularly staphylococci, which dominate PJI, may persist in a metabolically active but partially suppressed state. This may create conditions that favour the selection of tolerant or a-priori resistant bacteria. This is of concern in two-stage revision arthroplasty, where spacers often remain in situ for several weeks; if the elution profile of a cement fails to sustain concentrations above the MIC, antibacterial efficacy is compromised, potentially allowing low-grade infection to persist within biofilm reservoirs on surrounding tissue surfaces.

These principles also extend to other cement brands. As demonstrated in the study by Kittinger et al. (2024b) Palacos® R+G (1.25% gentamicin) released significantly more antibiotic than CMW® 1G (2.5% gentamicin), despite the higher nominal antibiotic content in CMW® 1G.

The observed differences in antibiotic release between Palacos® R+G and Simplex® T reflect fundamental differences in cement composition, polymer architecture, viscosity type, and handling characteristics. Palacos® R+G is formulated with a PMMA-MA-MMA copolymer matrix, whereas Simplex® T contains a PMMA-styrene copolymer (Kühn, 2014; Kühn et al., 2016). The incorporation of styrene in the cement matrix is known to reduce hydrophilicity, thereby limiting water absorption and restricting the formation of aqueous diffusion channels that are essential for antibiotic transport through the cement matrix. Since water uptake is a primary determinant of antibiotic elution, the higher hydrophilicity of Palacos® likely accounts for its superior release capacity (Kühn, 2014). Sterilisation methods may further contribute to these differences (Weisman et al., 2000). Simplex® is sterilised using gamma irradiation, while Palacos® undergoes ethylene oxide sterilisation (Kühn, 2014). As previously described, gamma irradiation can alter the chemical structure of the polymer backbone and consequently reduce matrix hydrophilicity, further limiting elution capacity.

Viscosity also appears to influence release behaviour. Palacos® R+G is classified as a high-viscosity cement, whereas Simplex® T is low-viscosity. Although low-viscosity cements generally require higher antibiotic loading to achieve release levels comparable to high-viscosity formulations, even a doubled antibiotic load did not enable Simplex® T to approach the elution performance of Palacos® R+G (Kühn, 2014). This indicates that viscosity interacts with polymer composition, hydrophilicity, and porosity rather than functioning as an isolated determinant. It's also common practice in joint replacement surgeries that Simplex® is applied a bit too early in the sticky phase (Fölsch et al., 2024). The sticky phase is known to be the highest phase of antibiotic elution so this could in clinical reality result in a potential antibiotic loss, reducing the amount available for sustained release over time. Interestingly, despite its widespread global use, Simplex® is frequently chosen for spacer fabrication even though experimental results consistently indicate that its elution profile is less favourable for sustained antimicrobial delivery. It's even recommended to clearly increase the antibiotic loading in a Simplex® spacer, but this always comes with an increased risk for mechanical failure (Tseng et al., 2022).

These considerations highlight that antibiotic elution behaviour is deeply dependent on the intrinsic characteristics of the cement formulation. Consequently, antibiotic type or dosage alone cannot predict elution performance, reinforcing the necessity for cement selection based on material science principles rather than solely on clinical familiarity.

3.3. Limitations and opportunities for further research

Intra-articular conditions differ markedly from the controlled environment created by PBS. Synovial fluid is a protein rich, viscous medium containing albumin, hyaluronic acid, lipids, electrolytes, and enzymes, and its pH and composition may fluctuate in the presence of inflammation or infection. These factors influence antibiotic solubility, protein binding, diffusivity, and chemical stability, and therefore have the potential to substantially modify elution behaviour in-vivo compared with laboratory experiments using PBS. Additionally, the native joint environment is characterised by continuous synovial turnover, cyclic mechanical shear, and the structural presence of biofilm on tissue surfaces which is difficult to be reproduced adequately in static in-vitro models. Given these considerations, further refinement of the IZT setup may be of interest. Future studies could include synovial fluid media or employ methodologies such as in-vivo micro dialysis to obtain more reliable pharmacokinetic data. Moreover, evaluating antibiotic efficacy against mature biofilms, rather than planktonic bacteria, would improve the translational relevance of such studies. This could be achieved using an in-vivo biofilm model, for instance by establishing PMMA cement spacers in the *Galleria mellonella* biofilm model.

4. *Galleria mellonella* as suitable in-vivo biofilm model for testing ALBCs

Using a combination of in-vitro assays and *Galleria mellonella* infection models, we tried to translate the findings from our in-vitro research into a simple in-vivo biofilm model. DALBCs consistently demonstrated superior antimicrobial performance compared with SALBC, providing significantly enhanced protection against MDR *Staphylococcus aureus* and *Enterococcus faecalis*. The *Galleria mellonella* implant infection model allowed us to directly assess whether the antibiotic concentrations eluted from Palacos® R+G, Copal® G+V, and Copal® G+C were sufficient to prevent or reduce early implant-associated infections caused by resistant bacteria.

4.1. Enhanced antimicrobial performance of DALBCs over SALBCs

Across all in-vitro assays, ALBCs exhibited a characteristic elution pattern consisting of a pronounced first day burst release followed by a lower sustained phase. DALBCs consistently released greater total amounts of active drug than SALBC, which was reflected in larger inhibition zones and more pronounced antibiofilm activity. Quantitatively, Palacos® R+G released approximately 25–35 µg gentamicin over five days, whereas Copal® G+C eluted nearly twice this amount of gentamicin in addition to substantial clindamycin concentrations. Copal® G+V displayed a comparable burst and sustained release profile for gentamicin alongside vancomycin. These differences in antibiotic elution translated into markedly stronger disruption of *S. aureus* biofilms and significant reductions in *E. faecalis* biofilm burden in DALBC compared with SALBC.

Consistent with these in-vitro observations, the in-vivo experiments demonstrated that cements containing two antibiotics (Copal® G+C and Copal® G+V) achieved significantly superior antimicrobial efficacies relative to Palacos® R+G. This occurred despite the pathogens exhibiting resistance to gentamicin and clindamycin, underscoring the capacity of locally delivered, high-concentration antibiotics to exceed MIC and MBC thresholds and achieve bacterial killing under conditions where systemic therapy would fail (Kühn et al., 2017). These findings highlight the fundamentally different pharmacodynamic environment created by ALBC, in which eluted antibiotics can reach concentrations several orders of magnitude higher than those achievable through systemic dosing (Steadman et al., 2023; Walenkamp 2007).

Among the DALBC formulations, Copal® G+V demonstrated the greatest overall efficacy, particularly against *E. faecalis*, consistent with the known potency of vancomycin against Gram-positive bacteria. Copal® G+C performed especially well against *S. aureus*, likely attributable to its higher gentamicin loading and robust early elution. In-vitro data clearly confirmed these differences: Copal® G+C produced the largest cumulative antibiotic release and the largest inhibition zones for both *S. aureus* and *E. faecalis*, followed by Copal® G+V, whereas Palacos® R+G demonstrated only modest antimicrobial activity in alignment with its single antibiotic loading and lower total release.

The *Galleria mellonella* biofilm model provided a relevant platform to test whether these in-vitro differences translated into in-vivo performance. In both the biofilm model and the haematogenous infection model, the in-vivo results strongly mirrored the in-vitro release characteristics. DALBC formulations showed markedly greater larval survival than Palacos® R+G: in the biofilm model, Copal® G+V yielded survival rates of 90% for *S. aureus* and 80% for *E. faecalis* and with Copal® G+C achieved 70% survival. In contrast, Palacos® R+G provided only moderate protection (23–36%), when the bacteria were resistant to gentamicin. Similar trends were observed in the haematogenous infection model, where DALBC again significantly outperformed SALBC, highlighting the critical influence of antibiotic combination on antimicrobial efficacy.

4.2. High local antibiotic concentrations overcome resistance

We also examined whether the high local antibiotic concentrations generated by ALBC are capable of overcoming resistance. Our findings strongly support the concept of local antibiotic usage in infections where the access of systemically administered antibiotics is limited. Despite resistance of *S. aureus* and *E. faecalis* to gentamicin and clindamycin, both pathogens were substantially inhibited by Copal® G+C. Even Palacos® R+G demonstrated measurable activity against planktonic *S. aureus*. Once again, these observations confirm that locally eluted antibiotic concentrations are easily capable of exceeding MIC and MBC thresholds by several orders of magnitude. This is particularly relevant for an antibiotic such as gentamicin, that has a strict bactericidal and concentration-dependent mode of action. Overall, these observations are consistent with the principle that locally delivered, high-dose antibiotic therapy, particularly during the initial release phase, can overcome established resistance mechanisms (Kühn et al., 2017; Walenkamp, 2007). The superior performance of DALBCs further suggests additive or synergistic interactions between agents with distinct mechanisms of action, enhancing bacterial eradication even in MDR bacteria.

The superior efficacy of DALBC over SALBC aligns with clinical and experimental findings reported by Blersch et al. (2024) and clinical trials in the context of both infection prophylaxis and septic revision surgery (Blersch et al., 2024; Cara et al., 2022; Dias Carvalho et al., 2021); (Agni et al., 2023; Sprowson et al., 2016; Yang et al., 2026). These data reflect clinical reality, where pathogens labelled as "resistant" in standard antibiograms are nevertheless frequently eradicated when high local antibiotic concentrations are achieved through DALBC delivery (Blersch et al., 2023; Blersch et al., 2024; Fink & Tetsworth, 2025). These findings are of highest clinical relevance and mean in practice, that even gentamicin or clindamycin-resistant organisms in routine antibiograms remain susceptible to local antibiotic delivery due to the concentration-dependent effect of aminoglycoside and lincosamide antibiotics. A combined approach involving systemic and local antimicrobial therapy is frequently the most effective strategy for managing infections caused by resistant pathogens. Gatin et al. (2019) demonstrated that MDR *Enterobacteriaceae* remained susceptible to the high local concentrations of colistin released from a PMMA cement spacer, and that infection eradication rates were highest when this local delivery was combined with systemic colistin therapy. Similar effects have been demonstrated for other local antimicrobial carrier systems. Metsemakers et al. (2015) reported that a doxycycline coated intramedullary nail effectively prevented *S. aureus* osteomyelitis in a rabbit model, although the infecting strain was classified as doxycycline resistant.

These findings also support prior conclusions that the use of SALBC or DALBC does not increase the risk of antibiotic resistance in the context of primary arthroplasty (Berberich & Sanz-Ruiz, 2019). The use of local antibiotics is frequently discussed within the framework of antimicrobial stewardship, particularly regarding whether local prophylaxis may select for resistant strains (Siljander et al., 2018). In addition to the safety profile, the economic implications of ALBC are increasingly addressed in the literature, with several studies aiming to demonstrate whether the use of ALBC is not only clinically safe but also cost-effective (Hoskins et al., 2020). Tootsi et al. (2021) demonstrated in a large multicentre cohort that low-dose gentamicin-loaded bone cement used in primary arthroplasty does not raise the prevalence of resistant bacteria in subsequent PJIs, even after adjustment for confounding variables. Their findings align with several previous reports showing no measurable increase in resistance attributable to ALBC, suggesting that the limited and short-term antibiotic exposure provided by cement is insufficient to create selective pressure strong enough to drive resistance development. Instead, broader ecological and systemic contributors, such as hospital antibiotic consumption patterns, high baseline rates of multi-drug-resistant organisms, horizontal gene transfer, and inappropriate systemic antibiotic use, are likely to play a far more decisive role in resistance development (Ventola, 2015).

4.3. Enhanced infection eradication by DALBC

By integrating quantitative release kinetics with in-vivo infection outcomes, this work provides a more comprehensive understanding of how different ALBC formulations behave within a biological environment. The data further reinforce the rationale for employing DALBCs when managing implant-associated infections caused by MDR pathogens. A key strength of the *G. mellonella* model is its ability to differentiate bacterial burden across distinct infection compartments, including planktonic bacteria in the haemolymph, biofilm on surrounding host tissue, and biofilm adherent to the implant surface.

This multidimensional assessment allowed us to reconstruct a more complete picture of early PJI. In these analyses, DALBC consistently reduced bacterial load across all larval compartments. In the pre-seeded implant biofilm model, DALBCs achieved >6-log reductions of *S. aureus* in both tissue and implant samples, while Palacos® R+G failed to produce comparable effects, indicating that SALBC release is insufficient to disrupt established biofilms. For *E. faecalis*, a pathogen with intrinsic tolerance to many antibiotics, Copal® G+V reduced bacterial burden in tissue and on implant surface by >2.4-log, while Copal® G+C produced smaller yet significant reductions. In contrast, Palacos® R+G showed no meaningful impact. These results highlight that reliance on planktonic susceptibility alone is inadequate for predicting clinical efficacy. This observation also aligns with the concept that high local antibiotic concentrations achievable with PMMA can partially overcome resistance, but that DALBCs combinations are required for robust antibiofilm efficacy.

Release kinetics analyses confirmed a pronounced burst release on day 1, followed by a lower sustained elution phase. When integrated with larval survival and bacterial burden data, it becomes evident that the initial high release phase is critical for antimicrobial protection, consistent with current understanding that bacterial adhesion and microcolony formation within the first 24 hours determine the path of PJI (Patel, 2023; Sauer et al., 2022). To evaluate whether implant geometry artificially enhanced elution, we considered the possibility that the relatively large surface area to volume ratio of the cemented K-wires might increase antibiotic release (Janssen et al., 2023). However, the release profiles closely matched those obtained from standardised cement discs, indicating that the elution kinetics are representative of clinically relevant PMMA formulations.

4.4. Establishment of the *Galleria mellonella* biofilm model

The development of *Galleria mellonella* as an in-vivo model for implant associated infections represents a relatively recent but rapidly advancing methodological innovation, driven by the need for ethically acceptable, cost-effective, and clinically relevant alternatives to vertebrate infection models. Although *G. mellonella* had long been used to investigate microbial virulence and antimicrobial therapies, its application to orthopaedic implant infection research required substantial adaptation and validation (Glavis-Bloom et al., 2012; Pereira et al., 2020; Tsai et al., 2016)

The suitability of *G. mellonella* for infection research is largely attributable to its innate immune system, which shows remarkable parallels to mammalian innate immunity including haemocyte mediated phagocytosis, opsonin, antimicrobial peptides, and a phenol oxidase driven melanisation cascade (Glavis-Bloom et al., 2012; Mannala et al., 2021). These defence mechanisms enable the larvae to respond to pathogens similarly to early vertebrate immune reactions. Early work established that the model can reliably differentiate virulence among *Staphylococcus aureus*, *Enterococcus spp.*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, which are all clinically relevant organisms in PJIs.

A major methodological advance was the demonstration that *G. mellonella* supports biofilm formation on implanted foreign materials, such as stainless steel and titanium. Mannala et al. (2021) showed that biofilms forming on metal implants inserted into larvae exhibit key characteristics known from mammalian models, including bacterial persistence, host tissue damage, and decreased antibiotic susceptibility, thereby extending the model from systemic infection to true device-associated infection research.

Building on these foundations, Zhao et al. (2024) developed the first dedicated PMMA-based implant infection model, using PMMA cement spacers. This model reproduces features of early PJI, including initial adhesion, biofilm maturation, and bacterial dissemination. It was further expanded to mimic a haematogenous implant infection, achieved by injecting bacteria after implant placement and early biofilm infection, produced by pre-colonising the implant prior to insertion.

The resulting implant infection model offers several important advantages. Its high throughput capability allows numerous implants and antimicrobial strategies to be evaluated in parallel, facilitating efficient experimental comparison. Because the model does not require formal animal licensing, it provides clear ethical and logistical benefits, enabling rapid iteration and screening. The system retains biological relevance, as the innate immune responses of *Galleria mellonella* closely mirror early mammalian immune processes. In addition, the model allows precise quantitative assessment of bacterial burden across multiple compartments, including planktonic populations, tissue-associated biofilms, and biofilms adherent to implant surfaces. Additionally, the larvae tolerate PMMA cement implants well, regardless of antibiotic loading, and that the model is sensitive enough to detect performance differences among ALBC formulations that align with clinical observations.

Despite its strengths, the model cannot replicate all aspects of human orthopaedic infection. It lacks adaptive immunity, does not reproduce bone-implant interface biology, and cannot model late phase osteointegration or chronic infection. Reproducibility can also be influenced by larval source, diet, and environmental conditions (Pereira et al., 2020; Tsai et al., 2016). Nevertheless, as a preclinical screening platform, it provides a valuable intermediate step between in-vitro assays and vertebrate studies.

4.5. Limitations and opportunities for further research

The *Galleria mellonella* model lacks key features of mammalian orthopaedic physiology, including adaptive immunity, a bone-implant interface, osteointegration processes, and the chronic inflammatory responses characteristic of PJIs. The model focuses on the early phase of infection dynamics, with quantitative cultures obtained after one day and survival monitored over 5 days only. Although these endpoints align with the initial release pharmacology of PMMA cements, they cannot fully address the complexity of biofilm and implant associated infections. Furthermore, the comparison between DALBCs and SALBC introduces differences in total antibiotic content; while this mirrors commercial products, it complicates interpretation by confounding synergistic effects with dose dependent effects.

These limitations directly point to several opportunities for further research. Future studies should incorporate PMMA formulations with comparable antibiotic loadings to reduce potential effects from antibiotic dosing. Integrating the *Galleria mellonella* biofilm data with mammalian pharmacokinetic and micro dialysis studies, for instance, quantifying local bone or synovial antibiotic concentrations, would help clarify how local antibiotic concentrations overcome bacterial resistance. Expanding the pathogen panel to include additional ESKAPE organisms is a logical next step, given their central role difficult to treat PJI cases. Further refinement could also include testing additional cement types and correlating in-vivo findings with clinical pharmacokinetic data, such as serum concentrations after SALBC versus DALBC implantation.

5. Daptomycin dose and cement matrix determine the efficacy of cement spacers

Two commercially available PMMA cements, Palacos® R+G and Simplex® T, were used as example formulations and were loaded with different amounts of daptomycin to determine the ideal dosage for cement spacers. While both cements can be manually loaded with daptomycin, their material properties, release kinetics, and antimicrobial efficacy differ substantially, with important implications for their clinical utility in the management of PJIs, particularly those caused by VRE.

5.1. Impact of daptomycin loading on mechanical and handling properties

Consistent with prior findings on the negative effects of antibiotic admixing on PMMA stability, we showed that manually adding daptomycin reduces ISO bending strength, ISO bending modulus, ISO compressive strength, and Dynstat (DIN) impact resistance across all cement types. The magnitude of mechanical impairment differed between cement brands, with Palacos® R+G remaining within ISO thresholds even after addition of 1.5 g daptomycin, whereas Simplex® T frequently fell below required minimum values. These findings indicate that the mechanical consequences of antibiotic admixing are dependent on the cement brand, and that Simplex® T may be less suitable for manual daptomycin supplementation when higher antibiotic loads are required.

Daptomycin reduced both doughing and setting times, with the most rapid setting observed in Palacos® R+G with 1.5 g daptomycin. Although predictable handling is crucial during spacer formation, both cement systems remained workable within a clinically acceptable window. Variability in setting times was more pronounced in Simplex® samples, which may complicate reproducibility during intraoperative use. These observations are consistent with established evidence that antibiotic admixture modifies polymerisation kinetics and should be considered when selecting PMMA cements for manual antibiotic admixing (Kühn, 2014).

5.2. In-vitro antimicrobial activity and release profile

The antimicrobial activity of daptomycin-loaded PMMA cements showed a clear dosage dependent effect. While 1 g daptomycin provided limited inhibition of VRE, incorporation of 1.5 g or 2 g produced robust antimicrobial zones during the early release phase, with measurable activity persisting even at day 42 for the highest dose.

Proliferation assays further confirmed that Palacos® R+G released higher amounts of daptomycin compared with Simplex® T, resulting in greater reduction of bacterial growth. This is consistent with known differences in the cement matrix: Palacos® contains MMA/MA copolymers conferring greater hydrophilicity, whereas Simplex® includes styrene components and a softener that reduce water uptake and thereby limit antibiotic elution as discussed in Chapter 3.3. (IV. Discussion, p. 145) (Kühn, 2014; Meeker et al., 2019). The prolonged antimicrobial activity observed for Palacos® R+G with 2 g daptomycin provides evidence that the brand specific polymer chemistry remains a critical determinant of elution kinetics and long-term antimicrobial activity.

5.3. In-vivo efficacy in the *Galleria mellonella* implant infection model

The *Galleria mellonella* model confirmed the in-vitro findings. Plain and SALBC samples were ineffective in both prevention and treatment settings, highlighting the inadequacy of only gentamicin-loaded PMMA cement against VRE. In contrast, daptomycin-loaded cements demonstrated significant protection against infection, with Palacos® R+G with 2 g daptomycin yielding the highest larval survival rates and the lowest bacterial loads on both tissue and implant surfaces. Although Simplex® T with 2 g daptomycin also improved survival, its performance was consistently inferior to Palacos® R+G at matching daptomycin loadings. These in-vivo findings reflect the release behaviour observed in our previous investigations (IV. Discussion, Chapter 3., pp. 142-145) and validate *G. mellonella* as an efficient preclinical model for assessing ALBC efficacy against MDR pathogens. Daptomycin represents a promising alternative antibiotic for local delivery in PMMA cements due to its potent activity against resistant Gram-positive pathogens. Our findings support the feasibility of daptomycin incorporation into PMMA cements, with Palacos® demonstrating superior performance: mechanical reliability, handling properties, antibiotic elution and antimicrobial activity. In summary, not only the antibiotic choice but also the choice of the cement matrix may influence the potential for infection eradication and infection prevention of a DALBC.

5.4. Limitations and opportunities for further research

The *Galleria mellonella* model is limited to a five-day observation period that only reflects short-term antimicrobial activity. Mechanical testing was conducted only at the 1.5 g dose. Building on the findings there are several options for further research. Comparative analyses of vancomycin-loaded and daptomycin-loaded PMMA cements would help to investigate on their differences in the management of VRE caused PJIs. In addition, a more detailed examination of daptomycin's biofilm penetration capacity, particularly in direct comparison to vancomycin, would be highly informative. Prior literature suggests that daptomycin may exhibit enhanced diffusion into staphylococcal biofilms and more rapid bactericidal activity within the biofilm matrix, but this phenomenon has not been systematically investigated for enterococcal biofilms.

A further limitation arises from the restricted range of PMMA formulations included in our study. Only Palacos®, Copal®, and, to a lesser extent, Simplex® were assessed, reflecting the limited availability of cement materials for experimental work. While this selection is common in the literature, it should be acknowledged that Palacos® and Copal® cements due to their unique composition, high porosity, and characteristic polymerisation behaviour, are widely regarded as the gold standard for antibiotic elution studies. Consequently, the favourable elution profiles observed here may not be directly transferable to PMMA cements with intrinsically lower release capacities. Future research should therefore expand beyond Palacos® based matrices to determine how formulation-specific differences influence antibiotic release, mechanical stability, and the overall suitability of daptomycin-loaded cements for clinical spacer applications.

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