Diplomarbeit

On the creation of PMMA spacers containing ß-lactam antibiotics

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Graz, den 23.03.2018

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Zusammenfassung

EINLEITUNG: Seit Jahrzehnten werden antibiotikahaltige Knochenzemente (ALBC) erfolgreich im chirurgischen/orthopädischen Bereich eingesetzt. Im Gegensatz zu systemischer Antibiotika Therapie erlaubt der lokale Einsatz solcher Wirkstoffe das Erreichen hoher Konzentrationen am Ort der Infektion und damit die vollständige Eradikation der jeweiligen Pathogene. Die dadurch zusätzlich erreichte Vermeidung hoher systemischer Konzentrationen mindert das Risiko und die Intensität systemischer Nebenwirkungen. Die Pro-Implant Foundation (PIF) veröffentlicht regelmäßig detaillierte und pathogen-spezifische Empfehlungen zum Einsatz von Antibiotika haltigen Knochenzementen für ein- oder zweistufige Protokolle zum Gelenksersatz. Aufgrund der fraglichen Kombinierbarkeit von ß-Lactam Antibiotika mit PMMA Zementen werden für derartige Kombinationen nur sehr selten Empfehlungen ausgesprochen. Es ist daher dringlich zu evaluieren ob und wie sich die Zugabe von ß-Lactam Antibiotika zu PMMA Zementen hinsichtlich der Stabilität und der antimikrobiellen Aktivität auswirkt um eine rationale und sichere klinische Anwendung zu ermöglichen.

MATERIAL UND METHODEN: Es wurden vier verschiedene PMMA Zemente (Palacos® R, Palacos® R+G, Copal® G+C, Copal® G+V) mit verschiedenen Konzentrationen an Ampicillin (2,5% und 7,5%), Piperacillin/Tazobactam (11,25% und 22,5%) und Cefuroxim (2,5% und 7,5%) vermischt und entsprechende Prüfkörper hergestellt. Zur Bestimmung der mechanischen Kenngrößen wurden Tests gemäß ISO 5833 und DIN 53435 durchgeführt. Die antimikrobielle Wirksamkeit wurde mittels Hemmhoftests in Dreifachbestimmung untersucht.

ERGEBNISSE: Alle getesteten Antibiotika konnten erfolgreich mit PMMA Zementen kombiniert werden ohne signifikante Inhomogenität zu bedingen. Der Einfluss auf das Biegemodul (ISO 5833) war vernachlässigbar, während sowohl für die Biegefestigkeit (ISO 5833) und die Schlagzähigkeit nach Dynstat (DIN 53435) signifikante Veränderungen beobachtet werden konnten. Insgesamt zeigte sich ein näherungsweise linearer Zusammenhang zwischen der zugegebenen Antibiotikamenge und der Abnahme der erwähnten mechanischen Kenngrößen. Die antimikrobielle Wirksamkeit zeigte eine starke Abhängigkeit vom jeweiligen Bakterienstamm, war jedoch für alle getesteten Stämme für zumindest 28 Tage nachweisbar.

DISKUSSION: ß-Laktam Antibiotika können ohne kritischen Wirkungsverlust mit PMMA Zementen kombiniert werden ohne dabei die mechanische Stabilität intolerabel zu mindern. Fallspezifische Empfehlungen für die klinische Anwendung können entsprechend des vorliegenden Antibiogramms getroffen werden. Dies ist besonders in Fällen, in welchen keine Allergien vorliegen und sonst andere Antibiotika zum Einsatz kommen würden von großer praktischer Bedeutung.

Abstract

INTRODUCTION: For decades, antibiotic loaded bone cement (ALBC) has been successfully used in surgical practise (1). In contrast to systemic antibiotic therapies, the use of ALBC enables high local concentrations and thus facilitates the total eradication of pathogens without risking systemic side effects. The Pro-Implant Foundation (PIF) periodically issues detailed and pathogen specific recommendations on which antibiotic agents are to be used as a PMMA supplement during one- or two-step revision protocols (2). Due to the rather fragile molecular structure of cephalosporin and ß-lactam antibiotics along with the questionable compatibility with PMMA cements, those antibiotics are only rarely or not at all recommended. Therefore, it is crucial to evaluate if cephalosporin and ß-lactam antibiotics are compatible with PMMA and how they impact on the mechanical and antimicrobial characteristics.

MATERIAL AND METHODS: The samples were produced using PMMA cements (Palacos® R, Palacos® R+G, Copal® G+C, Copal® G+V) in combination with ampicillin (2,5% and 7,5%), piperacillin/tazobactam (11,25% and 22,5%) and cefuroxime (2,5% and 5%) in different concentrations. In order to assess the mechanical characteristics, tests according to ISO 5833 and DIN 53435 were performed. The antimicrobial efficacy was determined by inhibition zone assays in triplicates.

RESULTS: All tested antibiotics could be successfully combined with PMMA without giving rise to significant inhomogeneities. The impact on the bending modulus (ISO 5833) was negligible, while significant differences could be observed for bending strength (ISO 5833) and Dynstat impact strength (DIN 53435). Generally, the higher the concentrations of the antibiotics were, the lower these values became. The antimicrobial efficacy showed strong strain-dependency, but was detectable for at least 28 days for the vast majority of tested strains.

DISCUSSION: ß-lactam antibiotics can be effectively and safely combined with PMMA without critically decreasing stability of the cement or impeding antimicrobial activity of the antibiotic. Depending on the respective antibiogram, specific recommendations on the use of ß-lactam antibiotics can be made. This especially applies to cases were no allergies against the respective antibiotic are present and were reserve antibiotics would have to be used otherwise.

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1 Introduction

1.1 Prosthetic joint infections

As the ability to move pain-free and independent from external help is absolutely essential for quality of life, total joint replacements (TJA) are medical procedures of tremendous importance on an individual level. With an ageing society, as found in nearly all western countries, the demand in such interventions is expected to strongly increase in the future. By 2030, the number of total hip and total knee arthroplasties in the US alone are expected to reach 572,000 and 3.48 million, respectively (1).

While most total joint arthroplasties are successful and provide a proper regain of function free of pain, a small percentage of patients will experience bacterial infection of the prosthesis and the surrounding tissue, commonly known as prosthetic joint infection (PJI) (2). PJIs are the leading cause for failure of total joint arthroplasty and since they are difficult to treat conservatively, costly revision arthroplasty becomes necessary in many cases. Thus, PJIs pose a serious threat on patient health and the global healthcare system and there is a strong demand for more efficient preventive and curative strategies (3).

In the cause of infection, the foreign material brought into the joint drastically increases the risk for bacterial infection and biofilm formation. Studies showed that the presence of prosthetic material decreased the number of invading microbial cells necessary to establish an PJI by up to 10,000-fold (4,5). This vast increase in infection risk is due to the ability of bacterial cells to efficiently bind foreign material and, in consequence, form biofilms. These biofilms are microbial communities present on surfaces and surrounded by a self-produced extracellular matrix that is composed of a complex mixture of polysaccharides and proteins to shield the bacteria from the surrounding. Any diffusing agent like antibiotics applied from the outside (in this case systemically) will not be able to sufficiently diffuse towards the cells and reach therapeutic concentrations. Furthermore, bacterial cells in biofilms are metabolically inactive and reside in a resting state, which makes them partially resistant towards any antibiotic targeting molecular mechanisms related to cell division and/or metabolic pathways. It is possible for individual cells to detach from the film, become planktonic again and initiate formation of a new biofilm in another location within the joint (see Figure 1) (6). After the initial infection, the establishment of a fully formed biofilm takes approximately 6 weeks. Since during that period of time, the extracellular matrix is not yet fully formed, treatment with biofilm-active antibiotics, like rifampicin or ciprofloxacin, is still effective. Hence, this six week interval is used to classify PJIs into early (<6 weeks) and late infections (>6 weeks) (5).

Early infections may allow the prosthetic material to reside in the joint if the stability is not impaired and radical debridement along with long-term antibiotic treatment is performed. Late infections, on the other hand, demand for radical debridement and complete removal of the prosthetic material. As treatment strategy, one- and two-stage protocols are available. While one stage procedures perform explantation, debridement and novel implantation in one surgical setting, two-stage protocols work with an intermediary spacer implant after removal of the old and before implantation of the new prosthesis. Importantly, one-step procedures are strictly limited to settings where exact knowledge of the infecting pathogen is available and the surgical prerequisites are met. Two stage protocols use temporary spacers that are commonly made of polymethylmethacrylate (PMMA) (7).

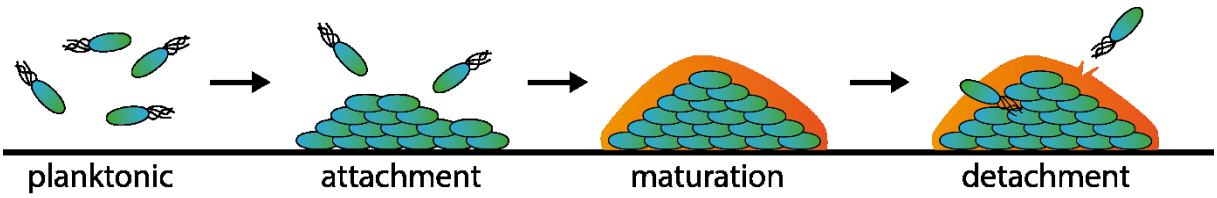

Figure 1: Stages during biofilm formation. Planktonic bacteria express surface antigens to enable attachment to surfaces and form colony-like structures. Subsequently, the cells secrete designated polysaccharides and proteins to form the extracellular matrix (orange). Individual cells are able to detach from the fully established biofilm and transition into planktonic cells.

1.2 PMMA cements

Bone cements have been in use for over 50 years now and they play a crucial role for a large number of orthopaedic surgeries including total shoulder arthroplasty, total hip arthroplasty and total knee arthroplasty (8,9). The vast majority of bone cements in use consist of polymethylmethacrylate (PMMA). PMMA is a polymer that is formed by radical substitution from methyl methacrylate (MMA) monomer. The widespread use of PMMA, also known as acrylic glass or Plexiglas®, brought extensive knowledge about its properties and the long history of its application in medical procedures made PMMA the prime selection for numerous settings.

PMMA cements are commercially distributed as two-component systems consisting of a powder component and a liquid component, which contain polymer and monomer, respectively. Furthermore, both components may contain additives that help to stabilize the components and to initiate the

polymerization once the components are combined. In clinical routine, both components are combined and the cement is prepared right before usage by the surgeon or the staff (10).

The formation of PMMA is carried out by radical polymerization. Radical polymerization works by the initial generation of free radicals using an labile initiator substance like for instance benzoyl peroxide (BPO) (see Figure 2a). Subsequently, the formed free radical is able to perform a nucleophilic attack on the C=C bond in methylmethacrylate (see Figure 2b), which in turn yields a radical adduct. Based on this scheme, polymerization continues and during each cycle, another MMA monomer is incorporated into the growing PMMA chain (see Figure 2c). The reaction terminates when two radicals react with each other to form a non-radical product (see Figure 2d).

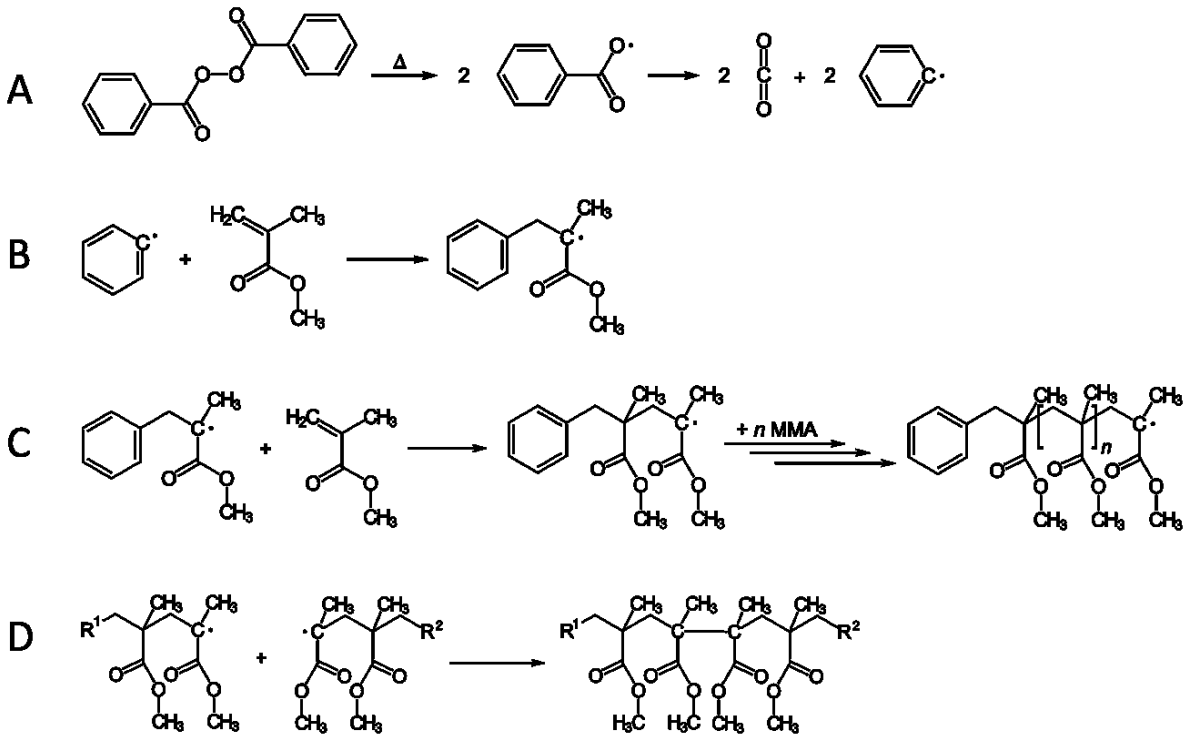

Figure 2: MMA polymerization. Panel A: Initiation and radical formation. Panel B: Chain starting reaction. Panel C: Chain propagation reaction. Panel D: Termination reaction. Adapted from reference (11).

Before the commencement of the European Medical Devices Act (MPG; Medizinproduktegesetz) in 1998, bone cements were considered drugs and had to meet the high requirements of the drug authorities. Till then, only a few products like Palacos®, Simplex® or CMW® were approved for clinical use. The re-classification of PMMA bone cements as medical devices by the MPG slackened those

requirements and led to a strong increase in commercially available products. While the old preparations are still available in unchanged quality and backed by solid scientific data, newer products may not have the same quality and lack reliable data. Therefore, close consideration should be paid when choosing the product, since available cements are not necessarily equivalent in quality (10).

1.3 Antibiotic loaded bone cements (ALBC)

In the late 1960s, it was the German surgeon Hans-Wilhelm Buchholz who for the first time attempted to combine PMMA cements with antibiotics (12). Based on the thought that added components, which are not to be incorporated into the polymer, may be gradually eluted from the hardened cement, he combined Palacos® R with gentamicin and witnessed the feasibility and efficacy of such an approach (12). After implantation, capillary forces cause soaking of the PMMA cement pores with synovial fluid and lead to the establishment of a continuous fluidic system between the joint cavity and the cement interior. Incorporated particles like antibiotics are then able to diffuse from the cement into the joint (see Figure 3). Compared to systemic administration of antibiotics, where a high systemic burden and only a low local concentration is reached, the local application enables high concentrations at the site of infection without the establishment of high systemic concentrations. Hence, local application allows for efficient infection treatment without risking severe side effects (13).

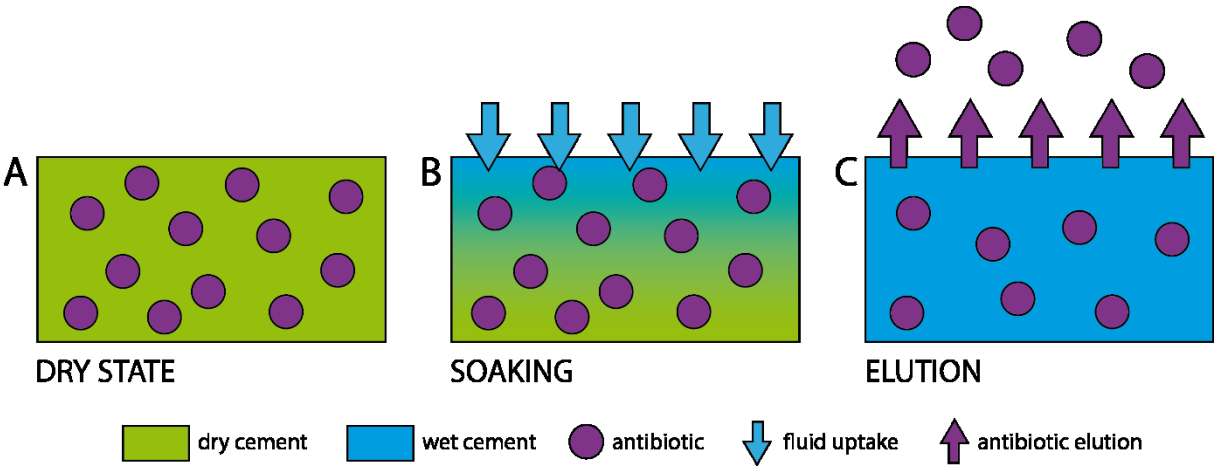

Figure 3: Antibiotic elution from PMMA cements. (A) In the dry state, antibiotic molecules are immobilized within the cement matrix. (B) Capillary forces cause synovial fluid to soak the cement and create a continuous fluidic system between the cement interior and the joint cavity. (C) Antibiotic particles are able to freely diffuse into the joint cavity. Adapted from reference (13).

The high local concentration within the joint reached by the usage of ALBC is potentially able to completely prevent biofilm formation and eradicate all bacterial cells present. In line with this, it has been shown that the usage of ALBC significantly reduces the incidence of PJI in arthroplastic procedures (9). Since the antibiotic concentration within the joint cavity and not within the cement conveys the antimicrobial efficacy, the elution properties of the used cement mixture are of prime importance. While commercially available PMMA cements may contain high amounts of antibiotics, not all of them will show efficient release and yield sufficient concentrations to fight off infections (see Figure 4). Again, available mixtures may seem equivalent from the outside but differ significantly in their clinical performance and close consideration should be paid on which one to use (14).

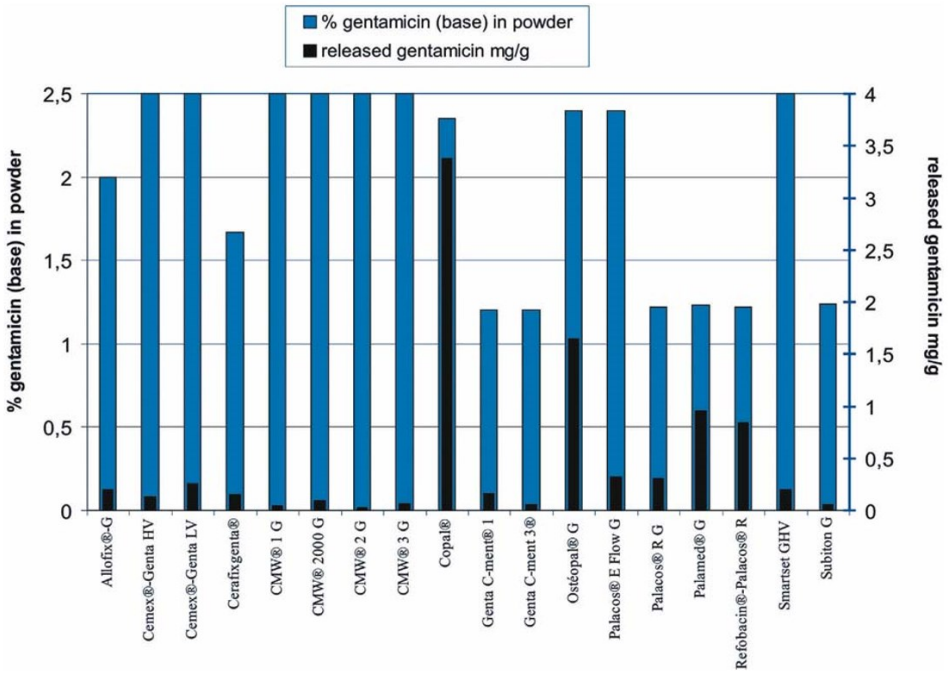

Figure 4: Commercially available PMMA cements and gentamicin release. Different preparations show stark differences in antibiotic release irrespective of the total amount incorporated. The elution properties of the PMMA cement limits the availability of antibiotics in the joint cavity and therefore directly impacts on the antimicrobial efficacy. Taken from reference (14).

1.4 Antibiotics

Antibiotics are commonly defined as substances derived from microorganisms that share the ability to inhibit the growth or to destroy other microorganisms. Although the term generally includes all kinds microorganisms like fungi or eukaryotic species, it is nowadays almost exclusively used for chemicals

effective against bacteria. Extensive research in this field lead to numerous discoveries and gave humanity an extensive set of antibiotic agents capable of treating a vast number of infectious diseases. Nevertheless, overuse in medical settings and especially application in livestock farming to increase productivity gave rise to an ever-growing number of resistant bacterial strains that pose a serious threat to our global health care system and to us, the patients. In order to understand how this ongoing arms race between bacterial resistances and biomedical research works, it is crucial to know about the history of antibiotics and their mechanism of action.

1.4.1 Antibiosis and the discovery of penicillin

The first written trace of antibiosis dates back to 1874 when William Roberts – a welsh physician practicing in Manchester, England – published his work on the growth of bacteria in the presence of *Penicillium glaucum*. He observed that liquids contaminated with this mould fungus were very hard to infect with bacteria and postulated the antagonistic nature of this affiliation without providing further molecular insights (15).

About 30 years later, the French physician Ernest Duchesne performed ground-breaking experiments on the potential therapeutic usability of these interactions. He infected guinea pigs with virulent bacterial cultures of *Salmonella enterica sp.* and *Escherichia coli.* He then used media containing *P. glaucum* to successfully treat the infection. Although giving conclusive information about the antibacterial potential of fungus moulds, Duchesne´s research remained almost completely unnoticed (16).

It was Alexander Fleming in the 1920s who shed light on the molecular nature of the observed antagonism and who thereby laid the cornerstone for the antibiotic era. During summer 1928, Fleming was performing experiments on the relationship between the colony morphology and the virulence of *Staphylococcus spp*. When leaving for holidays that year he forgot some inoculated plates on the corner of his laboratory bench and found them to be contaminated with moulds when he finally returned to the lab. Surprisingly, he found that the mould colonies changed the morphology of the staphylococcal colonies in close proximity. The clear appearance of those affected colonies reminded him of lytic processes he observed in earlier experiments on the antimicrobial activity of lysozyme. This observation gave rise to the idea that moulds might secrete some kind of antibacterial molecule (17). After carefully cultivating the mould, he identified as *Penicillium notatum*, Fleming and colleagues were able to isolate the effect causing component and termed it penicillin. Nevertheless, the inability to isolate or produce enough active substance kept Fleming from performing any clinical experiments or studies (18).

In 1939 the joint efforts of Howard Walter Florey and Ernst Chain at the University of Oxford established an efficient method to generate large amounts of penicillin (19). Only one year later, the first successful clinical studies were performed. Since Florey and colleagues were not able to produce enough penicillin for larger studies in their laboratory, they went seeking for investors in the US. The vast interest of numerous large pharmaceutical companies paved the way for large scale production and extensive clinical studies. In 1946, one year after Fleming, Florey and Chain received the Nobel Prize for medicine, penicillin was finally available on the open market (17).

1.4.2 ß-lactam rings and structural alternations

The first structural data on penicillin was published in 1949. Interestingly, the penicillin molecules isolated from the English and the American *Penicillium* species - despite both being effective - differed in molecular structure. It became evident that the shared structural feature that conferred bactericidal activity was the so-called ß-lactam ring, which was eponymous for the soon to be established group of penicillin antibiotics (see Figure 5) (20).

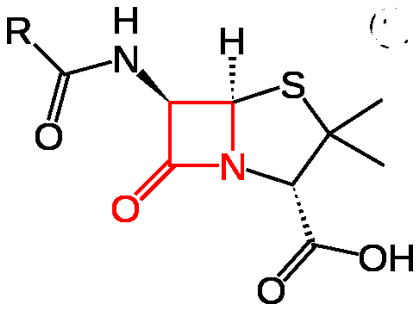

Figure 5: ß-lactam ring. The shown structure represents a typical penicillin antibiotic with the ß-lactam moiety coloured red.

Based on the fact that structural modifications may alter the efficacy of penicillin antibiotics, tremendous efforts were taken to synthesize new and active derivates. In 1959, J.C. Sheehan discovered that the ß-lactam ring containing 6-aminopenicillanic acid (6-APA) (see Figure 7a) constituted the perfect starting point for modifications (21,22). This important discovery marks the

starting point for the development of a vast number of clinically relevant ß-lactam antibiotics like aminopenicillines or ureidopenicillines.

Although chemical modifications of 6-APA held great potential, the search for other natural antibiotics continued. In 1961, Abraham and Newton published their discovery of a novel ß-lactam antibiotic isolated from *Cephalosporium acremonium* that contained 7-aminocephalosporinic acid (7-ACA) instead of 6-ACA as a core component (see Figure 7b). In line with this, they termed this new group of ß-lactam antibiotics cephalosporins (23). By using 7-ACA as a starting point, several generations of cephalosporins were created and successfully introduced into clinical practice.

Notably, penicillines and cephalosporins both possess a ß-lactam ring but constitute two distinct subgroups in the group of ß-lactam antibiotics.

1.4.3 ß-lactam antibiotics: mechanism of action

The key feature of antibiotics is the ability to harm bacterial cells while exhibiting minimal effect on the eukaryotic cells. Such a selective effect can only be achieved by targeting structures that are present in bacterial but absent from eukaryotic cells.

In case of ß-lactam antibiotics, the bacterial cell wall and especially its synthesis is targeted. During bacterial growth and cell division it is essential for the cell to modify its otherwise rigid cell wall by making and releasing bonds between its constituents. The bacterial cell wall can be described as a huge macromolecule of peptidoglycan. Peptidoglycan consists of backbones made up by alternating residues of N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) connected via β(1→4) glycosidic bonds. These elongated polysaccharide molecules are interconnected via short oligopetide chains that can differ in composition depending on the respective bacterial species. To connect one polysaccharide strand to another, an enzyme known as DD-transpeptidase is needed to establish a covalent bond between the D-alanine residues and other amino acids frequently present in the oligopeptides. Figure 6 depicts the relevant structures of peptidoglycan.

Chemically, the ß-lactam ring to be found in penicillines and cephalosporins is a dipeptide formed by the two amino acids L-cysteine and D-valine. As to be seen in Figure 7, the molecular structure of the ß-lactam ring in 6-APA and 7-ACA closely resembles the natural conformation of a D-alanyl-D-alanine residue. All three structures harbour the highly reactive CO-N motif needed for the reaction to performed by DD-transpeptidases. The reaction cannot be completed in case of ß-lactam antibiotics and the antibiotic will remain irreversibly bound to the DD-transpeptidase causing its complete inactivity. Ultimately, the shortage in cell wall modifying enzymes will cause osmotic vulnerability and

cause the lysis of the bacterial cell. Although DD-transpeptidases show the greatest vulnerability towards ß-lactam antibiotics, there are numerous other ß-lactam interacting proteins known as penicillin binding proteins (PDBs) that may convey antibiotic effects (17).

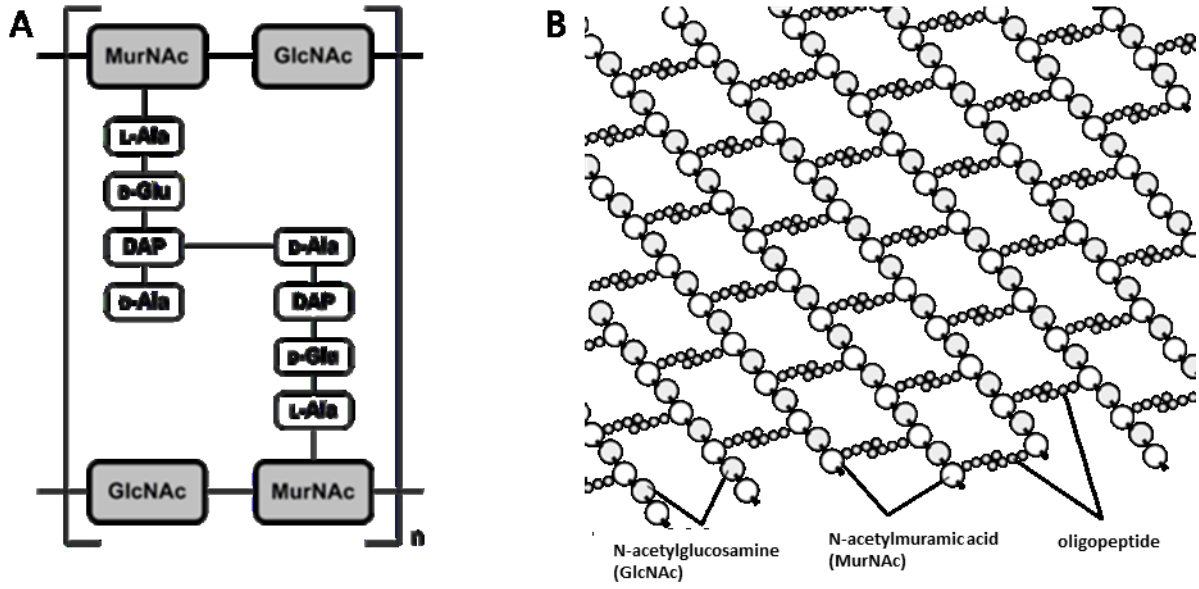

Figure 6: Cell wall structure. Panel A shows the typical oligopeptide connection between the polysaccharide backbones formed by of N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc). The bond between both peptide strands is formed by the DD-transpeptidase, which in turn is inhibited by ß-lactam antibiotics. The brackets imply the polymeric nature of the shown complex. Panel B provides an overview of the bacterial cell wall structure with polysaccharide backbones interconnected by oligopeptide bridges.

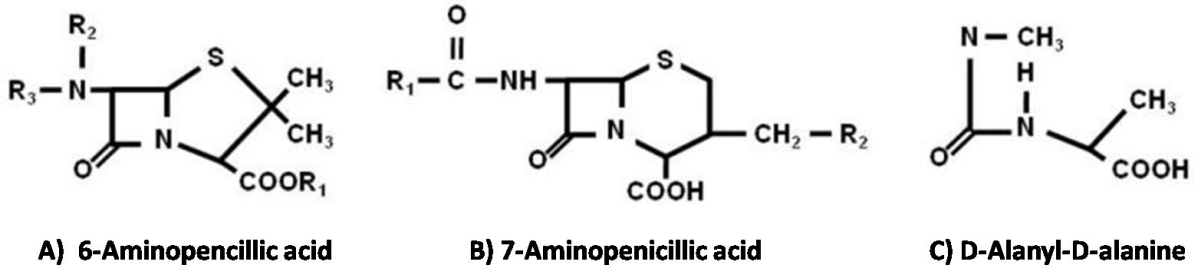

Figure 7: Core structures of ß-lactam antibiotics. Panel A shows 6-aminopenicillanic acid (6-APA), which forms the core of penicillin antibiotics. Panel B shows the 7-aminocephalosporinic acid (7-ACA) that forms the core of cephalosporin antibiotics. Panel C depicts the structure of the D-alanyl-D-alanine dimer found in bacterial cell walls. The striking similarity towards the other shown structures elucidates the mechanism in which ß-lactam antibiotics inhibit the DD-transpeptidase.

1.4.4 Theoretical considerations on the usage of ß-lactam antibiotics in PMMA bone cements

The combination of antibiotics with PMMA cements often is hampered by the fact that a large number of antibiotic agents are susceptible to non-enzymatic degradation induced by external factors like temperature or pH. While being the most important structural feature for antimicrobial activity, the ß-lactam ring of penicillins and cephalosporins is susceptible to spontaneous hydrolysis in aqueous environments. As depicted in Figure 8, in a first step the bond between the carbonyl group and the tertiary amine is hydrolysed, leaving a secondary amine and a carboxyl group, of which the latter undergoes spontaneous decarboxylation. The cleavage of the bond results in complete loss of antimicrobial activity, irrespective of side chains or other molecular features.

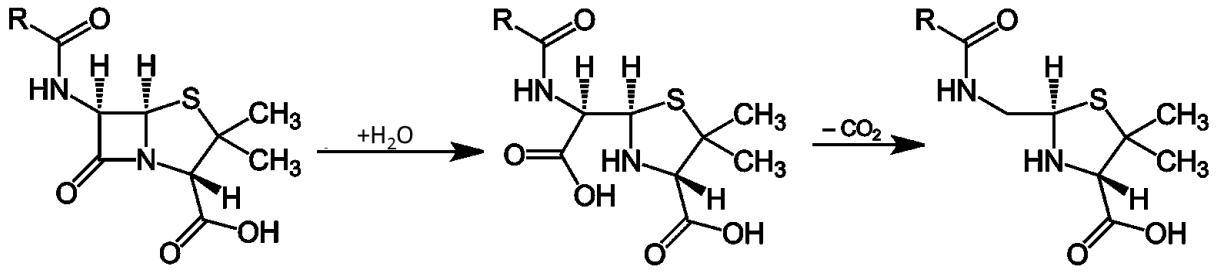

Figure 8: spontaneous hydrolysis of ß-lactam antibiotics in aqueous environments. Step 1: Hydrolysis between the carbonyl group and the tertiary amine, leaving a secondary amine and a carboxyl group. Step 2: Decarboxylation of the carboxyl group.

The reaction speed is strongly dependent on temperature and pH-value. Figure 9a shows an Arrhenius blot of ampicillin hydrolysis in dependence of temperature and pH. The x-axis shows the reciprocal temperature (lowest temperature right, highest left) and the y-axis the negative decadic logarithm of the reaction rate constant k. Since the temperature leads to exponential increments of the reaction speed, an Arrhenius blot serves to linearize the graph and allows a better visualization of dependencies. For each tested pH value an exponential increase of the reaction time with the temperature can be observed although the individual slopes show some degree of variation. Overall reaction speeds were the highest at a pH of 9.78 followed by pH 1.35 and the lowest at pH 4.93. Figure 9b shows the reactions speeds in different buffer systems in dependence on the pH. For all tested conditions, a global minimum can be observed in the range between 4.5 and 7.5 (24).

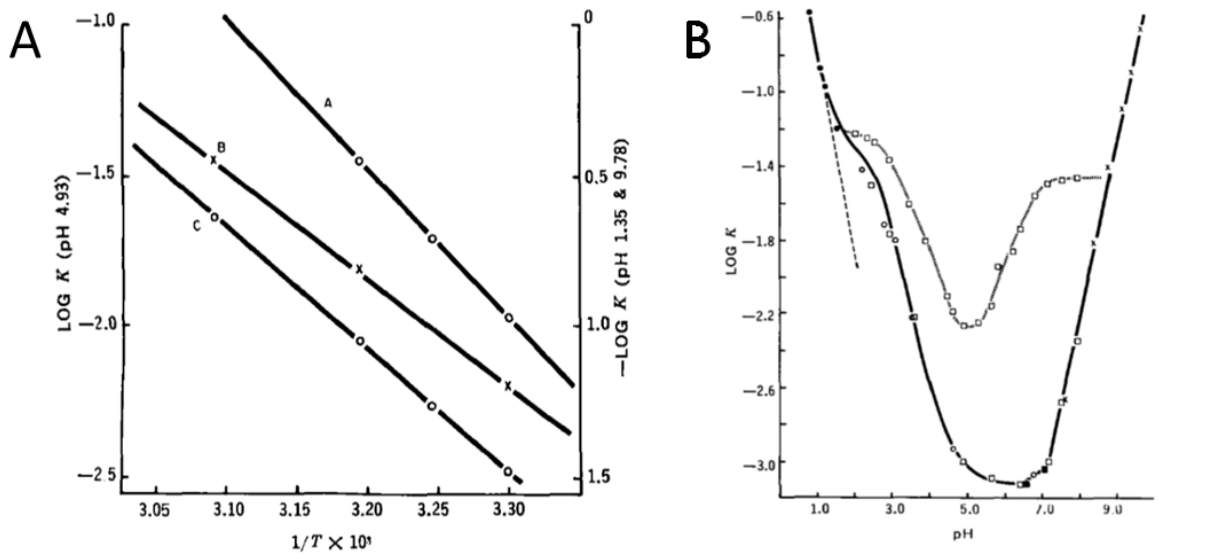

Figure 9: The impact of pH and temperature and pH on spontaneous ß-lactam hydrolysis. Panel A: The Arrhenius blot displays the influence of temperature and pH on the spontaneous hydrolysis of ampicillin (A: pH=9.78; B: pH=1.35; C: pH=4.93). The higher the temperature, the higher the reaction speed of hydrolysis in all tested conditions. The reaction speed increased in the following order: pH=4.93 < pH=1.35 < pH=9.78. Panel B: The graph shows the influence of the pH on the reaction speed in different butter systems. In all tested buffer systems, the reaction speed showed a global minimum between pH 4.5 and 7.5. Taken from reference (24).

The polymerisation of MMA takes place at slightly acidic pH values of around 6 and gives rise to temperatures of up to 60°C. Given the fact that the reaction rate constant shows lowest values in such pH ranges, one can conclude this influence on ß-lactam degradation to be minor and not causative to any significant loss of antimicrobial activity. To evaluate the impact of the elevated temperature, Table 1 shows the MICs of all used antibiotics at RT, 56°C and 121°C. For nearly all tested conditions the MIC at 56°C does not differ from the one at room temperature (RT). Although the temperatures generated during MMA polymerization may be slightly higher, a significant impact on ß-lactam integrity can be regarded as unlikely.

In conclusion, neither temperature nor pH during MMA polymerisation should cause a significant rise in ß-lactam hydrolysis. Hence, there are no theoretical concerns about the stability of ß-lactam antibiotics in PMMA cements.

Table 1: MICs of ampicillin, cefuroxime and piperacillin/tazobactam at RT, 56°C and 121°C. Individual samples of the antibiotics were incubated at room temperature (RT) and 56°C for 30min and at 121°C for 15min. The MIC was determined using inhibition zone assays. All depicted values are mg/l. Adapted from reference (25).

	S. aureus (ATCC 25923)			*S. aureus* (ATCC 29213)			*E. coli* (ATCC 25922)			*E. coli* (ATCC 35218)			*B. subtilis* (ATCC 6633)		
	RT	56°C	121°C	RT	56°C	121°C	RT	56°C	121°C	RT	56°C	121°C	RT	56°C	121°C
Ampicillin	<0,125	<0,125	0,5	1	1	4	4	4	16	NA	NA	NA	<0,125	<0,125	<0,125
Piperacillin/Tazobactam	0,5	1	16	1	1	16	2	2	32	4	8	32	0,5	0,5	2
Cefuroxime	1	1	>256	1	1	>256	4	4	256	2	2	>256	0,5	0,5	128

1.4.5 Ampicillin

Ampicillin is a ß-lactam antibiotic derived from 6-APA and therefore belongs in the group of penicillin antibiotics. As depicted in Figure 10, its structure only differs from benzylpenicillin (penicillin G) by a primary amine group introduced at the methylene part of the benzyl group. Due to this modification penicillin G´s problem of acid lability was overcome and ampicillin could be administered orally but remained ß-lactamase sensitive. It was the first broad-spectrum antibiotic to be introduced to the open market. Compared to penicillin G, the added amine group caused a slight loss of efficacy against Gram-positive bacteria but made ampicillin way more effective against Gram-negative germs (26). Nowadays, ampicillin is used extensively to treat a large number of infections (27).

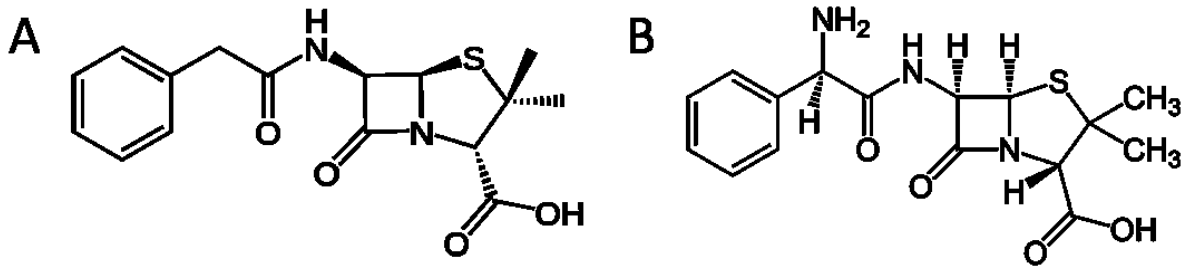

Figure 10: Structures of benzylpenicillin (penicillin G) and ampicillin. Panel A: Penicillin G. Panel B: Ampicillin. The primary amine group at the methylene part of the benzyl group in ampicillin is the only structural difference to penicillin G.

1.4.6 Cefuroxime

Cefuroxime is an antibiotic derived from 7-ACA and therefore is part of the group of 2nd generation cephalosporin antibiotics (see Figure 11). Cefuroxime was the first cephalosporin to become widely available for i.m. and i.v. applications. Due to its low pKa of 2.5, the carboxylic function located at the

central heterocycle will entail a negative charge in physiologic pH and impede any membrane passage. Hence, the prodrug cefuroxime axetil, the acetyloxyethyl ester of cefuroxime, was introduced to increase bioavailability after oral administration. During the intestinal passage, the ester bond between the carboxylic function of cefuroxime and the acetyloxyethyl spacer will be hydrolysed spontaneously or by esterases to convert the prodrug into its active form (28). The major benefit accounting for the huge clinical relevance of cephalosporins is their high stability towards ß-lactamases. Compared to cephalosporin C, the first cephalosporin discovered, numerous structural modifications enable cefuroxime´s broad efficacy against Gram-positive as well as Gram-negative bacteria (29,30).

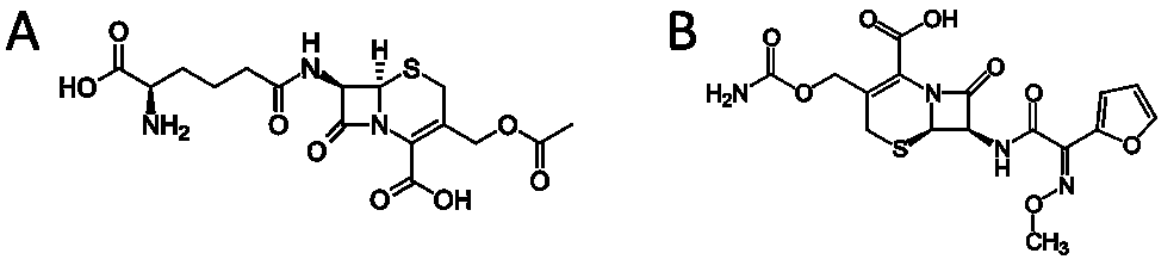

Figure 11: Structures of cephalosporin C and cefuroxime.

1.4.7 Piperacillin + tazobactam

Piperacillin is a semi synthetic ß-lactam antibiotic derived from 6-APA with a structure somewhat similar to ampicillin. Among a few others, piperacillin is classified as an ureidopenicillin. Members of this group are derivates of ampicillin that possess a number of cyclic urea repetitions attached to the amine group (see Figure 12). Compared to other penicillin antibiotics, piperacillin has the broadest spectrum by far (31,32). Especially its efficacy against *Pseudomonas sp.* paved its way to great clinical relevance although it is not ß-lactamase stable. In order to overcome this problem and to extend the spectrum towards ß-lactamase producing strains, piperacillin is often used in combination with tazobactam, an ß-lactamase inhibitor with negligible antibacterial properties (33,34).

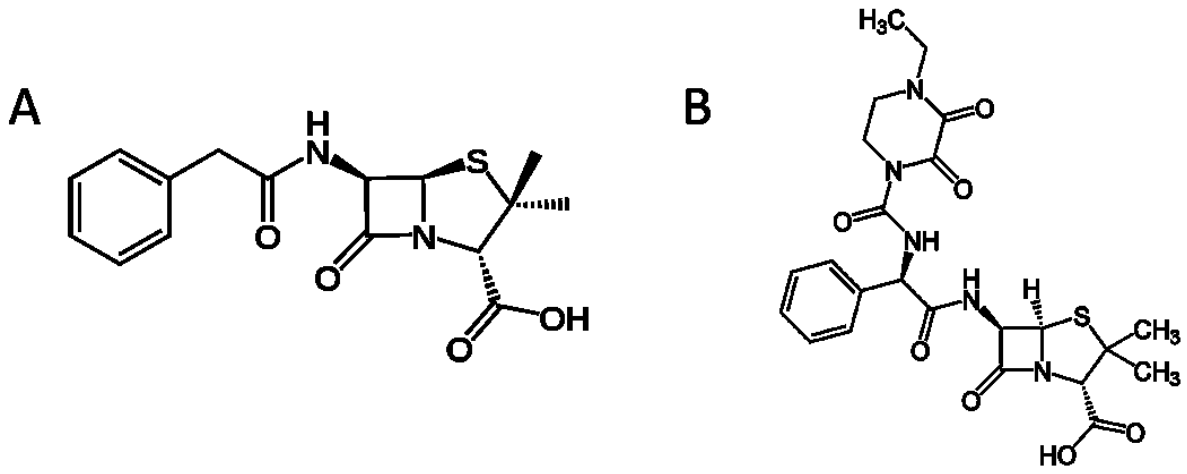

Figure 12: Structures of benzylpenicillin (penicillin G) and piperacillin.

1.4.8 Other relevant antibiotics

1.4.8.1 Gentamicin

Unlike the other mentioned antibiotics, gentamicin does not describe one defined component but a mixture of related molecular species. Gentamicines are aminoglycosides produced by the bacterium *Micromonospora purpurea* and related species, that exhibit bactericidal effects by inhibiting bacterial protein synthesis. While there is a plethora of different gentamicines, only the sulfate salts of type C gentamicines, namely C_1, C_{1a} and C_2, are used as antibiotic drugs (see Figure 13) (35). Gentamicin is a broad spectrum antibiotic, that is effective against a broad range of Gram-positive and especially Gram-negative species. Due to its severe side effects is mostly used as an systemic emergency treatment (36) or locally as additives in supporting materials like PMMA bone cements (37). Interestingly, *in vitro* and *in vivo* studies found synergistic effects between gentamicin and ß-lactam antibiotics (38).

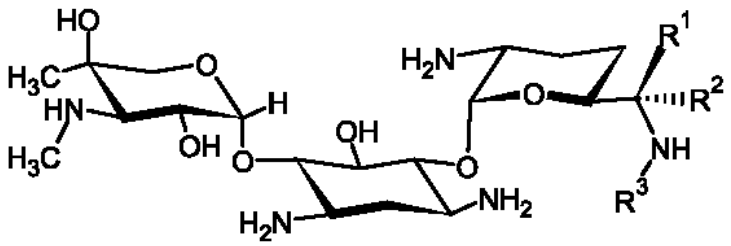

Figure 13: Structure of gentamicin type C.

1.4.8.2 Vancomycin

Vancomycin is a glycopeptide antibiotic (see Figure 14) initially isolated from *Streptomyces orientalis* in 1956 (39), and vancomycin hydrochloride is used ever since to treat infections with multi-resistant, Gram-positive species like for example MRSA (40). While being effective against Gram-positives, it exhibits no significant effect on Gram-negative strains. Mechanistically, vancomycin is able to bind and chelate the pentapeptide portion of peptidoglycan. This impairs transpeptidation and leads to a lethal inhibition of the bacterial cell wall synthesis (41).

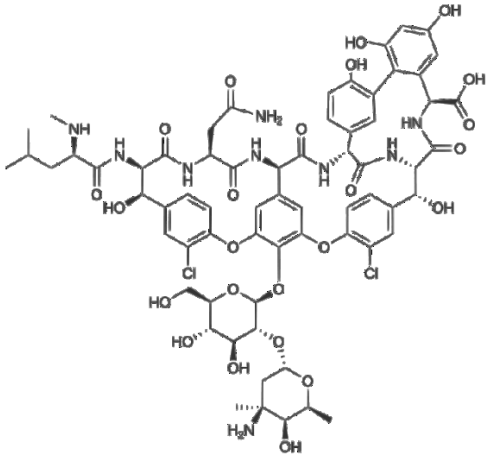

Figure 14: Structure of vancomycin.

1.4.8.3 Clindamycin

Clindamycin is an semi-synthetic antibiotic derived from lincomycin, which was originally discovered and isolated from *Streptomyces lincolnensis* (42). Both substances belong to the group of licosamide antibiotics with clindamycin being the chlorinated form of lincomycin (see Figure 15). Licosamides act bacteriostatic in moderate concentrations but bactericidal in high concentrations by binding the 50S subunit of prokaryotic ribosomes and inhibiting protein synthesis. Clindamycin is frequently used to treat infections with Gram-positive as well as Gram-negative anaerobic germs (43). Interestingly, studies found that this inhibitory stimulus on protein synthesis was sufficient to reduce of the expression of ß-lactamases in otherwise ß-lactam resistant strains to render them more sensitive towards ß-lactam antibiotics (44). Since macrolides and licosamide antbiotics share the same molecular target without having any significant structural relation, cross resistances often develop between these two classes (43).

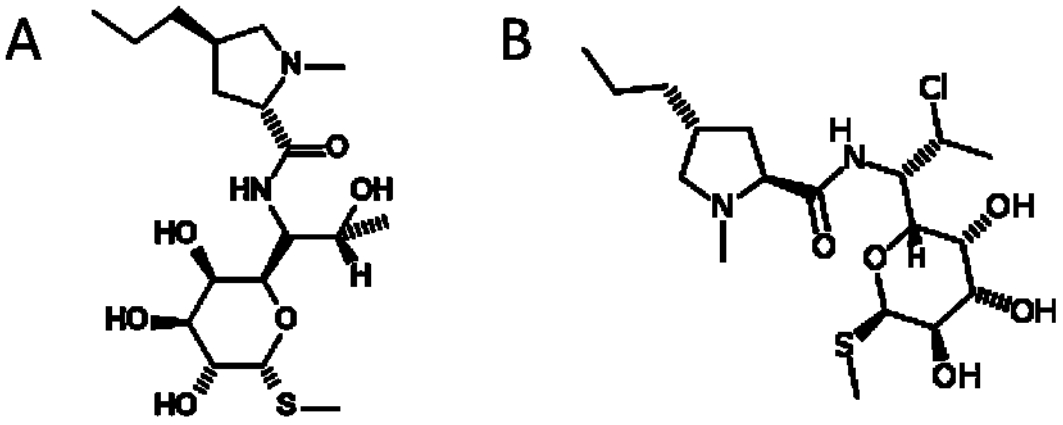

Figure 15: Structures of lincomycin and clindamycin.

1.5 Currently available data

The Pro-Implant Foundation (PIF) periodically issues detailed and pathogen specific recommendations on which antibiotic agents are to be used as a PMMA supplement during one- or two-step revision protocols. Due to the lack of high quality data on the safety and efficacy and based on worries about the general compatibility with PMMA cements, ß-lactam antibiotics are only rarely or not at all recommended (45). A recent systematic review on ALBC found that there are only few studies using ß-lactam antibiotics and that most of those are of poor quality (46). Since, on the other hand, available data strongly favours the general use of ALBC in order to prevent infections in primary TJAs or to treat infections in secondary TJAs (47,48), it is important to generate solid data for ß-lactam antibiotics that may allow the establishment of recommendations for this group of antibiotics.

Table 2 provides an overview of the currently available data on the use of ampicillin, cefuroxime and piperacillin in PMMA cements.

Table 2: Available data on the use of ampicillin, cefuroxime and piperacillin in PMMA cements.

Antibiotic	study type	PMID	cement	concentration	elution	microbiological efficacy	mechanical assessment	tested strains	key finding
Ampicillin	in vitro	24231380	CMW 3	10%/20%	YES	YES	NA	NA	almost no elution from cement observed
	in vitro	22110086	NA	NA	NA	NA	NA	E. faecalis	the combination of ampicillin with gentamicin is beneficial in the prevention of PJI
	in vitro	16464896	NA	NA	NA	NA	NA	E. faecalis / E. faecium	ampicillin alone is not sufficient to treat established biofilms
Cefuroxime	in vitro	17042225	Simplex P	3,75%/7,5%/ 11,25%	NA	NA	YES	NA	mechanical stability critically impaired above 4%w/w
	systematic review	PMC3861452	NA	NA	NA	NA	NA	NA	cefuroxime containing ALBC efficiently prevents PJI
	clinical study	12004017	NA	NA	NA	NA	NA	NA	cefuroxime containing ALBC efficiently prevents PJI
Piperacillin/ Tazobactam	in vitro	16340189	Simplex P	1,60%	YES	NA	NA	Micrococcus luteus	piperacillin shows low elution from Simplex P cements
	in vitro	PMC3809714	Simplex P	2,50%	YES	YES	NA	P. aeruginosa	piperacillin shows low elution from Simplex P cements with low antimicrobial activity

1.5.1 Ampicillin

A study using CMW® medium viscosity bone cement (DePuy, Inc Warsaw, IN, USA) tested ampicillin containing beads (10% and 20%w/w) for elution and antimicrobial efficacy. The HPLC elution profile (see Figure 16) revealed that in both cases almost no antibiotic was eluted and only very small inhibition zones were observed (49). In contrast, chitosan microspheres showed elevated ampicillin elution profiles, when combined with PMMA (50). An *in vitro* Study without usage of PMMA cement evaluated ampicillin´s effect on *E. faecali*s isolates from 15 PJIs with MIC- and MBEC-values of 0.5 mg/l (0.25–2) and 256 mg/l (128–512), respectively. Furthermore, it was shown that ampicillin is not able to completely eradicate *E. faecalis* biofilms, even if it´s still in a nascent stage (51). Additionally, it was shown that the combination of ampicillin with gentamicin may significantly decrease MICs and MBECs for some PJI isolates, rendering this combination attractive for ALBC applications (52). A retrospective study on musculoskeletal infection found resistances against gentamicin and ampicillin not to be strictly coinciding (53) and, in line with this, a case report covered the successful treatment of a PJI with gentamicin ALBC and systemic ampicillin during a two-stage revision protocol (54).

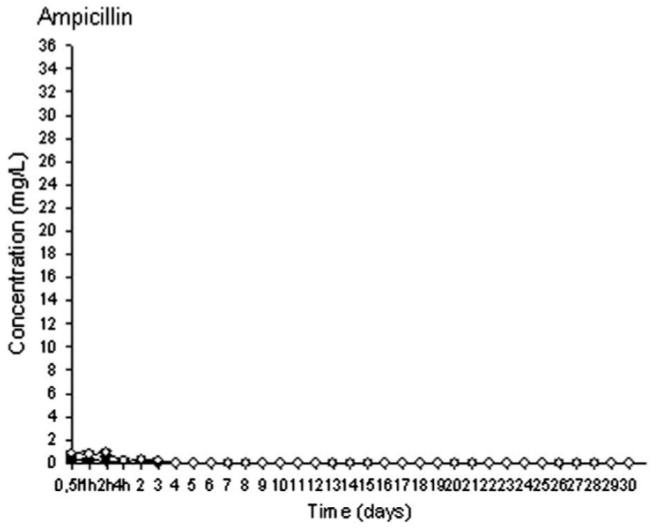

Figure 16: Ampicillin elution form CMV® beads. Both concentrations (10%w/w black circles and 20%w/w white circles) showed only very limited elution of ampicillin from the beads. Taken from reference (49).

1.5.2 Cefuroxime

An *in vitro* study using Simplex® P cement (Stryker) recommends not to add more than 4%w/w without assessing antimicrobial efficacy (55). No *in vitro* data on antimicrobial efficacy was provided. A systematic review on the usage of ALBC containing cefuroxime in primary TJA found reduced rates in deep infections, while the rate of superficially infections was not significantly altered compared to systemic antibiosis or the usage of antibiotic-free bone cement (48). Another study comparing cefuroxime containing bone cement against antibiotic-free cement found similar results (56). Furthermore, it was shown that cefuroxime containing ALBC alone was as efficient as systemic cefuroxime application in the prevention of PJI, notably, with minimal systemic burden (57). In another study, the combination of gentamicin containing ALBC with systemic cefuroxime was effective in 90% of cases (58), rendering the combination of antibiotics suitable for PJI treatment. Conversely, an *in vitro* study found that a combination of gentamicin containing ABLC and systemic cefuroxime administration failed to eradicate established biofilms but was effective in early stages where the inoculum was lower (59).

1.5.3 Piperacillin/tazobactam

A study comparing piperacillin/tazobactam release form capsules and Simplex® P PMMA cement beads (Stryker) found that elution from PMMA is low and averaged in the range from 12 to 50 µg/ml. Figure 18 shows the elution profile with the black circles representing release from the PMMA beads (60). Another in vitro study using Simplex® P PMMA cement (Stryker) only found relevant elution of piperacillin during the first day with maximal concentrations of 68.4 µg/ml. Accordingly, the growth of *P. aeruginosa* was only inhibited during day 1 (see Figure 17) (61).

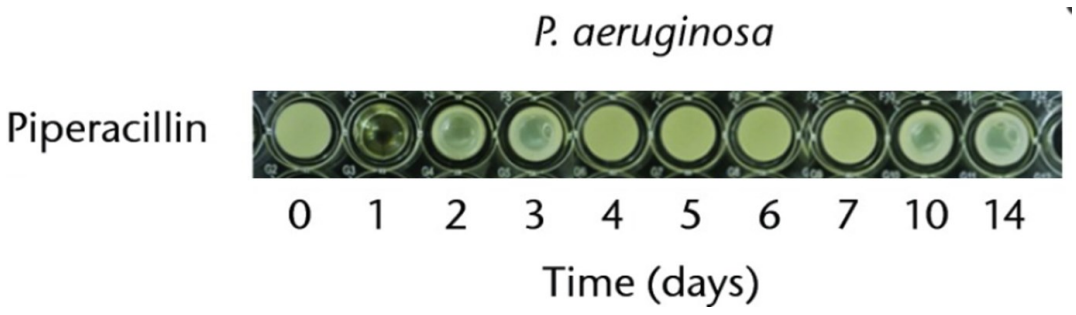

Figure 17: *P. aeruginosa* growth inhibition by piperacillin. Relevant growth inhibition was only seen for day 1. This finding harmonizes with the finding that only on day 1 relevant elution from the cement was observed.

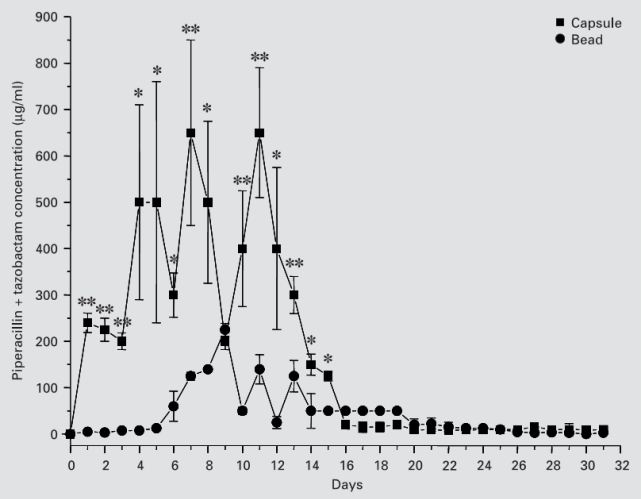

Figure 18: Piperacillin elution profiles. Black circles represent release from PMMA beads while the black squares represent release from capsules. Taken form reference (60)**.**

1.6 Experimental approach

The aim of this study was to generate reliable data on the usability of ampicillin, piperacillin/tazobactam and cefuroxime in PMMA cements to enable the establishment of informed recommendations based on this data. Therefore, cement bodies containing different concentrations of ß-lactam antibiotics were produced and subsequently tested for mechanical stability and antimicrobial efficacy. Figure 19 shows the PMMA cements used in this study.

Palacos® R	**high-viscosity standard cement** **contains no antibiotic**
Palacos® R+G	**high-viscosity standard cement** **contains 0.5g gentamicinsulfate**
Copal® G+C	**revision cement** **contains 1g gentamicinsulfate** **1g clindamycinhydrochloride**
Copal® G+V	**revision cement** **contains 0.5g gentamicinsulfate** **2g Vancomycinhydrochlorid**

Figure 19: Composition of used Palacos® and Copal® cements.

2 Material & Methods

2.1 Media, buffers and devices

a. Chemicals

Name	firma	cat#	LOT
Palacos® R	Heraeus		8703
Palacos® R + G	Heraeus		8713
Copal® G + C	Heraeus		8395
Copal® G + V	Heraeus		8674
Cefuroxime-Natrium	MIP Pharma		2723800
Ampicillin-Natrium	Ratiopharm		GU8446
Piperacillin/Tazobactam	Fresenius Kabi		5FL17106DE
Müller Hinton Agar	Oxoid	CM0337	2114571
Tryptone Soya Agar (TSA)	Oxoid	CM0131	1986191
Sheep Blood	Thermo scienfitic	SR0051E	32682200
PBS tablets	Amresco	E404-200TABS	103C412
Petri dishes			

b. Buffers and Media

Müller-Hinton Agar

Müller-Hinton Agar with 5% sheep blood

TSA

TSA with 5% sheep blood

c. Strains

Streptococcus agalactiae	ATCC 13813
Enterococcus faecalis	ATCC 29212
Proteus mirabilis	ATCC 12453
Escherichia coli	ATCC 25922
Klebsiella pneumoniae	ATCC 13883
Pseudomonas aeroginosa	ATCC 27853

2.2 Tested strains

2.2.1 Streptococcus agalactiae

Streptococcus agalactiae is a chain-forming, Gram-positive coccus with a diameter of approximately 2µm (see Figure 20). It lives facultatively anaerobic, grows at 37°C and belongs to Lancefield´s group B streptococci (62).

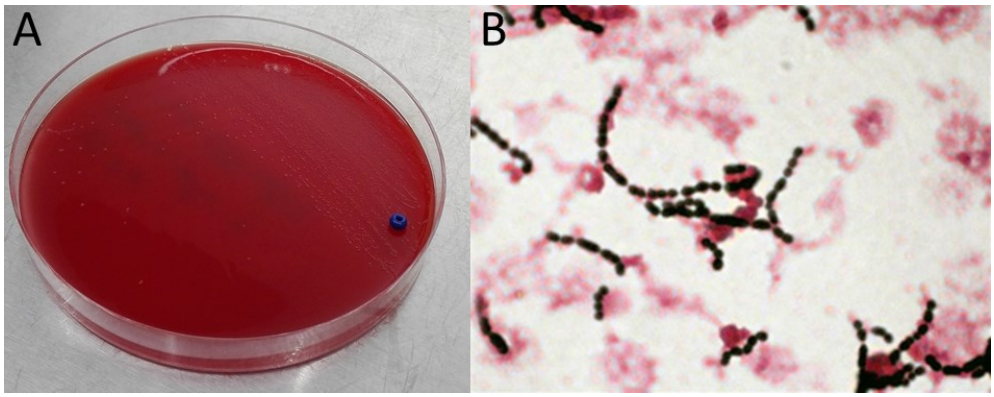

Figure 20: *S. agalactiae* morphology. Panel A: *S. agalactiae* colonies on TSA agar with 5% sheep blood. Panel B: microscopic picture of *S. agalactiae* cells. Taken from reference (63).

2.2.2 Enterococcus faecalis

Enterococcus faecalis is a chain-forming, Gram-positive coccus (see Figure 21). It is facultatively anaerobic, grows at 37°C and is to be found in the human and animal intestine (64).

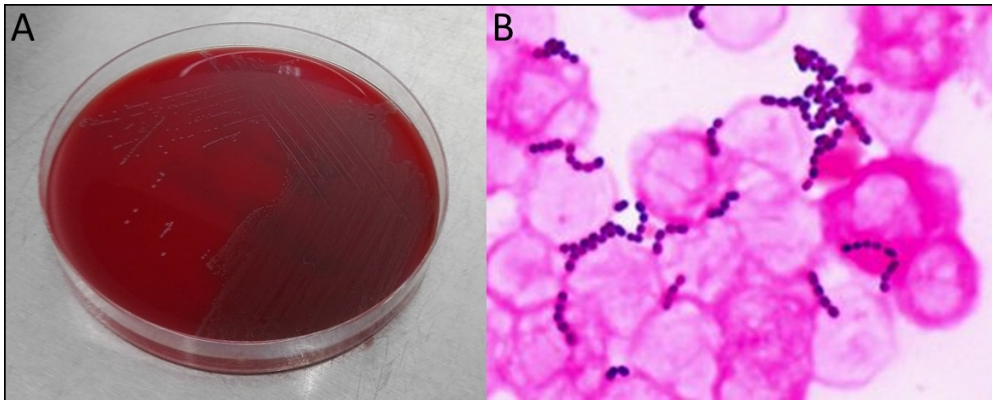

Figure 21: *E. faecalis* morphology. Panel A: *E. faecalis* colonies on TSA agar. Panel B: microscopic picture of *E. faecalis* cells. Taken from reference (65).

2.2.3 Proteus mirabilis

Proteus mirabilis is a chain-forming, Gram-negative, flagella-rich and therefore highly motile rod (see Figure 22). It is facultatively anaerobic and grows at 37°C (66,67).

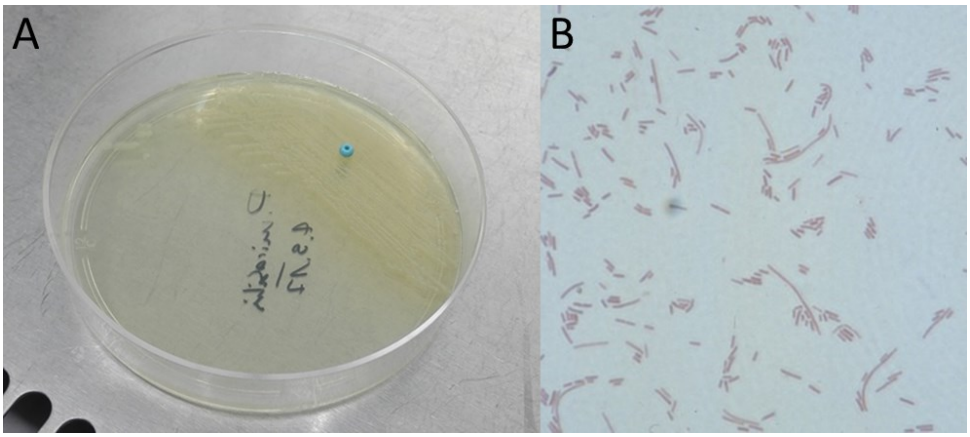

Figure 22: *P. mirabilis* morphology. Panel A: *P. mirabilis* colonies on TSA agar. Panel B: microscopic picture of *P. mirabilis* cells. Taken from reference (68).

2.2.4 Escherichia coli

Escherichia coli is a Gram-negative, peritricous and motile rod (see Figure 23). It is facultatively anaerobic, grows at 37°C and is to be found in the human and animal colon (69).

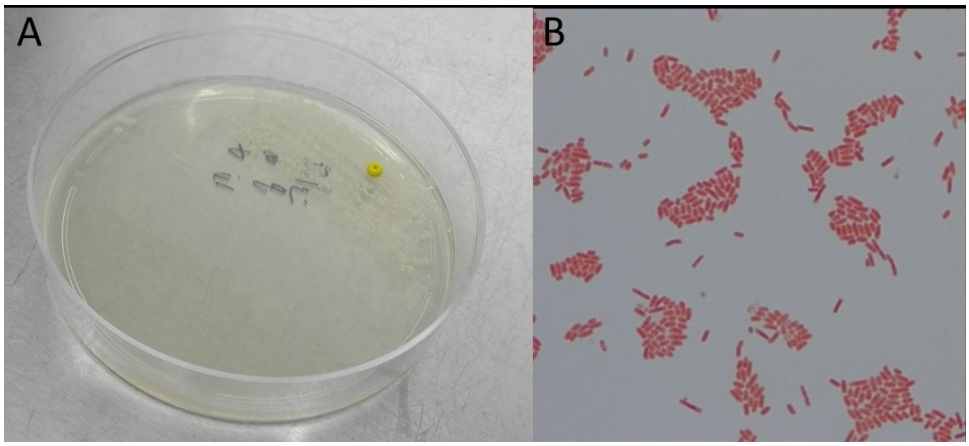

Figure 23: *E. coli* morphology. Panel A: *E. coli* colonies on TSA agar. Panel B: microscopic picture of *E. coli* cells. Taken from reference (70).

2.2.5 Klebsiella pneumoniae

Klebsiella pneumonia is a non- or short chain-forming, non-motile, Gram-negative rod (see Figure 24). It is facultatively anaerobic and growth at 37°C (71,72).

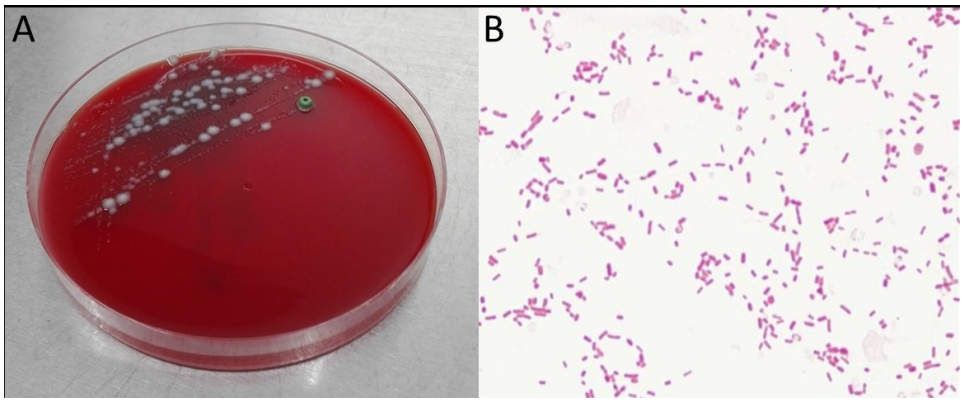

Figure 24: *K. pneumoniae* morphology. Panel A: *K. pneumoniae* colonies on TSA agar. Panel B: microscopic picture of *K. pneumoniae* cells. Taken from reference (73).

2.2.6 Pseudomonas aeruginosa

Pseudomonas aeruginosa is a small, monotrichous, motile, Gram-negative rod (see Figure 25). It is aerobic and grows at 37°C (see Figure 25)(74,75).

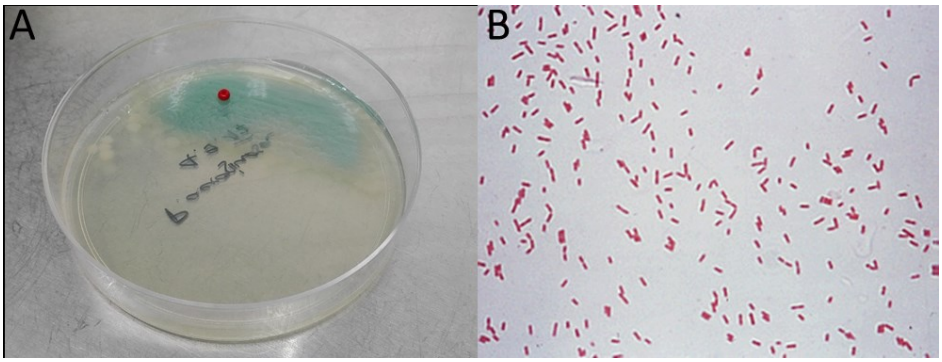

Figure 25: *P. aeruginosa* morphology. Panel A: *P. aeruginosa* colonies on TSA agar. Panel B: microscopic picture of *P. aeruginosa* cells. Taken from reference (76).

2.3 Preparation of cement bodies

To produce the cement bodies needed for subsequent experiments on mechanical properties and microbicidal efficacy only products commercially available from Heraeus were used.

One unit of the respective bone cement (~40g dry mass) was mixed with 20ml of monomer containing liquid in a single-use plastic cup. In order maximize reproducibility and homogeneity, the liquid was poured before the cement was added and a timer was used to standardize the preparation procedure. After adding the powder, both components were stirred using a metal spatula for approximately 30sec. Afterwards the cement rested for 20sec until the surface became non-sticky. After removing the cement from the cup it was hand kneaded for approximately 60sec and filled into the moulds (Figure 26). The filled moulds were pressed for 30min at 3bar using a hydraulic press. Subsequently, the cement bodies for microbiological testing were removed using a plastic hammer, the DIN 53435 bodies were removed by hammering using a pin punch and ISO 5833 bodies were cut in shape using a laser cutter. See Table 3 for an overview over all produced cement-antibiotic combinations and the performed assessments, where stability indicates testing for Dynstat impact strength and four-point bending and efficacy indicates testing for microbiocidal activity via inhibition zone assays.

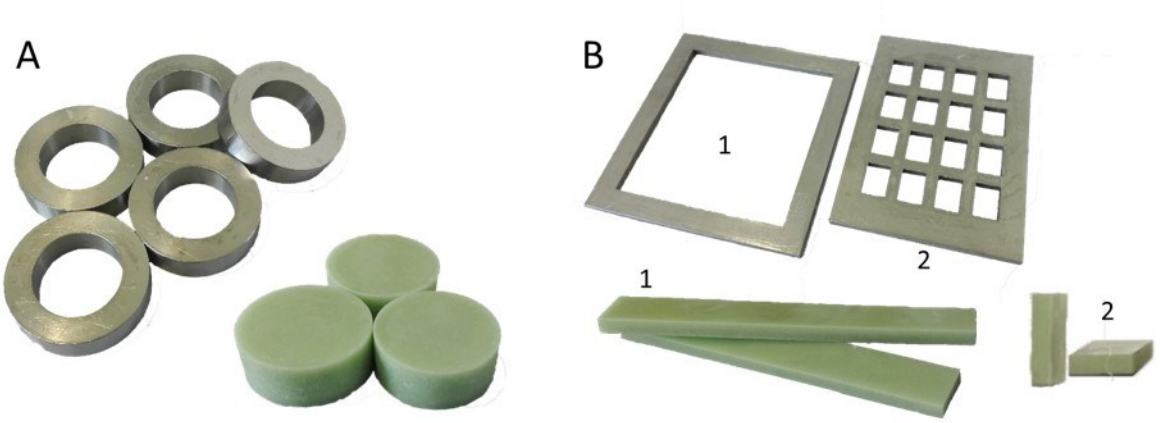

Figure 26: Preparation of cement bodies. Metal moulds were used to cast the cement bodies needed for mechanical and microbiologic assessments. Panel A: cylindrical cement bodies for microbiologic assessments and the corresponding moulds. Panel B: cement bodies for mechanical assessments and the corresponding moulds. 1: ISO 5833 bodies and moulds; 2: DIN 53435 bodies and moulds.

Table 3: Produces cement bodies and performed assessments. Mechanics indicates testing for Dynstat impact strength, bending modulus and bending strength. Microbiology indicates testing for antimicrobial activity via inhibition zone assays.

cement type	antibiotic	mechanics	microbiology
Palacos® R	ampicillin 1 g		X
	cefuroxime 1 g		X
	piperacillin 4 g + tazobactam 0.5 g		X
	piperacillin 8 g + tazobactam 1 g		X
Palacos® RG	ampicillin 1 g	X	X
	ampicillin 3 g	X	X
	cefuroxime 3 g	X	X
	piperacillin 4 g + tazobactam 0.5 g	X	X
	piperacillin 8 g + tazobactam 1 g	X	X
Copal® GV	ampicillin 1 g		X
Copal® GC	piperacillin 4 g + tazobactam 0.5 g		X

2.4 Dynstat impact strength

The tests for Dynstat impact strength were performed according to DIN 53435. A device with an 0.5 Joule weight (see Figure 27) was used. Before every measurement all bodies were measured for broad- and thickness before being placed into the mount. Special attention was paid on a close contact of the bodies with the latter fixation to prevent any systemic error and variability. The measurement was performed by manually readjusting the pointer to 0.3 J on the 5 J scale, engaging the swing arm and releasing it using the lever. For every cement preparation, eight replicates were measured and the results were used to calculate the Dynstat impact strength according to equation (1).

$$a_N = 1000 * \frac{A_N}{B * T} \qquad (1)$$

a_N = Dynstat impact strength
A_N = impact in J
B = broadness in mm
T = thickness in mm

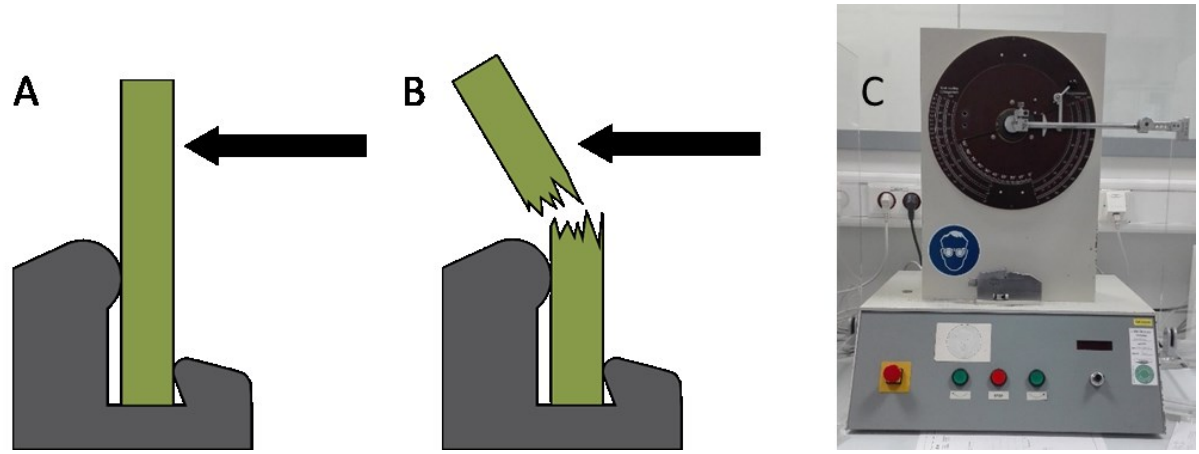

Figure 27: DIN 53435 (Dynstat bending strength) testing procedure. Panel A: The cement bodies were put into the mount with direct contact to the latter fixation. Panel B: The force exerted by the swing arm breaks the body and will cause the arm to loose kinetic energy. Panel C: The used apparatus.

2.5 Four-point bending test

The four-point bending tests were performed according to ISO 5833. A Zwick/Roell device running the testXpert® II software was used. Prior to every measurement all cement bodies were measured for broad- and thickness. Special attention was paid that the bodies were placed central on the device and that the distance probe was located centrally. For each cement preparation, five replicates were measured and the bending modulus as well as the bending strength were calculated and documented by the software.

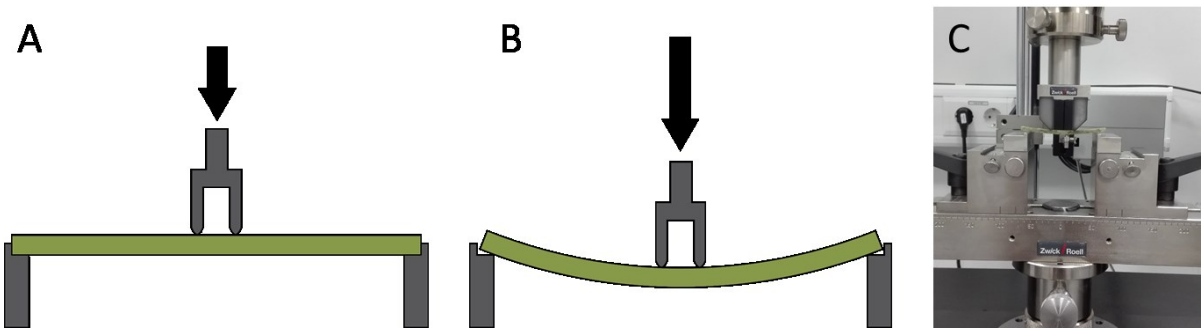

Figure 28: ISO5833 (bending modulus and bending strength) testing procedure. Panel A: The cement bodies (green) were put into the mount (grey). Panel B: The force exerted by the probe caused bending of the cement bodies. Deformation was automatically detected and processed. Panel C: The used apparatus.

2.6 Inhibition zone assay

To test for the antimicrobial efficacy of the different cement preparations a standard agar diffusion assay was performed (77). Since the cement bodies cannot be placed directly on the agar, we used eluates for the test. Unless indicated otherwise, all of the following steps were performed aseptically in a laminar flow-hood. Table 1 provides an overview over the used strains and the tested antibiotic-cement combinations. Figure 29 shows an overview of the whole process.

Table 4: Used strains and tested cement-antibiotic preparations.

strain	tested cement-antibiotic preparations
S. agalactiae	- Palacos® R + 1 g ampicillin
	- Palacos® RG + 1 g ampicillin
	- Palacos® RG + 3 g ampicillin
	- Copal® GV + 1 g ampicillin
E. faecalis	- Palacos® R + 1 g ampicillin
	- Palacos® RG + 1 g ampicillin
	- Palacos® RG + 3 g ampicillin
	- Copal® GV + 1 g ampicillin
P. mirabilis	- Palacos® R + 1 g ampicillin
	- Palacos® RG + 1 g ampicillin
	- Palacos® RG + 3 g ampicillin
	- Copal® GV + 1 g ampicillin
	- Palacos® R + 1 g cefuroxime
	- Palacos® RG + 3 g cefuroxime
K. pneumoniae	- Palacos® R + 1 g cefuroxime
	- Palacos® RG + 3 g cefuroxime
E. coli	- Palacos® R + 1 g cefuroxime
	- Palacos® RG + 3 g cefuroxime
P. aeruginosa	- Palacos® R + 4 g piperacillin + 0.5 g tazobactam
	- Palacos® R + 8 g piperacillin + 1 g tazobactam
	- Palacos® RG + 4 g piperacillin + 0.5 g tazobactam
	- Palacos® RG + 8 g piperacillin + 1 g tazobactam
	- Copal® GC + 4 g piperacillin + 0.5 g tazobactam

2.6.1 Elution

To elute the antibiotic agents, three cement bodies of every cement preparation were individually placed in a 50ml Falcon® tube containing 20ml of sterile 1x PBS. The tubes were placed up-side down and sealed using Parafilm® to prevent leakage. All tubes were incubated at room temperature. After 1h the cement bodies were removed from the tubes using a sterile forceps, gently dabbed off on a sterile tissue and put into a fresh falcon containing 20ml 1x PBS. The remaining liquid in the now cement body-free tubes (from now on referred to as eluate) was used for the inhibition zone assays performed on the same day. This process was repeated after 24h, 7d, 14d, 28d and 42d of incubation (see Figure 29).

2.6.2 Inhibition zone assay

For all of the used strains except *S.agalactiae* Müller-Hinton Agar plates were used. *S.agalactiae* was grown on Müller-Hinton Agar plates containing 5% of sheep blood. In accordance with the standard protocol for inhibition zone assays (77) all strains were adjusted to an McFarland turbidity standard of 0.5 and streaked out trebly. After the spreading of bacteria, the plates were kept half-open for approximately 10min to allow proper ingression of the bacterial suspension. Subsequently, a sterile glass Pasteur pipette´s broad end was used to punch a central hole on each plate into which 60µl of the respective eluate were pipetted. All plates except those containing *S.agalactiae* were incubated at 37°C under aerobic conditions for 24h. The plates containing *S.agalactiae* were incubated at 37°C under anaerobic conditions for 24h. After incubation the diameter of the inhibitory zones were measured and documented.

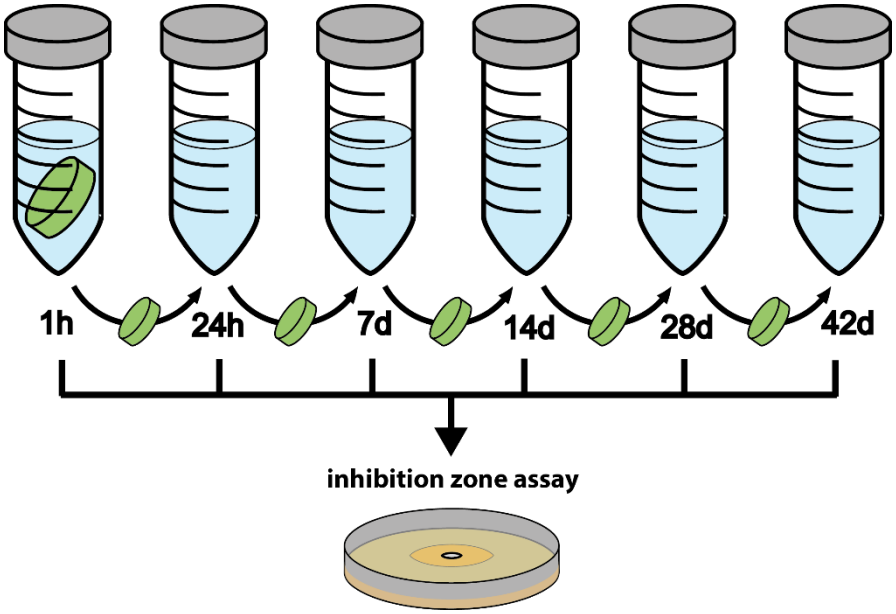

Figure 29: Inhibition zone assay. The cement bodies were incubated in 20ml 1x PBS until the indicated time points. After each time point the bodies were moved to a fresh tube and the previous eluates were used to run the inhibition zone assay.

3 Results

3.1 Visual evaluation

Inhomogeneities pose significant threads to the stability of bone cements. Any constituents like e.g. antibiotics that are not capable of being incorporated into the polymer may cause decreased stability and therefore limit the clinical application.

All antibiotics were used in powder form and are depicted in Figure 30. Ampicillin tended to form small aggregates, while piperacillin/tazobactam and cefuroxime remained rather fine-grained. Accordingly, the moulds containing ampicillin showed the highest amount of visible impurities relative to the other antibiotics. Figure 31 and Figure 32 show the respective moulds and observed impurities. Notably, also Palacos® R+G without antibiotics showed a few white grain-like impurities while Palacos® R was free of any impurities.

Figure 30: Antibiotic powders. Panel A: Ampicillin. Panel B: Cefuroxime. Panel C: Piperacillin/tazobactam.

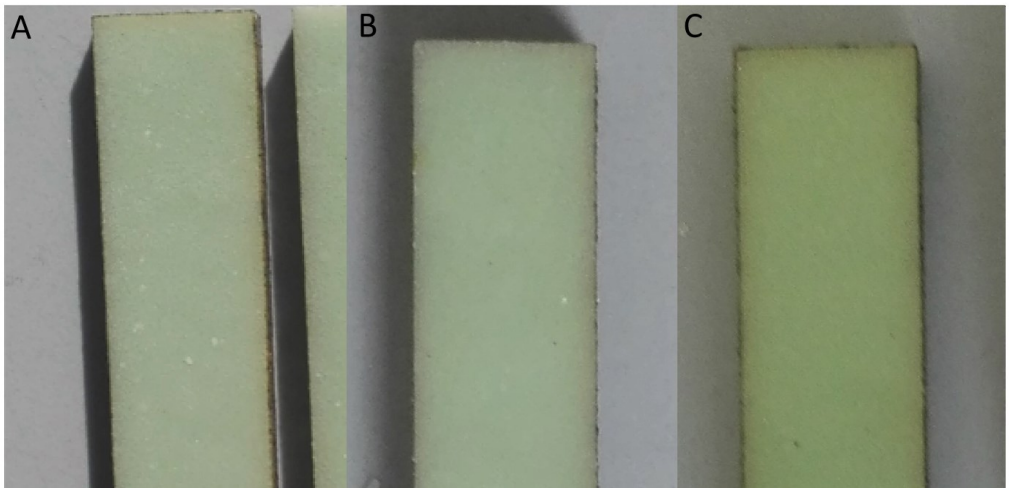

Figure 31: ISO 5833 cement body impurities. Panel A: Palacos® R+G with 3 g ampicillin. Panel B: Palacos® R+G with 3 g cefuroxime. Panel C: Palacos® R+G with 8 g piperacillin / 1 g tazobactam. Ampicillin addition led to clearly visible, multiple white spots, while the addition of 3 g cefuroxime resulted in fewer white spots. Piperacillin/tazobactam addition did not give rise to visible impurities.

Figure 32: Cement bodies for microbiologic assessments. Panel A: Palacos® R+G with 3 g ampicillin. Panel B: Palacos® R+G with 3 g cefuroxime. Panel C: Palacos® R+G with 4 g piperacillin/0.5 g tazobactam.

3.2 Mechanical assessments

3.2.1 DIN 53435 (Dynstat impact strength)

The tests for Dynstat impact strength produced the values depicted in Table 5. The directly measured impact values were used to calculate the respective Dysnstat impact strength values according to equation (1). The mean values were calculated according to equation (2), the standard deviation (s) according to equation (3) and the relative standard deviation according to equation (4).

$$\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i \qquad (2)$$

$\bar{x}$ = arithmetic mean
x = measurement
n = number of replicas

$$s = \sqrt{\frac{\sum(x - \bar{x})^2}{(n-1)}} \qquad (3)$$

s = standard deviation
x = measurement
$\bar{x}$ = arithmetic mean
n = number of replicas

$$\%s = \frac{s}{\bar{x}} \quad (4)$$

$\%s$ = relative standard deviation
$\bar{x}$ = arithmetic mean

Table 5: Dynstat impact strength raw data.

	replica	broadness (mm)	thickness (mm)	impact (J)	Dynstat impact strength (J/m^2)
Palacos® R+G reference	1	9.97	3.34	0.11	3.30
	2	10.02	3.36	0.09	2.67
	3	9.99	3.39	0.118	3.48
	4	10.04	3.43	0.087	2.53
	5	10	3.38	0.11	3.25
	6	9.99	3.39	0.103	3.04
	7	10	3.42	0.103	3.01
	8	10	3.35	0.091	2.72
	MW	**10.00**	**3.38**	**0.102**	**3.00**
	s	0.02	0.03	0.011	0.339
	%s	0.21	0.94	11.01	11.30
Palacos® R+G ampicillin 1 g	1	10.09	3.4	0.09	2.62
	2	10.07	3.4	0.11	3.21
	3	10.06	3.38	0.08	2.35
	4	10.09	3.4	0.101	2.94
	5	10.07	3.44	0.08	2.31
	6	10.08	3.41	0.085	2.47
	7	10.09	3.41	0.1	2.91
	8	10.03	3.42	0.105	3.06
	MW	**10.07**	**3.41**	**0.094**	**2.74**
	s	0.02	0.02	0.012	0.341
	%s	0.20	0.51	12.42	12.48
Palacos® R+G ampicillin 3 g	1	9.99	3.36	0.11	3.28
	2	10.04	3.4	0.08	2.34
	3	10.02	3.4	0.11	3.23
	4	9.99	3.37	0.085	2.52
	5	10.05	3.38	0.08	2.36
	6	9.99	3.39	0.105	3.10
	7	9.99	3.41	0.089	2.61
	8	9.99	3.43	0.09	2.63
	MW	**10.01**	**3.39**	**0.094**	**2.76**
	s	0.03	0.02	0.013	0.385
	%s	0.25	0.66	13.67	13.94
Palacos® R+G cefuroxime 3 g	1	9.98	3.43	0.08	2.34
	2	9.98	3.43	0.09	2.63
	3	9.99	3.42	0.1	2.93
	4	9.96	3.48	0.085	2.45
	5	10	3.42	0.105	3.07
	6	10.05	3.46	0.09	2.59
	7	10	3.34	0.082	2.46
	8	10	3.41	0.079	2.32
	MW	**10.00**	**3.42**	**0.089**	**2.60**
	s	0.03	0.04	0.009	0.273
	%s	0.26	1.20	10.63	10.51
Palacos® R+G piperacillin 4 g tazobactam 0.5 g	1	9.99	3.36	0.09	2.68
	2	10.02	3.39	0.079	2.33
	3	10	3.41	0.078	2.29
	4	10.02	3.4	0.087	2.55
	5	10.01	3.4	0.072	2.12
	6	9.98	3.41	0.073	2.15

	replica	broadness (mm)	thickness (mm)	impact (J)	Dynstat impact strength (J/m^2)
	7	9.99	3.37	0.098	2.91
	8	10	3.39	0.075	2.21
	MW	**10.00**	**3.39**	**0.082**	**2.40**
	s	0.01	0.02	0.009	0.284
	%s	0.15	0.53	11.34	11.80
Palacos® R+G piperacillin 8 g tazobactam 1 g	1	10	3.46	0.053	1.53
	2	10	3.49	0.072	2.06
	3	9.99	3.53	0.059	1.67
	4	10.03	3.4	0.06	1.76
	5	10	3.46	0.05	1.45
	6	10.02	3.38	0.068	2.01
	7	9.98	3.45	0.06	1.74
	8	10.01	3.45	0.067	1.94
	MW	**10.00**	**3.45**	**0.061**	**1.77**
	s	0.02	0.05	0.008	0.222
	%s	0.16	1.37	12.32	12.52

A student´s t-test was performed using the values from Table 5, the results are depicted in Table 6. Palacos® R+G combined with 1 g and 3 g ampicillin showed no significant decrease in Dynstat impact strength with percentual decreases relative to antibiotic free Palacos® R+G of -9% and -8%, respectively. Notably, increasing the amount of ampicillin from 1 g to 3 g had no effect on Dynstat impact strength at all. Addition of 3 g cefuroxime to Palacos® R+G caused a significant decrease of -13%. Piperacillin and tazobactam caused significant decreases for both concentrations used. While 4 g caused a drop of 20%, 8 g decreased the Dynstat impact strength by 41%. Figure 33 shows the corresponding bar graph.

Table 6: Dynstat impact strength results.

	mean value (J/m^2)	percentual decrease	p-value
Palacos® R+G reference	3.00		
Palacos® R+G ampicillin 1 g	2.74	-9%	0.14
Palacos® R+G ampicillin 3 g	2.76	-8%	0.20
Palacos® R+G cefuroxime 3 g	2.60	-13%	0.02*
Palacos® R+G piperacillin 4 g / tazobactam 0.5 g	2.40	-20%	0.0019**
Palacos® R+G piperacillin 8 g / tazobactam 1 g	1.77	-41%	$5*10^{-7}$***

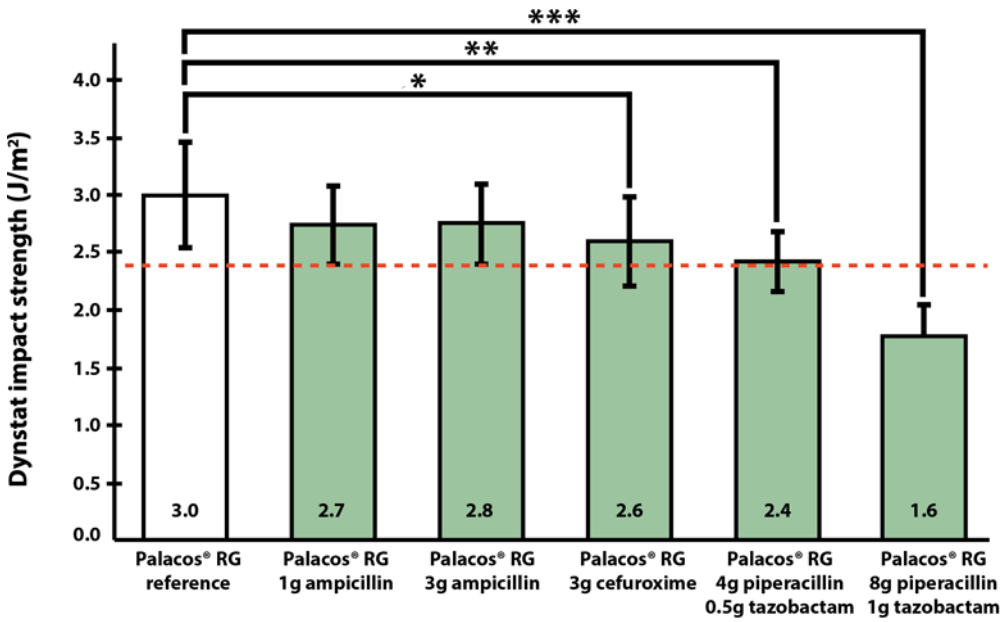

Figure 33: Dynstat impact strength results. The red dashed line represents 80% of the Palacos® R+G reference. Ampicillin showed no significant differences compared to the Palacos® R+G reference, while all other tested combinations did. * = p<0.05; ** = p<0.01; *** = p>0.001.

3.2.2 ISO 5833 (four-point bending test)

3.2.2.1 Bending strength

The performed four-point bending tests performed according to ISO 5833 yielded the results depicted in Table 7. The arithmetic mean was calculated according to equation (2), the standard deviation (s) was calculated according to equation (3) and a student´s t-test was applied to test for significance relative to Palacos® R+G as a reference.

Table 7: Bending strength results.

replica	Palacos® R+G reference	Palacos® R+G ampicillin 1 g	Palacos® R+G ampicillin 3 g	Palacos® R+G cefuroxime 3 g	Palacos® R+G piperacillin 4 g tazobacatam 0.5 g	Palacos® R+G piperacillin 8 g tazobacatam 1 g
1	69.8	70.6	59.3	61.1	59.5	51.7
2	70.5	70.3	59.6	65.7	58.8	52.9
3	73.1	67	62.4	63.4	57	48.9
4	70.09	69.8	61.3	62.1	59.2	53.5
5	72.1	68.7	59.5	64.6	57.7	49.1
mean	**71.1**	**69.3**	**60.4**	**63.4**	**58.4**	**51.2**
s	1.42	1.47	1.37	1.85	1.05	2.13
perc. dec.		-3%	-15%	-11%	-18%	-28%
t-test		0.2236	0.0001***	0.0007***	0.0003***	0.0002***

The addition of 1 g ampicillin was the only preparation that showed no significant difference compared to the reference, while all other combinations yielded highly significant differences. 1 g ampicillin decreased bending strength relative to the reference by 3%, 3 g ampicillin by 15%, 3 g cefuroxime by 11%, 4 g piperacillin with 0.5 g tazobactam by 18% and 8 g piperacillin with 1 g tazobactam by 28%. Figure 34 shows the corresponding bar graph.

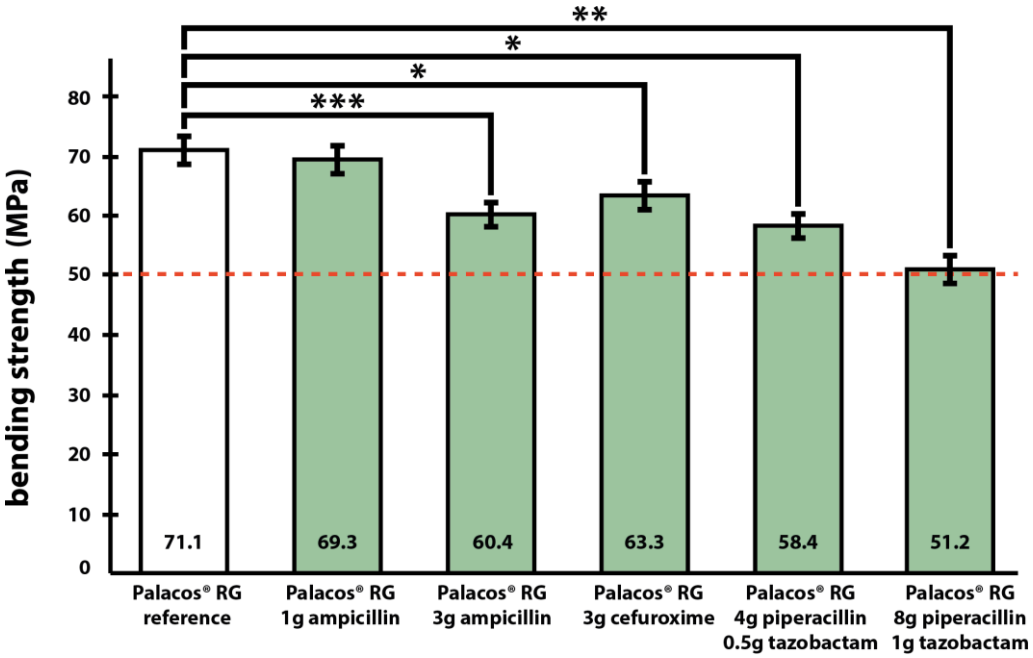

Figure 34: Bending strength results. The red dashed line represents the ISO threshold (50MPa). All combinations except Palacos® R+G with 1 g ampicillin showed significant differences compared to the Palacos® R+G reference. * = p<0.05; ** = p<0.01; *** = p>0.001.

3.2.2.2 Bending modulus

The performed bending modulus tests according to ISO 5833 yielded the results depicted in Table 8. The arithmetic mean was calculated according to equation (2), the standard deviation (s) was calculated according to equation (3) and a student´s t-test was applied to test for significance relative to Palacos® R+G as a reference.

Table 8: Bending modulus results.

replica	Palacos® R+G reference	Palacos® R+G ampicillin 1 g	Palacos® R+G ampicillin 3 g	Palacos® R+G cefuroxime 3 g	Palacos® R+G piperacillin 4 g tazobacatam 0.5 g	Palacos® R+G piperacillin 8 g tazobacatam 1 g
1	3097	3290	2993	3158	3269	3255
2	3047	3246	2988	3226	3245	3122
3	3175	3279	3127	3096	3175	3251
4	3094	3199	3053	3137	3194	3245
5	3115	3345	3019	3184	3268	3171
mean	**3106**	**3272**	**3036**	**3160**	**3230**	**3209**
s	46	54	57	49	43	60
perc. alt.		3%	-1%	1%	3%	5%
t-test		0.003**	0.006**	0.254	0.024*	0.008**

While there were significant differences according to the performed t-test, the relative percentual alterations compared to the Palacos® R+G reference were only +3% for 1 g ampicillin, -3% for 3 g ampicillin, +1% for 3 g cefuroxime, +3% for 4 g piperacillin with 0.5 g tazobactam and + 5% for 8 g piperacillin with 1 g tazobactam. The corresponding bar graph is shown in Figure 35.

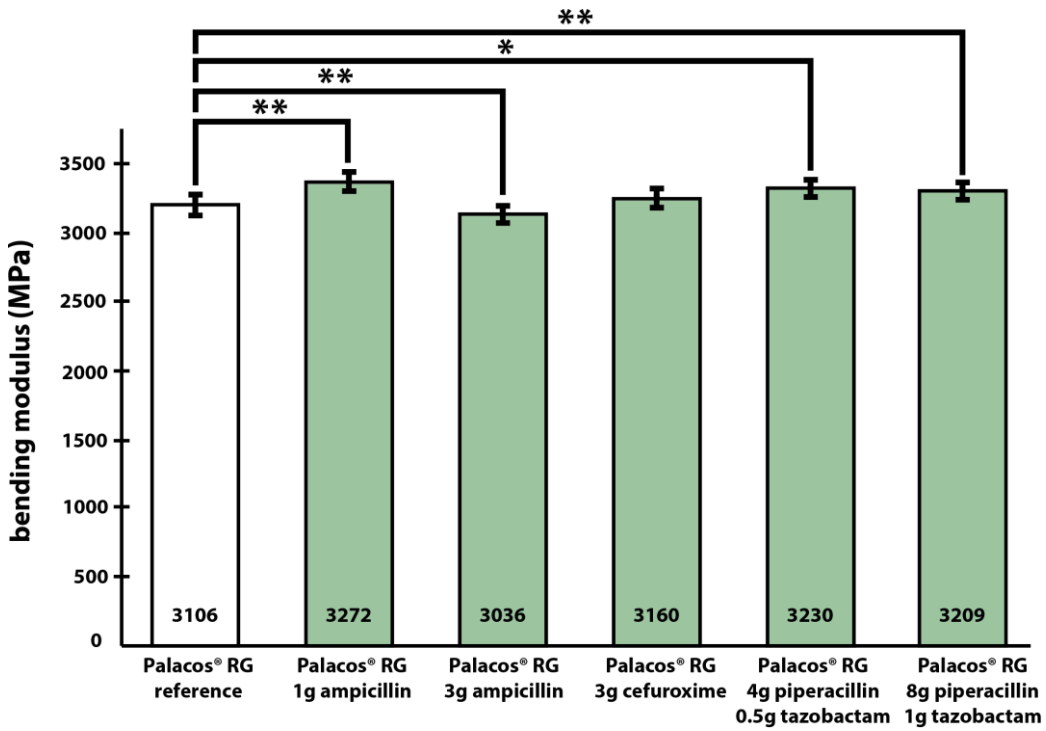

Figure 35: Bending modulus results. * = p<0.05; ** = p<0.01; *** = p>0.001.

3.3 Microbiologic assessments

3.3.1 Ampicillin

The arithmetic mean for all triplicates using ampicillin was calculated according to equation (2). The results are displayed in Table 9 with colours corresponding to the size of the inhibition zones (green = large, yellow = medium, red = small/none).

S. agalactiae showed no inhibition zones after 14 days for the Palacos® R and Palacos® R+G combinations with ampicillin. Copal® G+V combined with 1 g ampicillin showed slight inhibition up to day 42.

E. faecalis growth was not inhibited after day 7 when using combinations of ampicillin with Palacos® R or Palacos® R+G. Copal® G+V with 1 g ampicillin yielded small inhibition zones up to day 28.

P. mirabilis showed inhibition zones until day 7 when treated with Palacos® R containing 1 g ampicillin. Palacos® R+G with 1 g ampicillin or 3 g ampicillin as well as Copal® G+V showed medium sized inhibition zones up to day 42.

Table 9: Ampicillin inhibition assay results. The numbers represent the mean inhibition zone sizes, with green indicating a strong, yellow an intermediate and red a weak antimicrobial activity. R Amp 1 g = Palacos® R with 1 g ampicillin, RG Amp 1 g = Palacos® R+G with 1 g ampicillin, RG Amp 3 g = Palacos® R+G with 3 g ampicillin, CV Amp 1 g = Copal® G+V with 1 g ampicillin.

		1h	24h	7d	14d	28d	42d
S. agalactiae	**R Amp 1g**	34	30	22	16	0	0
	RG Amp 1g	32	29	21	21	0	0
	RG Amp 3g	39	34	29	22	10	0
	GV Amp 1g	34	31	22	13	13	11
E. faecalis	**R Amp 1g**	32	26	15	0	0	0
	RG Amp 1g	31	25	13	8	0	0
	RG Amp 3g	35	33	24	9	0	0
	GV Amp 1g	30	25	16	11	11	9
P. mirabilis	**R Amp 1g**	30	23	13	0	0	0
	RG Amp 1g	30	24	18	14	18	15
	RG Amp 3g	33	28	22	14	18	15
	GV Amp 1g	29	25	19	16	16	16

3.3.2 Piperacillin/tazobactam

The arithmetic mean for all triplicates using piperacillin/tazobactam against *P. aeruginosa* was calculated according to equation (2). The results are displayed in Table 10 with colours corresponding to the size of the inhibition zones (green = large, yellow = medium, red = small/none).

Palacos® R with 4 g piperacillin / 0.5 g tazobactam and 8 g piperacillin / 1 g tazobactam yielded medium sized inhibition zones up to days 7 and 28, respectively.

Palacos® R+G with 4 g piperacillin / 0.5 g tazobactam and 8 g piperacillin / 1 g tazobactam showed medium growth inhibition until days 28 and 42, respectively.

Copal® G+C showed medium sized inhibition zones until day 42, comparable to Palacos® R+G combined with 8 g piperacillin / 1 g tazobactam.

Table 10: Piperacillin/tazobactam inhibition assay results. The numbers represent the mean inhibition zone sizes, with green indicating a strong, yellow an intermediate and red a weak antimicrobial activity. R+Pip 4 g = Palacos® R with 4 g piperacillin and 0.5 g tazobactam, R+Pip 8 g = Palacos® R with 8 g piperacillin and 1 g tazobactam, RG+Pip 4 g = Palacos® R+G with 4 g piperacillin and 0.5 g tazobactam, RG+Pip 8 g = Palacos® R+G with 8 g piperacillin and 1 g tazobactam, GC+Pip 4 g = Copal® G+C with 4 g piperacillin and 0.5 g tazobactam.

		1h	24h	7d	14d	28d	42d
P. aeruginosa	R + Pip 4g	26	24	22	9	0	0
	R + Pip 8g	31	30	29	22	15	0
	RG + Pip 4g	27	24	22	17	16	8
	RG + Pip 8g	30	27	27	22	20	15
	GC + Pip 4g	26	24	24	18	18	17

3.3.3 Cefuroxime

The arithmetic mean for all triplicates using Cefuroxime was calculated according to equation (2). The results are displayed in Table 11 with colours corresponding to the size of the inhibition zones (green = large, yellow = medium, red = small/none).

E. coli showed no significant inhibition when treated with Palacos® R containing 1 g cefuroxime. Palacos® R+G with 3 g cefuroxime showed slight inhibition up to day 42.

K. pneumoniae was not significantly inhibited by Palacos® R containing 1 g cefuroxime. Palacos® R+G containing 3 g cefuroxime yielded medium to small sized inhibition zone assays up to day 42.

P. mirabilis displayed small inhibition zones up to day 42 when treated with Palacos® R containing 1 g cefuroxime and large inhibition zones when treated with Palacos® R+G with 3 g cefuroxime.

Table 11 Cefuroxime inhibition assay results. . The numbers represent the mean inhibition zone sizes, with green indicating a strong, yellow an intermediate and red a weak antimicrobial activity. R+Cef 1 g = Palacos® R with 1 g cefuroxime, RG+Cef 3 g = Palacos® R+G with 3 g cefuroxime.

		1h	24h	7d	14d	28d	42d
E. coli	**R Cef 1g**	11	7	0	0	0	0
	RG Cef 3g	20	16	14	13	16	11
K. pneumoniae	**R Cef 1g**	11	8	0	0	0	0
	RG Cef 3g	20	18	16	12	22	12
P. mirabilis	**R Cef 1g**	28	23	20	15	13	13
	RG Cef 3g	33	27	25	24	22	22

4 Discussion

The general usage of ALBC is still controversially discussed within the scientific community with worries ranging from mechanical impairment over toxic effects to bacterial resistance. While numerous studies have proven its safety and efficacy, there is a general lack of solid data arguing against the use of ALBC (78). Furthermore, evaluation of available clinical data strongly supports the usage of ALBC as a preventive measure for PJI (79). According to the available data on this topic, the risk/benefit ratio of ALBC in primary or secondary TJA strongly supports its clinical application, with some authors even calling for it to be incorporated as a standard procedure in TJA procedures and PJI management.

The fact that local application of antibiotics allows for quick establishment of high local concentrations without greatly elevating the systemic burden further adds to this picture. The formation of biofilms is a critical step during PJI, which exhibits enormous impact on the treatability. While planktonic bacteria and nascent biofilms are properly manageable, most antibiotics fail to completely eradicate fully formed biofilms. Therefore, it is crucial to completely eradicate pathogens as early as possible and to prevent biofilm formation. In this scenario, ALBC has a great advantage over systemic antibiotics since it will cause sufficient concentrations at the site of infection for prolonged periods of time, while commonly used systemic administration schemes may fail to establish such concentrations. The finding that gentamicin in combination with cefuroxime was only effective in early stages of infection with low bacterial burden and no fully established biofilm present (59), supports this view. Interestingly, a study showed that the onetime adhesion of *Staphylococcus epidermidis* to antibiotic-free PMMA cement or other foreign materials was sufficient to significantly decrease its susceptibility towards a broad range of ß-lactam antibiotics in the PMMA-bound cells as well as in planktonic cells (80,81). This finding further underlines the importance of early pathogen eradication by early establishment of high antibiotic concentration as achieved with ALBC.

However, the amount of antibiotic released from the PMMA spacer is a function of the total surface area exposed to synovial fluid, the molecular properties of the incorporated antibiotic and, importantly, also of the properties of the cement mixture used. While different commercially available mixtures with similar compositions show significant differences in their mechanical properties (7), the same applies for their ability to release added antibiotics. As an example, it has been shown that the release of vancomycin and tobramycin greatly differ between Simplex® P or Palacos® R bone cement. While Simplex® P eluted only low levels for a short period of time, Palacos® R lead to high concentration for prolonged periods of time (82). This clearly states, that besides the choice of antibiotic, the choice of the cement is of prime importance.

4.1 Mechanical assessments

As expected, the addition of components not capable of participating in the polymerization reaction lowered the stability in each case. This is due to the fact that in general any impurity within the cement matrix will prevent crosslinking between the polymer entities in its proximity by steric hindrance. In rare cases, added foreign molecules may interact with the polymerization reaction by being incorporated into the polymer or by terminating the reaction. For ß-lactam antibiotics, the main molecular feature to be targeted by radicals is the carbonyl group of the ß-lactam ring, which is essential for antimicrobial efficacy. Reports on the usage of radical species to degrade contaminations of ß-lactam antibiotics in aqueous environments showed general vulnerability of such antibiotics towards radicals (83). Since there was no global loss of antimicrobial activity and the impact of radical antibiotic degradation is considered to be of minor significance, no further discussion on this issue will be provided.

Based on the high similarity of the molecular structure, no individual differences in the impact of the used antibiotics on the stability of the PMMA cements are expected. In order to allow better comparisons, Table 12 lists the percentual proportion of the antibiotics in relation to total amount of solid material.

Table 12: Proportions of cement antibiotic combinations.

	PMMA [g]	gentamicinsulfate [g]	added foreign [g]	total foreign [g]	total solid [g]	per. amount
Palacos® R+G	40	0.8	0	0.8	40.8	**2%**
Palacos® R+G 1 g ampicillin	40	0.8	1.1	1.9	41.9	**4%**
Palacos® R+G 3 g ampicillin	40	0.8	3.2	4	44	**9%**
Palacos® R+G 3 g cefuroxime	40	0.8	3.2	4	44	**9%**
Palacos® R+G 4 g piperacillin / 0.5 g tazobactam	40	0.8	4.7	5.5	45.5	**12%**
Palacos® R+G 8 g piperacillin / 1 g tazobactam	40	0.8	9.4	10.2	50.2	**20%**

4.1.1 DIN 53435 (Dynstat impact strength)

Dynstat impact strength decreased with the amount of added antibiotics in all cases except Palacos® R+G with 3 g ampicillin (see Table 6). When blotting the percentage of foreign material in the cement mixture against the percentual decrease relative from the Palacos® R+G reference an apparent linear correlation can be observed (see Figure 36). In line with this, linear regression yields a coefficient of determination (R^2) of 0.9315 and strongly argues for a linear correlation. Based on R^2 being close to 1, it is justifiable to use equation (5) to calculate the expected decrease in Dynstat impact strength when adding a certain amount of ß-lactam antibiotics to Palacos® R+G.

$$y = -2.1296x + 5.0647 \qquad (5)$$

y= decrease in % relative to standard
x = foreign material in %

While all other used antibiotics fit well into this regression model, ampicillin shows unexpected results. Instead of further lowering the stability, increasing the amount of added ampicillin from 1 g to 3 g leads to a subtle increase in Dynstat impact strength. Figure 36 shows that the value corresponding to Palacos® R+G with 1 g ampicillin was lower and the value for Palacos® R+G with 3 g ampicillin was higher as expected according to the linear regression model. Since every step in the manufacturing process was double-checked and all cement bodies were correctly and unambiguously labelled, any confusion of probes is rendered unlikely. Given the results, ampicillin may not exhibit a linear impact on Dynstat impact strength and might be used in higher concentrations without impeding with stability. Since this interpretation is purely of theoretical nature, additional experiments need to be performed in order to validate this hypothesis.

Taken together, the percentage of added ß-lactam antibiotic should not exceed 15% to ensure Dynstat impact strength values of at least 70% relative to the Palacos® R+G standard.

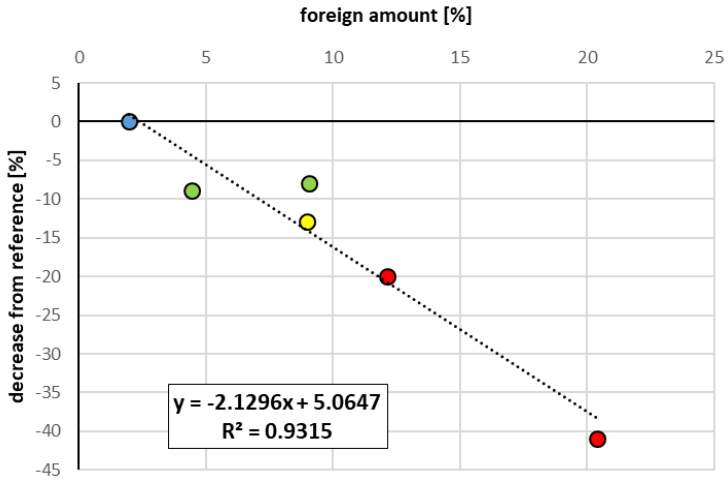

Figure 36: ß-lactam antibiotics impact linearly on Dynstat impact strength. The percentage of foreign material in the cement mixture shows linear correlation with the percentual decrease in Dynstat impact strength relative to the Palacos® R+G reference. The black line represents the linear regression with the corresponding equation and the coefficient of determination shown in the white box. Palacos® R+G: blue dot; Palacos® R+G ampicillin: green dots; Palacos® R+G cefuroxime: yellow dot; Palacos® R+G piperacillin/tazobactam: red dots.

4.1.2 ISO 5833 (four-point bending test)

4.1.2.1 Bending strength

Bending strength showed a clear dependency on the amount of incorporated foreign material. Figure 37 shows the percentage of foreign material in the cement mixture blotted against the percentual decrease relative to the Palacos® R+G reference. The linear regression yielded a coefficient of determination (R^2) of 0.9658 and argues for a strictly linear dependency. Equation (6) can be used to calculate the expected decrease in bending strength when adding ß-lactam antibiotics to Palacos® R+G. Based on this, it is justifiable to assume that only the amount of added ß-lactam antibiotic but not the structural details are relevant for the impact on bending strength. According to equation (6) the foreign material should not exceed 10% in order to guarantee adequate bending strength.

Furthermore, the close linear fit for both ampicillin preparations (green dots) strongly argues against any concerns about probe confusion previously raised in the Dynstat impact strength data discussion.

$$y = -1.5604x + 2.3235 \qquad (6)$$

y= decrease in % relative to standard
x = foreign material in %

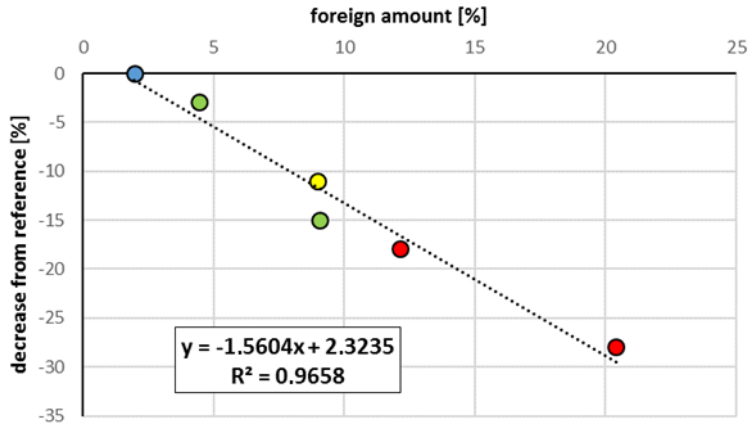

Figure 37: ß-lactam antibiotics impact linearly on bending strength. The percentage of foreign material in the cement mixture shows linear correlation with the percentual decrease in bending strength relative from the Palacos® R+G reference. The black line represents the linear regression with the corresponding equation and the coefficient of determination shown in the white box. Palacos® R+G: blue dot; Palacos® R+G ampicillin: green dots; Palacos® R+G cefuroxime: yellow dot; Palacos® R+G piperacillin/tazobactam: red dots.

4.1.2.2 Bending modulus

The addition of ß-lactam antibiotics showed no negative impact on the bending modulus as most preparations showed increased values relative to the Palacos® R+G standard. The linear regression (see Figure 38) showed poor correlation as indicated by a coefficient of determination (R^2) of only 0.4296. Taking the other mechanical assessment into account the possibility of increasing stability by adding antibiotics can be ruled out. Therefore, the bending modulus must be declared as unsuitable to assess mechanical stability in this setting and will not be considered for further discussion.

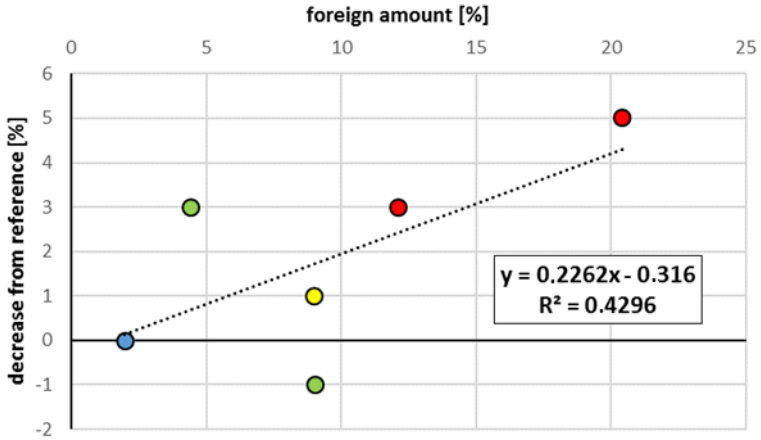

Figure 38: ß-lactam antibiotics do not negatively impact on the bending modulus. The percentage of foreign material in the cement mixture shows no clear correlation with the percentual decrease in bending strength relative from the Palacos® R+G reference. The black line represents the linear regression with the corresponding equation and the coefficient of determination shown in the white box. Palacos® R+G: blue dot; Palacos® R+G ampicillin: green dots; Palacos® R+G cefuroxime: yellow dot; Palacos® R+G piperacillin/tazobactam: red dots.

4.1.3 Literature comparison

While other studies found significant impairment of stability for ß-lactam concentrations of under 5%w/w (55), this study found that concentrations as high as 15%w/w did not critically impair stability. The main difference between the studies was the choice of PMMA cement used. While other studies used Simplex® P or CMW® cements, this study only used Palacos® R+G for mechanical tests. Apparently, Palacos® R+G is not as susceptible towards stability impairment caused by foreign material as other cements are. Therefore, concerning stability, the use of Palacos® R+G can be recommended for combination with ß-lactam antibiotics.

4.2 Microbial efficacy

The preformed assays with inhibition zone sizes as the readout allow for semi quantitative analyses. For surgical considerations the single most important fact is the antimicrobial activity after a certain time to reliably prevent biofilm formation. Therefore, interpretation of inhibition zone sizes is considered to be valid as the diameter of the inhibition zone directly correlates with antibiotic concentration in the eluate and hence, with antimicrobial activity. Since every tested strain was used in a McFarland of 0.5 the datasets can be considered as comparable.

It is important to note, that each time point represents a completely new elution process and no antibiotic from previous time points will have an impact on the following measurement. Hence, the displayed inhibition zone assays depict conservative estimates and can be considered as minimal values. Continuous elution would have resulted in higher concentrations and therefore larger inhibition zones. Due to the experimental set-up only inhibition zones above 9mm can be interpreted as antimicrobial activity since the central whole in the agar plate has a diameter of 8 to 9mm itself.

4.2.1 Ampicillin

In stark contrast to the observed low elution from CMW® cement (49), this study found strong elution form Palacos® R containing only 1 g of ampicillin (2,5%w/w) for up to 14 days, as indicated by the antimicrobial activity. Apparently, Palacos® R provides a better matrix for ampicillin elution than CMW® does. This finding underlines the significance of the used PMMA cement and justifies the recommendation of Palacos® cements when using ampicillin as antibiotic.

To treat infections with *S. agalactiae* or *E. faecalis* the combination of Palacos® R or Palacos® R+G with ampicillin is not to be preferred since both germs show no more significant growth inhibition at 28 and

14 days, respectively. Taking the mechanistic assessments into consideration, the addition of greater amounts of ampicillin poses no immediate risks and could help overcome the problem of short lasting antimicrobial activity. The combination of ampicillin with Copal® G+V leads to weak activity up to day 42. Although this finding indicates superior antimicrobial activity, no mechanical data for this combination is available. The established regression models cannot be used to estimate the decrease in stability because all models work with Palacos® R+G and allow no conclusions for Copal® cements. Nevertheless, the data suggests the usage of Copal® G+V combined with ampicillin against *S. agalactiae* or *E. faecalis*.

To prevent infections with *P. mirabilis* all combinations except Palacos® R with 1 g ampicillin can be recommended. Since Palacos® R+G with 1 g ampicillin showed no inferior efficacy compared to Palacos® R+G with 3 g ampicillin, the lower amount of added antibiotic should be preferred in order to ensure stability and reduce costs. Palacos® R+G with 1 g ampicillin is also to preferred over Copal® G+V with 1 g ampicillin since using fewer types of antibiotics lowers the amount of possible resistances to be developed during the treatment.

Overall addition of ampicillin showed greater effect on *P. mirabilis* compared to *S. agalactiae* or *E. faecalis.* This is unexpected since *P. mirabilis* is Gram-negative and ampicillin as a penicillin antibiotic should exhibit greater activity against Gram-positive germs like *S. agalactiae* and *E. faecalis.* Furthermore, the combination of ß-lactam and aminoglycoside antibiotics like gentamicin is known exhibit synergistic effects. This is because aminoglycosides penetrate the bacterial cell membrane at low rates but ß-lactam antibiotics interfere with membrane integrity and therefore facilitate aminoglycoside entry into the cell (84). The finding that ampicillin was more efficient on Gram-negative germs may be explained by the individual sensitivity of *S. agalactiae* and *E. faecalis* towards gentamicin. As Dressel et al. report, synergism between ampicillin and gentamicin is only to be observed with germ showing high sensitivity towards gentamicin (85). As the kill curves shown in Figure 39 imply, gentamicin combined with ampicillin has no improved efficacy compared to ampicillin mono-treatment when the treated strain has a low sensitivity towards gentamicin.

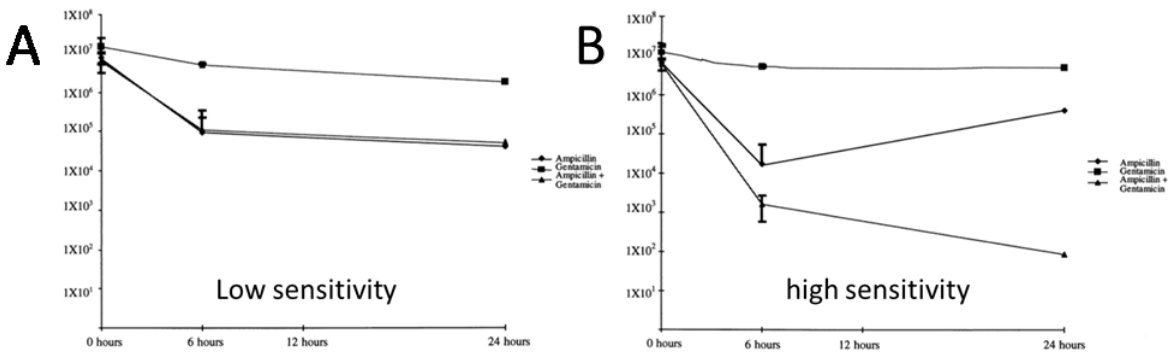

Figure 39: Synergism between ampicillin and gentamicin is dependent on gentamicin sensitivity. Panel A: Kill curves of lowly gentamicin sensitive enterococci. Panel B: Kill curves highly gentamicin sensitive enterococci. Synergism between ampicillin and gentamicin is only to be observed with highly gentamicin sensitive enterococci.

4.2.2 Piperacillin/tazobactam

The low elution profiles and weak antimicrobial activity found in the studies using Simplex® P cement (60,61) may be explained be the nature of the cement or by the fact that the studies only used relatively low antibiotic concentrations in the cement bodies (1.6% and 2.5%, respectively). Due to the lack of reliable data on other cements, Palacos® or Copal® cements are to be recommended.

4 g piperacillin / 0.5 g tazobactam in combination with Palacos® R was not able to suppress bacterial growth longer than 14 days and therefore should not be considered first choice. Nevertheless, it exhibited good antibacterial inhibition during the first 7 days and one would expect the stability to sufficient based on the rather small amount of foreign material.

Palacos® R with 8 g piperacillin / 1 g tazobactam was as efficient as Palacos® R+G with 4 g piperacillin / 0.5 g tazobactam. The latter makes use of the synergism between piperacillin and gentamicin and therefore is able to establish equal efficacy with less incorporated foreign material. Therefore, and in connection with the expectedly better mechanical properties, Palacos® R+G with 4 g piperacillin / 0.5 g tazobactam is to be preferred.

Palacos® R+G with 8 g piperacillin / 1 g tazobactam as well as Copal® G+C with 4 g piperacillin / 0.5 g tazobactam show superior efficacy with medium sized inhibition zones up to day 42. Nevertheless, Palacos® R+G 8 g piperacillin / 1 g tazobactam showed significantly reduced stability.

Taken together, it must be closely evaluated how long the antimicrobial activity is needed and if the respective mixture is intended as a spacer or as a permanent fixture. Palacos® R+G 4 g piperacillin / 0.5 g tazobactam constitutes a good compromise between antimicrobial efficacy and mechanical stability.

4.2.3 Cefuroxime

As opposed to the finding that the stability of Simplex® P cements is critically impaired with cefuroxime concentrations above 4%w/w (55), this study shows superior stability for Palacos® R+G cements in combination with cefuroxime. Taking the lack of data on antimicrobial properties of other cements into account, Palacos® cements are the prime choice for combination with cefuroxime.

While Palacos® R with 1 g cefuroxime did not establish and sustain antimicrobial efficacy against *E. coli* and *K. pneumoniae*, Palacos® R+G with 3 g cefuroxime was able to show medium to weak growth inhibition up to day 42 in both cases. It is important to note that the increase in antimicrobial activity may be due to synergism between gentamicin and cefuroxime, due to the increased cefuroxime concentration or due to a combination of both. Taking the stability data into account, Palacos® R+G with 3 g cefuroxime can be recommended to treat or prevent infections with *E. coli* and *K. pneumoniae*.

For *P. mirabilis* Palacos® R with 1 g cefuroxime as well as Palacos® R+G with 3 g cefuroxime exhibited antimicrobial activity up to day 42. In conclusion, both combinations can be recommended. To increase the antimicrobial activity, it can be considered as safe to increase the amount of cefuroxime in Palacos® R up to 3 g without risking intolerable instability.

5 Conclusion

The performed experiments clearly prove the usability of ß-lactam antibiotics in PMMA cements. In order to yield best results, the choice of cement, antibiotic and its concentration needs to be evaluated with respect to the respective pathogen and the planned surgical approach. Generally, two step revision processes with the use of temporarily inserted spacers allow for higher amounts of added antibiotics since spacer do not have to withstand extensive mechanical stress. Figure 40 lists the most effective cement-antibiotic combination for each tested strain.

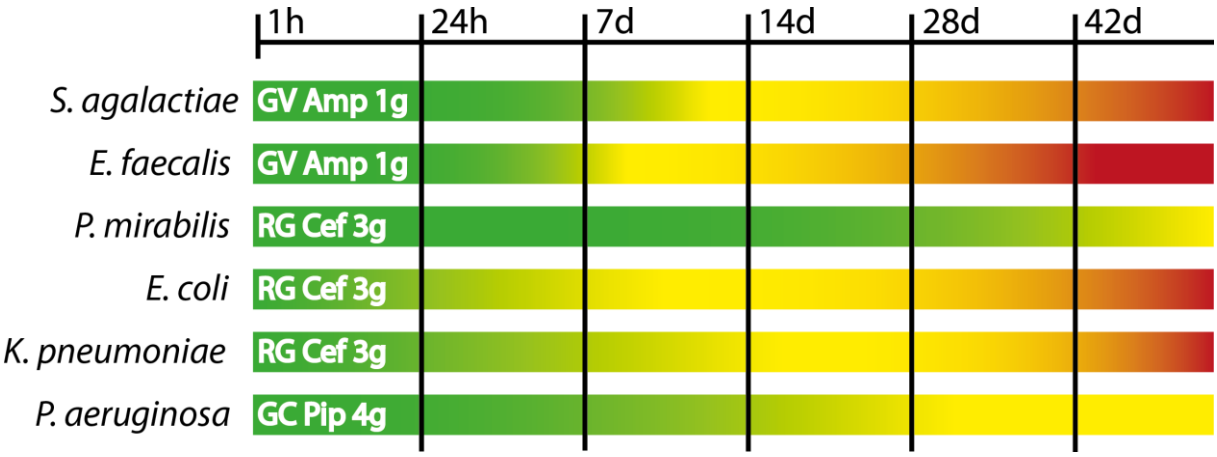

Figure 40: Most efficient cement-antibiotic combinations. The recommendations were made based on the antimicrobial efficacy and the amount of total foreign material. Hereby, it was assumed that the smaller the percentage of foreign material is, the higher the mechanical stability. Green indicates good antimicrobial activity, yellow intermediate and red no antimicrobial activity. GV Amp 1 g = Copal® G+V with 1 g ampcillin; RG Cef 3 g = Palacos® R+G with 3 g cefuroxime; GC Pip 4 g = Copal® GC with 4 g piperacillin and 0.5 g tazobactam.

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