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In vitro efficacy of antibiotic loaded calcium sulfate beads (Stimulan Rapid Cure) against polymicrobial communities and individual bacterial strains derived from diabetic foot infections

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Abstract

Introduction. Diabetic foot infection (DFI) is the main reason for diabetes-related hospitalisation and is a major cause of diabetes-related amputation. DFIs are often complicated by ischaemia in the affected limb, the presence of polymicrobial biofilms and increasingly the occurrence of antibiotic resistant bacteria.

Hypothesis/Gap statement. Antibiotic loaded beads could inhibit the growth of polymicrobial DFI communities with differing compositions *in vitro*.

Aim. This study investigates the *in vitro* efficacy of antibiotic loaded calcium sulfate beads (Stimulan Rapid Cure, Biocomposites Ltd., UK) against polymicrobial DFI communities and individual bacterial strains derived from DFIs.

Methodology. Debrided tissue obtained from the base of infected diabetic foot ulcers was homogenised and spread over the surface of Columbia blood agar (CBA) and fastidious anaerobe agar (FAA) plates. Calcium sulfate beads containing a combination of vancomycin and gentamicin were then placed on the surface of the agar and following incubation, zones of inhibition (ZOI) were measured. For individual bacterial strains isolated from the infected tissue, calcium sulfate beads containing vancomycin, gentamicin, flucloxacillin or rifampicin and beads containing a combination of vancomycin and gentamicin or flucloxacillin and rifampicin were tested for their ability to inhibit growth.

Results. Calcium sulfate beads loaded with a combination of vancomycin and gentamicin were able to inhibit bacterial growth from all polymicrobial tissue homogenates tested, with ZOI diameters ranging from 15 to 40 mm. In the case of individual bacterial strains, beads containing combinations of vancomycin and gentamicin or flucloxacillin and rifampicin were able to produce ZOI with Gram-positive facultatitive anaerobic strains such as *Staphylococcus aureus* and *Enterococcus faecalis*, Gram-negative facultative anaerobic strains such as *Pseudomonas aeruginosa* and obligate anaerobic strains such as *Finegoldia magna* even where acquired resistance to one of the antibiotics in the combination was evidenced.

Conclusion. The local use of calcium sulfate beads containing a combination of two antibiotics demonstrated high efficacy against polymicrobial DFI communities and individual DFI bacterial strains in *in vitro* zone of inhibition tests. These results show promise for clinical application, but further research and clinical studies are required.

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Keywords: infection; local release; calcium sulfate; antibiotics; zone of inhibition.
Abbreviations: CBA, Columbia blood agar; DFI, diabetic foot infection; DFO, diabetic foot osteomyelitis; DFU, diabetic foot ulcer; FAA, fastidious anaerobe agar.
Three supplementary tables are available with the online version of this article.
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INTRODUCTION

In 2014 an estimated 422 million people globally were living with diabetes with a prevalence of 8.5% amongst the adult population [1]. The prevalence of diabetes is predicted to rise to 10.4% by 2040 affecting an estimated 642 million adults worldwide [2]. Diabetic foot ulcers (DFU) are a common and serious complication of diabetes with up to 25% of diabetic individuals experiencing a DFU in their lifetime [3]. Living with DFUs can have a significant physical, social and psychological impact on individuals [4]. DFUs can limit mobility, ability to work and ultimately may become life threatening. These open wounds, which can take weeks or months to heal, provide an easy point of entry for microorganisms which can lead to colonisation and subsequent diabetic foot infection (DFI). The mortality and amputation rates associated with DFI are high. In a large prospective study of patients with DFIs, 15% of patients died and 17% of patients underwent at least partial lower extremity amputation during the 12 month follow up period [5]. It is estimated that every 20 seconds an individual living with diabetes loses a limb due to foot complications, 60% of which are due to DFIs [6–8]. Diabetic foot ulceration and infection places an enormous burden on healthcare systems. It is estimated that NHS spending in England and Wales on DFU and amputation for 2014–15 was in the region of £1 billion [9]. As the incidence of diabetes escalates globally, there is an urgent need for an improved understanding of DFIs and new therapeutic approaches.

Current clinical practice guidelines for the treatment for DFIs include a regime of debridement, alleviating pressure on the wound and systemic antibiotic therapy [7, 10, 11]. However, in a proportion of patients, infection resolution may not be achieved and there are many factors that contribute to treatment failure. Over half of patients with a DFI exhibit ischaemia in the affected limb [12]. As well as impaired tissue repair and regeneration, poor tissue perfusion may result in sub-inhibitory concentrations of systemically administered antibiotics reaching the wound site, resulting in inefficient clearance of the infection and conditions which can encourage the development of antibiotic resistance [13]. A complicating factor in DFI may be the presence of bacterial aggregates and biofilms, which can cause chronic and persistent infection. Using microscopy techniques, biofilms have been demonstrated in DFUs [14, 15] and diabetic foot osteomyelitis (DFO) [16]. It is widely accepted that bacteria within biofilms exhibit a greater tolerance to the effects of antibiotics [17, 18]. An additional problem in the treatment of DFIs is the development of resistance to antibiotics. In recent years there has been a dramatic increase in the emergence of resistant strains and strains demonstrating intermediate susceptibility. This shift towards higher minimum inhibitory concentrations (MICs) has meant that higher dosing regimens are required to achieve clinical success. Local release of antibiotics from a suitable carrier material may provide the benefit of high bio-availability at a site of infection, achieving higher concentrations when compared with serum plasma levels [19]. A further advantage of local delivery is that it may be possible to avoid the use of or reduce the length of treatment with systemically administered antibiotics [20]. Such an approach would help limit the co-lateral effects of systemic antibiotics such as those on the gut microbiome.

Antibiotic loaded carriers are widely used as an adjunct to systemic antibiotic therapy in the treatment of periprosthetic joint infections (PJI) and may also be placed prophylactically during surgery [21]. Currently the most widely used carrier for the local delivery of antibiotics is poly(methyl methacrylate) (PMMA) with a range of antibiotic-loaded PMMA bone cements commercially available [22]. However, this material is not absorbed by the body and a further procedure is required for its removal, due to the risk of remaining material acting as a nidus for infection [23–25]. There are a number of alternative materials that can be used to release antibiotics locally with purified calcium sulfate showing high potential. Calcium sulfate is biocompatible and is fully absorbed by the body. In addition, calcium sulfate can be mixed with a wide range of antibiotics [22] and its low curing temperature means that thermosensitive antibiotics can also be included. *In vitro* studies have demonstrated that antibiotics are released from calcium sulfate beads over extended periods and that the antibiotic concentrations achieved, exceed the MIC for common PJI pathogens [26]. Additionally, antibiotic loaded calcium sulfate beads have been shown to prevent bacterial colonisation and reduce biofilm formation of PJI pathogens *in vitro* [27, 28].

The application of calcium sulfate antibiotic loaded beads in the treatment of DFIs and DFO is an area of increasing interest with a number of preliminary clinical studies showing promising results. Antibiotic-impregnated calcium sulfate beads have been shown to prevent the recurrence of DFO in patients undergoing infected bone resection [29]. In patients with forefoot ulcers complicated with underlying osteomyelitis, the placement of vancomycin and gentamicin loaded calcium sulfate beads at the ulcer site led to healing in all cases, potentially avoiding the need for more radical surgery [30]. *In vitro* studies also support the use of antibiotic loaded calcium sulfate beads in the treatment of DFIs. Tobramycin or gentamicin loaded calcium sulfate beads were able to completely eradicate *Pseudomonas aeruginosa* biofilms in a collagen wound model used to mimic an infected DFU. One $\log_{10}$ reductions in the viable counts of multi-drug resistant *Staphylococcus aureus* biofilms were also reported [31]. Additionally 5–8 $\log_{10}$ order reductions in the viability of DFI-derived polymicrobial biofilms were observed when calcium sulfate beads loaded with vancomycin or gentamicin were added to the wound model [32, 33].

Commercially available antibiotic loaded PMMA bone cements and biomaterials such as calcium sulfate and collagen are typically combined with vancomycin, gentamicin, tobramycin or clindamycin either alone or in combination [22]. In the current study, the effect of calcium sulfate (Stimulan Rapid Cure, Biocomposites Ltd., UK) beads containing a combination of vancomycin and gentamicin on the growth of polymicrobial communities present in homogenised DFI tissue is investigated. For the treatment of DFIs in the

Table 1. Bead preparation and antibiotic content

Bead	Antibiotic manufacturer	Bead preparation 5 cc pack of Stimulan Rapid Cure combined with	Antibiotic amounts per 6 mm bead
unloaded		mixing solution	none
vancomycin	AAH Pharmaceuticals (Coventry, UK)	250 mg vancomycin plus mixing solution	3.4 mg vancomycin
gentamicin	Amdipharm (London)	3 ml gentamicin solution (40 mg ml^{-1})	1.6 mg gentamicin
vancomycin-gentamicin		250 mg vancomycin plus 3 ml gentamicin solution (40 mg ml^{-1})	3.4 mg vancomycin and 1.6 mg gentamicin
flucloxacillin	Wockhardt (Wrexham, UK)	250 mg flucloxacillin plus mixing solution	3.4 mg flucloxacillin
rifampicin	Mylan (Potters Bar, UK)	300 mg rifampicin plus mixing solution	4.1 mg rifampicin
flucloxacillin-rifampicin		300 mg rifampicin and 250 mg flucloxacillin plus mixing solution	3.4 mg flucloxacillin and 4.1 mg rifampicin

UK, National Institute for Health and Care Excellence (NICE) guidelines suggest flucloxacillin as a first-choice oral antibiotic for the treatment of mild infections, and this can be combined with gentamicin for moderate to severe infections. Where methicillin-resistant *S. aureus* is suspected vancomycin can be added [34]. There is growing clinical evidence that adding rifampicin to the antibiotic regime improves outcomes in DFO [8]. Both vancomycin and flucloxacillin inhibit cell wall synthesis in Gram-positive bacteria whilst gentamicin and rifampicin are broad spectrum antibiotics which act by inhibiting protein synthesis. In the current study a vancomycin and gentamicin combination was tested against a panel of strains isolated from the DFI to reflect commercially available products and a flucloxacillin and rifampicin combination was selected as a more tailored approach to the treatment of DFIs.

METHODS

Preparation of antibiotic loaded calcium sulfate beads

Unloaded beads were prepared by thoroughly mixing a 5 cc pack of Stimulan Rapid Cure (Biocomposites Ltd., UK) with the mixing solution provided for 30 s. The mixture was then pressed into the provided flexible mould mat, moulding the beads into 6 mm hemispherical shapes. Beads were allowed to set for 30 min at room temperature, then removed from the mould mat and stored at 4 °C until required. Bead preparation details and the antibiotic content for each bead type is shown in Table 1. Beads were used within 30 days of preparation.

Participant selection and ethics

Recruitment and sample collection was facilitated through the Royal Devon and Exeter Tissue Bank (RDETB). This is an ethically approved tissue bank (REC reference number 16/SC/0162) set up to collect and store tissue available at the time of routine procedures. Potential participants attending a specialist diabetic foot clinic at the Macleod Diabetes and Endocrine Centre (MDEC), were identified by clinicians during routine clinical care. Inclusion in the study was based on the presence of an infected foot ulcer requiring wound debridement. Infection was confirmed by the presence of clinical signs and symptoms of infection according to Infectious Diseases Society of America (IDSA) guidelines [10]. Over a 15 month period, 20 participants were recruited to the RDETB. All participants were given a unique study identifier (ID) and all associated data and samples were de-identified with this ID before being transferred to the study team. Debrided material from the ulcer base was collected and placed in a sterile container. The tissue sample was transported to the research laboratory and placed in an anaerobic cabinet within 1 h of sampling.

Preparation of polymicrobial DFI tissue homogenate

Within the anaerobic cabinet, the tissue sample was transferred to a sterile 40 µm nylon mesh filter (ThermoFisher Scientific, Waltham, MA, USA) in a sterile petri dish. Amies transport solution (3 ml) was added and the tissue was ground against the mesh filter using the flat, ridged end of a sterile syringe plunger. The resulting tissue homogenate was transferred to a sterile universal bottle containing glass beads (E and O Laboratories, Bonnybridge, UK) and the suspension was vortexed at maximum velocity for 2 min.

Evaluation of calcium sulfate beads containing antibiotics against polymicrobial DFI tissue homogenates

Modified Kirby-Bauer assays were used to determine the efficacy of the antibiotic loaded beads in inhibiting bacterial growth from the tissue homogenates. Freshly prepared tissue homogenate (50 µl) was spread over the surface of a Columbia blood agar (CBA) plate and a fastidious anaerobe agar (FAA) plate. Columbia blood agar and anaerobe basal agar (both Oxoid, ThermoFisher Scientific,

Waltham, MA, USA) were supplemented with 5% v/v defibrinated horse blood (E and O Laboratories, Bonnybridge, UK) and 25 ml was dispensed per petri-dish. Three beads containing a combination of vancomycin and gentamicin were placed onto the surface of each inoculated plate using sterile forceps. Three unloaded beads were placed on the surface of an additional inoculated CBA and FAA plate as a negative control. Plates were incubated at 37 °C aerobically or anaerobically as appropriate. Zones of inhibition (ZOI) were measured after 4 days to allow sufficient time for slow growing facultative anaerobic species on the CBA plates and obligate anaerobes on the FAA plates. The diameter of the ZOI was measured to the nearest mm from the innermost limit of all growth that could be detected by the naked eye. Tissue samples were collected from 20 different patients on separate days. It is important to note that this assay is not designed to determine resistance or susceptibility of bacterial species to a particular antibiotic. A ZOI demonstrated that antibiotic was released from the bead and that the antibiotic retained activity under the test conditions.

Obtaining individual strains from polymicrobial DFI tissue homogenates

Serial dilutions (undiluted through to 10^{-6}) of the freshly prepared tissue homogenate were plated out onto a range of selective and non-selective agars (Table S1). Following incubation, individual colonies with distinct colony morphology types were picked and sub-cultured onto the appropriate agar and incubated accordingly. Pure cultures of individual strains were stored at −80 °C using Microbank vials (Pro-Lab, Bromborough, UK). Strains were numbered according to participant number [1–20] followed by sequential numbering for each successive strain obtained from that DFI (e.g. 5.1, 5.2, 5.3 representing the first three strains obtained from DFI 5). Strains were identified by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS) using the Vitek MS (bioMérieux, Marcy l'Etoile, France). Spectral fingerprints were compared with the bioMérieux database (VITEK MS v3.2.0). *Escherichia coli* NCTC 12923 (National Collection of Type Cultures, UK Health Security Agency, Salisbury, UK) was used as the control organism.

Evaluation of calcium sulfate beads containing antibiotics against individual strains

An extended range of antibiotic loaded beads (Table 1) were tested against individual strains derived from the DFI tissue homogenates. A panel of 52 Gram-positive facultative anaerobic strains (including *Staphylococcus aureus*, *Enterococcus faecalis* and *Enterococcus faecium*), Gram-negative facultative anaerobic strains (including *Pseudomonas aeruginosa*) and obligate anaerobic strains (including *Finegoldia magna*) were tested (). A suspension of the strain was prepared in sterile saline and adjusted to a McFarland density of 0.5. The suspension was used to inoculate the surface of Mueller-Hinton (MH) agar (Oxoid, ThermoFisher Scientific, Waltham, MA, USA) using a cotton swab. In the case of fastidious organisms, MH agar was supplemented with nicotinamide adenine dinucleotide (NAD, 20 mg l^{-1}) and 5% v/v horse blood (MH-F). For obligate anaerobic species, FAA was used. Plates contained 25 ml agar per plate. Beads were placed on the surface of the inoculated agar as described previously and plates were incubated at 37 °C either aerobically, anaerobically or in a CO_2-enriched atmosphere according to the growth requirements of each strain. ZOI were measured after 24 h for facultative anaerobic species (except *Oligella urethralis* which was read at 3 days) or 3–7 days for anaerobic species. This assay is not designed to determine resistance or susceptibility of a bacterial strains to a particular antibiotic. However, as a defined inoculum was used and a range of antibiotic combinations tested, it was possible to make comparisons between the different beads for a particular strain, with a larger ZOI indicating a greater potency for that bead formulation.

Broth microdilution assays

*A*ntimicrobial susceptibility testing was carried out on a small number of *E. faecalis* and *E. faecium* strains using a 96 well broth microdilution assay. A 0.5 McFarland suspension was prepared in sterile saline and 10 µl was added to 11 ml of cation adjusted MH broth with TES buffer (ThermoFisher Scientific, Waltham, MA, USA). The resulting suspension was used to inoculate (50 µl per well) Sensititre GPALL1F plates (ThermoFisher Scientific, Waltham, MA, USA) containing pre-loaded concentrations of antibiotics. Following incubation for 18–24 h at 37 °C, growth in each well was recorded and the minimum inhibitory concentration (MIC) determined.

RESULTS

Effect of antibiotic loaded beads on microbial growth from polymicrobial DFI tissue homogenates

Calcium sulfate beads loaded with vancomycin and gentamicin had an inhibitory effect on bacterial growth from all polymicrobial tissue homogenates where growth was obtained (Fig. 1). Participant one did not meet the criteria of having active markers of infection present at the time of sampling and was therefore excluded from the study. No growth was obtained for participant seven on both CBA and FAA and only a small number of individual colonies were observed for participant two. For participant five only anaerobic growth on FAA was observed with no growth obtained on CBA incubated aerobically. The ZOI for each sample varied according to growth conditions with generally smaller zones observed on plates incubated anaerobically compared to plates incubated aerobically (Fig. 1). The smallest zone diameter observed was 14 mm (participant ten) which, with a bead diameter of 6 mm, represents a clearance of 4 mm between the edge of the bead and microbial growth. For participant six, a clear ZOI could be observed on anaerobic plates, however colonies were present on the aerobic plates

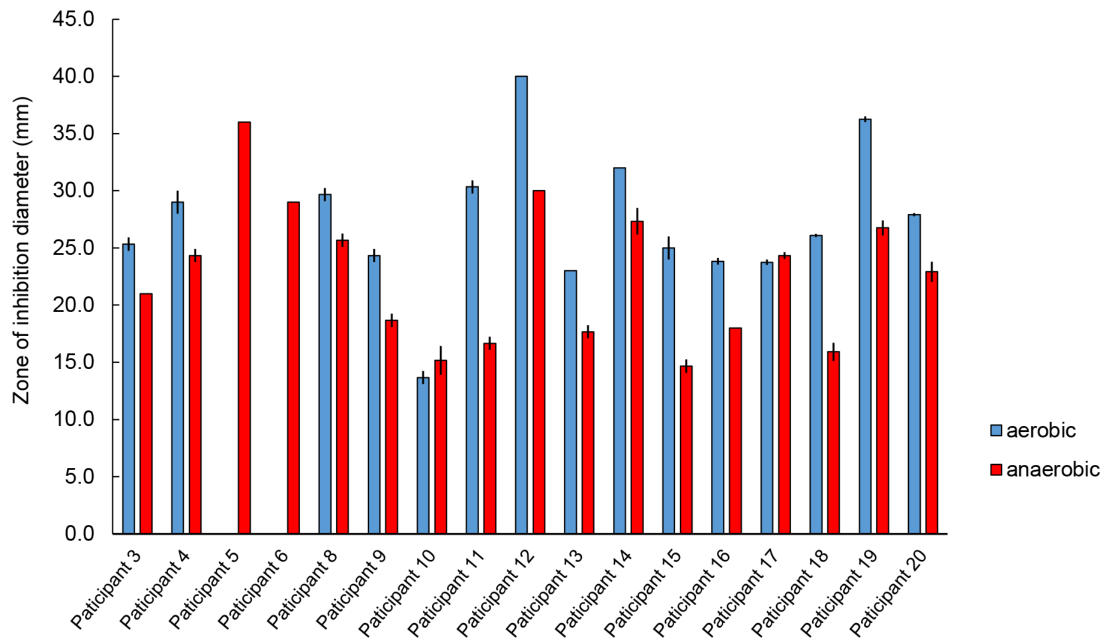

Fig. 1. Zones of inhibition of microbial growth from diabetic foot infection tissue homogenates in the presence of vancomycin-gentamicin loaded beads at day 4. Data are expressed as means of three replicates ± standard deviation.

which grew in contact with the antibiotic containing beads. Upon Gram-staining and microscopic investigation, these colonies were noted to be yeasts and given that growth was observed on the Brilliance Candida agar for this DFI tissue homogenate, these colonies were assumed to be *Candida* spp. Fig. 2 shows the polymicrobial lawn obtained on CBA following aerobic incubation of tissue homogenate (participant eight) in the presence of calcium sulfate beads loaded with vancomycin and gentamicin. Differential ZOI could be observed, with some species present in the polymicrobial lawn being inhibited to a greater extent than others (Fig. 2). No inhibition of microbial growth was observed with unloaded beads.

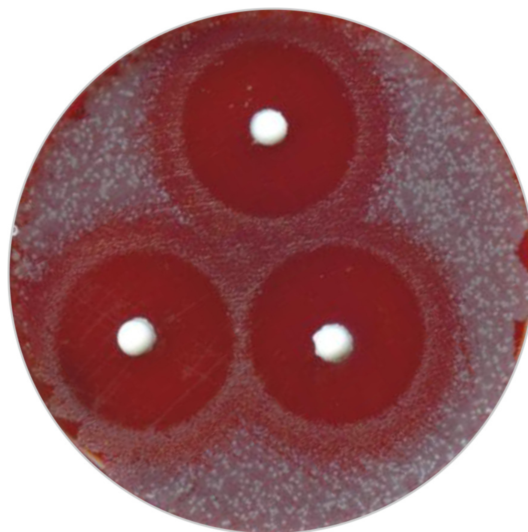

Fig. 2. Zones of inhibition of microbial growth from diabetic foot infection tissue homogenate (participant 8) on Columbia blood agar incubated aerobically.

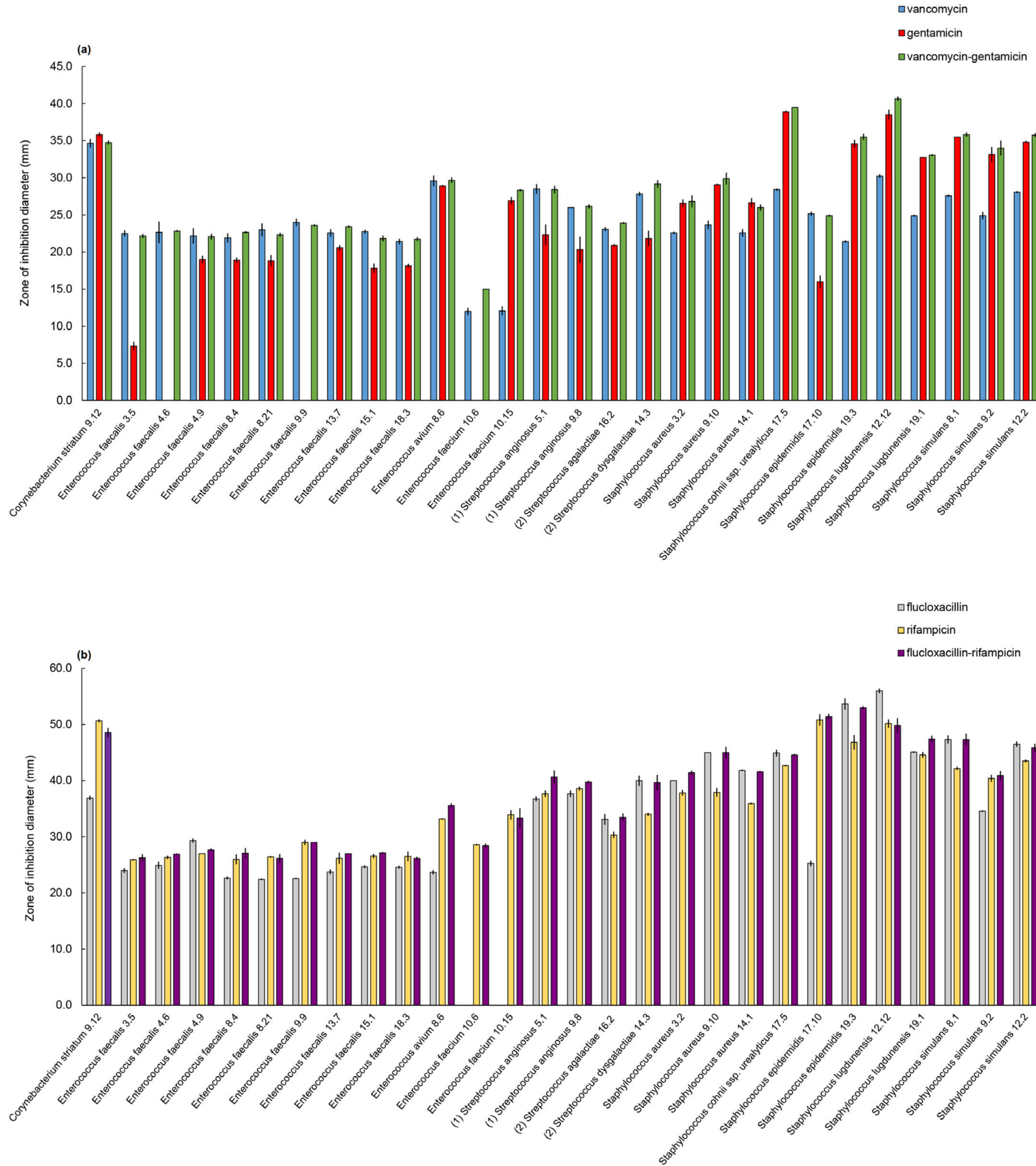

Fig. 3. Zones of inhibition observed with Gram-positive facultative anaerobic strains incubated with beads containing (a) vancomycin, gentamicin or vancomycin-gentamicin combined and (b) flucloxacillin, rifampicin or flucloxacillin-rifampicin combined. [1,2]Screened on Mueller-Hinton agar supplemented with defibrinated horse blood and β-NAD incubated in a [1]CO_2-enriched atmosphere, or [2]incubated aerobically. All other Gram-positive strains were screened on Mueller-Hinton agar incubated aerobically. Data are expressed as means of three replicates ± standard deviation.

Effect of antibiotic containing beads on individual bacterial strains from DFI tissue homogenates

All DFIs, that demonstrated growth, were polymicrobial with an average of 5.4 species (±2.2) per DFI . (Table S3) Gram-positive facultative anaerobic species were present in all of the DFIs sampled and Gram-negative species were present in 11/18 (61%). Obligate anaerobic species were present in 13/18 (72%) of DFIs. A panel of the individual bacterial strains isolated from the DFI tissue homogenates (Table S2) were screened for their growth in the presence of an extended range of antibiotic loaded beads. Four strains of *E. faecalis* (3.5, 4.6, 9.9 and 10.6) demonstrated no, or virtually no ZOI in the presence of gentamicin loaded beads (Fig. 3). However vancomycin and vancomycin-gentamicin loaded beads did inhibit the growth of these strains, although the ZOI for one of the strains (10.6) was small. Two *E. faecium* strains from the same patient (10.6 and 10.15) demonstrated differing patterns of inhibition in the presence of calcium sulfate beads loaded with single antibiotics (Fig. 3). Both strains demonstrated a ZOI of 12 mm with the vancomycin loaded beads, however whilst *E. faecium* 10.15 demonstrated a ZOI of 27 mm with the gentamicin loaded beads, no ZOI was observed for *E. faecium* 10.6 with

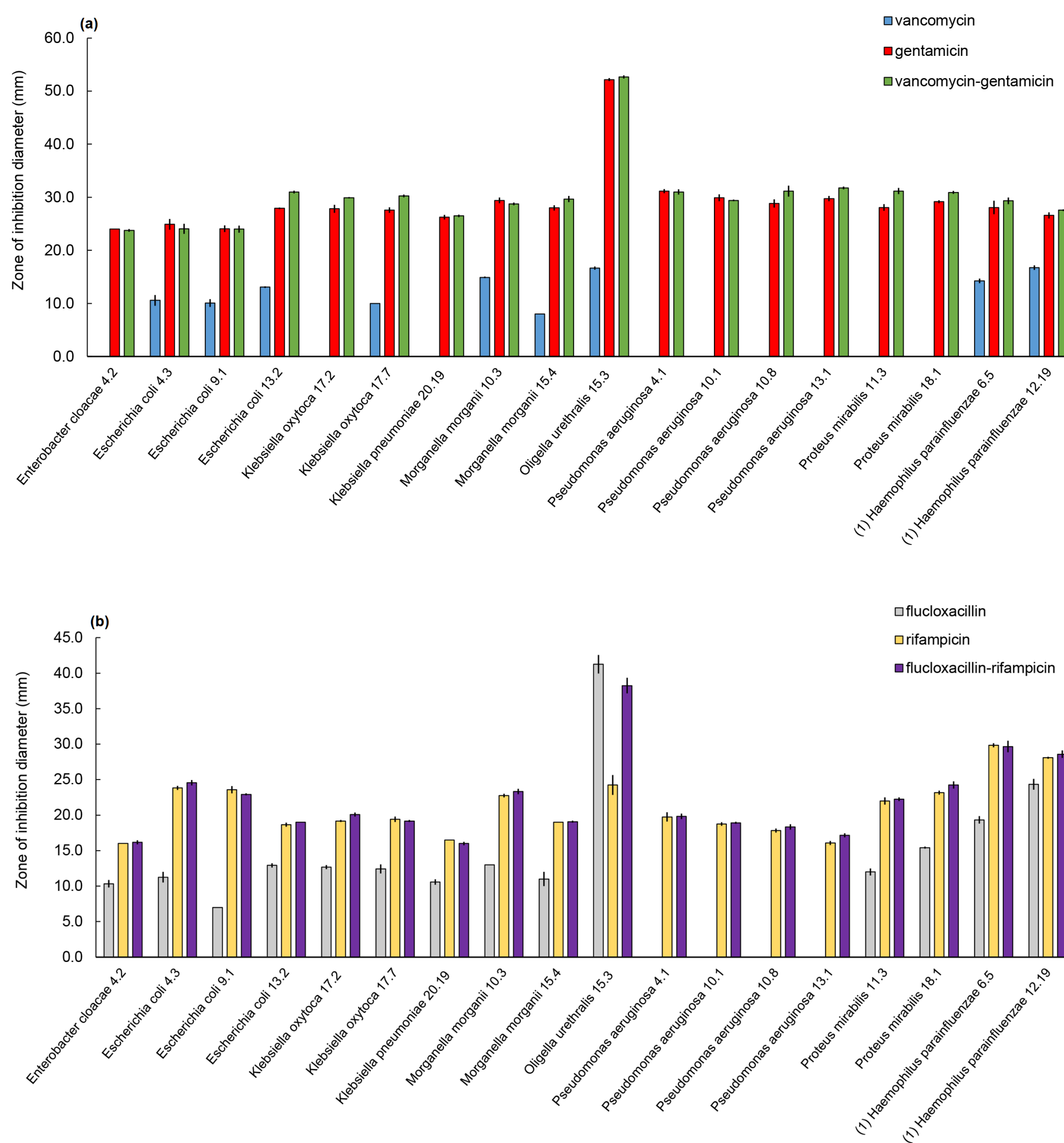

Fig. 4. Zones of inhibition observed with Gram-negative facultative anaerobic strains incubated with beads containing (a) vancomycin, gentamicin or vancomycin-gentamicin combined and (b) flucloxacillin, rifampicin or flucloxacillin-rifampicin combined. [1]Screened on Mueller-Hinton agar supplemented with defibrinated horse blood and β-NAD incubated in a CO_2-enriched atmosphere. All other Gram-negative strains were screened on Mueller-Hinton agar incubated aerobically. Data are expressed as means of three replicates ± standard deviation.

the gentamicin loaded beads (Fig. 3). For the vancomycin-gentamicin loaded beads, *E. faecium* 10.15 demonstrated a ZOI of 28 mm whilst for *E. faecium* 10.6, the ZOI with these beads was 15 mm (Fig. 3). Flucloxacillin produced large ZOI (22–56 mm) with the Gram-positive strains tested, with the exception of the *E. faecium* strains 10.6 and 10.15 where no ZOI was obtained. Rifampicin was the only single antibiotic loaded bead that was able to achieve a ZOI with all the Gram-positive strains tested (26–51 mm, Fig. 3). For the Gram-negative strains, no ZOI or a reduced ZOI was observed with vancomycin loaded beads (Fig. 4). However, gentamicin and vancomycin-gentamicin loaded beads produced ZOI≥24 mm for all the Gram-negative strains tested. Flucloxacillin loaded beads were ineffective against all four *Pseudomonas aeruginosa* strains tested (Fig. 4). Apart from three strains (*E. coli* 4.3 and *Haemophilus parainfluenzae* 6.5 and 12.19), the vancomycin-gentamicin loaded beads produced larger ZOI than the flucloxacillin-rifampicin loaded beads (Fig. 4). ZOI for the obligate anaerobic strains incubated in the presence of antibiotic loaded beads are shown in Fig. 5. Gentamicin is inactive under anaerobic conditions and three of the anaerobic strains tested showed no inhibition of growth in the presence of gentamicin loaded beads (*Bacteroides fragilis* 11.7, *Finegoldia magna* 9.21 and *Porphyromonas asaccharolytica* 16.12). In general, the anaerobic strains were highly sensitive to vancomycin, flucloxacillin and rifampicin loaded beads and large ZOI were observed in the presence of these beads.

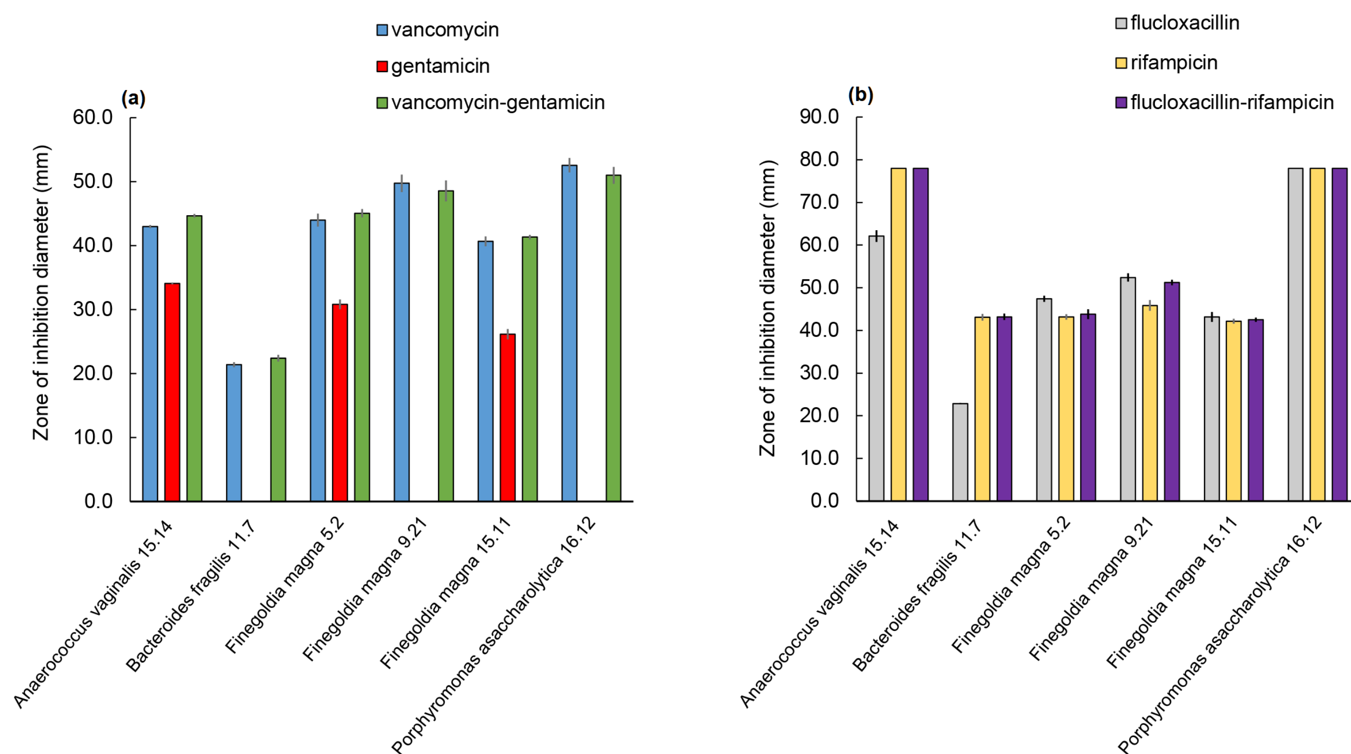

Fig. 5. Zones of inhibition observed with obligate anaerobic strains incubated with beads containing (a) vancomycin, gentamicin or vancomycin-gentamicin combined and (b) flucloxacillin, rifampicin or flucloxacillin-rifampicin combined. Screened on fastidious anaerobe agar incubated anaerobically. Data are expressed as means of three replicates ± standard deviation.

B. fragilis 11.7 was less sensitive to vancomycin and flucloxacillin loaded beads compared to the other anaerobic strains tested, with ZOI diameters of 21 mm and 22 mm for vancomycin and flucloxacillin loaded beads respectively.

Broth microdilution assays

The antimicrobial susceptibilities of six *E. faecalis* and *E. faecium* strains were determined using broth microdilution assays. Four of these strains had shown reduced ZOI with vancomycin and/or gentamicin loaded beads. For comparison, two strains from participant eight that had shown no evidence of reduced ZOI were also included. Three strains (*E. faecalis* 4.6, 9.9 and *E. faecium* 10.6) demonstrated acquired resistance to gentamicin (MIC >128 mg l^{-1}) (Table 2). Gentamicin loaded beads were ineffective in inhibiting the growth of these three strains and no ZOI were observed. *E. faecalis* 9.9 was shown to be resistant to vancomycin

Table 2. Minimum inhibitory concentration for vancomycin and gentamicin with DFI strains

	MIC (mg l^{-1})		Zone of inhibition diameter (mm)			
	Vancomycin 0.5–32 mg l^{-1}	Gentamicin 2–16 mg l^{-1} 500 mg l^{-1}	Vancomycin loaded beads	Gentamicin loaded beads	Vancomycin-gentamicin loaded beads	Unloaded beads
E. faecalis 4.6	0.5=S	>500=R	23	0	23	0
E. faecalis 8.4	1=S	8=S	22	19	23	0
E. faecalis 8.21	1=S	8=S	23	19	22	0
E. faecalis 9.9	32=R	>500=R	24	0	24	0
E. faecium 10.6	>32=R	>500=R	12	0	15	0
E. faecium 10.15	>32=R	8=S	12	27	28	0

Susceptible (S) or resistant (R) attributed from clinical breakpoints (EUCAST Clinical Breakpoint Tables v10.0 (2020), values expressed as mg l^{-1}).
Enterococcus spp. gentamicin (high-level aminoglycoside resistance) S≤128, R>128; vancomycin S≤4, R>4.

(MIC=32 mg l^{-1}). However a typical ZOI of 24 mm was observed with the vancomycin containing beads suggesting that the concentration of vancomycin released by the beads was high enough to inhibit the growth of this clinically resistant strain. *E. faecium* 10.6 and 10.15 demonstrated a higher level of resistance to vancomycin (MIC >32 mg l^{-1}) compared to *E. faecalis* 9.9 (Table 2), and this corresponded with smaller ZOI of 12 mm for both 10.6 and 10.15 with the vancomycin loaded beads. Even though strain 10.6 demonstrated a high level of resistance to both vancomycin and gentamicin in the broth microdilution assay, it was still possible to achieve a small ZOI of 15 mm with the vancomycin-gentamicin loaded beads. The ZOI obtained with vancomycin-gentamicin loaded beads for strain 10.6 was greater (15 mm) compared to the ZOIs obtained with beads containing the individual antibiotics (12 mm for vancomycin loaded beads and no ZOI for gentamicin loaded beads) suggesting that when combined the two antibiotics may have acted synergistically.

DISCUSSION

DFIs can greatly affect an individual's quality of life and in the event of treatment failure they can become limb and even life threatening. New treatments offering improved outcomes are needed to address the complications associated with DFIs.

In this *in vitro* study, calcium sulfate beads loaded with a combination of vancomycin and gentamicin were able to inhibit polymicrobial bacterial growth from homogenised DFI tissue as evidenced by a clear ZOI around the beads. The size of the ZOI is likely to depend on a number of factors including the microbial composition and the sensitivities of the individual components of the microbial community. In addition, the microbial load will have an effect on the size of the ZOI observed. A higher bacterial density will produce a smaller ZOI diameter in disc diffusion assays and a lower density will result in an increased ZOI diameter. In this study, the inoculum was not defined and will have varied depending on the microbial load present in the infected tissue sample. Environmental conditions may also influence the size of the ZOI detected. In most cases where growth was observed both aerobically and anaerobically, smaller ZOI were observed under anaerobic conditions. Gentamicin is inactive under anaerobic conditions [35] and in the absence of oxygen it is likely that the inhibitory effect of the vancomycin-gentamicin loaded beads is mainly due to their vancomycin content.

A broad range of Gram-positive and Gram-negative facultative anaerobic species and obligate anaerobic species were isolated from the DFI tissue samples. This is consistent with numerous other studies that have demonstrated the polymicrobial nature of microbial communities associated with DFIs [36–40]. Culture and sensitivity testing in the clinical microbiology laboratory may highlight the presence of one or more specific pathogens within DFI. However, the contribution of other components of the microflora to the disease process is not clear. For example, anaerobic species often make up a large proportion of the DFI microbial community [39] and yet their potential contribution to disease is little studied. Thus, antibiotic therapy would need to target all of the species that contribute to disease or aim to restore a microflora which is more closely associated with healing. An advantage of calcium sulfate beads is they can be tailored according to the species present and in addition combinations of antibiotics with differing modes of action can be incorporated. The current study has demonstrated that beads containing combinations of vancomycin and gentamicin or flucloxacillin and rifampicin inhibited the growth of all of the Gram-positive and Gram-negative facultative anaerobic strains and all of the obligate anaerobic strains tested. Oral or intravenous flucloxacillin is often given alone for mild DFIs. The advantage of including gentamicin in a local release carrier in addition to antibiotics targeting Gram-positive strains is three-fold. As disease progresses Gram-negative strains are increasingly detected [36] and the inclusion of gentamicin would target any potentially pathogenic Gram-negative species that may be present. In addition there will be the benefit of the synergistic effect of using antibiotics that target cell wall synthesis and antibiotics that inhibit protein synthesis. Furthermore local release of gentamicin may mean that intravenous delivery and its associated toxicity is avoided. Incorporation of a third antibiotic such as rifampicin into local release calcium sulfate beads might also be indicated and in the case of rifampicin this could have anti-biofilm benefits [41].

A further difficulty in treating DFIs empirically is the increasing prevalence of antibiotic resistance [42]. This study has demonstrated the presence of vancomycin resistant enterococci (VRE) and high-level aminoglycoside (gentamicin) resistant enterococci (HLARE).

An advantage of antibiotic loaded beads containing two different antibiotics with differing modes of action is that the presence of resistance to both antibiotics is less likely. In a panel of six enterococcal strains tested, two (*E. faecalis* 4.6 and *E. faecium* 10.15) were shown to have resistance to either vancomycin or gentamicin. In both cases the combination beads were able to produce a ZOI≥23 mm. Spontaneous development of resistance to both antibiotics *in vivo* is a much rarer event than development of resistance to one antibiotic [19]. In polymicrobial biofilm infections, resistant strains (innate or acquired) may afford passive protection to neighbouring species within the community [43] and again this is less likely where a combination of antibiotics with differing modes of action is used. Local release of antibiotics from a carrier material may achieve concentrations high enough to inhibit the growth of resistant strains [19]. Both *E. faecalis and E. faecium* are intrinsically resistant to clinically achievable serum concentrations of aminoglycosides due to their low cell wall permeability for this antibiotic [44]. To distinguish between intrinsic and acquired gentamicin resistance susceptibility testing of *E. faecium* and *E. faecalis* is carried out with high concentrations of gentamicin. Two of the enterococcal strains (*E. faecalis* 9.9 and *E. faecium* 10.6) demonstrated acquired resistance to both vancomycin and gentamicin with MICs above the clinical breakpoints for these antibiotics with *Enterococcus* spp. However, beads containing vancomycin and gentamicin in combination were able to produce a clear ZOI for both strains. Though the ZOI for strain 10.6 was much reduced, the ZOI for strain 9.9 was similar to that achieved with fully susceptible strains.

In addition to variations in antibiotic susceptibilities amongst a particular species across different DFIs, variations in antibiotic susceptibilities for a particular species also occurred within the same wound. Strains of *E. faecium* from the same DFI (participant ten) demonstrated differing susceptibility patterns to vancomycin and gentamicin. Both strains were resistant to vancomycin, however only strain 10.6 demonstrated high level aminoglycoside (gentamicin) resistance with strain 10.15 being susceptible to gentamicin. Whilst this result only hints at the intraspecies heterogeneity that may occur within a single wound, it certainly warrants further investigation as the complexity of DFI communities continues to be illuminated [39, 40]. These findings also illustrate the risk of carrying out antimicrobial susceptibility testing on selected colonies. The occurrence of heteroresistance within a bacterial isolate can result in routine lab testing falsely attributing sensitivity when resistant colonies are missed [45]. Higher local concentrations and use of combinations of antibiotics with different modes of action may inhibit the proliferation of resistant subpopulations *in vivo*.

In the treatment of DFIs, antibiotic loaded beads could be packed into the wound site following debridement. In order to achieve consistently high local concentrations of antibiotics a number of factors need to be considered including the residence time of the beads and their packing density in relation to microbial load [46, 47]. In a soft tissue site with a high fluid exchange, the residence time for calcium sulfate beads is around 3–6 weeks [48, 49]. There is a burst release of antibiotics initially followed by a gradual reduction in concentration as the calcium sulfate absorbs [26, 50, 51]. Patients with DFIs are seen on a regular, often weekly, basis for wound debridement and dressing change. This would provide a regular opportunity to check the integrity of the beads and replace them as required.

This study has shown that calcium sulfate beads loaded with combinations of antibiotics with different modes of action are effective in inhibiting the growth of polymicrobial DFI communities on a simple agar plate model *in vitro*. In the treatment of DFIs, antibiotic loaded calcium sulfate beads could deliver high local concentrations of antibiotics to the target site even where vascularisation is poor. Further work is required to establish the efficacy of antibiotic loaded beads *in vivo* where environmental conditions such as the presence of biofilms and potential removal of antibiotics form the local environment by exudate may influence the outcome.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

All participants gave informed, written consent prior to inclusion in the study. Tissue samples were anonymised in the clinic prior to transport to the research laboratory.

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