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Dear Healthcare provider:

Engineered Tissue Solutions ("ETS") encloses the following reprint, "*Antimicrobial effects of a borate-based bioactive glass wound matrix on wound-relevant pathogens,"* from the Journal of Wound Care, Vol. 32, No. 12 (December 2023) to provide peer-reviewed, scientifically sound and clinically relevant information concerning the use of a borate-based bioactive glass matrix (BBBGM) to reduce the concentration of aerobic pathogens commonly associated with acute and hard-to-heal (chronic) wounds. This study was conducted at an independent test lab and financially sponsored by ETS. ETS and MOSCI, LLC, the manufacturer of BBBGM, are both subsidiaries of the Heraeus Group. Steven Jung is the Chief Technology Officer at ETS and MOSCI.

The study uses the in vitro AATCC 100 test method to evaluate the reduction of clinically relevant pathogens, including Gram-negative and Gram-positive bacteria, yeasts, and moulds, on the borate-based bioactive glass matrix over 7 days. The authors present log reduction data from the in vitro study and conclude as follows: "*The [borate-based bioactive glass matrix] proved to have a robust antimicrobial effect against several bacteria and fungi (and to a lesser extent mould), that are known to complicate acute and hard-to-heal wound healing*." Reprint, p.9.

The limitations of the study are detailed in the reprint. The antimicrobial effects of the borate-based bioactive glass matrix were studied in vitro and independently on individual aerobic pathogens within the matrix product. The authors recommended BBBGM be studied in a more complex environment with mixtures of pathogens and anaerobic pathogens. Finally, while the in vitro analysis is an indication of potential in vivo performance, an in vivo assessment is required to confirm the antimicrobial effects of borate-based bioactive glass matrix.

Mirragen® Advanced Wound Matrix is composed of biocompatible and resorbable borate glass fibers and particulate, and is cleared for use in the management of wounds (K161067). Mirragen Advanced Wound Matrix has not been cleared for an antimicrobial claim, and the safety and effectiveness of Mirragen for uses based on such a claim have not been established. FDA's clearance is restrictive, and we cannot discuss the uses or properties of Mirragen that fall outside of the cleared indications. FDA's Guidance documents, however, allow us to provide reputable and clinically relevant scientific information for your evaluation and review.

For your convenience, please find below links to the Mirragen product labeling and 510(k) clearance summary, as well as an attached relevant scientific bibliography. For additional information regarding Mirragen Advanced Wound Matrix please contact ETS at ets.support@heraeus.com or 573-202-2550.

Labeling (IFU): https://engineeredtissue.com/wp-content/uploads/2023/03/Mirragen-IFU_LBL_0007-REV04.pdf
510(k) summary (K161067): https://www.accessdata.fda.gov/cdrh_docs/pdf16/K161067.pdf

Antimicrobial effects of a borate-based bioactive glass wound matrix on wound-relevant pathogens

Objective: The antimicrobial effects of a borate-based bioactive glass matrix (BBBGM) on clinically relevant microorganisms was investigated for up to seven days in vitro.
Method: A total of 19 wound-relevant pathogens were studied using the in vitro AATCC 100 test method.
Results: The reduction of viable Gram-negative and Gram-positive bacteria and yeasts at days 4 and 7 post-culture on the BBBGM was significant ($>4\log_{10}$) in most cases. Mould counts were reduced ($<2\log_{10}$) during the seven-day assessment, indicating that mould viability and reproduction was inhibited. The cell count of each organism was reduced at seven days indicating that the BBBGM not only reduced the viable cell count, but that the cell count did not recover during the seven-day period, indicating a sustained reduction in pathogenic activity.
Conclusion: Based on the present results, the use of a BBBGM as a pathogenic barrier should be considered as a tool for combating pathogenic colonisation and infection in acute and hard-to-heal (chronic) wounds.
Declaration of interest: Steven Jung is the Chief Technology Officer of MO-SCI LLC, the manufacturer of BBBGM.

antimicrobial • bioactive glass • borate-based bioactive glass matrix • synthetic • wound • wound care • wound dressing • wound healing • wound infection

The microbial bioburden is frequently highly elevated in many hard-to-heal (chronic) skin wounds, and it is well-established that it is a major factor in impairing wound healing.[1] This is due in large part to the patient's immune response to a persistently elevated microbial bioburden 'locking' the wound bed into a chronic inflammatory state. Proteins and molecules that are essential for healing are damaged by elevated levels of proteases (matrix metalloprotease and neutrophil elastase) and reactive oxygen (ROS).[2,3]

Both planktonic and biofilm phenotypes of bacteria are well-recognised as major components of wound microbial bioburden.[4] However, a clinical study revealed that yeasts and moulds are also important constituents of the wound total microbial bioburden.[5] Complete identification of all microbes in individual biopsies of hard-to-heal wounds using DNA sequencing analysis revealed that yeast and mould species were present in at least 23% of hard-to-heal wounds at the start of advanced wound care when wounds had healed after 4–6 weeks.[5] Thus, consensus guidelines for the treatment of acute infected wounds and hard-to-heal wounds have included the importance of using DNA identification to detect the presence of yeast and mould species.[2] Additionally, DNA identification can be used for detecting bacterial species that are difficult to detect using standard agar plate culture techniques because of inherently slow growth rates or fastidious nutritional requirements for these 'difficult to culture' species.[3]

In addition to recognising that yeasts and moulds are frequently important components of the wound total microbial bioburden, clinicians need more information on the ability of wound dressings and advanced wound care products to rapidly and effectively kill pathogenic yeasts and moulds that are frequently present in hard-to-heal wounds.

Bioactive glass was first developed in 1969 as a synthetic bone grafting material that would support bone formation and promote healing.[6] Bioactive glass is a water-soluble material that is composed of biologically useful elements that are released into ionic constituents when dissolved in body fluids. Ions, such as calcium and phosphorus, react in vivo to form a calcium apatite material similar to hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$. Hydroxyapatite is the mineral component found in pH basic hard tissues, such as bone and teeth.[6,7]

Interest in using bioactive glasses for soft tissue and wound repair and burns has grown rapidly.[7–12] Soft tissues, such as skin, have a neutral to acid pH which prevents the formation of the calcium apatite mineral.[13] As bioactive glass dissolves in soft tissues, instead of forming hydroxyapatite, free calcium ions promote a variety of functions including haemostasis and chemotaxis as a cell sensing receptor and recruiter to aid in the healing process.[14,15]

An interesting property of bioactive glass is its direct or indirect effect on inhibiting microbial growth.[16]

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Several mechanisms for the antimicrobial response of bioactive glass have been proposed and include: a basic pH environment; changes in osmotic pressure; cellular damage caused by physically damaging the cell wall of the pathogen; or the ionic concentration of the adjacent environment.[17–19] Previous work studying the antimicrobial activity of bioactive glass focused on in vitro assessments of planktonic bacteria, or the inhibition of biofilm formation.[20,21] Studies demonstrating effectiveness against pre-existing biofilms are limited at the present time. Jung et al.[17] reported that therapeutically active metal ions, including copper and zinc, added to a borate-based bioactive glass matrix were found to further enhance the antibiofilm activity in an in vitro peg-biofilm assay.

The dissolution behaviour of a bioactive borate glass was studied for reaction time and pH change in a dilute phosphate solution.[22] The pH rose rapidly from pH 7.3 to approximately pH 9.0 in 24 hours, then buffered at this level until the glass was fully dissolved at day 4.[23] The glass was found to dissolve congruently, releasing its ions into the phosphate solution and from beginning to end did not change the dissolution mechanism.[24] The rapid change in both ion concentration of the surrounding fluids and the pH likely contribute to the antimicrobial effects of the borate-based bioactive glass.

Table 1. List of clinically significant microorganisms tested in the study

Category	Species	Strain
Gram positive	*Bacillus subtilis*	ATCC 6633
	Enterococcus faecium (VRE)	ATCC 51559
	Staphylococcus aureus	ATCC 6538
	Staphylococcus aureus (MRSA)	USA300
	Streptococcus pyogenes	ATCC 12344
Gram negative	*Acinetobacter baumannii*	AB5075-UW
	Enterobacter cloacae	ATCC 13047
	Escherichia coli	ATCC 8739
	Klebsiella pneumoniae	ATCC 4352
	Pseudomonas aeruginosa	ATCC 27312
Yeast	*Candida albicans*	ATCC 18804
	Candida auris	AR-0384
	Candida krusei	ATCC 14243
	Candida tropicalis	ATCC 13803
Mould	*Aspergillus brasiliensis*	ATCC 16404
	Cladosporium herbarum	MYA-4682
	Curvularia lunata	ATCC 14595
	Fusarium dimerum	ATCC 16553
	Trichophyton mentagrophytes	ATCC 28145

The main cause of pathogen susceptibility will be pathogen-specific. Some pathogens may be majorly affected by pH while others may be affected more by the local ion concentration.[24] The overall effect is most likely a combination of these contributions as they are not mutually exclusive.

To further understand the potential effects of borate-based bioactive glass matrix (BBGFM) on yeast and mould species frequently present in hard-to-heal diabetic foot ulcers (DFU), the BBGFM was assessed for its antimicrobial effects on microbial species, including yeasts, moulds, and Gram-negative and Gram-positive bacteria using the standard AATCC 100 test method.[25] This is a quantitative test method used to determine the efficacy of antibacterial finishes applied to textile materials.

Primary and secondary objectives

The primary objective of the present work was to determine the antimicrobial effects of the BBBGM on clinically relevant microorganisms. The secondary objective was to understand that if an antimicrobial effect was observed in the first four days, whether or not there was a resurgence of the organisms between days 4 and 7, as can occur with wound cleansing products.

Methods

Test microorganisms

In this study, a total of 19 microbial strains, including five Gram-positive, five Gram-negative, four yeast, and five mould strains of clinical significance were investigated (Table 1). *Candida auris* was obtained from the US Centers for Disease Control (CDC) antibiotic resistance isolate bank (CDC, US), while all other species were obtained from the American Type Culture Collection (ATCC, US). All microbial strains were stored as 20% glycerol stocks at –80°C. Bacteria and yeasts were streaked on tryptic soy agar (TSA) (Becton Dickinson, US) plates and stored at 4°C for up to two weeks. However, mould stocks were stored for up to four weeks at 4°C.

Ethical approval

The present work did not require animal or human ethical approval as only in vitro cell testing of bacteria, mould, and fungi were used for this study.

Bacteria

Bacterial cultures were prepared by inoculation of 5ml of tryptic soy broth (TSB) (Becton Dickinson, US) in a 15ml culture tube with one colony of the respective bacterial strains, followed by incubation for 18–24 hours at 37°C with shaking (125rpm).

Yeast

Yeast cultures were prepared by inoculation of 30ml of yeast mold broth (YMB) (Becton Dickinson, US) in a 150ml baffled flask with one colony of the respective yeast strain. Yeasts were incubated for 48 hours with

shaking (125rpm) at 25°C (*Candida albicans, Candida krusei, Candida tropicalis*) or at 37°C (*Candida auris*).

Mould

Refrigerated mould stocks were prepared in 0.9% saline. Briefly, all mould strains were cultured on potato dextrose agar (PDA) (Becton Dickinson, US), except for *Aspergillus brasiliensis* which was cultured on sabouraud dextrose agar (SDA) (Becton Dickinson, US). After inoculation with the respective strains, the agar plates were incubated under stationary conditions at 25°C until significant sporulation was observed. After incubation, the mould was removed from the agar by washing with 5000ppm Tween-80 in 0.9% saline. The mould solution was filtered twice through sterile cotton balls to remove hyphal debris, then centrifuged at 2300 x g for 20 minutes and resuspended in 0.9% saline. An aliquot of the stock was serially diluted in phosphate buffered saline (PBS, 0.8% saline, 2.7mM potassium chloride (KCl), 10.1mM disodium phosphate (Na_2HPO_4), 1.8mM monopotassium phosphate (KH_2PO_4), pH 7.4) and enumerated on the appropriate agar. Stocks with a minimum concentration of 1×10^7 colony forming units (CFU)/ml were deemed acceptable for use.

Test materials

A BBBGM, consisting of bioabsorbable GL1550 borate bioactive glass fibres and particles (MO-SCI LLC, US), was evaluated in this study. The target composition of the GL1550 glass in % weight was: 53% boric oxide (B_2O_3); 20% calcium oxide (CaO); 12% potassium oxide (K_2O); 6% sodium oxide (Na_2O); 5% magnesium oxide (MgO); and 4% phosphorus pentoxide (P_2O_5).

A total of 312 BBBGM samples from two separate lots were studied for antimicrobial activity. Curity all-purpose sponges (non-woven Polyester/Rayon, 4-ply, 10.2×10.2cm, Lot E22021009N) (Covidien, Ireland) were stacked to achieve 8-ply thickness, sterilised via autoclave, and used for comparative purposes. Prior to use, all samples were cut aseptically from a single 10.2×10.2cm pad to approximately 5.1×5.1cm in size. Curity all-purpose sponges were used as the control because they have no antimicrobial efficacy.

Simulated wound fluid preparation

Simulated wound fluid (SWF) was prepared by mixing one-part sterile fetal bovine serum (FBS) (Mediatech, Inc., US) with one-part minimal recovery diluent (MRD). MRD was prepared by adding 1.0g peptone (Fisher BioReagents, US) and 8.5g sodium chloride (NaCl) (Avantor, Inc., US) to 1l sterile deionised (DI) water. After proper mixing, the pH of the solution was adjusted to approximately 7.0 and sterilised. After preparation, SWF was stored at 4°C for up to one month.

Modified AATCC TM100

Antimicrobial efficacy of the BBBGM was determined according to a modified version of AATCC TM100-2019.[1] The samples were placed in individual sterile 100×15mm Petri dishes, followed by the addition of 6ml of SWF, and then transferred to a stationary humidified incubator at 37°C (bacteria and yeast) or 25°C (mould) for seven days to precondition. Following the seven-day preconditioning period, the samples were inoculated with 1.5ml SWF containing approximately 10^6 CFUs of bacteria, yeast or mould. Following inoculation, the day 0 samples were recovered by transferring them into individual 50ml centrifuge tubes containing 30ml of Dey Engley broth (D/E) (Becton Dickinson, US). To remove the residual sample on the Petri dishes, each dish was washed with 5ml of D/E broth from the corresponding centrifuge tubes. The samples were vortexed at maximum speed for 10 seconds, sonicated for five minutes, and vortexed for another 10 seconds immediately prior to removing an aliquot from each tube. Aliquots were used to make 10-fold serial dilutions in PBS and were plated on the appropriate agar for enumeration. The plates for enumeration were incubated at 37°C (bacteria and yeast) or 25°C (mould) until colonies were countable.

The remaining samples were incubated for an additional one day, four days or seven days, then transferred individually to 50ml centrifuge tubes containing 30ml of D/E broth for sonication, recovery, and plating as described above. Each material was evaluated in triplicate, and the experiment was performed once per species. The seven-day model was used because the advanced skin substitute model for care is typically seven-day applications.

Statistical methods

Data were recorded in Microsoft Excel (Microsoft Corp., US). Microbial reduction was expressed as mean log (CFU/sample)±standard deviation (SD). To determine microbial reduction after day 1, day 4 and day 7, the data were compared with the same material at the start of the experiment (day 0). A two-way analysis of variance (ANOVA) followed by a Tukey post hoc test was performed to evaluate multiple comparisons using statistical software JMP for Windows ver. 17.0.0 (SAS Institute Inc., US). A p-value of <0.05 was considered statistically significant.

Results

The modified AATCC TM100 revealed that the BBBGM showed antimicrobial activity against a broad range of clinically relevant wound pathogens. The average logarithmic value of microbial growth reduction is summarised in Table 2, while logarithmic survival is plotted in Fig 1a–d. The test results indicated that, after respective contact time intervals (i.e., day 1, day 4, and day 7), the growth of the majority of the pathogens tested was significantly reduced in a time-dependent manner ($p<0.05$). However, no significant ($p>0.05$) difference in antimicrobial efficacy between the two lots of BBBGM was observed.

Table 2. Log reduction of test microorganisms in bioactive glass wound matrix at t=day 1, day 4, and day 7. Data are presented as log(CFU/sample). Reduction was calculated relative to the same material at day 0. Average reductions >4 logs are in bold. CFU–colony forming unit

		Reduction (log CFU/sample)		
Category	**Species and strain**	**day 1**	**day 4**	**day 7**
Gram-positive	*Bacillus subtilis ATCC 6633*	2.02	**6.22**	**5.86**
	Enterococcus faecium ATCC 51559	0.20	2.61	3.43
	Staphylococcus aureus ATCC 6538	2.85	**6.69**	**6.69**
	Staphylococcus aureus USA300	0.39	3.46	**6.15**
	Streptococcus pyogenes ATCC 12344	**6.93**	**6.93**	**6.93**
Gram-negative	*Acinetobacter baumannii AB5075-UW*	1.44	**5.56**	**6.02**
	Enterobacter cloacae ATCC 13047	1.31	**5.97**	**5.97**
	Escherichia coli ATCC 8739	**4.02**	**6.22**	**6.22**
	Klebsiella pneumoniae ATCC 4352	1.62	**6.00**	**6.00**
	Pseudomonas aeruginosa ATCC 27312	**6.32**	**6.32**	**6.32**
Yeast	*Candida albicans ATCC 18804*	0.22	**5.88**	**5.88**
	Candida auris AR-0384	0.07	0.92	2.71
	Candida krusei ATCC 14243	2.33	**6.43**	**6.43**
	Candida tropicalis ATCC 13803	0.71	5.84	5.84
Mould	*Aspergillus brasiliensis ATCC 16404*	0.83	0.92	2.13
	Curvularia lunata ATCC 14595	–0.15	0.21	0.21
	Cladosporium herbarum MYA-4682	0.25	0.49	1.62
	Fusarium dimerum AATCC 16553	0.27	1.06	1.49
	Trichophyton mentagrophytes AATCC 28145	0.11	0.19	0.27

In total, four of the five Gram-positive bacteria showed a reduction of >4log$_{10}$ after day 7 of contact. After day 1 of contact, the BBBGM achieved a reduction of 6.93-log$_{10}$ for *Streptococcus pyogenes* ATCC 12344, providing a short-term antimicrobial effect. However, a reduction of >6log$_{10}$ was achieved for *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 6538 after day 4 of contact. In addition, the BBBGM showed antimicrobial efficacy against the methicillin-resistant strain *Staphylococcus aureus* USA300, reducing to 6.15-log$_{10}$ after day 7 of contact. As for viable microbes, at day 0, the log$_{10}$ values ranged between 5.90–6.93, which decreased significantly with progression of time. No viable cells were detected for *Streptococcus pyogenes* ATCC 12344, *Streptococcus aureus* ATCC 6538, and *Streptococcus aureus* USA300 after days 1, 4 and 7 of contact, respectively.

The BBBGM also demonstrated strong antimicrobial efficacy against all five Gram-negative bacteria tested. *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 27312 achieved a reduction of >4log$_{10}$ after day 1 of contact, with no viable microbes detected after day 4 of contact. The remaining three microbes, including *Acinetobacter baumannii* AB5075-UW, *Enterobacter cloacae* ATCC 13047, and *Klebsiella pneumoniae* ATCC 4352, showed a reduction of 5.6–6.0log$_{10}$ after day 4 of contact.

In this study, three of the four yeasts showed a strong susceptibility to the BBBGM, particularly at longer timepoints. *Candida albicans* ATCC 18804, *Candida krusei* ATCC 14243, and *Candida tropicalis* ATCC 13803 showed no detectable survival after days 4 and 7 of contact, achieving a >5log$_{10}$ reduction. *Candida auris* AR-0384 was less susceptible, with a 2.71 log$_{10}$ reduction at seven days.

Although most of the microorganisms tested demonstrated sensitivity to the BBBGM, the moulds were relatively tolerant. *Cochliobolus lunata* ATCC 14595 and *Trichophyton mentagrophytes* ATCC 28145 showed the most tolerance among the molds, with a seven-day reduction of 0.21 and 0.27 log$_{10}$, respectively. *Fusariosis dimerum* ATCC 16553, *Cladosporium herbarum* ATCC MYA-4682 and *Aspergillus brasiliensis* ATCC 16404 showed more susceptibility, with an average log reduction ranging from 1.49–2.13 log$_{10}$ at seven days.

Fig 1. Log survival of test microorganisms in borate-based bioactive glass wound matrix versus standard cotton gauze at day 0, day 1, day 4 and day 7. Data are presented as log (CFU/sample). CFU–colony forming unit

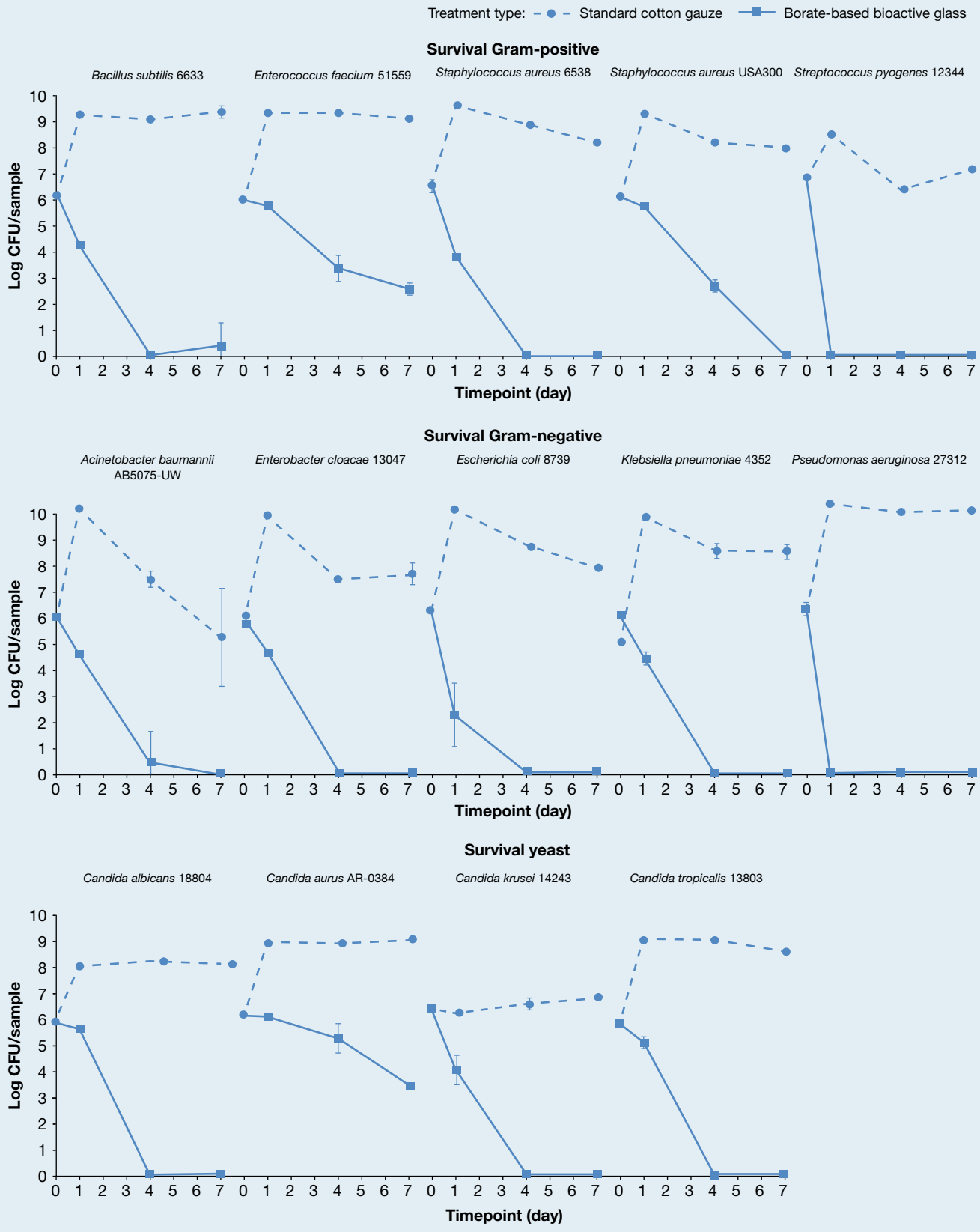

Fig 1. (Continued) Log survival of test microorganisms in borate-based bioactive glass wound matrix versus standard cotton gauze at day 0, day 1, day 4 and day 7. Data are presented as log(CFU/sample). CFU-colony forming unit

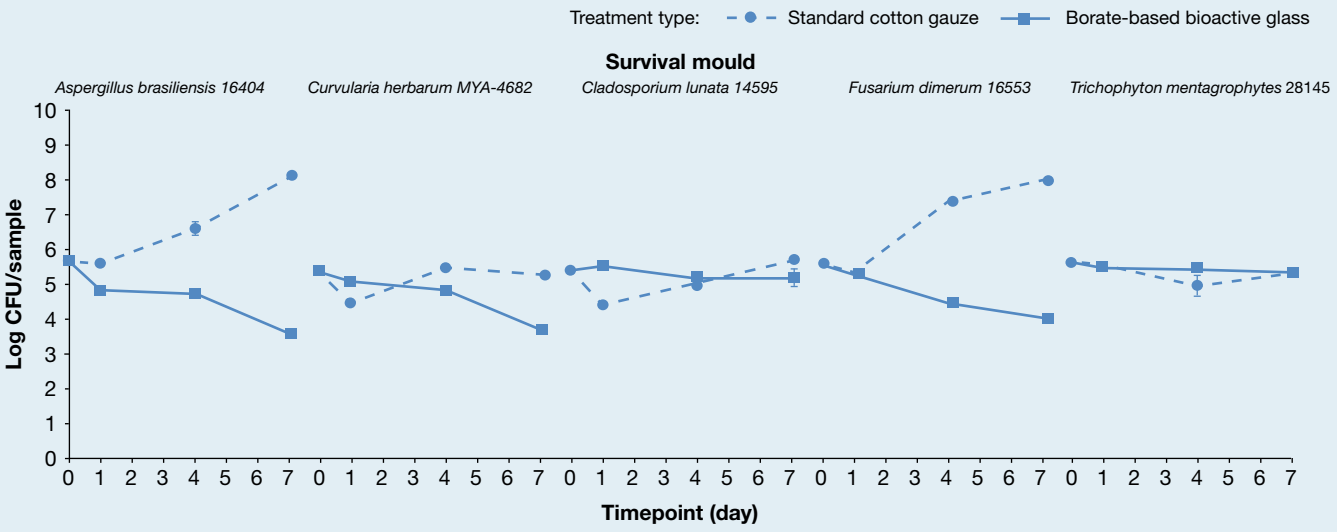

Table 3. Fundamental differences between types of fungi (moulds versus yeasts)

	Moulds	Yeasts
Structure	Filamentous and exist as multicellular structures	Typically single-celled fungi
Tolerance to environment	Broad range of tolerance to pH levels, temperature and humidity	Better adapted to high sugar concentrations and low oxygen levels
Reproduction	Formation of spores (several days to weeks)	Budding (occurs in a few days)

Discussion

A multicentre, single-blinded randomised controlled clinical trial evaluating the effect of BBGFM, (n=40, patients in the clinical trial) , in the treatment of hard-to-heal DFUs found that 70% of the BBGFM-treated DFUs healed compared with 25% treated with standard of care (SOC) alone (adjusted p=0.006). At 12 weeks, mean percentage area reduction of wounds was 79% in the BBGFM group compared with 37% in the SOC group (adjusted p=0.027). The mean number of BBGFM applications was 6.0. Adding BBGFM to SOC significantly improved wound healing and lead to no adverse events related to treatment compared with SOC alone. The authors of this study hypothesised that the difference in the adverse events between the SOC and BBGFM test groups could be due to reducing levels of yeasts, moulds, and bacterial species in the BBGFM treated wounds.[26]

The pathogens in this present study were selected because of their relevance to acute and hard-to-heal wound infection. The survival of the different pathogen groups shown in Fig 1. illustrates the overall reduction of viable cells from day 0–7. While some organisms such as *Streptococcus pyogenes* and *Pseudomonas aeruginosa* decreased rapidly after just one day, most bacteria and yeast did not have a significant reduction until day 4.

In the mould group, there was some reduction of the overall viable cell count for certain pathogens, such as *Aspergillus brasilienis* and *Fusarium dimerum*. Interestingly, these two moulds both had measurable increases in viable cell count in the control group during the seven-day assessment while the other three moulds in the control group were essentially the same count at day 7. The overall effect of the BBBGM on the three moulds (*Cladosporium herbarum*, *Curvularia lunata*, and *Trichophyton mentagrophytes*) with negligible change in the control group cell count was inconclusive. The growth rate was flat and the cell counts between the control and the BBBGM were similar. It is clear that the ability of the BBBGM to reduce the number of viable inoculated mould cells was limited. Based on the data in Fig 1, the moulds were slower to reproduce than the other pathogen groups but more resilient to the presence of the BBBGM.

Moulds and yeasts are both classified as fungi, but they have differences that are important to understand (Table 3). Moulds are filamentous fungi that exist as multicellular structures whereas yeast are typically single-celled fungi. Moulds generally have a broader range of tolerance for environmental factors compared to yeasts, which include pH levels, temperature, and humidity.[27,28] Yeasts are better adapted to environments including high sugar concentrations and low oxygen levels.[29,30] Moulds and yeasts reproduce differently as well. Moulds reproduce through the formation of spores which can be relatively slow compared to yeast which reproduce asexually through a process called budding. Budding is a process where a small daughter cell is formed and then detached from the parent cell. The reproduction rate of the yeasts in the study were markedly higher than the moulds as it can take moulds

several days to a few weeks to start producing spores.[31]

The BBBGM is composed of small diameter fibres (1–2µm) and larger diameter microspheres ranging in size from 100–500µm. The BBBGM is water soluble, so when in an aqueous environment, such as SWF or human body fluids, the glass dissolves into its ionic constituents.[23] The ions being released from the glass can affect the local pH and the local overall ion concentration, which can subsequently affect the viability of pathogens.[16] The GL1550 BBBGM contains ions of boron, calcium, potassium, magnesium, sodium, and phosphorus. Each of these elements are natural to the body and typically introduced through food or vitamin supplements.

The presence of these bioactive glass ions on the local environment of the BBBGM can have beneficial effects on decreasing pathogen viability. The known effects of bioactive glass ions on pathogens include: ion release of biologically active ions, such as boron,[32,33] calcium,[14] magnesium,[34] potassium,[35] sodium[36] and phosphate;[37] pH modulation;[38] surface interactions directly with pathogens;[35] generation of ROS which can lead to oxidative damage to microbial cells;[39] protein adsorption which can lead to the inactivation or alteration of pathogenic proteins;[40] and antibiofilm activity through the disruption of the stability or formation of biofilms.[7,16]

The major components of the bioactive glass all become ionic in nature when dissolved in aqueous environments. These ions have been shown to have a significant effect on pathogens. Boron is the single largest component of the BBBGM composition and its biological effects as an essential trace element have been studied for its contributions to bone health, hormone regulation, brain function, wound healing and antioxidant activity.[33] Boron is released as boric acid (H_3BO_3), and the effects of boric acid on a variety of pathogens have been widely published.[41,42] Boron has been shown to effectively disrupt cell walls in a variety of pathogens. It does so by inhibiting the synthesis of certain cell wall components, such as peptidoglycan in bacteria and chitin in fungi.[43] This weakens the cell wall, leading to cell lysis and death.

Microbial growth and survival require enzymes (protein catalysts) to function correctly to facilitate and accelerate specific chemical reactions in living organisms by lowering the activation energy required for the reactions to occur. Enzymes play a critical role in metabolism, regulation of biochemical processes, and maintaining the overall cellular function. Boron has the capability to modify or inhibit this activity.[44] Essential metabolic pathways, such as carbohydrate metabolism and energy production, can be modified or disrupted by enzyme inhibition. By inhibiting the enzymes in pathogens, the cells lose the capability for regular activity and die. It has also been reported that boron can inhibit the formation and stability of pathogenic biofilm.[42] Boron can interfere with the extracellular matrix (ECM) of the biofilm, and prevent the attachment and colonisation of pathogens.[16]

Boric acid has been found to be beneficial to wound healing and was found to have no toxic effect in Sprague Dawley rats at a concentration of 126mg/kg/day.[45] This is the equivalent to 170g of BBBGM applied to a human weighing just over 45kg. The 5.1×5.1cm pads used in this present study weighed approximately 2.25g each, and amounted to 75 test articles, with a total weight of 170g. These test articles would cover an area no smaller than 1935cm². This is relevant because the average DFU upon presentation to a physician is less than 5cm²,[46,47] or approximately 1/400th the amount of glass previously found to have no identifiable toxicity.

Elements, such as calcium and magnesium, have been shown to disrupt or kill pathogens in a variety of ways. The generation of ROS, such as hydrogen peroxide (H_2O_2) and hydroxl radicals (OH-) is one such way.[48] These ROS are highly reactive and cause oxidative damage to pathogenic cells including lipid peroxidation, protein denaturisation and DNA fragmentation.[49] High calcium concentration was linked to reduced degree of infection, biofilm formation and resistance to oxidative stress of *Pseudomonas aeruginosa* in vitro.[50] These ions can disrupt the ECM of biofilms which weakens the overall structure and makes them more susceptible to antimicrobial agents. Calcium and magnesium also play critical roles in immunomodulation of immune cells. Cells, such as T-cells, natural killer (NK) cells, neutrophils and macrophages which fight microbial infections, are first activated by calcium ions.[51] Interestingly, T-cells and NK cells were found to become more potent when the magnesium concentration increased in vitro.[51] Calcium-mediated chemotaxis (migration) contributes to the movement of immune cells, such as neutrophils, T-cells, macrophages, dendritic cells and mast cells, towards the site of infection or inflammation.[52,53]

Sodium and potassium interact with pathogens differently, which aids in the overall impact these ions can have. Alkali elements, such as sodium and potassium, can induce osmotic stress on pathogenic cells when present in high concentrations.[54] They do so by disrupting the osmotic balance by competing with calcium and magnesium in the cell wall-cytoplasm membrane interface. High concentrations of sodium have been shown to decrease the ability of *Geobacillus* to grow biofilm.[54] Ionic interference from both sodium and potassium by competition over binding sites and transport mechanisms within the pathogen can disrupt cellular processes including enzyme activity, metabolism and osmoregulation, all contributing to inhibited pathogenic growth. For example, potassium ions are involved in regulating the electrical potential across the pathogen cell membrane. Changing the potassium electrical potential in either direction can have negative effects for the pathogen.[35]

Phosphorus has the ability to interfere with energy metabolism of pathogens. It can contribute to and disrupt adenosine triphosphate (ATP) synthesis which is essential for cellular energy production.[55] By

inhibiting ATP synthesis, the pathogen will not have sufficient energy and reducing its likelihood of survival. Phosphorus can modulate pH through the formation of phosphoric acid. Phosphoric acid can inhibit the growth of pathogens and disrupt cellular processes, ultimately reducing pathogenic survival.[56]

To gauge the impact of the microbial survival data from this present study, it is important to compare with other antimicrobial wound materials found in the literature. A commonly studied wound pathogen is *Pseudomonas aeruginosa*, due to its prevalence in wound infections and biofilms. An in vitro assessment of four antimicrobials (polyhexanide (PHMB), octendine di-hydrochloride (OCT), cadexomer-iodine (C-IOD), and ionic silver (Ag)) were compared for antimicrobial efficacy against biofilms of *Pseudomonas aeruginosa* over the course of six days. Overall, the Ag and the OCT dressings did not achieve sufficient antimicrobial efficacy. The C-IOD dressing was the only one to achieve a $7\log_{10}$ reduction in three days and performed the best of the four dressings. The PHMB dressing achieved a significant antimicrobial effect over OCT and Ag from days 2–6.[57]

Another antimicrobial concept includes coating dressing materials with a compound called dialkylcarbamoyl chloride (DACC) which imparts a hydrophobic surface on the substrate, and when hydrophobic bacteria make contact, they are bound to the surface. DACC-coated dressings achieved a $>6\log_{10}$ reduction of *Pseudomonas aeruginosa* for a period of seven days, even with daily reinoculations.[58] Additionally, superabsorbent polymer (SAP) dressings have been studied for antimicrobial activity over seven days against *Pseudomonas aeruginosa*.[59] The growth of *Pseudomonas aeruginosa* was reduced after four days and completely inhibited after seven days. Wiegand et al.[59] mentioned that the antimicrobial behaviour was inhibited once the fluid present in the dressing exceeded the fluid capacity of the dressing.

In the present study, *Pseudomonas aeruginosa* was undetectable after 24 hours with planktonic cells seeded on the BBBGM. When Jung et al.[16] injected an extract of deionised water with dissolved ions from the GL1550 BBBGM onto an established biofilm of *Pseudomonas aeruginosa*, a $3\log_{10}$ reduction was reported after 24 hours, and was non-detectable at 48 hours. The BBBGM has proved to be highly effective against *Pseudomonas aeruginosa* and to elicit robust antimicrobial behaviour consistent with the most effective commercial antimicrobial technologies in wound care.[16]

Each of the pathogens in this present study are aerobic organisms. Interestingly, anaerobic organisms have also been found to play a key role in biofilm formation and wound infection.[60] The ions present in bioactive glass make it an interesting material for future studies on its impact against medically significant anaerobic organisms and biofilms. The ions present in BBBGM have been shown to impact several of these factors, as described in this in vitro study, which makes it an interesting material for future studies to evaluate its impact on medically significant anaerobic organisms and biofilms.

Limitations

The limitations of the present work are related to the pathogens. Each pathogen is aerobic in nature, therefore the effects of BBBGM on anaerobic pathogens requires future investigation. Each pathogen was studied independently to the others. Mixtures of pathogens of a type, or mixtures of multiple pathogen types similar to those found in wounds would be insightful. Additionally, in vitro analysis is an indication of potential in vivo performance, but further controlled in vivo assessments are required to confirm the antimicrobial effects of BBBGM.

Conclusion

The BBBGM proved to have a robust antimicrobial effect against several bacteria and fungi (and to a lesser extent mould), that are known to complicate acute and hard-to-heal wound healing. The overall pathogen viability was reduced at seven days with no intermediate spikes in pathogen growth. This sustained reduction in pathogen concentration is essential for the wound healing process. The beneficial effects of the BBBGM as a barrier to a broad spectrum of pathogens has been identified and should be studied in more complex environments with multiple pathogens, both in vitro and in vivo. **JWC**

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