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Efficacy and safety of biofilm dispersal by glycoside hydrolases in wounds☆

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ABSTRACT

Novel anti-biofilm and dispersal agents are currently being investigated in an attempt to combat biofilm-associated wound infections. Glycoside hydrolases (GHs) are enzymes that hydrolyze the glycosidic bonds between sugars, such as those found within the exopolysaccharides of the biofilm matrix. Previous studies have shown that GHs can weaken the matrix, inducing bacterial dispersal, and improving antibiotic clearance. Yet, the number of GH enzymes that have been examined for potential therapeutic effects is limited. In this study, we screened sixteen GHs for their ability to disperse mono-microbial and polymicrobial biofilms grown in different environments. Six GHs, α-amylase (source: *A. oryzae*), alginate lyase (source: various algae), pectinase (source: Rhizopus sp.), amyloglucosidase (source: *A. niger),* inulinase (source: *A. niger*), and xylanase (source: *A. oryzae*), exhibited the highest dispersal efficacy *in vitro*. Two GHs, α-amylase (source: Bacillus sp.) and cellulase (source: *A. niger*), used in conjunction with meropenem demonstrated infection clearing ability in a mouse wound model. GHs were also effective in improving antibiotic clearance in diabetic mice. To examine their safety, we screened the GHs for toxicity in cell culture. Overall, there was an inverse relationship between enzyme exposure time and cellular toxicity, with twelve out of sixteen GHs demonstrating some level of toxicity in cell culture. However, only one GH exhibited harmful effects in mice. These results further support the ability of GHs to improve antibiotic clearance of biofilm-associated infections and help lay a foundation for establishing GHs as therapeutic agents for chronic wound infections.

1. Introduction

Chronic wounds include diabetic foot ulcers, pressure ulcers, and venous ulcers and are estimated to affect 1 to 2% of the entire population of developed countries [1]. As the comorbidities (age, diabetes, and obesity) associated with chronic wounds continue to rise, their incidence is expected to exhibit a similar trend [2–4]. Chronic wounds have a significant impact on quality of life and are associated with high morbidity and mortality [5]. The chronicity of wounds is due in part to an inability of the host to clear infection and heal naturally. The development of a biofilm in the wound impedes host clearance of bacteria and can confer an increased tolerance to antibiotics [6,7]. A biofilm is a community of microorganisms that are protected by an extracellular polymeric substance (EPS) [8]. The EPS is composed of various substances including polysaccharides, proteins, lipids, extracellular DNA, and various host components such as fibrin and collagen [8]. To improve the outcome of chronic wound infections, agents targeting the EPS have been proposed as therapeutic agents [9,10].

The current standard of care for chronic wound infections is mechanical debridement of the infected tissue. This mode of treatment is often painful and not highly effective, as biofilm cells present deep in the tissue may not be completely removed by debridement [11]. To improve the treatment process, biofilm dispersal agents have been proposed to induce bacterial dispersal either by acting as a signal that initiates active dispersal or by disrupting the EPS scaffolding, causing passive dispersal. In theory, these newly planktonic cells would then be more susceptible to antimicrobials or immune cell clearance. As proof of concept, we successfully showed that by targeting the polysaccharide component of the EPS we could improve antibiotic efficacy *in vitro* and *in vivo* [12,13]. Our previous studies utilized the glycoside hydrolases (GHs) α-amylase

☆ Author order was determined on the basis of contribution.

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(from *Bacillus subtilis*) and cellulase (from *Aspergillus niger*) to target the α-1,4 and β- 1,4 linkages, respectively, of exopolysaccharides in the bacterial EPS and induce dispersal from the biofilm [12–14]. However, polysaccharides in the EPS can vary substantially, based on the species present in the biofilm and include varying linkages such as α-1,6, β-1,2, β-1,3, and β-1,6. Thus, it is likely that effective dispersal may require a cocktail of different GHs, especially for highly polymicrobial infections. This study focused on screening sixteen different GHs that have a variety of targets (Table 1) to induce bacterial dispersal in biofilms established by either *Pseudomonas aeruginosa, Staphylococcus aureus,* or both together, in a variety of models.

Our long-term goal is to provide new treatment options for difficult to treat biofilm-related infections by determining the most efficacious GHs that improve bacterial clearance when used in conjunction with antibiotics. To this end we evaluated the efficacy of GHs in two *in vitro* models, an *ex vivo* mouse model, and an *in vivo* mouse model. As a major concern for the development of antimicrobial and anti-biofilm agents is toxicity to the host, we also screened GHs for toxicity in both representative cell lines and mice. Our results suggest a lack of toxicity for five of the GHs tested.

2. Results

2.1. Several GHs disperse mono-species biofilms in vitro

In order to compare the efficacy of the selected GHs to disperse bacteria, we first tested them against two bacterial species commonly found in chronic wounds, in a commonly utilized *in vitro* well-plate dispersal assay [14]. *P. aeruginosa* or *S. aureus* were grown for 48 h and then the biofilms were treated with PBS (vehicle control) or 500 U/mL of different GH enzymes. 500 U/mL was selected for the GHs of interest based on the concentration of DNase used to disrupt biofilms in previously published work [15]. One enzyme, pectinase (source: Rhizopus sp.), was tested at 125 U/mL due to the inability of a higher concentration to go into solution. After 2 h, the dispersed cells in the supernatant were removed and the remaining biofilm was sonicated. Both the supernatant and biofilm cells were serially diluted and plated to measure CFU and calculate 'percent dispersed' (Fig. 1).

Using this simple, well-plate biofilm model we saw that alginate lyase (source: various algae), α-amylase (source: *A. oryzae*), β-amylase (source: barley), amyloglucosidase (source: *A. niger)* and xylanase (source: *A. orzyae*) significantly dispersed *P. aeruginosa* from mono-species biofilms (Fig. 1A). Alginate lyase (source: various algae) and xylanase (source: *A. oryzae*) target β-1,4 linkages, which are commonly found in the *P. aeruginosa* exopolysaccharide alginate [16]. β-amylase (source: barley), amyloglucosidase (source: *A. niger)* and xylanase (source: *A. orzyae*) target α-1,4 linkages, which are found in the *P. aeruginosa* exopolysaccharide Pel [17]. This suggests that the *P. aeruginosa* biofilms grown in this model possess alginate and/or Pel in their EPS, and that degrading these two exopolysaccharides is sufficient to disperse a significant number of bacterial cells. Although, it is clear that not all α-1,4 or β-1,4-targeting enzymes had the same effect.

S. aureus was significantly dispersed in this model by all tested GHs except for the two pectinases (sources: *A. niger*, Rhizopus sp.) (Fig. 1B). This is interesting because PNAG, which is composed of β-1,6 linkages [18], is thought to be the main exopolysaccharide produced in *S. aureus* biofilms [19], but none of these GHs target β-1,6 linkages. This result suggests the possibility that some of the GHs exhibit promiscuous binding to other polysaccharide linkages or perhaps there are polysaccharides present in the *S. aureus* EPS that have yet to be described. Notably, we also saw that the dispersal caused by the PBS control was lower for *S. aureus* biofilms than we have previously reported [14]. These results illustrate the variability of this model between replicates and studies. However, we still support the use of this model for early screening of dispersal agents prior to advancing to *in vivo* studies.

2.2. GHs are less effective in dispersing polymicrobial biofilms

The majority of biofilm-associated chronic infections are polymicrobial [20,21], with *P. aeruginosa* and *S. aureus* frequently found in the same wounds [22]. It has been well documented that many phenotypic bacterial traits, including virulence, biofilm formation and antimicrobial tolerance, can change when bacteria are grown in the presence of other species [23,24]. To determine if the efficacy of the selected GHs differed when *S. aureus* and *P. aeruginosa* were grown together, versus alone, wells were inoculated with a 1:1 ratio of *P. aeruginosa* and *S. aureus*. After 48 h of bacterial growth, non-attached cells were removed with a PBS wash, and the biofilms were then treated and processed the same way as the mono-species *in vitro* well-plate biofilms (Fig. 2A).

In the *in vitro* well-plate model, there were differences in dispersal for both bacterial species when comparing mono-to poly-microbial biofilms. α-amylase (source: *A. oryzae*), β-amylase (source: barley), amyloglucosidase (source: *A.* niger), lichenase (source: *B. subtilis*), alginate lyase (source: various algae), and xylanase (source: *A. oryzae*) were less efficient at dispersing *P. aeruginosa* from polymicrobial biofilms as compared to mono-microbial biofilms (Supplemental Figure 1A). However, the efficacy of amyloglucosidase (source: Rhizopus sp.), pectinase (source: Rhizopus sp.), inulinase (source: *A. niger*) and cellulase (source: *A. niger*) to disperse *P. aeruginosa* from polymicrobial biofilms was higher than from mono-microbial. Interestingly, there was a significant loss of efficacy by several GHs to disperse *S. aureus* from polymicrobial biofilms as compared to the mono-species biofilms (Supplemental Figure 1B). In the mono-species *S. aureus* biofilms, almost

Table 1
GHs utilized for this study.

Enzyme	Source	Commercial Source	Catalog #	Activation Temperature (°C)
Alginate lyase	Various algae	Sigma Aldrich	A1603	37
α-Amylase	Bacillus sp.	MP Biomedicals	100447	37
α-Amylase	*Aspergillus oryzae*	Sigma Aldrich	A9857	37
α-Amylase	Porcine Pancreas	Sigma Aldrich	A4268	37
β -Amylase	Barley	Sigma Aldrich	A7130	37
Amyloglucosidase	*Aspergillus niger*	Sigma Aldrich	10113	60
Amyloglucosidase	Rhizopus sp.	Sigma Aldrich	A9228	60
Cellulase	*Aspergillus niger*	MP Biomedicals	150583	37
Diastase	*Aspergillus oryzae*	Sigma Aldrich	9962	37
Inulinase	*Aspergillus niger*	Sigma Aldrich	57620	37
Invertase	*Saccharomyces cerevisiae*	Sigma Aldrich	14504	60
Invertase	*Candida utilis*	Sigma Aldrich	14753	60
Lichenase	*Bacillus subtilis*	Megazyme	E-LICHN	60
Pectinase	*Aspergillus niger*	Sigma Aldrich	17389	60
Pectinase	Rhizopus sp.	Sigma Aldrich	P2401	37
Xylanase	*Aspergillus oryzae*	Sigma Aldrich	X2753	37

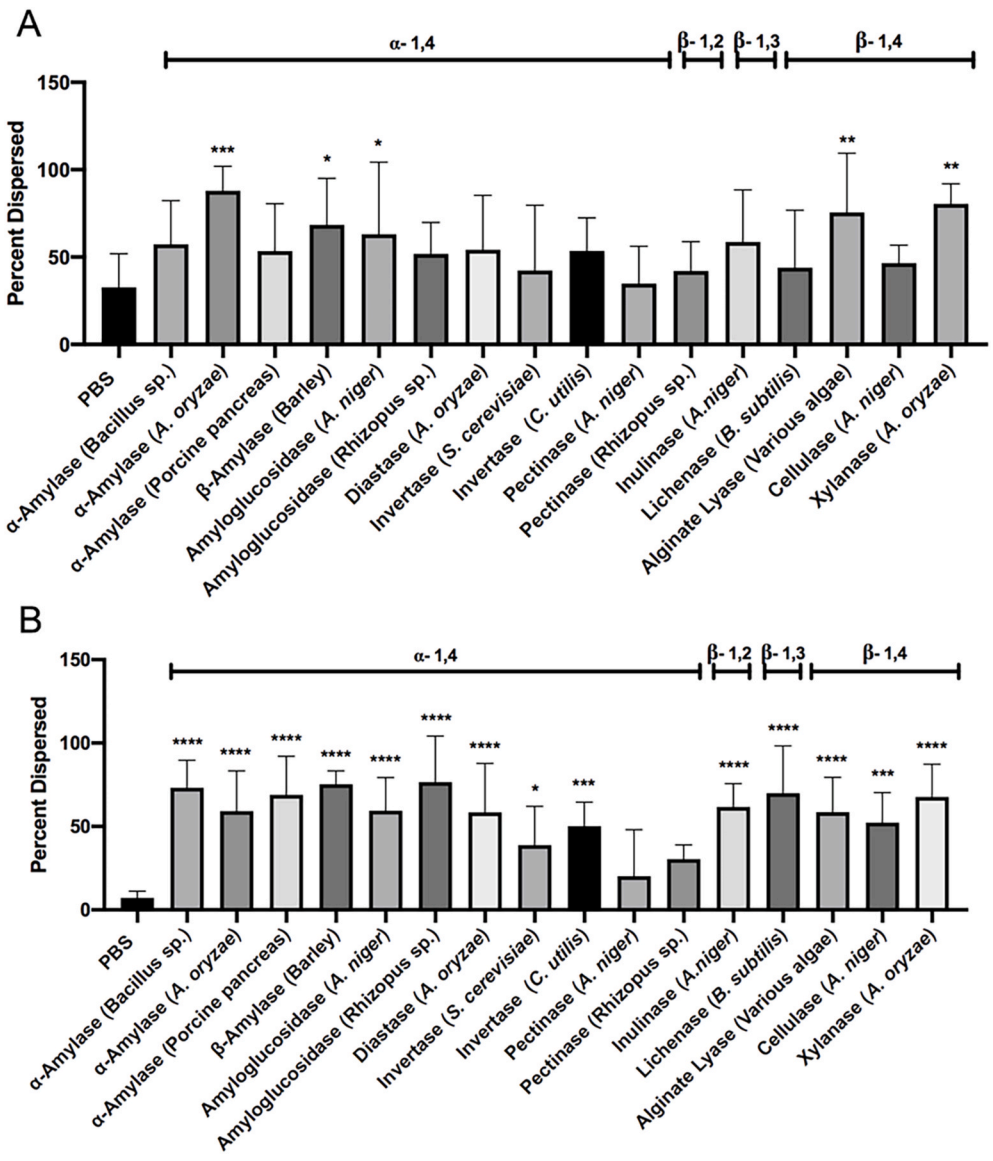

Fig. 1. GHs disperse *P. aeruginosa* and *S. aureus* from mono-species biofilms grown in well-plates. PAO1 **(A)** or SA31 **(B)** biofilms were grown in 24-well cell culture plates for 48 h and then treated with PBS (vehicle control) or the indicated GH for 2 h. Enzymes are grouped according to the bonds they target. Percent dispersal was calculated as follows: (CFU in supernatant/CFU in supernatant + CFU in biofilm). One-way analysis of variance and a Tukey-Kramer multiple –comparison test were used to test for differences between results. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Mean±standard deviation is shown, n = 5/group.

all of the GHs exhibited significant dispersal while only α-amylase (source: *A. oryzae*), inulinase (source: *A.* niger), alginate lyase (source: various algae), and xylanase (source: *A. oryzae*) significantly dispersed *S. aureus* from polymicrobial biofilms.

Theoretically, the amyloglucosidases (sources: *A. niger* and Rhizopus sp.) should exhibit similar results as they target the same linkages. However, enzymes from different sources have been documented to have different levels of activity or quality level [25]. Amyloglucosidase (source: *A. niger*) has a reported quality level of MQ "100" while amyloglucosidase (source: Rhizopus sp.) exhibits a higher quality level of MQ "200". These numbers are based on Sigma Aldrich M-Clarity™ Program to provide transparency in the product quality attributes such as testing sites, discriminating features, and regulation ranging from MQ 100 (low) to MQ 600 (high). Nonetheless, amyloglucosidase (source: *A. niger*) has a 3x higher activity level (120,000 U/g) compared to the other amyloglucosidase (40,000 U/g). However, a 500 U/mL concentration was used for both amyloglucosidases. Differences in purity, activity level, and enzymatic properties could explain this discrepancy amongst the same enzyme from different sources.

Inulinase (source: *A. niger*) was the only GH that caused significant dispersal of both *P. aeruginosa* and *S. aureus* in the polymicrobial well-plate model. These findings suggest that when *P. aeruginosa* and *S. aureus* are grown together, either the biomass of biofilm increases significantly, thwarting degradation, or the exopolysaccharide composition of the biofilm differs from that of mono-microbial biofilms. Data from an experiment comparing the overall biomass of *P. aeruginosa* and *S. aureus* mono- and polymicrobial biofilms indicated no significant difference (Supplemental Figure 2). So potentially, when *P. aeruginosa* and *S. aureus* are grown together *in vitro, S. aureus* contributes less to the EPS, and *P. aeruginosa* becomes the main polysaccharide producer. This also suggests that the increased complexity of highly polymicrobial biofilms will likely require a cocktail of enzymes that target many different polysaccharide bonds.

To assess GH therapy in a more clinically relevant model, *P. aeruginosa* and *S. aureus* were grown together for 48 h in an *in vivo* murine chronic wound model, which we have previously described [12, 26–28]. Wounds were infected with a 1:1 ratio of 10^4 CFU of *P. aeruginosa* and *S. aureus*. After 48 h of infection, mice were euthanized and the infected wound tissue was collected for *ex vivo* treatments. *Ex vivo* treatments were performed to reduce the number of animals used, as each wound bed is cut into four pieces for four separate treatments. The infected tissue was treated for 2 h with PBS (vehicle control) or different GH solutions at 500 U/mL or 125 U/mL for pectinase (source: Rhizopus sp.). After the allotted time, the dispersed cells in the

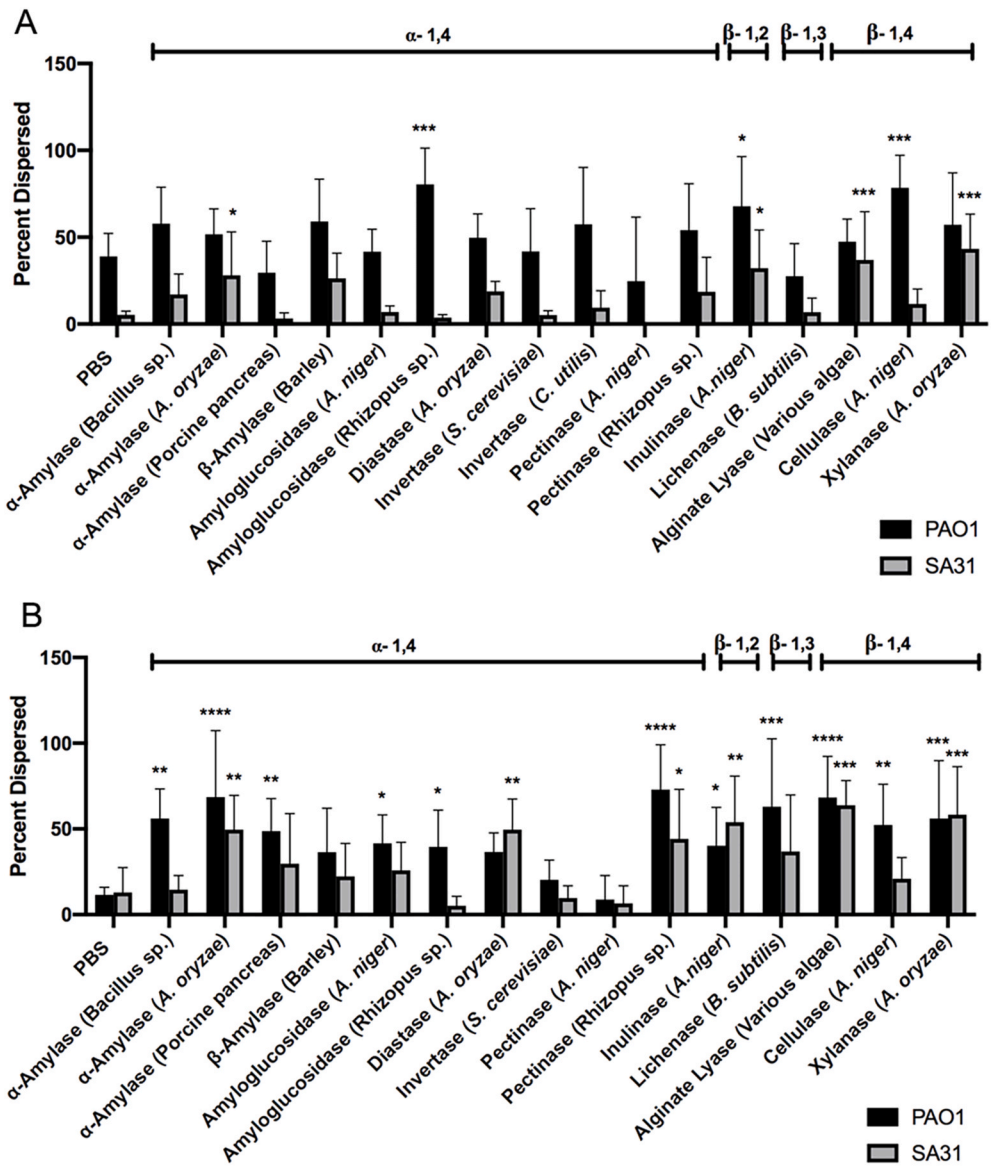

Fig. 2. GHs disperse polymicrobial biofilms *in vitro* (A) or from infected wound tissue *ex vivo* (B). PAO1/SA31 biofilms were grown in 24-well cell culture plates (A) or murine wounds (B) for 48 h and then harvested and treated with PBS (vehicle control) or the indicated GH for 2 h. Enzymes are grouped according to the bonds they target. Percent dispersed was calculated as follows: (CFU in supernatant/CFU in supernatant + CFU in biofilm) x 100. Two-way analysis of variance and a Tukey-Kramer multiple –comparison test were used to test for differences between results. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Mean±standard deviation is shown, n = 5/group.

supernatant were removed, serially diluted, and plated to determine CFU. The tissue containing the remaining biofilm was homogenized in PBS, serially diluted in PBS, and plated to enumerate CFU and calculate 'percent dispersed' (Fig. 2B).

Interestingly, GH therapy was more efficacious overall in dispersing bacteria from biofilms grown *in vivo* versus in well-plates (Fig. 2B). α-amylase (source: *A. oryzae*), pectinase (source: Rhizopus sp.), inulinase (source: *A. niger*), alginate lyase (source: various algae), and xylanase (source: *A. oryzae*) all significantly dispersed both *P. aeruginosa* and *S. aureus* from infected wound tissue, as opposed to only inulinase that significantly dispersed both species from biofilms grown *in vitro*. α-amylase (source: Bacillus sp.), α-amylase (source: porcine pancreas), amyloglucosidases (sources: *A. niger,* Rhizopus sp.), lichenase (source: *B. subtilis*) and cellulase (source: *A. niger*) were efficacious in dispersing *P. aeruginosa* but not *S. aureus*, and diastase (source: *A. oryzae*) was the only GH that significantly dispersed *S. aureus*, but not *P. aeruginosa* in this model. The most striking difference was the number of enzymes that successfully dispersed *P. aeruginosa* from tissue as compared to the number that dispersed *P. aeruginosa* from *in vitro* biofilms (five for monospecies and three for polymicrobial). Taken together, six GHs exhibited the best therapeutic dispersal potential: α-amylase (source: *A. oryzae*), amyloglucosidase (source: *A. niger*), pectinase (source: Rhizopus sp.), inulinase (source: *A. niger*), alginate lyase (source: various algae), and xylanase (source: *A. oryzae*). These GHs were selected to test dispersal efficacy *in vivo* as well as determine their ability to improve antibiotic clearance *in vivo*.

2.3. GH treatment coupled with meropenem lowers bacterial load in *P. aeruginosa*-infected wounds

In order to test the therapeutic potential of the selected GHs, we utilized our murine chronic wound model. Wounds were infected with 10^4 CFU of *P. aeruginosa* carrying the pQF50-lux plasmid. After 48 h of infection, the wound beds were irrigated 3 times with either PBS (vehicle control), meropenem (antibiotic control), GHs, or GHs + meropenem for 30 min. The wound beds were imaged pre-treatment, immediately post-treatment, and 5- and 20-h post-treatment. Mice were then euthanized and the wound beds and spleens were collected to enumerate CFU per gram of tissue.

There were no statistically significant differences detected in the bacterial loads from the wound beds of mice treated with enzyme versus PBS (Fig. 3A). However, bacteria were detected in the spleens of mice treated with some GHs, but not PBS (Fig. 3B). This suggests that some of the GHs caused the systemic spread of *P. aeruginosa,* which is consistent

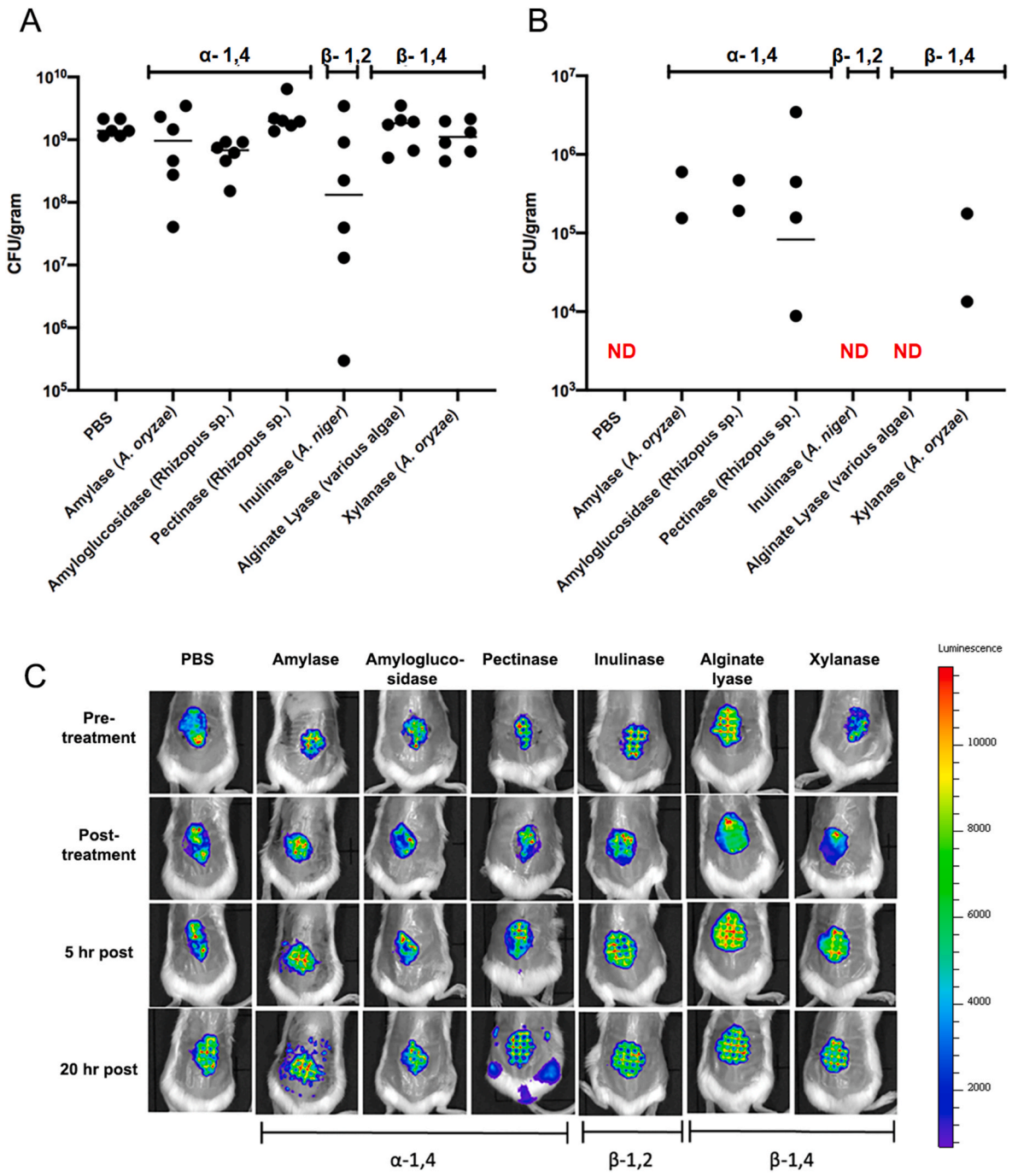

Fig. 3. GHs induce dispersal *in vivo*. Wounds were infected with 10^4 bioluminescent PAO1. At 48 h post-infection, mice were treated with 3 × 30 min irrigations of PBS (negative control) or the indicated GHs. At 20 h post-treatment, mice were euthanized and their wound beds (**A**) and spleens (**B**) were collected and homogenized. The homogenates were then serially diluted and plated to enumerate CFU per gram of tissue. ND indicates no detection of bacteria in the tissue samples for that treatment. Mice were imaged pre-treatment, 0 h, 5 h, and 20 h post-treatment by IVIS (**C**). Each mouse is represented by a dot on the graph. n = 5 or 6/group.

with our previously published data [13]. This was also supported by the visualization of bacteria spreading outside the wound bed by IVIS at 20 h post-treatment (Fig. 3C).

In order to test whether the dispersed cells were more effectively killed by antibiotics, mice were infected as above, but treated with either meropenem plus PBS or meropenem plus GH. Collectively, adding GHs did not significantly increase the ability of meropenem to kill bacteria within the wound beds (Fig. 4A). However, we saw that the number of mice within each treatment group whose wound bed bacterial load fell at or below 10^5 CFU/g of tissue (often used as a threshold for clinical infection [29–31]), was higher for some of the treatment groups versus others. Two of the treatment groups possessed at least one mouse that had lower bacterial load than the clinical level of infection after only one treatment. Inulinase + meropenem reduced the bacterial load to below clinical level of infection in 50% of the animals. While this seems apparent in some of the representative IVIS images (Fig. 4B), it should be noted that in general, IVIS has a detection limit of 10^6 CFU per gram of tissue [32]. We also saw that none of the mice in these treatment groups (n = 5 or 6/group) had bacteria in their spleens. This indicates that any bacteria that were dispersed due to the GH treatment were likely killed by meropenem. Overall, inulinase and xylanase were able to disperse bacteria *in vivo* and potentiate the efficacy of meropenem in some mice.

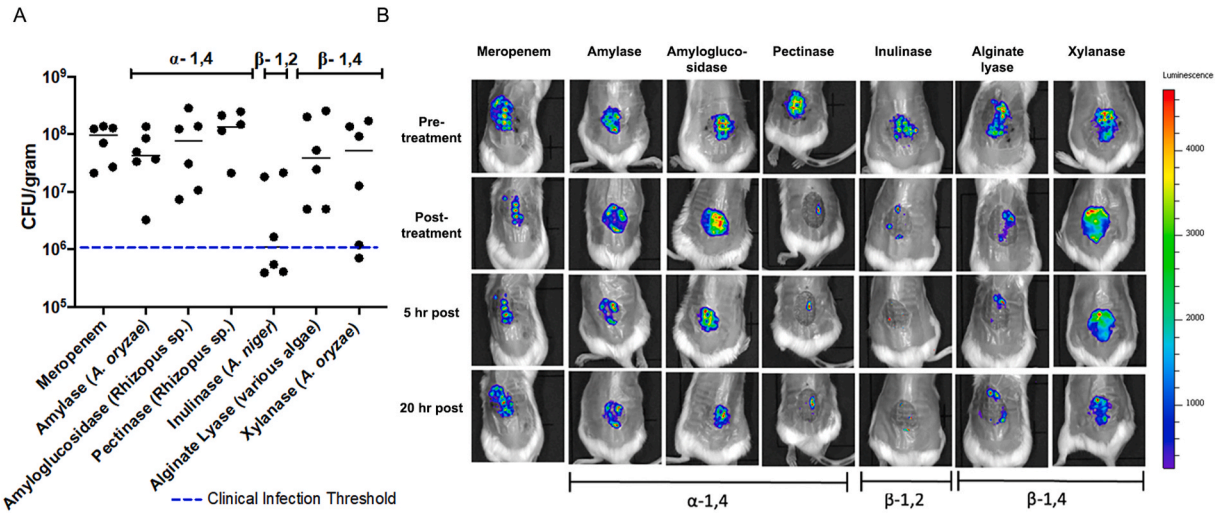

Fig. 4. GHs improve antibiotic efficacy *in vivo*. Wounds were infected with 10^4 bioluminescent PAO1. After 48 h, wounds were treated with 3 × 30 min irrigations of 3 mg/mL meropenem or 500 or 125 U/mL of meropenem + GHs. At 20 h post-treatment, mice were euthanized and their wound beds (**A**) were collected and homogenized. The homogenates were then serially diluted and plated to enumerate CFU per gram of tissue. Mice were imaged pre-treatment, and at 0, 5, and 20 h post-treatment (**B**). Each mouse is represented by a dot on the graph, n = 5 or 6/group.

Taken together, these results reaffirm that GHs should always be used in conjunction with antibiotics to prevent septic events. We have shown that some of these GHs have the ability to reduce bacterial load to below the clinical level of infection after just one treatment. It is possible that with repeated treatments, GHs in combination with antibiotics, can successfully clear biofilm-associated wound infections completely.

2.4. GHs are also effective in diabetic mice

Individuals with diabetes mellitus are more prone to developing biofilm-associated infections including diabetic foot ulcers, pressure ulcers, and venous ulcers [33]. Approximately 15% of individuals with diabetes mellitus develop an ulcer on the lower extremity, with 14–24% of those ulcers leading to amputation [34,35]. Studies in mice have demonstrated that biofilms in the wounds of diabetic mice are more robust and prevalent [28,36]. Thus it is possible that in the diabetic wound environment, the abundance and/or type of exopolysaccharides expressed is different than in a non-diabetic wound. If true, the efficacy of GHs could also be different in diabetic vs non-diabetic wounds. Furthermore, there is the possibility that bacterial dispersal by GHs could have a more dire effect on individuals immunocompromised by diabetes. As diabetic patients would likely represent a large sector of those receiving a future GH therapeutic, it is important to test whether GHs are safe and effective in dispersing the biofilms present in the wounds of a diabetic host. To test this, we utilized the $Lep^{db/db}$ mouse strain. This strain is derived from the C57BL/6 mouse with an autosomal recessive mutation of the leptin receptor on chromosome 4 to mimic Type 2 diabetes [37]. *db/db* mice become obese and insulin resistant due to beta cell failure in the pancreas [38].

For these experiments, wild-type C57BL/6 and *db/db* mice were wounded and infected with *P. aeruginosa.* At 48 h post-infection, PBS, 10% GH (5% amylase and 5% cellulase combination in weight (grams) per volume (mL), or 10% GH + meropenem was administered as 3 × 30 min irrigations. Mice were imaged prior to treatment, immediately after treatment, and 5 h and 20 h post-treatment (Fig. 5A). Upon euthanasia, the wound beds and spleens were collected and processed to enumerate CFU per gram of tissue (Fig. 5B and C). As seen in the IVIS images (Fig. 5A), PBS did not induce dispersal in either mouse strain. There were also no bacteria enumerated in the spleens of PBS-treated mice post-euthanasia. Conversely, the 10% GH treatment induced bacterial dispersal from the wound bed in both mouse strains. This dispersal event can be visualized in the representative IVIS images as well as the CFU enumeration from the spleen (Fig. 5A,C). In the *db/db* mice, mass dispersal can be imaged as soon as 5 h post-treatment. There was also a higher bacterial load in the spleen of the *db/db* mice (5.7×10^5 CFU/g) compared to their parent strain, C57BL/6 mice (1.4×10^4 CFU/g) that were treated with 10% GH.

Next, we wanted to determine if combining GHs with an antibiotic would prevent the systemic spread of *P. aeruginosa* in diabetic mice as we have seen before in non-diabetic mice. Experiments were conducted as above, with the addition of meropenem to the GH irrigations. The addition of meropenem to 10% GH reduced the bacterial loads in the wound beds of both mouse strains at 20 h post-treatment as demonstrated by IVIS imaging (Fig. 5A) and CFU enumeration from tissue homogenates (Fig. 5B). The bacterial load in the wounds of all the *db/db* mice that were treated with GH plus meropenem fell below the threshold of clinical infection (Fig. 5B). The addition of meropenem also protected all of the mice from dispersal-induced bacteremia, as there were no bacteria detected in the spleen homogenates. There were also no bacteria detected in the spleens treated with PBS alone. However, there were bacteria detected in the spleens of mice treated with GH alone (Fig. 5C), indicating systemic bacterial dispersal from the wound bed. These results suggest that not only does GH treatment disperse *P. aeruginosa* from wounds in a diabetic host, but that the addition of antibiotics prevents sepsis, even in an immunocompromised host.

2.5. Some GHs exhibit signs of toxicity

Next, the safety of GH enzymes was examined. We first tested GHs on the human fibroblast cell line CCD110 and the human epithelial cell line CCD841. We exposed cells to GHs for 30 min, 90 min, or 3 h to replicate the 30-min dwell time for the irrigations we perform when treating mice with GHs. Therefore, 30-min exposure represents one irrigation, 90-min exposure represents the entire 3, 30-min irrigation treatments, and 3-h exposure represents a treatment maximum of two times the normal exposure rate. The GHs utilized in this study are listed in Table 1. Cells were treated with either MEM (vehicle control) or 500 U/mL or 125 U/mL of GH enzymes. After exposure, the treatments were removed and cell viability was assessed.

Overall, as the exposure time increased, the percentage of viable cells decreased. After the maximum 3- hour exposure, there were eleven enzymes that caused more than 50% of cell death to the fibroblast cells (Fig. 6A) and nine enzymes that exhibited the same percentage of cell death to the epithelial cells (Fig. 6B). Fibroblasts exposed for 30 min to

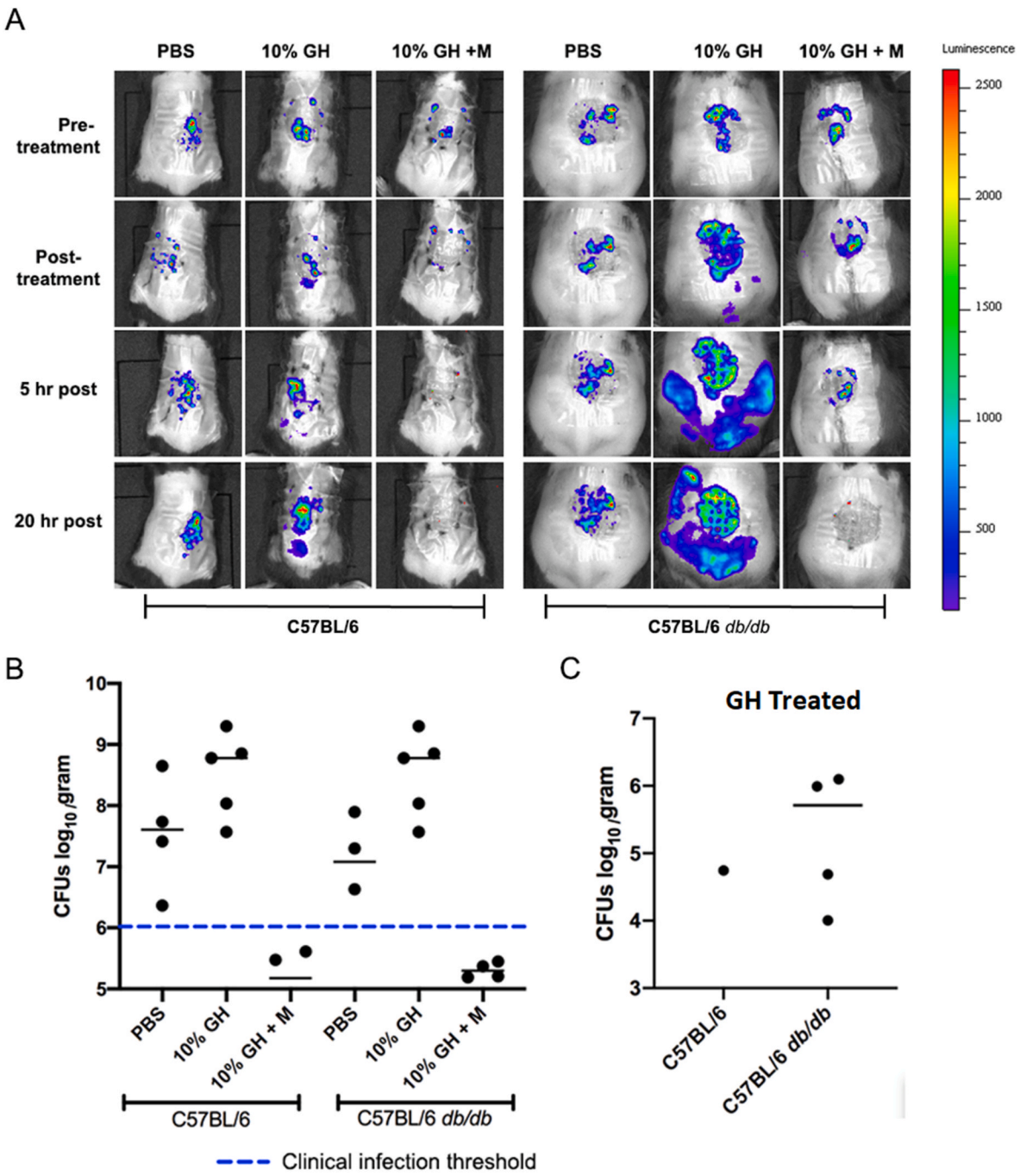

Fig. 5. GHs are effective in diabetic mice. Wounds were infected with 10^4 bioluminescent PAO1. At 48 h post-infection, mice were treated with 3 × 30 min irrigations of PBS (negative control), a 10% GH solution made with equal parts of amylase (source: *Bacillus* sp.) and cellulase (source: *A. niger*), or 10% GH + meropenem (M). Mice were imaged pre-treatment, 0 h, 5 h, and 20 h post-treatment by IVIS (A). At 20 h post-treatment, mice were euthanized and their wound beds (B) and spleens (C) were collected and homogenized. The homogenates were then serially diluted and plated to enumerate CFU per gram of tissue. Each mouse is represented by a dot on the graph, n = 5 or 6/group.

lichenase (source: *B. subtilis*) exhibited a lethal dose above 50% (Fig. 6A). Lichenase was the only β-1,3 targeting enzyme so it is possible that this linkage could be present in the fibroblast cell matrix, which includes collagen type I and III [39]. Interestingly, pectinase (source: *A. niger)*, which was toxic to both cell lines, targets α-1,4 linkages, but the remaining GHs that target this linkage did not exhibit toxic effects. It is unclear as to why some of the α-1,4 targeting GHs exhibited toxicity, while others did not. While these results support the utilization of some GHs is potentially safe, clearly others have the capacity to damage human cells. Thus, future studies should be conducted to explain these toxic effects *in vitro* by testing various concentrations, and treatment exposure times.

2.6. Most candidate GHs do not demonstrate toxicity in vivo

We next assessed GH safety in murine wounds. Mice were wounded as we have previously described [12,26–28], and the uninfected wounds were administered treatments in 3 × 30 min irrigations. The GHs that exhibited the highest dispersal potential were selected. These included α-amylase (source: *A. oryzae*), amyloglucosidase (source: *A. niger*),

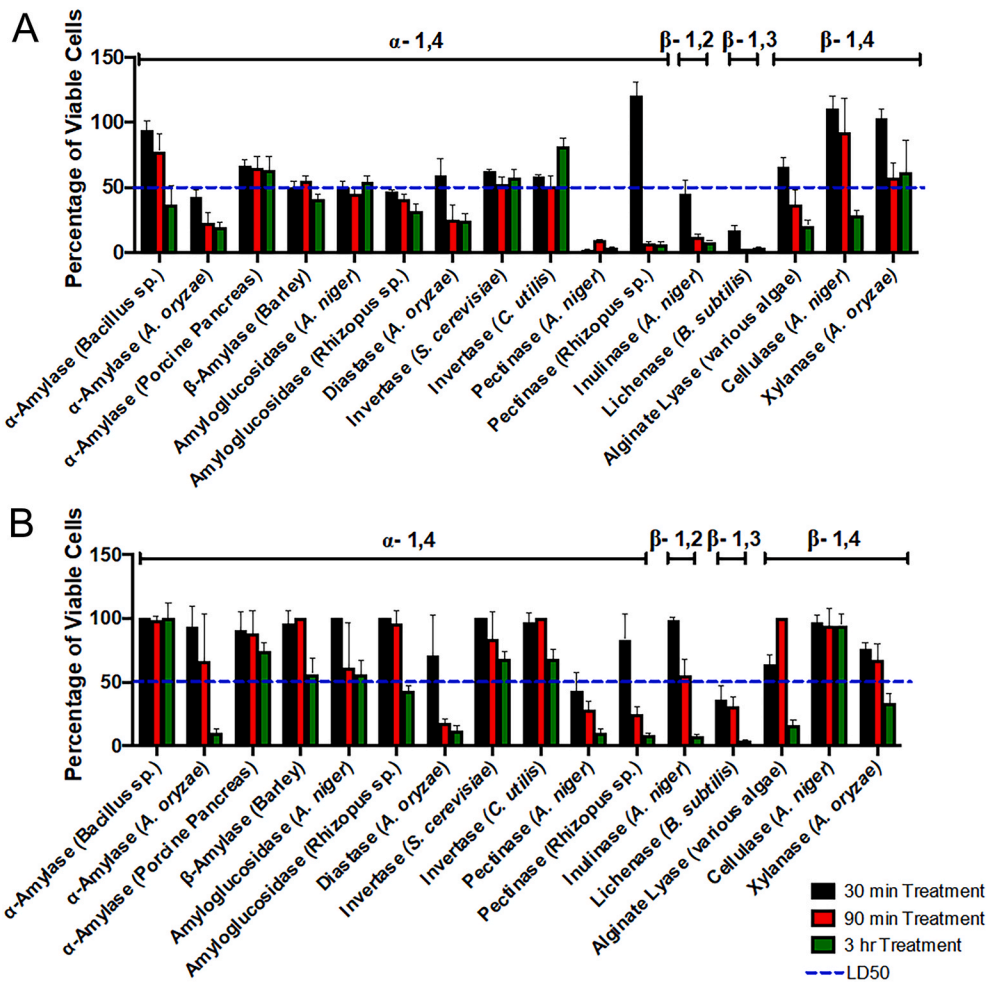

Fig. 6. Some GHs exhibit toxic effects to human cells in culture. 10,000 human epithelial fibroblasts (CCL110) (**A**) or 20,000 human normal colonic cells (CCD841) (**B**) were seeded into a 96-well plate for 48 h. After attachment, cells were exposed to various GHs for 30 min, 90 min, or 3 h. After exposure, 10% AlamarBlue + MEM + FBS was added and plates were incubated for 6 h. Viable cells remaining on the plate were quantified via absorbency and percent viability was calculated as [absorbency of treated cells/absorbency (average of untreated cells)] x 100. Values below LD50 were considered toxic. Mean±standard deviation is shown.

pectinase (source: Rhizopus sp.), inulinase (source: *A. niger*), alginate lyase (source: various algae) and xylanase (source: *A.* oryzae). The wounds were imaged every 48 h using a SilhouetteStar™ wound imaging and documentation system, and body weights were recorded. After 12 days of repeated treatments, mice were euthanized and the spleens were collected and weighed. There was no significant reduction in wound closure after 7 days of treatment compared to the PBS vehicle control (Fig. 7A and B). Wounds treated with alginate lyase (source: various algae) exhibited the lowest percentage wound closure (27.6%) and those treated with pectinase (source: Rhizopus sp.) exhibiting the highest (53.1%), but these differences were not significantly different from the control. After 7 treatments, the mice receiving the xylanase treatments (source: *A. oryzae*) were euthanized on day 8 due to lethargy and the development of swelling at the wound site. After 12 days of treatment, all wounds were over 80% closed. Body weight was monitored throughout the study since a decrease is often associated with illness. There was no significant decrease in the body weights of GH-treated mice during the 12 days of treatments as compared to the PBS control (Fig. 7C). Lastly, there was no significant difference in spleen weight between the groups, except for the xylanase (source: *A. oryzae*) group (Fig. 7D). Xylanase (source: *A.* oryzae) treated mice exhibited almost double the spleen weight (0.263 g) of those in the PBS group, suggesting severe splenomegaly. It should be noted though that the spleens from the xylanase treated mice were collected after 7 days of treatment, while the spleens from all other mice were collected after 12 days of treatment.

Overall, our results suggest that repeated exposure to GHs did not cause toxic effects to the mice, with the exception of xylanase (source: *A. oryzae*), which caused multiple severe effects that required euthanasia. We realize that the scope of our toxicity studies were narrow, and more thorough testing would be required before application to humans, but these results are promising nonetheless. Another limitation of our study was the very limited treatment regime. We only tested one concentration of each enzyme, in one vehicle, with one treatment schedule (one topical application per day). It is quite possible that GH treatments could be optimized by varying one or more of these parameters.

3. Discussion

Biofilms provide shelter for their constituent bacteria. This shelter inherently provides increased tolerances to desiccation, nutrient limitation, antibiotics, and phagocytosis [8,40]. These advantages potentially make biofilm infections difficult for the immune system to clear, even with clinical intervention, encouraging the pursuit of alternative treatment methods for biofilm infections to improve antibiotic potency. We, and others, have previously demonstrated the ability of GHs to disrupt biofilms in a variety of both *in vitro* and *in vivo* models [12–14, 41–43]. However, the specific GH enzymes that have been explored for therapeutic purposes are very limited. The overall goal of this study was to test the efficacy and safety of an expanded panel of agents from this class of enzymes. We began with 16 enzymes, screening them first in a simple well-plate *in vitro* model against *P. aeruginosa* and *S. aureus* biofilms. This experiment demonstrated that the efficacy of GHs changes based on the composition of the biofilm; enzymes that effectively disrupt mono-species biofilms may lose their effectiveness when additional species are added. We have observed this interesting phenomenon previously with cellulase and α-amylase [14], and are still unclear of the

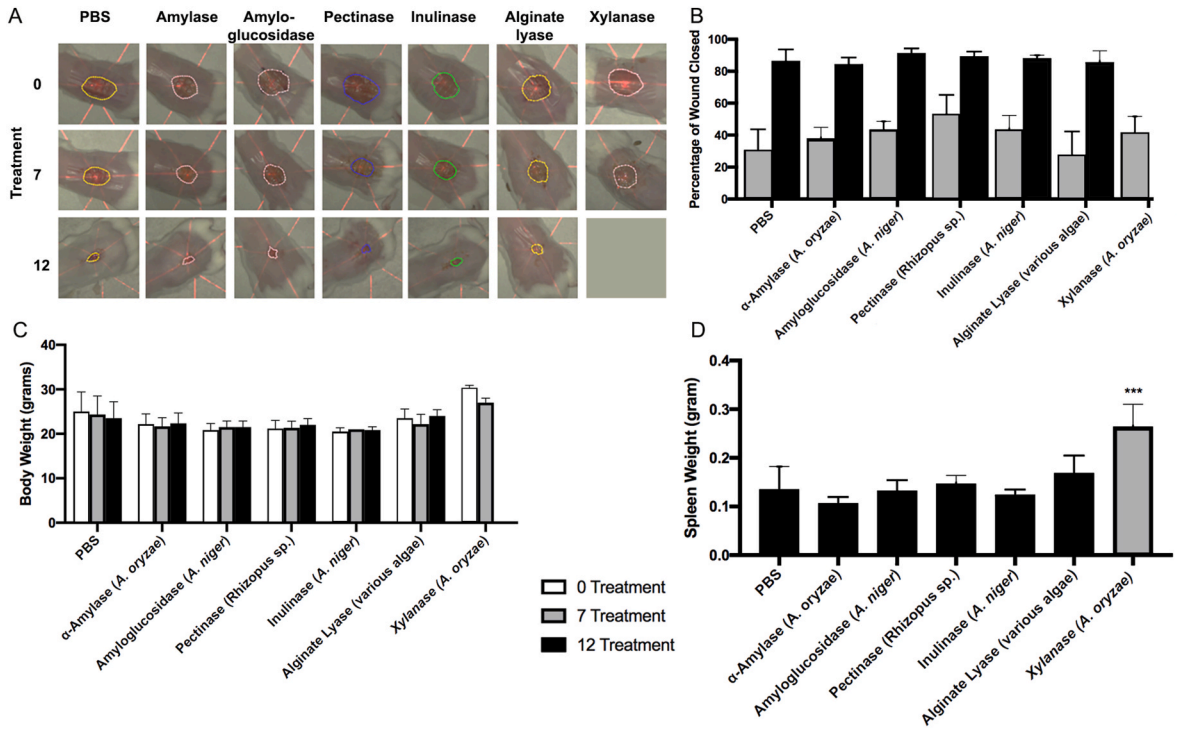

Fig. 7. Only xylanase treatment causes toxic effects in mice. Uninfected wound beds were irrigated with 3 × 30 min treatments of PBS (vehicle control) or GHs every 24 h for 12 days. Wounds were imaged every 48 h (**A**) to monitor wound closure (**B**). Mice were also weighed every 48 h to monitor body weight (**C**). Lastly, the spleens were collected and weighed 24 h after the last treatment. Xylanase (source: *A. oryzae*) treated mice were euthanized after 7 treatments because they exhibited signs of toxicity. One-way (**D**) or two-way (**B, C**) analysis of variance and a Tukey-Kramer multiple-comparison were used to test for differences between results. ***, $P < 0.001$. Mean±standard deviation is shown, n = 6 or n = 3 for xylanase (source: *A. oryzae*)/group.

mechanism responsible. It is possible that the addition of other species alters the biofilm matrixome produced by bacteria, in particular by *P. aeruginosa.*

As GHs target specific linkages within the EPS, the dispersal efficacy should reflect the linkages present and therefore allow insight into the polysaccharides that are produced. In the *in vitro* mono-microbial biofilms (Fig. 1), GHs that target the α-1,4 and β-1,4 linkages exhibited significant dispersal, suggesting *P. aeruginosa* produces the polysaccharides Pel and alginate in this model. Previous studies have shown contradictory results when determining the importance of alginate in biofilm formation *in vitro* [44,45]. These varying results from us [14] and others suggest that *P. aeruginosa* is utilizing various combinations of polysaccharides, which is dependent upon the environment, other species present, and the model. In this study, GH treatments that targeted the linkage found in alginate and Pel caused significant dispersal of *P. aeruginosa*, suggesting the utilization of these polysaccharides in biofilm formation in this model. However, when *S. aureus* was added in this model (Fig. 2A), the enzymes that target the β-1,4 linkages were unable to disperse *P. aeruginosa,* suggesting the absence of alginate. Conversely, when *P. aeruginosa* and *S. aureus* were grown together *in vivo* (Fig. 2B), the GHs that target Pel, Psl, or alginate all dispersed *P. aeruginosa,* suggesting that all three polysaccharides are being utilized or are not produced at all. Another possibility is that these enzymes are breaking up host components in the EPS, therefore freeing the bacteria. As EPS-targeting therapeutics continue to be investigated, future studies need to be conducted to evaluate environmental conditions that result in modification of the matrixome and its potential effect on therapeutic effectiveness.

We advanced the GHs that were most effective *in vitro* to testing *in vivo*. Our results demonstrate the variable ability of translating *in vitro* results to more clinically relevant *in vivo* models. For example, pectinase (source: Rhizopus sp.) induced biofilm dispersal *in vivo* but did not significantly disperse *P. aeruginosa* in the simple well-plate *in vitro* model. Inulinase (source: *A. niger)* did not exhibit significant dispersal *in vitro* but improved the efficacy of antibiotics *in vivo.* However, some of the *in vitro* results were translational *in vivo.* For example, xylanase (source: *A. oryzae*) dispersed *P. aeruginosa in vitro* and improved antibiotic efficacy *in vivo,* and α-amylase (source: *A. oryzae)* induced biofilm dispersal in both models. So while no *in vitro* model will be completely predictive of *in vivo* efficacy, using a simple well-plate biofilm model to screen multiple dispersing agents does have utility.

In order to gauge the potential safety of using GHs therapeutically, we evaluated their toxicity on human cells in culture, as well as in our mouse wound model. These experiments revealed the potential toxicity of a number of different GH enzymes, but also suggested some may be both safe and effective. To the best of our knowledge, this study is the first to describe the safety of GHs for biofilm dispersal during infection and while these studies are a good start, GHs will need to be further tested for safety in larger animals prior to clinical studies. It is also likely that GH treatments can be improved by optimizing concentration, delivery vehicles, purity of enzymes, routes of administration and dosing schedules. Taken together, our data, and the work of others, suggest a great utility for GHs both as anti-biofilm agents as well as tools to explore the dynamic nature of the biofilm matrixome.

4. Materials and methods

4.1. Bacterial strains

P. aeruginosa wild-type strain PAO1 [46], *S. aureus* wild-type strain SA31 [47] and a bioluminescent PAO1 strain carrying the luminescence reporter plasmid pQF50-lux [48] have all been previously described. *P. aeruginosa* and *S. aureus* were grown in baffled 125 mL Erlenmeyer flasks, with shaking at 200 rpm in Luria-Bertani (LB) broth at 37 °C for 16–18 h 100 μL of the overnight culture was used to inoculate sub-cultures containing 10 mL of LB broth and was grown at 37 °C at 200 rpm for 2.5 h. Planktonically grown cells in the sub-culture were

then used to initiate infection and inoculate the *in vitro* model. All CFU were quantified by serial dilution and plating on *Staphylococcus* medium 110 (Difco) and *Pseudomonas* isolation agar (Difco).

4.2. Glycoside hydrolases

Enzymes listed in Table 1 were prepared by dissolving lyophilized powder or diluting liquid solutions in 1 x phosphate buffered saline (PBS) at 37 °C or 60 °C according to manufacturer's instructions for 30 min 1 x PBS was used as a vehicle control. Enzyme solutions were prepared fresh immediately before use. All enzymes were used at a concentration of 500 U/mL except for pectinase (source: Rhizopus sp.) that was used at 125 U/mL. A 10% GH solution was made by combining 5% amylase (source: Bacillus sp.) and 5% cellulase (source: *A. niger*) weight per volume solution.

4.3. In-vitro well-plate biofilm model

To measure biofilm dispersal *in vitro,* the wells of a 24-well, non-tissue culture-treated, plate (Falcon) were inoculated with 800 μL of sub-culture, containing 10^5 *S. aureus, P. aeruginosa,* or *S. aureus* and *P. aeruginosa.* The correct inoculating dose was established by diluting the sub-cultures with LB. For polymicrobial biofilms, 10–20% adult bovine serum (B9433 Sigma-Aldrich®) was added to the inoculating solution to prevent *P. aeruginosa* from outcompeting *S. aureus* as previously shown [49]. Biofilms were grown for 48 h at 37 °C with shaking at 80 rpm. Following incubation, the supernatant was removed, and each well was gently rinsed with 1 mL PBS to remove any nonattached cells. Subsequently, wells were treated with 1 mL enzyme solution or vehicle control (1 x PBS) for 2 h at 37 °C with shaking at 80 rpm. Following treatment, the supernatant was removed and serially diluted in PBS. CFU were enumerated to calculate the 'dispersed' fraction. 1 mL of PBS was added to the remaining biofilms, which were broken up by 30 min of sonication, then serially diluted and plated for CFU enumeration to determine the 'biofilm' fraction. Percent dispersal was calculated by dividing the dispersed CFU by the total CFU (biofilm-associated plus dispersed). Two biological replicates with three technical replicates were performed for each treatment.

4.4. In vivo and ex vivo biofilm dispersal

Our murine chronic wound model has been previously described [12,26–28]. Briefly, mice were anesthetized by intraperitoneal injection of sodium pentobarbital. After a surgical plane of anesthesia was reached, the backs were shaved and mice were administered a full thickness, dorsal, 1.5- by 1.5-cm excisional skin wound to the level of the panniculus muscle with surgical scissors. Wounds were then covered with a semi-permeable polyurethane dressing (Opsite dressing; Smith and Nephew), under which 10^4 CFU of bioluminescent *P. aeruginosa (in vivo)* or 1:1 ratio of *P. aeruginosa* and *S. aureus* (*ex vivo*) bacterial cells were injected into the wound bed. Infection was allowed to proceed for 48 h, a time at which we have demonstrated the presence of biofilm in wounds [28,47,50]. After 48 h, mice were euthanized, and wound beds were harvested for *ex vivo* GH treatments. Tissue from the wound bed was cut into 4-sections, with each section receiving a different treatment. Tissue was submerged in 1 mL of treatment and incubated for 2 h at 37 °C, with shaking, at 80 rpm. Afterwards, the supernatant containing the dispersed cells was serially diluted. 1 mL of PBS was added to the remaining tissue and homogenized at 5 m/s for 60 s in FisherScientific™ 2 mL Pre-Filled Bead Mill Tubes using a FastPrep-24™ MP Biomedical Benchtop Homogenizer. The supernatant and biofilms were serially diluted, plated, and the percent dispersal was calculated as described above.

For *in vivo* studies, mice were treated with 3 × 30 min irrigations of either PBS, 3 mg/mL meropenem, GHs, or GHs + meropenem 48 h post-infection. Mice receiving meropenem were administered an intraperitoneal injection of 300 mg/kg of meropenem 4 h prior and 8 h post-treatment. Mice were imaged pre-treatment, immediately post-treatment (0 h), and 5 h, and 20 h post-treatment with IVIS. Mice were euthanized at 20 h post-treatment and their spleens and wound beds were collected and placed into pre-weighed FisherScientific™ 2 mL homogenizing tubes containing 1 mL of PBS. The tissue was homogenized at 5 m/s for 60 s two times. The homogenates were serially diluted and plated to enumerate CFU per gram of tissue. Two biological replicates with three technical replicates were performed for each treatment.

4.5. Cell culture

The human fibroblast and colonic epithelial cell lines (CCL110 and CCD841, respectively) were purchased from ATCC which utilizes STR technology for Cell Authentication, and were used in a low passage (<20) within 6 months or less after receipt or resuscitation. Both cell lines were cultured in Eagle's Minimum Essential Medium (MEM) (Gibco™ Cat # 67-008-6) supplemented with 10% fetal bovine serum (Gibco™ Cat# A4766801) and 1% penicillin/streptomycin (Gibco™ Cat# 15070063) with 5% CO_2, at 37 °C.

4.6. Cell toxicity assay

10,000 CCL110 cells or 20,000 CCD841 cells were seeded into a 96-well tissue cultured treated polystyrene plate (Falcon) for 48 h at 37 °C with 5% CO_2. Upon attachment, the MEM media was removed and cells were rinsed with PBS pH 7.4 (Gibco™ Cat# 10010023). 200 μL of GHs reconstituted in supplemented MEM media was administered to the cell lines for 30-min, 90-min, or 3-h at 37 °C, with 5% CO_2. The GH treatments were removed, and adhered cells were rinsed with PBS pH 7.4 (Gibco™) prior to adding 10% AlamarBlue® diluted with MEM + FBS media for 6 h. The living cells that remained were quantified via absorbency at 590-nm in a Synergy H1 Hybrid Reader (Biotek). Percent viability was calculated [absorbency of treated cells/absorbency (average of untreated cells)] x 100. One biological replicate with six technical replicates was performed for each treatment.

4.7. In vivo toxicity screening

Mice were administered surgical excision wounds as described above. 48 h after injury, wounds were irrigated three times with PBS (vehicle control) or GHs, with 30-min dwell time, every 24 h. Wounds were imaged and measured every 48 h using SilhouetteStar™ (ARANZ Medical). Body weights were also recorded every 48 h. After 12 days of repeated treatments, mice were euthanized. Upon euthanasia, spleens were collected and weighed to screen for splenomegaly. Two biological replicates with three technical replicates were performed for each treatment.

4.8. Statistical analysis

All statistical analysis was performed utilizing GraphPad Software, Inc. Multivariant analyses were performed by either one-way or two-way analysis of variance (ANOVA) by Tukey's multiple comparison test.

CRediT authorship contribution statement

Whitni K. Redman: Methodology, Data curation, Writing – original draft. **Garrett S. Welch:** Data curation, Writing – review & editing. **Avery C. Williams:** Data curation. **Addyson J. Damron:** Data curation. **Willem O. Northcut:** Data curation. **Kendra P. Rumbaugh:** Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Kendra Rumbaugh reports financial support was provided by National Institutes of Health. Kendra Rumbaugh reports financial support

was provided by US Department of Defense.Given her role as Editor, dr. Kendra RUMBAUGH had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to dr. Tom COENYE.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biofilm.2021.100061.

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