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Pre-clinical Evaluation of a New Antimicrobial Enzyme for the Control of Wound Bioburden

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Abstract: A new, optimized, antimicrobial enzyme system was developed for the control of wound bioburden. This Glucose oxidase-Lactoperoxidase-Guaiacol (GLG) system was analyzed for antimicrobial activity and cytotoxicity. The susceptibility of a wide range of antibiotic-resistant bacterial strains to the GLG-enzyme system was analyzed using minimum inhibitory concentration (MIC90), minimum bactericidal concentration (MBC) determination, and growth kinetics analysis. Additionally, challenge tests and cytotoxicity tests were performed with a new hydroactive alginate gel dressing with antimicrobial activity obtained by the presence of the GLG-enzyme system (Flaminal® Forte, Flen Pharma, Kontich, Belgium). All bacterial strains were susceptible to the GLG-enzyme system at low concentrations. The exact concentration required for growth arrest and cell death was dependent on the experimental design. Further, a 20% (w/v) GLG dilution showed no cytotoxicity toward fibroblasts and keratinocytes. Conversely, other antimicrobial wound-care products applied with the same dilution showed a high degree of cytotoxicity. With increasing concerns about bacterial resistance to antibiotics, this study shows that low concentrations of the GLG-enzyme system are successful in killing antibiotic-resistant bacterial strains. Furthermore, results show that GLG-enzyme system combines strong antimicrobial activity with non-cytotoxicity and promotes optimal wound healing.

Microbial drug resistance is a growing problem. The rise in multiresistant strains of bacteria observed over recent decades emphasizes the importance of the development of new, innovative, antimicrobial drugs. There is a great deal of interest in naturally occurring antimicrobial products, and intensive research aimed at the development of drugs based on naturally occurring antimicrobial peptides (AMPs) of various origins is being performed.[1-3] Despite such efforts over the past 2 decades, clinical success has been limited.[4] Other naturally occurring antimicrobial products with clinical potential are enzymes, which produce antimicrobial products. Hydrogen peroxide (H_2O_2) is such a potent antimicrobial product of enzyme activity that killing microorganisms through the oxidation of bio-

logically important molecules readily diffuses across cell membranes. H_2O_2-mediated cellular damage is caused by the oxidation of membranes and enzymes, DNA damage and mutation, and the inhibition of membrane transport.[5] It is broadly used as a disinfective agent across a wide range of industries.[6] Furthermore, H_2O_2 is used *in vivo* in a wide range of biological systems in the defense against pathogenic microorganisms.[7-11] The disadvantage of the use of pure H_2O_2 in clinical practice is its toxicity and the fact that it can cause embolism.[12-17] Therefore, a subtle approach in the use of H_2O_2 is suggested. This can be accomplished through the use of enzyme systems that produce H_2O_2 as an intermediate with the generation of other, less dangerous and less toxic, antimicrobial molecules.

Table 1. Bacterial strains used in this study.

Gram Stain	Species	Strain	Specifications
Positive	*Staphylococcus aureus*	07/0374	MRSA
	Staphylococcus aureus	07/0375	MRSA
	Staphylococcus aureus	07/0376	MRSA
	Enterococcus faecium	DIV5511	VREF
	Enterococcus faecium	2/03/2006	VREF
	Enterococcus faecalis	ATCC51299	VREF
Negative	*Escherichia coli*	07/0162	ESBL+
	Escherichia coli	07/0338	ESBL+
	Klebsiella oxytoca	07/0336	ESBL+
	Enterobacter cloacae	MYST07/189	ESBL+
	Enterobacter aerogenes	CXC613	ESBL+
	Burkholderia multivorans	06/0546	Multi-resistant
	Burkholderia multivorans	07/0199	Multi-resistant
	Pseudomonas aeruginosa	07/0247	Multi-resistant
	Pseudomonas aeruginosa	07/0224	Multi-resistant
	Stenotrophomonas maltophilia	07/0023	Multi-resistant
	Pandoraea apista	07/0284	Multi-resistant
	Achromobacter denitrificans	06/1465	Multi-resistant
	Achromobacter denitrificans	06/1031	Multi-resistant
	Achromobacter denitrificans	06/0871	Multi-resistant

The oxidase-peroxidase system, which is part of the innate salivary defence system, is such a naturally occurring antimicrobial enzyme system and is constituted of glucose oxidase, lactoperoxidase, and iodide (I^-) or thiocyanate (SCN^-).[18] Glucose oxidase catalyzes the oxidation of β-D-glucose to glucono-β-lactone, concomitantly producing hydrogen peroxide. Consequently, in the presence of hydrogen peroxide, lactoperoxidase produces the antimicrobial agents hypoiodite or hypothiocyanate by oxidation of iodide or thiocyanate, respectively.[11,19,20] The bactericidal effect of this system has been shown in various studies.[21,22] Recent research has shown that the addition of guaiacol—a naturally occurring, colorless, aromatic oil derived from guaiacum—markedly enhances the antimicrobial activity of the enzyme system. This antimicrobial enzyme system was named the GLG-enzyme system.[23]

The study investigated the susceptibility of various antibiotic-resistant, clinically relevant, strains to this new antimicrobial enzyme system. Further, the cytotoxicity of this enzyme system with an alginate-based wound dressing was analyzed and compared to other wound dressings.

Materials and Methods

Cell lines and strains. Human keratinocytes (HaCat) were kindly provided by Professor Dr. J. Merregaert (Molecular Biotechnology Lab, University of Antwerp, Belgium). Mouse fibroblasts (3T3) were obtained from ECACC (Salisbury, UK). All bacterial strains were obtained from the Department of Microbiology and Hospital Hygiene of the University Hospital of Brussels and were selected to enable analysis of a broad range of species and for high clinical relevance. All bacterial strains were clinical isolates (Table 1).

MIC_{90} and MBC analysis. Minimum inhibitory concentrations (MIC_{90}) were determined using the broth microdilution assay. MIC_{90} concentration was determined as the concentration of the GLG-enzyme system, which inhibited growth of bacteria by at least 90% compared with the control. Minimum bactericidal concentrations for the GLG-enzyme system were assessed by plating the entire volume from the broth microdilution MIC_{90} well and from the $\log_2$ dilutions above the MIC_{90} onto plates. The lowest concentration of the enzyme system, which killed ≥ 99.9% of the starting inoculum, was defined as the MBC endpoint. The experiment was performed at least in triplicate.

Growth kinetics analysis. Bacteria were grown until early log-phase and diluted to obtain an OD_{600} of 0.05. Growth kinetics were recorded by further growing these cultures at 37°C (98.6°F) in the presence of indicated concentrations of the GLG-enzyme system. As a

control, a culture was grown in the absence of the GLG-enzyme system. Growth was monitored for 8 hours by measuring OD_{600}. Results are the mean and standard deviation of at least 3 independent experiments.

Challenge test. Challenge tests were performed according to the European Pharmacopoeia 5.1.3 (v. 6.0) with slight modifications.

Cytotoxicity analysis. The cytotoxicity analysis was based on the European standard EN ISO10993-5:2003. Cells were seeded at subconfluency, re-suspended in growth medium, and incubated at 37°C/5% CO_2 for 24 hours. Dilutions of an alginate-based dressing system—the GLG-enzyme (Flaminal® Forte, Flen Pharma, Kontich, Belgium)—were made by dissolving 2 g into 8 mL of growth medium (1:5 dilution for a final concentration of 20% [w/v]).A further ½ dilution was made in the growth medium (10% [w/v]). Phenol 1:500, 1:1000, and 1:2000 dilutions were used as the positive control and the growth medium was used as the negative control. The growth medium was replaced by the dilutions of the alginate-based dressing containing the GLG-enzyme system, and the positive and negative controls. Cells were incubated at 37°C/5% CO_2 for a further 24 hours, after which the viability of cells was determined by the MTT cytotoxicity assay. The MTT assay was performed as follows: following incubation, cells were washed with PBS in order to remove gel-derived particles that would otherwise interfere with the spectrophotometric analysis. Next, cells were incubated for 3 hours with a 0.5-mg/mL MTT solution dissolved in growth medium. Viable cells will form insoluble formazan crystals. A mixture of isopropanol/0.04 N HCl was added in order to dissolve the formazan crystals. Finally, the absorption of the solution was measured at 570 nm with reference absorption at 630 nm. Results are the mean and standard deviation of 5 independent experiments. For comparison of the alginate-based dressing containing the GLG-enzyme system with other antimicrobial wound dressings, a 10% (w/v) solution of all dressings was prepared. Incubation of the cells with these solutions and an MTT assay were performed as described above. Results are the mean and standard deviation of 3 independent experiments.

Table 2. MIC_{90} and MBC values (%) of the GLG-enzyme system versus the antibiotic-resistant bacterial strains used in this study.

Organism	Strain	MIC_{90}	MBC
Staphylococcus aureus	07/0374	0.008	0.008
Staphylococcus aureus	07/0375	0.008	0.008
Staphylococcus aureus	07/0376	0.008	0.008
Enterococcus faecium	DIV5511	0.015	0.015
Enterococcus faecium	2/03/2006	0.03	0.03
Enterococcus faecalis	ATCC51299	0.06	0.06
Escherichia coli	07/0162	0.06	0.06
Escherichia coli	07/0338	0.13	0.13
Klebsiella oxytoca	07/0336	0.06	0.06
Enterobacter cloacae	MYST07/189	0.03	0.03
Enterobacter aerogenes	CXC613	0.06	0.06
Burkholderia multivorans	06/0546	0.015	0.015
Burkholderia multivorans	07/0199	0.015	0.015
Pseudomonas aeruginosa	07/0247	0.06	0.06
Pseudomonas aeruginosa	07/0224	0.25	0.25
Stenotrophomonas maltophilia	07/0023	0.03	0.03
Pandoraea apista	07/0284	0.015	0.015
Achromobacter denitrificans	06/1465	0.008	0.008
Achromobacter denitrificans	06/1031	0.015	0.015
Achromobacter denitrificans	06/0871	0.015	0.015

Results

MIC_{90} and MBC analysis. MIC_{90} and MBC values of all 20 strains were determined using a broth microdilution assay. For all strains, the MIC_{90} and MBC values were identical (Table 2). The MRSA strains and an *Achromobacter denitrificans* strain (06/1465) were the most sensitive to the GLG-enzyme system. The presence of only 0.008% (v/v) GLG-enzyme in the growth medium caused growth inhibition and cell death in these strains. The MIC_{90} and MBC values for most of the other strains ranged between 0.015%–0.06% (v/v) of GLG-enzyme. Two strains seem to be more resistant than other strains—an Extended Spectrum β-Lactamase-positive (ESBL+) strain (*Escherichia coli* 07/0338), with a MIC_{90} and MBC value of 0.13% (v/v), and *Pseudomonas aeruginosa* (07/0224), were the most resistant. The *P aeruginosa* strain had a MIC_{90} and MBC value of 0.25% (v/v).

Growth kinetic analysis. Growth kinetics of 9 strains were analyzed in order to determine whether the sensitivity of these strains to the GLG-enzyme system was still valid under other experimental settings. The results of the growth curve analysis show that all strains are sensitive to the GLG-enzyme system and that inhibition of growth is concentration dependent (Figure 1). However, the concentrations needed to obtain complete growth arrest were higher compared with the MIC_{90} val-

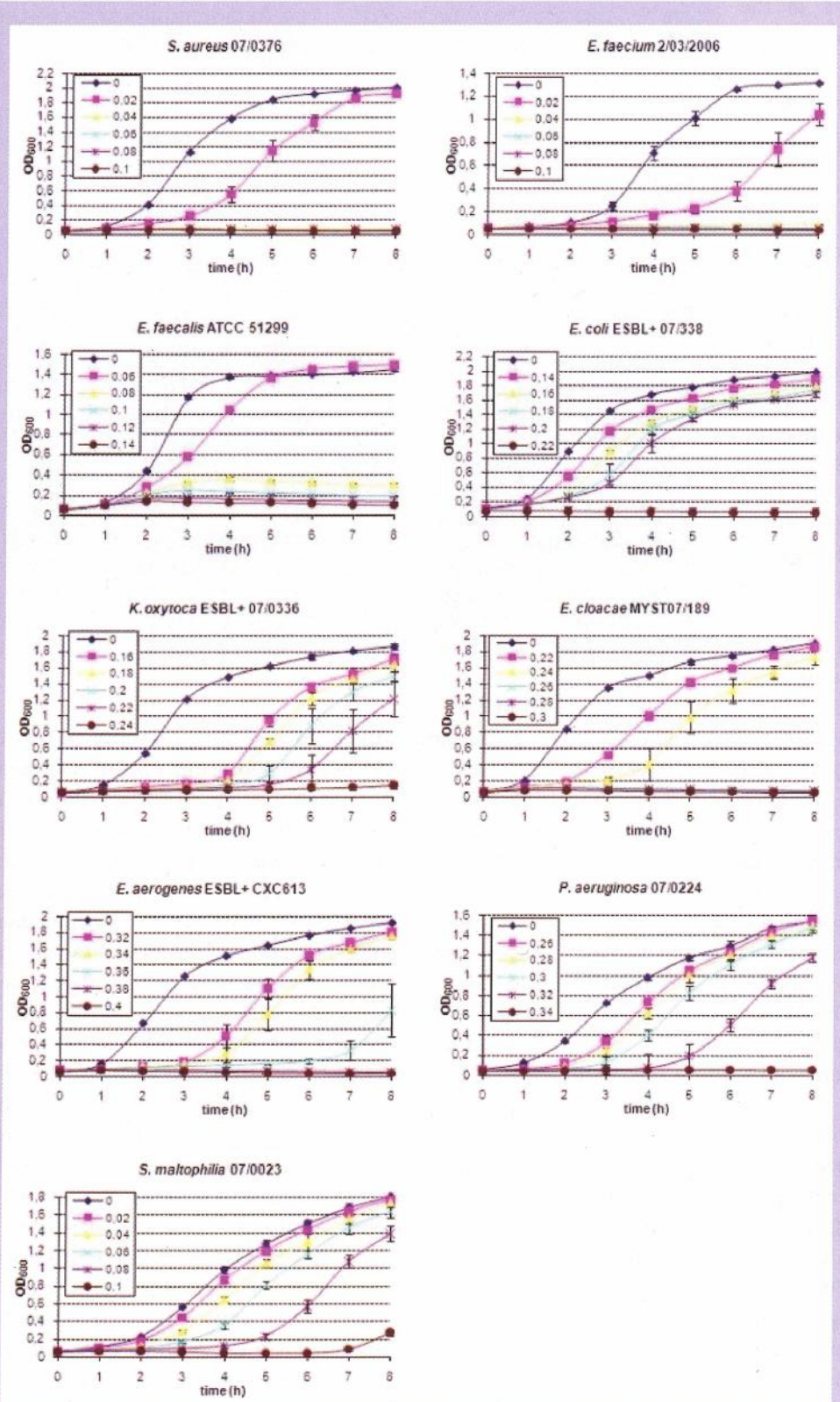
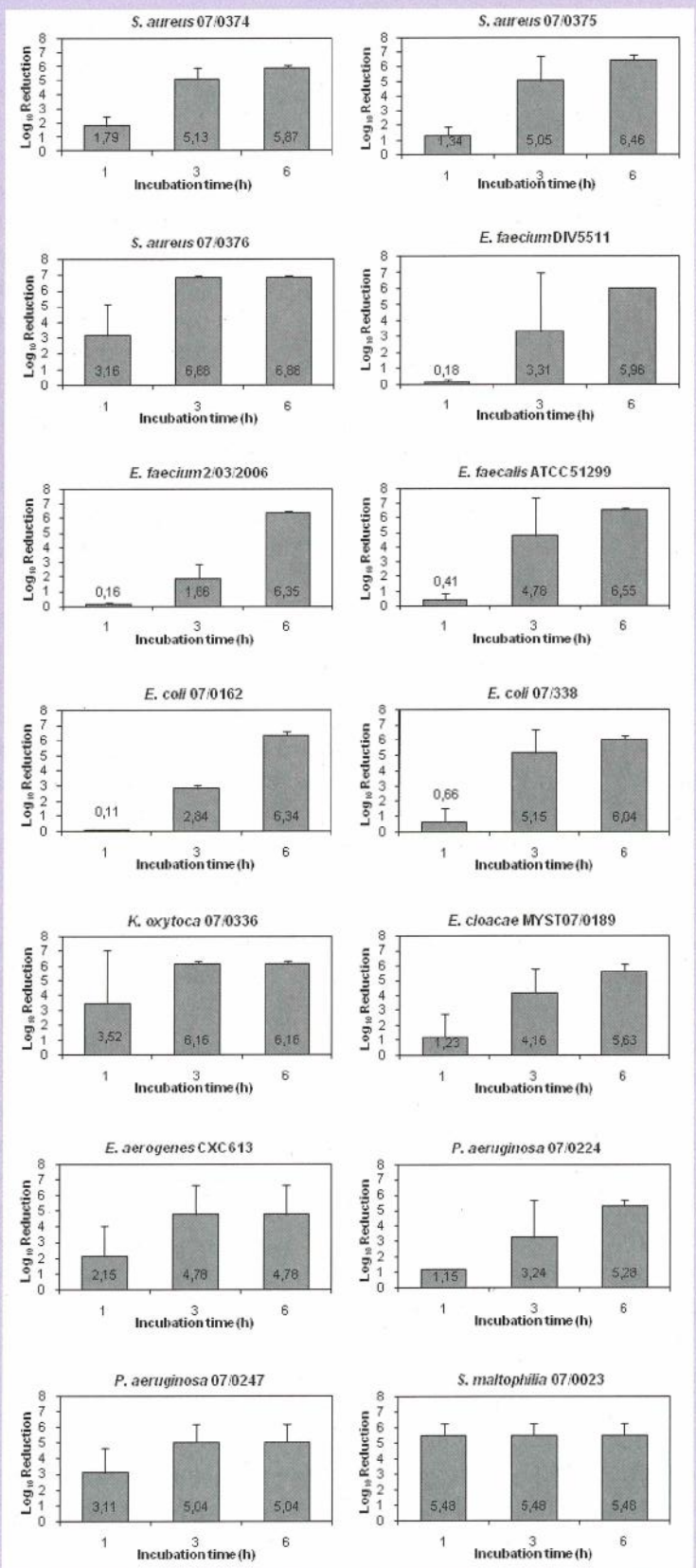


Figure 1. Growth kinetic analysis of 9 bacterial strains in the absence and presence of a concentration range (%) of the GLG-enzyme system.

ues of the strains. Despite this difference, the trend in sensitivity stayed the same—the most sensitive strains in the MIC_{90} analysis were also the most sensitive strains in the growth kinetic experiment. The difference for some strains between the two experimental designs was minor, and for others, the difference was more significant.

Challenge test. To determine whether the antimicrobial activity of the GLG-enzyme system is retained in a wound dressing used in clinical practice, challenge tests were performed using an alginate-based hydrogel containing the GLG-enzyme system. The results of these challenge tests show that all bacteria are killed within 6 hours of incubation, resulting in $\log_{10}$ reductions ranging from 4.78 to 6.88 (Figure 2). Four strains were killed within 3 hours of incubation and complete eradication within 1 hour of incubation was noted for Stenotrophomonas maltophilia ($\log_{10}$ reduction of 5.48; Figure 2). Interestingly, the two strains (*E coli* 07/0338 and *P aeruginosa* 07/0224) that showed a relatively high resistance in the MIC_{90} and MBC analysis were as sensitive as the other strains when challenged with this algi-



nate-based dressing (log_{10} reduction of 6.04 and 5.28, respectively).

Cytotoxicity analysis. Cytotoxicity analysis was performed using a MTT cytotoxicity assay measuring the metabolic activity of the cells, which is an indicator for cell survival. The data show that a 1/500 dilution of phenol in growth medium is cytotoxic for all tested cell lines and that 1/1000 and 1/2000 dilutions show no cytotoxicity ($P < 0.05$ compared to negative control [DMEM]). The dilutions of the alginate-based dressing containing the GLG-enzyme show no difference in cytotoxicity for 3T3 fibroblasts and HaCaT keratinocytes compared with the negative control (growth medium alone; Figure 3). Therefore, it can be concluded that the GLG-enzyme system in this alginate dressing is non-cytotoxic in an experimental set-up analogous to that described by the European standard EN ISO10993-5:2003.

When comparing the cytotoxicity of the GLG-enzyme with other antimicrobial wound dressings in the same experimental set-up, now in a 1/10 dilution, again the GLG-enzyme showed no cytotoxicity toward 3T3 fibroblasts (Figure 4A) or HaCat keratinocytes ($P < 0.05$; Figure 4B). Furthermore, when compared with wound dressings without an antimicrobial agent, the alginate-based dressing with GLG-enzyme system performed as well as these non-antimicrobial dressings in regard to cytotoxicity for fibroblasts (Figure 4A) and performed even better than a non-antimicrobial wound dressing (control hydrogel) when the products were tested on keratinocytes (Figure 4B). All other antimicrobial wound dressings showed significant cytotoxicity towards 3T3 fibroblasts and HaCat keratinocytes ($P < 0.05$).

Figure 2. Bactericidal activity of the GLG-enzyme system in an alginate gel formulation used in clinical practice (GLG-enzyme). Fourteen bacterial strains are challenged with an alginate-based dressing containing the GLG-enzyme system. Log_{10} reduction of viable colony forming units is plotted against time.

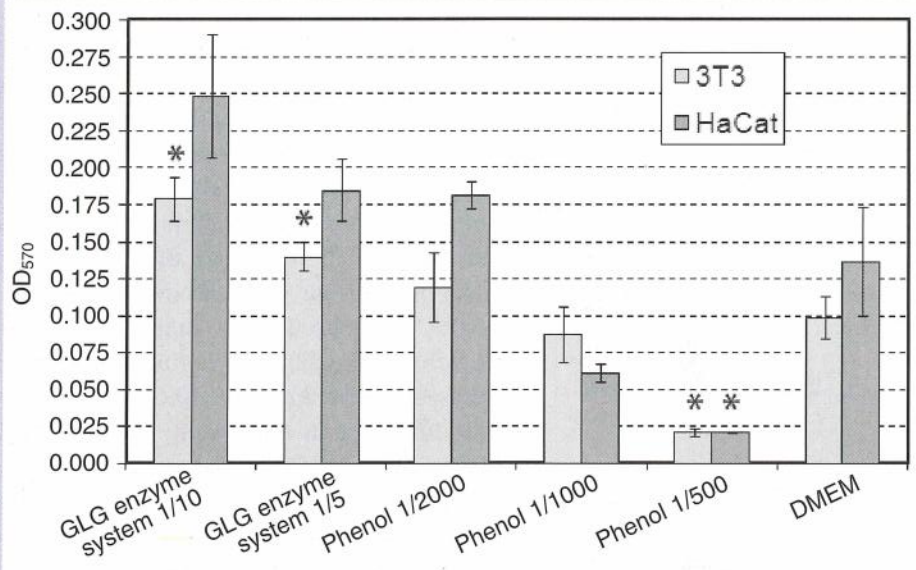


Figure 3. Cytotoxicity analysis of GLG-enzyme dilutions on 3T3 fibroblasts and HaCat keratinocytes compared to positive (phenol dilutions) and negative control (DMEM). *$P < 0.05$ compared to negative control (DMEM).

Discussion

This study intended to determine the sensitivity of a broad range of antibiotic-resistant bacterial strains to a new antimicrobial enzyme system. We also wanted to determine the cytotoxicity of this enzyme system as part of a new antimicrobial wound dressing. The cytotoxicity of this wound dressing was analyzed and compared with other antimicrobial wound dressings as well as with wound dressings with no antimicrobial activity.

Through different experimental set-ups we aimed to determine the susceptibility of a broad range of antibiotic-resistant clinical isolates to the GLG-enzyme system. First, MIC_{90} and MBC concentrations were determined. All strains were inhibited in their growth by the GLG-enzyme system. Further, the same concentration that inhibited growth also killed the bacteria (MIC_{90} = MBC). Two strains seem to be more resistant to the GLG-enzyme system. An Extended Spectrum β-Lactamase-positive (ESBL+) strain (*E coli* 07/0338) was isolated from blood and shows resistance to various antibiotics (ampicillin, cefuroxime, ceftriaxone, cefazoline, ceftazidime, cefepime, aztreonam, and ciprofloxazin; Prof. S. Lauwers, personal communication, March 2007). *P aeruginosa* 07/0224 turned out to be the most resistant strain. This multiresistant strain was also isolated from blood and shows antibiotic resistance to ampicillin, piperacillin, tazobactam, cefuroxime, ceftriaxone, cefazoline, ceftazidime, amoxicillin, temocillin, and aztreonam (Prof. S. Lauwers, personal communication, March 2007). It had a MIC_{90} and MBC value of 0.25%. The most sensitive strains were all MRSA strains tested and an *A denitrificans* strain. One MRSA strain was isolated from a brain biopsy, the other two from a swab. The *A denitrificans* strain was isolated from sputum. Although all of these strains have a broad range antibiotic resistance (Prof. S. Lauwers, personal communication, March 2007), all were sensitive to low concentrations of the GLG-enzyme system. Nine strains were further analyzed in a growth kinetic experiment. In this second experimental set-up the susceptibility of the strains to the GLG-enzyme system was retained. However, the concentration needed to obtain growth arrest was higher when compared with the MIC_{90} concentration. The susceptibility of the two strains that showed the highest MIC_{90} values were more moderate compared to the other strains in the second experimental set-up. For some strains the difference between the concentrations inducing growth arrest in both experimental settings was negligible (*Enterococcus faecium* 2/03/2006, *Enterococcus faecalis* ATCC51299 and *P aeruginosa* 07/0224), whereas for other strains this difference was substantial (*Staphylococcus aureus* 07/0376: 5 times difference, *Enterobacter cloacae* MYST07/189: 8.7 times difference and *Enterobacter aerogenes* CXC613: 6.3 times difference). No clear correlation between the two different experiments was found. The difference in concentration needed to obtain growth arrest is a result of the dissimilar design of both experiments. This lack of correlation in different experimental set-ups for determining antimicrobial activity is already reported.[24,25] Factors that have an influence on the susceptibility of the strains to the GLG-enzyme system in both experimental settings are the aeration of the cultures during growth (constant shaking versus no

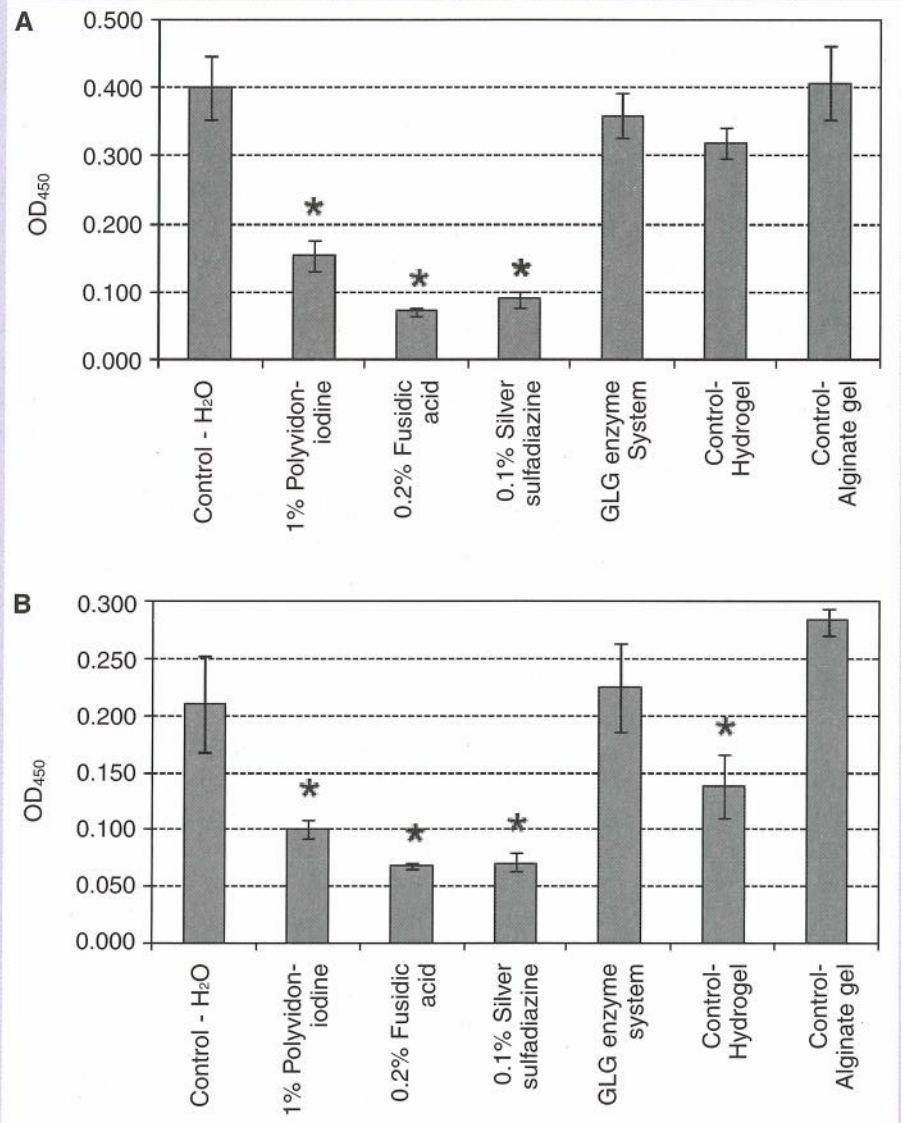


Figure 4. Comparison of the cytotoxicity on 3T3 fibroblasts A) HaCat keratinocytes, B) commonly used wound dressings (all in a 10% dilution in growth medium). The samples are named with the final concentration of the antimicrobial ingredient of the wound dressing. The final concentration of the GLG-enzyme system was 0.15%. Two wound dressings (a hydrogel and an alginate gel) without antimicrobial activity (10% dilution) and water (10% dilution) were used as control.
*$P < 0.05$ compared to control (H_2O).

shaking) and the amount of bacteria at the start of the experiment (OD_{600} of 0.05 versus OD_{600} of 0.001).

To determine whether the antimicrobial GLG-enzyme system retains its strong antimicrobial activity in a dressing used in clinical practice, challenge tests were performed with an antimicrobial alginate dressing, comprising the GLG-enzyme system. In this experimental design, the (somewhat considerable) differences in susceptibility between the strains to the GLG-enzyme system encountered in the other experimental settings disappeared. For most of the strains, all bacterial cells were killed within 6 hours of incubation, and 4 strains took 3 hours, resulting in a $\log_{10}$ reduction of between 4.78 and 6.88. It took only 1 hour to kill all *S maltophilia* cells, obtaining a $\log_{10}$ reduction of 5.48 in this short time span. These results clearly show that the antimicrobial activity of the GLG-enzyme system, as determined in the microdilution assay and the growth kinetic experiment, is retained in an alginate-based wound dressing—a dressing that has been established as beneficial to wound healing.[26,27]

Several conditions are important in order to achieve good wound healing. Specifically, an ideal moist environment should be present, wound infection needs to be controlled, and dressings need to show a low degree of cytotoxicity to avoid impairing cellular growth and normal healing.[28,29] In addition to the antimicrobial activity, the cytotoxicity of the alginate-based wound dressing containing the GLG-enzyme system was also analyzed. The cytotoxicity of this gel dressing was compared with other wound dressings with or without an antimicrobial agent. The survival of the cells challenged with the GLG-enzyme system in the alginate dressing showed no difference when compared with the survival of the cells challenged with the normal growth medium or with wound dressings without an antimicrobial agent. All other antimicrobial dress-

ings showed significantly lower cell survival. Surprisingly, challenging keratinocytes with the control hydrogel proved to be more cytotoxic compared to the alginate-based dressing containing the GLG-enzyme system, underlining the non-cytotoxicity of this antimicrobial alginate-based, hydroactive, colloid gel dressing *in vitro*.

The results of this study show that the GLG-enzyme system combines effective control of microbial bioburden with non-cytotoxicity. Low concentrations of the GLG-system are successful in killing a wide range of antibiotic-resistant bacterial strains and are not cytotoxic for fibroblasts and keratinocytes. Combining these properties of the GLG-enzyme system with a dressing regulating the moist environment of the wound would promote optimal wound healing.[26-30] Concerns regarding bacterial resistance to antibiotics are increasing. This study demonstrates that the antimicrobial GLG-system could be considered as a valid alternative to the use of topical antibiotics and other antimicrobial wound dressings.

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