In-vitro study of the synergistic effect of an enzyme cocktail and antibiotics against biofilms in a prosthetic joint infection model

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Abstract

Prosthetic Joint Infections (PJI) are frequent complications of arthroplasties. Their treatment is made complex by the rapid formation of bacterial biofilms, limiting the effectiveness of antibiotic therapy. In this study, we explore the effect of a tri-enzymatic cocktail (TEC) consisting of an endo-1,4-β-D-glucanase, a β-1,6-hexosaminidase, and an RNA/DNA non-specific endonuclease combined with antibiotics of different classes against biofilms of *S. aureus*, *S. epidermidis*, and *E. coli* grown on Ti6Al4V substrates. Biofilms were grown in TSB with 10g/L glucose and 20g/L NaCl (TGN). Mature biofilms were assigned to a control group or treated with the TEC for 30 minutes, then either analyzed or reincubated for 24h in TGN or TGN with antibiotics. The cytotoxicity of the TEC was assayed against MG-63 osteoblasts, primary murine fibroblasts, and J-774 macrophages using the LDH release test. The TEC dispersed 80.3 to 95.2% of the biofilms' biomass after 30 minutes. The reincubation of the treated biofilms with antibiotics resulted in a synergistic reduction of the total culturable bacterial count (CFU) compared to biofilms treated with antibiotics alone in the three tested species (additional reduction from 2 to more than 3 $\log_{10}$ CFU). No toxicity of the TEC was observed against the tested cell lines after a 24h incubation. The combination of pretreatment with TEC followed by a 24h incubation with antibiotics had a synergistic effect against biofilms of *S. aureus*, *S. epidermidis*, and *E. coli*. Further studies should assess the potential of the TEC as an adjuvant therapy in in-vivo models of PJI.

Introduction

Prosthetic Joint Infections (PJI) are catastrophic complications of joint replacement surgeries. With a prevalence of 2.0% to 2.4% in patients following a total knee or total hip replacement (1), PJIs represent one of the leading failure causes of arthroplasties (2).

The most prevalent species encountered in PJIs are *Staphylococcus aureus*, coagulase-negative staphylococci, streptococci, and Gram-negative bacilli (3). These species are known to adhere to the surfaces of implants and to form complex communities embedded in an extracellular matrix called biofilms. In these structures, bacteria become tolerant to antibiotics up to concentrations 1000 times the MIC of planktonic cells (4-6).

Surgical Debridement, Implant Retention, and Antibiotic therapy (DAIR) is the preferred treatment strategy of acute (less than 3 weeks of symptoms) PJI (7), with better functional outcomes (8) and lower costs (9, 10) than alternative strategies. However, the observed failure rate is considerable (11, 12). These failures are partly explained by an insufficient biofilm removal during the debridement surgery, as was shown in-vitro by Urish *et al.* (13) and Poilvache *et al.* (14).

As a consequence, the need for new adjuvant treatments to facilitate the dispersal of biofilms has been recognized in the literature (15-19). Among mechanical and pharmaceutical approaches, the use of hydrolytic enzymes targeting specific components of the biofilm matrix has been suggested (19). The dispersal potential of single enzymes targeting individual matrix components, such as extracellular DNA, exopolysaccharides, or extra-cellular proteins has been demonstrated by previous authors in in-vitro models (20-23). Moreover, the synergy of the combination of these enzymes with antibiotics (24, 25) or antiseptics (26, 27) against biofilms has been reported in previous studies.

However, these previous studies did not address the variety in matrix composition amongst bacterial species as they focused on the effect of single enzymes on selected species. For this reason, a broad-spectrum tri-enzymatic cocktail (hereafter referred to as TEC for Tri-enzymatic cocktail), composed of an endo-1,4-β-D-glucanase, a β-1,6-hexosaminidase and a recombinant RNA/DNA non-specific endonuclease (see Materials and Methods), was tested, targeting the matrix of *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* biofilms (patents n°WO2017/211737 A1 and n°BE2020/5726).

In this study, we investigated the dispersal effect of this tri-enzyme cocktail against *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Escherichia coli* mono microbial biofilms grown in vitro on titanium alloy surfaces similar to prosthetic implants and examined its synergy in combination with a broad selection of antibiotics belonging to different classes recommended for PJI treatment (7) at concentrations relevant to human use.

Results

Antibiotics susceptibility of planktonic bacteria and of biofilms

The minimal inhibitory concentrations (MIC) and minimal biofilm eradication concentrations (MBEC) are shown in Table 1. The strains were susceptible to all tested antibiotics according to the CLSI breakpoints, except for the ATCC 33591 MRSA strain which was resistant to doxycycline. For this reason, doxycycline was not used against ATCC 33591 biofilms for the next experiments. The MIC in MHB-ca and TGN were within two dilutions of each other. Most of the MBECs exceeded the MICs by a hundred or thousand-fold factor, to the notable exception of daptomycin, linezolid and moxifloxacin against the strain ATCC 33591 in MHB-Ca. The MBECs could not be established in TGN for the strain ATCC 47076 as we observed inconsistent results across replicates. All MBECs were found to be higher than the concentrations chosen for the coupons assays (Table 2).

Kinetics of TEC effect

The effect of TEC on ATCC 33591 MRSA biofilms is illustrated in Figure 1 for CFU counts (a) and biomass (b and c). The CFU counts decreased modestly after 15 minutes of incubation (-0.68 $\log_{10}$ CFU, p = 0.0365 from time 0) and no statistically significant differences were observed between the subsequent incubation times. A sharp decrease in the total biomass was observed after a 15 minutes incubation (-72.0% from time 0, p = 3.3e-06) and appeared to be maximal after 30 minutes (-95.82% from time 0, p = 7.0e-07; -19.45% from 15 min, p = 0.014). These effects were corroborated by macroscopic observation of the crystal violet stained samples (fig. 1.c). Based on these results, an incubation time of 30 minutes with TEC was adopted for the next experiments.

Effect of individual enzymes compared to the TEC

In a preliminary experiment, the effect of the individual enzymes composing the TEC was tested against biofilms of each of the three species in comparison with the cocktail (Figure 2). The TEC was found to be at least equivalent to the most effective single enzyme against all three species. While we did not observe any significant biofilm dispersal when treating MRSE ATCC 35984 biofilms with the individual enzymes, exposure to the TEC resulted in a 93% reduction of the biomass ($p<0.0001$). The RNA/DNA endonuclease was found to be equivalent to the TEC against MRSA ATCC 33591 and the β-1,6-hexosaminidase was equivalent to the TEC against *E. coli* ATCC 47076.

Effect of TEC and of antibiotics alone or in combination against biofilms

Biofilms exposed to TEC for 30 minutes were washed and reincubated in medium supplemented or not with antibiotics at clinically relevant concentrations (Table 2).
The results are illustrated in Figures 3 (MRSA ATCC 33591), 4 (MRSE ATCC 35984), and 5 (*E. coli* ATCC 47076) for CFU counts (panels A), biomass (panels B) and fluorescence microscopy (panels C). The details of the comparisons are shown in Tables 2 to 7 of the supplementary materials.

A 30 min exposure of the coupons to TEC significantly reduced the CFU counts and biomass of all strains. The CFU counts were reduced by 2.04 $\log_{10}$ in strain ATCC 33591, 2.54 $\log_{10}$ in strain ATCC 35984, and 1.24 $\log_{10}$ in strain ATCC 47076. Biomass was reduced by 95.2% in strain ATCC 33591, 88.6% in strain ATCC 35984, and 80.3% in strain ATCC 47076.

A 24h reincubation of control samples was not associated with significant changes in CFU counts. Their biomass remained stable in strains ATCC 35984 and ATCC 47076 but increased by 24.4% in strain ATCC 33591.
Similarly, the CFU counts of the samples treated with the enzyme cocktail and then

reincubated for 24 h in TGN were similar to those of non-treated samples for all three strains. The biomass was however significantly lower than for control group samples for ATCC 33591 (-19.1%) and ATCC 35984 (-34.7%) biofilms.

The effect of a 24h exposure to antibiotics on control biofilms was variable *vs*. CFU counts and generally weak or inexistent *vs.* the biomass when compared to initial controls (statistical analyses shown in supplemental materials Tables 2 to 7).

Against staphylococci, vancomycin (20 mg/L) caused a 1.84 $\log_{10}$ CFU increase in the CFU counts of MRSE ATCC 35984 biofilms but no changes MRSA ATCC 33591. Conversely, the biomass of MRSA samples increased by 22.3% but did not change significantly in MRSE samples. Rifampin (2.5 mg/L) caused a significant reduction of the CFU counts in both strains (ATCC 33591: -1.59 $\log_{10}$ CFU; ATCC 35984 -3.39 $\log_{10}$ CFU) but did not affect the biomass of either strain. Linezolid (5 mg/L) had a limited effect on the CFU counts of strain ATCC 33591 (-1.50 $\log_{10}$) and did not affect the CFU counts of strain ATCC 35984, nor the biomass of both strains. Moxifloxacin (1.5 mg/L) impacted both strains differently despite similar MICs. In ATCC 33591 samples, the CFU counts did not change, and the biomass increased by 29.7%, while in ATCC 35984, a 2.52 $\log_{10}$ CFU decrease was observed and the biomass remained stable. Daptomycin (30 mg/L) had a strong effect on the CFU counts of both strains (ATCC 33591: -4.99 $\log_{10}$; ATCC 35984: -3.50l og_{10}), as well as a moderate effect on the biomass of strain 33591 (-20.2%), while the biomass of strain ATCC 35984 remained unchanged. Doxycycline (1.5 mg/L) was used against MRSE ATCC 35984 only as MRSA ATCC 33591 was resistant. No significant effect was observed for either CFU counts or biomass.

Against *E. coli* ATCC 47076 biofilms, ceftazidime (3.8 mg/L) and ciprofloxacin (1.625 mg/L) had a significant effect on CFU counts (ceftazidime: -2.06 $\log_{10}$; ciprofloxacin: -3.21

141 log_{10}) and biomass (ceftazidime: -61.7%; ciprofloxacin: -48.33%), while colistin (2.5 mg/L)
142 and meropenem (0.25 mg/L) did not.

143 Turning now to the results for biofilms of the three strains successively exposed to TEC and
144 antibiotics at set concentrations, we observed significant reductions of CFU counts and
145 biomass when compared to samples treated only with antibiotics, except for the CFU counts
146 of MRSA ATCC 33591 samples treated with daptomycin (which were already markedly
147 decreased by the antibiotic alone) and the biomass of *E. coli* ATCC 47076 treated with
148 ceftazidime (for which the difference between samples preexposed or not to the TEC cocktail
149 did not reach significance). The difference in CFU counts between the samples preexposed to
150 the enzymes and the control group samples was always higher than 2 log_{10} and exceeded 3
151 log_{10} in MRSA ATCC 33591 samples treated with vancomycin (-4.22 log_{10}, $p<0.0001$) or
152 moxifloxacin (-3.87 log_{10}, $p<0.0001$), in MRSE ATCC 35984 samples treated with
153 vancomycin (-6.84 log_{10}, $p<0.0001$), and in all *E. coli* ATCC 470476 samples exposed to any
154 of the 4 tested antibiotics (ceftazidime: -5.23 log_{10}, $p<0.0001$); ciprofloxacin: -3.98 log_{10},
155 $p<0.0001$; colistin: -5.57 log_{10}, $p<0.0001$; meropenem: -6.11 log_{10}, $p<0.0001$).
156 The difference in biomass between samples treated with antibiotics alone or with the
157 combination of TEC and antibiotics exceeded 80% in most conditions, except for strain
158 ATCC 33591 samples treated with daptomycin and strain ATCC 47076 samples treated with
159 ciprofloxacin, and colistin.
160 The two-way ANOVA analysis (supplementary materials Tables 8 to 13) showed significant
161 interaction parameters in all cases when considering the exposure to the enzyme cocktail and
162 the reincubation of the samples as factors. This suggests that the tri-enzyme cocktail and the
163 antibiotics have a synergistic effect on both CFU counts and biomass of all three strains.
164 The analysis of biofilms using fluorescence microscopy after live/dead staining (figures 3, 4,
165 5, panels C) illustrates the observed CFU counts and biomass assays. The treatment of

samples with the TEC dispersed an important proportion of the bacterial cells, which where enough to regrow a biofilm after 24h of reincubation in TGN. This recovery appeared to be inhibited by the reincubation with antibiotics. The aspect of control samples exposed during 24h to vancomycin, ciprofloxacin, and meropenem appeared unchanged from the samples incubated in TGN, keeping a majority of live cells (green), while the samples incubated with daptomycin exhibited a high proportion of dead cells (red).

Cytotoxicity tests

The cytotoxicity of the tri-enzyme cocktail for different types of eukaryotic cells was then evaluated by measuring the release of the cytosolic enzyme LDH in the culture medium after 1 h and 24 h exposures to TEC at two concentrations (concentration used against biofilms and a 1:1 dilution). At 1 h, no significant toxicity was detected (values <5% in all cases). At 24 h, the release of the cytosolic enzyme lactate dehydrogenase (LDH) reached 3.0% (IC 95%: -1.1;7.0) for primary murine fibroblasts; -1.6% (IC 95%: -4.7%;1.4) for MG-63 human osteosarcoma cells and -3.2% (IC 95%: -17.0;10.7) for J774 murine macrophages.

Discussion

This study found that a tri-enzymatic cocktail containing two glycoside hydrolases (an endo-1,4-β-D-glucanase and a β-1,6-hexosaminidase) and a non-specific RNA/DNA endonuclease had a significant dispersal effect on mature biofilms of *S. aureus, S. epidermidis*, and *E. coli* reference strains grown on titanium alloy and that the successive treatment of these biofilms with this enzyme cocktail and antibiotics at concentrations relevant to the clinical setting was synergistic, resulting in significantly lower residual CFU counts than with either individual treatment.

While the potential of glycoside hydrolases and DNase I as biofilm dispersal agents has been previously described in the literature (19, 22), the originality of our work resides in the association of enzymes targeting biofilm matrix components found in biofilms of varied species (28), which is hypothesized to help sidestep the limited spectrum of individual enzymes caused by the significant variations in matrix contents observed among bacterial species and strains (29, 30). Indeed, the results of individual enzymes assays performed in this study underline the added value of combining enzymes as done in the TEC. The β-1,6-hexosaminidase used in this study is a Dispersin B homolog targeting the poly-β-1,6-*N*-acetylglucosamine (PNAG) component of the biofilm matrix (31, 32). PNAG is an important constituent of the matrix of multiple species' biofilms and is required for the formation of biofilm by *S. epidermidis* (30, 33, 34) and *S. aureus* strains expressing the *icaABCD* operon (30, 35) and of *E. coli* strains expressing the *pgaABCD* locus (29, 36). Dispersin B was shown to disperse the biofilms of PNAG-producing strains of multiple species, including those included in our study (20, 37, 38). Cellulase is as endo-1,4-β-D-glucanase targeting cellulose, which is an important constituent of the biofilm matrix of *Enterobacter* spp. (39), including *E. coli* (40). It has been shown to disperse *E. coli* (27) and *Pseudomonas aeruginosa* (21) biofilms in-vitro. The recombinant non-specific endonuclease targets both DNA and RNA

phosphodiester bonds (41). DNA is a common component of the biofilm matrix of most bacterial species, including *S. aureus*, *S. epidermidis*, and *E. coli* (42). While the biofilm dispersal properties of this enzyme had not been previously investigated, it has been favorably compared to the widely studied DNAse I (22) for the hydrolysis of DNA in sputum (43).

The dispersal of biofilms by enzymes similar to the ones constituting TEC was shown in previous publications to reduce the tolerance of bacterial biofilms to antibiotics (21, 25, 44) and antiseptics (27). However, limitations related to the use of single enzymes have been reported. Izano *et al.* observed that DNAse I did not disperse biofilms of *S. epidermidis* contrarily to *S. aureus* and that Dispersin B had an inverse effect by dispersing *S. epidermidis* biofilms and leaving *S. aureus* biofilms intact. Accordingly, they found that the dispersal of these biofilms by the appropriate enzyme (DNAse I for *S. aureus* of Dispersin B for *S. epidermidis*) improved the effect of the detergent cetylpyridinium chloride on CFU counts (37).

Our results illustrate the broad spectrum of the tri-enzymatic cocktail, which stems from the combination of enzymes with different activity spectrums (28). Furthermore, these results support the hypothesis of a synergy of enzymatic dispersal and a broad selection of antibiotics at concentrations clinically relevant to the treatment of PJIs against biofilms. As could be expected from the observed MBEC values, the antibiotic concentrations used for the coupons assays had a minimal effect on control biofilms, but their combination with an enzymatic pre-treatment resulted in significant reductions of culturable cell numbers as well as total biomass in nearly all combinations of bacterial species and antibiotics.

The cytotoxicity of TEC to different cell lines relevant to the joint environment (macrophages, fibroblasts, osteoblasts) was found to be negligible after a 24h exposure. An explanation to this observation could be that the targets of these enzymes are either not

produced by mammalian cells (cellulose), or irrelevant to their proliferation (eDNA and PNAG).

These results should however be interpreted with caution as our study presents some limitations. We limited ourselves to the study of biofilms of reference strains. While this facilitates the replication of our results by other teams in comparison to clinical strains, the loss of diversity in gene expression could mask the effect of the variability in matrix composition observed in some species (45). The antibiotic concentrations considered in our study did not take into account the serum protein binding. Among the drugs used in this study, doxycycline and daptomycin show a high serum protein binding, which could lead to an over-estimation of their effects in our experimental setting. However, the albumin concentration in synovial fluid is three to four times inferior to the serum concentration (46). This difference in albumin concentrations could lead to a higher free fraction of drugs in the synovial fluid than in serum. For this reason, the total serum concentration appeared to be a reasonable estimate that limited the risk of underestimating the effects of antibiotics. The in-vitro nature of our experiments limits the extrapolation to the clinical setting as it does not represent the diversity of the situations encountered in a clinical setting, nor does the use of set concentrations of antibiotics reproduce the complex pharmacokinetics observed in the bone and joint environment (47).

However, these observations support our hypothesis that a treatment approach combining a local administration, such as an intra-articular injection, of a broad-spectrum enzymatic cocktail and antibiotics could prove to be beneficial in a PJI setting where the variety of pathological bacterial species encountered is important (3). Further studies in more refined models such as animal PJI models should therefore be undertaken to confirm our in-vitro results and determine an adequate strategy around this approach in complement to DAIR.

Materials and Methods

Bacterial strains, antibiotics, and enzymes

Strains

The methicillin-resistant *Staphylococcus aureus* ATCC 33591, methicillin-resistant *Staphylococcus epidermidis* ATCC 35984, and the *Escherichia coli* ATCC 47076 were used as representatives of the bacterial species usually encountered in PJI.

Antibiotics

The following antibiotics were used as laboratory standards: ciprofloxacin HCl (potency: 93.9%), and moxifloxacin HCl (potency: 90.9%) from Bayer AG, Leverkusen, Germany, colistin (potency: 79.6%) and doxycycline (potency: 90.2%), from Sigma-Aldrich, Saint-Louis, MO, USA, and linezolid (potency: 99.2%; RibX Pharmaceuticals, Morristown, NJ, USA). The following antibiotics were used as powders for injections in humans: ceftazidime (potency: 72.5%; Panpharma France, Luitré, France), daptomycin (potency: 100%; MSD, Puteaux, France), meropenem (potency: 92%; Hospira Benelux BVBA, Brussels, Belgium), rifampin (potency: 100%; Aventis, Schiltigheim, France), vancomycin (potency: 97.5%; Mylan, Hoeilaart, Belgium). The antibiotic concentrations were set on the basis of the PK/PD characteristics of the antibiotics studied and their pharmacokinetic parameters in humans described in the EUCAST rationales for clinical breakpoints or in the literature (Table 2).

Tri-enzymatic cocktail (TEC)

The tri-enzymatic cocktail (TEC; OrthenzyQure) used in this study was designed and provided by OneLife SA (Louvain-la-Neuve, Belgium). It consits in a sterile Tris-buffered (pH=7) aqueous solution containing 400 U/ml of aspecific DNA/RNA endonuclease from *Serratia marcescens* (c-lecta GMBH, Liepzig, Germany), 50U/ml of endo-1,4-β-D-glucanase from *Aspergillus niger* (Sigma) and 0.06 U/ml of β-*N*-acetylhexosminidase from

Actinobacillus pleuropneumoniae (GIGA, Liège, Belgium) (composition described in patent publication numbers WO2017/211737 and BE2020/5726). The TEC was stored at 4 °C and was heated at 37°C for 30 minutes before use. The TEC is available upon request at OneLife SA for research purposes (under R&D reference OL-RD1000/OrthenzyQure).

In order to assay the biofilm dispersal effect of the individual enzymes, OneLife provided solutions of the individual enzymes at concentrations identical to those used in the TEC.

Susceptibility testing

MICs were determined by microdilution following the CLSI protocol (48) in MHB-ca and TGN (TSB + 2% NaCl + 1% glucose), i.e. the medium used for growing biofilms. MICs of daptomycin were tested in MHB-ca and TGN containing 50 mg/L of Ca^{2+}.

MBECs were assayed by growing biofilms in Calgary Biofilm Devices (CBD, Innovotech, Edmonton, Canada) in MHB-ca and TGN. Bacterial suspensions were prepared in PBS, starting from overnight cultures on TSA from frozen stocks. The suspensions were adjusted to a turbidity of 0.5 McFarland and diluted 1:10 in medium. 150µL were added per well of the CBDs. The CBDs were incubated for 24h at 37°C, under a continuous rotation of 50rpm to allow biofilm formation. Antibiotic dilutions in the two media – with 50mg/L of Ca^{2+} when assaying daptomycin - were prepared in new 96 wells plates at concentrations ranging from 1024mg/L to 0.5 mg/L (256 mg/L to 0.125 mg/L for meropenem). The lids of the CBDs were rinsed twice in PBS before being transferred to the plates containing the antibiotics. The plates were then incubated for 24h at 37°C with a continuous rotation of 50 rpm. At the end of the antibiotic challenge, the lids were rinsed twice in PBS before being transferred to new plates containing plain media. The plates were then sonicated for 10 minutes (Branson 5510 Ultrasonic bath, Emerson Electric, Saint-Louis, MO, USA). New steriles lids were placed and the plates were incubated for 24h at 37°C without agitation. At the end of the incubation

period, the plates were examined for bacterial growth and optical density measures at 650nm were performed using a Spectramax M3 spectrophotometer (Molecular Devices, San Jose, CA, USA).

Biofilm culture

Bacterial suspensions were prepared in PBS, starting from overnight cultures on TSA from frozen stocks. The suspensions were adjusted to an OD_{620nm} of 0.5 (~8.5 $\log_{10}$ CFU/mL) using a CECIL 2021 spectrophotometer (Cecil Instruments Ltd, Milton, United-Kingdom). The suspension was then diluted 1:100 in TGN. The biofilms were grown on titanium alloy (Ti-6Al-4V) coupons (Biosurface, Bozeman, MN, USA) in 12 wells plates using 3mL of the final suspension per coupon. The plates were incubated for 24 h (ATCC 33591 and ATCC 35984) or 48 h (ATCC 47076) at 37°C, with a constant orbital shaking of 50 rpm. In the case of strain ATCC 47076 biofilms, the medium was changed after 24 h. These culture times were selected as they allowed the biofilms to reach steady states over the study period and were determined in previous growth kinetic experiments for *S. aureus* (14). The culture duration period for *S. epidermidis* was derived from these results. Growth kinetic experiments were performed in this study for *E. coli* biofilms and showed that a steady state was reached after 48h of culture. Samples were analyzed for CFU counts and biomass every 24h for the growth kinetic experiments.

Biofilm treatments

Individual enzymes assay

The biofilms samples were rinsed twice with sterile PBS. They were then transferred to 24 wells plates filled with either 1mL of TEC or 1mL of one of the individual enzymes at 37°C. The plates were then incubated for 30 minutes at 37°C. At the end of the incubation period, the biofilm were rinsed twice in sterile PBS and assayed for total biomass (see below).

Combination of enzymatic cocktail and antibiotics

The biofilm samples were separated into a control group and an enzyme cocktail group. The coupons of both groups were rinsed twice in sterile PBS before being submitted to the treatments. The tri-enzyme cocktail group samples were transferred to a 24 wells plate, which wells were previously filled with 1mL of enzyme cocktail at 37°C. The plates were then incubated at 37°C with no agitation for either 15, 30, and 60 minutes (when studying the kinetics of activity) or 30 minutes (other experiments). After incubation, the samples were rinsed twice in sterile PBS before either immediate analysis (see below for analysis techniques descriptions) or reincubation. Control group samples were directly rinsed in sterile PBS. All samples were then reincubated for 24 h at 37°C, under a constant orbital shaking of 50 rpm in TGN or TGN containing antibiotics at set concentrations (TGN + 50 mg/L of Ca^{2+} for samples exposed to daptomycin) (see Table 2) in 12 wells plates containing 3 mL of medium per well.

Biofilms analysis

The coupons were rinsed twice in sterile PBS and then analyzed using the following techniques:

CFU counts

Coupons were placed in 15 mL conical tubes (Greiner Bio-One International GmbH, Kremsmünster, Austria) filled with 2 mL of sterile PBS. In order to detach the biofilms and suspend the bacteria, the tubes were then vortexed for 30 s at maximum intensity (Vortex-Genie 2, Scientific Industries Inc., Bohemia, NY, USA), sonicated for 5 min (Branson 5510 Ultrasonic bath, Emerson Electric, Saint-Louis, MO, USA), and vortexed again for 30 s. The suspension was then sampled and serially diluted, before plating on TSA. The TSA plates were then incubated for 24 to 48 h at 37°C until the colonies reached an adequate size. CFU

counts were then obtained using an automated method (image acquisition using Gel Doc XR+ and image processing using Quantity One (BioRad, Hercules, CA, USA).

Biomass assay

Coupons were dried overnight at 60°C. They were then stained with 1 mL of 1% crystal violet solution for 10 min (Sigma-Aldrich Corp.). The coupons were then rinsed twice using deionized water to remove the excess dye. Afterward, they were placed for 1 h in 24 wells plates filled with 1 mL of 66% acetic acid solution (Merck KGaA, Darmstadt, Germany) to resolubilize the dye. The coupons were removed from the wells and the absorbance of the solution was read at 570 nm using a Spectramax M3 spectrophotometer (Molecular Devices, San Jose, CA, USA).

Fluorescence microscopy

Coupons were rinsed twice in sterile deionized water to avoid interference from phosphate ions. They were then stained using the Filmtracer LIVE/DEAD Biofilm Viability Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. Coupons were then rinsed again twice in sterile deionized water before imaging using an AxioImager.Z1 microscope fitted with an ApoTome1 attachment (Zeiss, Oberkochen, Germany) at a 20x magnification. The images were post-processed using FIJI (49, 50), using the maximum intensity projection technique and by adjusting the brightness of the channels.

Cytotoxicity assays

Cell Culture

J774 murine macrophages (Sandoz Forschung Laboratories, Vienna, Austria) (51), MG-63 human osteoblasts (ATCC CRL-1427), and primary murine fibroblasts (52) were used for the cytotoxicity assays. The cells were grown in RPMI 1640 or Dulbecco's Modified Eagle

Medium supplemented with 10% of fetal bovine serum (all products from Thermo Fisher Scientific Inc., Waltham, MA, USA) at 37°C in a 5% CO_2 atmosphere. The cells were used for the assays between passages 2 and 5.

The cells were cultured initially in 250 cm^3 cell culture flasks before being transferred to 96 wells plates, at a concentration of 10^4 cells per well. The plates were incubated for 24 h at 37°C with 5% CO_2 to allow cellular adhesion to the wells before testing.

Assay

Cytotoxicity was assessed by measuring the release of the cytoplasmic enzyme lactate dehydrogenase (LDH) in the culture medium, using the Cytotoxicity Detection Kit (Sigma-Aldrich). The wells were rinsed with sterile PBS before adding 100µL of medium with 1% decomplemented fetal bovine serum. 100µL of the following test substances were then added to the wells (final concentrations): TEC (concentration used against biofilms and half of this concentration), Triton X-100 solution (1%, positive control), medium with 1% decomplemented serum (negative control). Three empty wells per experiment were filled with 200µL of medium with 1% decomplemented serum to measure the background signal.

The plates were then incubated for 1 h or 24 h, at 37°C in a 5% CO_2 atmosphere. 100µL of the supernatant of each well was aspirated and transferred to a new 96 wells plate and added by 100 µL of the extemporaneously prepared LDH assay solution, as per the manufacturer's instructions. The plates were then incubated for 30 min at room temperature in the dark. The light absorbance was then measured at 490 nm and 650 nm using a Spectramax M3 spectrophotometer (Molecular Devices, San Jose, CA, USA).

Data transformation and statistical analysis

CFU counts were normalized using a base 10 logarithmic transformation before analysis. Absorbance values of the biomass assays were transformed into percentages of the T0

controls after subtracting the average value of negative controls. The absorbance values of the LDH assays were transformed in percentages using the following equation:

$$Cytotoxicity\ (\%) = \frac{Abs.exp.value_{420nm-650nm} - Abs.control\ (-)_{420nm-650nm}}{Abs.control\ (+)_{420nm-650nm} - Abs.control\ (-)_{420nm-650nm}} \times 100$$

The statistical analysis was then performed using R (version 3.6.1) and the package car (version 3.0.6) or in GraphPad 8.4.3 (GraphPad Software, San Diego, CA).The TEC kinetics experiments data were analyzed using repeated measure ANOVA followed by paired t-tests with Holm correction. The data relating to the combined effect of TEC and antibiotics were analyzed using two-way ANOVA, after assessing the normality of residues with QQ plots and the equality of variances using the Levene test. The Tukey HSD test was used to perform pairwise comparisons. The synergy of treatments was defined as a statistically significant interaction parameter of the two-way ANOVAs (53). The threshold for alpha errors is of 0.05. The data reported in the text are the means with the 95% confidence interval.

Data Availability Statement

The data of the experiments reported in this publication are available upon request to the corresponding author.

Authors Contributions

HP designed and performed the experiments and data analysis, the other authors provided helpful suggestions regarding experiments' design or performance.

All authors contributed to the writing of the manuscript.

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Conflicts of Interest

The authors declare that they have no affiliations or involvement with any entity with financial interests related to the content of this manuscript.

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Tables

Table 1. Minimal Inhibitory Concentrations and Minimal Biofilm Eradication Concentrations (mg/L)

Antibiotic	Assay	ATCC 33591		ATCC 35984		ATCC 47076	
		MHB-Ca	TGN	MHB-Ca	TGN	MHB-Ca	TGN
Daptomycin	MIC	1	1	1	1	-	-
(DAP)	MBEC	4	>1024	>1024	>1024	-	-
Doxycycline	MIC	**16**	**16**	0.25	0.25	-	-
(DOX)	MBEC	-	-	128	512	-	-
Linezolid	MIC	2	1	2	1	-	-
(LZD)	MBEC	8	>1024	>1024	>1024	-	-
Moxifloxacin	MIC	0.125	0.125	0.06	0.125	-	-
(MXF)	MBEC	2	>1024	>1024	>1024	-	-
Rifampin	MIC	0.004	0.004	0.004	0.004	-	-
(RIF)	MBEC	8	2	128	4	-	-
Vancomycin	MIC	1	8	2	8	-	-
(VAN)	MBEC	>1024	>1024	>1024	>1024	-	-
Ceftazidime	MIC	-	-	-	-	0.25	0.125
(CEF)	MBEC	-	-	-	-	>1024	*
Ciprofloxacin	MIC	-	-	-	-	0.016	0.03
(CIP)	MBEC	-	-	-	-	128	*
Colistin	MIC	-	-	-	-	0.5	0.25
(CST)	MBEC	-	-	-	-	>1024	*
Meropenem	MIC	-	-	-	-	0.03	0.06
(MEM)	MBEC	-	-	-	-	>256	*

Values in bold indicate an MIC superior to the CLSI breakpoint values (48).

*: The MBEC assays results for the ATCC 47076 strain were unreliable and were not included.

Table 2. Antibiotics experimental concentrations and rationales.

Antibiotic	Concentration (mg/L)	Rationale
Daptomycin (DAP)	30	$\frac{AUC_{0-24h}}{24h}$
Doxycycline (DOX)	1.5	$\frac{AUC_{0-24h}}{24h}$
Linezolid (LZD)	5	C_{min}
Moxifloxacin (MXF)	1.5	$\frac{AUC_{0-24h}}{24h}$
Rifampin (RIF)	2.5	$\frac{AUC_{0-24h}}{24h}$
Vancomycin (VAN)	2	Target trough concentration (20 mg/L)
Ceftazidime (CEF)	3.8	C_{min}
Ciprofloxacin (CIP)	1.625	$\frac{AUC_{0-24h}}{24h}$
Colistin (CST)	2.5	$\frac{AUC_{0-24h}}{24h}$
Meropenem (MEM)	0.25	C_{min}

Note: Pharmacokinetic and PK/PD informations obtained from the EUCAST rationales for clinical breakpoints documents or from the literature (references in supplementary materials Table 1).

Figures

Figure 1

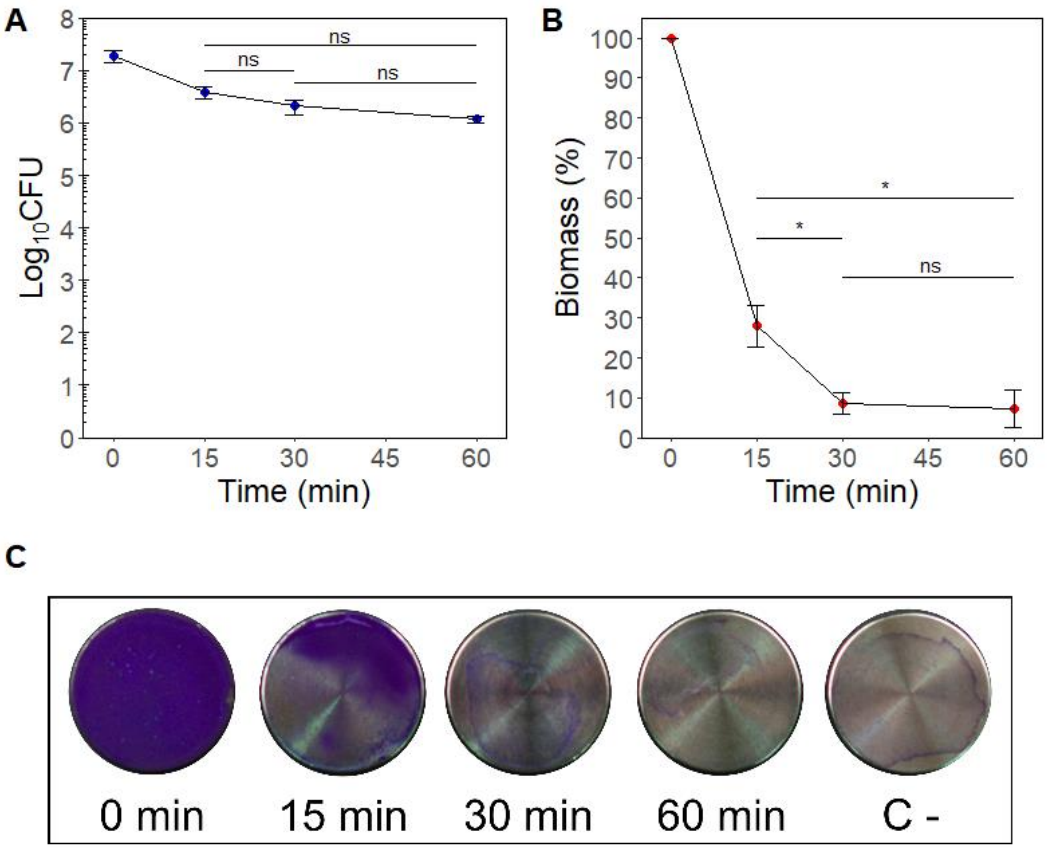


Figure 1. Kinetics of biofilm dispersal by TEC against MRSA *S. aureus* ATCC 33591 biofilms. A: Log_{10} CFU counts per coupon; B: Total biomass assay (expressed as the percentage of Time 0 control); C: Pictures of biofilm samples exposed to TEC for the indicated times (C-: negative control [coupon incubated in sterile culture medium]) stained with crystal violet). The values indicate the means of triplicate experiments (+/- standard error). Statistical analysis: repeated measure ANOVA, followed by paired t-tests with Holm correction. *: $p<0.05$.

Figure 2

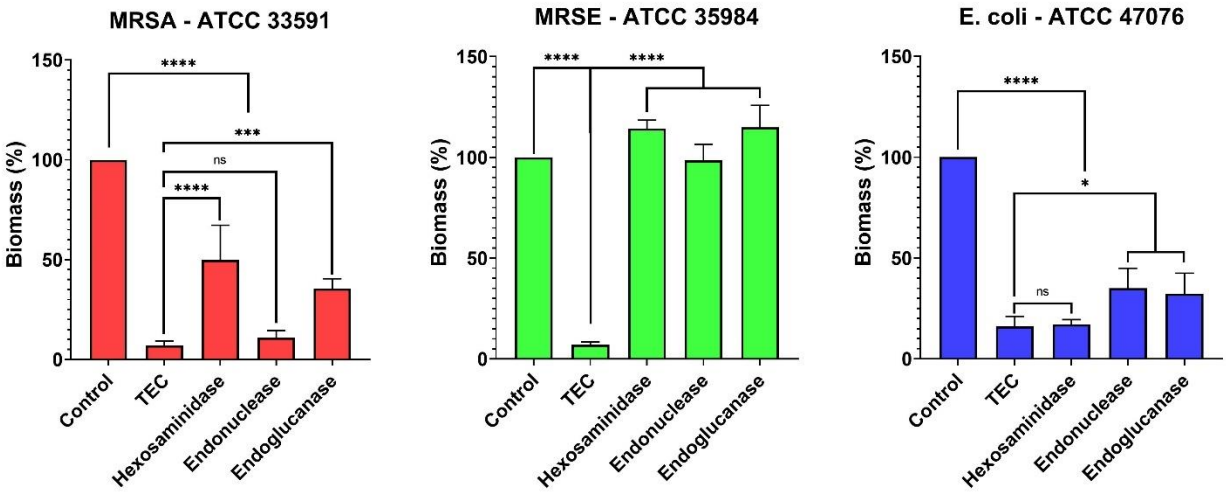


Figure 2. Effect of a 30 minutes treatment with the individual enzymes on the biomass of ATCC 33591, ATCC 35984, and ATCC 47076 biofilms and comparison to the TEC. Hexosaminidase: β-*N*-acetylhexosaminidase; Endonuclease: DNA/RNA endonuclease; Endoglucanase: endo-1,4-β-D-glucanase. Data expressed as percentage of the control. The values indicate the means of triplicate experiments (+/- standard error). Statistical analysis: two-way ANOVA followed by Holm-Sidàk post-hoc test. NS: no significant difference *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$

Figure 3

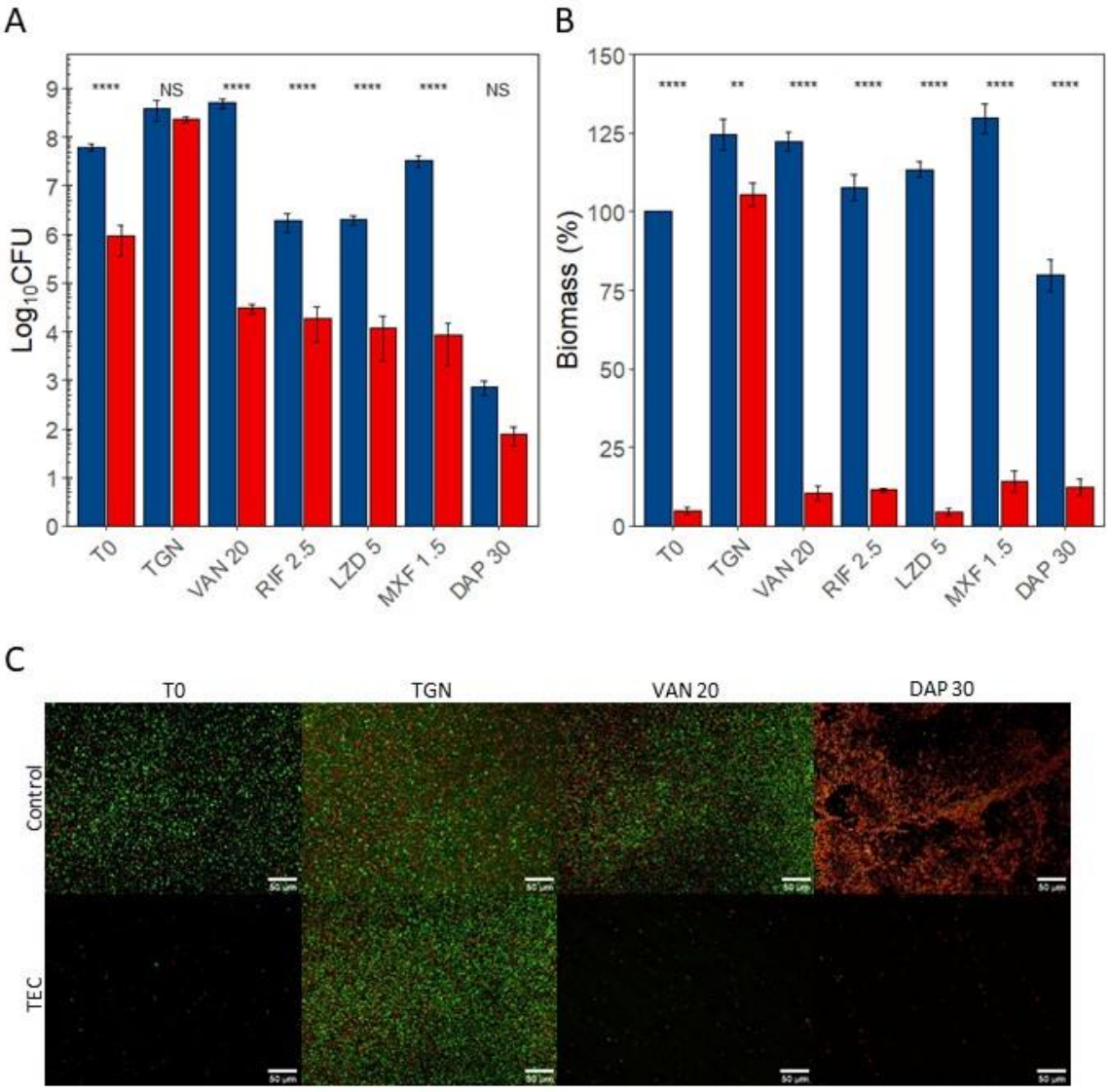


Figure 3. Effect of the combination of the TEC with antibiotics on MRSA ATCC 33591 biofilms. A: CFU counts; B: Biomass; C: Fluorescence microscopy. A & B: blue: controls, red: samples treated for 30 minutes with TEC. T0: no reincubation; TGN: 24h reincubation in TGN; Others: 24h reincubation in TGN with antibiotics at the indicated concentrations (see Table 1). CFU counts expressed in $\log_{10}$ CFU per coupon, biomass expressed in percentage of T0 controls. The values indicate the means of triplicate experiments (+/- standard error).

Statistical analysis: two-way ANOVA followed by Tukey HSD comparing control samples to samples exposed to the enzymatic cocktail. NS: no significant difference *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$. C: Green: living cells (SYTO 9); red: dead cells (Propidium Iodide). 20x magnification. Scale bars represent 50µm.

Figure 4

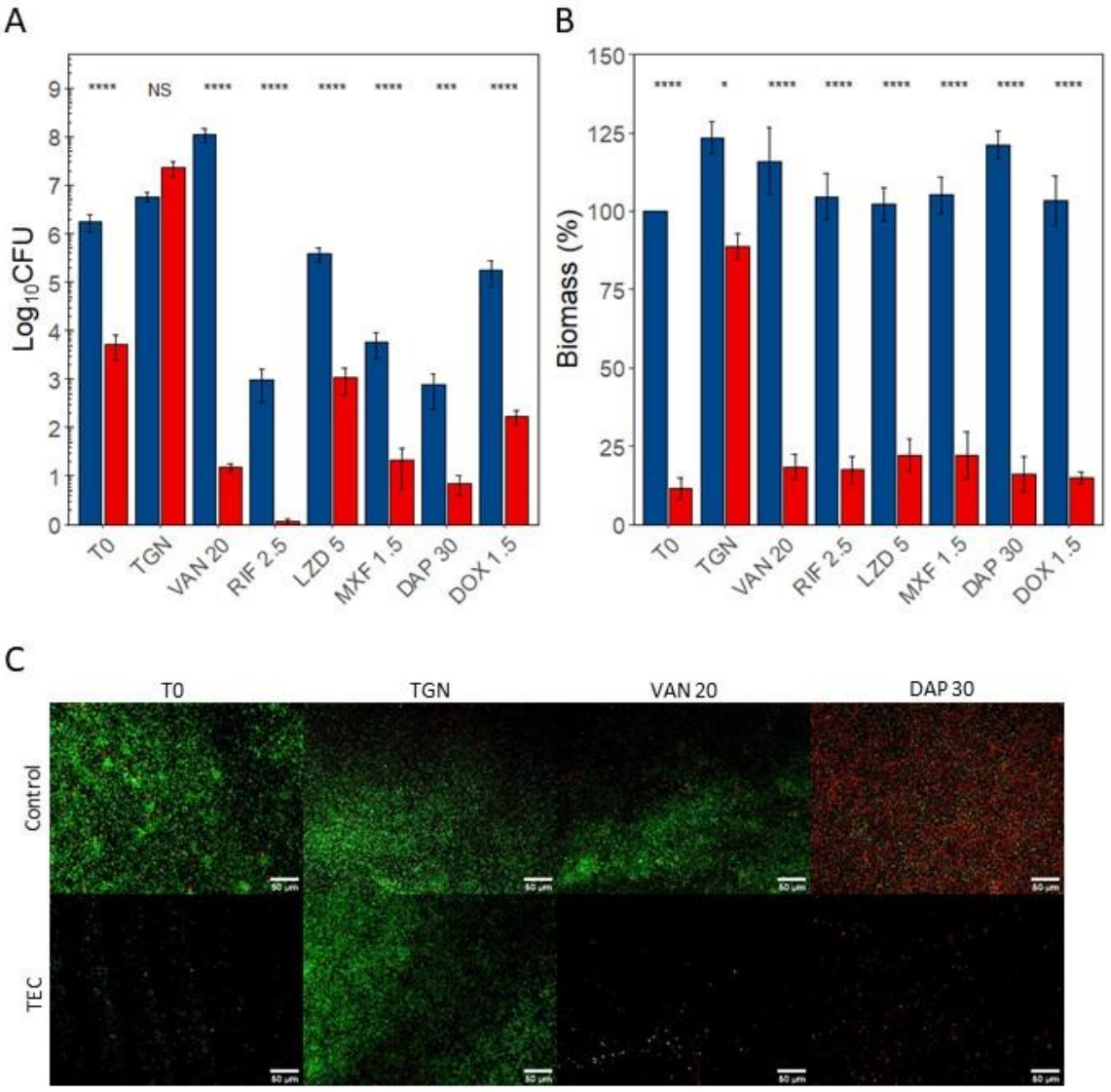
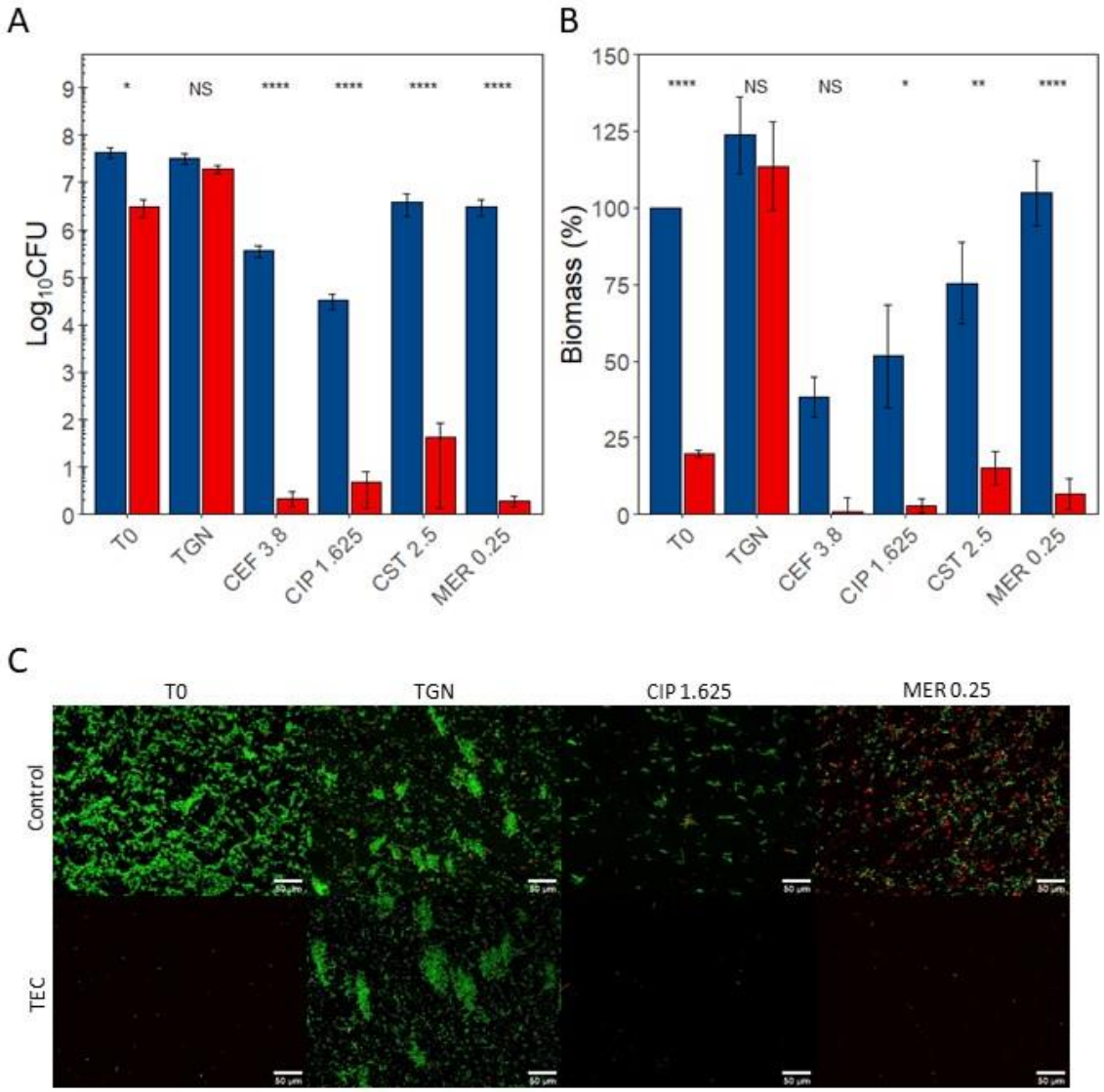


Figure 4. Effect of the combination of the TEC with antibiotics on MRSE ATCC biofilms. A: CFU counts; B: Biomass; C: Fluorescence microscopy.A & B: blue: controls, red: samples treated for 30 minutes with TEC. T0: no reincubation; TGN: 24h reincubation in TGN; Others: 24h reincubation in TGN with antibiotics at the indicated concentrations (see Table 1). CFU counts expressed in log_{10} CFU per coupon, biomass expressed in percentage of T0 controls. The values indicate the means of triplicate experiments (+/- standard error).

Statistical analysis: two-way ANOVA followed by Tukey HSD comparing control samples to samples exposed to the enzymatic cocktail. NS: no significant difference *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$. C: Green: living cells (SYTO 9); red: dead cells (Propidium Iodide). 20x magnification. Scale bars represent 50µm.

650 **Figure 5**

651



652

653 Figure 5. Effect of the combination of the TEC with antibiotics on *E. coli* ATCC 47076
654 biofilms. A: CFU counts; B: Biomass; C: Fluorescence microscopy. A & B: blue: controls,
655 red: samples treated for 30 minutes with TEC. T0: no reincubation; TGN: 24h reincubation in
656 TGN; Others: 24h reincubation in TGN with antibiotics at the indicated concentrations (see
657 Table 1). CFU counts expressed in $\log_{10}$ CFU per coupon, biomass expressed in percentage of
658 T0 controls. The values indicate the means of triplicate experiments (+/- standard error).

659 Statistical analysis: two-way ANOVA followed by Tukey HSD comparing control samples to
660 samples exposed to the enzymatic cocktail. NS: no significant difference *: $p<0.05$; **:
661 $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$. C: Green: living cells (SYTO 9); red: dead cells
662 (Propidium Iodide). 20x magnification. Scale bars represent 50µm.

663