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Destruction of single species biofilms of *Escherichia coli* or *Klebsiella pneumoniae* subsp. *pneumoniae* by dextranase, lactoferrin, and lysozyme

Cynthia L. Sheffield,* Tawni L. Crippen, Toni L. Poole, Ross C. Beier

Food and Feed Safety Unit, Southern Plains Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, College Station, Texas, USA

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Summary. The aim of this work was to determine the destructive activity of dextranase, lactoferrin, and lysozyme, against single species biofilms composed of either *Klebsiella pneumoniae* subsp. *pneumoniae* or *Escherichia coli* using the MBEC Assay. Luminescence measurements based on quantitation of the ATP present were used to determine the amount of biofilm elimination and correlated with quantity of live bacteria present in the sample. The data were analyzed employing a two-way ANOVA and Bonferroni post-test. Treatments resulted in percentage reductions of *E. coli* biofilms ranging from 73 to 98 %. Lactoferrin (40 µg/ml) produced a significantly higher-percentage reduction than lysozyme (10 µg/ml) ($P < 0.05$), no other significant differences occurred. Similar treatments resulted in percentage reductions of *K. pneumoniae* subsp. *pneumoniae* biofilms ranging from 51 to 100 %. Dextranase treatments produced a significantly lower percentage reduction than all other materials ($P < 0.05$), no other significant differences occurred. No material was capable of complete destruction of both single species biofilms; however, low concentrations of lactoferrin and lysozyme each removed 100 % of the *K. pneumoniae* subsp. *pneumoniae* biofilm. Low concentrations of lactoferrin or lysozyme might be beneficial to prevent biofilm formation by *K. pneumoniae* subsp. *pneumoniae*. [**Int Microbiol** 2012; 15(4):183-187]

Keywords: *Escherichia coli* · *Klebsiella pneumoniae* subsp. *pneumoniae* · dextranase · lactoferrin · lysozyme · biofilms · food safety

Introduction

Approximately 99 % of the microorganisms on Earth exist as microbial communities known as biofilms [5]. Bacterial biofilms occur in a wide variety of natural and human-made environments and have been implicated as a constant source of pathogens that cause infection and contamination in medical and food processing devices [12]. This has led to an increased interest in probing the molecular mechanisms fundamental to the formation and maintenance of these communities [10]. These factors result in serious economic and environmental impacts and consequently, a growing need exists for effective treatments focusing on biofilm reduction.

Ölmez and Temur [15] have examined the effect of ozone, chlorine and organic acid treatments on the removal of *Escherichia coli* and *Listeria monocytogenes* embedded inside biofilms on the surface of lettuce leaves. Unfortunately, none of these sanitizing treatments are effective in eradi-

***Corresponding author:** C.L. Sheffield
USDA-ARS-SPARC
2881 F & B Rd
College Station, TX 77845, USA
Tel. +1-9792609221. Fax +1-9792609332
E-mail: cindy.sheffield@ars.usda.gov

cating the bacteria. Furukawa et al. [6] have reported that some strong alkaline or acidic decontamination agents are markedly effective for disinfecting *Staphylococcus aureus* biofilm, as well as *E. coli* biofilm. Starek et al. [18] have found that toluene reduces average biofilm biomass and thickness, and diminishes the diversity of amplifiable 16S rRNA sequences. Regardless of their respective effectiveness, these materials are either highly corrosive or not suited to numerous environmental decontamination situations. Furukawa et al. [6] have also tested the relatively mild agents EDTA (ethylenediaminetetraacetic acid), SDS (sodium dodecyl sulfate) and Tween 20. These safer compounds, however, are ineffective against *S. aureus* biofilms and remove only partiallly *E. coli* biofilms.

No ideal biofilm decontamination protocol presently exists and more innovation is needed to develop an effective, relatively robust, and non-corrosive material or cocktail of materials. These treatments must be novel, and for successful treatment, the materials must both kill the bacteria and detach the dead biofilm by removing the extracellular polymeric substances (EPS). Simple disruption of the biofilm without significant cell death permits relocation of viable remnants and eventual formation of a new biofilm [17].

To that end, our research examined three milder decontamination substances (dextranase, lactoferrin, and lysozyme) for their efficacy in the destruction of single species biofilms composed of either *Klebsiella pneumoniae* subsp. *pneumoniae* or *E. coli.*

Materials and methods

Bacterial biofilm. *Escherichia coli (*ATCC 4157) and *Klebsiella pneumoniae* subsp. *pneumoniae (*ATCC 4352) were cultured on nutrient agar (NA) plates overnight at 37 °C. Using a sterile cotton swab, a sample of each bacterium was removed from the surface of the overnight culture. The bacteria were resuspended at approximately 10^7 colony forming units (CFU)/ml in sterile phosphate-buffered saline (PBS) for use as inoculum in the MBEC (minimum biofilm eliminating concentration) Assay (Innovotech, Edmonton, Canada) as per the manufacturer's directions. Actual inoculum range as determined by serial dilution was 3.15 to 6.00×10^7 CFU/ml for *E. coli* and 2.35 to 3.40×10^7 CFU/ml for *K. pneumoniae* subsp. *pneumoniae*. Each bacterium was run on two-columns of every MBEC plate without any treatments applied as controls. One-column was quantified by serial dilution plating of the biofilm after dislodging via sonication from the growth peg to determine biofilm growth rate, and the second-column was quantified in the same manner as the treated biofilms after dislodging via sonication of biofilm from the growth peg.

Treatment material. Treatment concentrations of dextranase (1, 2, 3, and 4 U/ml; A, B, C, D, respectively); lactoferrin (20, 40, 60, and 80 µg/ml; A, B, C, D, respectively) and lysozyme (5, 10, 25, and 50 µg/ml; A, B, C, D, respectively) dissolved in PBS were evaluated. PBS was run independent of the test material to determine its effect if any on the growth of *E. coli* and *K. pneumoniae* subsp. *pneumoniae*, and quantified in the same manner as the treated biofilms after dislodging via sonication of biofilm from the growth peg.

Experimental setup. The experimental design is based on the manufacturer's suggested protocol; the MBEC assay has also been described in detail elsewhere [4,20]. Briefly, an aliquot of 135 µl of bacterial inoculum was added per well to the MBEC plate and incubated at 37 °C while shaking at 150 rpm overnight. Biofilm formation was viewed as complete after 24 h. At that point, a biofilm colony concentration of $\geq 10^7$ CFU/ml was achieved as determined by serial dilution plating of the biofilm removed from the growth peg by sonication. After incubation, the peg lids containing the biofilm were transferred into a rinse plate containing 200 µl/well (PBS) at pH 7.2, and incubated for 2 min without agitation at room temperature. The peg lids were then transferred into another plate containing 135 µl/well of each treatment. Each test material concentration was run in quadruplicate wells. The peg lids were then incubated at 37 °C for 1 h.

After treatment, the peg lids were transferred into a rinse as described above, and incubated for 2 min without agitation at room temperature before being placed into the recovery wells containing 135 ml/well PBS. The recovery wells and MBEC peg lids were then subjected to 5 min of sonication to dislodge the biofilm from the peg lids. BacTiter-Glo reagent (100 µl/well; Promega, Madison, WI, USA) was added to the suspension, and the samples were mixed for 30 s at 150 rpm and then incubated for 5 min at room temperature. The luminescence was quantified using a VICTOR^3 V plate reader (PerkinElmer, Waltham, MA, USA). The resulting live bacterial counts were correlated with a standard curve calculated from known bacterial quantities. Each experiment was replicated three times.

Statistical analysis. Data were analyzed using commercially available statistical software (Prism ver. 5.01, GraphPad Software, La Jolla, CA, USA). Within each treatment and bacterial type, a means comparison of concentration was performed using a two-way ANOVA followed by Bonferroni post tests to determine least square means ($P < 0.05$).

Results

Figure 1 shows the percentage of reduction in a 24 h old *E. coli* biofilm achieved after a 1-h exposure to three individual substances at increasing concentrations. Lactoferrin treatments produced the highest-reduction in biofilm of the three test substances, with a range in reduction of 95 to 98 %. Dextranase treatments were the second most effective, reducing biofilm from 81 to 84 %. Lysozyme treatments showed no significant difference when compared to the dextranase treatment, reducing the biofilm from 73 to 84 %.

Figure 2 shows the percentage of reduction in a 24-h-old *K. pneumoniae* subsp. *pneumoniae* biofilm achieved after a 1-h exposure to the three substances at increasing concentrations. Lactoferrin and lysozyme treatments each produced a 100 % reduction in biofilms. Dextranase treatments resulted in biofilm reductions ranging from 51 to 65 %.

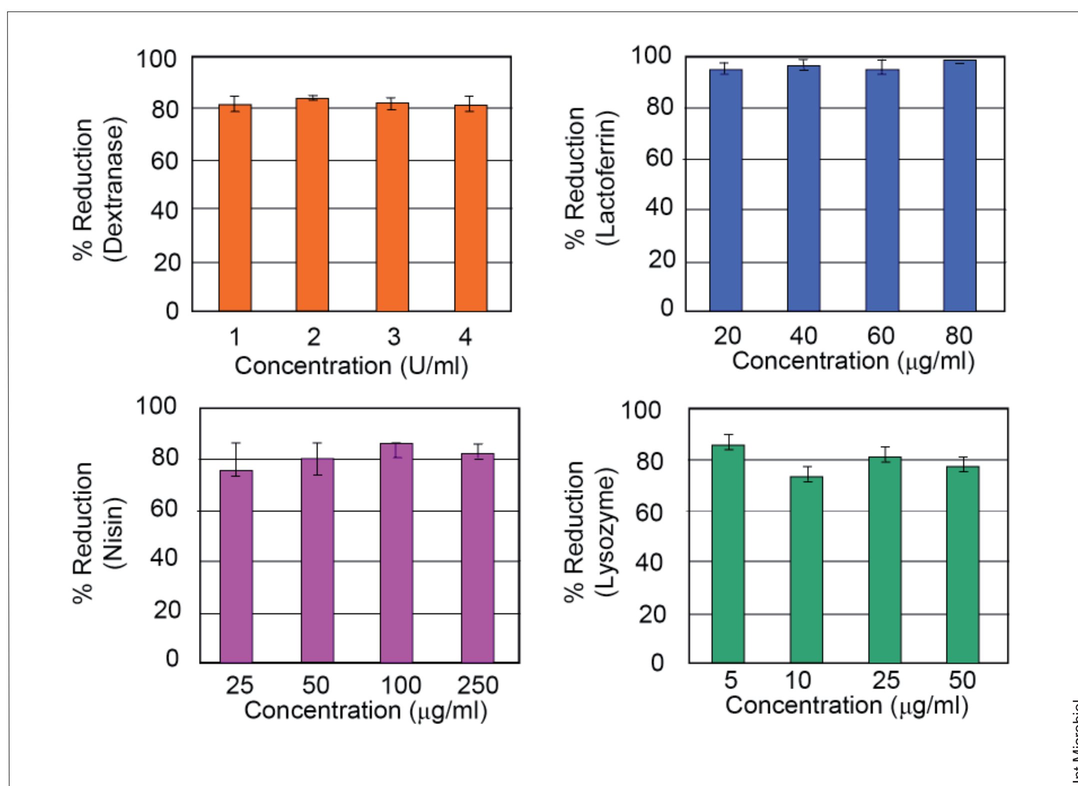

Fig. 1. Percent reduction of the 24-h-old *Escherichia coli* biofilm resulting from dextranase, lactoferrin and lysozyme treatments at four dosage levels with one 1 h treatment exposure.

Escherichia coli and *K. pneumoniae* subsp. *pneumoniae* biofilms were compared; *K. pneumoniae* subsp. *pneumoniae* was significantly more resistant to removal by dextranase than was *E. coli*. Conversely, *E. coli* was significantly more resistant to removal by lysozyme than was *K. pneumoniae* subsp. *pneumoniae*. Unfortunately, none of the materials tested were able to completely eradicate individual biofilms of both *E. coli* and *K. pneumoniae* subsp. *pneumoniae*. PBS had no effect on the growth of either of the two bacteria (data not shown).

Discussion

Dextranase, lactoferrin, and lysozyme were selected because they are capable of destroying the physical integrity of the matrix, interfere with bacterial adhesion or initiate cell detachment from surfaces in addition to destroying the individual bacterial cells. As such, they are good alternatives to biocides and/or disinfectants which can contribute to the propagation and spread of resistant strains and may have restricted use because of environmental regulations. Dextranase is an enzyme produced by several bacteria and molds which catalyzes the endohydrolysis of 1,6-α-glucosidic linkages in dextran resulting in damage to the biofilm where dextran is employed as a key component [7,9]. Lactoferrin is a globular glycoprotein widely found in secretory fluids, whose antimicrobial activity results from its iron-binding properties which oxidize the bacteria via the formation of peroxides—which in turn deprives the bacteria of this essential element for growth; disruption of the cell membrane; and targeting of H^+-ATPase [2,16]. Lysozyme is a glycoside hydrolase enzyme found in a number of secretions that disrupts bacterial cell walls by catalyzing hydrolysis of 1,4-β-linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues in peptidoglycan and between *N*-acetyl-D-glucosamine residues in chitodextrins [11]. Dextranase, lactoferrin, and lysozyme were used at different concentrations to determine their effectiveness at eliminating mature single species biofilms of *E. coli* or *K. pneumoniae* subsp. *pneumoniae*. To our knowledge, this is the first report of these substances being utilized against *K. pneumoniae* subsp. *pneumoniae* biofilms.

Yano et al. [21] have found that dextranase at 0.25 % (v/v) produces no significant reduction of *Streptococcus mutans* or

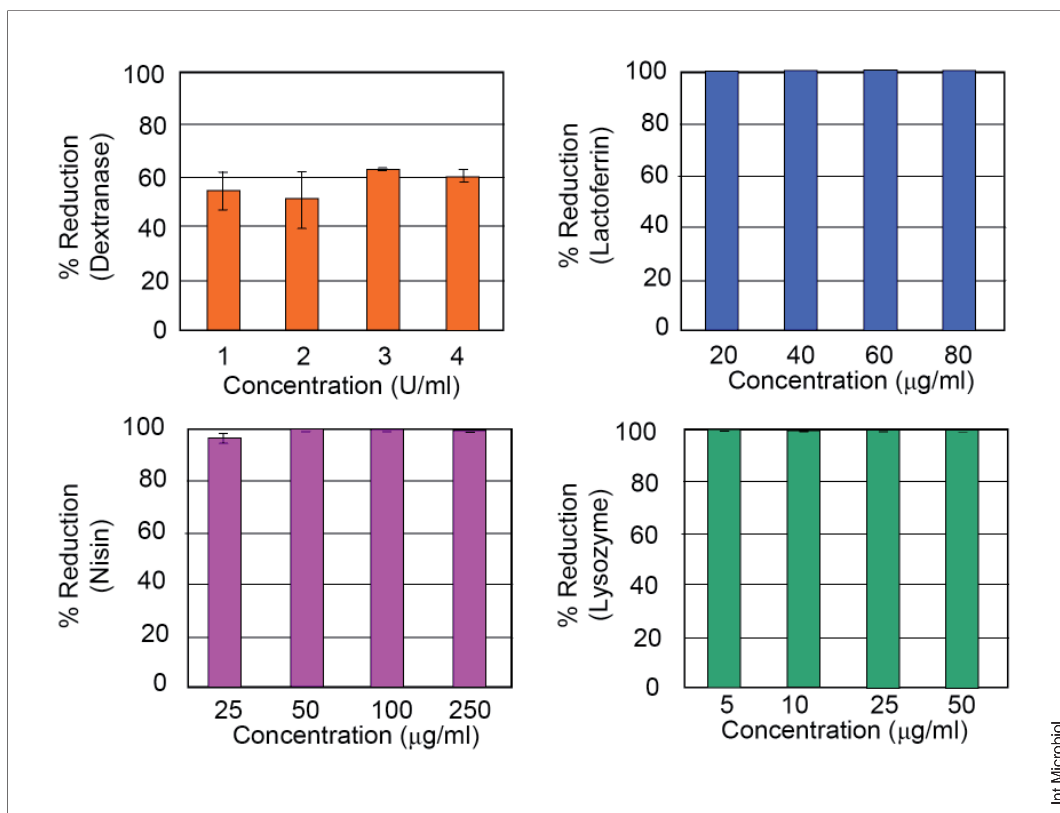

Fig. 2. Percent reduction of the 24-h-old *Klebsiella pneumoniae* subsp. *pneumoniae* biofilm resulting from dextranase, lactoferrin and lysozyme treatments at four dosage levels with 1 h treatment exposure.

S. sobrinus biofilms. Our results demonstrated that dextranase was the least effective overall of the three substances tested; however, against *E. coli* and *K. pneumoniae*, it did achieve some biofilm destruction. This difference in findings can be explained by the content of dextran and glucans within the biofilm matrix, which is linked to the bacterial species, present [1,13,19].

Earlier work with *E. coli* O157:H7 biofilms has determined that lactoferrin alone is not bacteriostatic, and that, at concentrations between 20 and 40 mg/ml, it does not prevent the growth of *E. coli* O157:H7 in tryptic soy broth [3,14]. In contrast, we found that lactoferrin was very effective to destroy both single species *K. pneumoniae* subsp. *pneumoniae* and *E. coli* biofilms. The difference in outcome could partially be explained by the observations of Jenssen and Hancock [8], who have reported lactoferrin acting on *E. coli* by damaging the bacterial membrane and disrupting the bacterial type III secretion system. These actions would be expected to affect the hardiness of a biofilm formed by this bacterial species and thus have a more detrimental effect on biofilms than on planktonic cells.

Previous work has demonstrated the differential effect of lysozyme against various bacterial species. Branen and Davidson [3] have found that lysozyme has a mean inhibitory concentration <500 μg/ml on the growth of *E. coli* O157:H7. In our work, lysozyme at levels as low as 5 μg/ml was completely effective (100 %) against *K. pneumoniae* subsp. *pneumoniae* biofilms and partially effective (73 %) against *E. coli* biofilms. The differences between our results and those of Branen and Davidson [3] could be caused by the difference in species or variant and testing in a planktonic vs. biofilm format.

The results of our study demonstrate the potential of lactoferrin as an agent to eradicate mature biofilms of *K. pneumoniae* subsp. *pneumoniae*. Further, low concentrations of lysozyme or lactoferrin might be beneficial to prevent biofilm formation by gram-negative bacteria, such as *E. coli* and *K. pneumoniae* subsp. *pneumoniae*, thus providing better hygiene in both agricultural and medical arenas. While dextranase achieved biofilm reduction, it was only partial, which minimizes its potential as a control product since biofilms are structured to resist and overcome incomplete degradation. In

future work we will examine the effectiveness of these compounds against mature gram-positive biofilms and mixed-species biofilms.

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Competing interests. None declared.

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