Middlesex University London

Middlesex University Research Repository

An open access repository of

Middlesex University research

http://eprints.mdx.ac.uk

Islam, Mohammad, Durie, Ian, Ramadan, Reham, Purchase, Diane ORCID logoORCID: https://orcid.org/0000-0001-8071-4385 and Marvasi, Massimiliano ORCID logoORCID: https://orcid.org/0000-0001-8525-7232 (2020) Exploitation of nitric oxide donors to control bacterial adhesion on readytoeat vegetables and dispersal of pathogenic biofilm from polypropylene. Journal of the Science of Food and Agriculture, 100 (7) . pp. 3078-3086. ISSN 0022-5142 [Article] (doi:10.1002/jsfa.10340)

Final accepted version (with author's formatting)

This version is available at: https://eprints.mdx.ac.uk/29152/

Copyright:

Middlesex University Research Repository makes the University's research available electronically.

Copyright and moral rights to this work are retained by the author and/or other copyright owners unless otherwise stated. The work is supplied on the understanding that any use for commercial gain is strictly forbidden. A copy may be downloaded for personal, non-commercial, research or study without prior permission and without charge.

Works, including theses and research projects, may not be reproduced in any format or medium, or extensive quotations taken from them, or their content changed in any way, without first obtaining permission in writing from the copyright holder(s). They may not be sold or exploited commercially in any format or medium without the prior written permission of the copyright holder(s).

Full bibliographic details must be given when referring to, or quoting from full items including the author's name, the title of the work, publication details where relevant (place, publisher, date), pagination, and for theses or dissertations the awarding institution, the degree type awarded, and the date of the award.

If you believe that any material held in the repository infringes copyright law, please contact the Repository Team at Middlesex University via the following email address:

eprints@mdx.ac.uk

The item will be removed from the repository while any claim is being investigated.

See also repository copyright: re-use policy: http://eprints.mdx.ac.uk/policies.html#copy

Exploitation of nitric oxide donors to control bacterial adhesion on ready-to-eat vegetables and dispersal of pathogenic biofilm from polypropylene.

Mohammad Islam [1], Ian Durie [2], Reham Ramadan [1], Diane Purchase [1], Massimiliano Marvasi [3]*

[1] Middlesex University London, Department of Natural Sciences, London, UK.

[2] University of Florida, Gainesville, Soil and Water Department, FL, USA.

[3] University of Florence, Department of Biology, Florence, Italy.

*Corresponding author: Massimiliano Marvasi, University of Florence, Department of Biology. Via Madonna del Piano 6, 50019, Sesto Fiorentino, Florence, Italy.

Email: massimiliano.marvasi@unifi.it

ABSTRACT

Background. Nitric oxide (NO) donors have been used to control biofilm formation. NO can be delivered *in situ* using organic carriers and act as a signaling molecule. Cells exposed to NO shift from biofilm to the planktonic state and are better exposed to the action of disinfectants. In this study, we investigate the capability of the NO donors molsidomine, MAHAMA NONOate, NO-aspirin and diethylamine NONOate to act as anti-adhesion agents on ready-to-eat vegetables, as well as dispersants to a number of pathogenic biofilms on plastic.

Results. Our results showed that 10pM molsidomine reduced the attachment of *Salmonella enterica* sv Typhimurium 14028 to pea shoots and coriander leaves of about 0.5 Log(CFU/leaf) when compared with untreated control. The association of 10 pM molsidomine with 0.006% H_2O_2 showed a synergistic effect, obtaining a significant reduction in cell collection on the surface of the vegetable of about 1 Log(CFU/leaf). Similar results were obtained for MAHMA NONOate.

We also showed that the association of diethylamine NONOate at 10mM and 10pM with the quaternary ammonium compound diquat bromide improves the effectiveness of biofilm dispersal by 50% when compared with the donor alone.

Conclusions. Our findings reveal the dual role of NO compounds in biofilm control. Molsidomine, MAHMA NONOate and diethylamine NONOate are good candidates in either preventing biofilm formation or dispersing biofilm, especially when used in conjunction with disinfectants. NO compounds have the potential to be developed into tool-kit for pro-active practices for GAPs, HACCP and Cleaning-in-place (CIP) protocols in industrial settings where washing is routinely applied.

Keywords: antimicrobial, food safety, ready-to-eat vegetables, nitric oxide donors, hydrogen peroxide, quaternary ammonium.

INTRODUCTION

Disinfection of surfaces in agriculture and food industry is pivotal to prevent outbreaks of human pathogens. As the main risk arises from uncontrolled biofilm formation, mechanical and chemical removal of biofilms are the main strategies adopted by agricultural and food industries [1,2]. The use of the signaling molecule nitric oxide (NO) has been proposed as dispersal agent of preformed biofilm. This sensing system is quite effective as demonstrated in pioneering studies by Barraud and collaborators (2006) and further studies by others [3-5]. NO acts as a signal molecule rather than a disinfectant stimulates the detachment from a surface via the cyclic diguanosine monophosphate (c-di-GMP) signal [6-10]. A feed-forward loop between response regulators with phosphodiesterase domains and phosphorylation-mediated activation regulates c-di-GMP levels that leads ultimately to biofilm dispersal [8,9,11]. Despite the toxicity of gaseous NO, it can be released safely *in situ* by using specific organic donor molecules: this practice has been regularly used in medical applications [12].

Nitric oxide is a signaling molecule and it has an excellent potential to avert cell adhesion and control biofilm formation. Therefore, we were interested in the effectiveness of selected NO donors in preventing biofouling with focus on ready-to-eat vegetables.

The risk of outbreaks in the produce industry due to bacterial adhesion to vegetables is well-known [13]. Recent research has shown that NO donors can be used to prevent adhesion to abiotic surfaces; e.g. S-nitroso-N-acetylpenicillamine incorporated hydrophobic polymer (silicone rubber), this polymer can reduce bacterial adhesion and could be used as a highly efficient antifouling agent for biomedical applications [14,15]. In this study we investigated a number of NO compounds, including: N-(Ethoxycarbonyl)-3-(4-morpholino)sydnone imine (molsidomine), 6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-hexanamine (MAHAMA NONOate), 2-(acetyloxy)ben-zoic acid 4-(nitroxymethyl) phenyl ester (NO-aspirin) and 1,1-Diethyl-2-hydroxy-2-nitroso-hydrazine sodium

(diethylamine NONOate), in preventing adhesion of *Salmonella enterica* sv Typhimurium strain 14028 to leaf tissues in combination with 0.006% H_2O_2 or by using the NO donors alone. To our knowledge, this is the first study aiming to measure the capability of NO donors to prevent adhesion directly on food surfaces such as pea shoots or microgreens. In addition to prevent adhesion, we were also interested in dispersal: cells in planktonic state can be easily killed in association with disinfectants. Dispersal capability of others NO donors has been recently established: examples of donors previously examined include molsidomine, MAHAMA NONOate, NO-aspirin and diethylamine NONOate sodium showing a synergistic effect with diquat (at 4ºC), peracetic acid, hydrogel composed of cellulose nanocrystals (CNC) [3-5,16-18].The ability of these donors to disperse different preformed biofilms have been demonstrated using *Pseudomonas spp.*, *Salmonella enterica*, *Listeria spp.* [3-5,10,11,16-18].

Despite this knowledge, the development of this new technology is still in its infancy, in particular the applications of molecules commonly used in industry, such as the quaternary ammonium compounds. For instance, diquat dibromide is routinely used for desiccation of seed crops and it has antibacterial properties [19,20].

The objective of this study is to address this knowledge gap by conducting a systematic analysis of a number of selected NO donors in association of diquat dibromide, peracetic acid and Pheno-Tek II to disperse preformed biofilms on polypropylene, showing the potentiality of this system.

We propose the use of NO donors as a dual effector, both able to prevent biofilm formation and to disperse preformed biofilm. In this paper we discuss and speculate implementation of this technology in a tool-kit for food safety practices such as good agricultural practices (GAPs), HACCP and Cleaning-in-place (CIP) protocols in industrial settings where washing is routinely carried out.

MATERIALS AND METHODS

Bacterial strains. Biofilm dispersal was studied by using a cocktail of strains isolated from produce outbreaks. The strains are in a collection at the University of Florida and further information are available in references 21 and 22. The following strains were used: pathogenic *E. coli* O157:H7 LJH0537, *E. coli* O157:H7 LJH1186, *E. coli* O157:H7 LJH643, *E. coli* O145 RM12333 [21]; *Salmonella enterica*, sv. Braenderup 04E01347, Braenderup 04E01556, Braenderup 04E00783, sv. Montevideo LJH519, sv. Javiana ATCC BAA-1593 and sv. Newport C6.3 [22]; *Listeria innocua* ATCC33090; the plant pathogen *Pectobacterium carotovorum* SR38. *S. enterica* sv Typhimurium ATCC14028 was used to test cell adhesion to plant tissue.

Nitric oxide donors. Nitric oxide donors were selected taking in account low toxicity (0.1 % of probable, possible or confirmed human carcinogenicity according to the International Agency for Research on Cancer), low/moderate cost and commercial availability. Nitric oxide donors used in this study were: NO-aspirin, MAHMA NONOate, molsidomine, and diethylamine NONOate sodium salt hydrate (abbreviated as diethylamine NONOate) (all from Sigma-Aldrich, St. Louis, MO, USA).

As stock solution, each donor was prepared at a concentration of 1 mM in sterile 1X phosphate buffered saline (PBS) (9.8 g/L, pH 7.3) (Fisher, Waltham, MA, USA) (PBS) and small volume aliquots were stored at −80 °C for maximum of 3 weeks and used once after thawing. For the assays, serial dilutions were freshly prepared using PBS just before the experiments and used immediately.

Cells adhesion on plant tissue. After purchase at the local grocery store, ready-to-eat pea shoots or coriander leaves (Sainsbury's, UK) were stored at 4°C for not longer than

48 hours and used immediately once opened. Undamaged leaves of equal size and morphology were chosen for the experiment. Overnight culture of *Salmonella enterica* sv Typhimurium 14028 in Lysogenic broth (LB) (Fisher Scientific, Hampton, NH, USA) (10^9 cells/mL) was washed three times with sterile 1X PBS, 25µL of the washed cells were added to 7mL of 10µM, 10nM, 10pM of each NO donor in 1X PBS and 1X PBS alone as a control [23]. Three leaves per Petri plate were submerged in the 7 mL *Salmonella* suspension with the appropriate NO donor and incubated for 2 hours at 22ºC. Similarly, for the experiments in association with H_2O_2, a final concentration of 0.006% H_2O_2 (Camlab, UK) was added to the solution and incubated for 2 hours at 22^0C. After incubation, the leaves were washed in gently agitation for 15 seconds in three consecutive 50mL sterile 1X PBS washing solutions. After the third wash, leaves were put in a plastic bag for blending (one leaf per bag) with 25mL 1X PBS and blended at 225 rpm for 60 seconds in a stomacher (Fisher Scientific, Hampton, NH, USA). A 30µL suspension was plated onto xylose lysine deoxycholate agar (OXOID, Basingstoke, UK) and incubated overnight at 37ºC. Black colonies were counted and normalized per leaf. Five replicas were done for each experiments.

Recovery of cells on XLD upon exposure to 0.006% H_2O_2 and 10pM molsidomine. We also tested to what extent the selective XLD medium was able to recover bacterial cells upon exposure to H_2O_2. To this end, 1mL of overnight *S.* Typhimurium 14028 cells grown in LB were diluted in sterile physiological solution (0.89 % w/v NaCl) up to a dilution of 10^{-6} . The tubes were split in two, one treated with 0.006% H_2O_2 and 10µM molsidomine, the other was physiological solution alone. After 10 minutes of incubation at 25ºC, 20µL of each suspension were plated in LB and XLD and incubated overnight at 37ºC. After incubation the colonies were counted. Four replicas were done.

Biofilm formation and dispersal on polypropylene. Biofilm formation and dispersal were tested according with previously described protocols, with the following modifications [16]. Briefly, for *Salmonella* and *E. coli*, overnight cultures (10^9 CFU/mL) were grown in colonizing-factor antigen medium (CFA) [24], the culture was then diluted to 1:100 in CFA and 200µL were aliquoted into each well of a polypropylene 96-well plate (Fisher, Waltham, MA, USA). When a cocktail was used, 10^9 cell/mL from each strain culture were mixed in the same proportion and processed as a mono culture in 1:100 CFA. Same protocol as before was used for the other strains. Nutrient broth (Fisher, Waltham, MA, USA) was used for *Pectobacterium carotovorum* SR38 and Brain heart infusion broth (Fisher, Waltham, MA, USA) with 1 % glucose was used for *L. innocua*. Plates with bacteria were incubated in static for 18 h at 37 °C for *Salmonella*, *E. coli*, *L. innocua* and 48 h at 30 °C for *P. carotovorum* SR38. Plates with bacteria were incubated as above inside a Ziploc bag to prevent dehydration. Upon completion of the incubation, the medium with planktonic bacteria was removed by aspiration and 200 µL aliquots of 10µM, 10nM, 10pM of NO donors in 1X PBS were added to the biofilms and incubated for 6 hours at 22ºC. Upon completion of the incubation, the entire volume of each well was aspirated, then 200 µL of selected disinfectants were added to each well according to the following final concentrations: 500 mg/L diquat bromide (Nufarm, Morrisville, NC, USA), or 10% (v/v) peracetic acid (Sigma-Aldrich, St. Louis, MO, USA), or Pheno-Tek II (Bio-Tek, Atlanta, GA, USA) diluted 1:256 in water as per user manual. The 96-well plate was incubated with the disinfectant for 10 min at 22°C. In the controls, where disinfectants were applied alone, the experiments were repeated as mentioned above excluding the addition of NO donors. After this second incubation, the remaining biofilm was measured by staining with 1 % (w/v) crystal violet in ethanol and destained with acetic acid 33 % (v/v), as described previously [25,26]. Two biological and four technical replicates for each experiment were done.

Statistical analysis. The analysis was performed by using JMP (SAS, Buckinghamshire, UK) for Tukey's test, t-test and ANOVA when required. Significance level was $p<0.05$. Prism (GraphPad, La Jolla, USA) was used to edit the graphs.

RESULTS

Nitric oxide donors prevent cell adhesion on leaf tissue

Pea shoots were exposed to 10^5 *Salmonella* cell/mL and selected donors at different concentrations. *Salmonella* adhesion was also tested in combination with 0.006% H_2O_2 to identify any synergistic effect. With reference to MAHMA NONOate, untreated pea shoots (only PBS) showed 5.39±0.14 Log(CFU/leaf) while a significant less recovery was observed using 10pM MAHMA NONOate to 4.97±0.08 Log(CFU/leaf) (Figure 1, A), showing a difference of about 0.4 Log(CFU/leaf). The same treatment in association with H_2O_2 showed a synergistic effect: the treatment with 0.006% H_2O_2 alone resulted in 5.00±0.06 Log(CFU/leaf) while in the association with 10pM MAHMA NONOate were recovered 3.99±0.18 Log(CFU/leaf) (Figure 1, A), showing a reduction of attached cells of about 1 Log(CFU/leaf). Similar results were obtained for molsidomine (Figure 1, B), showing a decrease of about 0.4 Log(CFU/leaf) when the donor alone was applied and up to 1 Log(CFU/leaf) when 0.006% H_2O_2 was added.

NO-aspirin and diethylamine NONOate also showed a significant prevention of truly attached cells on pea shoots. NO-aspirin showed the higher reduction in adhesion for both NO-aspirin alone or in association with H_2O_2 (Figure 1, C). For this donor the recovery was from 5.40±0.10 Log(CFU/leaf) to 5.01±0.07 Log(CFU/leaf) for 10pM NO-aspirin on the treatment with NO-aspiring only (Figure 1, C). The association of 10pM NO-aspirin with 0.006% H_2O_2 showed a decrease in attachment from 4.99±0.10 Log(CFU/leaf) to

4.10±0.16 Log(CFU/leaf) (Figure 1, C). Similar results were obtained for diethylamine NONOate, with a reduction of 0.4 Log(CFU/leaf) with 10pM alone (Figure 1, D) and a reduction of about 1 Log(CFU/leaf) when 10pM concentration was associated with 0.006% H_2O_2 (Figure 1, D). To confirm this effectiveness on another leafy produce, experiments were repeated at the most effective concentrations (10pM) on coriander leaf (Figure 2). Initially, we tested the truly attached cells when treated with 0.006% H_2O_2, showing a reduction of attachment from 5.42±0.09 Log(CFU/leaf) to 4.81±0.18 Log(CFU/leaf) (Figure 2, A). When donors were tested, 10pM concentrations associated with 0.006% H_2O_2 showed a further reduction on average of about 0.6 Log(CFU/leaf) (Figure 2, B).

As XLD is a selective medium, it could underestimate the recovery of *Salmonella* cells treated with H_2O_2 as the bacteria might be struggling to grow in XLD. However, we found that XLD showed similar cell recovery compared to those grown on LB agar medium (Supplementary materials, Figure S1). Therefore, these results show that NO donors can be used as agents able to control biofouling, limiting cellular adhesion.

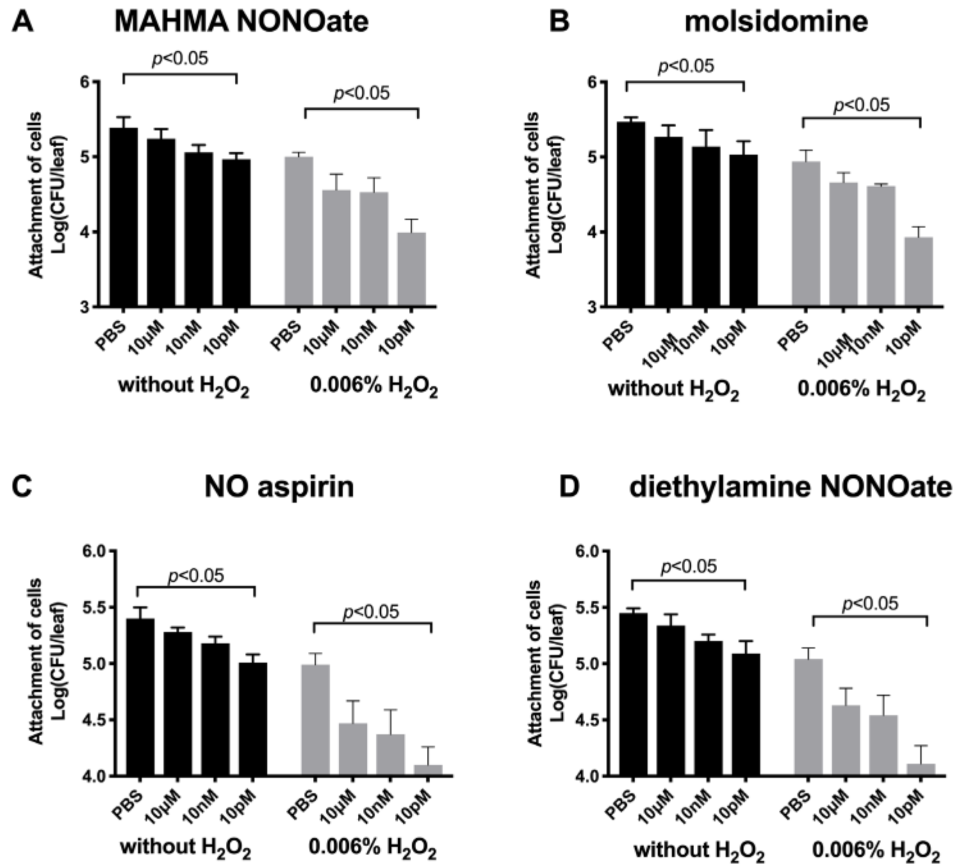

Figure 1. Absolute number of attached cells of *S.* Typhimurium 14028 on pea shoots treated with MAHMA NONOate and molsidomine. Panel A) Pea shoots treated with different concentrations of MAHMA NONOate and 0.006% H_2O_2. Panel B) Pea shoots treated with different concentrations of molsidomine and 0.006% H_2O_2. Panel C) Pea shoots treated with different concentrations of NO aspirin and 0.006% H_2O_2. Panel D) Pea shoots treated with different concentrations of diethylamine NONOate and 0.006% H2O2. The horizontal lines indicate two significant different means. Error bars represent standard error.

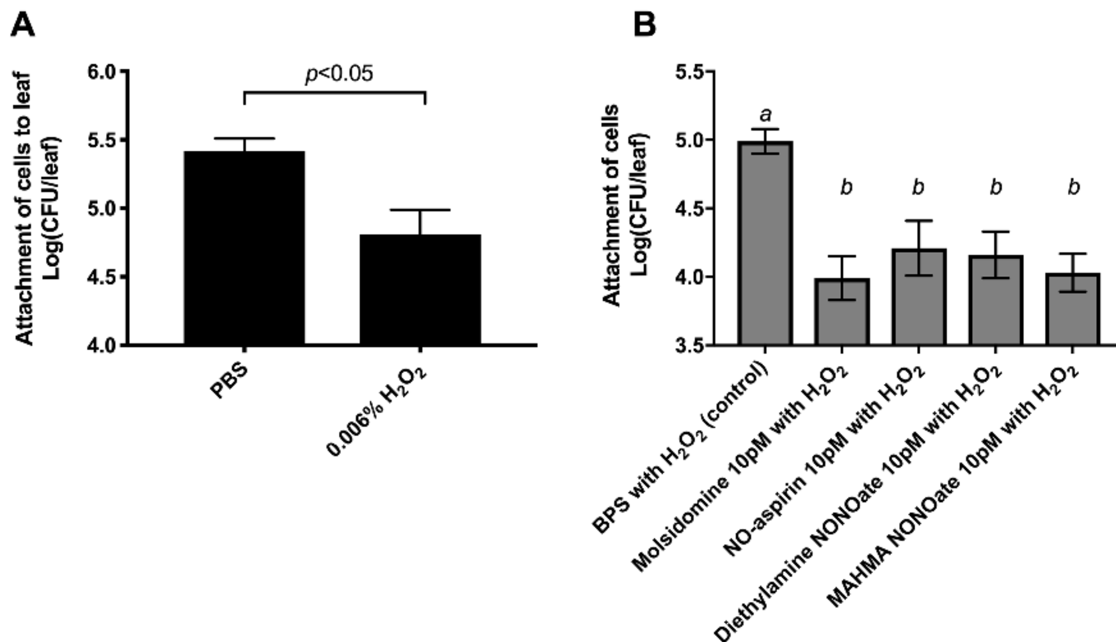

Figure 2. Absolute number of attached cells of *S.* Typhimurium 14028 on coriander leaves treated with selected NO donors. Panel A) Reduction of *S. enterica* cell adhesion on coriander leaves upon exposure to 0.006% H_2O_2. The horizontal lines indicate two significant different means. Panel B) Reduction of cell adhesion on coriander leaves treated with molsidomine, MAHAMA nonoate, NO-aspirin and diethylamine NONOate in association with 0.006% H_2O_2. Different letters represent different means. Error bars represent standard error.

Association of selected nitric oxide donors with diquat bromide improves effectiveness of biofilm dispersal at low concentration of the donor

Preliminary studies showed a general capability of the selected donors molsidomine, MAHAMA nonoate, NO-aspirin and diethylamine NONOate to significantly disperse biofilm on both polystyrene and polypropylene at pico-molar concentrations [16-18].

To advance this knowledge, the association of the same selected donors with the disinfectant diquat bromide was examined in-depth. Preformed biofilms of cocktails of

pathogenic *E. coli* and *S. enterica* and single-strain biofilm of *L. innocua*, and *Pectobacterium* on polypropylene were tested in combination of MAHMA NONOate and molsidomine (Figure 3). The experiments showed that diquat+PBS was effective inducing a significant dispersal of all preformed biofilms, as expected by a quaternary ammonium compound (Figure 3, panels A to H). When molsidomine and MAHMA NONOate were tested, only one significant combination was observed: the combination of diquat with 10nM of molsidomine on preformed *L. innocua* strain (Figure 3, panel G) was significant. When NO-aspirin was tested in association with diquat bromide, the dispersal of preformed *S. enterica* cocktail was significant at a concentration of 10pM (Figure 4, A). Diethylamine NONOate was the only NO donor to show effective synergistic dispersal on multiple biofilms. Diethylamine NONOate was significantly effective with *S. enterica* cocktail at a concentration of 10nM (Figure 4, E) and on pathogenic *E. coli* cocktail at concentrations of 10nM and 10pM (Figure 4, F). On preformed biofilm of *L. innocua* the lowest concentrations required to achieve significant reduction were 10μM and 10nM (Figure 4, G). None of the NO donors were effective in dispersing the preformed biofilms of *Pectobacterium*.

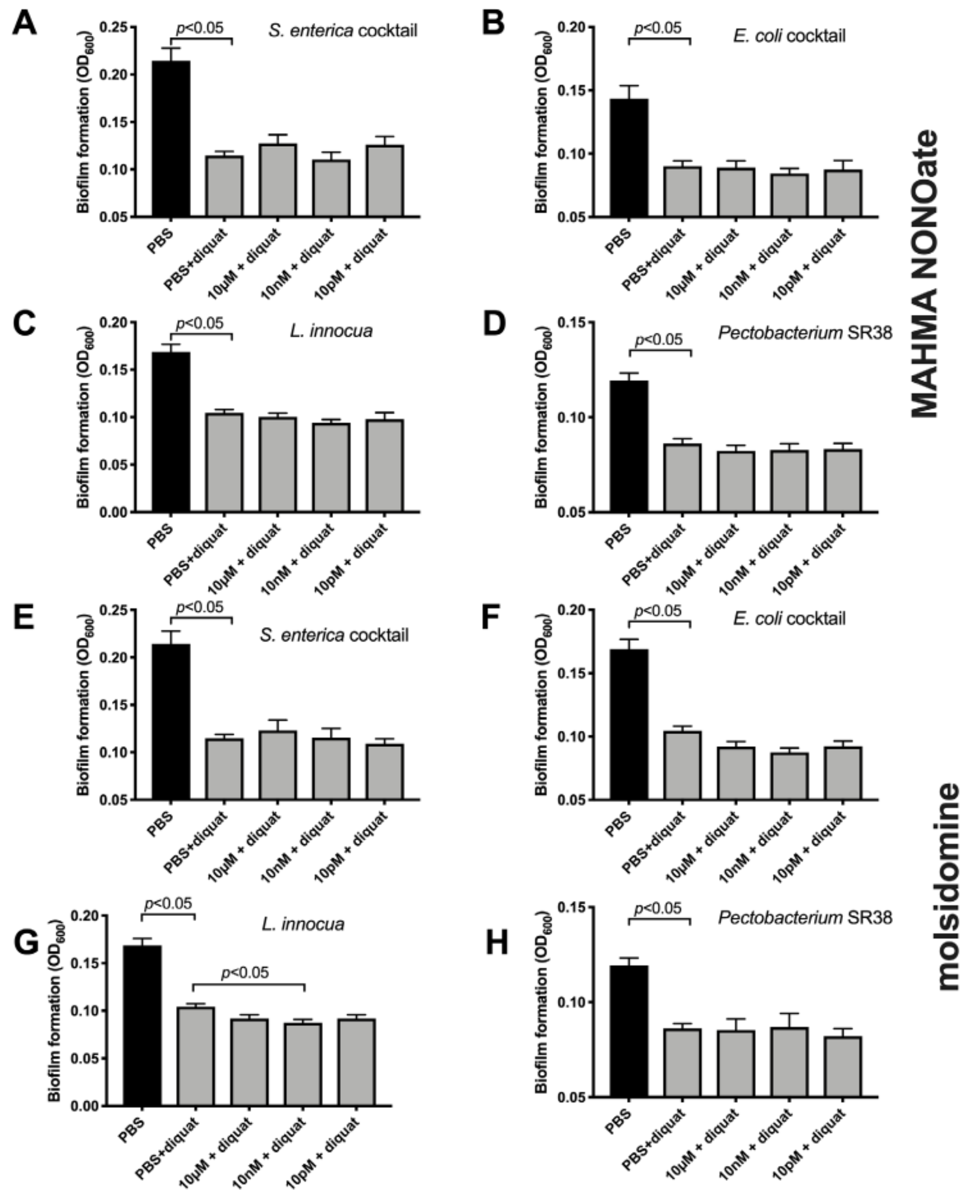

Figure 3. Dispersal of biofilms by MAHMA NONOate and molsidomine on polypropylene in association with diquat bromide. Panel A to D) Treatments with different concentrations of MAHMA NONOate on different preformed biofilms, highlighted on each panel. Panel E to H) Treatments with different concentrations of molsidomine on different preformed biofilms, strains are highlighted on each panel. The horizontal lines indicate two significant different means. Error bars represent standard error.

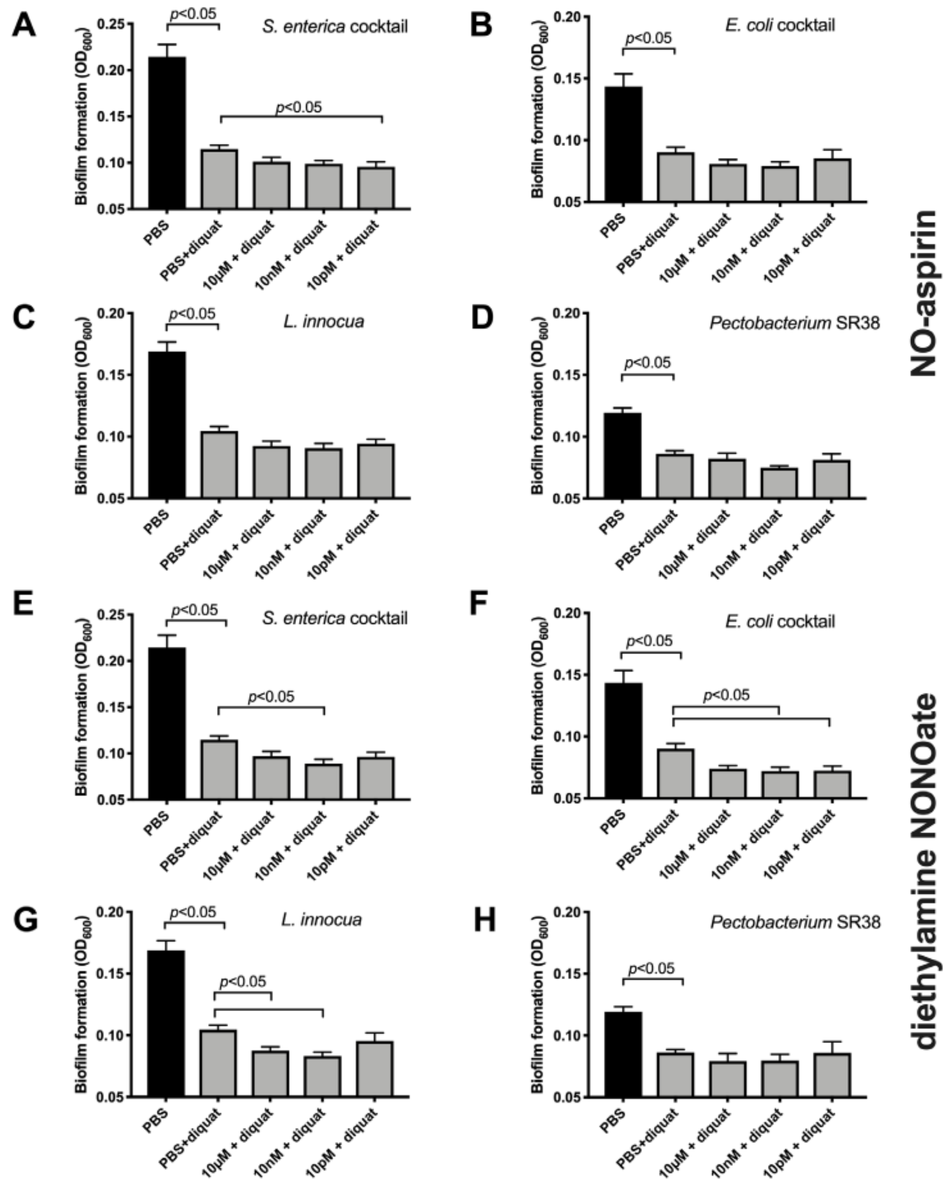

Figure 4. Dispersal of biofilms by NO-aspirin and diethylamine NONOate sodium salt hydrate on polypropylene in association with diquat bromide. Panel A to D) Treatments with different concentrations of NO-aspirin on different preformed biofilms, strains are highlighted on each panel. Panel E to H) Treatments with different concentrations of diethylamine NONOate on different preformed biofilms, highlighted on each panel. The horizontal lines indicate two significant different means. Error bars represent standard error.

To test potential toxicity of the donors, *Salmonella* cells were exposed to the four NO donors for 6 hours showing no significant decrease of its viability. Molsidomine had not effect on its ability to generally disrupt metabolism or respiration of *Salmonella* [17].

The association of molsidomine and diethylamine NONOate with diquat have shown the most significant results among the entire screening. Therefore the other two disinfectants, the peracetic acid and Pheno-Tek II, were tested with these two NO donors (Figure 5 and 6). Both disinfectants were very effective showing significant dispersal on preformed biofilms of cocktails of *Salmonella*, *E. coli* and *Listeria* within 10 minutes of application, but there was no significant synergistic effect when they were applied together with the NO donors. Peracetic acid and Pheno-Tek II can therefore be used to significantly reduce preformed biofilm produced by human pathogens on polypropylene without the addition of NO donors.

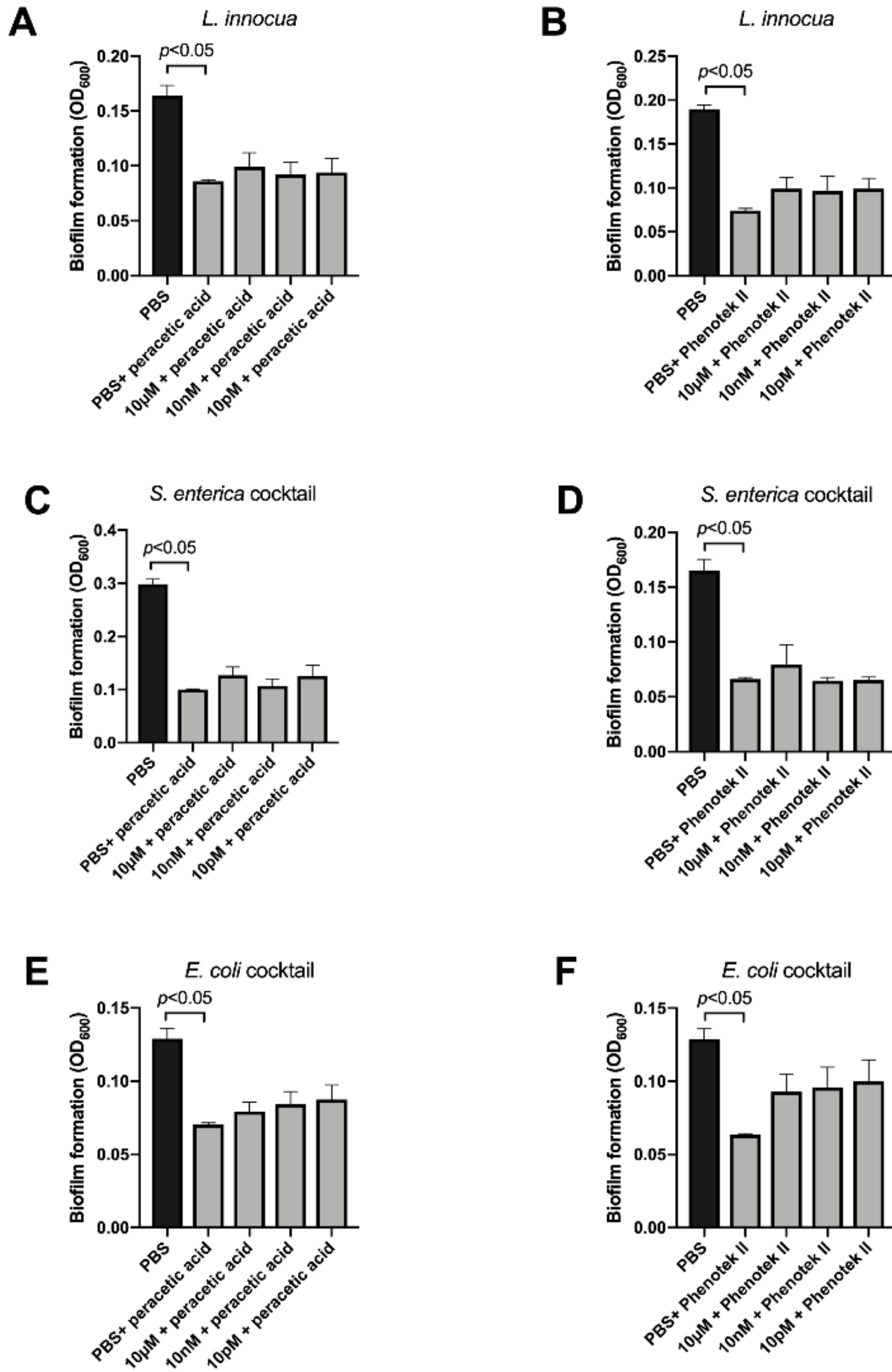

Figure 5. Dispersal of biofilms by molsidomine on polypropylene in association with peracetic acid and Pheno-Tek II. Treatments with different concentrations of molsidomine on different preformed biofilms. The horizontal lines indicate two significant different means. Error bars represent standard error.

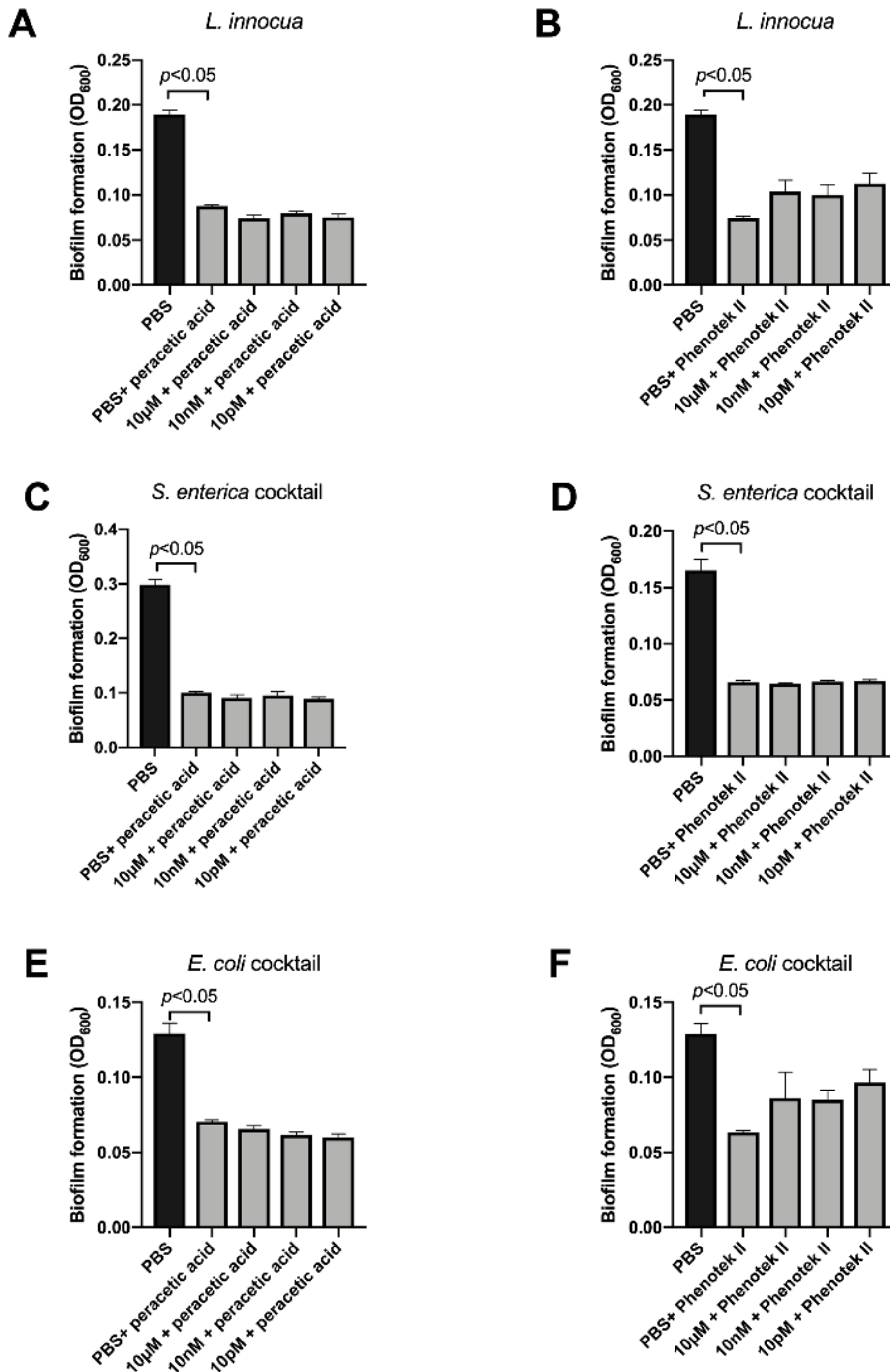

Figure 6. Dispersal of diethylamine NONOate sodium salt hydrate on polypropylene in association with peracetic acid and Pheno-Tek II. Treatments with different concentrations of molsidomine on different preformed biofilms. The horizontal lines indicate two significant different means. Error bars represent standard error.

DISCUSSION

In this study we propose a comprehensive application of nitric oxide donors in association with disinfectants to control biofouling and to disperse preformed biofilm. We present encouraging results for *in vitro* applications to support further development in real industrial settings. To our knowledge, this is the first report showing the capability of NO donors to prevent cellular adhesion on ready-to-eat leaves. We therefore can compare only similar treatment performed on materials for medical devices, such as plastics and rubber. However, this could be of interest when speculating for construction of plastic boxes for food storage. For example, NO-releasing coatings on (poly(ethylene terephthalate) (PET) and silicone elastomer (SE) has shown to significantly reduce *Pseudomonas aeruginosa* adhesion over 24 h with a reduction measured to be 1 Log (CFU/sample) [27]. Superhydrophobic nitric oxide (NO)-releasing xerogels supported a reduction in viable *P. aeruginosa* adhesion by >2 Log (CFU/sample) [28]. Cell adhesion has also been prevented by coating medical-grade stainless steel with sol-gel film of 40% N-aminohexyl-N-aminopropyltrimethoxysilane and 60% isobutyl-trimethoxysilane showing significant less adhesion of *P. aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* at 25ºC and 37ºC [29]. NO-aspirin at a concentration of 125 μM also significantly inhibited adhesion to polystyrene of *Candida albicans* preformed biofilms [30]. For obvious reasons coating cannot be used with food, nevertheless as previously mentioned, food containers, storage boxes can be produced with this technology, preventing adhesion and facilitate disinfection. The results reported in these papers showed higher reduction of adhesion ranging 1 to 2 Log (CFU/sample) on coating materials [27,28]. In our study the reduction is limited to 0.5 Log(CFU/sample) when the NO donors were used alone (Figure 1), however it has to be considered that the leafy produce has uneven or irregular surface, including sites where cells can easily hide (such as stomata, trichomes), while plastic and stainless steel are smooth or levelled. Interestingly, when 0.006% H_20_2 was added the adhesion

decreased up to 1 Log (CFU/sample) (Figures 1 to 2). We speculate that these donors could be used for washing produce in association with low dose disinfectants, allowing the planktonic cell to be more exposed to the diluted disinfectant. The application of this synergy will require a lower exposure to disinfectants for consumers and workers.

With reference to the biofilm dispersal, a number of papers are available for further comparisons and for an effective strategy development to advance this technology to a further level. It has to be noted that all the NO donor proposed here (molsidomine, MAHAMA NONOate, NO-aspirin and diethylamine NONOate) have been tested as biofilm dispersal in certain conditions and have shown good potential. Molsidomine is effective to disperse preformed biofilms of *Escherichia coli* and *Salmonella enterica.* Molsidomine induced dispersal of up to 50% when compared with the not treated polystyrene and polypropylene [17]. MAHMA NONOate has been tested alone and in association with cellulose nanocrystals showing dispersal correlated with a reduction in both bacterial cells and exopolymeric substances (EPS) [18,31]. NO-aspirin has shown potential activity at 4ºC for use in refrigerated conditions and as antifungal/antibiofilm *in vitro* on *Candida albicans* isolates from denture stomatitis patients [16,30]. Diethylamine NONOate sodium salt hydrate has showed strongest dispersion of *S. enterica* 14028 up to 50% of biofilm reduction [17]. It is clear that all these molecules are effective as dispersants, however when the association with the quaternary ammonium diquat bromide was tested, the synergistic effect was evident for diethylamine NONOate (Figure 4, E to F) with a significant dispersal of the *E. coli* cocktail and *L. innocua* at the lowest dilutions (nM and pM). NO is a signaling molecule therefore it is not a surprise that it is active at low concentration, as demonstrated by recent research [3-5,17]. The release of NO from the organic donor is mediated by a number of factors, including pH, chemical environment, light [32,33]. Therefore, these types of systematic screenings are pivotal for identifying the best combination to achieve an effective strategy. Diethylamine NONOate spontaneously release NO in a pH dependent

manner, which is faster when compared with other donors. Its half-life in 0.1 M phosphate buffer at pH 7.4 is 16 min at 22-25 °C. It liberates 1.5 moles of NO per mole of parent compound, reaching 2 minutes at 37ºC (product specification sheet) [34]. Therefore the quick release typical of diethylamine NONOate seems to be more effective in co-application with diquat. The association diquat and Diethylamine NONOate is particularly interesting to prevent attachment of pathogens and algal biofilm formation, in fact diquat can be associated with copper sources for algae control or possibly stone cleaning [35,36].

Previous research has shown that *Salmonella enterica* preformed biofilms were further reduced by using peracetic acid and Pheno-Tek II when associated with MAHMA NONOate at 4ºC [16]. In this study we did not observe any significant synergistic effect, therefore the interaction of the disinfectants with the NO donors should be further investigated. As mentioned previously, a synergistic effect can be promoted by the penetration in the biofilm layers, which is dependent on the type of biocide used [37]. Recent research showed that peracetic acid allowed a linear loss of cell viability in *Pseudomonas aeruginosa* biofilm [37].

The variability obtained in recent literature while application of NO donors with disinfectants may be attributed to the highly difficult task in predicting the release of nitric oxide, redox processes, showing that further research should be carried out to stabilize and control NO release for industrial purposes. In this context nanotechnology seems to be the new frontier in using NO donors for food safety [38].

Conclusions

In this study we compare for the first time the capability of NO donors to prevent attachment on leafy produce, all tested donors revealed good capabilities to prevent attachment of *Salmonella* in particular in association with low concentrations of H_2O_2. We

also propose the dual effect of donors as biofilm dispersant and to prevent biofouling in association with the disinfectant and herbicide diquat. In addition we showed the capability of diquat, peracetic acid and Pheno-Tek II as biofilm dispersant without the association with NO donors. Further studies of NO donor in agriculture and food safety should be better explored to develop a tool-kit for food safety practices such as GAPs, HACCP and Cleaning-in-place (CIP) protocols in industrial settings where washing is routinely applied [39].

REFERENCES

1. Chmielewski RAN, Frank JF. Biofilm formation and control in food processing facilities. *Comp Rev Food Sci F* **2**:22-32 (2006).
2. Moretro T, Langsrud S. Residential Bacteria on Surfaces in the Food Industry and Their Implications for Food Safety and Quality. *Comp Rev Food Sci Food Safety* **16**(5):1022-1041 (2017).
3. Barraud N, Hassett DJ, Hwang SH, Rice SA, Kjelleberg S, Webb JS. Involvement of nitric oxide in biofilm dispersal of Pseudomonas aeruginosa. *J Bacteriol* **188**:7344-7353 (2006).
4. Charville GW, Hetrick EM, Geer CB, Schoenfisch MH. Reduced bacterial adhesion to fibrinogen-coated substrates via nitric oxide release. Biomaterials **29**:4039-4044 (2008).
5. Barnes RJ, Low JH, Bandi RR, Tay M, Chua F, Aung T, Fane AG, Kjelleberg S, Rice SA. Nitric Oxide Treatment for the Control of Reverse Osmosis Membrane Biofouling. *App Environ Microbiol* **81**(7):2515-2524 (2015).

6. Rinaldo S, Giardina G, Mantoni F, Paone A, Cutruzzola F. Beyond nitrogen metabolism: nitric oxide, cyclic-di-GMP and bacterial biofilms. *FEMS microbiol letters* **365**(6):fny029-fny029 (2018).

7. Ryan RP, Lucey J, O'Donovan K, McCarthy Y, Yang L, Tolker-Nielsen T, Dow JM. HD-GYP domain proteins regulate biofilm formation and virulence in Pseudomonas aeruginosa. *Env microbiol* **11**(5):1126-1136 (2009).

8. Plate L, Marletta MA. 3361614; Nitric oxide modulates bacterial biofilm formation through a multicomponent cyclic-di-GMP signaling network. *Mol Cell* **46**:449-460 (2012).

9. Liu N, Xu Y, Hossain S, Huang N, Coursolle D, Gralnick JA, Boon EM. Nitric oxide regulation of cyclic di-GMP synthesis and hydrolysis in Shewanella woodyi. *Biochemistry* **51**(10):2087-2099 (2012).

10. Barraud N, Schleheck D, Klebensberger J, Webb JS, Hassett DJ, Rice SA, Kjelleberg S. Nitric oxide signaling in Pseudomonas aeruginosa biofilms mediates phosphodiesterase activity, decreased cyclic di-GMP levels, and enhanced dispersal. *J Bacteriol* **191**:7333-7342 (2009).

11. Barraud N, Storey MV, Moore ZP, Webb JS, Rice SA, Kjelleberg S. Nitric oxide-mediated dispersal in single- and multi-species biofilms of clinically and industrially relevant microorganisms. Microb Biotechnol **2**:370-378 (2009).

12. Yang C, Jeong S, Ku S, Lee K, Park MH. Use of gasotransmitters for the controlled release of polymer-based nitric oxide carriers in medical applications. *J Control Rel* **279**:157-170 (2018).

13. Barak Y, Schreiber F, Thorne SH, Contag CH, Debeer D, Matin A. Role of nitric oxide in Salmonella typhimurium-mediated cancer cell killing. *BMC Cancer* **10**:146 (2010).

14. Pant J, Gao J, Goudie MJ, Hopkins SP, Locklin J, Handa H. A multi-defense strategy: Enhancing bactericidal activity of a medical grade polymer with a nitric oxide donor and surface-immobilized quaternary ammonium compound. *Acta Biomaterialia* **58**:421-431 (2017).

15. Singha P, Pant J, Goudie MJ, Workman CD, Handa H. Enhanced antibacterial efficacy of nitric oxide releasing thermoplastic polyurethanes with antifouling hydrophilic topcoats. Biomaterials Science **5**(7):1246-1255 (2017).

16. Marvasi M, Durie IA, Henríquez T, Satkute A, Matuszewska M, Prado RC. Dispersal of human and plant pathogens biofilms via nitric oxide donors at 4 °C. *AMB Express* **6**(1):49 (2016).

17. Marvasi M, Chen C, Carrazana M, Durie I, Teplitski M. Dispersal of biofilms formed by Salmonella enterica and Escherichia coli O157:H7 by Nitric Oxide donors. *AEM Express* **4**:42 (2014).

18. Marvasi M, Durie IA, McLamore ES, Vanegas DC, Chaturvedi P. Salmonella enterica biofilm-mediated dispersal by nitric oxide donors in association with cellulose nanocrystal hydrogels. *AMB Express* **5**:28 (2015).

19. Maris P. Modes of action of disinfectants. *Rev sci tech Off int Epiz* **14**(1):47-55 (1995).

20. Dvorak G. Disinfection 101. Ames, IA, USA: Center for Food Security and Public Health; 2008.

21. Selma MV, Ibáñez AM, Allende A, Cantwell M, Suslow T. Effect of gaseous ozone and hot water on microbial and sensory quality of cantaloupe and potential transference of *Escherichia coli* O157:H7 during cutting. Food Microbiol **25**:162-168 (2008).

22. Noel JT, Arrach N, Alagely A, McClelland M, Teplitski M. Specific responses of *Salmonella enterica* to tomato varieties and fruit ripeness identified by in vivo expression technology. PLoS One **5**:e12406 (2010).

23. Marvasi M, Cox CE, Xu Y, Noel JT, Giovannoni JJ, Teplitski M. Differential regulation of Salmonella Typhimurium genes involved in O-antigen capsule production and their role in persistence within tomato fruit. *Mol Plant Microbe Interact* **26**:793-800 (2013).

24. Teplitski M, Al-Agely A, Ahmer BMM. Contribution of the SirA regulon to biofilm formation in Salmonella enterica serovar Typhimurium. *Microbiol* **152**:3411-3424 (2006).

25. O'Toole GA, Kolter R. Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol* **28**:449-461 (1998).

26. Merritt JH, Kadouri DE, O'Toole GA. Growing and analyzing static biofilms. *Curr Protoc Microbiol*;Chapter 1:Unit 1B 1 (2005).

27. Fleming G, Aveyard J, Fothergill JL, McBride F, Raval R, D'Sa RA. Nitric Oxide Releasing Polymeric Coatings for the Prevention of Biofilm Formation. *Polymers* **9**(11):601-601 (2017).

28. Storm WL, Youn J, Reighard KP, Worley BV, Lodaya HM, Shin JH, Schoenfisch MH. Superhydrophobic nitric oxide-releasing xerogels. *Acta Biomaterialia* **10**(8):3442-3448 (2014).

29. Nablo BJ, Rothrock AR, Schoenfisch MH. Nitric oxide-releasing sol-gels as antibacterial coatings for orthopedic implants. *Biomaterials* **26**(8):917-924 (2005).

30. Madariaga-Venegas F, Fernandez-Soto R, Fernanda Duarte L, Suarez N, Delgadillo D, Jara JA, Fernandez-Ramires R, Urzua B, Molina-Berrios A. Characterization of a novel antibiofilm effect of nitric oxide-releasing aspirin

(NCX-4040) on Candida albicans isolates from denture stomatitis patients. *Plos One* **12**(5):e0176755-e0176755 (2017).

31. Barnes RJ, Bandi RR, Wong WS, Barraud N, McDougald D, Fane A, Kjelleberg S, Rice SA. Optimal dosing regimen of nitric oxide donor compounds for the reduction of Pseudomonas aeruginosa biofilm and isolates from wastewater membranes. *Biofouling* **29**:203-212 (2013).
32. Cai TB, Wang PG, Holder AA. NO and NO Donors. In: Wang PG, Cai TB, Taniguchi N, editors: Wiley-VCH Verlag GmbH & Co. KGaA; 2005. p 1-31.
33. Wang PG, Cai TB, Taniguchi N. Nitric oxide donors. Wiley-VCH Verlag GmbH & Co. KGaA; 2005.
34. Abacam I. DEA NONOate (Diethylamine nonoate) datasheet. Abacam, Inc; 2018.
35. Garlich N, Da Cruz C, Da Silva AF, Carraschi SP, Malaspina IC, Pitelli RA, Bianco S. Diquat associated with copper sources for algae control: Efficacy and ecotoxicology. *J Environ Sci Health Part B*-Pesticides Food Contaminants and Agricultural Wastes **51**:215-221 (2016).
36. Marvasi M, Donnarumma F, Frandi A, Mastromei G, Sterflinger K, Tiano P, Perito B. Black microcolonial fungi as deteriogens of two famous marble statues in Florence, Italy. *Int Biodet Biodeg* **68**:36-44 (2012).
37. Bridier A, Briandet R, Thomas V, Dubois-Brissonnet F. Resistance of bacterial biofilms to disinfectants: a review. Biofouling **27**:1017-32 (2011).
38. Han G, Friedman AJ, Friedman JM. Nitric oxide releasing nanoparticle synthesis and characterization. Methods Mol Biol 2011;704:187-95.
39. Marvasi M. Potential use and perspectives of nitric oxide donors in agriculture. J Sci Food Agric 2017;97(4):1065-1072.