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In situ electrochemical investigation of the interaction between bacteria *Xylella fastidiosa* DNA and copper(II) using DNA-electrochemical biosensors

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ABSTRACT

New emerging bacterial plant diseases are spreading fast in different geographical regions, and effective safety measures have not been found. *Xylella fastidiosa* (*Xf*) is a Gram-negative bacterial plant pathogen classified as a priority quarantine pest, which affects economically important agricultural crops. Although there is no treatment for diseases caused by *Xf*, copper-based compounds are widely applied to its hosts.

A *Xf*-DNA-electrochemical biosensor based on bacterial DNA from *Xf,* is reported here for the first time, and the Cu(II)-*Xf*-dsDNA interaction was investigated in situ. The Cu(II)-DNA interaction was also evaluated using ct-dsDNA-, poly[G]- and poly[A]-electrochemical biosensors. The Cu(II)-*Xf*-dsDNA interaction occurs by Cu(II) binding at different sites, independent of the bacterial DNA sequence, leading to the condensation/aggregation of *Xf*-DNA strands due to the formation of a rigid Cu(II)-*Xf*-dsDNA complex structure. Cu(II) did not cause oxidative DNA damage. Resistance of the bacterial *Xf*-dsDNA to Cu(II) was observed.

1. Introduction

Bacteria are biological cells found in most habitats, which play important roles in many areas, such as agriculture, but which can also cause problems. Bacterial diseases can affect various types of agricultural crops [1].

At the end of last year, the European Commission published a list of 20 regulated quarantine pests that qualify as priority pests, whose economic, environmental, and social impact on the EU's territory is the most severe [2]. Among them is the pathogenic bacterium *Xylella fastidiosa* (*Xf*), a Gram-negative of the *Gammaproteobacteria* class in the *Xanthomonadaceae* family, which colonizes the large xylem vessels of plants, causing a variety of diseases in over 100 species [3]. The infection occurs via vectors, such as leafhoppers, forming biofilms that block the transport of water and nutrients from the roots to the leaves [3,4] and causing leaf scorch, dwarfing, matchsticking and chlorotic spots [3].

First discovered in association with Pierce's disease in the USA (1973) [3], *Xf* was only detected in Europe many years later in Italy (2013) [5]. Since then, many outbreaks have been observed in different crops in France (2015) [6], Spain (2016) [7], and Germany (2016) [8]. In early 2019, *Xf* was detected for the first time in Portugal, and after one year, 60 zones were infected, where more than 3000 samples were collected and more than 11,000 plants of the different host species had already been destroyed [9].

Unfortunately, there is no treatment for diseases caused by *Xf*, as it colonizes the internal niche (xylem), and normal methods for applying antibacterial treatments, such as foliar sprays, cannot easily reach the pathogen. Therefore, the main goal is to prevent the spread of this bacterium.

Copper-based compounds have been widely used as antimicrobials in plant disease control [10]. The copper is toxic to bacteria, causing the disruption of essential iron-sulphur cluster proteins [11], and the production of reactive oxygen species that lead to lipid and protein damage that can trigger plant defence responses [12]. Although Cu(II)-based antimicrobials are not generally used directly in the management of diseases caused by *Xf*, these compounds are widely applied to its hosts.

The copper accumulation in the soil, resulting from thousands of tons being sprayed on the fields year after year, is relatively high, which leads to exposure of the bacterium to high copper concentrations taken up from the soil by the host plant roots and distributed via the xylem. A problem related to this is that the bacteria could, theoretically, acquire copper resistance from this copper-rich environment [13].

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A very interesting approach to improving understanding of the Cu (II)-*Xf* interaction, and consequently Cu(II) resistance, is to investigate the molecular interactions between Cu(II) and *Xf* double-strand DNA (*Xf*-dsDNA), which may occur inside the bacteria.

DNA-electrochemical biosensors have been successfully used to detect small perturbations of the double-helical structure and oxidative DNA damage, allowing the unravelling of detailed mechanistic interactions in situ and in real time [14].

The use of electrochemical methods in the investigation of hazardous compounds-DNA interaction is very relevant, since electrochemical techniques have a fast response, high sensitivity and use only a small amount of sample. In addition, electrochemical methods have a better signal-to-noise ratio than steady-state techniques and, in most cases, also have greater selectivity.

The hazardous compound-DNA interaction can be electrochemically investigated in incubated solutions or by using a DNA-electrochemical biosensor. The development of DNA-electrochemical biosensors involves the immobilization of DNA at an electrode surface. The immobilisation methodology is based on the controlled *bottom-up* self-assembly of nucleic acid nanostructures [14].

In this context, the purpose of this paper is to develop a *Xf*-dsDNA-electrochemical biosensor and investigate the interaction between *Xf*-dsDNA and Cu(II) using this DNA-electrochemical biosensor. The interactions of Cu(II) with calf thymus dsDNA, polyguanosine, and polyadenosine were also investigated using electrochemical biosensors in order to better understand the dsDNA-Cu(II) interaction.

2. Experimental procedures

2.1. Materials and reagents

Copper(II) acetate monohydrate (229601, purity 99.99%), double-stranded deoxyribonucleic acid sodium salt from calf thymus (ct-dsDNA, 10,000–15,000 bp) (D1501), polyguanylic acid potassium salt (poly[G]) (P4404) and polyadenylic acid potassium salt (poly[A]) (P9403), potassium hexacyanoferrate(II) ($K_4[Fe(CN)_6]$) (P4066, purity 99.98%) and potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$) (P8131, purity ~99%) were obtained from Sigma-Aldrich (Spain).

Stock solutions of 10.0 mM Cu(II), 300 μg mL^{-1} dsDNA, poly[G] and poly[A], and 10.0 mM $K_4[Fe(CN)_6]$ and $K_3[Fe(CN)_6]$ were prepared in deionised water and diluted to the desired concentrations prior to use. In all experiments, a 0.1 M acetate buffer (pH 4.5) prepared with analytical grade reagents and deionised water (Millipore Milli-Q Ultrapure Water system) was used [15].

The pH measurements were carried out with a Crison 2001 micropH-meter with an Ingold combined glass electrode (Crison Instruments, Barcelona, Spain). Microvolumes were dispensed by a Pipetman® single chancel electronic pipette (Gilson Co, Inc., France).

All experiments were performed at room temperature (25 ± 1 °C).

2.2. Bacterial culture and DNA extraction

The bacterial culture of *Xf* subsp. *multiplex* and its dsDNA (approximately 2700 bp) extraction procedure, performed by the Centre International de Resources Microbiennes of the Institut National de la Recherche Agronomique (France), is described in the Supplementary Material.

2.3. Electrochemical procedures

Voltammetric experiments were performed using a μAutolab running GPES 4.9 software (Metrohm/Autolab, The Netherlands). A three-electrode system was used, with a glassy carbon electrode (GCE) ($d = 1.0$ mm), Pt counter wire, and Ag/AgCl (3 M KCl) reference, in a one-compartment 2 mL electrochemical cell (eDAQ, Europe). The GCE surface was cleaned and pre-treated as described in previous reports [16,17]. The differential pulse (DPV) conditions were pulse amplitude 50 mV, pulse width 100 ms, step potential 2 mV and scan rate 5 mV s^{-1}.

Electrochemical impedance spectroscopy (EIS) experiments were performed using an IVIUM potentiostat running IviumSoft 2.219 software (Ivium Technologies, Eindhoven, The Netherlands). A sinusoidal perturbation of amplitude 10 mV rms was applied in the frequency range 60 kHz to 0.1 Hz with 7 frequency steps per decade. The EIS measurements were obtained from CV experiments in 0.1 M acetate buffer (pH 4.5) containing 5.0 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$, at the hexacyanoferrate(III)/(II) midpoint potential, $E_{ap} = +0.25$ V, applied to the GCE.

UV–vis spectrophotometry unfortunately did not provide a significant signal, bearing in mind the very low bacterial DNA concentration (820 ng mL^{-1}) used and the UV–vis detection limits.

2.4. Preparation and incubation of the Xf-dsDNA-, ct-dsDNA, poly[G]- and poly[A]-electrochemical biosensors

The *Xf*-dsDNA-electrochemical biosensors were prepared by depositing 5.0 μL of 820 ng mL^{-1} *Xf*-dsDNA solution on the GCE. The biosensor surface was dried under a constant flow of $N_{2(g)}$.

The ct-dsDNA-electrochemical biosensor was prepared by successively covering the GCE with three drops, each of 5.0 μL, of 50 μg mL^{-1} ct-dsDNA in 0.1 M acetate buffer (pH 4.5), and drying the GCE surface under $N_{2(g)}$ after each drop. A similar procedure was used for the poly [G]- and poly[A]-electrochemical biosensors [16,17].

The DNA-electrochemical biosensors were incubated for different periods of time in different concentrations of Cu(II) solution: the *Xf*-dsDNA-electrochemical biosensor was incubated in 1.0 mM Cu(II), and the ct-dsDNA-, poly[G]- and poly[A]-electrochemical biosensors were incubated in 25.0 μM Cu(II), Fig. 1. Control experiments were also performed for all of the DNA-electrochemical biosensors, (Supplementary Material Fig. 1S), which were incubated in 0.1 M acetate buffer (pH 4.5) solutions without Cu(II) for the same periods of time.

3. Results and discussion

3.1. Voltammetric behaviour of Xf-dsDNA, ct-dsDNA, poly[G] and poly [A]

Although the bacterial DNA genomic sequence of *Xf* is known [18,19], there are no reports of its electrochemical behaviour. The direct electrochemistry of *Xf*-dsDNA is investigated here for the first time, using a *Xf*-dsDNA-electrochemical biosensor. DPVs for the ct-dsDNA-, poly[G]- and poly[A]-electrochemical biosensors were also obtained under the same experimental conditions, Fig. 1 (▬) and Supplementary Material Fig. 1S.

The DPV for the *Xf*-dsDNA-electrochemical biosensor showed two oxidation peaks corresponding to the DNA nucleosides, desoxyguanosine (dGuo), at $E_{pa} = +1.06$ V, and desoxyadenosine (dAdo), at $E_{pa} = +1.29$ V. The results are similar to those obtained for the ct-dsDNA-electrochemical biosensor, dGuo, at $E_{pa} = +0.99$ V, and dAdo, at $E_{pa} = +1.26$ V.

The small current from the dGuo and dAdo peaks for both dsDNA-electrochemical biosensors can be explained by the greater difficulty of electron transfer from the purine bases within the DNA double strand to the GCE surface [14].

The DPV of poly[G]- and poly[A]-electrochemical biosensors showed, as expected, only one oxidation peak each. The poly[G] homopolynucleotide contains only guanine (Gua) residues and the oxidation occurred at the dGuo residue, at $E_{pa} = +0.98$ V, while poly[A] homopolynucleotide contains only adenine (Ade) residues and the oxidation occurred at the dAdo residues, at $E_{pa} = +1.25$ V.

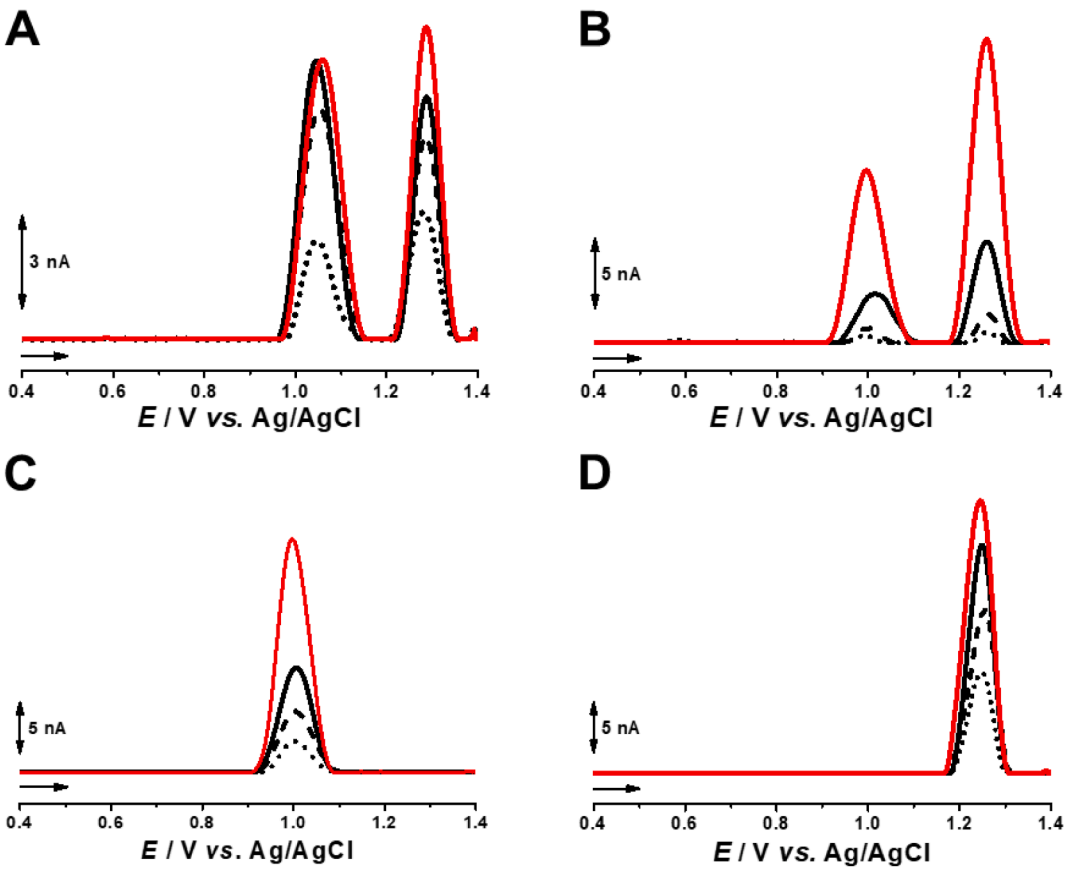

Fig. 1. Baseline-corrected DPV in 0.1 M acetate buffer pH 4.5: (▬) control, (A) *Xf*-dsDNA- and (B) ct-dsDNA-, (C) poly[G]- and (D) poly[A]-electrochemical biosensors; and in 0.1 M acetate buffer pH 4.5 after incubation with Cu(II) for different time periods, 30 (▪), 60 (– – –) and 120 (●●●) minutes: (A) *Xf*-dsDNA-electrochemical biosensor incubated in 1.0 mM Cu(II), (B) ct-dsDNA-, (C) poly[G]- and (D) poly[A]-electrochemical biosensors incubated in 25.0 μM Cu (II), $v = 5\ \text{mV s}^{-1}$.

3.2. *Cu(II)-dsDNA interaction using Xf-dsDNA- and ct-dsDNA-electrochemical biosensors*

3.2.1. *Differential pulse voltammetry*

dsDNA-electrochemical biosensors make it possible to study the Cu (II)-dsDNA interaction in situ and in real time, following the changes in the dsDNA, the appearance of free purine bases, and oxidative DNA damage biomarkers, oxidation peaks.

The DPV for the *Xf*-dsDNA-electrochemical biosensor after 30 min incubation in 1.0 mM Cu(II) ions, Fig. 1A, showed no significant changes in the dGuo oxidation peak current, at $E_{pa} = +1.06$ V, while a decrease in the dAdo oxidation peak, at $E_{pa} = +1.29$ V, was observed. New *Xf*-dsDNA-electrochemical biosensors were incubated in 1.0 mM Cu(II) for 60 and 120 min, and both *Xf*-dsDNA oxidation peaks continue to decrease, showing a greater and more effective interaction between Cu (II) and the bacterial DNA. A conformational change in the *Xf*-dsDNA structure occurred, which is related to Cu(II) binding to *Xf*-dsDNA.

However, due to base pairing of nucleic acid residues, a limited number of binding sites are available on the DNA double helix [20]. The interaction of metals with phosphate groups will stabilize the DNA structure, while metals binding to the nucleotide bases destabilize the double helix.

The biochemical nature of Cu(II)-dsDNA binding sites is related to properties of the metal and is still unknown. Proton NMR experiments suggested that Cu(II) binds to the phosphate groups in the DNA, the data from melting temperature and viscosity suggested that Cu(II) binds to DNA bases, the absorption spectra indicated that the metal binds to DNA pyrimidine bases, and the IR spectra demonstrated that a preferential binding of Cu(II) to guanine and cytosine nucleosides occurred [20,21].

Thus, according to the results obtained using the *Xf*-dsDNA-electrochemical biosensors, the Cu(II)-*Xf*-dsDNA interaction can be described by the formation of a Cu(II)-*Xf*-dsDNA complex, in which different binding sites in the dsDNA are occupied by Cu(II) ions. The formation of the Cu(II)-*Xf*-dsDNA complex will cause a conformational change in the *Xf*-dsDNA structure.

For incubations with ct-dsDNA-electrochemical biosensors, a concentration of 25.0 μM Cu(II) was used. Bearing in mind that the normal level of copper in human blood is approximately 16.0 μM [20], this represents an environment with an excess of copper. The DPV for the ct-dsDNA-electrochemical biosensor after 30 min incubation in 25.0 μM Cu (II), Fig. 1B, showed a large decrease in the oxidation peak currents for both dGuo, at $E_{pa} = +0.99$ V, and dAdo, at $E_{pa} = +1.26$ V. New ct-dsDNA-electrochemical biosensors were incubated in 25.0 μM Cu(II), for 60 and 120 min, and revealed a progressive and continuous decrease in ct-dsDNA oxidation peak currents. The formation of the Cu(II)-ct-dsDNA complex caused conformational changes in the structure of the ct-dsDNA.

Finally, experiments with longer incubation times were also conducted, but no significant changes in the *Xf*-dsDNA and ct-dsDNA oxidation peak currents were observed, suggesting a resistance to Cu (II). In addition, Cu(II) ions did not induce oxidative DNA damage to bacterial *Xf*-dsDNA, or to ct-dsDNA.

Under the experimental conditions used, no oxidation peak of the biomarkers 8-oxoGua and/or 2,8-oxoAde at $E_{pa} = +0.45$ V, corresponding to oxidative DNA damage of the DNA structure, was electrochemically detected.

The *Xf* grows inside the xylem vessels of the host plant and the foregut of the vector, forming a biofilm [22]. The interaction of *Xf*

biofilms with 7 mM copper, the inhibitory concentration for cell growth in culture, showed that high-quality RNA was obtained and the expression of genes associated with copper resistance was verified, meaning that copper was toxic to the *Xf* cells but failed to kill the whole population [23].

In the case of *Xf*-dsDNA, there are no studies that correlate the interaction between Cu(II) and *Xf*-dsDNA. However, as expected, the Cu (II)-*Xf*-dsDNA interaction goes through a more complex pathway, since Cu(II) needs to cross the entire cell membrane of the bacterium to reach its nucleoid, and then interact with the *Xf*-DNA.

Considering the concentration of copper compounds normally used in the treatment of diseases in agriculture, the Cu(II) concentration used in these experiments is appropriate for understanding the Cu(II)-*Xf*-dsDNA interaction mechanism.

The Cu(II)-ct-dsDNA interaction was more sensitive and effective than the Cu(II)-*Xf*-dsDNA interaction, since the variation in the dsDNA oxidation peak currents was higher, even for a lower Cu(II) concentration.

The small variation in peak currents observed for the *Xf*-dsDNA-electrochemical biosensors incubated in Cu(II) shows the resistance of the bacterial DNA to the antimicrobial compound, as shown for *Xf* biofilms [23]. Although Cu(II) causes a change in the morphological structure of the *Xf*-DNA, due to its binding to DNA and formation of the Cu(II)-*Xf*-dsDNA complex, there is no oxidative DNA damage or breakdown of the bacterial DNA structure, thus explaining the non-death of the whole population, as well as its continuous proliferation.

3.2.2. Electrochemical impedance spectroscopy

EIS complex plane impedance plots were obtained for both the bare GCE, *Xf*-dsDNA- and ct-dsDNA-electrochemical biosensors, and after their interaction with Cu(II) ions for different time periods (Fig. 1S). A Randles-type equivalent electrical circuit was used to fit each EIS spectra and the changes in the semicircle diameters in the Nyquist plots, which are related to the charge-transfer resistance (R_{ct}), were investigated (Table 1S).

The complex plane impedance obtained for the *Xf*-dsDNA-electrochemical biosensor showed an increase in the semicircle, reflected in the increase in R_{ct} when compared to the bare GCE, due to the immobilization of *Xf*-dsDNA molecules, which hinders the charge transfer of the redox process from the $[Fe(CN)_6]^{4-/3-}$ probe.

For the *Xf*-dsDNA-electrochemical biosensor incubated for 30 min in 1.0 mM Cu(II), a small increase in R_{ct} was observed, which can be explained by the metal binding to the dsDNA structure, forming the Cu (II)-*Xf*-dsDNA complex on the electrode surface, in agreement with the small decrease in the dGuo and dAd oxidation peak currents. After 60 and 120 min incubation in Cu(II), a progressive and more significant increase in the R_{ct} values was observed, corroborating the fact that a conformational change in the structure of *Xf*-dsDNA occurred due to Cu (II) binding to dsDNA.

For the ct-dsDNA-electrochemical biosensor, as expected, the complex plane impedance showed an increase in the semicircle and consequently in R_{ct} when compared to a bare GCE, which is related to the immobilization of ct-dsDNA on the GCE surface. After incubation for 30 min in 25.0 μM Cu(II), an increase in R_{ct} was observed due to the binding of Cu(II) to the immobilized ct-dsDNA on the GCE surface. The Cu(II)-ct-dsDNA complex makes charge transfer from the redox probe to the electrode surface more difficult. Finally, after 60 and 120 min incubation in Cu(II), a progressive increase in R_{ct} was observed, consolidating the strong interaction between Cu(II) and ct-dsDNA.

The results obtained by EIS were complementary and confirm those obtained by DPV concerning the interaction of Cu(II)-dsDNA with either *Xf*-dsDNA- or ct-dsDNA-electrochemical biosensors.

3.3. Cu(II)-poly[G] and Cu(II)-poly[A] interaction using poly[G]- and poly[A]-electrochemical biosensors

The poly[G]- and poly[A]-electrochemical biosensors were used in order to clarify the purine base with which a more specific preferential Cu(II)-dsDNA interaction occurred.

The poly[G]-electrochemical biosensor incubated in 25.0 μM Cu(II), Fig. 1C, showed the dGuo oxidation peak current continuously decrease, as was observed for the *Xf*-dsDNA- and ct-dsDNA-electrochemical biosensors.

The poly[A]-electrochemical biosensors incubated in 25.0 μM Cu(II), Fig. 1D, also showed the dAdo oxidation peak current progressively decreasing, corroborating the observations with the *Xf*-dsDNA- and ct-dsDNA-electrochemical biosensors.

The homopolynucleotide poly[G] in the poly[G]-electrochemical biosensor surface consists of a mixture of single-, double-stranded and G-quadruplex configurations, while the homopolynucleotide poly[A] in the poly[A]-electrochemical biosensor surface consists of a mixture of single- and double-stranded configurations, due to partially protonated Ade residues that are able to form AH+–AH+ double bonds [24]. Consequently, Cu(II) binds to different sites in the poly[G] and poly[A] structures, forming rigid Cu(II)-poly[G] and Cu(II)-poly[A] complex structures, causing a decrease in the dGuo and dAdo oxidation peak currents.

The results of the *Xf*-dsDNA-, ct-dsDNA-, poly[G]- and poly[A]-electrochemical biosensors agree and provide important information for understanding the molecular mechanism involved in the Cu(II)-*Xf*-DNA interaction.

4. Conclusions

The development of a *Xf*-DNA-electrochemical biosensor based on DNA from the bacteria *Xylella fastidiosa*, a phytopathogen that causes diseases in many economically important crops worldwide, was reported for the first time.

Considering the action of Cu(II) as phytosanitary agent in the treatment of several diseases related to bacteria and fungus in agriculture, the Cu(II)-*Xf*-dsDNA interaction was investigated in situ. This occurs by Cu (II) binding at different sites independent of the bacterial DNA sequence, leading to the condensation/aggregation of *Xf*-DNA strands, due to the formation of a rigid Cu(II)-*Xf*-dsDNA complex structure, but without causing oxidative DNA damage. The results obtained also suggest resistance of the bacterial *Xf*-dsDNA to Cu(II).

Finally, this work shows the importance of the development of bacterial DNA-electrochemical biosensors for the investigation of potential pesticide agents, in order to understand the molecular interaction mechanisms, to enable the discovery of treatments for the diseases caused by bacteria, and to reduce the negative impacts on agriculture.

Credit authorship contribution statement

W.B.S. Machini: Methodology, Writing - review & editing. **A.M. Oliveira-Brett:** Conceptualization, Methodology, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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