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The use of optical oxygen sensing and respirometry to quantify the effects of antimicrobials on common food spoilage bacteria and food samples

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ABSTRACT

Microbial spoilage and foodborne diseases cause significant economic and productivity losses. There is a need for novel approaches and antimicrobial treatments to extend shelf life of products, improve quality and microbial safety, and reduce spoilage and waste, and new assessment methods. Traditional assays for testing the toxicity of antimicrobials are time consuming, labour intensive, give crude estimations of toxicity, and cannot analyse complex samples such as crude food homogenates. Using a model antimicrobial compound Lauroyl Arginate Ethyl Ester (LAE), we describe a new analytical methodology based on optical oxygen sensing and respirometry to investigate the effects of various antimicrobial treatments on pure bacterial cultures, meat microbiota and packaged meat samples. By measuring and analysing the time profiles of O_2 probe signal (phosphorescence lifetime) in incubating test samples, we were able to visualise the toxic effects of LAE on the different bacterial specie, generate time and dose response curves, calculate EC50 and generation times of test organisms. The new multi-parametric toxicity testing platform allows for rapid, automated and parallel analysis of multiple samples under a range of antimicrobial concentrations and conditions.

1. Introduction

Microbial spoilage is responsible for the global loss of ~25 % of all food produced [1,2], causing significant economic and environmental burden for producers [3,4]. Alongside microbial spoilage, foodborne diseases caused by bacteria such as *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella spp*., have also become a widespread concern both for public health and productivity losses [4]. In meat products, the main bacterial culprits associated with spoilage are *Enterobacteriaceae*, *Pseudomonas*, *Brochothrix thermosphacta*, and *Lactobacillus* spp. [4,5]. There is also a continuous demand from producers to extend shelf life of food products, improve their quality and microbial safety, reduce spoilage and waste [6]. To tackle all these problems, various strategies are pursued, including the new packaging processes [7], packaging materials with antimicrobial treatments [6], antimicrobial additives to foods [8], smart packaging systems [7] and (bio)sensor technologies [9].

Thus, the traditional antimicrobials used to combat spoilage and pathogenic bacteria in meat are nitrites, sulphates, organic salts and acids, parabens [4,10]. However, the application of these antimicrobials is limited due to their effects on product taste and colour (in the case of organic acids and salts) or possible toxic and carcinogenic effects (in the case of nitrites, sulphates, and parabens) [4,10]. This in conjunction with consumer demand has created a need for new antimicrobials with minimal to no effects on food quality and consumer health. Essential oils, the aromatic and volatile plant extracts, have gained popularity as a natural alternative due to their strong antimicrobial properties [4]. Nevertheless, they tend to have a strong odour, which can drastically modify the organoleptic properties of the treated product [6]. Another promising antimicrobial compound is the cationic surfactant Lauroyl Arginate Ethyl Ester (LAE) [8,11]. LAE interacts with the charged proteins present in microbial cell membranes and enzymatic systems, which leads to protein denaturation, increased cell permeability [12] inhibition of growth and ultimately cell death, but without cell lysis [13]. Although synthetically produced, LAE is metabolised by humans into natural components lauric acid and arginine, and has thus been classified as Generally Recognised as Safe (GRAS) by the FDA and EFSA [14,15]. Being odourless and tasteless [12], it looks as an ideal antimicrobial for many food products.

The use of antimicrobials with food products, in turn, requires

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adequate analytical approaches to assess their efficiency on treated foods, protective effects and overall safety for consumers. Such approaches should be simple and robust, affordable and usable with various types of food products and antimicrobials. Currently, several assays are used to test the toxicity of antimicrobials. In disc diffusion method, a disc with a known concentration of antimicrobial is placed on a pre-inoculated agar plate and incubated to determine a zone of inhibition [16]. Similarly, the agar diffusion test involves the making wells of a known diameter in agar plates, filling them with different concentrations of the compound in question and incubating to also determine a zone of inhibition [31]. Alternatively, chromogenic substrates such as Purple broth and INT (p-iodonitrotetrazolium chloride) can be used to visually assess toxicity. Purple broth, which changes colour in the presence of acids, relies on the metabolic activity of bacteria to break down carbohydrates [17]. INT dye is added post overnight incubation and changes colour from colourless to pink in the presence of growing bacteria [17]. Optical Density (OD600) can also be used to monitor bacterial growth in the medium [17]. However, these assays have relatively long time to result (18–20 hours), crude estimation of toxicity, and lack of automation. In addition, all of these assays are ill-suited for complex food samples such as crude homogenates, which tend to have plenty of debris, colour and reduced transparency.

Oxygen respirometry [18] offers a promising, user-friendly, and quick alternative to the conventional microbial testing. This method relies on dedicated sensor materials (solid-state coatings or soluble probes), phosphorescence of which is quenched by sample oxygen, such that low phosphorescent signals correspond to high levels of oxygen and vice versa [19]. Furthermore, monitoring of the phosphorescence over time on a fluorescent reader in standard 96-well plates allows robust tracing of bacterial growth and respiration [20,21]. Such contactless, high-throughput monitoring of dissolved O_2 levels has been used to quantify microbial load and enumerate bacteria in pure bacterial cultures, their mixtures and crude food homogenates [18,22,21]. The assay offers a high degree of automation and sensitivity, simple set-up, fast, quantitative and real-time readout. However so far, such assays have had limited use in testing of food antimicrobials.

In this study, we describe further development of oxygen microrespirometry technique, particularly its new application in the multiparametric assessment of toxicity of food-grade antimicrobials in both pure cultures and real food samples such as meat homogenates and packaged meat samples. By analysing the O_2 probe signal (phosphorescence lifetime) over time, we can not only visualise the effects of the antimicrobial, but also produce dose-response curves, calculate EC50 and generation time for tested bacteria.

2. Materials and methods

2.1. Materials

Ethyl Lauryl Arginate (LAE; IUPAC name: Ethyl N5-(diaminomethylene)-N2-dodecanoyl-L-ornithinate) and Plate count agar (PCA) were purchased from Sigma-Aldrich Merck (Dublin, Ireland). Nutrient Broth (NB), Luria-Bertani (LB) Broth, Maximum Recovery Diluent (MRD), and Mueller-Hinton Broth (MHB) were from Fisher Scientific Oxoid (Dublin, Ireland). Phosphorescent O_2-sensing probe MitoXpress®-Xtra was from Agilent (Cork, Ireland).

Stock cultures of Gram-negative *Escherichia coli* NCIMB 11943 (*E. coli*) and *Pseudomonas fluorescens* DSM 50091 (*P. fluorescens*), and Gram-positive *Staphylococcus aureus* ATCC 1448 (*S. aureus*) and *Bacillus cereus* NCIMB 9373 (*B. cereus*) were obtained from the School of Microbiology, University College Cork and Teagasc Food Research Centre (Ashtown, Dublin), and stored at -80 °C in 80 % glycerol in LB broth. Working cultures were prepared by inoculating 50 µL of semi-defrosted stock into 5 mL of LB broth, and incubating it at either 37 °C (*S. aureus* and *E. coli*) or 30 °C (*P. fluorescens* and *B. cereus*) on a rotary shaker at 250 rpm until an OD600 of ~ 0.8 was reached (typically overnight).

2.2. Determination of total aerobic viable counts (CFU/mL) by agar plating

1:10 serial dilutions of overnight cultures were prepared using NB and 100 µL aliquots of these suspensions were spread-plated onto PCA in duplicate. Plates were incubated at either 37 °C or 30 °C overnight and then grown colonies were enumerated using an automatic counter pen (VWR International, Dublin, Ireland). Concentrations of the initial bacterial stocks were calculated (typically 10^9 CFU/mL) and then diluted to the desired working concentrations (10^7-10^5 CFU/mL) using NB or LB broth for the respirometric assays.

The microbial load of the meat samples was calculated in accordance with the standard method (ISO4833-1:2013). A 10 g sample was taken from a different part of the meat, placed in a sterile mesh lined stomacher bag (Filter Bag, 400 Series, Grade; Spark Lab Supplies, Dublin, Ireland) to which 90 mL of MRD was added and then stomached for 2.5 min on a Colworth Stomacher 400 (Colworth, UK). The resulting meat homogenate was serially diluted using 900 µL of NB, plated on PCA plates in duplicate, incubated for 48 − 72 hrs at 30 °C and then counted.

2.3. Respirometric assays

Working dilution of MitoXpress®-Xtra in NB was prepared and dispensed in 100 µL aliquots to each test well of a 96-well plate. In-plate serial (1:3) dilutions of LAE stock (2 mg/mL) were prepared to produce concentrations ranging from 166.7 µg/mL to 2.1 µg/mL. 15 µL of bacterial stock was added to assay wells to give a final concentration of 10^4, 10^5 or 10^6 CFU/mL in the well. For each condition, 3 replicates were included on the plate, plus positive (bacteria with no antimicrobial) and negative (media only) controls. Then 40 µL of mineral oil was added to each well to seal the samples from ambient air, the plate placed in Victor4 (PerkinElmer) reader pre-heated to 30 °C or 37 °C and monitored in time resolved fluorescence (TR-F) mode for 10 h measuring probe signal each well every 5 min in kinetic mode. TR-F settings were: excitation filter – 340 nm, emission filter - 642 nm, Delay times of 30 µs and 70 µs (two windows), gate time - 100 µs (for each delay window). For each reading and sample well, the phosphorescence lifetime values (LT) were calculated in Excel using the formula: $LT = (t_2\text{-}t_1)/\ln(F_1/F_2)$ where t_1 and t_2 are the delay times (30 and 70 µs) and F_1 and F_2 are the corresponding intensity signals [18]. The resulting LT profiles were plotted and analysed to determine the time required (time to result) to reach the threshold LT signal, which was set at 35 µs. For consistent results preparation time for the plate was kept under 15 min.

2.4. Reference antimicrobial test

A reference method for Minimum Inhibitory Concentration (MIC) determination (adapted from Eloff [17] and Wiegand et al. [23]) was applied on two representative microorganisms, *E. coli* (Gram-negative) and *S. aureus* (Gram-positive). 100 µL aliquots of MHB were added to each assay well and LAE was serially diluted (1:3) on the plate to give the same range of concentrations as mentioned previously. 100 µL of suspension of bacterial cells were added to the wells to give a final concentration of 10^5 CFU/mL, with each condition set up in triplicate. The plate was sealed with mineral oil (40 µL) and an initial reading of optical density was measured at a wavelength of 600 nm using a plate reader. Following an overnight incubation (18 h) at 37 °C, the optical density was measured again. MIC was determined using the interpretation suggested by Wiegan et al. [23], in which the lowest concentration of the antimicrobial that inhibits visibile growth is said to be its MIC.

2.5. Analysis of LAE action on meat microflora

Samples of fresh raw beef obtained from a local butcher were kept for 5 days at 5 ± 1.0 °C in air atmosphere to increase their bacterial load (from approximately 10^2 CFU/g to 10^7 CFU/g). Following the standard method (ISO4833-1: 2013), a 10 g sample was cut out, homogenised with 90 mL of MRD in a sterile mesh lined stomacher bag. The homogenate was added in triplicate to the assay microplate and the respirometric assay was performed on Victor reader as described in Section 2.3.

2.6. Shelf life study of vacuum packed raw meat treated with LAE

Two beef slivers were cut into 54 pieces (27 pieces from each sliver, ~200 g for each cut). One third of the cuts was vacuum packed with no treatment applied (18 samples, coded as NT). The other two thirds were each dipped into concentrated solution of LAE (2 mg/mL in water - 18 samples, coded SCon) or LAE diluted 1:1 with water (18 samples, coded S5050), then squeezed off to remove excess liquid and vacuum packed. The packs were stored in a cold room set at +3 °C for 80 days. Every 10 days, two meat samples of each category were taken and their microbial load was determined by destructive sampling and oxygen respirometry assay (as described in Section 2.5). Microbial load of meat samples was calculated from measured Threshold Time (TT) values using the following equation [24]:

$$\text{Log (CFU/g)} = 8.2 - (0.43 \times \text{TT}),$$

where TT is signal threshold time (in hours) determined from the corresponding respiration profile.

2.7. Statistics and analysis

All respirometric experiments were repeated at least 2–3 times, to ensure consistency of the data. The dose-response graphs and EC50 values were calculated by plotting the reciprocal of time to result values vs the log of LAE concentration and processing the data in Quest Graph™ EC50 Calculator [25]. Results are presented as the mean ± standard deviation.

For the analysis of differences in microbial load (Log(CFU/g)) between the control and two series of LAE-treated vacuum packed meat samples over 80 days period, Mann-Whitney *U* test (two-tailed) was applied (https://www.socscistatistics.com/tests/); p values ≤ 0.01 were deemed significant. Results are visualised using http://shiny.chemgrid.org/boxplotr/ and Adobe Illustrator.

3. Results and discussion

3.1. Design of the O_2 sensing method for toxicological assessment of antimicrobials

Oxygen micro-respirometry based on the optical O_2 sensing is a versatile detection platform, which has already proven its high utility in the analysis of mammalian [26,27] and bacterial [18] cells and environmental toxicants [28]. However, its potential with respect to the assessment of antimicrobials remains under-explored. To address this, we have designed a dedicated testing platform, which can provide advanced, multi-parametric assessment of antimicrobials. The general concept of this sensing method is shown in Fig. 1, which illustrates method flexibility and applicability to various compounds, bacteria, sample types and testing conditions. Furthermore, the biosensing platform can be configured based on the user requirements, to produce a number of different (but complementary) readouts. These readouts can be used individually or combined, to report on compound's toxicity in highly quantitative and accurate, detailed and comprehensive manner.

Practical use of this toxicity testing platform is further illustrated with a particular set-up for multi-parametric toxicological assessment described in Fig. 2. Together with the experimental procedure (Section 2.3), the plate map on Fig. 2 shows that in one respirometric assay on a 96-well plate two sets of variables - compound concentration and bacteria concentration - can be assessed to reveal the corresponding dependences and quantitative relationships. Similar 2D layouts of respirometric assays of antimicrobials can be designed and used for other main variables, such as bacterial specie, growth media, temperature, sample type, etc. Corresponding experimental results and their interpretation are presented in the following sections.

3.2. Determination of antimicrobial activity of LAE on pure cultures using respirometry

Respirometry of microbial cultures usually produces sigmoidal time profiles of the sensor/probe optical signal (phosphorescence intensity or lifetime). The transition from the low to high optical signal reflects the steep change from sample air-saturated condition to deoxygenated condition, when bacteria reach certain cell density during their exponential growth [18]. The onset time of this transition correlates with sample's *initial* load of viable cells, and it can be used for enumeration of bacteria in unknown samples or quantifying the effects of different factors on test cells [21]. Respirometric readout also provides high sample throughput, speed and automation, quantitative and accurate readout, so that many different samples and conditions can be tested on one plate in parallel.

For the testing of antimicrobial activity of LAE, samples of two Gram-positive and two Gram-negative species were prepared according to the plate layout in Fig. 2, at known initial concentrations (10^4, 10^5 or 10^6 CFU/mL) on a 96-well plate in suitable media containing the O_2 probe and known concentrations of LAE (varied from 166.7 μg/mL to 2.1 μg/mL as 1:3 serial dilutions). Then the plate was incubated at 37 °C or 30 °C for ~10 h while measuring each sample well every 5 min. These assay settings allowed us to assess the dose and time dependence of LAE toxicity via EC50 calculation, and effects of LAE on metabolism and growth of different cells cultured under different conditions.

Representative respiration profiles of the different cultures are shown in Fig. 3. From each respiration profile the Threshold Time (TT), i.e. time to reach threshold LT value (set at 35 μs), was determined, converted into its reciprocal value, plotted vs log [LAE] and processed to determine EC50 values for each culture. For the curves which did not produce signal changes characteristic to cell growth and respiration, zero reciprocal TT values were assigned (i.e. TT = ∝).

The dose-response curves for the different bacterial species used at several different seeding concentrations are presented in Fig. 4. The EC50 values calculated by sigmoidal fitting of these semi-logarithmic plots are compared in Fig. 5. One can see that the Gram-negative bacteria, *P. fluorescens* showed a lower sensitivity to LAE and higher EC50 values than *E. coli* (Figs. 4AC and 5). This higher resistance of *P. fluorescens* to LAE is most likely due to extracellular polysaccharide in the cell wall which is used for biofilm formation and which has shown to confer stronger resistance to antimicrobials [6,32,34]. By changing the initial concentration of bacteria, it was also possible to vary the exposure time for the antimicrobial, which correlates with signal onset time that can range from < 1 h to ~ 8 h (see Supplementary Materials, Figs. S1–S4). This parameter can also be used to optimise the time to result for the respirometric toxicity assay. Fig. 4A shows that *E. coli* gives practically identical EC50 values for the different initial cells numbers, which indicates that LAE has fast toxic action on these cells. In contrast, *P.fluorescencs* showed a 2-fold increase in EC50 when cell numbers increased from 10^5 to 10^6 CFU/mL and assay time decreased from 8 h to 3.5 h (Fig. S3). Hence, the toxic action of LAE on *P. fluorescencs* cells is slower than on *E. coli*.

For the Gram-positive bacteria *S.aureus* and *B.cereus,* LAE action appeared to be similar (Figs. 4,5). Interestingly, Gram-positives are known to be more sensitive to antimicrobials than Gram-negative

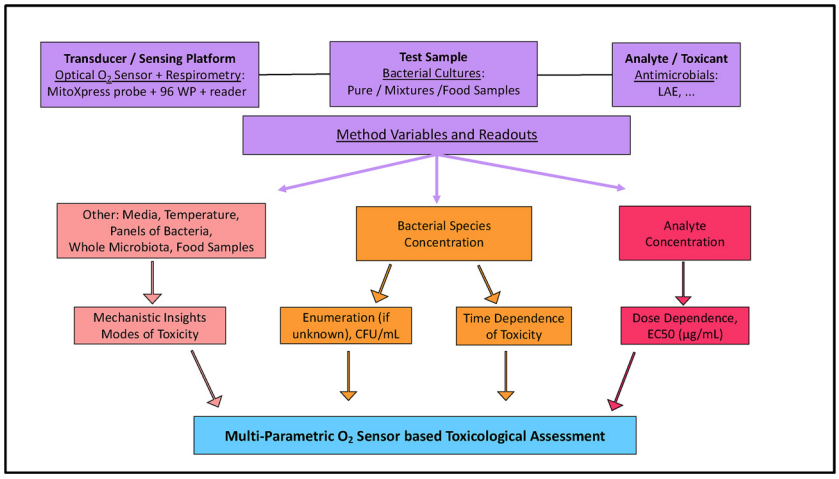

Fig. 1. General design of the O_2 sensing method for multi-parametric toxicological assessment of antimicrobials, showing the main components of the assays, controllable variables, analytical readouts, and their inter-connection.

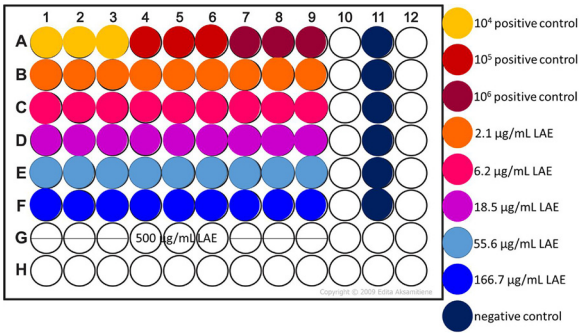

Fig. 2. Typical plate layout used in assays with concentrations of LAE ranging from 166.7 µg/mL to 2.1 µg/mL, bacterial cultures at 10^4, 10^5 and 10^6 CFU/mL in wells, the required replicates, positive and negative controls.

bacteria, as the latter have an additional outer lipid membrane restricting the diffusion of molecules [29,33]. However, using LAE and respirometry readout we did not observe this difference: both *S.aureus* and *B.cereus* produced similar EC50 values to *E.coli* (Fig. 5). Also spore-forming Gram-positive bacteria are typically more resistant to antimicrobials [33]. However, our *B.cereus* strain was in a vegetative state and did not show this effect with LAE. The antimicrobial effects of LAE were also seen to be temperature dependent. For *E.coli* the EC50 increased 2-fold when changing from 30 °C to 37 °C (Fig. 5). This increase can be related to the additional stress imposed by sub-optimal growing conditions.

For comparison, we analysed LAE toxicity on Gram-negative *E.coli* and Gram-positive *S.aureus* cells by the established densitometry method, which involved OD600 measurement and MIC determination [23]. For both bacteria, MIC of 18.5 µg/mL was obtained, which is consistent with literature values for *E.coli,* but not for *S. aureus* [13,29].

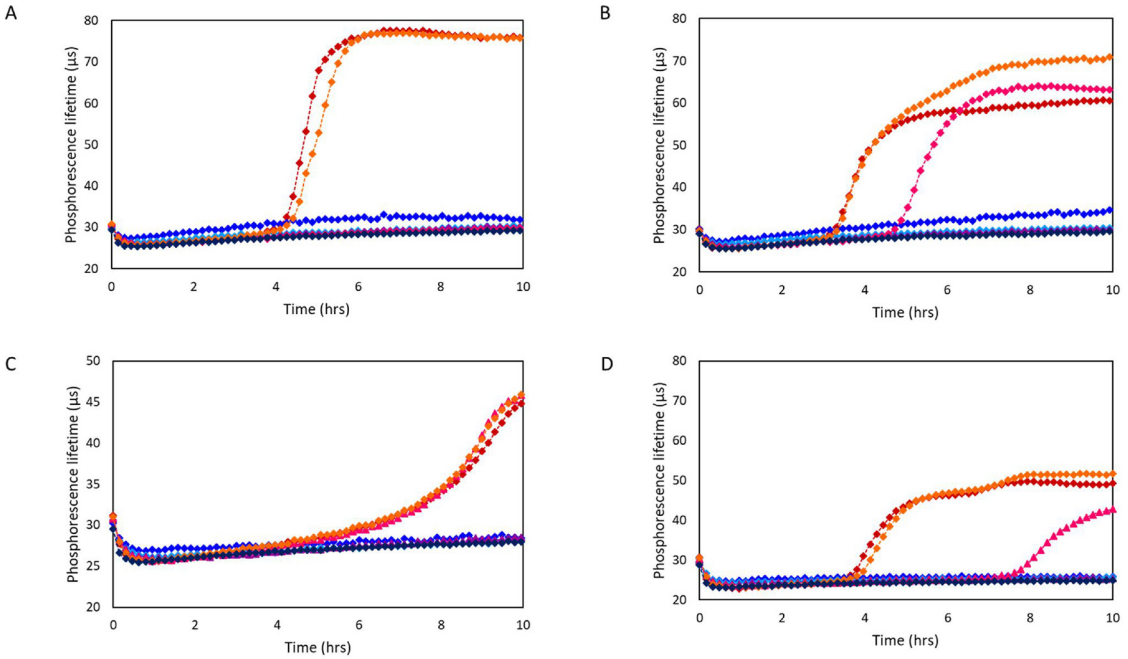

Fig. 3. Phosphorescent signal profiles of pure bacterial cultures at 10^5 CFU/mL exposed to LAE (166.7 µg/mL to 2.1 µg/mL) over 10 h: (**A**): *Escherichia coli*. (**B**): *Bacillus cereus*. (**C**): *Pseudomonas fluorescencs* (**D**): *Staphylococcus aureus*. Labels: positive control (---◆---), 166.7 µg/mL (---◆---), 55.6 µg/mL (---◆---), 18.5 µg/mL (---◆---), 6.2 µg/mL (---◆---), 2.1 µg/mL (---◆---), and negative control (---◆---). Additional bacterial concentrations can be seen in the Supplementary Materials (Figs. S1–S4).

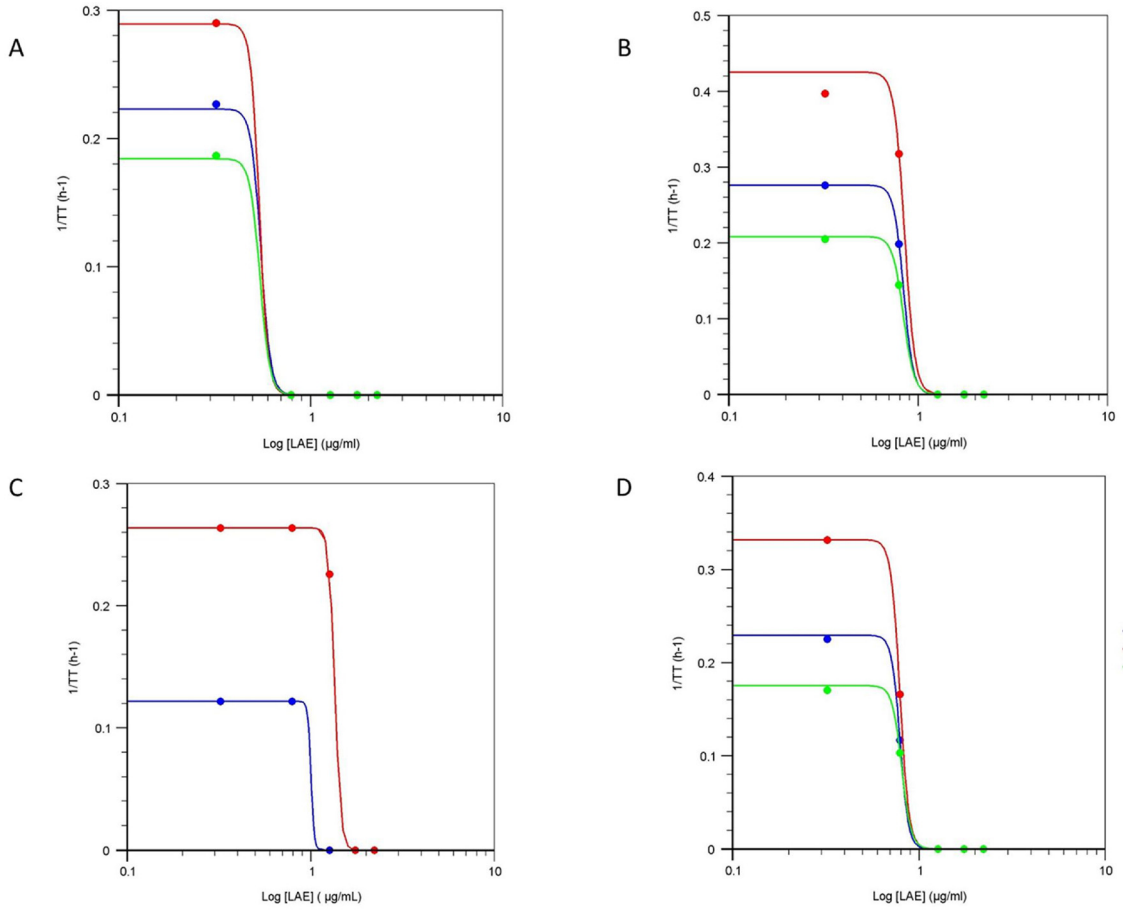

Fig. 4. Dose-response curves for pure bacterial cultures exposed to LAE (166.7 µg/mL to 2.1 µg/mL) (**A**): *Escherichia coli*. (**B**): *Bacillus cereus*. (**C**): *Pseudomonas fluorescens* (**D**): *Staphylococcus aureus,* at seeding concentrations 10^4 (green), 10^5 (blue) and 10^6 (red) CFU/mL. More statistical data on the determination of the EC50 values is shown in Supplementary Information, Tables S1-S4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

The MICs were also significantly higher than the EC50 values produced in the respirometric assays (Fig. 5), even though MIC assays are inherently more sensitive than EC50 assays. Thus, we can conclude that respirometric assays provide more sensitive detection of antimicrobial action, as they can detect subtle and sub-lethal changes in cell metabolism. Comparison of the different toxicity assays is given in Table 1.

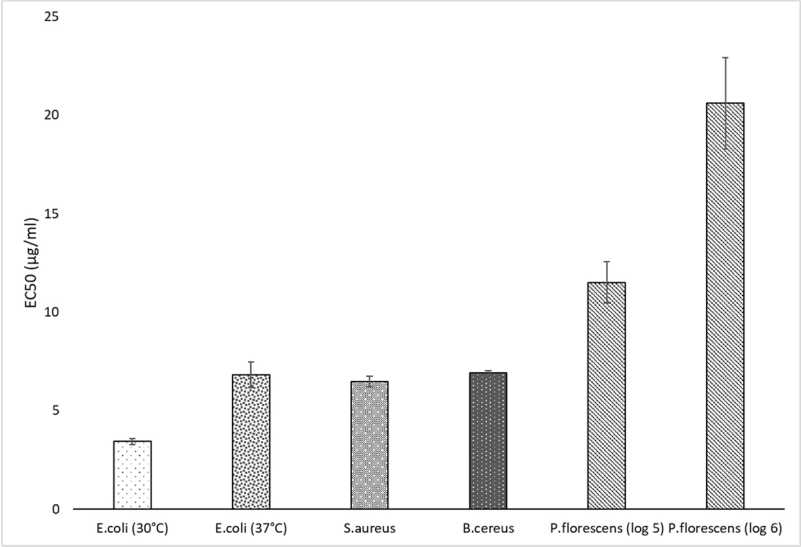

Fig. 5. Graphical representation of EC50 values (µg/mL) for the pure cultures of *E. coli, S. aureus, B. cereus* and *P. fluorescens*. Effects of testing conditions (T, cell concentration) are also shown.

Table 1
Summary of EC50 and MIC values of pure cultures obtained via respirometric and OD600 assays.

Microorganism	Bacterial Strain	EC50 (μg/mL) (MitoXpress)	Exposure Time Dependent	MIC (μg/mL) (OD600)
Gram-negative	*E.coli* (30 °C) (log 4–6)	3.43 ± 0.16	No	
	E.coli (37 °C) (log 4–6)	6.81 ± 0.64		18.5
	P.florescens (log 5)	11.51 ± 1.04	Yes	
	P.florescens (log 6)	20.61 ± 2.33		
Gram-positive	*S.aureus* (log 4–6)	6.47 ± 0.27	No	18.5
	B.cereus (log 4–6)	6.93 ± 0.10	No	
Natural meat spoilage microbiota		39.99	No	

3.3. Effects of LAE on whole microbiota of meat samples

Most of the existing toxicity tests can only operate with 'clean' samples such as pure bacterial cultures and media. In contrast, respirometric assays with quenched-phosphorescence detection and lifetime readout are more robust as they are internally referenced and not dependent on the intensity signal and probe concentration which are affected by sample optical properties [21]. As a result, such assays can analyse reliably complex biological samples, including crude homogenates of food products, coloured, opaque and particulate samples [21]. Knowing this feature, we applied O_2 respirometry to assess the effects of LAE on the whole microbiota of fresh red meat.

Since fresh meat samples usually have low microbial loads, we used a spoiled beef steak sample, which was pre-incubated at 4 °C in air atmosphere for five days. Based on the Total Viable Count (TVC) assay, the microbial load of the meat sample was determined to be 7.4 $\log_{10}$ CFU/g, which is well within the range for spoilage [30]. Then, using the standard ISO procedure (ISO4833-1: 2013), we produced a crude homogenate of the meat and tested on it the antimicrobial effectiveness of LAE. The effects of LAE concentration on respiration profiles of meat homogenates are shown in Fig. 6. The testing of whole meat microbiota revealed the pattern of toxicity and range of effective concentrations of LAE, resembling those produced by the pure cultures (Figs. 3–5), but not quite the same.

The respiration profiles of spoiled beef homogenate are shown in Fig. 6A, for which EC50 was calculated using the same method as for pure cultures. In the beef homogenate, we observed a significantly higher resistance to LAE, with EC50 of 39.99 μg/mL (Fig. 6B). This difference can be attributed to partial adsorption of LAE molecules on fine particles of meat homogenate, and to a higher diversity of bacteria present in the sample. This diversity can be seen by the bi-phasic respiration profiles in Fig. 6A, which suggests the presence of two populations of bacteria. The first, which is more abundant or fast growing, but more sensitive (either to low O_2 levels or LAE) produced the first step in the profile, but then cease to grow and consume oxygen. The second population is slower growing or more resistant to LAE [30], can respire at lower levels of oxygen than the first population and thus produce the second LT step.

Although we did not directly analyse the microbiota of the spoiled beef homogenate, Stellato et al. [5] showed using 16sRNA sequencing that *Pseudomonas* spp. are predominant in meat microbiota. As seen in Fig. 5, pure culture of *P.fluorescens*, showed relatively high levels of LAE resistance. This in combination with other bacterial species not analysed in this paper *(Streptococcus* spp., *Brochonthrix* spp., etc.) could attribute to the observed higher level of resistance of LAE in the spoiled beef homogenate.

Based on the results from the respirometric antimicrobial action assay, as low as 80 μg/mL of LAE can be applied to fresh beef products, which is well below the human consumption levels stated by the FDA (200 ppm or 200 μg/mL) and EFSA (160 ppm or 160 μg/mL) [14,15]. This will be highly beneficial since the maximum antimicrobial activity will be achieved with the minimum application of LAE. Moreover, this will fit the trend for both producers and consumers by extending shelf-life while maintaining quality produce with minimal additives.

3.4. The antimicrobial effects of LAE on packaged meat samples: a shelf life study

Lastly, we analysed the effects of LAE treatment of fresh meat samples packaged under industrial settings. The three groups of meat cuts - untreated samples, treated with concentrated LAE solution (2 mg/mL) or with LAE diluted 1:1 with water - were prepared, packaged on a vacuum packager, then stored at +3 °C for 80 days and periodically tested by destructive sampling for their total microbial load by O_2 respirometry (see Section 2.6).

From Fig. 7 we can see that in all groups, bacterial contamination was not detected at Day 10 and Day 20 time points. Starting from Day 30, bacterial growth was detected with varying frequency in each group. Due to the low number of samples, we did not observe any time dependent increase in CFU/g values (NT vs Conc, $p = 0.0074$; NT vs S5050, $p = 0.0044$). Basic inspection of the meat samples undergoing the CFU/g testing also revealed off-tone odours for the untreated group – from Day 40 onwards. These results are in agreement with the known

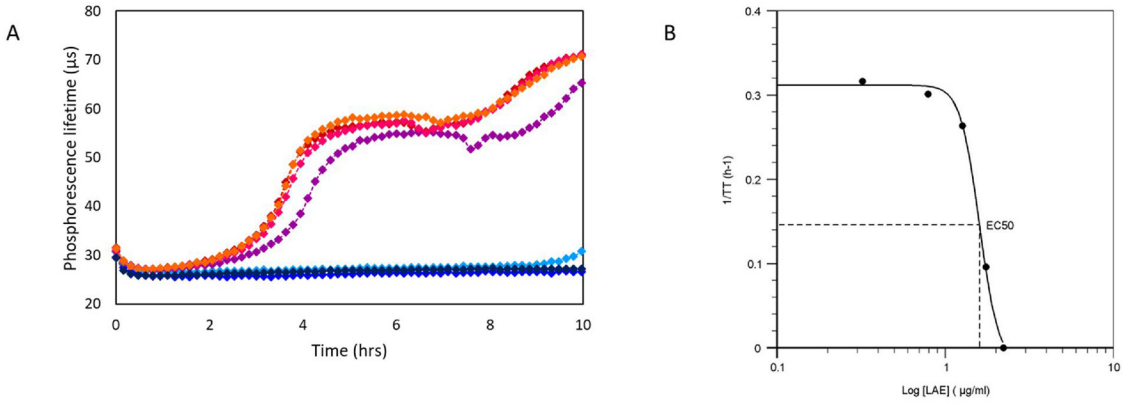

Fig. 6. Effects of LAE on microbiota of spoiled meat sample. (**A**): Phosphorescent lifetime profiles of undiluted spoiled beef homogenate. Labels: positive control (---◆---), 166.7 μg/mL (---◆---), 55.6 μg/mL (---◆---), 18.5 μg/mL (---◆---), 6.2 μg/mL (---◆---), 2.1 μg/mL (---◆---), and negative control (---◆---). (**B**): Dose-response curve of spoiled beef homogenate treated with LAE.

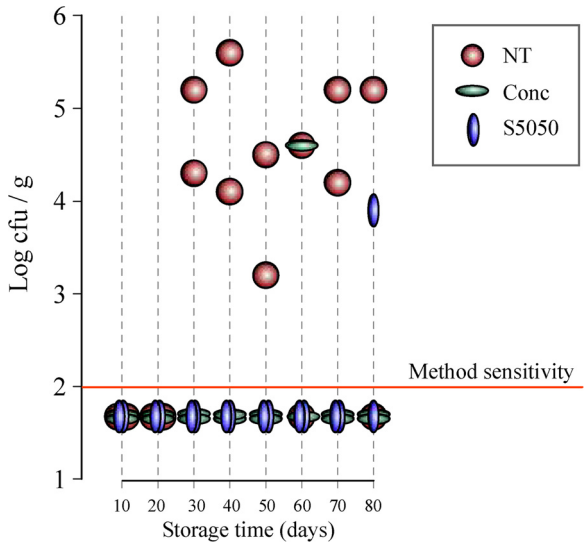

Fig. 7. Microbial load (Log(CFU/g) in the untreated vacuum packed meat samples (NT) and samples treated with undiluted (Conc) and 1:1 diluted (S5050) LAE solution, monitored over 80 days shelf life. At each time point N = 2 for each group (conducted in duplicate). Method sensitivity – 10^2 CFU/g, safety threshold – 10^5- 10^6 CFU/g. The corresponding raw data is also presented in Table S5.

shelf-life of this product.

At the same time, compared to the NT samples, microbial growth in LAE-treated groups Conc and S5050 was significantly inhibited: positive CFU/g values were detected just in two samples: one on Day 60 for Conc and one on Day 80 for S5050 group (Fig. 7). On the other hand, Conc and S5050 samples showed only insignificant difference in their odour: very slight off-tones were observed on days 70 and 80, respectively. Overall, these results prove that anti-microbial treatment of fresh meat samples with LAE was beneficial with respect to their microbial safety. In addition, LAE treatment also improved odour characteristics, appearance and shelf life of this product. Thus, the respirometric assay also provided valuable information on the effects of treatments with antimicrobial compounds of meat samples prepared, packaged and stored under standard industrial settings.

4. Conclusions

The respirometric testing platform, based on the commercial phosphorescent O_2-sensing probe MitoXpress-Xtra and standard fluorescent reader, was applied to the assessment of antimicrobial activity of LAE on pure bacterial cultures, whole meat microbiota, and vacuum packaged meat samples surface-treated with LAE. The platform allowed rapid, automated and parallel analysis of panels of different samples under a range of antimicrobial concentrations and conditions (temperature, exposure time, growth media, and concentration of bacteria). It provided robust, real-time and sensitive readout and generated consistent and reproducible results (Table 1), in as little as 3 – 10 hours. The assay allowed for the analysis of complex homogenates with little interference to the primary signal of the probe (phosphorescence lifetime) and measured signal onset time. The assay can be used to analyse various antimicrobials and various microbiological and food samples in a quick, straightforward manner giving accurate and multi-parametric assessment of antimicrobial action and mode of toxicity. It can supersede and replace the more tedious and less robust antimicrobial testing systems that are currently in use. At the same time, this methodology is not limited to MitoXpress probe and can be realised with other suitable O2 probes and detectors.

CRediT authorship contribution statement

Sophia Elisseeva: Writing - original draft, Writing - review & editing, Data curation. **Caroline Kelly:** Data curation. **Malco Cruz-Romero:** Conceptualization, Supervision. **Alexander V. Zhdanov:** Methodology. **Joe P. Kerry:** Conceptualization, Supervision, Writing - review & editing. **Dmitri B. Papkovsky:** Conceptualization, Supervision, 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

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.snb.2020.128572.

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