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Repurposing blood glucose test strips for identification of the antimicrobial colistin

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ABSTRACT

The presence and fate of antimicrobial residues in the environment is a subject of growing concern. Previous researchers have demonstrated the persistence of residues in soil and water. Additionally, antimicrobial resistance is a growing concern, particularly to public health, animal health and economic development. In this study, a low cost, commercial blood glucose meter was explored as the basis for detecting antimicrobial residues in conjunction with a microorganism sensitive to this residue. A microbial bioassay was developed based on the metabolic response of *Geobacillus stearothermophilus*, a sensitive bacteria used in the determination of antimicrobial residues in food products, by measuring changes in glucose as a result of metabolic activity. After optimizing experimental conditions, this sensing strategy was tested using bacterial cultures in the presence of colistin, a last-resort antibiotic used for human and animal health. Growth of *G. stearothermophilus* was measurable as a change in glucose concentration after 2–4 h incubation at 60 °C, when LB media was supplemented with 100 mg/dL of glucose. The lowest measured colistin concentration that resulted in inhibition of growth was 1 mg/L colistin and an increase in lag phase resulted at 100 μg/L colistin. To increase the sensitivity of the assay, we then added a sub-inhibitory concentration of chloramphenicol to the media and found that growth inhibition could be achieve at a lower colistin concentration of 8 μg /L. These results provide a promising basis for a future low-cost sensor to identify antimicrobial residues from environmental samples in the field.

1. Introduction

Environmental antimicrobial residues are a source of great concern globally and have been shown in previous studies to drive antimicrobial resistance (AMR) [1], including resistance against antibiotics of last resort [2]. AMR is problematic because without effective drugs, infections cannot be treated in humans or animals resulting in high rates of morbidity and mortality. Nearly 5 million deaths were attributed to AMR in 2019, highlighting the problems urgency today [3]. The COVID 19 pandemic has also impacted the spread of AMR as antimicrobial stewardship programmes were relaxed globally [4]. Antimicrobial residues find their way into the environment through several routes, including irresponsible handling of pharmaceuticals during production, excretion of unmetabolised drugs by humans and animals, and improper disposal of unused drugs. Because of these routes, residues accumulate and persist within the environment. Larsson et al. reported high concentrations of fluoroquinolone antibiotics close to the pharmaceutical industry near Hyderabad in central India [5]. In addition, evidence of environmental resistance to drugs on the WHO watch and reserve list, such as vancomycin and colistin respectively, is particularly concerning [6–8]. Vancomycin resistance genes have been reported within several environmental reservoirs associated with anthropogenic activity, such as livestock feces, wastewater treatment plants, landfill sites and food processing sites [9–13]. Similarly, colistin-resistant *Escherichia coli* has been reported in many of the same environmental samples and is viewed as a serious public health threat [14].

Typically, quantifying residues require sophisticated laboratory equipment and procedures such as HPLC which is expensive and time-consuming. As an alternative to HPLC and mass spectroscopy approaches, bioassays can be used to assess the toxicity of a substance or a combination of compounds in aquatic and terrestrial environments [15]. A bioassay measures the response of the bacterial culture in the presence of toxic compounds. The response is measured as substrate consumption, turbidity, or change of other conditions such as pH [16,17]. This

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technique has been employed successfully within the food sector to test for antimicrobial residues within milk, in conjunction with a pH indicator dye [18]. Several biosensors have also been developed to identify antimicrobial residues, including fluorescent, electrochemical and colorimetric techniques [19,20].

Although several biosensors exist for the detection of antimicrobial residues, predominantly driven by the food industry, few sensors have been developed for the detection of colistin [21–24]. Building upon an established microbial inhibition assay, we present the principal of a simple sensor system that could be used for identification of antimicrobial residues in environmental samples, without the need for extensive laboratory infrastructure (Fig. 1). Blood glucose test strips have been used previously to measure changes in the concentration of glucose as a measure of bacterial activity in the presence of metals [25] and other toxic chemicals [26], but to the best of our knowledge, they have not been used in this manner for the detection of antimicrobial residues, particularly colistin.

First, we explored the ability of *Geobacillus stearothermophilus* to metabolize glucose and verified that this could be characterised using a blood glucose test strip. *G. stearothermophilus* is widely used to identify antimicrobial residues in milk for human consumption, using a colorimetric indicator [27]. Following this, we tested the growth of *G. stearothermophilus* in the presence of colistin. The results of this work show promise for a detection principle that could be used at a low cost to identify the presence of residues in environmental samples.

2. Materials and methods

2.1. Materials and reagents

Manganese sulfate $MnSO_4{\cdot}H_2O$, Colistin sulfate salt (≥ 15,000 U/mg), D (+)-glucose and meat peptone were all purchased from Sigma Aldrich. Nutrient Broth, Nutrient Agar, Luria Bertani (LB) Media, LB Agar and Plate Count Agar were purchased from Oxoid Ltd. NaCl (laboratory reagent grade) was purchase from Fisher Scientific. Sterile saline/peptone solution was created by mixing 8.5 g NaCl and 1 g peptone in 1 liter of dH_2O. All media was autoclaved at 121 °C for 20 min prior to use. Filter sterilised (0.2 μm) glucose was added to the media after autoclaving. Glucose measurements were performed using an ACCU-CHEK Performa Nano commercial blood glucose meter (Roche Diagnostics, Rotkreuz, Switzerland).

2.2. Bacteria strain

G. stearothermophilus (NCTC 10,339) was obtained from the Public Health England Culture Collections. The extraction of the test-organism and preparation of the bacterial suspension was carried out following the procedure described in DD ISO/TS 26,844 (2006). Initially, the freeze-dried bacterial cultures were suspended in 0.5 ml of nutrient broth and transferred from the ampoule to sterile slant agar tubes, containing 10 ml of nutrient agar and 207 μM manganese sulfate adjusted to pH 7.4 prior to autoclaving at 121 °C for 20 min. Next, the slant tubes were incubated for 48 h at 60 °C. After that, the tubes were refrigerated at 5 °C and used as stock cultures to prepare the test-organism suspension.

2.3. Measurement of glucose concentration with blood glucose meter

A commercial glucose meter and associated test-strips were used to measure the glucose consumption in the bacterial culture. According to the manufacturer, the ACCU-CHEK meter has a measurement range between 0.6 and 33.3 mmol-glucose/L (10 to 600 mg/dL). The test strips use a mutant enzyme variant of quinoprotein glucose dehydrogenase (Mut. Q-GDH) that reacts with the glucose in the sample. Through a redox couple, this reaction produces a current proportional to the glucose concentration in the blood.

Three sets of standard curve measurements were performed. Initial measurements were carried out across a wide glucose range to establish the upper and lower limit response of the meter and verify acceptable performance in a non-blood matrix. This was followed by a more detailed analysis of the performance over a narrower range (60 to 300 mg/dL) related to the growth experiments with G. stearothermophilus. A final standard curve was produced in extracted soil samples. To create the extracted soil sample, 200 mg of garden center topsoil was mixed with 1 ml of LB-glucose solution, or 1 ml of LB without glucose. This was vortexed for 1 min, then centrifuged at 1000 *x g* for 5 min. The supernatant was removed and the two aliquots, containing and not containing glucose, were used to create a series of dilutions for the standard curve.

2.4. Preparation and growth of test cultures

To prepare the bacterial suspension for the experiments, the *G. stearothermophilus* was transferred from the stock tubes into sterile 140 mm Petri dishes by extracting the cells with autoclaved dH_2O. The Petri dishes were placed in the incubator for 48 h. After cultivation, the cells were extracted from the plate with sterile saline/peptone solution

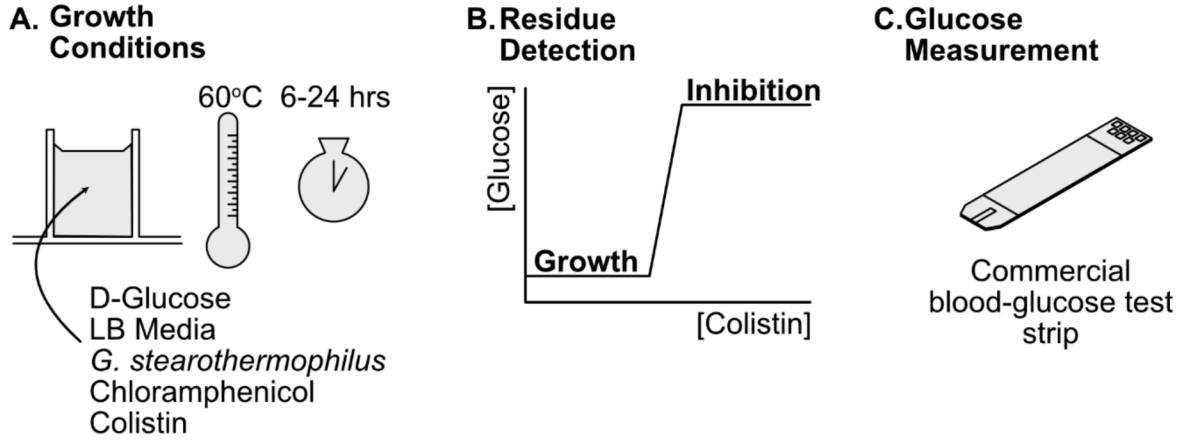

Fig. 1. Detection Scheme used in this study. (A) G. stearothermophilus is incubated in microbial growth media containing glucose. (B) Under normal growth conditions, the bacteria use glucose as a carbon source and the substrate is consumed during growth between 6 and 24 h. (C) In the presence of colistin, growth on *G. stearothermophilus* is inhibited and the glucose concentration remains constant. (D) These changes can rapidly and easily be measured using a commercial enzymatic test strip consisting of glucose dehydrogenase and a redox mediator to transduce glucose concentration to an electrochemically measurable current.

(8.5 g NaCl/1 g peptone/kg water), centrifuged at 5000 *x g* for 5 min twice and resuspended in saline/peptone solution. This bacterial suspension was heated at 80 °C for 10 mins to promote its sporulation (DD ISO/TS 26,844 2006).

The final solution was distributed in 2 ml graduated sterile tubes stored at −20 °C until the toxicity tests. The cell concentration was adjusted saline/peptone solution with a final inoculum concentration of 1×10^6 CFU/ml determined by colony count using the pour plate technique [28]. All growth experiments and toxicity tests were performed at 60 °C. Liquid cultures were shaken at 100 rpm. Samples were taken to measure glucose concentration and to explore the relationship between bacterial growth and substrate consumption. These consisted of blood-glucose meter measurements and optical density measurements at 600 nm (OD_{600}) with a UV–VIS spectrophotometer (Helios Zeta, ThermoFisher Scientific).

2.5. Biotoxicity assessment with colistin

Stocks solutions of colistin were prepared with ultra-pure water on the day of the experiments. A conical flask was filled with 50 ml of LB broth and 1×10^6 CFU/ml of *G. stearothermophilus* suspension (final concentration 2×10^4 CFU/ml). Colistin was added to the liquid broth to a final concentration of 10 μg/L or 10 mg/L. Additional cultures were prepared without colistin and used as controls in the experiments. All flasks were covered with sterile cotton stoppers and incubated at 60 °C with shaking at 100 rpm. Samples were collected at 0, 6 and 24 h to measure glucose concentration and OD600. All toxicity tests were conducted in triplicate.

2.6. High sensitivity microbial inhibition assay

Glucose stock solutions were created by dissolving 3 g/dL glucose into autoclaved LB media and then filter sterilizing to create a 10x stock solution. The was then diluted with LB to create a working stock solution of 300 mg/dL. Chloramphenicol was added to aliquots of LB-Glucose to a final concentration of 1.5 mg/L, G. stearothermophilus to a final concentration of 2×10^4 CFU/ml, and different concentrations of colistin between 400 μg/L and 4 μg/L by a series of three-fold serial dilutions. In addition, three control conditions were produced. The first consisted of 1500 μg/L and 400 μg/L of chloramphenicol and colistin respectively, without B. stearothermophilus (termed the negative control). The second consisted of 1500 μg/L chloramphenicol and 2×10^4 CFU/ml B. stearothermophilus (termed the CHL-Positive control). The final control consisted of 2×10^4 CFU/ml of B. stearothermophilus with neither chloramphenicol or colistin (termed the positive control). The assay was performed with 100 uL of growth media per condition in a 96 well plate, incubated at 100% RH, 60 °C for up to 24 h. Measurements were taken periodically during this time.

3. Results and discussion

3.1. Initial investigation of experimental conditions

The DD ISO/TS 26,844 (2006) protocol was used as a basis for designing the experiments because this assay is robustly used within the dairy industry to test for the presence of antimicrobial residues. However, this methodology determines the presence of antimicrobial agents by observing the pH change in the test tubes with agar. Therefore, the test was optimised here for media with a measurable glucose concentration for detection with the blood glucose meter. Three different liquid media were tested to evaluate bacterial growth, including nutrient broth with manganese sulfate (pH 7.2) from the ISO protocol, LB media [29] and nutrient broth [30]. The most significant change in glucose was observed when LB broth was used to grow the *G. stearothermophilus* (Table 1). Furthermore, turbidity of the culture was greatest during growth with LB broth. Therefore, LB broth was selected for use in subsequent experiments.

Table 1
Identification of optimum liquid growth medium G. stearothermophilus. Glucose concentrations were measured using the glucose test strips and reported by the blood glucose meter.

Liquid media	Initial ($t = 0$ h)		Final ($t = 24$ h)	
	Glucose (mg/dl)	OD600	Glucose (mg/dl)	OD600
LB Broth	216.8	0.006	34.2	0.588
Nutrient Media	205.4	0.006	121.9	0.314
Nutrient Media + MnSO4·H2O	206.0	0.006	92.5	0.359

3.2. Glucose meter performance in a non-blood matrix

Enzyme-based glucometers have been used successfully to measure glucose in different matrices including tears and non-clinical samples [31–33]. However, commercial blood glucose meters are tailored and calibrated precisely for capillary and venous blood samples. Therefore, measurements were carried out to understand the meter's performance in microbial growth media with glucose. Initially, we measured across a wide range of glucose concentrations from 600 mg/dL down to 50 mg/dL. Measurements of "high" were recorded above concentrations 400 mg/dL glucose, therefore we focused upon a lower measurement range for subsequent experiments. A standard curve between 300 mg/dL and 60 mg/dL shows that there is a linear relationship between the known glucose concentration within LB media and the concentration reported by the meter (Fig. 2A).

A potential problem with using an enzymatic biosensor is associated with interferents that could be found in environmental samples. Soil is rich in a wide range of biomolecules, macro and micro particles, inorganic particles and microorganisms. Therefore, we conducted a simple extraction on a standard topsoil sample to establish whether this had an inhibiting impact upon the performance of the glucose meter, and whether the glucose adsorbs to larger molecules within the soil to change the measurement observed with the meter. Very little difference was observed between the extracted soil and measurements in extracted soil (Fig. 2B). This indicated that for a more complex sample, the performance of an enzymatic glucose meter remains acceptable. Although this result is welcomed, further assessment is required to fully elucidate the performance of the sensors system through the implementation of environmental samples suspected of containing antimicrobial residues.

The glucose concentrations reported by the meter differed slightly from the actual concentrations found in the solution. This is likely caused by the difference in composition between LB media and human blood. Differences in salt concentrations between LB media and blood will have an impact upon the pseudo reference potential of the blood glucose meter and therefore impact the working electrode potential, driving a change in reaction rate and therefore measured current. Furthermore, non-specific interactions between Mut. Q-GDH could differ between the two matrices, resulting in differences to the measurements. Importantly, these standard curve measurements demonstrate that, within LB media, a low-cost commercially available glucose meter and test strip can be used to semi-quantitatively determine glucose concentration, or quantitatively determine glucose concentration (if a standard curve is created).

3.3. Growth characterisation in the presence of glucose

Growth of *G. stearothermophilus* was explored with two different starting concentrations of glucose (300 mg/dL and 200 mg/dL), added to sterile LB media. These concentrations were selected based on the standard curve as they sit well within the measured linear range (Fig. 2). The cultures were incubated for 22–24 h, with regular measurements

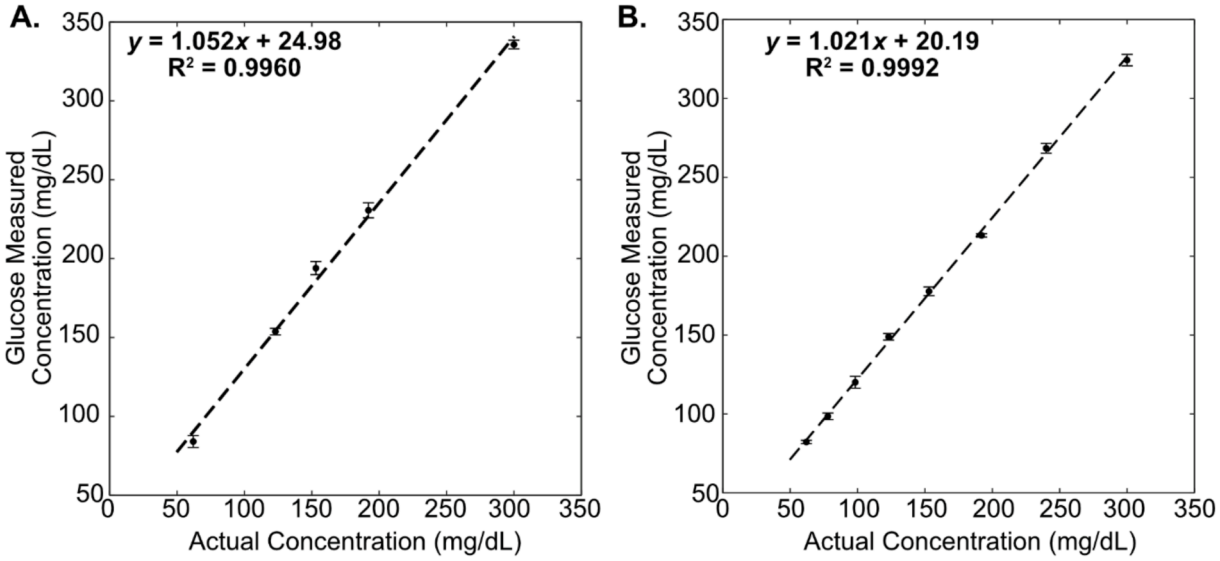

Fig. 2. Glucose test strip standard curves. (A)Sterile LB media containing different glucose concentrations from 62 mg/dL to 300 mg/dL. (B) Measurements in soil extracted using LB-glucose. Dashed line represents linear fit, equation inset. Error bars +/- 1SD, $n = 3$.

taken for the first eight hours. The lag phase, log phase and the start of the stationary phase all occurred during this time.

At 300 mg/dL of initial glucose, exponential growth started between 2 and 4 h of cultivation and was observable as a simultaneous increase in turbidity and a decline in glucose concentration (Fig. 3A). The mean glucose concentrations between the 8 h and 22 h were 113 mg/dL and 111 mg/dL respectively as reported by the glucose meter, suggesting that at a starting concentration of 300 mg/dL glucose remains available as a carbon source well beyond the end of log phase growth. This is most likely caused by the depletion of a critical nutrient required for growth [34]. In other studies, the stationary phase has been attributed to the complete depletion of oxygen within the culture [35], although this is unlikely to be the case here because the cultures were grown in an O_2 rich environment due to the use of conical flasks and shaking during incubation. Overall, the results demonstrate that the system developed can monitor the metabolism and growth of *G. stearothermophilus*.

Similar results were observed for growth with a 200 mg/dL starting culture, whereby glucose concentration changed inversely followed the turbidity (Fig. 3B). However, two key differences are apparent with this lower starting glucose concentration, contrasted with 300 mg/dL. Firstly, *G. stearothermophilus* takes longer to reach the stationary phase when the initial glucose concentration was lower (7 h vs 5 h for 200 mg/dL and 300 mg/dL, respectively). This suggests that a higher concentration of glucose increases the doubling rate of the bacteria and could be important from a fidelity perspective when using the *G. stearothermophilus* for an antimicrobial residue test. Secondly, the measured glucose concentration drops to 13.8 mg/dL at the 24-h measurement. This corresponds to an actual glucose concentration of zero and suggests that the substrate is fully exhausted after a 24-h incubation at a lower initial glucose concentration of 200 mg/dL, but when the higher glucose concentration of 300 mg/dL is used, some glucose remains into the stationary phase of growth.

3.4. Initial microbial inhibition testing with colistin

Colistin was selected as a model residue for detection using the glucose measurement system developed here, due to its use as a drug of last resort. G. stearothermophilus has been characterised previously in the context of a commercial test for multiple other antimicrobial residues in food, but this does not include Colistin [36] Cultures of *G. stearothermophilus* were prepared as described in Section 1.4 and grown in LB medium supplemented with 200 mg/dL glucose. Measurements were performed at 0, 6, and 24 h to measure glucose concentration using the blood-glucose meter and test strips. Two experiments were performed with different starting concentrations of colistin. In the first experiment, starting concentrations of 1 mg/L and 10 mg/L of colistin were tested (Fig. 4A). At 6 and 24 h, the glucose concentration of samples inoculated with 1 mg/L and 10 mg/L of colistin remained the same as measured at the experiment's start. In contrast, the negative control showed a significant drop in glucose concentration from 6 h for both 1 mg/L and 10 mg/L colistin (two--sample *t*-test, both $P = 0.00001$). A further drop in the negative control glucose concentration at 24 h resulted in a mean measured glucose concentration of 13.8 mg/dL, consistent with the growth curve measurements performed above.

Following these results, a further analysis was performed to determine the lower limit at which colistin could be detected. To achieve this, 10 μg/L and 100 μg/L of colistin were used as the starting inoculum within the measurements performed (Fig. 4B). At 24 h, the glucose concentration was exhausted in all electrode chambers. However, after six hours of incubation, a colistin concentration of 100 μg/L resulted in partial inhibition *G. stearothermophilus*, which increased the lag phase and thus the time required for growth and depletion of glucose.

Colistin is employed to treat infections mainly caused by Gram-negative pathogens [37]. Colistin destabilises the lipopolysaccharide (LPS) components in Gram-negative bacteria. In Gram-positive bacteria, the mode of action is still in debate [38]. Although role of colistin on *G. stearothermophilus* is not reported, its effect on other bacillus strains has. Yu et al. (2019) suggested that the presence of colistin may stimulate the respiratory chain in Gram-positive bacteria (including *Bacillus subtilis)*, leading to increased concentrations of NAD^+/NADH and oxidative stress which subsequently kill the cells [39]. Based on this, a similar inhibition mechanism may occur with *G stearothermophilus*, affecting metabolic activity, explaining the reduction of substrate utilization seen here as a delay to the start of growth with colistin concentrations below 1 mg/L. Further optimization of the experimental conditions, such as adjusting the amount of inoculum [22] or adding supplements to the media (DD ISO/TS 26,844 2006), may improve the sensitivity of the assay.

3.5. High sensitivity colistin assay

The inhibition results with colistin suggest a detection limit for colistin between 100 μg/L and 10 μg/L. To explore this further, and

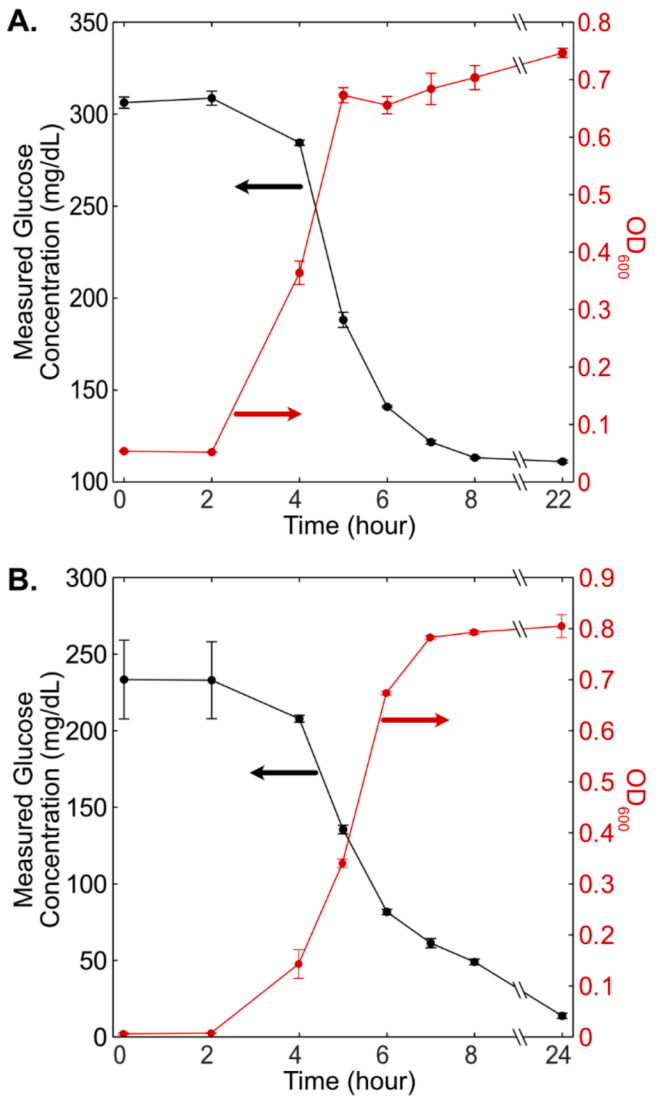

Fig. 3. Glucose metabolism during growth of *G. stearothermophilus* in LB media supplemented with glucose. (A) Initial glucose concentration: 300 mg/dL. (B) Initial glucose concentration: 200 mg/dL. Error bars +/- 1SD, $n = 3$.

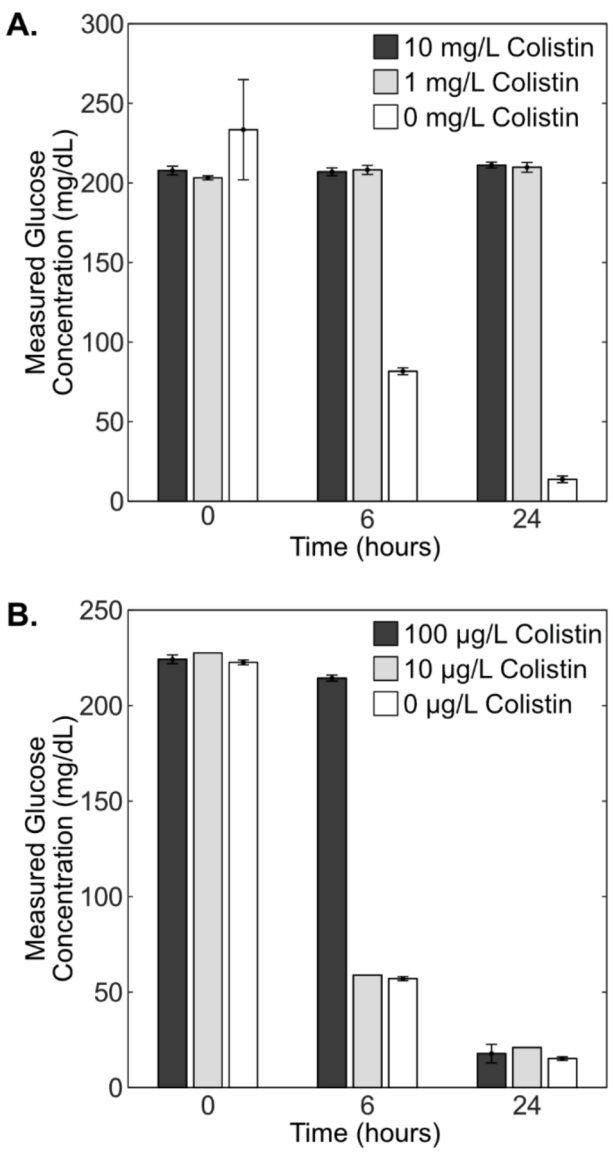

Fig. 4. Glucose metabolism by *G. stearothermophilus* in the presence of colistin. (A) measurements at 10 mg/L and 1 mg/L, showing full inhibition of growth (error bars +/- 1SD, $n = 3$). (B) measurements at 100 µg/Land 10 µg/L($n = 1$) showing partial inhibition of growth (error bars +/- 1SD, $n = 3$ unless otherwise stated).

attempt to increase the sensitivity of the assay, a series of experiments were performed in LB-glucose media supplemented with chloramphenicol, which is used to increase the sensitivity of G. stearothermophilus to other residues through the synergistic effect that multiple resides can have [27]. Following initial analysis of inhibition, guided by the ISO standard [27], we selected a chloramphenicol concentration of 1.5 mg/L which we found slowed but did not inhibit the growth of G. stearothermophilus. Additionally, adopting a 96 well plate format for the assay increased the ease with which multiple measurements across different concentrations of colistin could be performed. The use of chloramphenicol dramatically increased the sensitivity of the assay. Measurements performed at 3 h and at 5 h on the control measurements did not indicate that any growth had occurred. However, at the 24 h measurement point, it was seen that growth was inhibited down to a colistin concentration of 8 µg/L(Fig. 5).

4. Study limitations and future direction

The results described above indicate that the principle of using an enzymatic assay to test for antimicrobial residues. Whilst there are many studies and indeed commercial tests for the detection of antimicrobial residues within food products, little analysis has been carried out of colistin detection using biosensors [22]. Whole cell biosensor based approaches haven been adopted previously where *E. coli* has been genetically modified to include a plasmid with a luciferase reporter molecule which is expressed in the presence of colistin. It was found that the response was activated after the bacteria was exposed to more than 500 µg/L of colistin [23]. In terms of relevant detection limits, several countries mandate a Maximum Residue Limit for the presence of a wide range of drugs within food and drink. These are designed to protect consumers from harmful or adverse effects of drugs including antimicrobial residues. In the case of colistin, this is set between 50 ug/Kg and 300 ug/Kg for milk and eggs respectively [40].

Although the detection limit found within this study holds promise, a key question remains around the role of other inhibiting artefact that may exist in extracted environmental samples which could affect the accuracy of the assay. This was briefly explored in this study, by filter sterilizing extracted soil samples and measuring the change in glucose concentration before and after growth in LB-glucose. Whilst a twofold drop in glucose concentration was observed, comparable to that

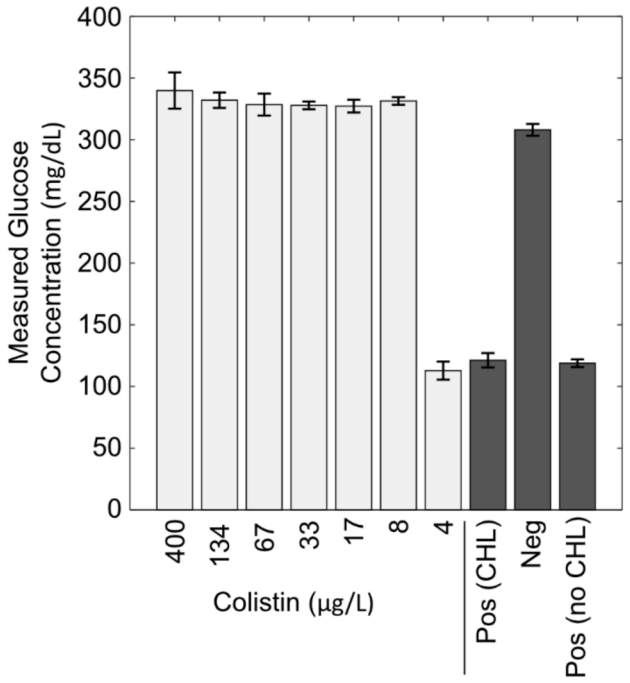

Fig. 5. High sensitivity assay for colistin. Growth chambers were supplemented with chloramphenicol (final concentration 1.5 mg/L) to increase the sensitivity of *G. stearothermophilus* of colistin. Pos(CHL) – wells contained 1.5 mg/L chloramphenicol and G. stearothermophilus; Neg – contained both colistin at 400 µg/L and chloramphenicol at 1.5 mg/L; Pos(no CHL) contained *G. stearothermophilus* and no antimicrobial residues. Error bars: +/- 1 SD, $n = 3$.

observed in other experiments reported here, a more in-depth study is required with a wide range of environmental samples to fully explore the extent and applicability of the principles described here.

5. Conclusions

In this study, we evaluated the use of *G. stearothermophilus,* towards screening antimicrobial residues in environmental samples. The principle of the bioassay consists of the assessment of bacterial activity using a commercial blood glucose meter to measure glucose changes as a metabolic response. We selected colistin, a last-resort antibiotic, in the experimental testing and compared the results with control cultures. The findings show that the presence of colistin at concentrations of 100 to 10 mg/L inhibited the glucose consumption of the *G. stearothermophilus.* cultures after 24 h. Furthermore, *G stearothermophilus* susceptibility to colistin can be enhanced by the use of chloramphenicol and under these conditions, an inhibition concentration of 8 µg/L of colistin is achievable. These findings suggest that this method can be applied to detect antimicrobial residues in samples, offering an streamlined and low-cost solution for screening antimicrobial agents in environmental matrices. Further work is required to optimize testing conditions to improve the method sensitivity and to create sample preparation processes that will remove other commonly encountered toxic chemicals, such as heavy metals, which can also inhibit *G stearothermophilus* growth.

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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