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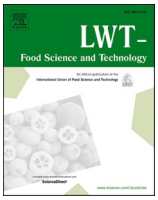

A modification of the resazurin cell viability assay, suitable for the quantification of lactic acid producing bacteria

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ABSTRACT

The conventional resazurin cell viability assay is based on the conversion of resazurin into the fluorescent molecule resorufin in the presence of metabolically active cells. Nevertheless, this method encounters limitations derived from medium acidification by lactic acid bacteria. Here we propose a two-step modification of the resazurin method for accurate lactic acid bacteria growth and prebiotic assessment without pH interference. Using a polyphenolic extract from *Cytisus scoparius* as a test prebiotic, this study demonstrates the efficacy of a two-step resazurin assay in quantifying lactic acid bacteria growth underscoring the importance of innovative methodologies for prebiotics screening.

1. Introduction

Resazurin is a blue, non-fluorescent, and non-toxic dye that becomes pink and fluorescent when reduced into resorufin by oxidoreductases within viable cells (Labadie et al., 2021; Travnickova et al., 2019). It is commonly used to evaluate cell growth, particularly for cytotoxicity assays (Pala et al., 2020) and antimicrobial evaluations (Barros et al., 2021; Jung et al., 2017). Despite extensive research on the potential applications of resazurin, few studies have focused on its compatibility with coloured natural products (Madushan et al., 2021) and its role in discovering new substrates that enhance the growth of beneficial bacteria, such as lactic acid bacteria (LAB) (Milutinović et al., 2021).

LAB constitute one of the most interesting bacterial groups related to the microbiota, not only because of the bacteriocin production (Zacharof & Lovitt, 2012) and antagonist activity with pathogens' growth (Mariam et al., 2014; Zhang et al., 2023), but also because of other metabolites production, such as short chain fatty acids (SCFA) and vitamins (Tang et al., 2023), closely related to microbiota and overall health. Hence, the demand for faster and more accurate methods to quantify and monitor the activity of LAB is growing. Currently, approaches such as plate culturing, turbidity measurement, and qPCR (Lee et al., 2021) are employed to assess the growth and proliferation of these microorganisms. Although colony counting on agar plates is the most common method for measuring viable cells (Patil et al., 2014), it is slow and subject to significant variations. Turbidity measurement methods offer a faster and easier approach, but they do not distinguish between living and dead bacteria. Furthermore, research on plant extracts and turbidity methods may not be compatible due to the colorimetric interference of dark-pigmented substances (Simeonov & Davis, n.d.).

Previous research has shown that resorufin can be reduced into dihydroresorufin in acidic or poorly buffered environments (Fig. 1), resulting in a loss of fluorescence emission and breaking the correlation between resazurin fluorescence and bacterial viability (Jung et al., 2017; Labadie et al., 2021). As the growth of LAB inevitably turns the medium acidic (Labadie et al., 2021), the previous detection method becomes unsuitable for assessing the viability of such cells.

To adapt the resazurin fluorescence methodology for quantifying LAB and to apply it to the discovery of new prebiotic sources, we propose an additional step to the standard resazurin protocol. This approach includes a second incubation step, in which the overnight bacterial culture is transferred into fresh culture medium containing resazurin. This step is designed to prevent the accumulation of acidic metabolites and to reduce interference with coloured compounds. We selected a prebiotic polyphenolic extract from *C. scoparius*, previously described as a potential antioxidant and antipathogenic agent (Calvo et al., 2023), to assess its influence on the growth of LAB and the compatibility of the method with resazurin fluorometric reading.

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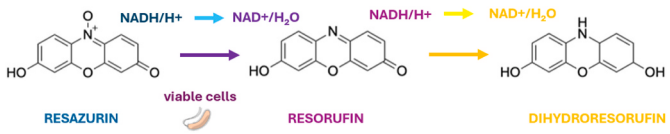

Fig. 1. Reduction of resazurin by viable cells and formation of resorufin product. Acidic environments enhance reduction of resorufin into dihydroresorufin. Resazurin, a non-fluorescent blue dye, is converted into resorufin, a pink product with high fluorescence emission. Fluorescence emission stops when resorufin is reduced into dihydroresorufin, a yellowish-white molecule.

2. Methodology

2.1. Reagents, media and bacterial strains

Bacterial strains were purchased from the Spanish Type Culture Collection (CECT). Bacterial strains were purchased from the CECT. *Lacticaseibacillus rhamnosus* CECT 275 was selected as a representative LAB probiotic strain. *Escherichia coli* ATCC 25922CECT was selected as a non-lactic acid bacteria control.

Culture media, TSA (Tryptone soja agar), and MRS (Man Rogosa Sharpe Agar) were purchased from Condalab (Madrid, Spain) and cation Adjusted Müller Hinton II broth (CAMHB) from Becton-Dickinson (BBL, Sparks, NV, USA). Commercial resazurin solution alamarBlue from ThermoFisher Scientific (Waltham, MA, USA) was employed as enzymatic substrate for the resazurin cell viability test, following the manufacturer's indications.

C. scoparius' extract was provided by i-Grape Laboratories and obtained as previously described by Calvo et al., 2023. Briefly, plant samples were collected and air-dried. Medium scale ambient temperature (MSAT) procedure using a hydro organic solvent as an eluting was employed to extract polyphenols from the plant-waste (Castillo et al., 2022; Lores et al., 2015).

2.2. Inoculum preparation

L. rhamnosus was grown on MRS medium plates, which were incubated for 48 h at 37 °C. TSA medium was used to culture E. *coli* at 37 °C for 24 h. A couple of colonies were transferred to 2 mL of CAMHB 2X or CAMHB-MRS 2X depending on the bacterial species. Following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations about fastidious bacteria medium supplementation, in the assays with LAB, CAMHB was minimally supplemented with 4% Man, Rogosa and Sharpe (MRS) medium, to achieve the correct bacterial growth. The optical density of the inoculum was measured at 600 nm wavelength and adjusted to match the 0.5 McFarland turbidity standard (A_{600nm} = 0.08–0.10, ca. 10^8 CFU/mL). The bacterial culture was then diluted to a final inoculum size of 10^6 colony forming units per mL (CFU/mL) in accordance with the EUCAST guidelines.

2.3. Calibration curves of resazurin reduction

To define the correlation between the bacterial concentration and the intensity of the resorufin fluorescence, resazurin reduction curves for all bacterial species were plotted. The bacterial suspensions were prepared following the methodology described by Manso et al., 2023. Briefly, bacterial cells recovered from overnight plates were resuspended in CAMHB or CAMHB-MRS to a final cell concentration of $5x10^5$ CFU/mL. Starting from this inoculum, two-fold serial dilutions with CAMHB medium were performed in a 96-multiwell plate and 20uL of alamarBlue resazurin were added to a final volume of 200uL in each well (resazurin solution (5mg/100 mL) was added to the wells to a final concentration of 0.5mg/100 mL). Bacteria were incubated at 37 °C for 30 min - 1 h until blue to pink colorimetric change was observed. Resazurin metabolization by bacteria was measured by fluorimetry at an excitation wavelength of 544 nm and emission wavelength of 590 nm using the FLUOstar microplate reader.

2.4. Plate preparation

Initially, 100 μL of a bacterial culture of 10^6 CFU/mL in 2X CAMHB (*E. coli*) and 2X CAMHB- MRS (*L. rhamnosus)* were added to the wells of a 96-multiwell microplate and mixed with 60 μL of phosphate buffered saline (PBS) 1M to control the extract's possible pH variations. Stock solutions of the extract were prepared in water, so that after addition of 40 μL of the corresponding stock in each well, the final concentrations of the extract were 0%, 0.625%, 1.25%, 2.5%, 5%, 10%, and 20%, being the final volume 200 μL Cell-free blanks of the extract were used, in order to detect plant extract fluorescence interferences.

2.5. Resazurin one-step assay

Fluorometric resazurin conventional method was employed to determine the effect of *C. scoparius*' extract on the growth of LAB and *E. coli*. For that purpose, 96-multiwell plates were prepared as described in section 2.4. Microplates were incubated overnight at 37 °C. After the overnight incubation, 20 μL of alamarBlue resazurin solution were added to each well. Once colorimetric change was observed (after 30 min for *E. coli* and 60 min for the lactobacilli), resazurin reduction to resorufin was measured using the FLUOstar microplate reader.

2.6. Resazurin two-step assay

In order to assess the effect of *C. scoparius*' extract on lactic acid bacteria avoiding the residual acidification produced by the metabolites of LAB strains' growth, a two-step viable cell account by fluorometric reading was used. The 96-multiwell plates were prepared and incubated overnight as specified in section 2.4. After the overnight incubation, 100 μL of fresh CAMHB 2X, 60 μL of phosphate-buffered saline (PBS, 1M), 20 μL of alamarBlue resazurin solution and 20 μL of each well from the overnight incubated plate were mixed in a new 96-well microplate. As described previously, alamarBlue resazurin method followed by fluorometric reading was performed to quantify viable cells.

2.7. Plate count validation assay

To test the accuracy of the fluorometric assays, bacterial strains were exposed to different extract concentrations as previously described. Subsequently, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-8} dilutions of the tested cultures were seeded on MRS agar or TSA, depending on the bacterial strain. The plates were incubated at 37 °C during 48-h in the case of *L. rhamnosus* and overnight in the case of *E. coli*. Final correlation between UFC/mL count and fluorescence values were setup.

2.8. Statistical analysis

All experiments were performed in triplicate. Media and standard deviation of all samples were calculated and represented graphically. Blank data interference was subtracted from the final measurement values. Bacterial growth tendency was expressed as a percentage, being the non-treated samples the reference control for data normalization. Statistical analyses were performed using the Software GraphPad Prism 9.0.

3. Results and discussion

3.1. Conventional resazurin assay

In recent years, colorimetric and fluorometric methods have been gaining attention as bacterial and fungal cell growth indicators (Monteiro et al., 2012). Among them, resazurin is one of the most

employed and well known (Schmitt et al., 2013), and it has been extensively used, especially for antimicrobial assessments and bacterial resistance studies (Jia et al., 2020). Although the resazurin conventional assay outstands as a fast and accurate alternative to conventional turbidity and cell or colony counting methods, its applicability is limited when studying prebiotics on acid-producing bacteria such as LAB (Jung et al., 2017).

The reduction of resazurin to resorufin is directly correlated with bacterial viability. However, this correlation is affected by the medium's pH (Elshikh et al., 2016). During their growth, LAB causes a dramatic decrease of the pH due to the release of large amounts of lactic acid as a fermentation byproduct.

To solve this pH interference problem, our work proposes a two-step modification of the conventional alamarBlue resazurin-based method that solves the afore mentioned limitation and offers the possibility to extend its application to the study of LAB growth.

Cation Adjusted Muller Hinton broth (CAMHB) was the culture medium used throughout these assays. This medium is a buffered version of Mueller Hinton broth, which is the standard medium recommended by EUCAST for susceptibility testing. This buffered version has comparable results and improves the assay quality when testing acid substances.

Firstly, in order to prove the correlation between the resazurin reduction and the number of viable cells, calibration curves for *L. rhamnosus* and *E. coli* were set up (Fig. 2).

Nevertheless, when we assayed the effect of *C. scoparius*' extract on *L. rhamnosus* and *E. coli*'s growth, the previously assessed correlation was only maintained in *E. coli*. Similar results were previously reported by Jung et al., 2017, who observed a blue to yellow color change when applying the conventional resazurin assay to the assessment of LAB's growth.

The explanation for this phenomenon, which is accompanied by a cancellation of the detectable fluorescence, is the acidification of the medium, due to the high production of LAB acidic metabolites, which promotes the reduction of resazurin into dihydroresorufin, instead of resorufin (Fig. 3A). When we applied the standard resazurin method to the detection of *L. rhamnosus*, the wells turned yellow in the presence of metabolically active cells, in spite of the use of buffered culture medium (Fig. 3D). This prevents the emission of fluorescence and therefore the detection of living cells (Fig. 3F). On the contrary, the method has proven to be suitable for *E. coli* and other non-LAB, since the buffered culture medium employed is enough to maintain a stable pH during the cell growth, and therefore to detect the fluorescence emission of resorufin (Fig. 3C). Fig. 3I and J shows the plate count and fluorescence detected on *E. coli* growth when exposed to different *C. scoparius* concentrations, respectively. A good correlation was observed between both methodologies when *E. coli* was evaluated. On the contrary, unrelated values were detected in the case of *L. rhamnosus*, (Fig. 3E and F). These results support the evidence of LAB metabolites accumulation and its interference with fluorescence detection.

3.2. Two-step resazurin assay

To adapt the well-known conventional one-step resazurin method to the growth of lactic acid bacteria, an additional step was added. This additional step consists in a second incubation of the overnight bacterial growth in fresh buffered medium with resazurin during the period of time needed to observe a color change (30 min for *E. coli*, 60 min for *L. rhamnosus*) (Fig. 4). By using this method, the incubation time can be extended, which is essential to assess the possible prebiotic effect of the extract on LAB, avoiding fluorescence interferences due to the acidic metabolites.

Fluorescence measurements during the growth of *L. rhamnosus* and *E. coli* using this two-step resazurin methodology are presented in Fig. 3G and K, respectively. Significant differences were observed between the conventional one-step method and the two-step approach when assessing the impact of *C. scoparius* extract on *Lactobacillus* (Fig. 3B), which supports the hypothesis of acid metabolite interference. The fluorescence data from the two-step resazurin assay are consistent with the results obtained through standard plate counting, (Fig. 3E–G), in contrast to the discrepancies observed with the conventional one-step assay (Fig. 3F). Correlation analysis supports the suitability of the proposed method for LAB growth detection in comparison to plate counting (Fig. 3I).

Finally, these assays offer initial evidence that *C. scoparius* extract promotes the growth of beneficial bacteria while inhibiting pathogenic strains. These findings, combined with prior research demonstrating the extract's inhibitory effects on foodborne pathogens (Calvo et al., 2023), highlight its selective action. This selectivity underscores the extract's potential as a prebiotic, with promising implications for gut microbiota modulation and pathogen control.

4. Conclusions

The alamarBlue assay is a well known method for viable cell quantification, based on the detection of fluorescence emitted when resazurin is reduced into resorufin by the cell metabolism. pH acidification by LAB results in resorufin reduction to non-fluorescent dihydroresofurin, leading to misinterpretations of the results. Using a *C. scoparius*' extract with a prebiotic effect, this research has resulted in an improvement of the resazurin cell viability protocol, making it suitable to assess the growth of acid producing microorganisms. Incompatibility of low medium pH with conventional resazurin method and its negative correlation with standard plate count validation when studying the growth of

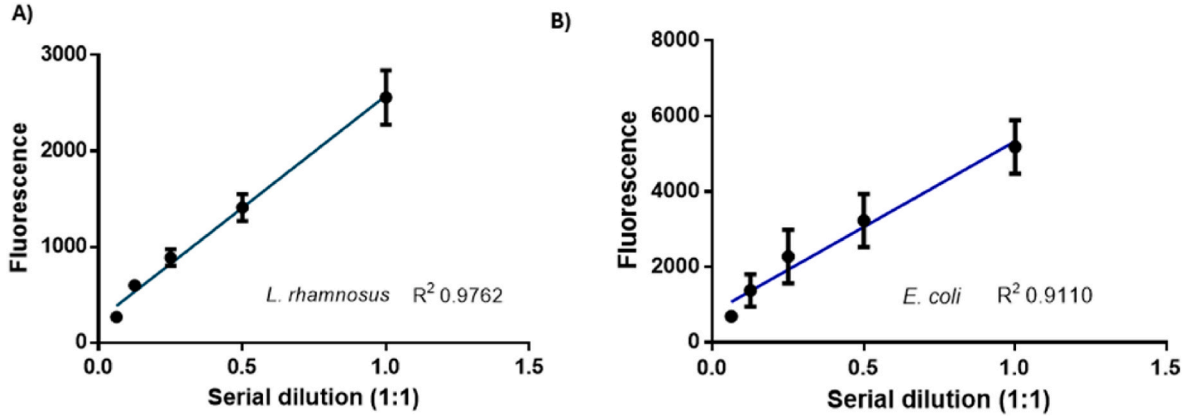

Fig. 2. Resazurin cell viability assay calibration. Overnight bacterial cultures were secuentially two-fold diluted and resazurin to resorufin fluorescece emission was measured. A) *L. rhamnosus* calibration curve. B) *E. coli* calibration curve.

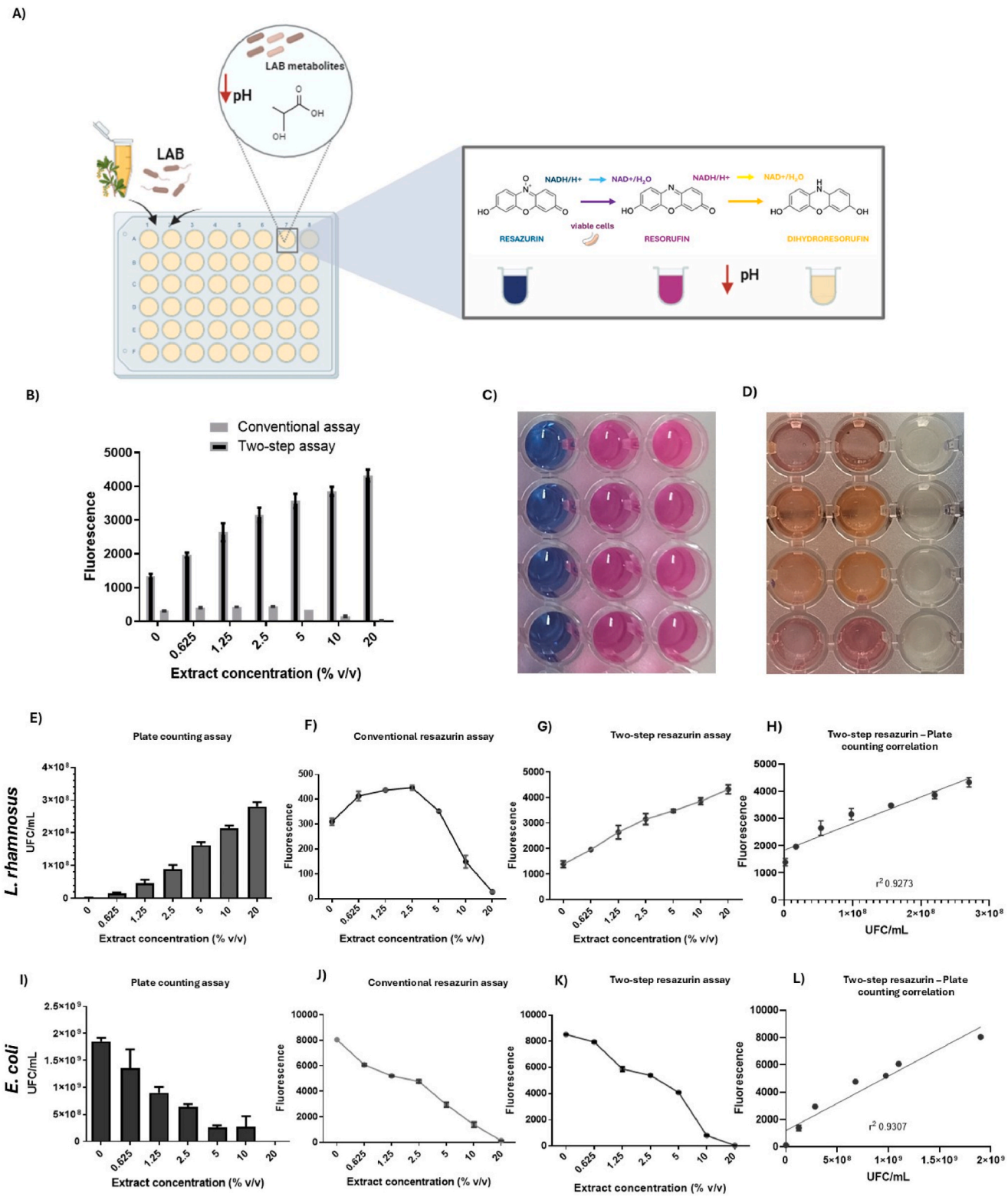

Fig. 3. A) Schematic explanation of resazurin overreduction to dihydroresorufin due to the growth of LAB. B) Comparison of the fluorescence emission under conventional one-step and the proposed two-step assays, during growth of *L. rhamnosus* exposed to different concentrations of *C. scoparius*' extract. C) One-step resazurin reduction assay: resazurin (Blue) reduction to resorufin (pink) by *E. coli*. D) One-step resazurin reduction assay: resazurin reduced to dihydroresorufin by acidic metabolites produced during LAB growth. Orange, yellowish, soft pink and transparent colours are associated with extreme resazurin reduction. E) MRS plate count of *L. rhamnosus*' growth tendency after being exposed to a wide range of extract concentrations. F) Conventional resazurin assay fluorescence variation during *L. rhamnosus*' growth. G) Two-step resazurin fluorescence variation during *L. rhamnosus* growth under different *C. scoparius* extract concentrations. H) *L. rhamnosus* viability regression between two-step fluorescence vs UFC/mL values. I) *E. coli* plate count growth tendency after being exposed to a wide range of extract concentrations. J) Conventional one-step resazurin fluorescence variation during *E. coli* growth under different *C. scoparius*'extract concentrations. K) Two-step resazurin fluorescence variation during *E. coli* growth under different *C. scoparius'* extract concentrations. L) *E. coli* viability regression between two-step fluorescence and UFC/mL. Fig. 3A was created with BioRender.com.

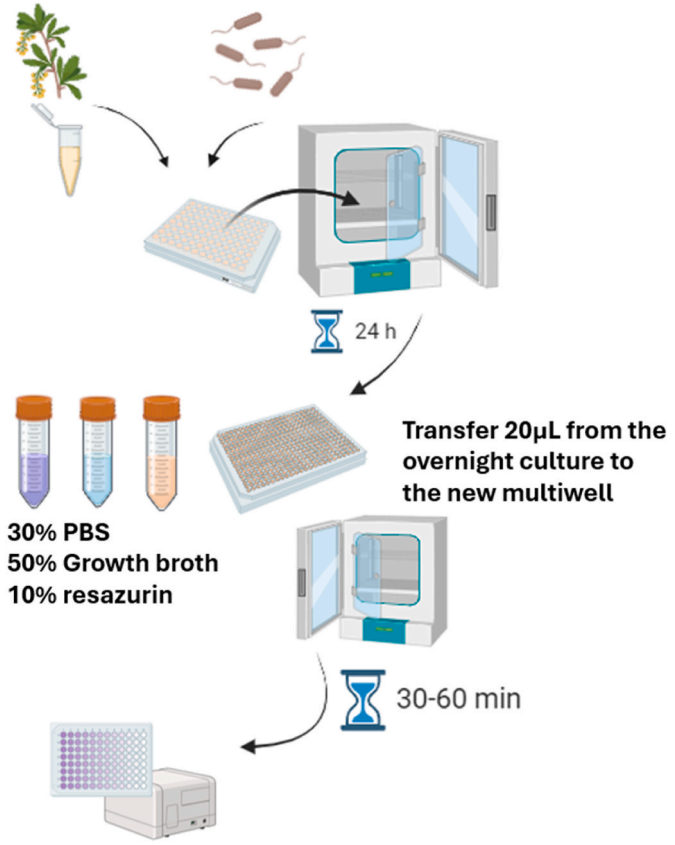

Fig. 4. Diagram of the two-step resazurin method. Created with BioRender.com.

LAB was assessed. The proposed two-step resazurin assay shows no pH interference with fluorescence emission, having a highly positive correlation with the plate count method recommended by the EUCAST guidelines. *E. coli* used as a non-lactic acid bacterium control, showed similar results using both methodologies, which supports the use of the proposed modification to assess the growth of different microorganisms. This newly developed two-step assay will allow a more accurate assessment of acid producing bacteria's growth and a faster natural prebiotics identification.

CRediT authorship contribution statement

Lorena G. Calvo: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Conceptualization. **Rosa-Antía Villarino:** Methodology, Formal analysis. **José Luis R. Rama:** Methodology, Conceptualization. **Ana G. Abril:** Writing – review & editing, Supervision, Investigation. **Trinidad de Miguel:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Conceptualization.

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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Data availability

No data was used for the research described in the article.

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