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Microcalorimetric study of the growth of *Enterococcus faecalis*, *Pseudomonas aeruginosa* and their mixtures in an enriched culture medium

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Abstract Microcalorimetry is a highly sensitive experimental technique that allows to determine the energy released by any process or transformation. In the field of medicine, it is interesting for investigations of microbial processes. The interaction relationship between *Enterococcus faecalis* and *Pseudomonas aeruginosa* was researched using a Tian–Calvet calorimeter equipped with two stainless steel cells (reference and experimental). Three samples from both bacteria were prepared in the following proportions: 20 + 80 % (0.2 mL *E. faecalis* + 0.8 mL *P. aeruginosa*), 50 + 50 % (0.5 mL *E. faecalis* + 0.5 mL *P. aeruginosa*) and 80 + 20 % (0.8 mL *E. faecalis* + 0.2 mL *P. aeruginosa*). Experiments were carried out at a concentration of 10^3 CFU mL^{-1} and a constant temperature of 309.65 K. Recording the heat voltage difference versus time, the growth curves for *E. faecalis*, *P. aeruginosa* and their mixtures were obtained. The differences in shape of curves of single microorganisms and their mixtures were compared. Also, the thermokinetic parameters of single microorganisms and their mixtures (growth constant, generation time, detection time and amount of heat released) were calculated.

Keywords Microcalorimetry · *Enterococcus faecalis* · *Pseudomonas aeruginosa* · Metabolism · Thermogram

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Introduction

Microcalorimetry is an analytical technique which allowing measurement of heat flow related to biological processes, which is proportional to the rate at which a given chemical or physical process takes place [1]. It is a non-destructive method, with high sensitivity, accuracy and simplicity, which has been extensively applied in physics, chemistry, life sciences and other fields. Also, it provides us some qualitative information, such as the real-time thermogenic fingerprints (heat flow–time curves), and many quantitative parameters, such as growth constant (k), the maximum thermal power (V_{max}), the appearance time of maximum thermal power (t_{max}), the generation time (G) and the amount of heat released (Q). Knowledge of these parameters facilitates the identification and study of the growth of bacterial species. However, this technique presents drawbacks, which are its lack of specificity and that it requires an initial equilibration time of approximately 2 h [1–3].

Microcalorimetry arouses interest in the biological sciences for its potential application in the study of the microbial activities in vitro [4]. Microorganisms produce small amounts of heat in a range of 1–3 pW per cell. Despite the low bacterial heat, their exponential growth in culture media permits their detection within a few hours, even from samples with a low concentration, e.g., 10 colony forming units (CFU) mL^{-1} [3, 5]. In the medical field, this method is very interesting due to the fast-growing nature of microorganisms and the resultant amount of heat that is soon produced [4]. In the last few years, it has been used to study the rapid detection of bacterial growth under different conditions [6–9] or microbial contamination [10–12]. Also, it has been employed to determine inhibitory effects and/or the minimal inhibitory concentration

for different antimicrobial substances [13–16] and antibiotics [17–19] in a few hours. Similarly, antibacterial coatings for implant materials have also been tested using this technique [20]. In addition, microcalorimetry permits rapid differentiation between two species of bacteria of the same genus [21] and rapid detection of resistant strains of a bacterial species within hours [22, 23].

However, despite all the references mentioned above, there are few studies that attempt to investigate the mechanism of interaction between bacteria of clinical relevance employing microcalorimetric techniques [24, 25]. In our study, we researched the interaction relationship between two pathogenic bacteria, *Enterococcus faecalis* and *Pseudomonas aeruginosa*.

E. faecalis is a Gram-positive cocci that grows in pairs or chains and ferment carbon sources to produce lactid acid. It is a commensal organism in the gastrointestinal tract of healthy individuals as well as many other animals. This bacterium can act as opportunistic pathogens in compromised patients with nosocomial infections. *E. faecalis* is intrinsically resistant to many antibiotics and often requires synergistic antibiotic combinations to successfully treat infection [26].

P. aeruginosa is a nonfermenting, aerobic, motile Gram-negative rod that is ubiquitous in the natural environment. It has minimal nutritional requirements and can easily contaminate wet surfaces in the hospital, as well as equipment, devices and solutions. The bacterium secretes a biofilm that protects it and allows it to adhere to tissues and expresses a wide range of virulence factors. *P. aeruginosa* is an important opportunistic pathogen that causes a variety of infections in immunocompromised patients and hospitalized patients. Also, it is resistant to many commonly used antibiotics [26].

Materials and methods

E. faecalis (ATCC 29212) and *P. aeruginosa* (ATCC 27853) were provided by American Type Culture Collection (ATCC). These bacterial strains were streaked onto blood agar plates and incubated at 309.65 K for 24 h. The blood agar plates with multiple bacterial colonies were then used to prepare a bacterial suspension with sterile saline (0.9 % NaCl) and adjusted to an optical density of 0.5 on the McFarland scale. This suspension was diluted with 0.9 % sterile saline to obtain final concentration of 10^3 CFU mL^{-1}. The inoculum concentration was confirmed using the plate count method. Later, mixtures of both bacteria were prepared in the following proportions: 20 + 80 % (0.2 mL *E. faecalis* + 0.8 mL *P. aeruginosa*), 50 + 50 % (0.5 mL *E. faecalis* + 0.5 mL *P. aeruginosa*) and 80 + 20 % (0.8 mL *E. faecalis* + 0.2 mL *P. aeruginosa*).

The measures were carried out using Tian–Calvet microcalorimeter equipment designed by Professor Paz Andrade [27]. It is equipped with a device allowing operation in the absence of vapor phase and has two Teflon® screw-capped stainless steel cells of approximately 10 cm^3 (Fig. 1). One of these cells contains the reference solution, named the reference cell, and the other one the sample, named the experimental cell. A Philips PM2535 multimeter and a data acquisition system were linked to the microcalorimeter. Calibration was performed electrically using a Setaram EJP30 stabilized current source. The precision in calorimetric signal was ±0.01 mW. Further details about the experimental method have been already published by [3, 28].

The external media of the calorimeter was maintained at a constant temperature of 309.65 K. The reference cell was filled with 7 mL of culture medium +1 mL of sterile saline, while the experimental cell was injected with 7 mL of culture medium. The culture medium used was a liquid enriched with soybean-casein (Becton, Dickinson and Company, USA), which is a mixture of processed water (40 mL), soybean-casein digest broth (2.75 % w/v), yeast extract (0.25 % w/v), animal tissue digest (0.10 % w/v), sodium pyruvate (0.10 % w/v), dextrose (0.06 % w/v), sucrose (0.08 % w/v), hemin (0.0005 % w/v), menadione (0.00005 % w/v), sodium

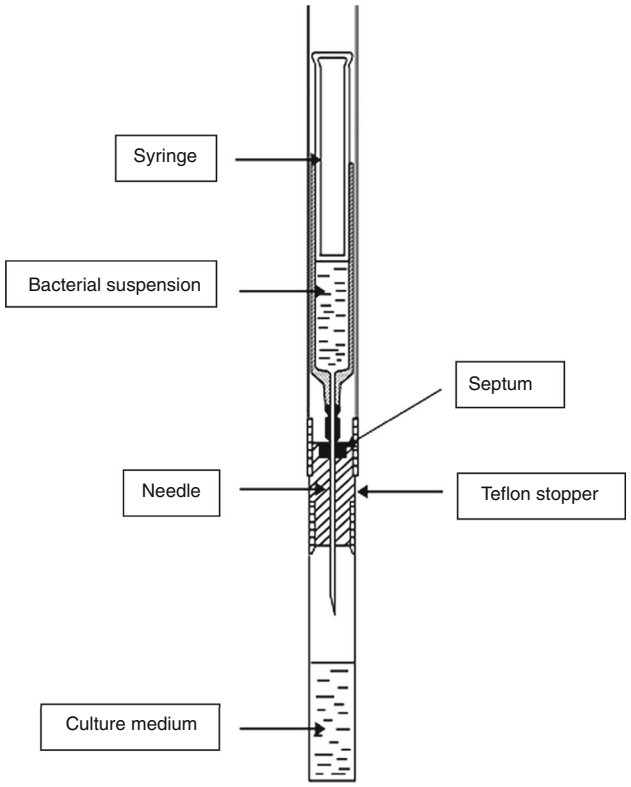

Fig. 1 Scheme of the experimental cell of Tian–Calvet microcalorimeter

polyanetholsulfonate (0.020 % w/v) and pyridoxal HCl (0.001 % w/v). Both cells were introduced from the upper part of the calorimeter in the internal thermopile chamber through two cylindrical holes aligned in parallel. The large distance that separates the cells from the entrance ensures the minimization of heat flow to the exterior. The system is then allowed to stabilize during 2 h, after which 1 mL of the sample is introduced into the experimental cell.

The experiment was also carried out with a sample not containing any bacteria (control). All experiments were realized in triplicate.

Both cells were cleaned and sterilized by autoclaving before using (20 min at 394.15 K).

A data collection and processing system were used to record the electrical signal at intervals of 20 s throughout the duration of the experiment.

Results

When the suspensions of single microorganisms and their mixtures are introduced into the reference cell, the heat voltage signals are recorded to form exothermic heat flow–time curves of *E. faecalis*, *P. aeruginosa* and the mixed microorganisms at different proportions.

The curve of *E. faecalis* [29] (Fig. 2) had one single energetic phase with four peaks of heat flow, and the signal was recorded for about 10 h, returning to baseline levels after this time. The curve of *P. aeruginosa* [3] (Fig. 3) also presented one phase, where the ascending part showed four discrete peaks and the descending one an exponential shape that was extended in time. Probably, this last phase of the curve is related to the ability of this organism to form biofilms, allowing survival in the culture medium, without a net increase in cell number. Such behavior has been

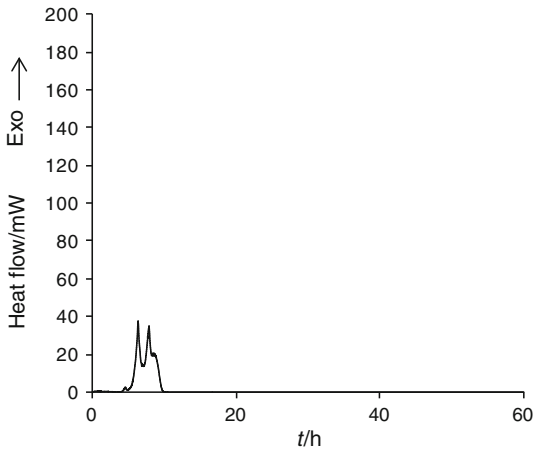

Fig. 2 Graphical representation of heat flow versus time for the *E. faecalis* at 10^3 UFC mL^{-1}

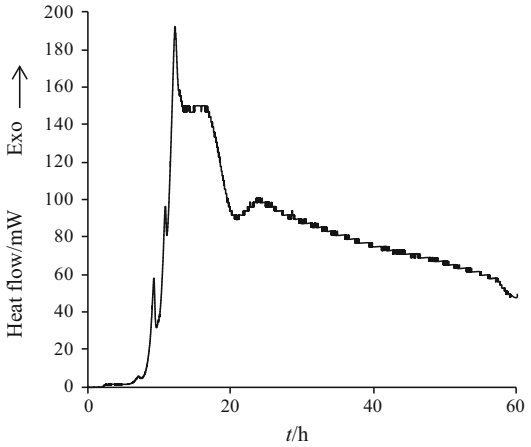

Fig. 3 Graphical representation of heat flow versus time for the *P. aeruginosa* at 10^3 UFC mL^{-1}

observed in various microorganisms that produce biofilms [30, 31]. Although *E. faecalis* also forms biofilm, *P. aeruginosa* is much more efficient at making biofilms.

In the graphs of mixtures of *E. faecalis* and *P. aeruginosa* (Fig. 4), we have seen that the curve of 20 % *E. faecalis* + 80 % *P. aeruginosa* presented one phase with four peaks of thermal power separated by a stretch of the concave curve. The curve of 50 % *E. faecalis* + 50 % *P. aeruginosa* consisted in one phase with two peaks of voltage. In both cases, *E. faecalis* modifies the growth profile of *P. aeruginosa*, even at low proportions in the sample. Finally, in the graph of 80 % *E. faecalis* + 20 % *P. aeruginosa,* two metabolic phases were observed, the first with four lower energy peaks which signal was recorded for about 10 h, and is characteristic of the *E. faecalis* curve; and the second of greater energy that is prolonged over time and is similar to the curve of *P. aeruginosa*.

The results were processed mathematically by adjusting them for exponential equations in ascending and descending phases of each peak of heat flow and polynomial equations in the areas of higher activity of the curve.

By means of the first derivative of the polynomial equations, we determine the value of the maximum thermal power (V_{max}) and the time of its registration (t_{max}) in the curves of *E. faecalis*, *P. aeruginosa* and their mixtures in different proportions. Although we cannot establish a quantitative proportionality relationship of the maximum peaks and their appearance time between pure cultures and their mixtures, all experiments show a maximum peak before 20 h (Table 1).

The detection time of the signal (t_d) of *E. faecalis*, *P. aeruginosa* and their mixtures are presented in Table 1. As can be seen, the sample 20 % *E. faecalis* + 80 % *P. aeruginosa* has a t_d higher than 50 % *E. faecalis* + 50 % *P. aeruginosa* and 80 % *E. faecalis* + 20 % *P. aeruginosa.*

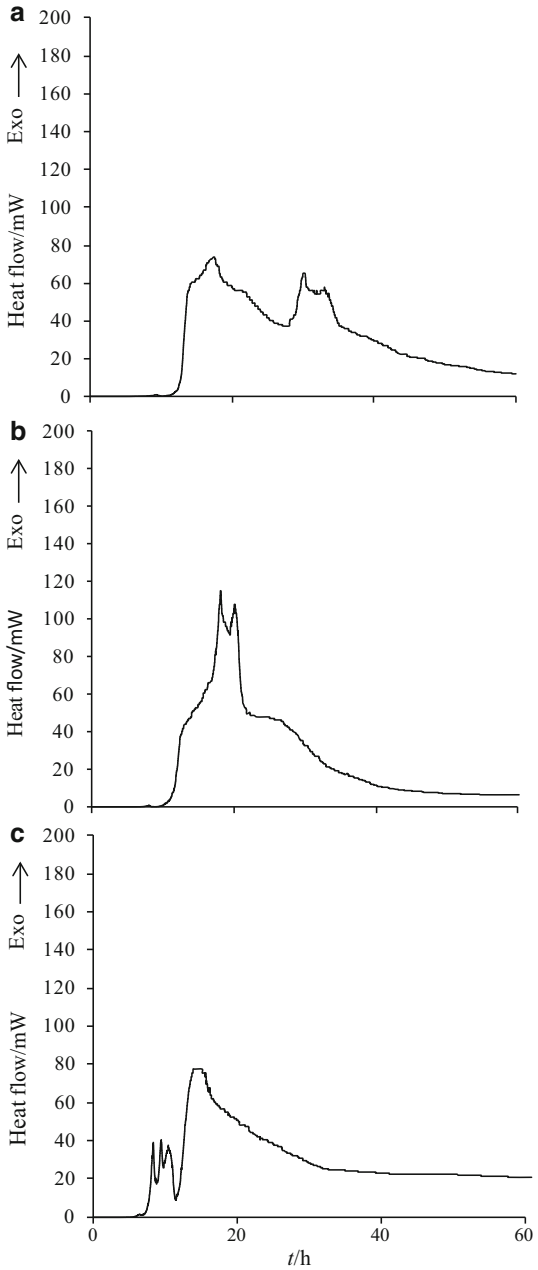

Fig. 4 Graphical representation of heat flow versus time for *E. faecalis* and *P. aeruginosa* at 10^3 UFC mL^{-1} for the different mixtures studied: **a** 20 % *E. faecalis* + 80 % *P. aeruginosa*; b 50 % *E. faecalis* + 50 % *P. aeruginosa* and **c** 80 % *E. faecalis* + 20 % *P. aeruginosa*

This is because *E. faecalis* has a t_d lower than *P. aeruginosa*. In all curves, growth can be detected in less than 8 h.

In the logarithmic phase of the growth curve, we fitted the data to an exponential equation that can be expressed as [32, 33]:

$$n_t = n_0 \times e^{k \cdot t} \quad (1)$$

Table 1 Detection time (t_d), maximum thermal power (V_{max}) and time registration of maximum peak (t_{max}) of *E. faecalis*, *P. aeruginosa* and the mixed microorganisms at 10^3 CFU mL^{-1}

Microorganism	t_d/h	t_{max}/h	V_{max}/mW
E. faecalis	3.63	6.37	37
P. aeruginosa	3.83	12.22	192
20 % *E. faecalis* + 80 % *P. aeruginosa*	7.56	17.02	74
50 % *E. faecalis* + 50 % *P. aeruginosa*	6.85	18.10	115
80 % *E. faecalis* + 20 % *P. aeruginosa*	4.57	13.88	77

where n_0 is the number of bacteria at time 0, n_t the number of bacteria at time t and k the growth constant.

If P_w is the thermal power of each cell, then:

$$n_t \times P_w = n_0 \times P_w \times e^{k \cdot t} \quad (2)$$

Taking into account that P_0 and P_t are the energies released at onset and at time t, respectively:

$$P_t = P_0 \times e^{k \cdot t} \quad (3)$$

$$\ln P_t = \ln P_0 + k \times t \quad (4)$$

Thus, the growth constant (k) can be obtained by selecting two points from the exponential phase of a bacterial growth curve.

The generation time (G) is defined as the time taken by a population to duplicate its number and is expressed as:

$$G = \frac{(\ln 2)}{k} \quad (5)$$

Therefore, deriving from the mathematical adjustment of the powers obtained from the microcalorimetric study of bacterial cultures, we can quickly and easily extrapolate the value of the growth constant and the generation time of the bacteria and their mixtures in different proportions. Methods generally used to achieve such parameters are quite complex, tedious and require a lot of time. In Table 2 are summarized the values of k and G of studied bacteria.

From the curve, we can also calculate the amount of heat released (Q) over the culture time:

$$Q = K \times A \quad (6)$$

where A (mW h) is the area, calculated by the trapezoidal method, and K represents a constant whose value is 33.1 J mW^{-1} h^{-1}, which was calculated from the electric calibration performed by the Joule effect on the equipment.

Also, we have compared the amount of heat exchanged by *E. faecalis*, *P. aeruginosa* and their mixtures during the first 24 h (Q_{24}). In Table 3, the Q_{24} by *E. faecalis* is approximately eight times less than *P. aeruginosa*, because the metabolic activity of *Enterococci* is shorter than *P. aeruginosa*. However, the results showed the mixture 50 % *E. faecalis* + 50 % *P. aeruginosa* had a Q_{24} lower than expected, due to the interaction between both bacteria.

Table 2 Growth constant (k), generation time (G) and standard deviation obtained from the heat flow–time curves of *E. faecalis*, *P. aeruginosa* and the mixed microorganisms at 10^3 CFU mL^{-1}

Microorganism	k/h^{-1}	G/min	s/mW
E. faecalis	2.817	14.64	0.56
P. aeruginosa	0.930	44.46	2.27
20 % *E. faecalis* + 80 % *P. aeruginosa*	2.617	15.78	1.15
50 % *E. faecalis* + 50 % *P. aeruginosa*	1.534	26.94	0.74
80 % *E. faecalis* + 20 % *P. aeruginosa*	2.579	16.02	0.51

Table 3 Area under the curve (AUC_{24}) and heat (Q_{24}) of *E. faecalis*, *P. aeruginosa* and the mixed microorganisms at 10^3 CFU mL^{-1} during 24 h in culture

Microorganism	AUC_{24}/mW h	Q_{24}/J
E. faecalis	135.9	4.1
P. aeruginosa	1140.5	34.4
20 % *E. faecalis* + 80 % *P. aeruginosa*	610.5	18.4
50 % *E. faecalis* + 50 % *P. aeruginosa*	761.2	22.9
80 % *E. faecalis* + 20 % *P. aeruginosa*	697.8	21.1

Discussion

In this paper, we have investigated the interaction relationship between two pathogenic bacteria, *E. faecalis* and *P. aeruginosa,* at a concentration of 10^3 CFU mL^{-1}. We have found that the growth fingerprints of *E. faecalis*, *P. aeruginosa* and the mixed microorganisms at different proportions could be real-time monitored using Calvet microcalorimeter equipment. From growth curves of pure and mixed cultures, we have calculated the thermokinetic parameters for evaluating the interaction between two bacteria. When both pathogens were put together, we have observed that in the curves 20 % *E. faecalis* + 80 % *P. aeruginosa* and 50 % *E. faecalis* + 50 % *P. aeruginosa,* we can detect the presence of *P. aeruginosa* as bacterial growth is recorded during the duration of the experiment, which is characteristic of this bacterium. However, in both cases, even at low rates of *E. faecalis* in the sample, the growth profile of *P. aeruginosa* is modified. Perhaps this is because *Enterococcus* can use nutrients more effectively or its growth is not completely inhibited by *Pseudomonas*. In the case of the curve of 80 % *E. faecalis* + 20 % *P. aeruginosa*, the growth profiles of both bacteria can be identified more clearly. In the first stretch, we could observe the growth curve of *E. faecalis*, with three peaks of heat flow, which signal was recorded during the first 10 h of the experiment, and the second stretch where an increase in potential is maintained for the duration of the experiment, which is characteristic of *Pseudomonas*.

In relation to this work, our group had previously published a study about the mechanism of interaction between *E. faecalis* and *Klebsiella pneumoniae* at a concentration of 10^3 CFU mL^{-1} [24]. Mixtures of both bacteria were prepared in three different proportions: 20 % *E. faecalis* + 80 % *K. pneumoniae*, 50 % *E. faecalis* + 50 % *K. pneumoniae* and 80 % *E. faecalis* + 20 % *K. pneumoniae*. In this case, unlike the present study, the results revealed that when *E. faecalis* and *K. pneumoniae* were mixed, in the curves of samples of 20 % *E. faecalis* + 80 % *K. pneumoniae* and 50 % *E. faecalis* + 50 % *K. pneumoniae*, the characteristic growth of *E. faecalis* was not present, due to *K. pneumoniae* completely inhibiting its growth. While in the sample 80 % *E. faecalis* + 20 % *K. pneumoniae,* we can see in the first part of the curve, the characteristic growth of *E. faecalis*, this is because 80 % of the sample is *Enterococci*, in the same result as seen in the sample of 80 % *E. faecalis* + 20 % *P. aeruginosa*.

Conclusions

Microcalorimetric analysis of heat flow–time curves allows us to investigate the growth of bacteria species as such *E. faecalis* and *P. aeruginosa* and their mixtures at low concentrations (10^3 CFU mL^{-1}). In our study, we have seen that although *E. faecalis* affects the growth of *P. aeruginosa* even at low proportions in the sample (20 % *E. faecalis* + 80 % *P. aeruginosa*). This could be due to *E. faecalis* using nutrients from the culture medium in a more effective way than *P. aeruginosa*, and thus affecting the growth profile. Therefore, microcalorimetry provides a new method to assess the relationship between different bacteria.

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