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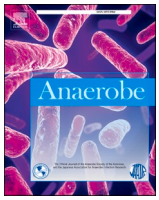

Original Article

Growth assessment of mixed cultures of probiotics and common pathogens

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ABSTRACT

Objectives: In this work, an isothermal microcalorimeter was applied to investigate the antipathogenic activity of three probiotics (*Lactobacillus acidophilus*, *Bifidobacterium lactis* and *Bifidobacterium bifidum*) against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* using the probiotics in mixed culture with the pathogenic microorganisms.

Methods: A microcalorimeter was used to monitor the growth of the microorganisms as pure cultures and as co-cultures at 37 °C. Relative growths of the probiotics and pathogenic species were determined after microcalorimetric measurements by serial dilution and plate incubation. Relative growth of mixed cultures of *E. coli* with *L. acidophilus* or *B. lactis* was also determined by traditional plate growth assay for 5.5 h.

Results: The results showed growth profiles of the microorganisms that were characteristic and showed different lag and peak times for the species. The pathogenic species grew faster than the probiotic species. In the co-cultures, the growth profile of both pathogenic species and probiotics could be identified with the microcalorimeter. Although the pathogenic species grew faster, at the end of the assay, the results showed that the pathogenic species were inhibited in growth by the probiotics as no viable growth of the pathogenic species was detected whereas 10^7–10^8 CFU/mL of the probiotics were enumerated after the microcalorimetric assay. Using the traditional plate assay, the data confirmed co-growth of the probiotics and *E. coli* although cell numbers of *E. coli* were higher than the probiotics during 5.5 h of co-culture incubation when both were inoculated at 10^6 CFU/mL.

Conclusion: The results demonstrate the antipathogenic effects of probiotics and highlights the potential of microcalorimetry in live mixed culture assays and its limitation.

1. Introduction

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health effect on the host" [1]. One of the primary effect of probiotics in humans is their antipathogenic effects [2–4]. They are believed to produce antimicrobial compounds which includes acidic metabolites and bacteriocins that inhibit pathogens [5,6]. They are also believed to reduce intestinal permeability, adhere onto the cell surfaces of its hosts, preventing pathogenic bacteria from attaching to the same surfaces and compete for nutrients with pathogens [7,8]. Another important mechanism in their antipathogenic effect is their ability to stimulate host's immunity [9].

Demonstration of direct antagonism through inhibitory substance production of probiotics is often assessed for potential probiotic candidates to establish antipathogenic activity. This is routinely demonstrated through diffusion tests or co-culture assays where test microorganism is co-cultured with potential probiotic supernatants or the live probiotic. Co-culture assays with live microorganisms could be laborious, requiring continuous sampling, serial dilutions, and plate assays if the experimenter wants to determine the profile of growth. Another disadvantage of the experiment is that data could be far from reality because of the retrospective nature of data collection. Furthermore, when molecular methods are combined, the assays become more expensive, requiring other skills and expertise. More experimenters therefore prefer the diffusion method for routine determination of antipathogenic activity of probiotics [10–14]. The diffusion test has its own limitation, for instance, the dependence of the inhibitory metabolite to diffuse irrespective of size or potential interaction in a solid medium [15,16]. In our previous study, we compared isothermal microcalorimetry with the agar diffusion and broth culture assays for assessing the inhibitory activity of

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probiotic culture supernatants. The results demonstrated that isothermal microcalorimetry could detect inhibitory activity of neutralised probiotic culture supernatant which could not be detected with the traditional broth culture and the agar well diffusion methods [15].

The importance of co-culture assays with live microorganisms cannot be ignored. For instance, they help us understand specific interactions and patterns that occur amongst microorganisms, which has led to some breakthroughs and discoveries and enhanced our insights in microbial ecology. The current approach in these co-culture assays with live microorganisms as mentioned, could be time intensive, labour intensive, require special equipment or expensive when molecular methods are combined. We propose an isothermal microcalorimetric method for determining growth profile of mixed cultures of microorganisms. Briefly, isothermal microcalorimetry is a technique based on the principle of measurement of heat. It measures heat flow of physical, chemical and biological processes. It has been previously used in the study of bacterial growth kinetics [15,17]. The technique is simple and offers the opportunity to monitor the growth of live microorganisms in real time. However, its application is often limited to monoculture bacterial assays. Its potential in mixed bacterial assays, and in application to live probiotic assays have not been clearly understood yet.

In this work, the microcalorimeter was explored to study the growth of *Lactobacillus acidophilus*, *Bifidobacterium lactis* and *Bifidobacterium bifidum* probiotic species in mixed culture with *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*.

2. Materials and methods

2.1. Microorganisms

Pure cultures of *Lactobacillus acidophilus* La-5® and *Bifidobacterium lactis* BB-12® were obtained from Chr. Hansen's Culture Collection (Hørsholm, Denmark). *Bifidobacterium bifidum* ATCC 11863 was purchased from American Type Culture Collection, USA.

Staphylococcus aureus NCIMB 9518 and *Pseudomonas aeruginosa* NCIMB 8628 were obtained from ConvaTec Ltd. *Escherichia coli* ATCC 25922 was purchased from American Type Culture Collection, USA.

2.2. Microbiological media and chemicals

MacConkey agar (MCA), cooked meat medium (CMM), cetrimide agar, mannitol salt agar (MSA), de man rogosa sharpe (MRS) broth and agar, agar powder, reinforced clostridial medium (RCM) and nutrient broth were obtained from Oxoid Ltd, Basingstoke, UK. L-cysteine hydrochloride and glycerol were purchased from Fisher Scientific, UK. Glucose was purchased from Sigma-Aldrich, UK.

2.3. Culture maintenance

B. bifidum, *B. lactis* and *L. acidophilus* were grown respectively in RCM and MRS broth supplemented with 0.05 % w/v L-cysteine hydrochloride under anaerobic conditions (anaerobic jar with AnaeroGen, Oxoid to generate the anaerobic environment) at 37 °C. *P. aeruginosa, S. aureus* and *E. coli* were grown in nutrient broth at 37 °C. The cells were harvested when they reached stationary phase of growth. The cells were washed twice in phosphate buffered saline (PBS) and resuspended in 15 % v/v glycerol at an organism density of 10^8 CFU/mL. The resuspended cells were frozen in 1.8 mL aliquots over liquid nitrogen [18] and stored under liquid nitrogen until required. Prior to use, they were removed from the storage liquid nitrogen container, thawed at 40 °C for 3 min and vortexed for 1 min.

2.4. Pure and mixed culture of probiotics and P. aeruginosa, S. aureus and E. coli

30 μL of the thawed culture of the probiotic species, *B. bifidum*, *L. acidophilus* and *B. lactis* was each inoculated into 2970 μL of pre-warmed CMM supplemented with 2 % w/v glucose in a sterile 3 mL calorimetric glass ampoule giving a density of 10^6 CFU/mL. The pathogenic species, *P. aeruginosa*, *S. aureus* and *E. coli* were also inoculated into 2970 μL of pre-warmed CMM supplemented with 2 % w/v glucose in a sterile 3 mL calorimetric glass ampoule to a density of 10^6 CFU/mL. The ampoules were sealed and vortexed for 10 s. They were placed in the thermal equilibration position of a Thermometric Thermal Activity Monitor 2277 (TAM 2277, TA Instruments Ltd., UK) set at 37 °C (±0.1 °C). The loaded ampoules were allowed to equilibrate at this intermediate position for 30 min before being lowered into the measurement position. The probiotic species were also co-inoculated with each of the respective pathogenic species to a density of 10^6 CFU/mL of each species in the 3 mL calorimetric ampoule and sealed. The ampoules were placed in the TAM 2277 and data captured with Digitam 4.1 every 10 s with an amplifier range of 1000 μW until the power-time data returned to baseline. Data were analysed with Origin Pro 8.6 (Microcal Software Inc.). The reference ampoule was loaded with 3 mL of sterile media. The instrument was calibrated at regular intervals. Cooked meat medium (CMM) can support mixed cultures of bacteria. The addition of glucose to the medium allows rapid, heavy growth of anaerobic bacteria. The microorganisms were inoculated into freshly prepared medium and sealed immediately upon inoculation, creating an anaerobic environment within the microcalorimetric ampoule.

The relative growths of the probiotic and pathogenic species were determined after microcalorimetric measurements by plating 50 μL of serially diluted cultures on MRS agar supplemented with 0.05 % w/v L-cysteine hydrochloride, RCM agar, cetrimide agar, mannitol salt agar and MacConkey agar. pH measurements (pHenomenal®, UK) were also done post calorimetric experiments. Relative growth of mixed culture of *E. coli* with *L. acidophilus* and *E. coli* with *B. lactis* was also monitored by traditional plate growth for 5.5 h by sampling the mixed culture every 30 min, serial dilution, plating and incubation.

3. Results and discussion

The isothermal microcalorimeter records power (μW or μJs^{-1}) as a function of time (t). The growth of microorganisms in the microcalorimeter typically results in an initial lag, then an exponentially increasing signal characterized with peaks and troughs, shown to be representative of the heat produced by the growing microorganism [17]. When the concentration of the energy source becomes limiting, and there is a build-up of toxic metabolites, the power signal approaches baseline. The power-time curves of 10^6 CFU/mL of pure cultures of the probiotic species and the pathogenic species inoculated into CMM supplemented with 2 % w/v glucose are shown in Fig. 1. The power-time curves of the different species were characteristic in the media showing differences in lag and peak time. The peak of the growth curves occurred at approximately 11 h for *B. bifidum*, 10 h for *B. lactis*, 7 h for *L. acidophilus*. Whilst it occurred at 4.5 h for *P. aeruginosa*, 6.5 h for *S. aureus* and 5 h for *E. coli*. The power-time curves suggest that *B. lactis* and *B. bifidum* are slower growing species relative to the other species in the medium. Although effectively, an anaerobic environment was created within the ampoule, the two *Bifidobacterium* species have longer generation times [19] and may have taken a longer time to adapt to the medium than the other species. Amongst the species, *L. acidophilus* showed maximum growth in the medium, generating the greatest heat outputs and reducing the pH of the medium the most, from an initial pH of 7.20 ± 0.20 to 4.00 ± 0.13 (Table 1). This superior growth may likely be because the environment generated in the ampoule suited its growth the most. Data from post microcalorimetric analysis of the cultures are presented in Table 1. All the species showed presence of viable cells at the end of the microcalorimetric analysis which suggest that the baseline part of the curve could be the period of stationary phase or briefly after the stationary phase. Amongst the microorganisms, the probiotic species reduced the pH of the medium the most, suggesting the likely production

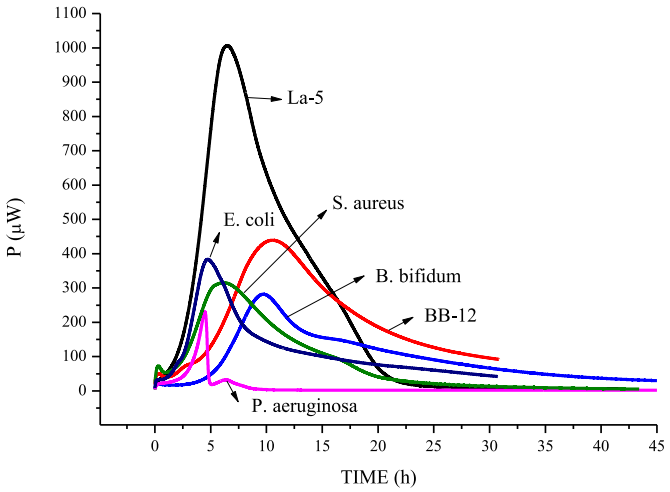

Fig. 1. Power-time curves of pure cultures of *L. acidophilus* La-5®, *B. lactis* BB-12®, *B. bifidum*, *P. aeruginosa*, *S. aureus* and *E. coli* in CMM supplemented with 2 % w/v glucose at densities of 10^6 CFU/mL of each.

Table 1
Comparison of the post microcalorimetric profile of the pure culture and mixed culture of probiotics with *P. aeruginosa*, *S. aureus* and *E. coli*.

Organism	pH post analysis	Plate count (CFU/mL) post analysis	
Pure Culture			
L. acidophilus	4.00 ± 0.13	3.6×10^8	
B. lactis	4.55 ± 0.01	8.0×10^8	
B. bifidum	4.79 ± 0.01	4.6×10^7	
P. aeruginosa	6.78 ± 0.09	2.8×10^7	
S. aureus	5.05 ± 0.05	1.8×10^8	
E. coli	5.39 ± 0.01	3.9×10^8	
Mixed Culture		**Probiotic**	***P. aeruginosa* or *S. aureus* or *E. coli***
L. acidophilus and *P. aeruginosa*	4.30 ± 0.01	2.5×10^8	0
L. acidophilus and *S. aureus*	4.12 ± 0.02	4.5×10^8	0
L. acidophilus and *E. coli*	4.20 ± 0.05	2.0×10^8	0
B. lactis and *P. aeruginosa*	4.35 ± 0.02	6.1×10^8	0
B. lactis and *S. aureus*	4.30 ± 0.06	9.2×10^8	0
B. lactis and *E. coli*	4.45 ± 0.02	1.8×10^8	0
B. bifidum and *P. aeruginosa*	4.48 ± 0.01	8.6×10^8	0
B. bifidum and *S. aureus*	4.49 ± 0.08	2.7×10^8	0
B. bifidum and *E. coli*	4.48 ± 0.01	4.6×10^8	0

of organic acids. *L. acidophilus* recorded the least pH whilst *P. aeruginosa* reduced the pH the least.

The power-time curves for the mixed cultures of the probiotic species: *L. acidophilus*, *B. lactis* and *B. bifidum* with *P. aeruginosa*, *S. aureus* and *E. coli* are shown in Figs. 2–4 respectively. The power-time profiles of co-cultures of *L. acidophilus* with the pathogenic species (Fig. 2) represented cultures being dominated by *L. acidophilus* as the power-time curves did not significantly differ from that of the pure culture of *L. acidophilus*. However, in co-culture with *P. aeruginosa,* the pathogenic species may have contributed a peak at 4.5 h. The total area under the control curve (total heat output) of *L. acidophilus* which was reproducible to 3.5 % (n = 3) was increased by almost 10 % by the presence of *P. aeruginosa*. Co-cultures of the pathogenic species with *B. lactis* and *B. bifidum* showed the presence of the pathogenic species in the co-cultures (Figs. 3 and 4 respectively). For instance, comparing a co-culture of *B. lactis* with *P. aeruginosa* (Fig. 5), it is very likely that *P. aeruginosa* contributed the first peak (peak 1) and the second growth peak (peak 2) contributed by *B. lactis*. The post microcalorimetric

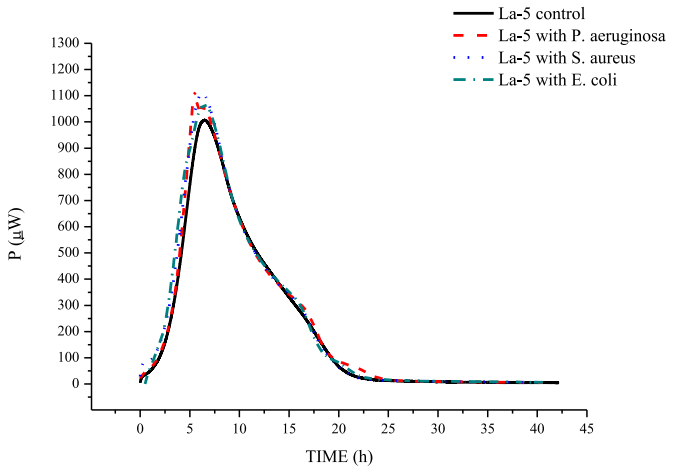

Fig. 2. Comparison of the power-time curves of *L. acidophilus* La-5® and co-cultures with *P. aeruginosa*, *S. aureus* and *E. coli* in CMM supplemented with 2 % w/v glucose. All species were inoculated to a density of 10^6 CFU/mL.

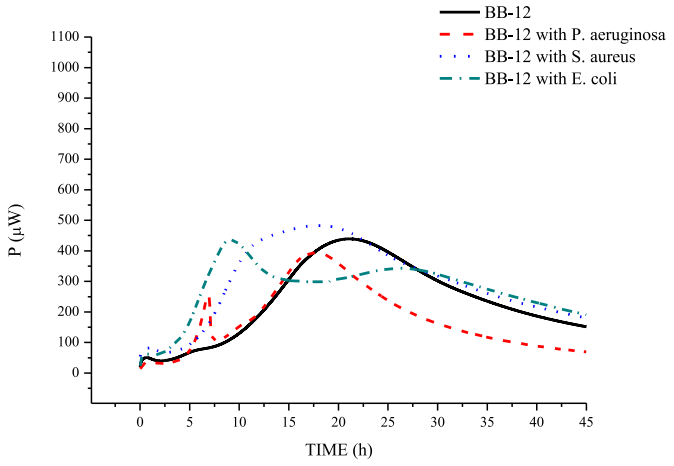

Fig. 3. Comparison of the power-time curves of *B. lactis* BB-12® and co-cultures with *P. aeruginosa*, *S. aureus* and *E. coli* in CMM supplemented with 2 % w/v glucose. Each species was inoculated to a density of 10^6 CFU/mL.

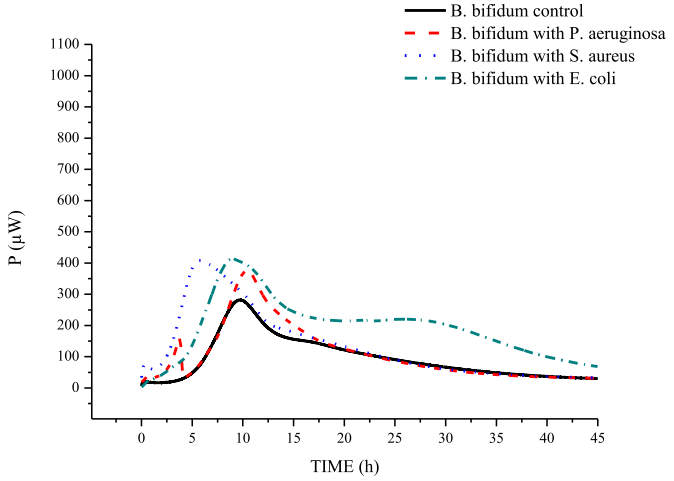

Fig. 4. Comparison of the power-time curves of *B. bifidum* and co-cultures with *P. aeruginosa*, *S. aureus* and *E. coli* in CMM supplemented with 2 % w/v glucose. Each species was inoculated to a density of 10^6 CFU/mL.

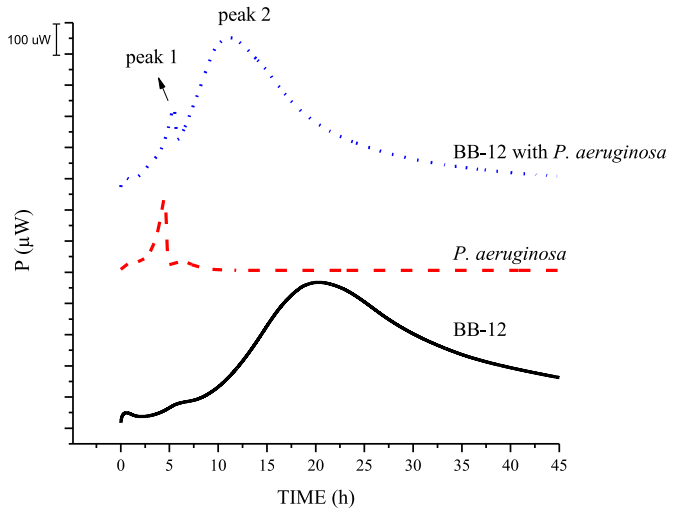

Fig. 5. Comparison of the power-time curves of *B. lactis* BB-12®, *P. aeruginosa* and their mixed culture.

measurements (Table 1) shows that whilst the probiotic species survived the co-cultures showing viable counts of 10^8 CFU/mL, all the pathogenic species lost viability after the test. The synchronous growth of the pathogenic species with the probiotic species noted in the microcalorimeter occurred because the probiotic species were slower growing, as evident by the late occurrence of the peak of the curves compared to the other species. Therefore, the pathogenic species could have attained exponential phase of growth before the probiotics could. The co-growth was confirmed by a plate count assay of the probiotic species with the pathogenic species. Table 2 shows viable count of *E. coli*, *L. acidophilus* and *B. lactis* when co-cultured, sampled and enumerated every 30 min for 5.5 h.

Lactic acid bacteria and other probiotics are known to produce two main varieties of antimicrobial compounds which may affect themselves as well as undesirable or pathogenic strains. They produce low molecular weight compounds such as organic acids which may have bactericidal or bacteriostatic effects and bacteriocins, which may have a narrow spectrum of activity [20–22]. The results suggest that when the probiotics were co-incubated with the pathogenic species, the probiotic species could only inhibit the other species after an active or exponential growth. Thus, the probiotics produced extracellular inhibitory substances after consumption of the nutrient substrates and accumulation of inhibitory substances. Prior to that phase, they coexisted with the other species. This observation agrees with a previous study by Apella et al. [23] who showed that *Shigella sonnei* could grow together with *Lactobacillus casei* or *L. acidophilus* in mixed culture for the first few hours but noted that inhibition of *S. sonnei* began at 6 h and death phase at 9 h of incubation [23]. Bendali et al. [24] also demonstrated that co-cultures of

Table 2
Plate count data of *B. lactis* - *E. coli* co-culture and *L. acidophilus* - *E. coli* co-culture within first 5.5 h of incubation.

Time (h)	Plate count of mixed culture of *B. lactis* and *E. coli* (CFU/mL)		Plate count of mixed culture of *L. acidophilus* and *E. coli* (CFU/mL)	
	B. lactis	*E. coli*	*L. acidophilus*	*E. coli*
0.5	9.5×10^5	1.2×10^6	2.4×10^6	9.5×10^5
1	8.8×10^5	1.1×10^6	2.1×10^6	9.5×10^5
1.5	1.1×10^6	1.2×10^6	2.4×10^6	9.3×10^5
2	9.5×10^5	1.4×10^6	2.4×10^6	1.2×10^6
2.5	2.3×10^6	4.0×10^6	2.1×10^6	1.9×10^6
3	4.8×10^6	1.6×10^7	1.0×10^7	1.0×10^7
3.5	1.9×10^7	2.8×10^7	6.7×10^7	4.2×10^7
4.5	9.7×10^7	2.0×10^8	1.9×10^7	3.0×10^8
5.5	1.2×10^8	6.4×10^8	1.1×10^8	8.1×10^8

Lactobacillus paracasei with either *E. coli* or *Salmonella enterica Typhimurium* showed inhibition at approximately 2 h and 6 h respectively when the growth kinetics of the mono-cultures of the pathogens were compared with the co-cultures. Another study by Voravuthikunchai et al. [25] showed inhibition of methicillin-resistant *S. aureus* (MRSA) by probiotic *Lactobacillus* strain L 22 6 h after co-culture incubation and no viability of *S. aureus* ATCC 25923 by 24 h of co-culture with L 22. *B. lactis* was also demonstrated to inhibit Avian Pathogenic *E. coli* (APEC) by 24 h of co-culture [26].

Previous studies in the microcalorimeter have shown that it can detect growth of mixed cultures of *P. aeruginosa* with *S. aureus* and *E. coli* [27] and probiotic mixed cultures [28]. In these studies, the species which were co-cultured had similar growth or detection time and the microcalorimeter could detect relative growth showing profiles which looked like the dominant species. In another study, where *Clostridioides difficile* was co-cultured with probiotics [29], the species had different detection times in the microcalorimeter. The probiotic species growth occurred faster than *C. difficile*. Therefore, after detection of growth of the probiotic species, *C. difficile* growth was not seen in the microcalorimeter likely due to the production of inhibitory substances by the probiotics which prevented the growth of *C. difficile*. In the present study, the growth or detection time of the pathogenic species occurred faster than the probiotic species. However, the probiotic species were able to grow whilst the growth of the pathogenic species was in progress and resulted in a growth profile contributed by the pathogenic and probiotic species, also confirmed with the plate assay (Tables 1 and 2). However, post microcalorimetric assay of the culture (Table 1) revealed that the pathogenic species were inhibited in growth most likely when the probiotic species had actively grown and produced pH lowering end products. This antipathogenic activity of the probiotics was shown with the plate count data after microcalorimetric measurements. The calorimetric ampoule is a closed system. It is interesting that, the probiotic species were able to grow after growth of the pathogenic species, since the pathogenic species may have utilised some of the nutrients available in the medium. Most bacteria can release exotoxins to inhibit others in mixed cultures. For instance, *E. coli* and *P. aeruginosa* have been shown to produce inhibitory substances including bacteriocins that can kill other bacteria [30–32]. For instance, *P. aeruginosa* was shown to inhibit *S. aureus* in mixed culture [27]. Both *E. coli* and *S. aureus* were able to produce acidic by-products as they significantly reduced the pH of the medium after sole cultures in the closed system of the calorimetric ampoule (Table 1). It is noteworthy that the pathogenic species were unable to retard growth of the probiotic species but were rather inhibited by the probiotic species. *E. coli* and *S. aureus* were able to thrive in the acidic conditions they created but could not survive the conditions created by the probiotic metabolism.

4. Conclusion

This study has demonstrated the antipathogenic effect of probiotics against *P. aeuginosa*, *S. aueus* and *E. coli*. Even though the pathogenic species tested were faster growing relative to the probiotic species, in a closed system, the probiotic species were still able to utilize remaining nutrients and produce inhibitory substances against the pathogenic species. The data demonstrates that the probiotics produced acidic metabolites to inhibit the pathogenic bacteria. It is also likely the probiotic species produced other inhibitory substances apart from the acidic metabolic products to inhibit the pathogenic species. In a system where nutrient is constantly supplied, the microcalorimeter could be useful in determining interspecies interaction, overcoming the tedium associated with traditional assays and other limitations. Future studies will determine mixed cultures with a flow throw set up in the microcalorimeter which would supply constant nutrient to the culture.

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.

Data availability

Data will be made available on request.

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