Microcalorimetric evaluation of a multi-strain probiotic: interspecies inhibition between probiotic strains

Mansa Fredua-Agyeman, Paul Stapleton, Abdul Basit, Simon Gaisford

UCL School of Pharmacy, University College London, 29-39 Brunswick Square, London, WC1N 1AX, United Kingdom

Corresponding author:

Simon Gaisford, PhD

Department of Pharmaceutics

UCL School of Pharmacy

University College London

29-39 Brunswick Square, London

WC1N 1AX

United Kingdom

Email: s.gaisford@ucl.ac.uk

Abstract

Lactobacillus plantarum, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus* and *Enterococcus faecium,* which are the component species of a commercially available probiotic mixture (Symprove®, P1), were grown in co-culture to determine whether they would inhibit each other *in vitro* using an isothermal microcalorimeter (IMC). The growth profiles in the IMC were characteristic and unique to each species while the growth profile of P1 was most similar to that of *L. plantarum,* suggesting this is the dominant organism in mixed-culture. Bacterial growth in the cell free supernatants (CFS) of the probiotic species were also evaluated by IMC and viable counts determined at the end of the incubation period. *L. plantarum* was found to be the most effective species at inhibiting *L. rhamnosus*. Conversely, *L. rhamnosus* was the most effective at limiting the growth of *L. plantarum*. Both *L. plantarum* and *L. rhamnosus* were inhibitory toward *L. acidophilus* and *E. faecium*. *E. faecium* was the least inhibitory towards all the other species. The study shows how complex, multi-species probiotic products can be analysed to determine the predominant species, and so provides a route to formulation of new products.

Keywords: probiotic, *Lactobacillus*, interspecies inhibition, isothermal microcalorimetry

1. Introduction

Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health effect on the host” [1]. Probiotics are claimed to improve digestibility and nutrition [2, 3], prevent the occurrence of diarrhoea [4], reduce cancer risk [5], prevent or alleviate allergies and atopic diseases [6, 7] and prevent and treat infectious diseases [8]. The mechanism by which their beneficial effects are achieved has been proposed to include competition for nutrients, production of antimicrobial substances, competition for adhesion receptors and stimulation of immunity [9, 10].

Probiotics are usually members of the genera *Lactobacillus* and *Bifidobacterium* (although some members of the genera *Streptococcus*, *Enterococcus, Lactococcus* spp*., Bacillus* spp*.* and some yeast, for example *Saccharomyces boulardii*, have been identified as probiotics). They are known as members of the microbiota, which significantly contribute to a beneficial health effect and have a long history of safe use [11]. Initially it was anticipated that single probiotic strains from these genera or species could produce the intended health benefits using the mechanisms underlined but as knowledge of probiotic use has developed it is becoming clearer that for optimal effect, mixed probiotics should be formulated. This resolution stemmed from the basis that it was unlikely a single probiotic strain could colonize the gut and achieve all therapeutic benefits and also because probiotics could be used for targeting a number of diseases; each targeted disease may require a specific probiotic property, which cannot be found in a single probiotic strain [12-15]. Multi-species probiotic products are therefore now commonly available and although some have not shown superior

benefits [16, 17], there exists some evidence on their greater efficacy compared with single strains [18-21]. For example, Chapman et al. [16] reported insignificant differences between single probiotics and mixtures when studying the effect of probiotics against the urinary pathogens *Escherichia coli* and *Enterococcus faecium*. Tejero-Sarinena et al. [17] also demonstrated better potency of inhibiton by some single probiotic species than mixtures against enteric pathogens (*Clostridium difficile* and *Salmonella* Typhimurium). Further, a previous study by Chapman et al. [18] demonstrated that 5 multi-species probiotic preparations had significantly greater inhibitions in 12 out of 24 cases towards *C. difficile*, *E. coli* and *S. typhimurium*, than 15 single-species probiotics. Apella et al. [21] and Drago et al. [20] have shown the superior potency of mixtures than single strains in inhibiting the growth of pathogens in co-culture.

However, very little is known about the growth behaviour of individual species in probiotic mixtures, i.e. whether there is the possibility of inhibition or promotion of growth [18, 19]. Also a previous evaluation of commercial probiotic products on the UK market [22] indicated that none of the multi-species products contained all the labelled species; a reason believed to be the likely result of inhibition amongst the species. In this study, the component species of a commercially available probiotic mixture (Symprove, P1) were tested against each other to determine whether some probiotic species could inhibit the growth of others *in vitro.* This product was selected because it is an aqueous suspension containing 4 probiotic species and as such is unique in the market.

Conventionally, the *in vitro* assessment for inhibition would involve two main

methods, although there are adaptations to these. The first is the observation of growth of the species as whole organism co-cultures or bioproduct/species co-culture on or within selective growth media; colony counting or turbidimetric measurements are used to determine the degree of inhibition [20, 21, 23, 24]. The alternative is to use diffusion assays for assessment of inhibition [18, 23, 25]. Both of these methods are well established and have several advantages but are labour intensive and time consuming. The plate technique allows data acquisition in a retrospective manner, with colony-forming ability being influenced by the plating procedure and morphological alteration during treatment. Turbidimetric measurements may also not distinguish viable cells from dead cells, while the diffusion method may be limited by the capacity of bioproducts to enter into and spread through the growth medium.

The use of isothermal microcalorimetry (IMC) has been shown to circumvent some of these limitations, offering many benefits; in particular, the experiments are simple to set up and because there is no requirement for optical clarity, growth of live organisms can be monitored in real time, non-destructively [26, 27]. Although successfully applied in the detection and characterization of bacteria and other microorganisms, IMC assays are usually done on pure cultures [27-30] and complex polymicrobial systems [31, 32] are rarely explored for detection of relative growth of two or more species. This study aimed to explore the potential of IMC to detect the relative growth of mixed culture of probiotic species to determine whether inhibition occurs amongst them.

2. Materials and methods

2.1. Probiotic strains and product

The probiotics used were *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus* and *Enterococcus faecium*. The species were obtained from the manufacturer of a commercially available combination product (Symprove®, P1) with these constituent species in the United Kingdom. The species were obtained as dehydrated cultures (the form in which the species are introduced to make the final product, P1).

2.2. Growth conditions and maintenance of strains

The probiotic species were cultured overnight in de Man Rogosa Sharpe (MRS) broth (Oxoid, Basingstoke, UK) supplemented with 0.05% w/v L-cysteine hydrochloride for 24 h at 37^{o}C under anaerobic conditions (anaerobic jar with AnaeroGen GasPak System; Oxoid, Basingstoke, UK). The cells were then harvested, washed in phosphate buffered saline (PBS), resuspended in 15% (v/v) glycerol at an organism density of 10^{8} CFU/mL and frozen in 1.8 mL aliquots over liquid nitrogen [33]. Bacterial concentration was determined by serial dilution and colony counting. Aliquots were stored under liquid nitrogen until required. Prior to use, they were thawed for 3 min by immersion in a water bath (40^{o}C) and vortexed for a period of 1 min.

2.3. Sample preparation and microcalorimeter experiments with strains and product

For pure culture studies, the probiotic species were each inoculated into pre-warmed Brain Heart Infusion (BHI) broth or MRS broth (Oxoid, Basingstoke,

137 UK) supplemented with 0.05% w/v L-cysteine hydrochloride (BHIc; MRSc) (in
138 3 mL calorimetric glass ampoules) to give individual population densities of
139 10^6 CFU/mL. The probiotic bacteria were also inoculated into the pre-warmed
140 medium to give a mixed culture of the individual species at concentrations of
141 10^6 CFU/mL of each organism in the ampoules. Samples of batches of P1
142 were inoculated into pre-warmed BHIc or MRSc in ampoules at 1 in 100
143 dilutions to give a final concentration of 10^6 CFU/mL. The ampoules were
144 sealed with crimped caps, vortexed for 10 s and loaded into the intermediate
145 position of a Thermometric Thermal Activity Monitor 2277 (TAM 2277) (TA
146 Instruments Ltd., UK). The temperature of the instrument was set at 37^{o}C (±
147 0.1^{o}C). The loaded samples were allowed to equilibrate thermally at the
148 intermediate position for 30 min before measurement. Data were collected
149 every 10 s, with an amplifier range of 1000 μW using the software package,
150 Digitam 4.1 and analysed using Origin Pro 8.6 (Microcal Software Inc.). The
151 reference ampoule was loaded with 3 mL of sterile media.

152 **2.4. Cell free supernatant and microcalorimeter experiments**

153 The cell free supernatant (CFS) obtained from each species was tested
154 against the producing organism and the other species. Culture supernatants
155 of the probiotic species were prepared by cultivating the respective species in
156 broth over 48 h anaerobically using an Oxoid anaerobic jar with an
157 AnaeroGen GasPak System (Oxoid, Basingstoke, UK). The cells were
158 removed by centrifuging at 3500 *g* for 10 min at 4^{o}C. The supernatant was
159 collected and filter-sterilized using a 0.22 μm membrane syringe filter. The
160 pHs of the supernatants were examined and recorded.

1.5 mL of CFS obtained from the species were homogeneously mixed with double fold concentrated medium. The probiotic species were individually inoculated into the respective CFS-broth mixture to a population density of 10^6 CFU/mL and placed in the TAM. Power-time measurements were then taken. A control experiment was done by replacing the CFS with sterile distilled water. Colony counts of serially-diluted samples of the bacteria were done after the TAM experiments.

3. Results and Discussion

As noted above, IMC is a technique widely used for monitoring bacterial growth [26, 34]. The raw data from IMC are a plot of power (μW or μJs^{-1}) as a function of time (*t*). The power-time data showing growth curves of 10 batches of P1 are shown in Figure 1. The power-time curves are complex, with peaks and troughs representing the growth phases of the individual species in the product [35]. The growth curves are generally reproducible but there are some variances in the lag period, which may reflect slight differences in the numbers of organisms loaded into the calorimeter; [36].

The power-time curves of the component species of P1 (*L. plantarum*, *L. rhamnosus*, *L. acidophilus* and *E. faecium)*, their mixed culture and a batch of the product in BHIc are compared in Figure 2. It is apparent that the curves are characteristic for individual species, with different onset times (increase in power from baseline) and areas under curve (AUC, representing heat output). It is important to note that the inoculum concentration for the species were the same; while it would be possible to explore the effect of different inoculum concentrations, the number of permutations and combinations would be vast.

185 The time-lag before growth for some of the species may indicate a period of
186 adaptation of the species to the medium [36]. AUC also varied amongst the
187 species with *E. faecium* producing the highest heat output and *L. acidophilus*
188 the least. The maximum power attained was also higher for *E. faecium*
189 relative to the other species, which could imply that growth of *E. faecium* in
190 the medium is favoured or the species adapts to the medium more quickly
191 than the others. Growth of the species and P1 in MRSc, Figure 3, showed
192 characteristic growth curves but did not show the fastest growth of *E. faecium.*
193 This result shows the importance of media selection when carrying out these
194 studies and suggests that the best in-vitro:in-vitro correlation will be obtained
195 in biorelevant media.

196 The growth curve of a mixed culture of all four species in BHIc appears to be
197 dominated by *E. faecium*, in line with the observation above. However, when
198 the growth curves are compared, the growth curve of the product (P1)
199 appears to share some similarities to that of the growth curve of *L. plantarum*
200 in both BHIc and MRSc. So while it is likely *E. faecium* rapidly adapted and
201 consumed the nutrients before the other species in BHIc, in the commercial
202 product it could have contrarily been inhibited by the other species; colony
203 counting at the end of the IMC study showed lower numbers of *E. faecium*
204 relative to the other species, supporting this hypothesis. The dominance of *L.*
205 *plantarum* in the growth curve of the product may reflect that it is very robust
206 and may have survived the product manufacturing process and/or storage
207 better relative to the other species or may have inhibited the other species
208 during growth. Notably, isolation and characterization of the species in the
209 product showed *L. plantarum* to be the numerically superior organism

between those isolated (*L. plantarum* and *L. rhamnosus*; [22]). Also, the power-time data of the species in the CFS of each other (Figure 4) and the plate count data at the end of the IMC-CFS experiment (Table 1) showed that both *L. acidophilus* and *E. faecium* did not grow in the CFS of *L. plantarum* and *L. rhamnosus* indicating inhibition of the former organisms by the other two probiotic species. Also, lower concentrations of viable cells (10^4-10^5 CFU/mL) were observed at the end of the IMC-CFS experiment with these species relative to 10^7 CFU/mL for the others. It could be reasoned that the CFS of *L. plantarum* and *L. rhamnosus* caused some cell death in *L. acidophilus* and *E. faecium*.

According to the IMC data and plate count data, the CFS of *L. plantarum* was the most inhibitory towards all the other species; that of *E. faecium* was the least inhibitory. The CFS of *L. rhamnosus* was the most effective against *L. plantarum* and had a greater capacity to inhibit other species than *L. acidophilus*. The data also showed that the species were inhibited by their own CFS, this being more profound in the case of *L. acidophilus* which had lower final concentration of 10^6 CFU/mL unlike the other species, which maintained cell count of 10^7 CFU/mL after incubation in their own CFS.

In this study, our goal was to explore the potential of IMC to determine whether some probiotics could inhibit others *in vitro*. The data show that some species inhibit others and therefore may consequently inhibit them when blended together as a formulation. This observation is consistent with previous findings of Be'er et al. [37] and Chapman et al. [18] who reported inhibition of closely related strains and species/genera respectively. For instance, Be'er et al. [37] reported mutual inhibition of sibling colonies of

Paenibacillus dendritiformis, observing that growth inhibition and cell death occurred if material extracted from the agar plate between the two growing colonies was introduced near a growing single colony [37]. Also, Chapman et al. [18] reported that among lactobacilli, bifidobacteria, *Streptococcus*, *Lactococcus* and *Bacillus* species tested, mutual inhibition was observed; however the degree of inhibition was reported to be genus-specific.

Lactobacilli were reported to be most effective in inhibiting species of other genera followed, by bifidobacteria. *Bacillus*, *Streptococcus* and *Lactococcus* species showed little ability to inhibit species from the other genera. Testing against strains of their own genus, they also reported that lactobacilli showed mutual inhibition amongst the species [18], which is consistent with the present findings. The inhibitory properties of lactobacilli may be due to the production of acids and other metabolites to which they themselves are susceptible. According to the present study, amongst the lactobacilli tested, *L. plantarum* had the greatest capacity to inhibit other bacteria followed by *L. rhamnosus* then *L. acidophilus*. The reason for the greater inhibitory profile of *L. plantarum* could be either the production of greater quantity of antimicrobial substances or a broader spectrum of activity of the antimicrobial substances produced. Indeed CFS produced by *L. plantarum* recorded the lowest pH indicating that it may have produced the highest quantity of acidic metabolites, which may have contributed to its inhibitory profile.

The results from the study have several implications, not least of which is the importance for research into intra and interspecies interaction of potential probiotic strains and species and the need for their characterization before they are put together as a product, submissions also echoed by Myllyluoma et

al. [38] when studying the effects of multispecies probiotic combinations on *Helicobacter pylori* infection *in vitro* and Grandy et al. [39] when studying two different probiotic preparations for treatment of acute rotavirus diarrhoea [38, 39]. One likely consequence of species inhibition in combination products is the probability that the species inhibited is the species offering the specific activity anticipated. Also, species may adversely react or the presence of a species could affect the potency of the other [18, 40].

In conclusion, the results from this study show that some probiotic species could be inhibitory to others and highlight the importance of characterizing probiotic species before putting them together as combination products.

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Figure 1. Power-time data of 10 batches of P1.

Figure 2. Comparison of the power-time curves of the individual species of P1 (*L. acidophilus*, *L. plantarum*, *L. rhamnosus*, and *E. faecium*), their mixed culture at equal cell density and a batch of the commercial product (P1) in BHIc.

Figure 3. Comparison of the power-time curves of the individual species of P1 (*L. acidophilus*, *L. plantarum*, *L. rhamnosus*, and *E. faecium*), their mixed culture at equal cell density and a batch of the commercial product (P1) in MRSc.

Figure 4. Power-time data of the species in the CFS of each other; the species in the CFS of [A], *L. plantarum*, [B], *L. rhamnosus*, [C], *L. acidophilus*, [D], *E. faecium*.

405 Table 1. Cell count of *L. plantarum*, *L. rhamnosus*, *L. acidophilus* and *E.*

406 *faecium* after incubation in the CFSs of each other (n=3).

CFS of species	Cell count (log CFU/mL) of species post CFS incubation			
	L. plantarum	*L. rhamnosus*	*L. acidophilus*	*E. faecium*
L. plantarum	7.28 ± 0.07	7.09 ± 0.04	5.30 ± 0.02	4.54 ± 0.03
L. rhamnosus	7.20 ± 0.05	7.26 ± 0.10	5.53 ± 0.06	5.36 ± 0.06
L. acidophilus	7.27 ± 0.13	7.38 ± 0.05	6.49 ± 0.03	7.08 ± 0.04
E. faecium	7.99 ± 0.03	7.58 ± 0.02	7.54 ± 0.07	7.34 ± 0.05

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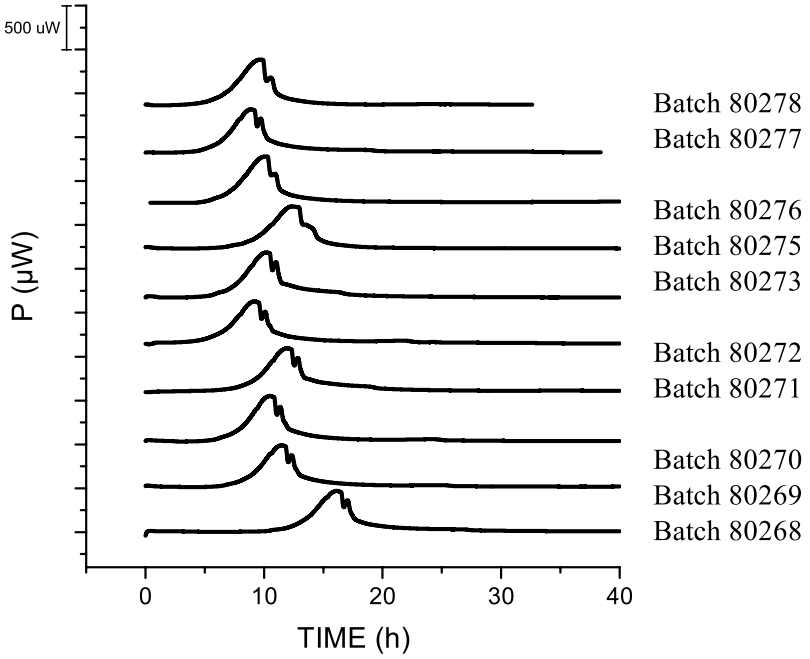

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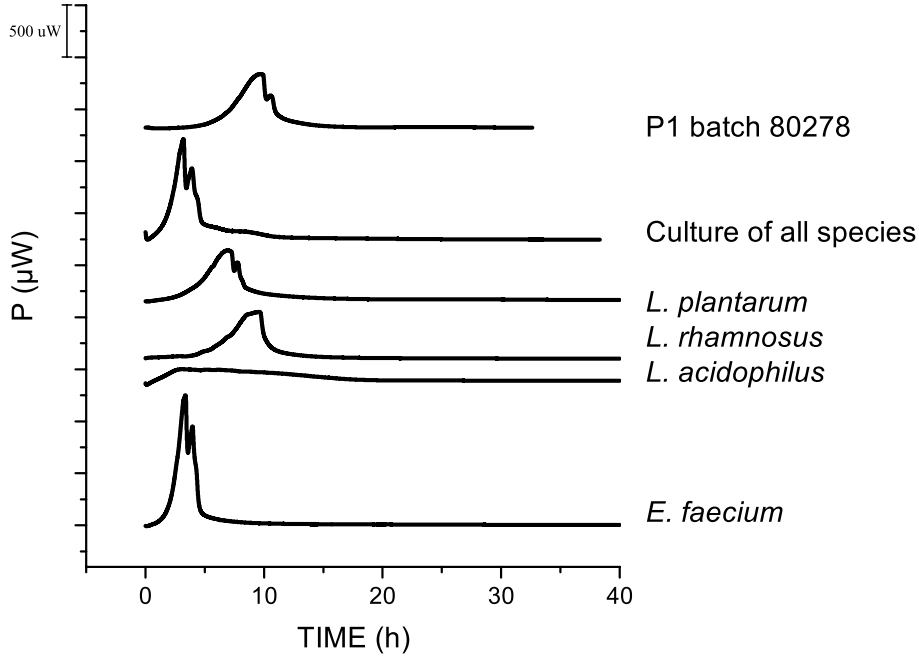
411 Figure 1. Power-time data of 10 batches of P1.



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413 Figure 2. Comparison of the power-time curves of the individual species of P1

414 (*L. acidophilus*, *L. plantarum*, *L. rhamnosus*, and *E. faecium*), their mixed

415 culture at equal cell density and a batch of the commercial product (P1).

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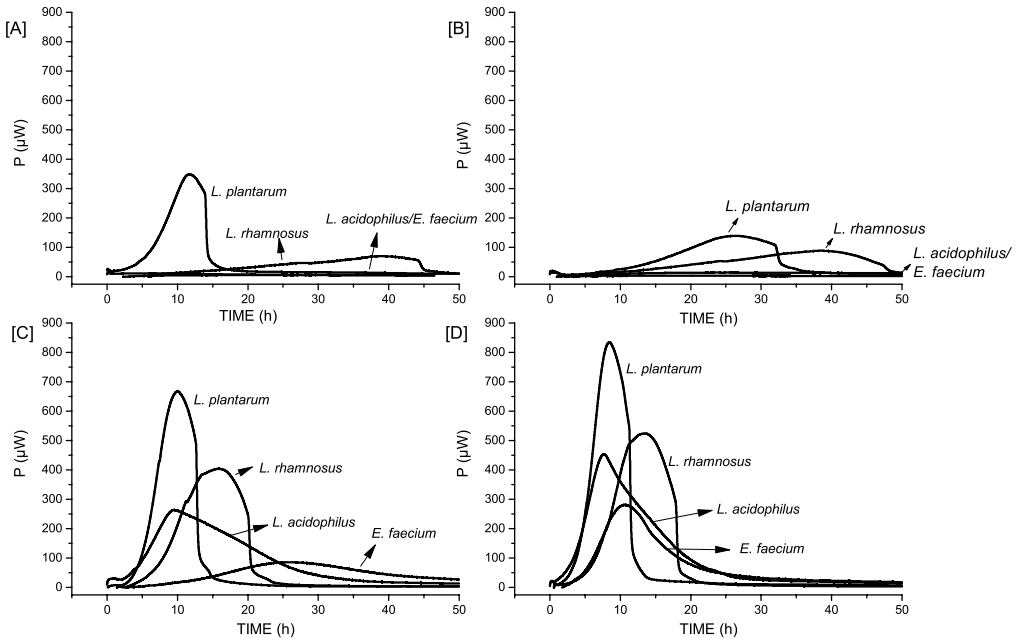

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418 Figure 3. Power-time data of the species in the CFS of each other; the

419 species in the CFS of [A], *L. plantarum*, [B], *L. rhamnosus*, [C], *L. acidophilus*,

420 [D], *E. faecium*.

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