

1 **Microcalorimetric evaluation of a multi-strain**
2 **probiotic: interspecies inhibition between probiotic**
3 **strains**

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17 **Abstract**

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

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

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40 **1. Introduction**

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

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

65 benefits [16, 17], there exists some evidence on their greater efficacy
66 compared with single strains [18-21]. For example, Chapman et al. [16]
67 reported insignificant differences between single probiotics and mixtures when
68 studying the effect of probiotics against the urinary pathogens *Escherichia coli*
69 and *Enterococcus faecium*. Tejero-Sarinena et al. [17] also demonstrated
70 better potency of inhibition by some single probiotic species than mixtures
71 against enteric pathogens (*Clostridium difficile* and *Salmonella* Typhimurium).
72 Further, a previous study by Chapman et al. [18] demonstrated that 5 multi-
73 species probiotic preparations had significantly greater inhibitions in 12 out of
74 24 cases towards *C. difficile*, *E. coli* and *S. typhimurium*, than 15 single-
75 species probiotics. Apella et al. [21] and Drago et al. [20] have shown the
76 superior potency of mixtures than single strains in inhibiting the growth of
77 pathogens in co-culture.

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

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

90 methods, although there are adaptations to these. The first is the observation
91 of growth of the species as whole organism co-cultures or bioproduct/species
92 co-culture on or within selective growth media; colony counting or
93 turbidimetric measurements are used to determine the degree of inhibition
94 [20, 21, 23, 24]. The alternative is to use diffusion assays for assessment of
95 inhibition [18, 23, 25]. Both of these methods are well established and have
96 several advantages but are labour intensive and time consuming. The plate
97 technique allows data acquisition in a retrospective manner, with colony-
98 forming ability being influenced by the plating procedure and morphological
99 alteration during treatment. Turbidimetric measurements may also not
100 distinguish viable cells from dead cells, while the diffusion method may be
101 limited by the capacity of bioproducts to enter into and spread through the
102 growth medium.

103 The use of isothermal microcalorimetry (IMC) has been shown to circumvent
104 some of these limitations, offering many benefits; in particular, the
105 experiments are simple to set up and because there is no requirement for
106 optical clarity, growth of live organisms can be monitored in real time, non-
107 destructively [26, 27]. Although successfully applied in the detection and
108 characterization of bacteria and other microorganisms, IMC assays are
109 usually done on pure cultures [27-30] and complex polymicrobial systems [31,
110 32] are rarely explored for detection of relative growth of two or more species.
111 This study aimed to explore the potential of IMC to detect the relative growth
112 of mixed culture of probiotic species to determine whether inhibition occurs
113 amongst them.

114 **2. Materials and methods**

115 **2.1. Probiotic strains and product**

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

122 **2.2. Growth conditions and maintenance of strains**

123 The probiotic species were cultured overnight in de Man Rogosa Sharpe
124 (MRS) broth (Oxoid, Basingstoke, UK) supplemented with 0.05% w/v L-
125 cysteine hydrochloride for 24 h at 37°C under anaerobic conditions (anaerobic
126 jar with AnaeroGen GasPak System; Oxoid, Basingstoke, UK). The cells were
127 then harvested, washed in phosphate buffered saline (PBS), resuspended in
128 15% (v/v) glycerol at an organism density of 10⁸ CFU/mL and frozen in 1.8 mL
129 aliquots over liquid nitrogen [33]. Bacterial concentration was determined by
130 serial dilution and colony counting. Aliquots were stored under liquid nitrogen
131 until required. Prior to use, they were thawed for 3 min by immersion in a
132 water bath (40°C) and vortexed for a period of 1 min.

133 **2.3. Sample preparation and microcalorimeter experiments with strains** 134 **and product**

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

137 UK) supplemented with 0.05% w/v L-cysteine hydrochloride (BHlc; 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 BHlc 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°C (\pm
147 0.1°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°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.

161 1.5 mL of CFS obtained from the species were homogeneously mixed with
162 double fold concentrated medium. The probiotic species were individually
163 inoculated into the respective CFS-broth mixture to a population density of 10^6
164 CFU/mL and placed in the TAM. Power-time measurements were then taken.
165 A control experiment was done by replacing the CFS with sterile distilled
166 water. Colony counts of serially-diluted samples of the bacteria were done
167 after the TAM experiments.

168 **3. Results and Discussion**

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

177 The power-time curves of the component species of P1 (*L. plantarum*, *L.*
178 *rhamnosus*, *L. acidophilus* and *E. faecium*), their mixed culture and a batch of
179 the product in BHIc are compared in Figure 2. It is apparent that the curves
180 are characteristic for individual species, with different onset times (increase in
181 power from baseline) and areas under curve (AUC, representing heat output).
182 It is important to note that the inoculum concentration for the species were the
183 same; while it would be possible to explore the effect of different inoculum
184 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 BHlc 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 BHlc and MRSc. So while it is likely *E. faecium* rapidly adapted and
201 consumed the nutrients before the other species in BHlc, 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

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

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

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

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

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

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

260 al. [38] when studying the effects of multispecies probiotic combinations on
261 *Helicobacter pylori* infection *in vitro* and Grandy et al. [39] when studying two
262 different probiotic preparations for treatment of acute rotavirus diarrhoea [38,
263 39]. One likely consequence of species inhibition in combination products is
264 the probability that the species inhibited is the species offering the specific
265 activity anticipated. Also, species may adversely react or the presence of a
266 species could affect the potency of the other [18, 40].

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

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390

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

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

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

394 culture at equal cell density and a batch of the commercial product (P1) in

395 BHlc.

396 Figure 3. Comparison of the power-time curves of the individual species of P1

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

398 culture at equal cell density and a batch of the commercial product (P1) in

399 MRSc.

400

401 Figure 4. Power-time data of the species in the CFS of each other; the

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

403 [D], *E. faecium*.

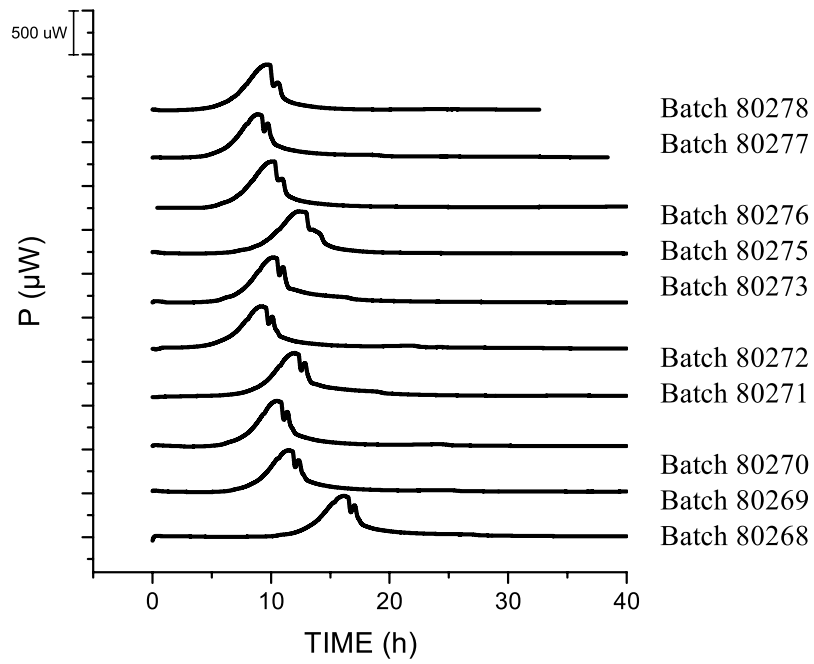
404

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			
	<i>L. plantarum</i>	<i>L. rhamnosus</i>	<i>L. acidophilus</i>	<i>E. faecium</i>
<i>L. plantarum</i>	7.28 ± 0.07	7.09 ± 0.04	5.30 ± 0.02	4.54 ± 0.03
<i>L. rhamnosus</i>	7.20 ± 0.05	7.26 ± 0.10	5.53 ± 0.06	5.36 ± 0.06
<i>L. acidophilus</i>	7.27 ± 0.13	7.38 ± 0.05	6.49 ± 0.03	7.08 ± 0.04
<i>E. faecium</i>	7.99 ± 0.03	7.58 ± 0.02	7.54 ± 0.07	7.34 ± 0.05

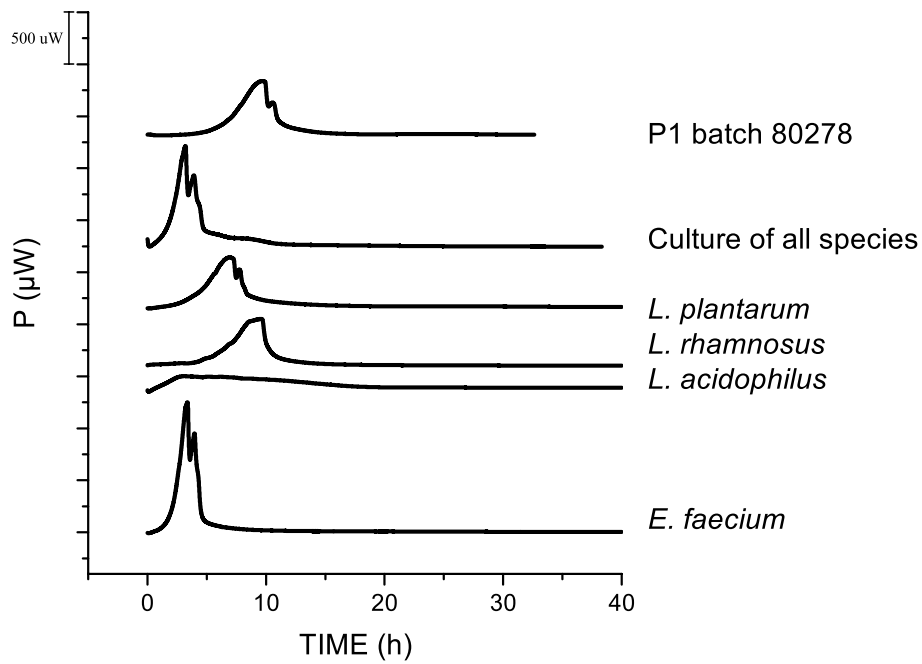
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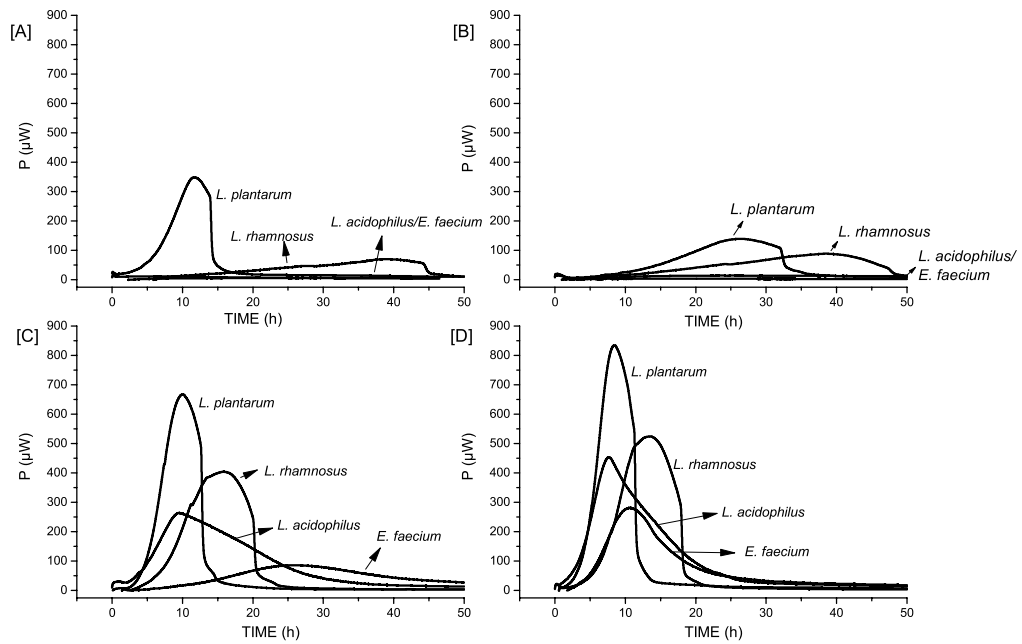
410

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



412

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).
416



417
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*.
421