In vitro inhibition of *Clostridium difficile* by commercial probiotics: a microcalorimetric study

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Abstract

The aim of the study was to investigate the influence of some commercial probiotics on the growth of *Clostridium difficile* using the isothermal microcalorimeter, an instrument which can monitor the real time growth of bacteria. Commercial probiotic strains and products, *Lactobacillus acidophilus* LA-5®, *Bifidobacterium lactis* BB-12®, Probio 7® and Symprove™ were co-cultured with *C. difficile* in Brain Heart Infusion (BHI) broth supplemented with 0.1% (w/v) L-cysteine hydrochloride and 0.1% (w/v) sodium taurocholate and monitored in the microcalorimeter. *Pseudomonas aeruginosa* NCIMB 8628 was also co-cultured with *C. difficile* and studied. The results indicated inhibition of *C. difficile* by the probiotics. The inhibition of *C. difficile* was shown to be pH-dependent using neutralized and unmodified cell free supernatant (CFS) produced by the probiotic strains. However, concentrated CFS of the probiotics also inhibited the intestinal pathogen in a non pH-dependent manner, likely due to specific antimicrobial substances produced. The results also indicated that *C. difficile* growth was greatly influenced by the presence of sodium taurocholate and by the pH of the medium. A medium pH of between 6.45 and 6.9 demonstrated maximum growth of the organism in the microcalorimeter.

Keywords: *Clostridium difficile*, probiotics, inhibition, isothermal microcalorimetry, co-culture

1. Introduction

Antibiotics can alter the composition of the microorganisms constituting the gut microbiota and result in complications such as Antibiotic-associated diarrhoea (AAD). One predominant opportunistic pathogen responsible for AAD is *Clostridium*

difficile; accountable for 20-30% cases of AAD and almost all cases of pseudomembranous colitis (Bartlett, 2002; Pochapin, 2000). It is known to be the most common nosocomial diarrhoeal pathogen in hospitalized patients and very important in terms of cost and extended stays in hospitals (Kuijper et al., 2006; Vonberg et al., 2008).

C. difficile is a Gram-positive, anaerobic spore forming bacillus, which can be cultured from the stools of 1-3% of healthy adults and up to 80% of healthy newborns and infants (Bartlett, 2002; Viscidi et al., 1981). The spore form of the organism is central in disease transmission (Cloud and Kelly, 2007; Fekety et al., 1981). It is resistant to heat, radiation, chemicals, and antibiotics (Gerding et al., 2008). When germinated, the spores produce enterotoxin (Toxin A), and cytotoxin (Toxin B) which mediate severe inflammatory response and epithelial damage resulting in the main clinical symptoms and signs of *C. difficile* infection: secretory diarrhoea and pseudomembranous colitis (Rupnik et al., 2009).

Standard treatment of *C. difficile* infection involves antibiotics, usually vancomycin and metronidazole. However, up to 24% of patients relapse from the infection within two months of first episode with the risk of additional recurrences increasing to 50-65% after two or more episodes of the infection (Pepin et al., 2005; Sunenshine and McDonald, 2006). There is also reported resistance and reduced susceptibility of *C. difficile* to both metronidazole and vancomycin (Baines et al., 2008; Brazier et al., 2008; Huang et al., 2009; Pelaez et al., 2002). An alternative approach for the management of the infection is therefore highly needed to ameliorate the current deficiency in management.

Some studies have supported the potential efficacy of some probiotics in the management of *C. difficile* infection but generally, the benefit of probiotics in the management of *C. difficile* infection is contentious (Allen et al., 2013; Goldenberg et al., 2013; Hickson, 2011; Na and Kelly, 2011; Pattani et al., 2013; Pillai and Nelson, 2008; Rainkie and Kolber, 2013). For instance, a review published by Pillai and Nelson (2008) in the Cochrane library, which initially investigated the effect of probiotics either used alone or in conjunction with antibiotics for the treatment of *C. difficile* infection, reported that only one study out of the four that met the inclusion criteria showed significant benefit of the probiotics (Pillai and Nelson, 2008). One main problem that has been raised concerning the clinical efficacy of probiotics in trials is the species (or strain) specificity. Thus, the potentially very large number of probiotics and combination of probiotics that could be assessed in clinical studies make the quest to find the true role of probiotics in the management of *C. difficile* infection a challenge due to the possible specific characteristics required for demonstrable effect which may not be possessed by some probiotics (Smith, 2013). *In vitro* studies prior to clinical tests could therefore be advantageous, providing a means of identifying probiotic species, strains or products with anticlostridial activity before further laboratory and clinical studies, saving the clinical and economic cost of inapt studies. *In vitro* testing could also provide an understanding into the mechanisms by which probiotics act against *C. difficile*.

The aim of the study was to investigate the influence of some commercial probiotics on the growth of *C. difficile* using the isothermal microcalorimeter (IMC), an instrument which has been established to measure bacterial growth in real time by monitoring net metabolic heat output, giving characteristic signatures for individual bacteria that are proportional to their growth (Beezer, 1980; Braissant et al., 2010;

Said et al., 2014). Noteworthy, traditional *in vitro* methods to study the effect of probiotics against intestinal pathogens involve either a broth culture or an agar diffusion assay (Lee et al., 2003; Naaber et al., 2004; Schoster et al., 2013). These methods only give endpoints, providing no information on the kinetics of inhibition. Also, the diffusion method may be subject to the diffusibility of inhibitory compounds. It is believed that IMC can overcome these limitations.

2. Materials and methods

Clostridium difficile NCTC 13565, purchased from the National Collection of Type Cultures (NCTC), Public Health England was used for the study. It is a toxigenic strain which had been previously isolated from the faeces of a patient with pseudomembranous colitis. *Lactobacillus acidophilus* LA-5® and *Bifidobacterium lactis* BB-12® from Chr. Hansen's Culture Collection (Hørsholm, Denmark) were the commercial probiotic strains used in the study. The commercial probiotic product, Probio 7® was purchased from a local pharmacy in Brunswick Centre (London, UK). Symprove™ was obtained from Symprove Ltd., UK. *Pseudomonas aeruginosa* NCIMB 8628 was obtained from ConvaTec Ltd., UK.

C. difficile and the probiotic strains were grown respectively in Reinforced Clostridial Medium (RCM, Oxoid) and de Man Rogosa Sharpe broth (MRS, Oxoid) supplemented with 0.05% (w/v) L-cysteine hydrochloride (MRSc) under anaerobic conditions (10% CO_2, 10% H_2 and 80% N_2; Don Whitley DG250 Scientific Anaerobic workstation, UK) at 37ºC. L-cysteine hydrochloride was used as a reducing agent, producing a pre-reduced culture medium suitable for anaerobic bacteria. *P. aeruginosa* was grown in Nutrient broth (NB, Oxoid) aerobically at 37ºC. Cells were

harvested when they reached the stationary phase of growth. The cells were washed in phosphate buffered saline (PBS), and 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 (Beezer et al., 1976). Glycerol was used as a cryoprotectant in all experiments reported in this work; it has been shown to have no effect on bacterial growth but to retain viability of organisms post-thawing (Morgan and Bunch, 2000). Aliquots were stored under liquid nitrogen until required.

Frozen aliquots of the strains were thawed for 3 min by immersion in a water bath (40^{o}C) and vortexed for 1 min before use. The strains and products were first studied as pure culture then co-cultured with *C. difficile*. For pure cultures, the thawed strains were inoculated into pre-warmed Brain Heart Infusion broth (BHI, Oxoid) supplemented with 0.1% (w/v) L-cysteine hydrochloride and 0.1% (w/v) sodium taurocholate (BHIct) to a population density of 10^6 CFU/mL in 3 mL calorimetric glass ampoules. Sodium taurocholate was added to induce germination of spores of *C. difficile* to recover them as vegetative cells. 30 µL of Symprove^{tm} or hydrated Probio 7® (in 10 mL of PBS) was also inoculated into 2970 µL of BHIct (3 mL calorimetric ampoules). For co-cultures, 10^6 CFU/mL of the probiotic strains or 30 µL of hydrated or liquid commercial probiotic products were mixed with *C. difficile.* The sealed ampoules were vortexed for 10 s and loaded into the intermediate position of a Thermometric Thermal Activity Monitor 2277 (TAM 2277) (TA Instruments Ltd., UK). Loaded samples were allowed to equilibrate thermally at the intermediate position for 30 min before measurement. Data were collected every 10 s, with an amplifier range of 1000 µW using the software package, Digitam 4.1. Data were analysed using Origin Pro 8.6 (Microcal Software Inc.). Culture observation, plate counts and pH measurements (pHenomenal®, UK; the pH meter was calibrated with

buffers of known pH and the electrode was rinsed with ethanol solution to sterilize it between experiments) were done post IMC experiments.

Culture supernatants of the probiotic strains were examined for their activity against *C. difficile*. The culture supernatants were prepared by cultivating the respective probiotic strain in MRSc at 37ºC for 48 h under anaerobic conditions. The cells were removed by centrifuging at 3500 *g* for 10 min at 4ºC. The supernatant was collected and filter-sterilized using a 0.22 µm membrane syringe filter. The pHs of the supernatants were examined and equal aliquots modified by adjusting the pH to 5, 6, 7 and 8 with 5 M NaOH. The supernatants were also concentrated by freeze-drying (Modulyo D-230, Thermo Scientific, UK) and reconstituted with sterile distilled water to achieve 2.5-fold, 5-fold, 10-fold and 20-fold concentration of the supernatant, pH adjusted. Modified supernatants were filter-sterilized before use.

1.5 mL of unmodified or modified cell free supernatants (CFS) of the probiotic strains, *L. acidophilus* LA-5®, *B. lactis* BB-12® was mixed with 1.5 mL of 2-fold concentrated BHIct (dsBHIct) in 3 mL calorimetric glass ampoule. The mixture was vortexed for 10 s. *C. difficile* was inoculated into this mixture and vortexed. Controls were conducted by replacing the CFS with MRSc broth. The ampoules were placed in the TAM 2277 and power-time measurements were taken.

CFS of *L. acidophilus* LA-5® and *B. lactis* BB-12® were also examined for their activity against *C. difficile* by the agar well diffusion assay. *C. difficile* lawn was made by seeding 1 mL of a thawed culture of the organism in 15 mL molten BHIct agar (45ºC). The agar was left to solidify. Wells of 9 mm diameter were made with a sterile borer and filled with 100 µL of unmodified and pH modified CFSs. The plates

were incubated at 37^{o}C in a Don Whitley DG250 Scientific Anaerobic workstation, UK. The zones of inhibition were measured after 48 h of incubation.

3. Results and discussion

IMC is an established technique for monitoring bacterial growth (Braissant et al., 2010). It produces net metabolic heat output proportional to growth and characteristic for different species of bacteria (Beezer, 1980; Braissant et al., 2010). Data obtained from the IMC is a plot of power (μW or μJs^{-1}) versus time (t). Fig. 1 shows the power-time curves of the pure cultures of the different strains and commercial probiotic products in BHIct. The curves are complex with peaks and troughs, which have been associated with the sequential utilization of major carbon sources typical for growth in complex media and also the diverse fermentation pathways utilized by a species (Beezer, 1980; Gaisford et al., 2009). The curves also show different onset time and area under the curve (AUC, depicting heat output) yielding individual signatures, which were used for strain or product identification. Experiments were performed in closed ampoules, which allowed anaerobic conditions to be maintained, mimicking the environment of the human gut.

It is important to note that for the purpose of differentiation of microorganisms in the IMC (and per this study for strain identification), one needs to have control over the repeatability of growth curves of the aliquots of the microorganisms. This is usually difficult to achieve using freshly grown cultures for each experiment due to natural batch-to-batch variability, which can occur. To ensure control over repeatability, the microorganisms were cryopreserved in aliquots using a standardized protocol so that they were sourced from the same batch. It was however observed that

cryopreservation of *C. difficile* resulted in spores of the organism. Whilst the spore is important in disease transmission (Cloud and Kelly, 2007; Gerding et al., 2008), spore germination is essential for *C. difficile* to cause disease (Burns et al., 2010). To ascertain the vegetative growth of *C. difficile* and consequently, the influence of the probiotic strains and products on such possible growth, we firstly examined the effect of medium supplements on the germination and growth of *C. difficile*. Fig. 2 shows the growth of *C. difficile* in different media conditions. Growth of *C. difficile* was delayed in BHIc, (BHI broth supplemented with 0.1% (w/v) of the reducing agent, L-cysteine hydrochloride) (this was shown as a long time-lag in the data). When further supplemented with 0.1% w/v sodium taurocholate, (BHIct) the data shows a significant faster growth, possibly due to the germination potential of sodium taurocholate (Giel et al., 2010; Sorg and Sonenshein, 2008). Growth was however not significantly influenced by further supplementation with 5mg/mL yeast extract (BHIcty). Previous studies have indicated germination of some strains of *C. difficile* in BHI broth (Paredes-Sabja et al., 2008) as others have shown the germination potential of taurocholate (Giel et al., 2010; Sorg and Sonenshein, 2008; Wheeldon et al., 2008; Wilson, 1983). For instance, a study by Paredes-Sabja et al. (2008) reported high levels of germination of six *C. difficile* strains in BHI broth, which they initially suggested to be due to the significant levels of inorganic phosphate ions and potassium ion content of the medium. Their results on the other hand differed from a study by Sorg and Sonenshein (2008) who reported a poor germination of two *C. difficile* strains in BHI medium. The spores from both studies were heat treated at 80^{o}C for 10 min or 60^{o}C for 20 min respectively before introduction into the medium. However in the case of the present study, they were directly incubated without heat activation. The germination of spores of *C. difficile* by taurocholate has also been

reported a number of times (Giel et al., 2010; Sorg and Sonenshein, 2008; Wheeldon et al., 2008; Wilson, 1983). Although no specific receptor binding sites have been recognized in *C. difficile* unlike in *Bacillus subtilis*, a well-studied spore forming organism, it has been suggested that *C. difficile* possibly encode unknown receptor proteins to bind sodium taurocholate to trigger germination (Ramirez et al., 2010).

The optical density (OD) measurements at 600 nm (spectrophotometer, Heλiosα, Thermo Scientific) (Table 1) show that germination and exponential growth of *C. difficile* occurred in BHI medium supplemented with sodium taurocholate but not in unsupplemented medium during 24 h of incubation. This correlates with the IMC data and strengthens the effect of sodium taurocholate on the organism.

Fig. 3 shows the power-time curves of co-cultures of *C. difficile* with the probiotic strains and products and with *P. aeruginosa*. When compared to the sole cultures of the strains and products, it can be observed that the power-time curves of the co-cultures of *C. difficile* with the probiotics lacked the characteristic curve of *C. difficile* and were superimposed on the sole cultures of the probiotics except in the case of Probio7® where the growth curve of *C. difficile* can be identified in the data (between ca.10-17 h). The growth curve of *C. difficile* could also be identified in the co-culture with *P. aeruginosa* (between ca. 10-19 h). When a plate count of vegetative cells of *C. difficile* was done post IMC experiments on selective medium, *Clostridium difficile* selective agar supplemented with *Clostridium difficile* selective supplement (cycloserine 250 mg/L, cefoxitin 8 mg/L) and 0.7% (v/v) defibrinated horse blood (CDSAsb), no recoveries of vegetative *C. difficile* was obtained on plates for the co-cultures with the probiotic strains and Symprove™ (Table 2). Growth of *C. difficile* was however observed for co-cultures with Probio 7® and *P. aeruginosa.* This made

us believe that the probiotic strains and Symprove^{tm} may have inhibited the germination and consequent growth of *C. difficile*. It is possible that one or more *C. difficile* exo-product may have caused some cell death of *P. aeruginosa* during co-culturing, since cell numbers of *P. aeruginosa* were lower after co-incubation with *C. difficile* relative to the other species.

The pH and appearance of the pure and co-cultures of the strains and products after IMC measurements are given in Table 3. A decrease in pH is assumed to mean proliferation of lactic-acid producing species, and so is reflective of inhibition of *C. difficile*. The probiotic strains and Symprove^{tm} appeared to decrease the pH of the medium the most while Probio 7® and *P. aeruginosa* decreased the pH of the medium the least although this pH reduction was more in respect to *C. difficile.* From Table 3, it appears that inhibition of *C. difficile* was pH-dependent. Thus, the probiotics may have firstly utilized the components of the medium and produced acidic metabolites, which may have prevented *C. difficile* spores from germinating and growing. In terms of appearance, where cloudiness for a co-culture appeared the same as cloudiness for a pure culture or product, this was assumed to indicate inhibition of *C. difficile*.

Figs. 4 A and B and 5A and B show inhibition of *C. difficile* by the supernatants of the probiotic strains, *L. acidophilus* LA-5® and *B. lactis* BB-12® respectively. The control experiments for Figures 4 and 5 were conducted in dsBHIct diluted with MRSc, which produced a pH of 6.30 ± 0.04 pre-inoculation. The presence of MRSc caused the organism to metabolize for longer and caused changes in the power-time curve of *C. difficile* relative to Fig. 1, where *C. difficile* was inoculated into BHIct. The pHs of the CFS produced by both strains in MRSc were 4.12 ± 0.01 and 4.62 ± 0.01 respectively. Even though the unmodified supernatants of both strains were able to

produce growth inhibition of *C. difficile*, the pH modified and concentrated supernatants showed different degree of inhibition inferring that degree of inhibition of *C. difficile* was species (or strain) specific. Also, while no growth of *C. difficile* was observed in the IMC for CFS of *B. lactis* BB-12® at pH 5, growth of *C. difficile* was observed with the CFS of *L. acidophilus* LA-5® (but with a long time-lag depicting inhibition). pH modified CFS of *L. acidophilus* LA-5® at pH 7 and 8, which produced final pHs of 6.71 ± 0.01 and 6.9 ± 0.04 respectively when mixed with dsBHIct enhanced growth of *C. difficile* significantly unlike that of *B. lactis* BB-12® which were still inhibitory to *C. difficile*. When mixed with dsBHIct, the CFS of *L. acidophilus* LA-5® at pH 6 produced a final pH of 6.45 ± 0.07 pre-inoculation and only enhanced growth of *C. difficile* slightly relative to the control (Fig. 4A). The observations with the CFS of *L. acidophilus* LA-5® suggest that the optimum pH for growth of *C. difficile* may lie between pH 6.45 and 6.9. This observation correlates with findings from Wheeldon et al. (2008) who reported that the optimum pH range for germination of *C. difficile* was 6.5-7.5 with decreased rate and extent of germination at pH 5.5 and 8.5 (Wheeldon et al., 2008). Complete inhibition of *C. difficile* in the IMC was also observed for a 5-fold concentrated CFS of *B. lactis* BB-12® and a 20-fold concentrated CFS of *L. acidophilus* LA-5®. Lower concentrations of both CFS decreased the AUC and added time-lag to the growth of *C. difficile* in the IMC. From Figs. 4 and 5, one can speculate the higher presence of other non-acidic metabolites produced by *B. lactis* BB-12® which may have resulted in superior inhibition.

In the study, the inhibitory effect of unmodified CFS and pH modified CFS were assessed to determine if inhibition of germination and growth of *C. difficile* was solely due to acidic metabolites or was also due to the presence of other non-acidic metabolites produced by the probiotic strains. Whilst some inhibition was observed

for the unmodified CFS, which suggest the likely production of organic acids such as lactic and acetic acids and hydrogen peroxide (Naaber et al., 2004; Tejero-Sarinena et al., 2012), upon pH modification, inhibition was reduced supporting that these substances indeed may have been acids. However there was a significant difference in the level of inhibition between *L. acidophilus* LA-5® and *B. lactis* BB-12® indicating inhibition was species (or strain) dependent, further supported by the use of the commercial products. Previous studies have also indicated the pH-dependency of inhibition of *C. difficile* by some probiotics. For instance, inhibition of *C. difficile* was reported by Schoster et al. (2013) to be only observed when the pH of the supernatants obtained from the probiotic species was not neutralized (Schoster et al., 2013). Trejo et al. (2006) also reported that the inhibition of isolated *Bifidobacterium* strains against *C. difficile* were dependent on their production of lactic and acetic acids (Trejo et al., 2006). Additionally, Naaber et al. (2004) also demonstrated the correlation of hydrogen peroxide and lactic acid production with inhibition of *C. difficile* by intestinal lactobacilli while Tejero-Sarinena et al. (2012) showed that these substances were lactic acid and acetic acid (Naaber et al., 2004; Tejero-Sarinena et al., 2012).

In the present study, there also seems to be the presence of other non-acidic substances, likely bacteriocins or bacteriocin-like compounds (Anand et al., 1984, 1985; Barefoot and Klaenhammer, 1983; Cheikhyoussef et al., 2008) that may account for the inhibitory activity seen in the study. Inhibitory activity was observed for pH modified CFS of *B. lactis* BB-12® and diluted CFS (results not shown) but was only observed when CFS obtained from *L. acidophilus* LA-5® was below pH 5 or was concentrated. Also whilst 5-fold concentrated CFS of *B. lactis* BB-12® produced total inhibition of *C. difficile* in the IMC, 20-fold concentration of CFS of *L. acidophilus* LA-

5® was needed for total inhibition. The purported bacteriocins or bacteriocin-like compounds could have therefore been produced in larger quantities in the *Bifidobacterium* strain than the *Lactobacillus*.

The diameters of growth inhibition zones of *C. difficile* (with the CFSs of the probiotic strains) are shown in Table 4. Data from the agar diffusion assay correlate with the IMC data but there was no indication that neutralized CFS of *B. lactis* BB-12® partially inhibited *C. difficile* or that of *L. acidophilus* LA-5® enhanced growth of *C. difficile* unlike the IMC data. In other words, the IMC data were more sensitive to subtle effects of co-culturing than the agar diffusion assays.

4. Conclusions

This study has shown that some commercial probiotics have inhibitory activity against *C. difficile* whilst others do not. Thus, inhibition and degree of inhibition may be species (or strain) dependent. Inhibition of *C. difficile* by the probiotic strains was pH-dependent, which suggest the likely production of organic acids, correlating with previous studies. The results further suggest that the probiotic strains may have produced other substances apart from the acids, which could have inhibited the germination and growth of *C. difficile.* These other substances, possibly bacteriocins or bacteriocin-like compounds, may have been produced in larger quantities in the *Bifidobacterium* strain than the *Lactobacillus* strain. The results also demonstrated that the germination and growth of *C. difficile* were influenced by pH and the presence of sodium taurocholate. pH of between 6.45 and 6.9 showed highest germination and growth of *C. difficile*; correlating with and lending weight to results obtained by Wheeldon et al. (2008).

In conclusion, this study has enhanced existing knowledge on factors that could influence the growth of *C. difficile* and showed the potential of some commercial probiotics in the possible management of *C. difficile* infection although further *in vitro* and *in vivo* animal studies will be needed to demonstrate the potential clinical benefit and the mechanism by which this will be achieved. The study has also showed the value of IMC in microbiological assays, demonstrating that it has the potential to address some microbiological problems.

Fig. 1. Power-time curves of pure cultures of *C. difficile*, *L. acidophilus* LA-5®, *B. lactis* BB-12®, *P. aeruginosa*, commercial probiotic products, Symprove™ and Probio 7® inoculated to culture densities of 10^6 CFU/mL or 1 in 100 dilutions in BHIct (representing ca. 10^6 CFU/mL). Power-time curve of each culture was characteristic in the medium.

Fig. 2. Power-time curves of *C. difficile* in different media condition: BHIc with and without supplementation with 0.1% w/v sodium taurocholate and 5 mg/mL yeast extract. Growth was significantly influenced with sodium taurocholate supplementation.

Fig. 3. Comparison of the power-time curves of pure and co-cultures of *C. difficile* with *L. acidophilus* LA-5®, *B. lactis* BB-12®, *P. aeruginosa*, commercial probiotic products: Symprove™ and Probio 7®, each inoculated to culture densities of 10^6 CFU/mL or 1 in 100 dilutions in BHIct. Power-time curves of co-cultures of *C. difficile* with *L. acidophilus* LA-5®, *B. lactis* BB-12® and Symprove™ lacked the characteristic curve of *C. difficile* and were superimposed on sole cultures of the probiotics. *C. difficile* growth curve can be identified in co-cultures with *P. aeruginosa* and Probio 7® depicting that its growth was not inhibited when co-cultured with these strain or product.

Fig. 4. Power-time curves of *C. difficile* in the CFS of *L. acidophilus* LA-5®, [A] unmodified and pH modified, [B] neutralized but concentrated 2.5-fold, 5-fold, 10-fold and 20-fold. Inhibitory activities of the CFS were lost upon neutralisation but inhibitory activities were observed for concentrated CFS. Total inhibition was observed in the microcalorimeter at 20-fold concentration.

Fig. 5. Power-time curves of *C. difficile* in the CFS of *B. lactis* BB-12®, [A] unmodified and pH modified, [B] neutralized but concentrated 2.5-fold and 5-fold. Inhibitory activities of the CFS were partially lost upon neutralisation. Total inhibition of *C. difficile* in neutralized CFS was observed in the microcalorimeter at 5-fold concentration.

Table 1. Optical density of *C. difficile* culture in different medium condition after 24 h of incubation

Medium	OD 600 nm at 24 h
BHIc	0.039
BHIct	1.213
BHIcty	1.323

Table 2. Cell counts after IMC measurements of both pure and co-cultures (n=3; The values are mean ± SD)

Culture	Cell count after IMC measurements (log CFU/mL)	
C. difficile	6.00 ± 0.60	
L. acidophilus LA-5®	5.19 ± 0.19	
B. lactis BB-12®	6.47 ± 0.07	
P. aeruginosa	8.00 ± 0.06	
Symprove™	8.35 ± 0.05	
Probio 7®	8.04 ± 0.05	
	Other species	*C. difficile*
L. acidophilus LA-5® + *C. difficile*	5.04 ± 0.09	0

B. lactis BB-12® + *C. difficile*	6.50 ± 0.03	0
P. aeruginosa + *C. difficile*	6.36 ± 0.39	6.45 ± 0.02
Symprove™ + *C. difficile*	8.36 ± 0.07	0
Probio 7® + *C. difficile*	8.01 ± 0.11	5.94 ± 0.66

Table 3. pH and appearance of cultures in BHIct post IMC measurements (n=3; The values are mean ± SD)

Culture	pH	Appearance
C. difficile	5.88 ± 0.05	Very cloudy
L. acidophilus LA-5®	5.13 ± 0.04	Slightly cloudy
B. lactis BB-12®	4.92 ± 0.01	Moderately cloudy
P. aeruginosa	5.30 ± 0.08	Moderately cloudy
Symprove™	4.72 ± 0.08	Very cloudy
Probio 7®	5.38 ± 0.01	Moderately cloudy
L. acidophilus LA-5® + *C. difficile*	5.24 ± 0.19	Slightly cloudy
B. lactis BB-12® + *C. difficile*	4.92 ± 0.03	Moderately cloudy
P. aeruginosa + *C. difficile*	5.38 ± 0.04	Very cloudy
Symprove™ + *C. difficile*	4.76 ± 0.06	Very cloudy
Probio 7® + *C. difficile*	5.39 ± 0.02	Very cloudy

Table 4. Zones of inhibition of unmodified and neutralized cell free supernatant (CFS) of *L. acidophilus* LA-5® and *B. lactis* BB-12® against *C. difficile*. (n=4. The values are mean ± SD)

Cell Free Supernatants (CFS)	Zone of inhibition of *C. difficile* (mm)
L. acidophilus LA-5® CFS unmodified	7 ± 0.0
L. acidophilus LA-5® CFS neutralized	0.0 ± 0.0
B. lactis BB-12® CFS unmodified	7.5 ± 0.72
B. lactis BB-12® CFS neutralized	0.0 ± 0.0

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