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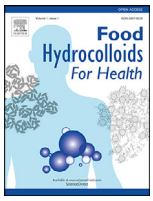

Real time microcalorimetric profiling of prebiotic inulin metabolism

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ABSTRACT

The *in vitro* assessment of prebiotics involves elaborate microbiological techniques or a combination of culture techniques and molecular methods. In this study, the isothermal microcalorimeter, an instrument which can monitor the real time growth of bacteria was applied to investigate the prebiotic effect of inulin in real time. Fresh and standardized frozen faecal slurries were prepared, placed and monitored in the isothermal microcalorimeter. The faecal samples and commercial probiotic strains *Lactobacillus acidophilus* LA-5®, *Bifidobacterium lactis* BB-12® were cultured in a mixed medium of cooked meat medium (CMM) and brain heart infusion (BHI) broth with and without supplementation with inulin and monitored in the microcalorimeter. The results showed power-time (*p-t*) curves that were characteristic for the samples. The *p-t* curves of the fresh and frozen faecal samples were similar. Augmented microbial activity was observed when the faecal sample was inoculated into CMM-BHI mixed broth with significant enhancement of microbial activity detected in the presence of inulin which was reproducible. Deconvoluted *p-t* curves showed multiple peaks with time and intensity variance depending on presence or absence of inulin suggesting possible differences in utilization of inulin by the different groups of bacteria in the polymicrobial sample. *P-t* curves of the pure species did not show any significant change when inulin was supplemented into the medium likely due to the inability of the bacteria to primarily utilize inulin.

1. Introduction

Prebiotics are non-digestible food ingredients that stimulate the growth of beneficial microorganisms of the microbiota and confer a health benefit. Gibson and Roberfroid (1995) first introduced the concept of prebiotic. They believed that unlike probiotics, the effect of prebiotics could be much more long lived due to their ability to incessantly feed and populate selected residents of the microbiota (Gibson et al., 2004; Gibson & Roberfroid, 1995). The beneficial effects of prebiotics are derived mainly from their saccharolytic fermentation which leads to short chain fatty acids (SCFA) such as acetic, propionic and butyric acids. These SCFA can be metabolized in the kidney, brain, heart or muscle tissue or utilized in the liver. They interfere with cholesterol production or serve as the energy source for the gastrointestinal cells (Cummings, 1981; Roberfroid et al., 2010; Ukai et al., 1976).

Traditionally, *in vitro* prebiotic potential of substrates is investigated by incubating potential substrates in simple batch cultures (Yun et al., 2021) or multi-stage continuous culture and gut models to mimic the complexity of the human large intestine (Duque et al., 2021;Van den Abbeele et al., 2013). The incubations are done with either pure culture of selected bacteria or faecal samples. Changes in microbial content or profile are analysed using either traditional microbiological techniques or molecular techniques or both to show the selective fermentation of a substrate or substrates (Duque et al., 2021; Li et al., 2021; Wei et al., 2020). Some of the *in vitro* studies also aim to measure and compare the production of SCFA and gases as a result of fermentation of different substrates (Cardelle-Cobas et al., 2012).

Although the traditional methods of assay are well established and have several advantages, their drawbacks are also known (Gracias & McKillip, 2004; Sohier et al., 2014). For instance, traditional culture methods are extremely laborious and time consuming. The use of turbidity assays also suffers from drawbacks such as inability to analyse heterogenous or complex samples and to distinguish viable cells from dead cells (O'Mahony & Papkovsky, 2006). Some molecular methods may fail to distinguish between viable cells from dead. They may also require special skills, reagents, pretreatment of the sample before analysis and generally tend to be costly.

Isothermal microcalorimetry, a technique based on heat measurement has great advantages in the characterization of the growth of microorganisms. The heat released by the microbial cultures during growth, presented as power–time (*p-t*) curves has been correlated quantitatively with for instance biomass generation and changes of numbers

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of cells (Braissant et al., 2010). The *p-t* profile has been used to characterize and differentiate the growth of different bacteria (Bonkat et al., 2012; Fredua-Agyeman et al., 2018; Zaharia et al., 2013). One main advantage of the microcalorimeter is its ability to monitor activities in real time unlike traditional culture assays which give data retrospectively. Although the microcalorimeter has application in microbiological assays, it is rarely used in mixed bacterial assays and is typically explored for pure species experiments like traditional microbiological assays. Microcalorimetry provides rapid detection apart from real time measurement. It has potential in metabolic profiling of polymicrobial samples and to study shifts that may occur in the presence of a metabolic modifier which could pave way for the routine study of prebiotic substrates and other metabolic modifiers in real time in a simple and cost-efficient way.

The aim of this study was to explore the microcalorimeter to observe in real time, the fermentability of inulin by a polymicrobial sample (faecal slurry) and pure probiotic species. This was to determine whether the microcalorimeter could detect in real time an increase in growth of pure probiotic species or specific members of colonic bacteria in the presence of the prebiotic substrate.

2. Materials and methods

2.1. Microbiological media and chemicals

Peptone water, yeast extract, brain heart infusion (BHI) broth, cooked meat medium (CMM) and de man rogosa sharpe (MRS) broth were obtained from Oxoid Ltd, UK. NaCl, $MgSO_4.7H_2O$ and $CaCl_2.6H_2O$ were obtained from VWR International, UK. L-cysteine hydrochloride and glycerol were purchased from Fisher Scientific, UK. K_2HPO_4, $NaHCO_3$, Ringer's solution tablets, sodium taurocholate, haemin, vitamin K, resazurin and Tween 80 were purchased from Sigma-Aldrich, UK. Inulin, from chicory, mw 522.5 g/mol was obtained from Sigma-Aldrich, UK.

2.2. Preparation of faecal slurry

Freshly voided human faeces from three healthy volunteers who had not been on any medications or antibiotics for a period of at least 6 months was obtained. The faecal material was transferred into an Anaerobic Workstation (10% CO_2, 10% H_2 and 80% N_2; Don Whitley DG250 Scientific Anaerobic workstation, UK) at 37°C and diluted with phosphate buffered saline (PBS) solution to obtain a 40% w/w slurry. The mixture was homogenized using an Ultra Turrax (IKA T18 Basic) at a speed of 18000 rpm until no large solid agglomerates could be observed. This was sieved through an open mesh fabric (Sefar Nitex^{TM}) with pore size of 350 μm to remove any unhomogenised fibrous material. A sterile basal medium was prepared with composition described by Hughes et al. (2008). Briefly, in per litre quantity it contained: peptone water 2 g, yeast extract 2 g, NaCl 0.1 g, K_2HPO_4 0.04 g, $MgSO_4.7H_2O$ 0.01 g, $CaCl_2.6H_2O$ 0.01 g, $NaHCO_3$ 2 g, haemin 0.005 g, L-cysteine hydrochloride 0.5 g, sodium taurocholate 0.5 g, Tween 80 2 mL, vitamin K 10 μL and resazurin solution 0.025% 4 mL. The sterile basal medium was added to the sieved homogenised faecal slurry to achieve a 1:1 dilution (Yadav et al., 2013). A 50% v/v glycerol in $\frac{1}{4}$ strength Ringer's solution was prepared. The faecal slurry obtained was added to the sterile 50% v/v glycerol in a 3:1 dilution. The attained faecal slurry was dispensed into 3 mL aliquots, sealed and frozen at -80°C. Frozen faecal slurry was thawed at 40°C in a water bath for 3 min and vortexed for 1 min prior to use.

2.3. Growth conditions and maintenance of pure probiotic strains

Lactobacillus acidophilus LA-5® and *Bifidobacterium lactis* BB-12® were obtained from Chr. Hansen's Culture Collection (Hørsholm, Denmark). The species were grown and maintained in MRS broth supplemented with 0.05% (w/v) L-cysteine hydrochloride. Pure cells were harvested when they reached the stationary phase of growth. The cells were washed in PBS and resuspended in 15% (v/v) glycerol in $\frac{1}{4}$ strength Ringer's solution at an organism density of 10^8 CFU/mL (Fredua-Agyeman et al., 2018) and frozen in aliquots over liquid nitrogen (Beezer et al., 1976). Frozen aliquots of the strains were thawed for 3 min by immersion in a water bath (40°C) and vortexed for 1 min before use.

2.4. Microcalorimetric fermentations

For microcalorimetric fermentation profiling, 3 mL of fresh faecal slurry was filled into a sterile 3 mL calorimetric ampoule, aseptically sealed and 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). It was left to equilibrate at that position for 30 min before being lowered into the measurement position. Data were captured with Digitam 4.1 every 10 s with an amplifier range of 1000 μW (Fredua-Agyeman et al., 2018). This was repeated for the frozen faecal slurry aliquots after thawing. Thawed faecal slurry was also inoculated in a 1 in 100 dilution in a 3 mL broth comprised of 50% v/v BHI in CMM (CMM-BHI) in a sterile 3 mL calorimetric ampoule. It was placed in the microcalorimeter and power-time measurements taken. CMM is a non-selective medium used for the cultivation and maintenance of a large spectrum of microorganisms. It contains beef heart, peptone and a small amount of glucose that provides the nutritional requirements needed by most bacteria for growth, maintaining their growth for a long time and supporting both saccharolytic and proteolytic microorganisms. BHI is also a highly nutritious medium that meets the growth requirements of many types of microorganisms supporting both fastidious and non-fastidious microorganisms. The two media were mixed with the assumption that the mixture will support the growth of a diversity of bacteria within the faecal slurry.

The fermentation of the faecal slurry in the presence of the potential prebiotic was studied by inoculating faecal slurry (1 in 100) into CMM-BHI mixed broth supplemented with 1% w/v inulin in a calorimetric ampoule. Data were recorded as described previously. Pure cultures of *L. acidophilus* LA-5® and *B. lactis* BB-12® were also inoculated into CMM-BHI broth supplemented with or without 1% w/v inulin and power-time measurements taken in the microcalorimeter. pH of the culture was taken post microcalorimetric measurements.

2.5. Statistical analysis

Results are expressed as mean ± standard deviation of triplicate experiments. Statistical analysis was performed in Origin Pro 8.6 (Microcal Software Inc.). Data were subjected to analysis of variance (ANOVA). Turkey's test was used for means comparison. P value less than 0.05 was regarded as significant difference between means.

3. Results and discussion

Faecal samples are crude; hence their composition cannot be standardized in replicate experiments. To standardize the inoculum and control reproducibility, slurries made from the faecal material were frozen down. In that way, instead of obtaining fresh faecal material to make fresh slurry for every experiment, which could introduce variability, the frozen samples from one batch of slurry was used. Frozen samples were comparable to the fresh sample as indicated by Fig. 1. The power-time curves showed a brief spike in heat production followed by a decline and plateau. The curves suggest organisms in a stationary phase. It is likely there was not enough energy source for growth of microorganisms present in the sample as a steady decay of the growth curve was observed. When the faecal slurry was inoculated into fresh medium (CMM-BHI) in a 1 in 100 dilution, there was a reproducible exponential heat production and output representing growth of the microorganisms in the

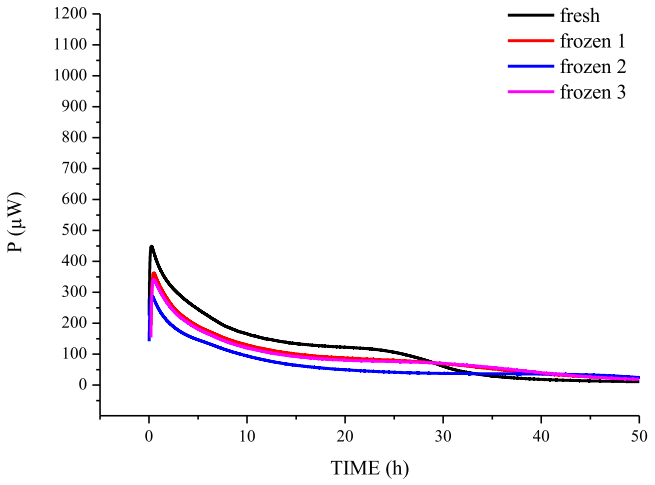

Fig. 1. Power-time curves of fresh and frozen faecal slurry.

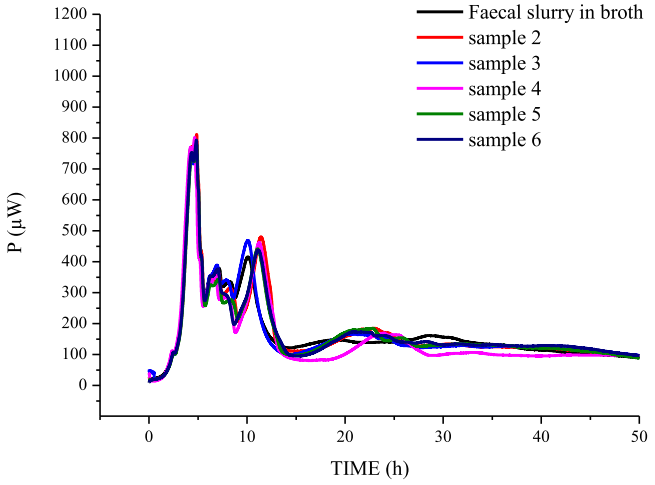

Fig. 2. Power-time curves of faecal slurry inoculated into fresh CMM-BHI medium.

Table 1
Final pH after microcalorimetric experiments with and without inulin supplementation of the culture.

Culture	Final pH
Faecal slurry in CMM-BHI without inulin	6.39 ± 0.03*
Faecal slurry in CMM-BHI with 1% w/v inulin	5.86 ± 0.02*
LA-5® in CMM-BHI without inulin	5.70 ± 0.10*
LA-5® in CMM-BHI with 1% w/v inulin	5.40 ± 0.04*
BB-12® in CMM-BHI without inulin	5.51 ± 0.01*
BB-12® in CMM-BHI with 1% w/v inulin	5.36 ± 0.04*

Data is expressed as mean ± standard deviation, (n=3). *Indicate significant differences ($p < 0.05$) between the control (uninoculated medium, pH 7.24 ± 0.01) and the different culture.

sample (Braissant et al., 2010) (Fig. 2), with a reduction of pH of the culture from an initial 7.24 ± 0.01 to 6.39 ± 0.03 after microcalorimetric measurement (Table 1). Greater growth was observed when 1% w/v inulin was added to the medium, evident with the significant increase of the area under the curve (AUC) or heat output (Fig. 3) and significant decrease ($p < 0.05$) of the pH of the medium to 5.86 ± 0.02 from the initial value of 7.24 ± 0.01. Changes in the *p-t* curves were observed when 1% w/v inulin was added to the medium. The deconvoluted peaks for the heat curves of faecal slurry in CMM-BHI medium without and with 1% w/v inulin supplementation are shown in Figs. 4 and 5. The

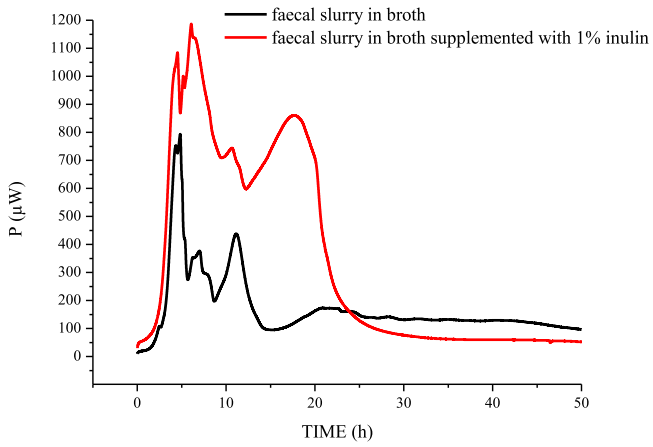

Fig. 3. Power-time curves of faecal slurry in CMM-BHI and medium supplemented with 1% w/v inulin.

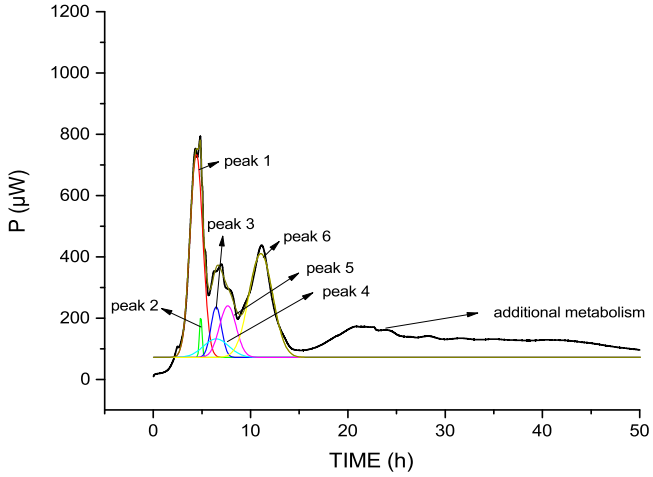

Fig. 4. Heat flow record and deconvulated peaks of faecal slurry in CMM-BHI medium.

deconvoluted peaks have been suggested to represent respiration and fermentation of different substrates (Braissant et al., 2010). The time of occurrence of the peaks varied without or with inulin supplementation. An additional peak was observed in the heat flow record when the sample was supplemented with inulin. The time of occurrence of the first two peaks without inulin supplementation was 4.31 h and 4.82 h whereas that with inulin supplementation was delayed, occurring at 4.54 h and 5.15 h respectively. Peaks 3 and 4 however occurred earlier for the sample with inulin supplementation, occurring at 6.08 h and 6.51 h relative to 6.19 h and 7.06 h for unsupplemented medium. The deconvoluted peaks also showed variances in intensities of the peaks suggesting possibly different growth profile of specific groups of microorganisms with and without inulin supplementation. Additional metabolic activity that occurred in the unsupplemented medium after the final peak, (Fig. 4) was absent in the inulin supplemented medium (Fig. 5). The power-time curves of *L. acidophilus* LA-5® and *B. lactis* BB-12® inoculated into CMM-BHI medium with and without inulin supplementation are shown in Figs. 6 and 7. Supplementation with inulin at 1% w/v did not change the *p-t* profile of the microorganisms significantly like the faecal sample. The *p-t* curves indicated that the growth of these species were not significantly enhanced.

Inulin is a known prebiotic and its prebiotic potential has been investigated extensively. For instance, Van den Abbeele et al., (2013) comparing inulin and long-chain arabinoxylan (a wheat derived fiber) and also two elaborate models, simulator of the human intestinal micro-

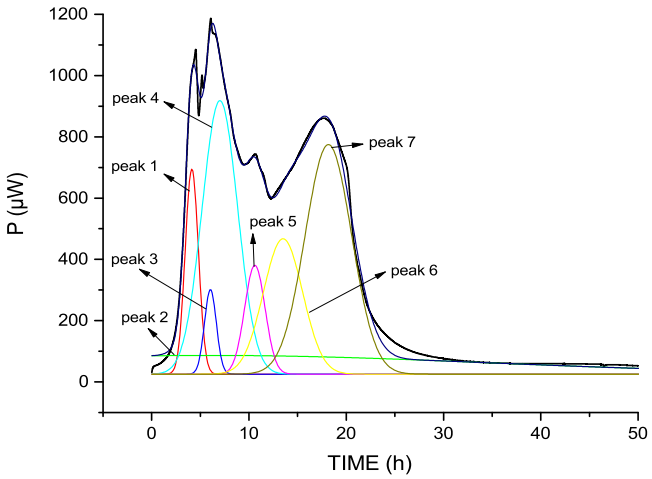

Fig. 5. Heat flow record and deconvulated peaks of faecal slurry in CMM-BHI medium supplemented with 1% w/v inulin.

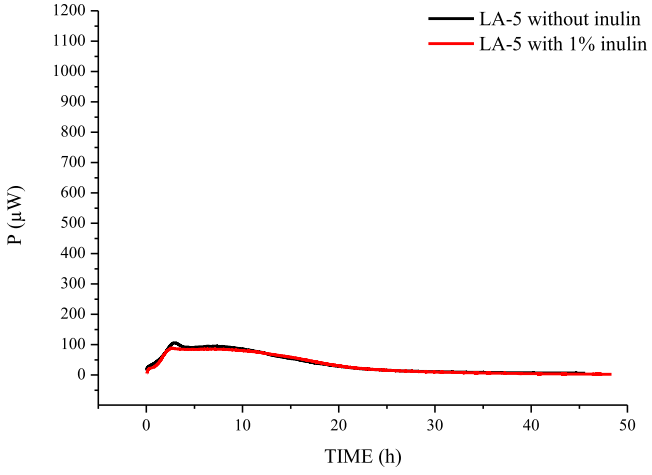

Fig. 6. Power-time curves of *L. acidophilus* LA-5® with and without 1% w/v inulin supplementation.

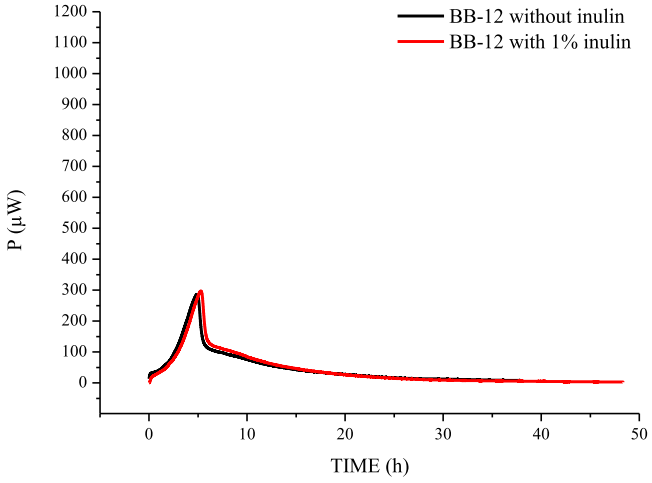

Fig. 7. Power-time curves of *B. lactis* BB-12® with and without 1% w/v inulin supplementation.

bial ecosystem model (SHIME) and dynamic TNO *in vitro* model of the colon (TIM-2) combined with conventional plate count, denaturing gradient gel electrophoresis (to monitor shifts within the SHIME microbiota) and the intestinal-chip (I-chip, a phylogenetic microarray, applied standardly to monitor microbial shifts in the TIM-2), reported increased abundance of bifodobacteria (including *B. catenulatum, B. adolescentis, B. angulatum* and *B. bifidum*) whilst members of the Bacteroidetes were decreased. Also, a significant reduction of *Ruminococcus bromii* was reported with inulin. Another study by Zhu et al. (2022) demonstrated the prebiotic effects of inulin and blends of polydextrose with inulin. Their data showed that inulin stimulated growth of *Bifodobacteria spp* at high levels (Zhu et al., 2022). In the present study, the microcalorimeter detected increased metabolic activity of the faecal culture with inulin supplementation. Notably, faecal slurry could contain different groups of bacteria. O'Donnell et al. (2016) reported faecal slurry to be dominated by *Firmicutes* and *Bacteriodetes* and to a lesser extent, *Actinobacteria* and *Proteobacteria* at the phyla level and by *Ruminococcaceae, Lachnospiraceae* and *Prevotellaceae* at the family level and by *Lachnospiraceae Incertae Sedis, Prevotella, Faecalibacterium* and *Bacteroides* at the genus level (O'Donnell et al., 2016). Whilst some groups, as exemplified by previous studies may be able to effectively metabolise inulin, other groups may not. Amongst groups, some species, and strains may not be able to metabolise inulin. For example, *Bifidobacterium breve* Yakult and *Bifidobacterium adolescentis* LMG 10734 were shown not to be able to degrade inulin whereas *Bifidobacterium longum* LMG 11047 and *Bifidobacterium angulatum* LMG 11039T could partially degrade inulin and also degrade oligofructose when grown in coculture fermentations with *Bacteroides thetaiotaomicron* LMG 11262, a strain able to metabolize both inulin and oligofructose (Falony et al., 2009). For the present study, the *p-t* profile obtained when faecal slurry was inoculated into CMM-BHI medium was generated by the dominant groups in the sample. A previous study in the microcalorimeter demonstrated that for polymicrobial samples, interspecies interaction tend to predominate and *p-t* curve could represent the dominant sample (Fredua-Agyeman et al., 2017). It is likely the dominant groups in the faecal sample utilized the supplemented inulin, enhancing their growth. However, some changes in the metabolism (changes in the heat peaks and intensity of peaks) was noted, likely due to differences in inulin metabolism amongst the groups of bacteria that dominated the sample.

In the case of the pure species, when inulin was supplemented into the growth media of *L. acidophilus* LA-5® and *B. lactis* BB-12®, the *p-t* curves did not change. A reduction in pH was however noted after microcalorimetric measurements. It must be noted that the pH of the medium with and without inulin supplementation preinoculation was not different. It is likely that inulin was not fermented by the pure species at 1% w/v supplementation. When inulin was however supplemented at 3%, a more enhanced reduction in pH was noted, thus 4.57 ± 0.15 and 5.12 ± 0.11 respectively for *L. acidophilus* LA-5® and *B. lactis* BB-12® and appearance of culture was more cloudy post microcalorimetric measeasurements (results not shown). The pure species may have partially degraded inulin or possibly degraded it when the other nutrient sources in the medium were exhausting at the higher concentration. For instance, for *L. acidophilus* LA-5®, the "inulin effect" was noticeable after 30 h of microcalorimetric incubation (results not shown). The reason for this may be that the pure species may have adapted on the other sources of nutrients supplied in the medium and may have therefore preferentially metabolised the other substrate (with possibly accumulated toxic waste) before switching to fermenting inulin. Such adaptations have been previously observed in microbiological assays (Garcia-Nunez et al., 2022).

In summary, the data shows that inulin was degraded by the faecal culture; possibly by a specific group, which dominated the culture. Inulin was not degraded by the pure species at 1% w/v supplementation but might have been partially degraded by the pure species at higher concentration. It is also likely degradation of inulin occurred after other carbohydrate sources in the medium were exhausting for the

pure species. Methods used to determine microbial changes when studying the prebiotic potential of substrates are based on conventional culture techniques (colony counts on selective agars) and molecular techniques. The conventional culture techniques, apart from being laborious and time consuming are unable to account for unculturable organisms (Liesack & Stackebrandt, 1992) hence differences exists between culture approaches and molecular tools (Sghir et al., 1998; Van de Wiele et al., 2004). Also, the conventional culture techniques are based on retrospective data unlike data from the microcalorimeter. The results obtained from this study suggest that microcalorimetry offers a potential to study the prebiotic potential of substrates and could complement the current methods, being advantageous in routine screening of multiple substrates. It has its own limitations, for instance, the influence of interspecies effect. Thus, the possibility of competition between species of polymicrobial samples pre-supplementation with a potential substrate since the microcalorimeter measures the overall activity of a culture embracing interspecies effect which occurs in natural habitats. Also, for the current study, the medium used contained other carbohydrates sources (for example glucose), which could have been preferentially utilised by the pure species. A way of surmounting this in future is to use inulin as the sole carbohydrate source so that the organisms would have been limited by the sole substrate for metabolism.

4. Conclusion

The microcalorimeter was explored to detect in real time the fermentability of inulin by faecal slurry and pure probiotic species. The fermentation of inulin was demonstrated in the microcalorimeter with the faecal sample, suggesting a possible impact on gut microbiome. However, the fermentation of inulin was not observed in the microcalorimeter at 1% w/v for the pure species. The study shows the importance of isothermal microcalorimetry in microbiological assays, particularly for the routine screening of substrates with potential prebiotic effects as it complements conventional methods. Future studies would explore limiting the prebiotic substrate as the sole carbohydrate source and test different pure bacteria species. Also, the potential mechanisms underlying the observed effects would be explored in a future study.

Ethical statement

Ethical approval was granted by the UCL Research Ethics Committee. Informed consent was received from all volunteers before sample collection.

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.

References

Beezer, A. E., Newell, R. D., & Tyrrell, H. J. (1976). Application of flow microcalorimetry to analytical problems: the preparation, storage and assay of frozen inocula of *Saccharomyces cerevisiae*. *Journal of Applied Bacteriology, 41*(2), 197–207. 10.1016/0039-9140(74)80176-1.

Bonkat, G., Braissant, O., Widmer, A. F., Frei, R., Rieken, M., Wyler, S., Gasser, T. C., Wirz, D., Daniels, A. U., & Bachmann, A. (2012). Rapid detection of urinary tract pathogens using microcalorimetry: principle, technique and first results. *BJU International, 110*(6), 892–897. 10.1111/j.1464-410X.2011.10902.x.

Braissant, O., Wirz, D., Gopfert, B., & Daniels, A. U. (2010). Use of isothermal microcalorimetry to monitor microbial activities. *FEMS Microbiology Letters, 303*(1), 1–8. 10.1111/j.1574-6968.2009.01819.x.

Cardelle-Cobas, A., Olano, A., Corzo, N., Villamiel, M., Collins, M., Kolida, S., & Rastall, R. A. (2012). *In vitro* fermentation of lactulose-derived oligosaccharides by mixed fecal microbiota. *Journal of Agricultural and Food Chemistry, 60*(8), 2024–2032. 10.1021/jf203622d.

Cummings, J. H. (1981). Short chain fatty acids in the human colon. *Gut, 22*(9), 763–779. 10.1136/gut.22.9.763.

Duque, A., Demarqui, F. M., Santoni, M. M., Zanelli, C. F., Adorno, M. A. T., Milenkovic, D., Mesa, V., & Sivieri, K. (2021). Effect of probiotic, prebiotic, and synbiotic on the gut microbiota of autistic children using an *in vitro* gut microbiome model. *Food Research International, 149*, Article 110657. 10.1016/j.foodres.2021.110657.

Falony, G., Calmeyn, T., Leroy, F., & De Vuyst, L (2009). Coculture fermentations of Bifidobacterium species and Bacteroides thetaiotaomicron reveal a mechanistic insight into the prebiotic effect of inulin-type fructans. *Applied and Environmental Microbiology, 75*(8), 2312–2319. 10.1128/aem.02649-08.

Fredua-Agyeman, M., Gaisford, S., & Beezer, A. E. (2018). Observation with microcalorimetry: Behaviour of *P. aeruginosa* in mixed cultures with *S. aureus* and *E. coli*. *Thermochimica Acta, 663*, 93–98. 10.1016/j.tca.2018.03.009.

Fredua-Agyeman, M., Stapleton, P., Basit, A. W., & Gaisford, S. (2017). Microcalorimetric evaluation of a multi-strain probiotic: Interspecies inhibition between probiotic strains. *Journal of Functional Foods, 36*, 357–361. 10.1016/j.jff.2017.07.018.

Gibson, G. R., Probert, H. M., Loo, J. V., Rastall, R. A., & Roberfroid, M. B. (2004). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutrition Research Reviews, 17*(2), 259–275. 10.1079/NRR200479.

Gibson, G. R., & Roberfroid, M. B. (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *The Journal of Nutrition, 125*(6), 1401–1412. 10.1093/jn/125.6.1401.

Gracias, K. S., & McKillip, J. L. (2004). A review of conventional detection and enumeration methods for pathogenic bacteria in food. *Canadian Journal of Microbiology, 50*(11), 883–890. 10.1139/w04-080.

Garcia-Nunez, I. M., Santacruz, A., Serna-Saldivar, S. O., Hernandez, S. L. C., & Amaya Guerra, C. A. (2022). Assessment of potential probiotic and synbiotic properties of lactic acid bacteria grown *in vitro* with starch-based soluble corn fiber or inulin. *Foods*, (24), 11. 10.3390/foods11244020.

Hughes, S. A., Shewry, P. R., Gibson, G. R., McCleary, B. V., & Rastall, R. A. (2008). *In vitro* fermentation of oat and barley derived beta-glucans by human faecal microbiota. *FEMS Microbiology Ecology, 64*(3), 482–493. 10.1111/j.1574-6941.2008.00478.x.

Li, C., Zhou, S., Fu, X., Huang, Q., & Chen, Q. (2021). *In vitro* digestibility and prebiotic activities of a bioactive polysaccharide from Moringa oleifera leaves. *Journal of Food Biochemistry, 45*(11), e13944. 10.1111/jfbc.13944.

Liesack, W., & Stackebrandt, E. (1992). Unculturable microbes detected by molecular sequences and probes. *Biodiversity and Conservation, 1*(4), 250–262. 10.1007/bf00693763.

O'Donnell, M. M., Rea, M. C., O'Sullivan, O., Flynn, C., Jones, B., McQuaid, A., Shanahan, F., & Ross, R. P. (2016). Preparation of a standardised faecal slurry for *ex-vivo* microbiota studies which reduces inter-individual donor bias. *Journal of Microbiological Methods, 129*, 109–116. 10.1016/j.mimet.2016.08.002.

O'Mahony, F. C., & Papkovsky, D. B. (2006). Rapid high-throughput assessment of aerobic bacteria in complex samples by fluorescence-based oxygen respirometry. *Applied and Environmental Microbiology, 72*(2), 1279–1287. 10.1128/AEM.72.2.1279-1287.2006.

Roberfroid, M., Gibson, G. R., Hoyles, L., McCartney, A. L., Rastall, R., Rowland, I., Wolvers, D., Watzl, B., Szajewska, H., Stahl, B., Guarner, F., Respondek, F., Whelan, K., Coxam, V., Davicco, M. J., Leotoing, L., Wittrant, Y., Delzenne, N. M., Cani, P. D., Neyrinck, A. M., & Meheust, A. (2010). Prebiotic effects: metabolic and health benefits. *British Journal of Nutriton, 104*(2), S1–63 *Suppl.* 10.1017/S0007114510003363.

Sghir, A., Chow, J. M., & Mackie, R. I. (1998). Continuous culture selection of bifidobacteria and lactobacilli from human faecal samples using fructooligosaccharide as selective substrate. *Journal of Applied Microbiology, 85*(4), 769–777.

Sohier, D., Pavan, S., Riou, A., Combrisson, J., & Postollec, F. (2014). Evolution of microbiological analytical methods for dairy industry needs. *Frontiers in Microbiology, 5*(10), 16 Article. 10.3389/fmicb.2014.00016.

Ukai, M., Tomura, A., & Ito, M. (1976). Cholesterol synthesis in germfree and conventional rats. *Journal of Nutrition, 106*(8), 1175–1183. 10.1093/jn/106.8.1175.

Van de Wiele, T., Boon, N., Possemiers, S., Jacobs, H., & Verstraete, W. (2004). Prebiotic effects of chicory inulin in the simulator of the human intestinal microbial ecosystem. *FEMS Microbiology Ecology, 51*(1), 143–153. 10.1016/j.femsec.2004.07.014.

Van den Abbeele, P., Venema, K., Van de Wiele, T., Verstraete, W., & Possemiers, S. (2013). Different human gut models reveal the distinct fermentation patterns of arabinoxylan versus inulin. *Journal of Agricultural and Food Chemistry, 61*(41), 9819–9827. 10.1021/jf4021784.

Wei, X., Fu, X., Xiao, M., Liu, Z., Zhang, L., & Mou, H. (2020). Dietary galactosyl and mannosyl carbohydrates: *In-vitro* assessment of prebiotic effects. *Food Chemistry, 329*, Article 127179. 10.1016/j.foodchem.2020.127179.

Yadav, V., Gaisford, S., Merchant, H. A., & Basit, A. W. (2013). Colonic bacterial metabolism of corticosteroids. *International Journal of Pharmaceutics, 457*(1), 268–274. 10.1016/j.ijpharm.2013.09.007.

Yun, E. J., Yu, S., Kim, Y. A., Liu, J. J., Kang, N. J., Jin, Y. S., & Kim, K. H. (2021). *In vitro* prebiotic and anti-colon cancer activities of agar-derived sugars from red seaweeds. *Marine Drugs, 19*(4). 10.3390/md19040213.

Zaharia, D. C., Popa, M. G., Steriade, A. T., Muntean, A. A., Balint, O., Micut, R., Popa, V. T., Popa, M. I., & Bogdan, M. A. (2013). Microcalorimetry - a new method for bacterial characterisation. *Pneumologia, 62*(4), 232–235.

Zhu, L., Guo, F., Guo, Z., Chen, X., Qian, X., Li, X., Li, X., Li, J., Wang, X., & Jia, W. (2022). Potential health benefits of lowering gas production and bifidogenic effect of the blends of polydextrose with inulin in a human gut model. *Frontiers in Nutrition, 9*, Article 934621. 10.3389/fnut.2022.934621.