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In vitro effects of Bifidobacterium lactis-based synbiotics on human faecal bacteria

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Henrique-Bana, F. C., Wang, X., Costa, G. N., Spinosa, W. A., Miglioranza, L. H. S., Scorletti, E., Calder, P. C., Byrne, C. D. and Gibson, G. In vitro effects of *Bifidobacterium lactis*-based synbiotics on human faecal bacteria.

Abstract

Synbiotics are dietary means of modulating gut microbiota composition and activities. This study aimed to investigate the *in vitro* potential fermentation properties of two synbiotic mixtures on human faecal bacteria from healthy persons. The selected synbiotics (fructo-oligosaccharide (FOS) (1%) combined with *Bifidobacterium lactis* BB12 or *Bifidobacterium lactis* HN019 (10^6 colony-forming units (CFU)/mL)) were added to 48-h pH-controlled anaerobic batch cultures reflective of the distal region of the human large intestine. Maltodextrin, FOS and the probiotic components were also tested alone. Effects upon major groups of the microbiota were monitored during a time course by fluorescence in situ hybridisation (FISH). Short-chain fatty acids (SCFA) were measured by gas chromatography (GC).

Quantitative analysis of bacterial populations revealed that there was a significant increase ($P<0.05$) in bifidobacterial numbers in vessels with FOS and the synbiotic mixtures compared to the negative control. Levels of *Clostridium coccoides*–*Eubacterium rectale* group significantly increased ($P < 0.05$) at 8 and 24 h of fermentation with maltodextrin, whereas *Atopobium* cluster increased after 8 h of FOS fermentation.

Additionally, vessels containing carbohydrates promoted shifts in the production of SCFA. Fermentation of FOS and synbiotic mixtures at all time points resulted in a greater production of acetate and butyrate compared to a negative control, whereas the concentrations of iso-valerate were lower in these three treatments. Lactate concentrations were higher only after 8 h of fermentation with maltodextrin, FOS and the synbiotic mixtures.

Conclusion: Probiotics alone had no significant effect upon relevant microbial populations and SCFA. The effects of synbiotic mixtures on human faecal bacteria were similar to FOS as a prebiotic.

Objective

To study the *in vitro* fermentation properties of two synbiotic mixtures (FOS + *Bifidobacterium lactis* BB12 and FOS + *Bifidobacterium lactis* HN019) on human faecal bacteria of healthy humans.

1) Investigate the growth of total bacteria and 11 functionally significant faecal microbial groups in a pH-controlled, stirred, batch-culture fermentation system reflective of the environmental conditions of the distal region of the human large intestine.

2) Analyse the production of selected short-chain fatty acid (SCFA).

Materials and methods

Methods

Substrates

Fructo-oligosaccharide with a degree of polymerisation < 10 and maltodextrin were supplied by University Hospital Southampton NHS Foundation Trust. Freeze-dried *Bifidobacterium animalis* subsp. *lactis* HN019 (DuPont-Danisco, Madison, USA) and *Bifidobacterium animalis* subsp. *lactis* BB12 (Chr. Hansen, Hørsholm, Denmark) were stored at -80 °C. Plates of de Man-Rogosa-Sharpe (MRS) agar (Oxoid Ltd, Basingstoke, Hampshire, UK) were inoculated with the bifidobacterial strains and incubated at 37°C in an anaerobic chamber (10% CO_2, 10% H_2 and 80% N_2, Don Whitley Scientific LTD, Shipley, West Yorkshire, UK). After incubation, bottles containing 9 mL of MRS broth were then inoculated with one colony from each plate. The cultured broths of probiotics were incubated for 24 h under the same conditions as mentioned above.

Faecal sample preparation

Faecal samples were collected from three separate individuals (2 women and 1 man aged between 25 and 35y) who were in good health, were not taking probiotic or prebiotic supplements and had not had antibiotics for at least 3 months before the study. Samples were collected, on site, on the day of the experiment and placed in an

anaerobic jar (AnaeroJar™ 2.5L, Oxoid Ltd), including a gas-generating kit (AnaeroGen™, Oxoid). Then, samples were weighed, diluted, 1:10 (w/v), with anaerobic sterile PBS (0.1 mol/L phosphate buffer solution, pH 7·4) and homogenised (Stomacher 400; Seward) for 2 min at 240 paddle/min. Resulting faecal slurries from each individual were used to inoculate the batch culture fermentation systems.

In vitro *batch culture fermentation*

Sterile stirred batch culture fermentation vessels (300 mL working volume) were prepared and aseptically filled with 135 mL of sterile basal nutrient medium (peptone water (2 g/l), yeast extract (2 g/l), NaCl (0.1 g/l), K_2HPO_4 (0.04 g/l), $NaCO_3$ (2 g/l), $MgSO_4.7H_2O$ (0.01 g/l), $CaCl_2.6H_2O$ (0.01 g/l), Tween 80 (2 ml/l), haemin (50 mg/l), vitamin K1 (10 ll/l), L-cysteine (0.5 g/l), bile salts (0.5 g/l), resazurin (1 mg/l) and distilled water). Once in the fermentation vessels, sterile medium was maintained under anaerobic conditions by sparging the vessels with O_2-free N_2 overnight. Temperature was held at 37 °C using a circulating water bath and pH values controlled between 6.7 and 6.9 using an automated pH controller (Fermac 260; Electrolab, Tewkesbury, UK) which added acid or alkali as required (0.5 M HCl and 0.5 M NaOH). Culture pH and temperature mimicked the conditions of the distal region of the human large intestine.

Seven gently stirred pH-controlled batch fermenters were run in parallel. Two vessels were inoculated with the carbohydrates FOS and maltodextrin (1% w/v) and another set of two vessels were inoculated with 1 mL of a suspension of *B. lactis* HN019 and *B. lactis* BB12 (10^6 CFU mL^{-1}), respectively. The two synbiotic vessels were inoculated with FOS combined with *B. lactis* HN019 and *B. lactis* BB12 at the same concentrations as above. Finally, a control with neither probiotics nor carbohydrates added (negative control) was also included.

The experiment was performed in triplicate, using one faecal sample given by a different donor for each run of seven batch fermenters. Probiotics and carbohydrates were added to each vessel just before the addition of 15 mL (1:10, w/v) of fresh faecal slurry, prepared as described above. Batch cultures were conducted for 48 h, and 4 mL samples obtained from each vessel at 0, 8, 24 and 48 h for analysis of bacterial populations by fluorescent I situ hybridization (FISH) and for SCFA analyses using gas chromatography (GC).

Enumeration of bacterial populations by FISH

Bacterial composition in the batch culture models was analysed by FISH with flow cytometry (FISH-FCM). Seven hundred and fifty microlitres of samples were centrifuged at 1136 × g for 5 min. Pellets were resuspended in 375 μL of filtered PBS and fixed in 1125 μL of 4% (v/v) paraformaldehyde. After 4 h of incubation at 4°C, samples were washed twice using 1 mL of PBS, resuspended in 600 μL PBS-ethanol (1:1, v/v) and stored at –20°C. Permeabilisation steps were performed using 30 μL of the fixed samples added to 500 μL PBS and centrifuged at 1136 × g for 3 min. Pellets were resuspended using 100 μL of filtered TE-FISH (Tris/HCl 1 M pH 8, EDTA 0.5 M pH 8, distilled H2O, 0.22 μm PVDF membrane) containing lysozyme (1 mg/mL of 50 000 U/mg protein) and incubated for 10 min at room temperature. Solutions containing the samples were then vortexed and centrifuged at 1136 × g for 3 min. Pellets were washed with 500 μL PBS and centrifuged (1136 × g, 3 min). Hybridisations were performed by resuspending the pellets in 150 μL of hybridisation buffer (5 M NaCl, 1 M Tris/HCl pH 8, 30% formamide, ddH2O, 10% SDS), vortexed and centrifuged (1136 × g, 3 min). Pellets were then resuspended in 1 mL of hybridisation buffer and 50 μL aliquoted into Eppendorf tubes.

Probes used (Sigma Aldrich Ltd, Poole, Dorset, UK) are shown in Table 1. Four microliters of each probe and 4 μL of Eub338 I-II-III were added to the working solution and incubated overnight at 35°C in a heating block. After 12 h of incubation, an aliquot of 150 μL hybridisation buffer was added to the working solution, vortexed and centrifuged (1136 × g, 3 min). One hundred and fifty microlitres of supernatant was removed from each sample and the remaining volume centrifuged (1136 × g, 3 min). The pellets were washed with 200 μL of washing buffer (5 M NaCl, 1 M Tris/HCl pH 8, 0.5 M EDTA pH 8, ddH2O, 10% SDS), homogenised by vortexing and incubated for 20 min at 37°C in a heating block. Afterwards, samples were centrifuged (1136 × g, 3 min) and supernatants removed. Negative control samples (no probes added) were screened by FCM to detect background before the probe samples were resuspended in an appropriate amount of PBS. Samples were stored at 4°C until determination. Numbers of specific and total bacteria were assessed taking into account dilution factor, calculated from different volumes used in samples preparation steps, and events/μL obtained from NON EUB338 and EUB338 I-II-III probes analysed by FCM.

SCFA analysis

Aliquots of 1 mL of samples supernatant were transferred into glass tubes, followed by the addition of 50 µL of internal standard (100 mM; 2-ethylbutyric acid), 500 µL of concentrated hydrochloric acid (HCl) and 3 mL of diethyl ether (Sigma Aldrich Ltd., Poole, Dorset, UK). Samples were then vortexed for 1 min before centrifugation at 3,000 x g for 10 min. The top ether layer was transferred from each tube into clean glass tubes. A second extraction step was then completed using a further 1mL of diethyl ether. The diethyl layer was again collected and pooled with the layer from the first extraction. Aliquots of 400µL of this pooled extract were transferred into glass vials, alongside 50 µL of N-methyl-N-tbutyldimethylsilyltrifluoroacetamide (Cheshire Sciences, Chester, UK). Samples were then incubated at 80°C in a water bath for 20 min and left at room temperature for 72 h to allow for the complete derivatisation of lactic acid.

The derivatised samples were run on a 5890 Series II Gas Chromatograph (HP, Crawley, West Sussex, UK) with flame ionisation detector, using an Rtx-1 10m×0.18mm column coated with a 0.20µm coating (Crossbond 100% dimethyl polysiloxane; Restek, Buckinghamshire, UK). Injector and detector temperature were set at 275°C and the column temperature programmed from 63°C for 3 min to 190°C at 10°C/min-1 and held at 190 °C for 3 min. Helium was used as the carrier gas (flow rate 1.2 mL/min; head pressure 90 MPa). External standards contained (mM): sodium formate, 10; acetic acid, 30; propionic acid, 20; iso-butyric acid, 5; n-butyric acid, 20; isovaleric acid, 5; n-valeric acid, 5; sodium lactate, 10; sodium succinate, 20. Chemstation B.03.01 (Agilent Technologies, Cheshire, UK) was used for calibration and calculation of the internal response factor for quantification of peak areas within samples.

Statistical analysis

One-way ANOVA tests were used to compare treatments data for bacterial counts and SCFA concentrations. The data of each treatment were compared over the same time of fermentation. Mean values were then analysed using the post hoc analysis Tukey test at $P \leq 0.05$ by Statistica software version 10.0 (Statsoft South America).

Table 1. Oligonucleotide probes used in this study for FISH-FCM analysis of bacterial populations. +: These probes are used together in equimolar concentration of 50 ng/µL.

Probe name	Sequence (5' TO 3')	Target group

Non Eub	ACTCCTACGGGAGGCAGC	
Eub338 I +	GCT GCC TCC CGT AGG AGT	Most bacteria
Eub338 II +	GCA GCC ACC CGT AGG TGT	Planctomycetales
Eub338 III +	GCT GCC ACC CGT AGG TGT	Verrucomicrobiales
Bif164	CAT CCG GCA TTA CCA CCC	Most *Bifidobacterium* spp. and *Parascardovia denticolens*
Lab158	GGTATTAGCAYCTGTTTCCA	Most *Lactobacillus*, *Leuconostoc* and *Weissella* spp.; *Lactococcus lactis*; all *Vagococcus*, *Enterococcus*, *Melisococcus*, *Tetragenococcus*, *Catellicoccus*, *Pediococcus* and *Paralactobacillus* spp,
Bac303	CCA ATG TGG GGG ACC TT	Most *Bacteroidaceae* and P*revotellaceae*, some *Porphyromonadaceae*
Erec482	GCT TCT TAG TCA RGT ACCG	Most of the *Clostridium coccoides-Eubacterium rectale* group (*Clostridium* clusters XIVa and XIVb)
Rrec584	TCA GAC TTG CCG YAC CGC	*Roseburia* subcluster
Ato291	GGT CGG TCT CTC AAC CC	*Atopobium*, *Colinsella*, *Olsenella* and *Eggerthella* spp.; *Cryptobacterium curtum*; *Mycoplasma equigenitalium* and *Mycoplasma elephantis*
Prop853	ATT GCG TTA ACT CCG GCAC	Clostridial cluster IX
Fprau655	CGCCTACCTCTGCACTAC	*Faecalibacterium prausnitzii* and related sequences
DSV687	TAC GGA TTT CAC TCC T	Most *Desulfovibrionales* (excluding *Lawsonia*) and many *Desulfuromonales*
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the *Clostridium histolyticum* group (*Clostridium* clusters I and II)
CFB286	GTAGGGGTTCTGAGAGGA	*Cytophaga-Flexibacter-Bacteroides*

Results

Bacterial Enumeration by FISH

In order to determine bacterial population changes, 16S-rRNA fluorescent *in situ* hybridisation probes were used to follow the changes in population of total bacteria and 11 functionally significant faecal microbial groups (at 0, 8, 24 and 48 h of incubation).

Trends to increases in maltodextrin, FOS and both synbiotic vessels were observed for total bacterial levels (detected by Eub I-II-III probe), however no significant changes were found, the highest number was detected in the fermentation with BB12 + FOS at 8 h (Log_{10} 8.34 ± 0.18 bacteria mL^{-1}).

A significant increase in *Bifidobacterium* spp. numbers (detected by Bif164 probe) was observed for FOS and synbiotic mixtures compared to probiotic vessels and the negative control ($P < 0.05$). Regarding *Lactobacillus-Enterococcus* group (detected by

Lab158 probe), no significant changes were observed at all time points, however, highest numbers were detected in the fermentation with FOS at 24 h (Log_{10} 6.24 ± 0.45 bacteria mL^{-1}).

Levels of *Clostridium coccoides–Eubacterium rectale* group (detected by Erec482 probe) significantly increased ($P < 0.05$) at 8 and 24 h of fermentation with maltodextrin (Log_{10} 6.61±0.23 to 7.63±0.53 bacteria mL^{-1}). An increase in *Atopobium* cluster (detected by Ato291 probe) was observed with FOS fermentation at 8h (Log_{10} 5.12±0.59 to 7.02±0.54 bacteria mL^{-1}). Because of high variations between the volunteers at 8 and 24 h, the increase in the *Atopobium* cluster was not significant for maltodextrin and synbiotic mixtures compared with 0 h.

No significant differences were found for the other bacterial groups analysed, including *Bacteroides* spp.-*Prevotella* group (Bac303 probe), *Roseburia* genus (Rrec584 probe), Clostridial cluster IX populations (Prop853 probe), *F. prausnitzii* group (Fprau655 probe), *Desulfovibrio* genus (DSV687 probe), *Clostridium histolyticum* group (Chis150 probe) and *Cytophaga-Flexibacter-Bacteroides* (CFB286 probe).

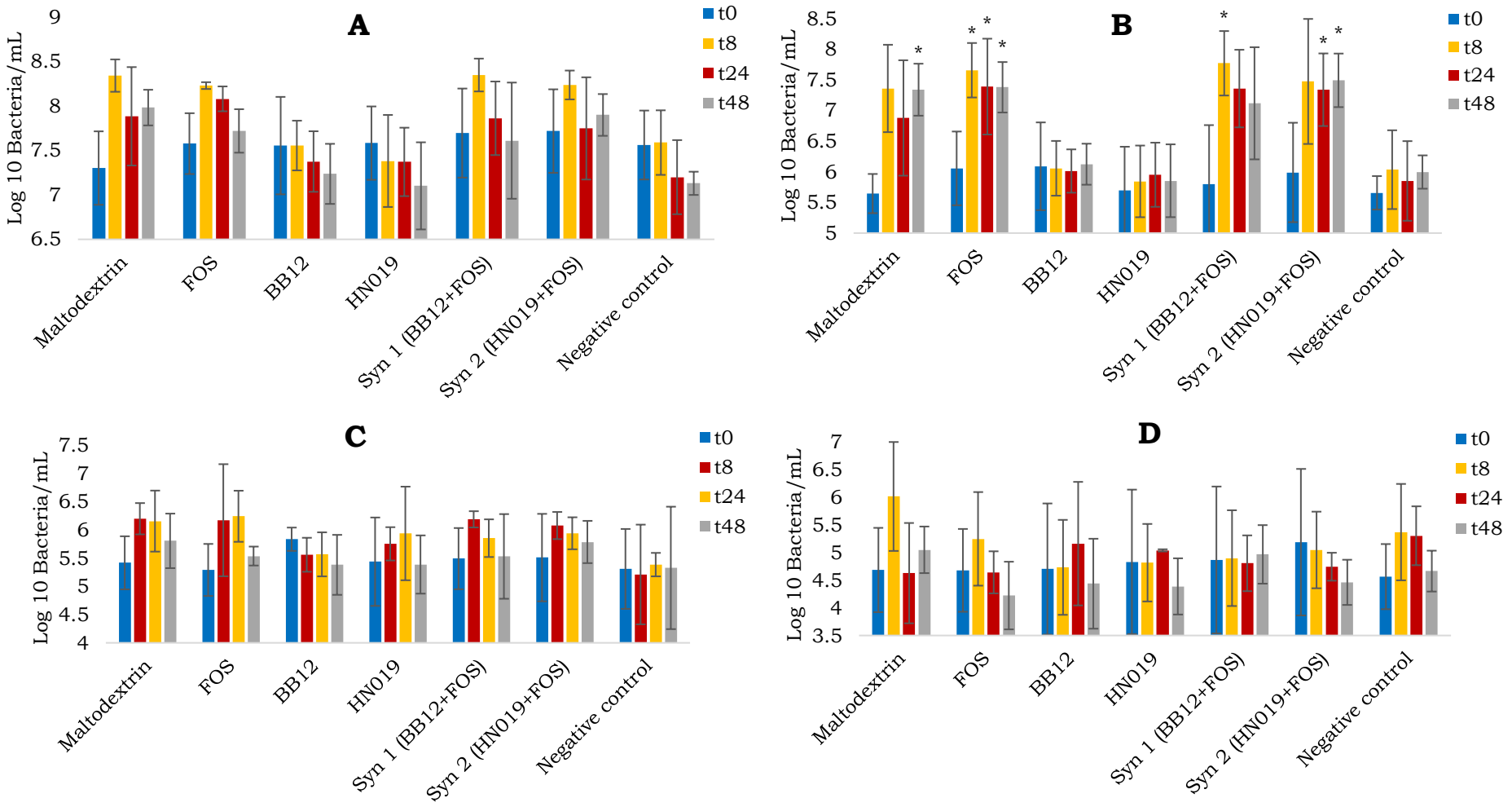

Figure 1. Total bacterial (A), Bif164 (B), Lab158 (C) and Bac303 (D) changes over time as $\log_{10}$ CFU/mL in the single stage batch culture as analysed by FISH. Values are mean values at four time points from three healthy faecal donor's ± standard deviation. * Mean values were significantly different from 0h fermentation ($p<0.05$).

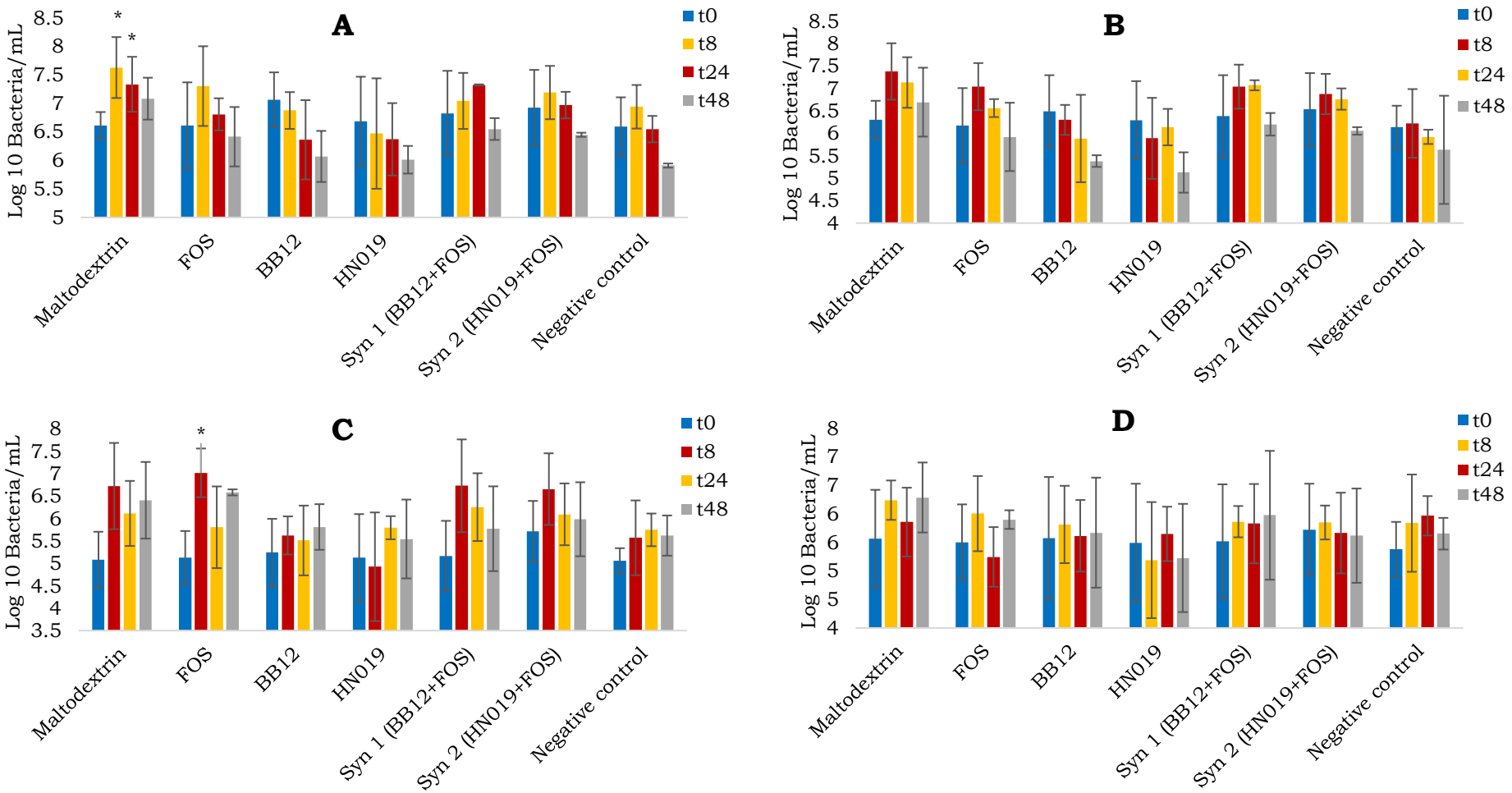

Figure 2. Erec482 (A), Rrec584 (B), Ato291 (C) and Prop853 (D) changes over time as $\log_{10}$ CFU/mL in the single stage batch culture as analysed by FISH. Values are mean values at four time points from three healthy faecal donor's ± standard deviation. * Mean values were significantly different from 0h fermentation ($p<0.05$).

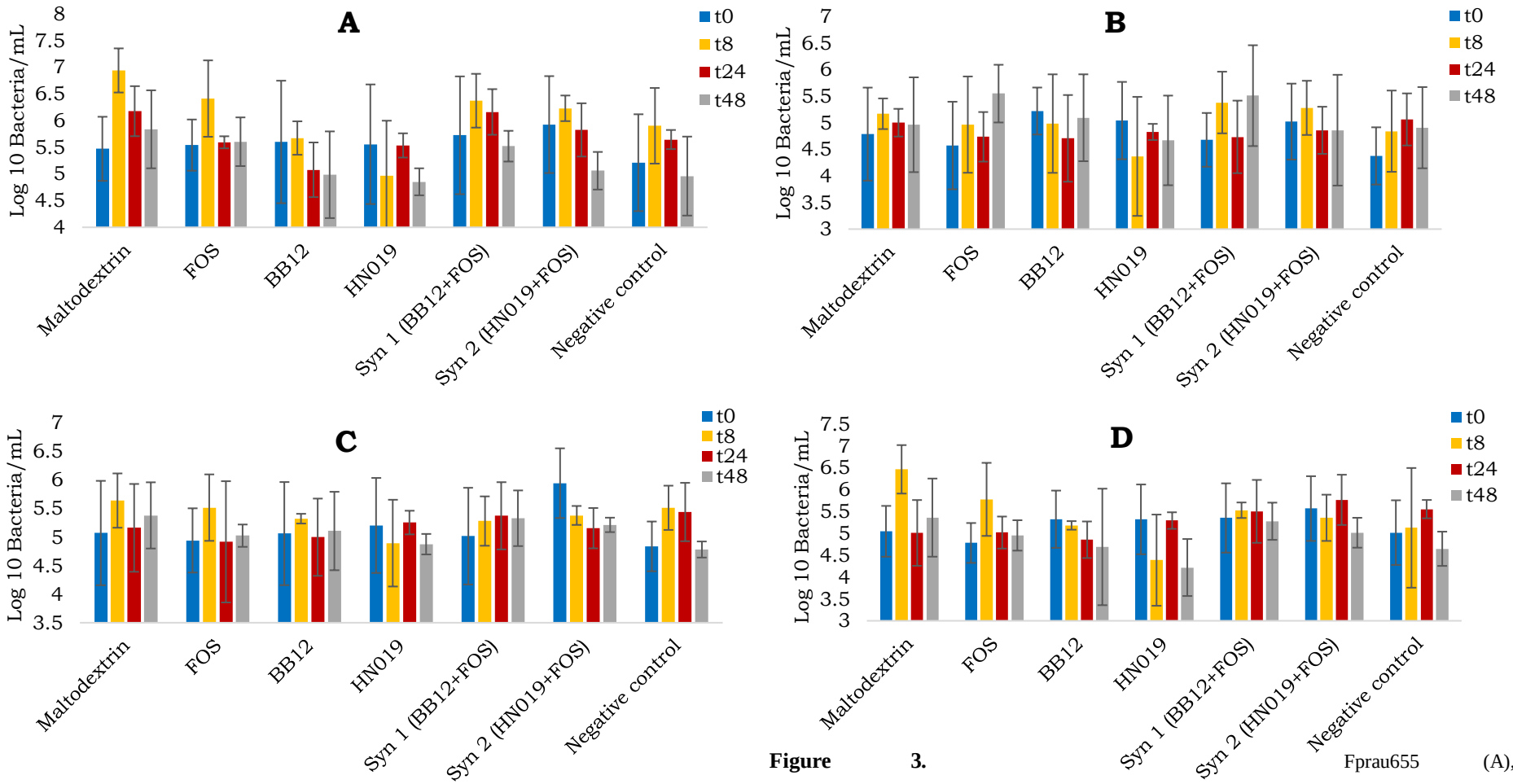

Figure 3. Fprau655 (A), DSV687 (B), Chis150 (C) and CFB286 (D) changes over time as log_{10} CFU/mL in the single stage batch culture as analysed by FISH. Values are mean values at four time points from three healthy faecal donor's ± standard deviation. * Mean values were significantly different from 0h fermentation ($p<0.05$).

SCFA production

SCFAs such as acetic, propionic and butyric acid can affect health positively. Folowing administration of all treatments, acetate was the main end product of microbial fermentation. Acetate concentrations increased ($P < 0.05$) on maltodextrin, FOS and synbiotic vessels compared to the negative control and the probiotics, however there were no significant differences.

Production of butyrate was also higher ($P < 0.05$) in the vessels with maltodextrin, FOS and synbiotic addition compared to the negative control. Propionate increased in all vessels compared with 0 h, however because of high variations between the volunteers at 24 and 48 h, the increase of this SCFA with maltodextrin, FOS and synbiotic fermentation was not significant compared to the negative control. Lactate concentrations were higher only after 8h of fermentation in the vessels containing carbohydrates – as it is an electron sink product.

On the other hand, isovalerate, a branched-chain fatty acids (BCFA) from amino acid metabolism was higher in the probiotic vessels and the negative control after 24 and 48 h of fermentation. The presence of maltodextrin and FOS may have inhibited production of this BCFA. No differences were found in valerate or isobutyrate.

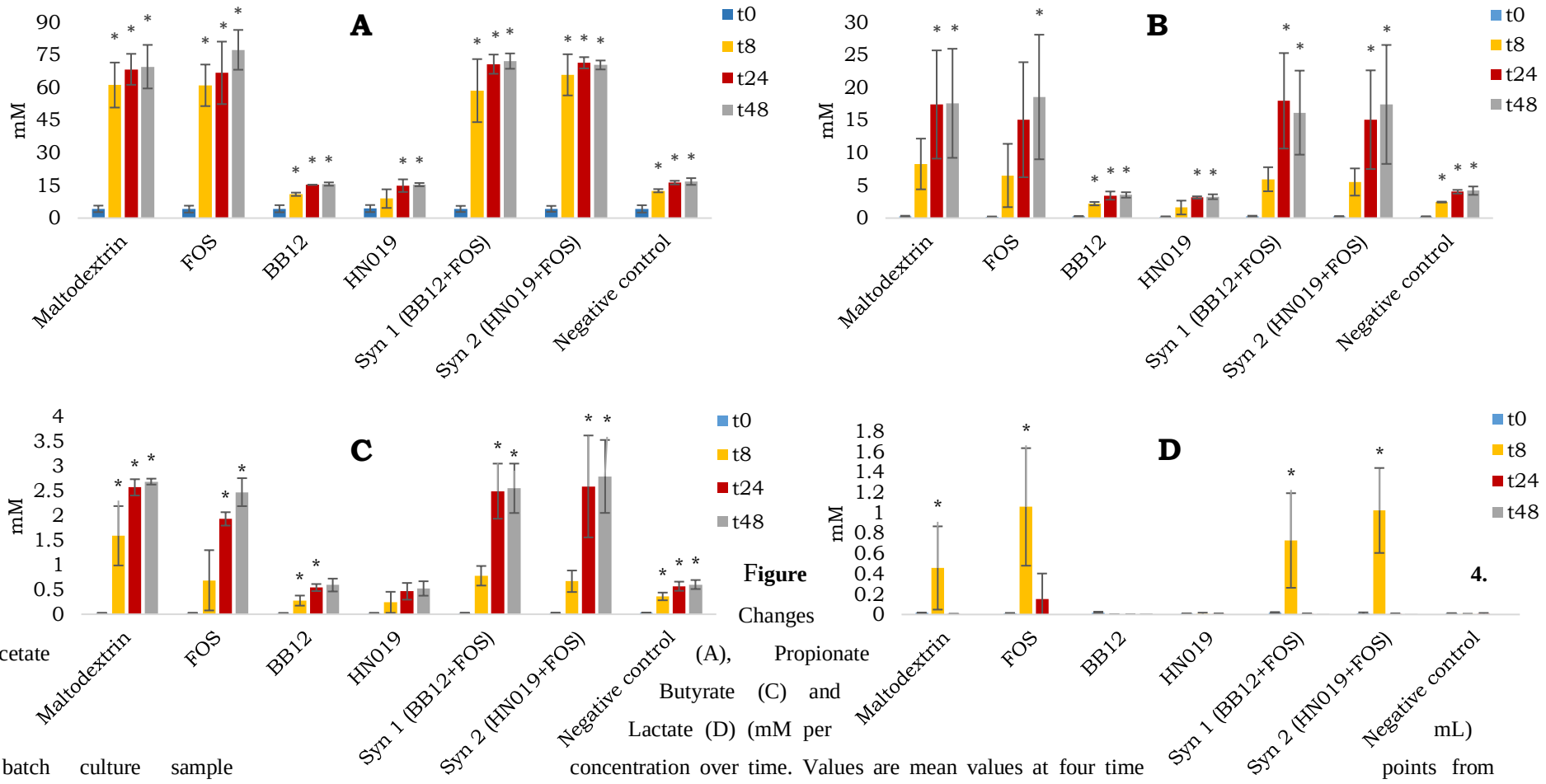

Figure 4. Changes in Acetate (A), Propionate (B), Butyrate (C) and Lactate (D) (mM per mL) of batch culture sample concentration over time. Values are mean values at four time points from three healthy faecal donor's ± standard deviation. * Mean values were significantly different from 0h fermentation ($p<0.05$).

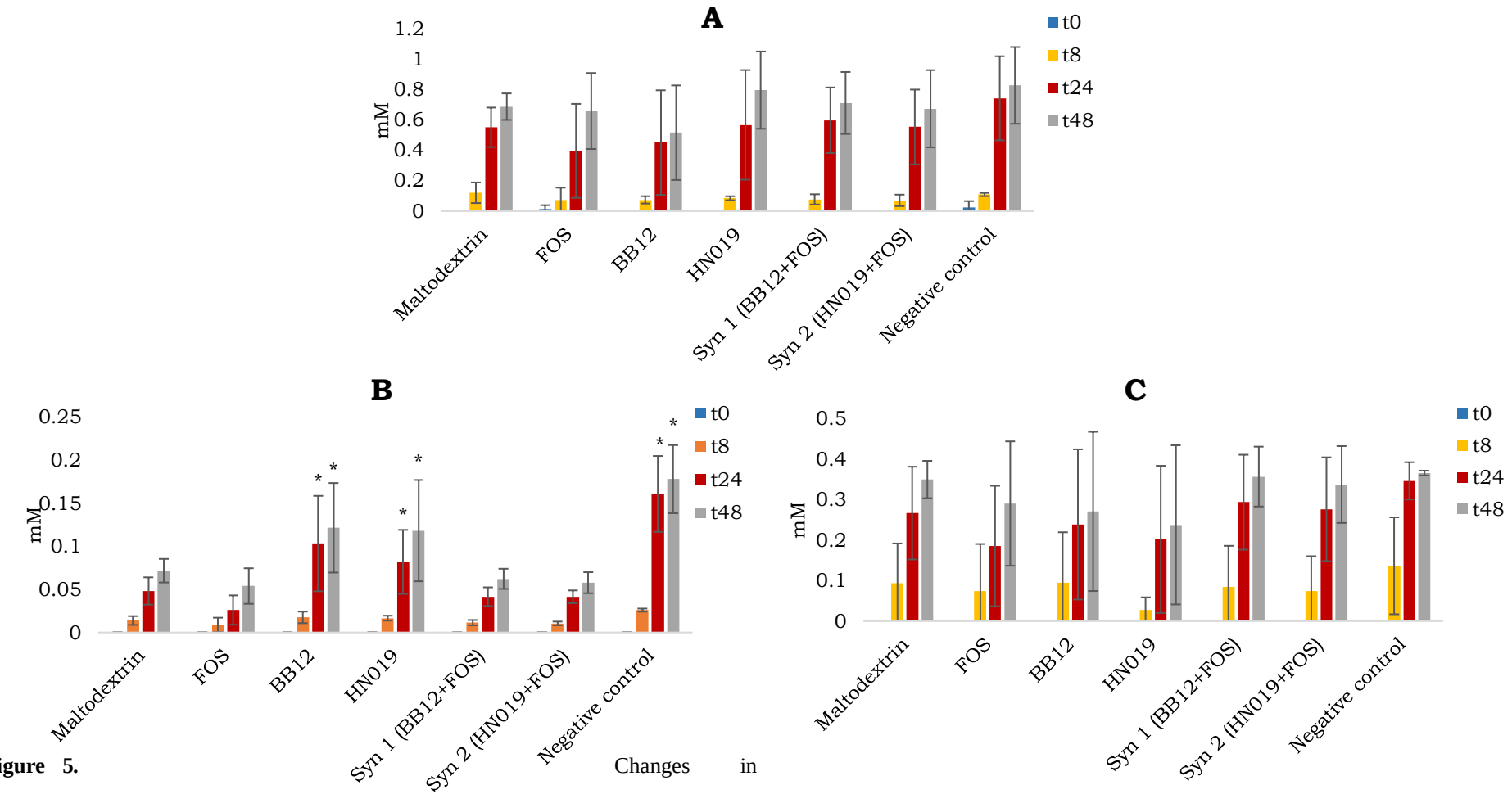

Figure 5. Changes in Isobutyrate (A), Isovalerate (B) and Valerate (C) and Lactate (D) (mM per mL) of batch culture sample concentration over time. Values are mean values at four time points from three healthy faecal donor's ± standard deviation. * Mean values were significantly different from 0h fermentation ($p<0.05$).