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Inulin-type fructan degradation capacity of *Clostridium* cluster IV and XIVa butyrate-producing colon bacteria and their associated metabolic outcomes

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RESEARCH ARTICLE

Abstract

Four selected butyrate-producing colon bacterial strains belonging to *Clostridium* cluster IV (*Butyricicoccus pullicaecorum* DSM 23266^T and *Faecalibacterium prausnitzii* DSM 17677^T) and XIVa (*Eubacterium hallii* DSM 17630 and *Eubacterium rectale* CIP 105953^T) were studied as to their capacity to degrade inulin-type fructans and concomitant metabolite production. Cultivation of these strains was performed in bottles and fermentors containing a modified medium for colon bacteria, including acetate, supplemented with either fructose, oligofructose, or inulin as the sole energy source. Inulin-type fructan degradation was not a general characteristic among these strains. *B. pullicaecorum* DSM 23266^T and *E. hallii* DSM 17630 could only ferment fructose and did not degrade oligofructose or inulin. *E. rectale* CIP 105953^T and *F. prausnitzii* DSM 17677^T fermented fructose and could degrade both oligofructose and inulin. All chain length fractions of oligofructose were degraded simultaneously (both strains) and both long and short chain length fractions of inulin were degraded either simultaneously (*E. rectale* CIP 105953^T) or consecutively (*F. prausnitzii* DSM 17677^T), indicating an extracellular polymer degradation mechanism. *B. pullicaecorum* DSM 23266^T and *E. hallii* DSM 17630 produced high concentrations of butyrate, CO_2, and H_2 from fructose. *E. rectale* CIP 105953^T produced lactate, butyrate, CO_2, and H_2, from fructose, oligofructose, and inulin, whereas *F. prausnitzii* DSM 17677^T produced butyrate, formate, CO_2, and traces of lactate from fructose, oligofructose, and inulin. Based on carbon recovery and theoretical metabolite production calculations, an adapted stoichiometrically balanced metabolic pathway for butyrate, formate, lactate, CO_2, and H_2 production by members of both *Clostridium* cluster IV and XIVa butyrate-producing bacteria was constructed.

Keywords: *Clostridium* cluster IV, *Clostridium* cluster XIVa, inulin-type fructans, butyrate, gases.

1. Introduction

The human colon harbours dense microbial communities that are mainly fed by non-digestible carbohydrates and proteins and have an essential impact on human physiology and health (Flint *et al.*, 2008; Koropatkin *et al.*, 2012; Scott *et al.*, 2013; Sommer and Bäcked, 2013). Notwithstanding their low representation in the human colon, bifidobacteria are essential members of the colon microbiota because of their selective fermentation of non-digestible carbohydrates that stimulates their growth and metabolic activities, the bifidogenic effect, and associated health-promoting activities (De Preter *et al.*, 2008; Dewulf *et al.*, 2013; Joossens *et al.*, 2012; Ramirez-Farias *et al.*, 2009; Van der Meulen *et al.*, 2004, 2006). Furthermore, they are responsible for several cross-feeding interactions with other members of the colon microbiota stimulating the metabolic activities of the latter, which is reflected in, for instance, the butyrogenic effect of inulin-type fructans and arabinoxylan-oligosaccharides (Damen *et al.*, 2012; De Vuyst and Leroy, 2011; De Vuyst *et al.*, 2013; Gibson *et al.*, 2004; Morrison *et al.*, 2006; Walton *et al.*, 2012). The butyrogenic effect of inulin-type fructans is of major importance for colon health, in particular because butyrate is the major energy source for renewal of the cells of the colon epithelium, inhibits inflammatory pathways, and stimulates anti-inflammatory pathways (Eeckhaut *et al.*, 2013; Hamer *et al.*, 2008).

Bifidobacterial fermentation of inulin-type fructans through the bifid shunt results in the production of acetate, lactate, formate, and ethanol (De Vuyst and Leroy, 2011; Falony *et al.*, 2009a,b; Rossi *et al.*, 2005; Van der Meulen *et al.,* 2004, 2006). The combined bifidogenic and butyrogenic effects of inulin-type fructans have been attributed to metabolic cross-feeding of acetate produced by bifidobacteria toward butyrate-producing colon bacteria, since reports on direct degradation of inulin-type fructans by the latter bacteria are scarce (De Vuyst and Leroy, 2011; De Vuyst *et al.*, 2013). Only when bifidobacteria (e.g. *Bifidobacterium longum*) initiate oligofructose degradation, with a concomitant production of acetate, some of the colon bacteria belonging to *Clostridium* clusters IV (e.g. *Faecalibacterium prausnitzii* and *Butyricicoccus pullicaecorum*) and XIVa (e.g. *Anaerostipes caccae*, *Anaerostipes hadrus*, *Anaerostipes butyraticus*, *Eubacterium hallii*, *Eubacterium rectale*, and *Roseburia* spp.) are capable of degrading simultaneously oligofructose and concomitant consumption of acetate, resulting in the production of butyrate (Allen-Vercoe *et al.*, 2012; Barcenilla *et al.*, 2000; Duncan and Flint, 2008; Duncan *et al.*, 2002a,b,c, 2004, 2006; Eeckhaut *et al.*, 2008, 2010; Falony *et al.*, 2006, 2009c; Schwiertz *et al.*, 2002). Lactate can only be converted into butyrate by members of *Clostridium* cluster XIVa (e.g. *A. caccae*, *A. hadrus*, *A. butyraticus*, and *E. hallii*) (Allen-Vercoe *et al.*, 2012; Duncan *et al.*, 2004; Eeckhaut *et al.*, 2010). This type of cross-feeding indicates the direct stimulation of butyrate-producing colon bacteria by inulin-type fructans, although not all members of species of *Roseburia* and *A. caccae* are capable to do so (Falony *et al.*, 2009c; Scott *et al.*, 2014). Hence, further research is needed to know which butyrate-producing colon bacteria, not only *A. caccae* and *Roseburia* spp. but also other important members belonging to *Clostridium* clusters IV and XIVa, are able to degrade inulin-type fructans.

It has been demonstrated that *E. rectale* and *F. prausnitzii* are dominant butyrate-producing colon bacteria accounting for up to 4% and 8% of the colon microbiota of individuals consuming a Western-type diet, respectively (Arumugam *et al.*, 2011; Tap *et al.*, 2009; Walker *et al.*, 2011). Furthermore, *E. rectale* and *E. hallii* represent prevalent colon bacterial species in healthy subjects (Louis *et al.*, 2010). Also, several studies have revealed that butyrate-producing colon bacteria, in particular *F. prausnitzii* and *B. pullicaecorum*, are present in decreased numbers in the colon of individuals suffering from inflammatory bowel disease (Eeckhaut *et al.*, 2013; Frank *et al.*, 2007; Jia *et al.*, 2010; Joossens *et al.*, 2011). Following these findings, it has now been established that the relative proportions of *F. prausnitzii* in the human colon have a direct impact on human health (Miquel *et al.*, 2013, 2014). More specifically, the metabolic activities of the type strains *F. prausnitzii* DSM 17677^T (a human colon isolate, Duncan *et al.*, 2002c) and *B. pullicaecorum* DSM 23266^T (a broiler chicken isolate, Eeckhaut *et al.*, 2008) display significant anti-inflammatory effects when tested in mouse colitis models (Eeckhaut *et al.*, 2013; Miquel *et al.*, 2015; Sokol *et al.*, 2008). Further, *B. pullicaecorum* DSM 23266^T tolerates the harsh conditions present in the human stomach and small intestine, which indicates that this strain can reach the colon in a viable and metabolically active state (Geirnaert *et al.*, 2014). Meanwhile, it has been shown that *B. pullicaecorum* DSM 23266^T can be safely administered to healthy individuals (Boesmans *et al.*, 2015). Therefore, these strains could represent ideal probiotic candidates for the treatment of inflammatory bowel disease (Van Immerseel *et al.*, 2010). The combination of these probiotic strains with prebiotics could improve their metabolic activity and survival once they colonize the gastrointestinal tract (Khan *et al.*, 2014). However, the breakdown of inulin-type fructans by these butyrate-producing colon bacteria was hardly investigated up to now (Duncan and Flint, 2008; Duncan *et al.*, 2002c; Eeckhaut *et al.*, 2008; Scott *et al.*, 2014). Furthermore, the latter studies did not link substrate consumption, organic acid production, and gas production (CO_2 and H_2) quantitatively. This is of particular importance because their ratios can be influenced by environmental factors such as pH and hydrogen partial pressure (Macfarlane and Macfarlane, 2003; Louis and Flint, 2009). Moreover, *F. prausnitzii* does not produce H_2, whereas other butyrate-producing colon bacteria (except for *Roseburia inulinivorans* DSM 16841^T) produce high levels of H_2, which may interfere with gut comfort (Falony *et al.*, 2009c).

The present study aimed at investigating the inulin-type fructan breakdown by four selected butyrate-producing colon bacteria of *Clostridium* clusters IV and XIVa to explain the possible stimulation of their growth and metabolic activity upon inulin-type fructan administration. Also, it aimed at constructing an adapted stoichiometric pathway for butyrate production to provide a quantitative link between substrate consumption and the production of organic acids and gases by these bacteria.

2. Materials and methods

Microorganisms and media

F. prausnitzii DSM 17677^T, *B. pullicaecorum* DSM 23266^T, and *E. hallii* DSM 17630 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Göttingen, Germany). *E. rectale* CIP 105953^T was obtained from the Collection de l'Institut Pasteur (CIP, Paris, France). All strains are of human faecal origin, except for *B. pullicaecorum* DSM 23266^T, which is from the caecal content of a broiler chicken but has been applied in human intervention studies successfully (Boesmans *et al.*, 2015). All strains were stored at -80 °C in reinforced clostridial medium (RCM; Oxoid Ltd., Basingstoke, UK), supplemented with 25% (v/v) of glycerol as a cryoprotectant.

In the case of *E. hallii* DSM 17630, Tween 80 (2 ml/l; Merck, Darmstadt, Germany) was added to RCM.

A modified version of a medium for colon bacteria (MCB; Van der Meulen *et al.*, 2006), further referred to as mMCB, was used during the screening and fermentation experiments (concentrations in g/l) to enable the growth of *F. prausnitzii* DSM 17677^T (optimization data of the medium formulation not shown): $CH_3COO^-Na^+.3H_2O$ (Merck), 6.8 g/l (corresponding to 50 mM acetate); bacteriological peptone (Oxoid), 6.5 g/l; soya peptone (Oxoid), 5.0 g/l; yeast extract (VWR International, Darmstadt, Germany), 3.0 g/l; Tryptone (Oxoid), 2.5 g/l; NaCl (VWR International), 4.5 g/l; K_2HPO_4 (Merck), 0.45 g/l; KH_2PO_4 (Merck), 0.45 g/l; $MgSO_4.7H_2O$ (Merck), 0.09 g/l; $CaCl_2.2H_2O$ (Merck), 0.09 g/l; cysteine-HCl (Merck), 0.4 g/l; $NaHCO_3$ (VWR International), 0.2 g/l; $MnSO_4.H_2O$ (VWR International), 0.05 g/l; $FeSO_4.7H_2O$ (Merck), 0.005 g/l; $ZnSO_4.7H_2O$ (VWR International), 0.005 g/l; hemin (Sigma-Aldrich, Saint Louis, MO, USA), 0.005 g/l; menadione (Sigma-Aldrich), 0.005 g/l; and resazurin (Sigma-Aldrich), 0.001 g/l. In the case of *E. hallii* DSM 17630, 2 ml/l of Tween 80 (Merck) was added to the medium to promote its growth (growth of *F. prausnitzii* DSM 17677^T was inhibited by 2 ml/l of Tween 80; data not shown). The pH of all media was adjusted to 6.3, mimicking the average pH of the colon, and the media were autoclaved at 210 kPa and 121 °C for 20 min. After sterilization, fructose (VWR), oligofructose (Raftilose P95; Beneo-Orafti NV, Tienen, Belgium), or inulin (OraftiHP; Beneo-Orafti) were added as the sole energy source aseptically, always at a final concentration of 50 mM fructose equivalents (FE), using sterile stock solutions. Oligofructose and inulin stock solutions were made sterile by membrane filtration using Minisart filters (pore size, 0.2 µm; Sartorius, Göttingen, Germany). For the cultivation experiments in bottles, stock solutions of fructose, oligofructose, and inulin were initially made anaerobic by sparging them with N_2 (Air Liquide, Paris, France). The solutions were subsequently filter-sterilized and transferred into the glass bottles, which were sealed with butyl rubber septa that were pierced with a Sterican needle (VWR) connected with a Millex-GP filter (Merck) permanently to ensure sterile conditions and avoid overpressure by allowing ingress of the surrounding atmosphere. For the cultivation experiments in fermentors, fructose stock solutions were autoclaved under the same conditions as the media.

OraftiP95 and OraftiHP are commercial powders of inulin-type fructans derived from chicory roots. OraftiP95 is obtained through enzymatic hydrolysis of chicory inulin. It consists mainly of oligofructose (≥93.2% [m/m]), with minor concentrations of glucose, fructose, and sucrose (<6.8% [m/m]). The degree of polymerisation (DP) of the oligofructose chain length fractions varies between 2 and 8, with an average of 4. OraftiHP contains inulin (≥99.5% [m/m]), with a DP ranging from 12 to 65, hence covering both short and long chain length fractions, and minor concentrations of glucose, fructose, and sucrose (<0.5% [m/m]). The average DP of the inulin chain length fractions exceeds 23, due to removal of the smaller molecules during processing.

Cultivation experiments in bottles

Cultivation of the strains under study in mMCB, without pH control, in stationary glass bottles was performed as to their capacity to degrade inulin-type fructans (fructose, oligofructose, and inulin) and to produce butyrate. Monoculture cultivations with all strains were carried out in glass bottles containing 100 ml of medium supplemented with the energy source under study. Inocula of *F. prausnitzii* DSM 17677^T, *B. pullicaecorum* DSM 23266^T, *E. hallii* DSM 17630, and *E. rectale* CIP 105953^T were prepared as follows. The strains were transferred from -80 °C to test tubes containing 10 ml of RCM (for *E. hallii* DSM 17630 and *E. rectale* CIP 105953^T) or to glass bottles containing 100 ml of RCM (for *F. prausnitzii* DSM 17677^T and *B. pullicaecorum* DSM 23266^T). Tubes and bottles were incubated anaerobically in a modular atmosphere-controlled system (MG anaerobic work station, Don Whitley Scientific Ltd., West Yorkshire, United Kingdom) that was continuously sparged with a mixture of 80% N_2, 10% CO_2, and 10% H_2 (Air Liquide) at 37 °C for 16 h (*E. hallii* DSM 17630) or 24 h (*F. prausnitzii* DSM 17677^T, *B. pullicaecorum* DSM 23266^T, and *E. rectale* CIP 105953^T). Subsequently, the strains were propagated twice at 37 °C for 12 h (*F. prausnitzii* DSM 17677^T) or 24 h (*B. pullicaecorum* DSM 23266^T, *E. hallii* DSM 17630, and *E. rectale* CIP 105953^T) in glass bottles containing 100 ml of mMCB supplemented with fructose as the sole energy source, and finally added to the glass bottles used for the screening experiments. During the inoculum build-up, the transferred volume was always 5% (v/v). All bottles were equipped as described above and incubated anaerobically (MG anaerobic work station), allowing introduction of the inocula using sterile needles and syringes. Samples were taken after 0, 6, 12, 24, and 48 h of incubation. All screening experiments were performed in duplicate. The results and figures presented onward are representative for both experiments.

In the case of *E. rectale* CIP 105953^T, a second set of cultivation experiments was performed in glass bottles containing 100 ml of mMCB supplemented with 50 mM FE of inulin. In this set of experiments, the butyl rubber septa of the glass bottles were not pierced with a needle permanently, except for introduction of the inocula, but were kept sealed during the whole experiment to avoid ingress of the surrounding atmosphere. This prevented entry of the gases N_2, CO_2, and H_2, which were present in the atmosphere of the anaerobic work station, into the bottles and the medium. Thus, the presence of CO_2 and

H_2 in the medium could only originate from bacterial metabolism. Samples were taken at the beginning of the experiments (0 h) and after 48 h of incubation. All experiments were performed in duplicate.

Cultivation experiments in fermentors

Additional cultivation experiments in mMCB in continuously sparged fermentors were performed to allow a detailed analysis of organic acid and gas production by the strains under study (see below). The data obtained allowed to construct a stoichiometrically balanced pathway for butyrate production. Monoculture fermentations with all strains were carried out in 2 l Biostat B-DCU fermentors (Sartorius) containing 1.5 l of medium supplemented with the energy source under study. Inocula of *F. prausnitzii* DSM 17677^T, *B. pullicaecorum* DSM 23266^T, *E. hallii* DSM 17630, and *E. rectale* CIP 105953^T were prepared as described above and finally added to the fermentors aseptically. Anaerobic conditions were assured by continuously sparging the medium with N_2 (Air liquide) at a flow rate of 70 ml/min. The fermentation temperature was kept constant at 37 °C. A constant pH of 6.3 was imposed and controlled automatically, using 1.5 M solutions of NaOH and H_3PO_4. To keep the medium homogeneous, a gentle stirring of 100 rpm was applied. Temperature, pH, and agitation speed were controlled online (MFCS/win 2.1 software, Sartorius). Fermentations were followed for 48 h. Samples were withdrawn at regular time intervals. All fermentations were performed in duplicate. The results and figures presented onward are representative for both fermentations. Fermentations with strains that did not show oligofructose or inulin degradation during the cultivation experiments in bottles were performed once for confirmation.

Analysis of bacterial growth

Optical density

During all cultivation experiments, bacterial growth was followed by measuring the optical density at 600 nm. The measurements were performed in triplicate.

Flow cytometry

During all cultivation experiments in fermentors, growth was followed by flow cytometry [expressed as Log (cells/ml)] as described previously (Moens *et al.*, 2016). The measurements were performed in triplicate.

Analysis of carbohydrate consumption and metabolite production

All samples were first rendered free of cells through centrifugation (4,618×*g* for 20 min at 10 °C).

Carbohydrate, organic acid, and ethanol concentration determinations

Residual concentrations of glucose, fructose, oligofructose, and inulin (the latter two expressed as mM FE), as well as concentrations of formate, acetate, butyrate, lactate, and ethanol were determined through high-performance liquid chromatography (HPLC) with refractive index (RI) detection, using a Waters chromatograph (Waters Corp., Milford, MA, USA) equipped with an ICSep ICE ORH-801 column (Transgenomic North America, Omaha, NE, USA), and applying external standards, as described previously (Moens *et al.*, 2016). Samples were prepared as described previously (Moens *et al.*, 2016). The samples were analyzed in triplicate.

Breakdown of oligofructose and inulin

Analysis of oligofructose and inulin breakdown was performed using high-performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD), using a DX500 chromatograph (Dionex, Sunnyvale, CA, USA) equipped with a CarboPac PA100 column (Dionex), as described previously (Moens *et al.*, 2016). The samples were analysed in duplicate. Qualitative fingerprints were generated by plotting the elution time (in min) against the detected signal (in nC).

During the cultivation experiments with oligofructose and inulin as the sole added energy source, concentrations of free glucose, fructose, and sucrose (initially present in the medium or released during inulin-type fructan breakdown) were measured through HPAEC-PAD using an ICS 3000 chromatograph (Dionex) equipped with a CarboPac PA10 column (Dionex). The mobile phase, at a flow rate of 1.0 ml/min, consisted of ultrapure water (0.015 µS/cm; eluent A), 260 mM NaOH (eluent B), and 736 mM NaOH (eluent C), with the following gradient: 0.0 min, 89% A and 11% B; 20.0 min, 89% A and 11% B; 21.0 min, 100% C; 27.0 min, 100% C; 27.2 min, 89% A and 11% B; and 30.0 min, 89% A and 11% B. Sample preparation involved initial dilution (1:20) with ultrapure water followed by deproteinization with acetonitrile (Sigma-Aldrich) containing 0.03 g/l of rhamnose (Merck) as an internal standard (1:2), centrifugation (21,912×*g*, 15 min, 4 °C), and filtration (0.2-µm filters; Minisart RC 4, Sartorius) prior to injection (10 µl) into the column. Calibration was performed with external standards. The samples were analysed in triplicate.

Based on the above mentioned analyses and the quantitative determination of the breakdown of oligofructose (expressed as mM FE) through HPLC-RI, a quantitative determination of the breakdown of the different chain length fractions of oligofructose was performed on relevant samples by gas chromatography (GC) with flame ionization detection (FID), using a 5300-HT high-resolution gas chromatograph

(Carlo Erba, Rodina, Italy) equipped with a SGE Aluminium Clad-5 capillary column (Achrom NV, Zulte, Belgium) and oligofructose, glucose, fructose, and sucrose as external standards, as described previously (Falony *et al.*, 2009b). Using this method, concentrations of fructose (F), glucose (G), sucrose (GF), the oligosaccharides inulobiose (F_2) up to inuloheptaose (F_7), and kestose (GF_2) up to kestoheptaose (GF_6) were determined separately. In the figures below, the concentrations of glucose, fructose, sucrose, and inulobiose are represented; the concentrations of the different chain length fractions of oligofructose with the same DP (starting from DP 3) are represented as the sum of their concentrations, i.e. GF_X and F_{X+1}. For cost effectiveness, the samples were analysed once; however, the method has shown to be highly reproducible and reliable (Falony *et al.*, 2009c; Joye and Hoebregs, 2000).

Analysis of gas production

During all cultivation experiments in fermentors, the concentrations of H_2 and CO_2 in the fermentor gas effluents were determined online through gas chromatography with thermal conductivity detection (TCD), using a CompactGC (Interscience, Breda, the Netherlands) equipped with a 10-m Molsieve 5A column (H_2; Varian Inc., Palo Alto, CA, USA), using N_2 (Air Liquide) as a carrier gas, and a 10-m PoraBOND Q column (CO_2; Varian), using He (Air Liquide) as a carrier gas, as described previously (Falony *et al.*, 2009c). The data are represented as dissolved metabolites (mM), corresponding with the concentrations of CO_2 or H_2 produced from 1 l of fermentation medium.

Carbon recovery and theoretical calculations

For all cultivation experiments in fermentors, the carbon recovery (CR; in percentage) was calculated by dividing the total concentration of carbon recovered in the bacterial metabolites by the total concentration of carbon present in the substrates added. Also, molar ratios of acetate consumption, CO_2 production, and/or H_2 production to energy source consumption were calculated to construct an adapted pathway for butyrate and gas production. Using this pathway, the metabolite concentrations measured could be compared with their theoretical values calculated. Finally, this pathway could be used to calculate the production of H_2 and CO_2 during the cultivation experiments in glass bottles.

3. Results

Cultivation experiments in bottles

B. pullicaecorum DSM 23266^T and *E. hallii* DSM 17630 fermented fructose in mMCB, which was accompanied by the consumption of acetate (Table 1). They did not degrade oligofructose or inulin. Initial growth of both strains on those substrates occurred through consumption of the free concentrations of fructose, glucose, and sucrose present in the commercial oligofructose and inulin substrates. *E. hallii* DSM 17630 consumed fructose completely after 24 h of incubation (data not shown), whereas residual fructose was still present after 48 h of incubation in the case of *B. pullicaecorum* DSM 23266^T (Table 1). Butyrate was the only organic acid produced from fructose by *B.*

Table 1. Substrate consumption and metabolite production by *Butyricicoccus pullicaecorum* DSM 23266^T, *Eubacterium hallii* DSM 17630, *Eubacterium rectale* CIP 105953^T, and *Faecalibacterium prausnitzii* DSM 17677^T after 48 h of incubation, during cultivation experiments in stationary glass bottles containing a modified medium for colon bacteria (mMCB), including 50 mM acetate, supplemented with 50 mM fructose equivalents (FE) of fructose, oligofructose, or inulin as the sole added energy source.

Strain	Energy source	Mean consumption ± standard deviation (mM) of substrates		Mean production ± standard deviation (mM) of metabolites		
		Energy source (FE)	Acetate	Butyrate	Lactate	Formate
B. pullicaecorum DSM 23266^T	fructose	17.0±0.8	28.0±1.1	32.0±0.0	-	-
E. hallii DSM 17630	fructose	52.7±1.2	34.1±1.6	62.2±1.3	-	8.6±0.1
E. rectale CIP 105953^T	fructose	40.1±0.6	16.5±1.0	26.6±0.5	34.3±1.5	-
	oligofructose	11.4±0.6	11.9±0.1	16.5±0.3	2.1± 0.1	-
	inulin	19.6±1.1	11.3±0.7	18.4±0.5	10.2±0.2	-
	inulin[1]	21.1±1.3	15.1±0.4	21.5±0.6	9.4±0.4	2.8±0.1
F. prausnitzii DSM 17677^T	fructose	13.9±0.3	17.2±0.3	21.7±0.2	1.2±0.1	3.0±0.1
	oligofructose	11.4±0.3	13.0±0.4	17.2±0.3	0.9±0.1	3.0±0.3
	inulin	9.8±2.7	11.8±2.4	13.7±1.2	-	0.4±0.2

[1] Cultivation experiments in sealed glass bottles, see Materials and methods.

pullicaecorum DSM 23266^T, whereas next to butyrate, formate was produced by *E. hallii* DSM 17630. Lactate and ethanol were not produced by these bacteria (Table 1).

E. rectale CIP 105953^T grew on fructose, oligofructose, and inulin, although they were not degraded completely after 48 h of incubation; these degradations were always accompanied by the consumption of acetate (Table 1; Figure 1A-C). Consumption of fructose resulted in the production of both butyrate and lactate in nearly equal concentrations (Table 1). Degradation of oligofructose and inulin mainly resulted in the production of butyrate. However, low concentrations of lactate were also produced during the degradation of these energy sources (Table 1). Quantitative determination of the breakdown of oligofructose by *E. rectale* CIP 105953^T revealed that all chain length fractions were degraded simultaneously, whereby the concentrations of fructose and inulobiose in the medium increased (Figures 1B and 2A). During the incubations with inulin as the sole added energy source, two separate growth phases could be distinguished. *E. rectale* CIP 105953^T initially degraded the monomers fructose and glucose present in the medium within the first 6 h of incubation. After 24 h, a second growth phase occurred, during which the strain degraded inulin. Qualitative determination of the breakdown of inulin revealed that *E. rectale* CIP 105953^T degraded all chain length fractions of inulin simultaneously. Despite residual inulin still being present in the medium after 48 h of incubation, *E. rectale* CIP 105953^T degraded not only the short chain length fractions of inulin but also its long chain length fractions, which resulted in the release of free fructose into the medium (Table 1; Figures 1C and 2B, 2C).

F. prausnitzii DSM 17677^T degraded fructose, oligofructose, and inulin, albeit not completely after 48 h of incubation; these degradations were always accompanied by the consumption of acetate (Table 1; Figure 3A-C). Consumption of fructose, oligofructose, and inulin resulted in the production of mainly butyrate, besides low concentrations of formate and lactate. Whereas only low concentrations of oligofructose were degraded, quantitative determination revealed that *F. prausnitzii* DSM 17677^T degraded all oligofructose chain length fractions simultaneously, whereby the concentrations of fructose in the medium increased after 48 h of incubation (Figure 4A). Qualitative determination of the breakdown of inulin revealed that its short chain length fractions were degraded. Even prolonged incubation did not result in the degradation of the long chain length fractions of inulin neither was fructose released into the medium (Figure 4B,4C).

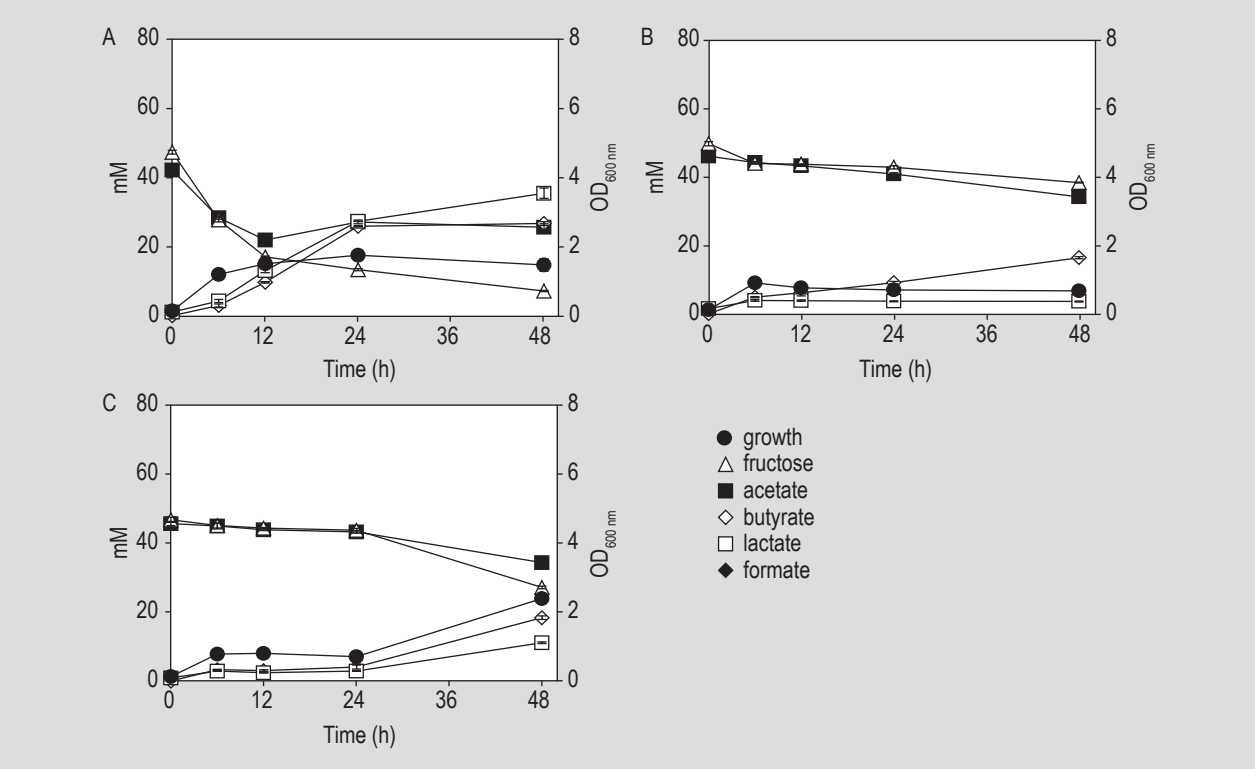

Figure 1. Growth, substrate consumption, and metabolite production by *Eubacterium rectale* CIP 105953^T during cultivation experiments (stationary glass bottles) in a modified medium for colon bacteria (mMCB), including 50 mM acetate, supplemented with (A) fructose, (B) oligofructose and (C) inulin.

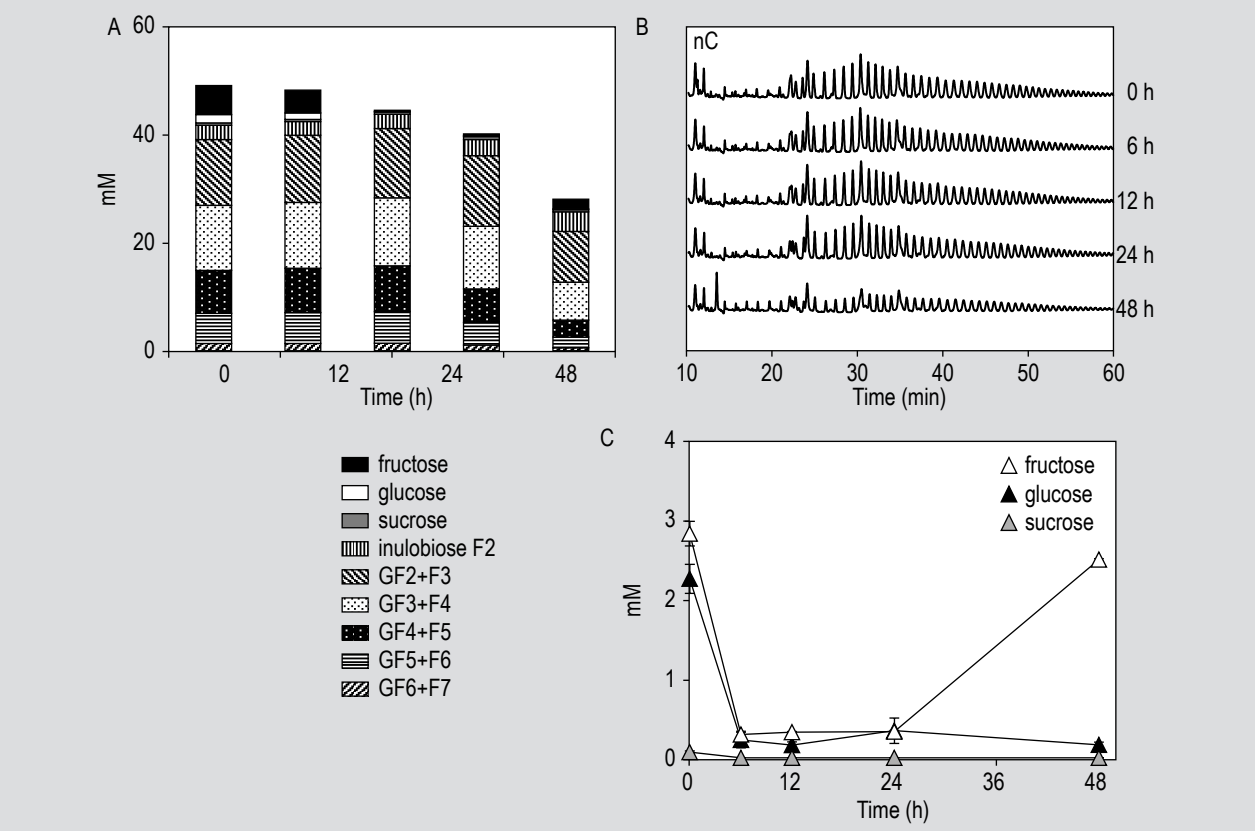

Figure 2. (A) Oligofructose degradation monitored through gas chromatography with flame ionization detection (GC-FID). (B) Inulin degradation monitored through high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). (C) Free fructose, glucose, and sucrose during inulin degradation.

Cultivation experiments in fermentors

To be able to construct a stoichiometrically balanced pathway for butyrate production, a detailed analysis of organic acid and gas production was studied through cultivation experiments in continuously sparged fermentors.

Fermentations with B. pullicaecorum *DSM 23266^T and* E. hallii *DSM 17630*

E. hallii DSM 17630 consumed all fructose within 15 h of fermentation, *B. pullicaecorum* DSM 23266^T consumed only 81% of the fructose within 48 h of fermentation (Table 2). Again, both strains did not degrade oligofructose or inulin. Initial growth of both strains on these substrates occurred through consumption of the free concentrations of fructose, glucose, and sucrose present in the commercial oligofructose and inulin substrates.

Both butyrate-producing bacterial strains consumed acetate during fructose degradation, albeit to a different extent, i.e., more acetate was consumed by *B. pullicaecorum* DSM 23266^T than by *E. hallii* DSM 17630 (Table 2). Both bacterial strains produced butyrate as the main organic acid from fructose and acetate. During the fermentation with *E. hallii* DSM 17630, also low concentrations of formate were produced (Table 2). Further, substantial concentrations of CO_2 and H_2 were produced by both bacteria (Table 2). *E. hallii* DSM 17630 produced higher concentrations of H_2 from fructose compared to *B. pullicaecorum* DSM 23266^T, whereas *E. hallii* DSM 17630 produced less CO_2 than *B. pullicaecorum* DSM 23266^T. The inverse relationships between acetate consumption and H_2 production (*E. hallii* DSM 17630) and between formate production and CO_2 production (*B. pullicaecorum* DSM 23266^T) were reflected in the molar ratios of acetate consumption, H_2 production, or CO_2 production to energy source consumption during the fructose fermentations (Table 3). All these data indicated that both strains must have followed a similar butyrate production pathway during their consumption of fructose (Table 2; see below). Based on the ratios mentioned above, this stoichiometric pathway for butyrate production could be constructed (Figure 5).

Fermentations with F. prausnitzii *DSM 17677^T*

F. prausnitzii DSM 17677^T consumed fructose, oligofructose, and inulin within 12, 15, and 48 h of fermentation, respectively (Table 2; Figure 6A-C). All three energy sources were not degraded till completion. Quantitative

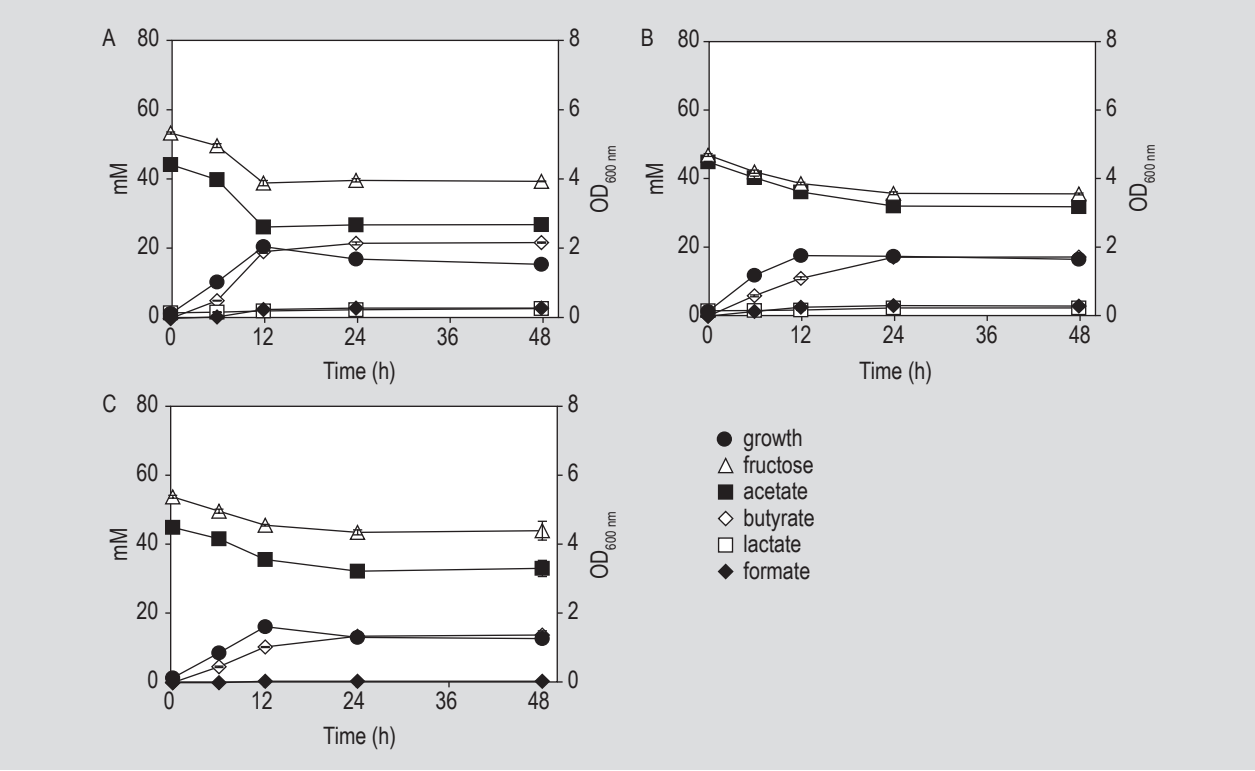

Figure 3. Growth, substrate consumption, and metabolite production by *Faecalibacterium prausnitzii* DSM 17677^T during cultivation experiments (stationary glass bottles) in a modified medium for colon bacteria (mMCB), including 50 mM acetate, supplemented with (A) fructose, (B) oligofructose and (C) inulin.

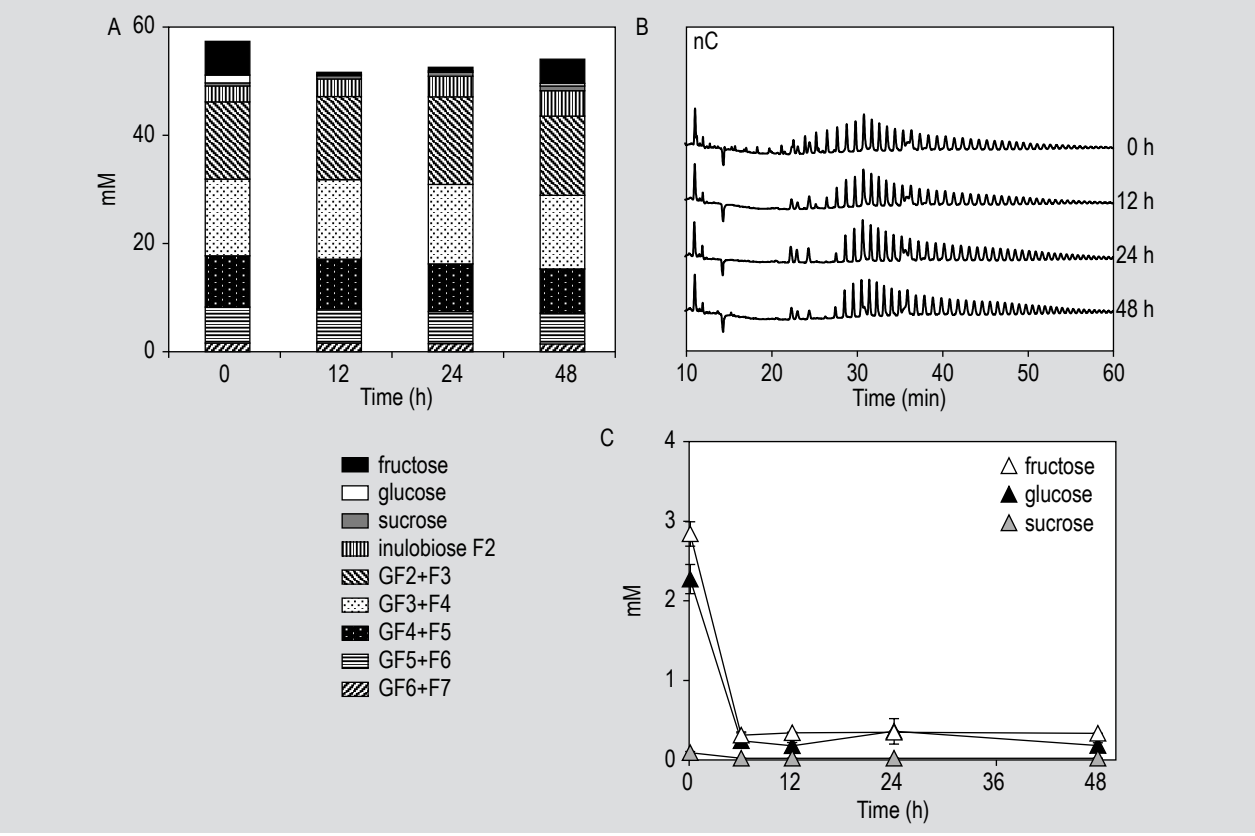

Figure 4. (A) Oligofructose degradation monitored through GC-FID. (B) Inulin degradation monitored through HPAEC-PAD. (C) Free fructose, glucose, and sucrose.

Table 2. Substrate consumption and metabolite production by *Butyricicoccus pullicaecorum* DSM 23266^T, *Eubacterium hallii* DSM 17630, *Eubacterium rectale* CIP 105953^T, and *Faecalibacterium prausnitzii* DSM 17677^T after 48 h of incubation, during cultivation experiments in fermentors containing a modified medium for colon bacteria (mMCB), including 50 mM acetate, supplemented with 50 mM fructose equivalents (FE) of fructose, oligofructose, or inulin as the sole added energy source.

Strain	Energy source	Mean consumption ± standard deviation (mM) of substrates		Mean production ± standard deviation (mM) of metabolites					Carbon recovery (%)
		Energy source (FE)	Acetate	Butyrate	Formate	Lactate	H_2	CO_2	
B. pullicaecorum DSM 23266^T	fructose	42.4±0.8	39.2±1.0	58.1±1.2	-	-	41.1±0.1	105.3±0.1	102
E. hallii DSM 17630	fructose	49.7±0.5	14.2±0.5	50.8±0.1	12.3±0.7	-	77.2±0.1	83.1±0.1	92
E. rectale CIP 105953^T	fructose	30.1±1.0	10.8±2.2	25.4±0.3	2.0±0.1	21.3±0.3	21.8±0.1	35.9±0.1	101
	oligofructose	12.9±0.5	11.5±0.6	18.3±0.1	-	1.4±0.1	14.6±0.1	22.1±0.1	99
F. prausnitzii DSM 17677^T	fructose	29.9±1.1	16.8±0.5	37.5±0.7	29.8±0.4	3.0±0.0	-	29.3±0.1	102
	oligofructose	25.6±0.5	18.2±0.9	33.1±0.7	27.2±0.9	3.1±0.6	-	27.5±0.1	103
	inulin	11.0±0.9	11.7±1.4	16.1±0.2	7.8±0.2	0.4±0.2	-	13.0±0.1	97

Table 3. Molar ratios of acetate consumption, H_2 production, and CO_2 production to energy source consumption after 48 h of incubation, during cultivation experiments in stationary glass bottles (SCR) and fermentors (FER) containing a modified medium for colon bacteria (mMCB), including 50 mM acetate, supplemented with 50 mM fructose equivalents (FE) of fructose, oligofructose, or inulin as the sole added energy source.

Strain	Energy source	Acetate consumption/ energy source consumption		H_2 production/energy source consumption		CO_2 production/energy source consumption	
		SCR[1]	FER	SCR	FER	SCR	FER
B. pullicaecorum DSM 23266^T	fructose	1.6	0.9	0.4	1.0	2.0	2.5
E. hallii DSM 17630	fructose	0.6	0.3	1.2	1.6	1.8	1.7
E. rectale CIP 105953^T	fructose	0.4	0.4	0.7	0.7	1.1	1.2
	oligofructose	1.0	0.9	0.8	1.1	1.8	1.7
	inulin	0.6	NR	0.9	NR	1.5	NR
F. prausnitzii DSM 17677^T	fructose	1.2	0.6	NR	NR	1.7	1.0
	oligofructose	1.1	0.7	NR	NR	1.7	1.1
	inulin	1.2	1.1	NR	NR	2.0	1.2

[1] Calculations of the concentrations of H_2 and CO_2 produced during the cultivation experiments in stationary glass bottles were based on the concentrations measured for fructose/oligofructose/inulin (expressed as mM FE) and acetate consumed and formate and lactate produced according to the proposed stoichiometric pathway. NR: not relevant.

determination of the degradation of oligofructose revealed that all chain length fractions were degraded simultaneously (Figures 6B and 7A). Moreover, all oligofructose chain length fractions were completely hydrolysed and converted into free fructose, which was released into the medium, within 24 h of fermentation (Figures 6B and 7A). Qualitative determination of the degradation of inulin revealed that *F. prausnitzii* DSM 17677^T hydrolysed the short chain length fractions of inulin during the first 12 h of fermentation (Figure 7B). After 24 h of fermentation, also the long chain length fractions of inulin were hydrolysed, which resulted in the release of fructose and short chain length fractions of inulin into the medium (Figures 6C, 7B and 7C). During this degradation, acetate was always consumed, albeit to a higher

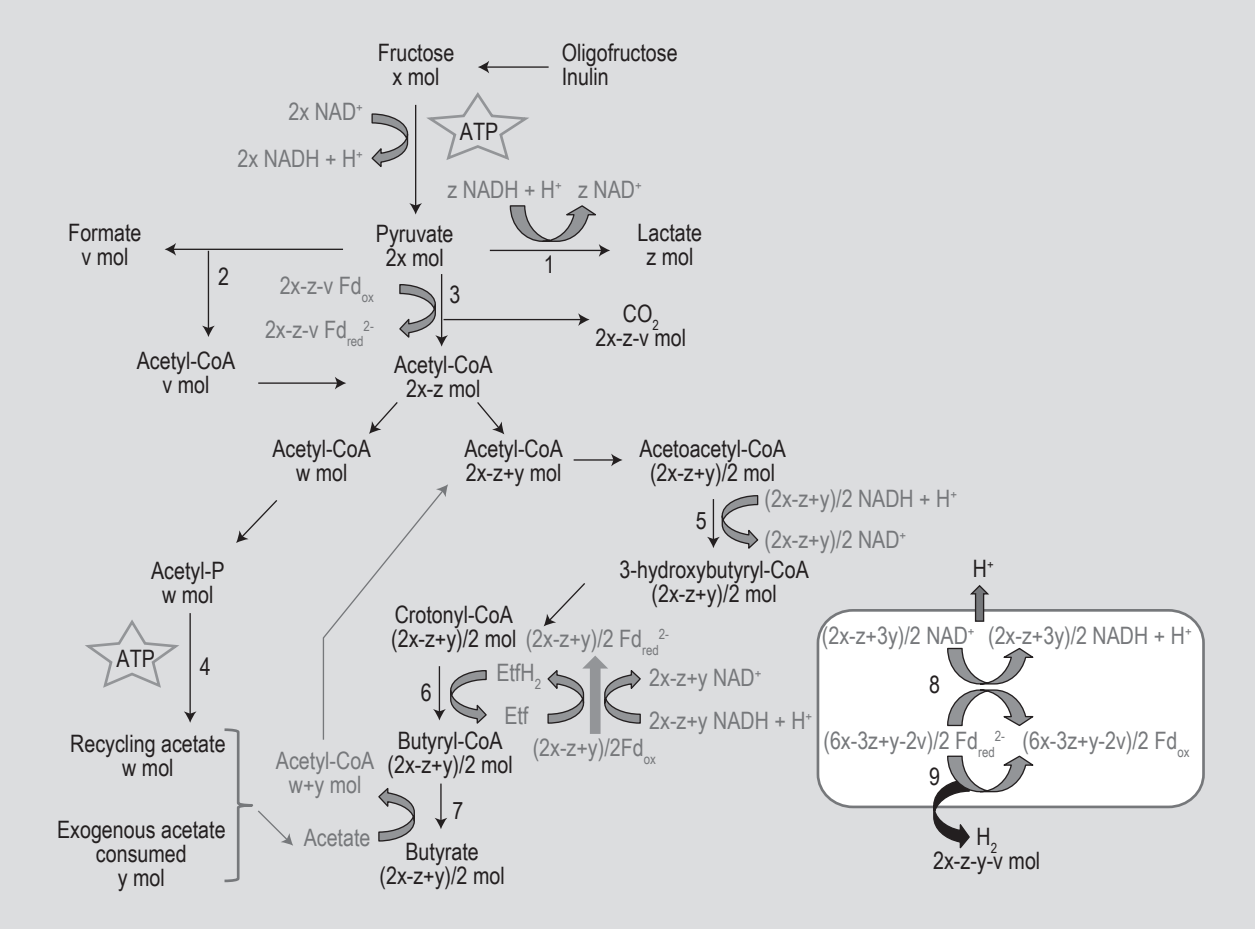

Figure 5. Proposed stoichiometric butyrate production pathway for butyrate-producing colon bacteria belonging to *Clostridium* clusters IV and XIVa. 1 = lactate dehydrogenase; 2 = pyruvate-formate lyase; 3 = pyruvate:ferredoxin oxidoreductase; 4 = acetate kinase; 5 = *β*-hydroxybutyryl-CoA dehydrogenase; 6 = butyryl-CoA dehydrogenase electron-transferring flavoprotein complex; 7 = butyryl-CoA:acetate CoA-transferase; 8 = membrane-associated NADH:ferredoxin oxidoreductase; 9 = hydrogenase.

extent during inulin degradation compared with fructose and oligofructose degradation (Table 2). Degradation of fructose, oligfructose, and inulin resulted in the production of mainly butyrate, formate, CO_2, and low concentrations of lactate, whereas H_2 was not produced (Table 2; Figure 6). The production of formate by *F. prausnitzii* DSM 17677^T, which was lower on inulin than on fructose and oligofructose, was accompanied by the production of higher concentrations of CO_2 (Table 2). The molar ratios of energy source consumption to acetate consumption plus formate production during the fermentations with fructose, oligofructose, and inulin were always 0.6.

Fermentations with E. rectale *CIP 105953^T*

E. rectale CIP 105953^T consumed 60% of the fructose and 24% of the oligofructose within 48 h of fermentation (Table 2). During the fermentations in mMCB supplemented with inulin, only the consumption of the monosaccharides present in the commercial inulin substrate resulted in a short initial growth phase. No further degradation of the short and long chain length fractions of inulin took place. The degradation of fructose and oligofructose by *E. rectale* CIP 105953^T was accompanied by the consumption of acetate and the production of mainly butyrate, lactate, CO_2, and H_2 (Tables 2 and 3). Only minor concentrations of formate were produced by *E. rectale* CIP 105953^T during the consumption of fructose (Table 2). During the fructose fermentations with *E. rectale* CIP 105953^T, nearly equal concentrations of butyrate and lactate were produced (Table 2). However, during the fermentations with oligofructose only low concentrations of lactate were produced compared to butyrate production (Table 2). Substantial production of lactate from the added energy source resulted in the production of less CO_2 and H_2 (Table 2 and 3). During the fermentations with fructose and oligofructose, the molar ratios of lactate production to butyrate production were 0.8 and 0.1, respectively.

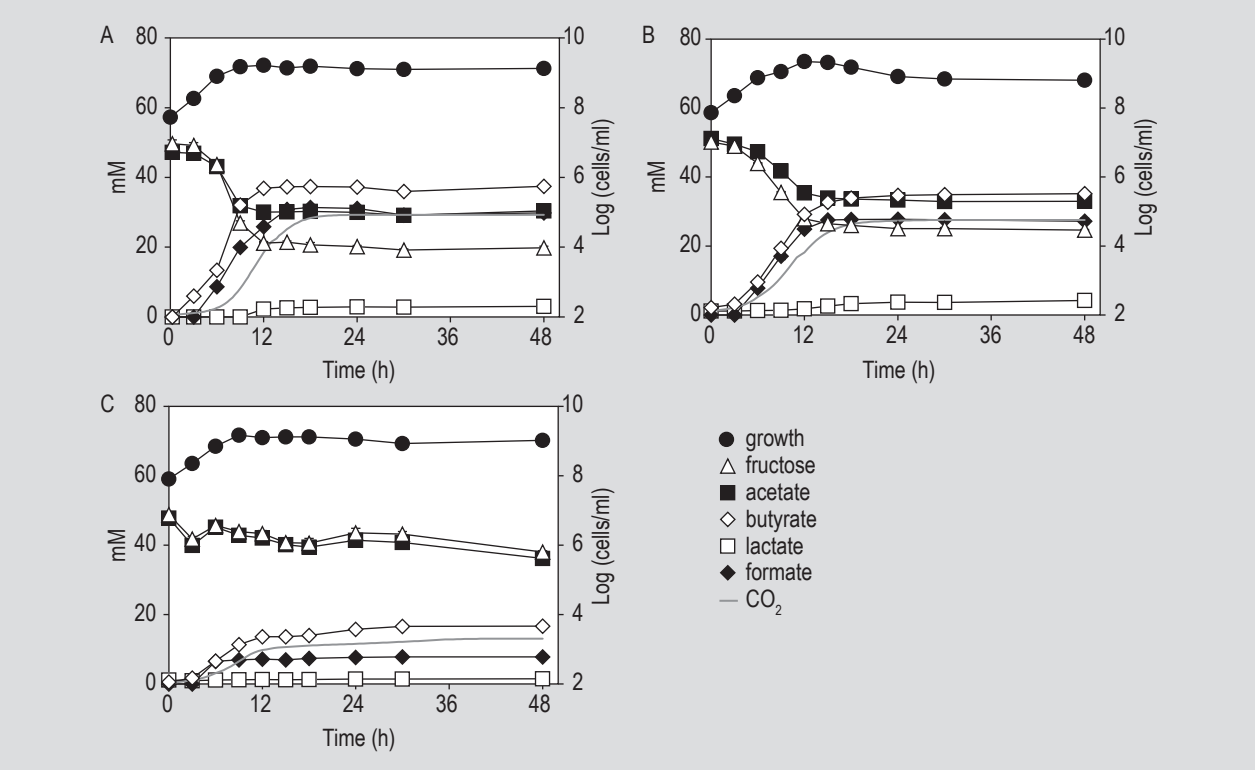

Figure 6. Growth, substrate consumption, and metabolite production by *Faecalibacterium prausnitzii* DSM 17677^T during cultivation experiments (fermentors) in a modified medium for colon bacteria (mMCB), including 50 mM acetate, supplemented with (A) fructose, (B) oligofructose, or (C) inulin.

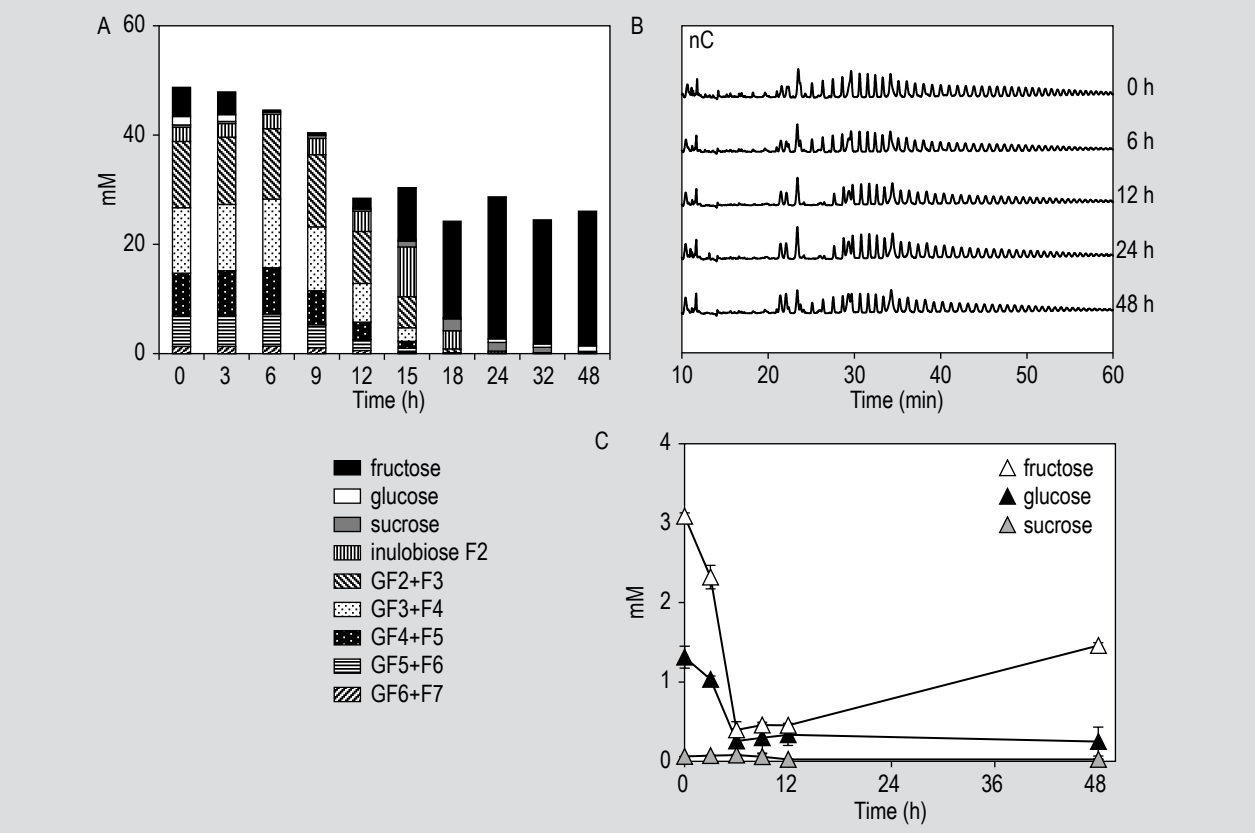

Figure 7. (A) Oligofructose degradation monitored through GC-FID. (B) Inulin degradation monitored through HPAEC-PAD. (C) Free fructose, glucose, and sucrose during inulin degradation.

Cultivation experiments in sealed bottles with *E. rectale* CIP 105953^T

The fact that *E. rectale* CIP 105953^T did not consume inulin during cultivation in fermentors was not in accordance with the results obtained for the cultivation experiments in glass bottles. During the latter cultivations, *E. rectale* CIP 105953^T showed initial growth on the monosaccharides present in the commercial inulin substrate (as in the fermentors), followed by degradation of all chain length fractions of inulin (Figure 1C,E,F). These divergent results could possibly indicate that substantial growth and consumption of inulin by *E. rectale* CIP 105953^T was dependent on the environmental conditions. Indeed, online measurement of the concentrations of CO_2 revealed that this compound was completely flushed out of the medium (due to continuous sparging of the fermentors with N_2) after the initial growth phase on the monosaccharides (data not shown). This possibly prohibited the subsequent growth on the inulin substrate. During the cultivation experiments in glass bottles with a sealed butyl rubber septum, *E. rectale* CIP 105953^T degraded inulin to a comparable extent as during the cultivation experiments in bottles with a pierced septum (Table 1). The initial growth of this strain on the monomers present in the commercial inulin substrate resulted in the production of low concentrations of CO_2 and H_2. However, the CO_2 produced in sealed bottles was kept in the medium, which allowed *E. rectale* CIP 105953^T to enter a second growth phase during which inulin was consumed (Table 1).

Calculated gas production

The constructed stoichiometric pathway for butyrate production mentioned above allowed theoretical calculations of the concentrations of butyrate, CO_2, and H_2 produced, based on the concentrations measured for fructose/oligofructose/inulin (expressed as mM FE) and acetate consumption and those of lactate and formate production (Table 4). These theoretical calculations showed a negative correlation between acetate consumption and H_2 production and between formate production and CO_2 production in the case of *B. pullicaecorum* DSM 23266^T and *E. hallii* DSM 17630. They confirmed the absence of H_2 production by *F. prausnitzii* DSM 17677^T and the production and consumption of adequate concentrations of formate and acetate, respectively. Also, they confirmed a negative correlation between lactate production and CO_2 and H_2 production in the case of *E. rectale* CIP 105953^T. Furthermore, this stoichiometric pathway enabled the calculation of the concentrations of the off-gases CO_2 and H_2 produced during the cultivation experiments in stationary glass bottles (Table 3). A negative correlation was found for acetate consumption and H_2 production for all strains tested. In general, during the cultivation experiments in stationary glass bottles, which were performed without pH control and in a gas atmosphere containing a higher partial hydrogen pressure than during the cultivation experiments in fermentors, higher concentrations of acetate were consumed, resulting in the production of lower concentrations of H_2 produced. Also, less formate was produced by *F. prausnitzii* DSM 17677^T during the cultivation experiments in stationary glass bottles, resulting in the production of higher concentrations of CO_2, as compared to the cultivation experiments in fermentors (Table 3).

Table 4. Comparison between the measured and calculated (according to the proposed stoichiometric pathway) productions of butyrate, H_2, and CO_2, based on the concentrations measured for fructose, oligofructose or inulin (expressed in mM FE) and acetate consumption and lactate and formate production after 48 h of incubation, during cultivation experiments in fermentors containing a modified medium for colon bacteria (mMCB), including 50 mM acetate, supplemented with 50 mM fructose equivalents (FE) of fructose, oligofructose, or inulin as the sole added energy source.

Strain	Energy source	Production of metabolites (mM)					
		Butyrate		H_2		CO_2	
		Measured	Calculated	Measured	Calculated	Measured	Calculated
B. pullicaecorum DSM 23266^T	fructose	58.1	62.0	41.1	45.5	105.3	84.7
E. hallii DSM 17630	fructose	50.8	56.8	77.2	72.9	83.1	87.1
E. rectale CIP 105953^T	fructose	25.4	24.9	21.8	26.1	35.9	36.9
	oligofructose	18.3	18.0	14.6	12.9	22.1	24.4
F. prausnitzii DSM 17677^T	fructose	37.5	36.8	0.0	10.1	29.3	27.0
	oligofructose	33.1	33.1	0.0	2.7	27.5	20.9
	inulin	16.1	16.6	0.0	2.1	13.0	13.7

4. Discussion

The cultivation experiments performed during this study revealed that degradation of inulin-type fructans is not a general characteristic among the butyrate-producing colon bacterial strains tested. Whereas *E. rectale* CIP 105953^T and *F. prausnitzii* DSM 17677^T were able to degrade fructose, oligofructose, and inulin, *B. pullicaecorum* DSM 23266^T and *E. hallii* DSM 17630 could only consume the monomer fructose as energy source. Similarly, it has been shown that not all species of the genera *Anaerostipes* and *Roseburia* are able to degrade inulin-type fructans (Falony *et al.*, 2009c). Growth and inulin-type fructan degradation by *E. rectale* CIP 105953^T were dependent on the availability of CO_2 in the medium, which either originated from the gas atmosphere or was produced through the fermentation of energy sources by the respective bacterial strain. As such, anaerobic growth only occurs when CO_2 is added to the culture medium or when endogenously produced CO_2 is not flushed out of the medium (Merlin *et al.*, 2003). In particular, *E. rectale* requires CO_2 for the biosynthesis of amino acids. In the presence of *Bacteroides thetaiotaomicron* (requires CO_2 too), *E. rectale* up-regulates the expression of its phosphoenolpyruvate carboxykinase, indicating competition for CO_2 uptake (Mahowald *et al.*, 2009). CO_2 assimilation by heterotrophic bacteria through anaplerotic CO_2 fixation pathways has been demonstrated before (Muthusamy *et al.*, 2014; Roslev *et al.*, 2004). The release of predominantly fructose and inulobiose (*E. rectale* CIP 105953^T) or fructose (*F. prausnitzii* DSM 17677^T) into the medium during oligofructose degradation has been shown for *Bacteroides* spp., *Roseburia* spp., and *Lactobacillus paracasei* 8700:2 before (Falony *et al.*, 2009c; Makras *et al.*, 2005; Van der Meulen *et al.*, 2006). In these previous studies, it has been suggested that this oligofructose degradation pattern is associated with extracellular polymer degradation. Extracellular β-fructofuranosidases are present in *Bacteroides fragilis* BF-1 and *L. paracasei* 1195 (Blatch and Woods, 1993; Goh *et al.*, 2007). Also, the release of fructose into the medium upon simultaneous degradation of all chain length fractions of inulin (*E. rectale* CIP 105953^T) or upon initial degradation of the short chain length fractions of inulin, followed by a further degradation of the longer ones and a concomitant accumulation of the short ones into the medium (*F. prausnitzii* DSM 17677^T), indicated an extracellular degradation of inulin. Such a degradation mechanism represents a competitive disadvantage for a colon bacterium in the highly competitive ecosystem of the human colon compared to intracellular degradation, which is typical for bifidobacteria (De Vuyst and Leroy, 2011; De Vuyst *et al.*, 2013; Falony *et al.*, 2006, 2009a,b,c; Makras *et al.*, 2005; Van der Meulen *et al.*, 2006). However, a β-fructofuranosidase gene lacking a signal peptide sequence is strongly induced during growth of *R. inulinivorans* on inulin (Scott *et al.*, 2011), suggesting an intracellular breakdown by the latter too. Hence, ingestion of inulin-type fructans will always result in a fierce competition between highly competitive bifidobacteria and less competitive butyrate-producing colon bacteria in the human colon ecosystem (De Vuyst *et al.*, 2013). The selective intracellular degradation of oligofructose or fast extracellular degradation of oligofructose and partial degradation of inulin by bifidobacteria makes them the primary beneficiaries of inulin-type fructan administration in the diet (De Preter *et al.*, 2008; Harmsen *et al.*, 2002; Kleessen *et al.*, 2007; Tuohy *et al.*, 2001). However, human studies have demonstrated that the growth of colon bacteria such as *F. prausnitzii* can be stimulated by inulin-type fructans too (Dewulf *et al.*, 2013; Ramirez-Farias *et al.*, 2009). Also, the *Roseburia/E. rectale* group is stimulated by these fructose polymers in gnotobiotic mice (Van den Abbeele *et al.*, 2011). This indicates that certain butyrate-producing colon bacteria can be stimulated upon inulin-type fructan supplementation, thereby avoiding competition for the available substrates and becoming involved in beneficial interactions with other colon bacteria.

This study underlined that inulin-type fructan-degrading butyrate-producing colon bacteria of *Clostridium* clusters IV and XIVa degrade oligofructose and inulin by the same mechanisms. However, fermentation of these energy sources by these bacteria resulted in the production of different organic acids and gases, whose molar ratios varied among different bacteria and under different environmental conditions. For instance, CO_2 and H_2 were not necessarily produced simultaneously, neither were butyrate and formate formed as end-metabolites simultaneously. The negative correlation between formate production and gas production can be explained as follows. Butyrate production results from the initial conversion of two molecules of acetyl-CoA into acetoacetyl-CoA (Duncan *et al.*, 2002a; Falony *et al.*, 2009c; Louis and Flint, 2009; Seedorf *et al.*, 2008). A functional metabolic map of *F. prausnitzii* shows that acetyl-CoA can be produced from pyruvate by both a pyruvate-formate lyase and a pyruvate:ferredoxin oxidoreductase (Heinken *et al.*, 2014). The former enzyme activity results in the production of formate next to acetyl-CoA, whereas the latter one results in the production of CO_2 and reduced ferredoxin next to acetyl-CoA. Reoxidation of reduced ferredoxin most likely occurs through the action of a ferredoxin-dependent [Fe-Fe] hydrogenase, as is the case in *Clostridium kluyveri* (Seedorf *et al.*, 2008) and *Clostridium pasteurianum* (Peters *et al.*, 1998). This may explain the production of H_2 by butyrate-producing colon bacteria. However, formate production could also be a source of H_2 and additional CO_2. The common gut bacterium, *Escherichia coli*, converts formate by a formate-hydrogen lyase complex, which couples the oxidation of formate into CO_2 by a formate dehydrogenase with the reduction of protons into H_2 by a [Ni-Fe] hydrogenase (McDowall *et al.*, 2014). During mixed-acid fermentation in *E. coli*, formate is produced and initially excreted out of the cell.

The accumulation of formate and the concomitant decrease of the pH of the extracellular medium below 6.8 results in a subsequent import of formate into the cell, where it is completely converted into CO_2 and H_2 by the formate-hydrogen lyase complex (Sawers, 2005; Vivijs *et al.*, 2015). However, mutants of *E. coli*, incapable of intracellular or periplasmic utilization of formate, secrete formate into the medium (Beyer *et al.*, 2013), as was the case with *E. hallii* DSM 17630 and *F. prausnitzii* DSM 17677^T during the cultivation experiments in stationary glass bottles (no pH control) and fermentors (constant pH of 6.3) of the present study. The latter strain did not produce H_2 at all neither was there conversion of formate and associated gas production in the case of both butyrate producers.

Butyrate-producing colon bacteria such as *F. prausnitzii* DSM 17677^T (this study), *E. rectale* CIP 105953^T (this study), and *R. inulinivorans* DSM 16841^T (Falony *et al.*, 2009c) maintain their redox balance by producing lactate along with butyrate. Lactate is produced from pyruvate by a lactate dehydrogenase, which contributes to the regeneration of NAD^+ (Falony *et al.*, 2009c; Heinken *et al.*, 2014). The production of lactate reduces that part of pyruvate that can be converted into acetyl-CoA and, therefore, negatively correlated with butyrate and gas production. However, a metabolic shift towards more butyrate production, at the expense of lactate, occurred when *E. rectale* CIP 105953^T (this study) and *R. inulinivorans* DSM 16841^T (Falony *et al.*, 2009c) grew on less easily degradable substrates. This indicates that the maintenance of the redox balance through butyrate production instead of lactate production was more favourable for these strains. Indeed, it has been shown that energy conservation in many anaerobes occurs through not only substrate-level phosphorylation but also a proton-motive force. For instance, it has been demonstrated that during the production of butyrate by *C. kluyveri*, crotonyl-CoA is reduced into butyryl-CoA by a butyryl-CoA dehydrogenase electron-transferring flavoprotein (Bcd-Etf) complex that couples the highly exergonic reduction of crotonyl-CoA with the endergonic reduction of ferredoxin, both mediated by $NADH + H^+$, through electron bifurcation, resulting in the formation of NAD^+. Subsequently, reduced ferredoxin can be reoxidized by a membrane-bound NADH:ferredoxin oxidoreductase (RnfA-G), which results in the regeneration of $NADH + H^+$ and the build-up of a proton-motive force (Buckel and Thauer, 2013; Li *et al.*, 2008; Seedorf *et al.*, 2008). The Rnf complex is an ion-motive electron transport complex, present in many prokaryotes, resulting in the development of an ion gradient (Na^+ or H^+) that is possibly the driving force for ATP biosynthesis (Biegel *et al.*, 2011). Indeed, all genes required for the biosynthesis of the Bcd-Etf complex are present in the genomes of butyrate-producing colon bacteria, such as *A. caccae*, *E. hallii*, *E. rectale*, and *Roseburia* spp. (Louis *et al.*, 2007). Further, it has been demonstrated that both the Bcd-Etf complex and the Rnf genes are highly expressed during butyrate production by *E. rectale* (Mahowald *et al.*, 2009). Also, the functional metabolic map of *F. prausnitzii* DSM 17677^T shows a coupling of the activity of Rnf with the production of a proton-motive force (Heinken *et al.*, 2014).

The negative correlation between acetate consumption and H_2 production can be explained by the consumption of exogenous acetate, which results in the generation of an extra pool of NAD^+. Indeed, the concentration of H_2 produced by *Clostridium* cluster IV and XIVa butyrate-producing colon bacteria is influenced by the action of Rnf, since this enzyme can reoxidize the reduced ferredoxin that is generated by pyruvate:ferredoxin oxidoreductase, without the production of H_2. This implicates that an extra pool of NAD^+ needs to be generated in the cell that serves as an electron acceptor. As the final step of butyrate biosynthesis consists of the conversion of butyryl-CoA into butyrate by a butyryl-CoA:acetate CoA-transferase, which transfers the CoA-fraction of butyryl-CoA to acetate, resulting in the production of acetyl-CoA and butyrate (Charrier *et al.*, 2006; Duncan *et al.*, 2002a; Louis *et al.*, 2004), the liberated acetyl-CoA can be incorporated into the butyrate biosynthesis pathway to form butyrate and NAD^+. Butyryl-CoA:acetate CoA-transferase can use both exogenous acetate and acetate that is generated in the cell through recycling of acetyl-CoA by means of an acetate kinase (Duncan *et al.*, 2004). The favoured consumption of acetate along with butyrate production during the cultivation experiments in stationary glass bottles (closed atmosphere, no pH control) compared with fermentors (flushed atmosphere, pH kept constant) may be ascribed to a decrease in the pH of the medium. Indeed, it has been shown that strains of *Roseburia intestinalis* and *Roseburia hominis* consume more acetate and produce less formate in response to a lower initial pH of the medium (Kettle *et al.*, 2015).

The inability of *F. prausnitzii* DSM 17677^T to produce H_2 is due to the absence of a functional hydrogenase (Heinken *et al.*, 2014). Based on the proposed stoichiometry, H_2 production can only be avoided when the molar ratio of energy source consumption to acetate consumption plus formate and lactate production equals 0.5. However, this ratio was higher during all cultivation experiments of the present study. *R. inulinivorans* DSM 16841^T (does not produce H_2) behaves similarly (Falony *et al.*, 2009c). The energy metabolism of both butyrate-producing strains requires a ferredoxin-oxidizing reaction for every ferredoxin-reducing reaction (Buckel and Thauer, 2013). This necessitates the consumption of exogenous acetate that equals the concentration of CO_2 produced by these strains. However, the concentration of acetate consumed was always lower than the concentration of CO_2 produced, indicating that the total concentration of acetate consumed was the sum of both exogenous acetate consumed and acetate

produced from intracellular acetyl-CoA. The production of acetate from intracellular acetyl-CoA by means of an acetate kinase is most likely linked to the glycolytic rate of energy source consumption, since acetate production by *F. prausnitzii* DSM 17677^T mainly occurred during fast consumption of fructose and oligofructose and not during slow consumption of inulin.

In conclusion, this study revealed that both short and long chain length fractions of oligofructose and inulin are degraded simultaneously by some selected butyrate-producing colon bacterial strains of *Clostridium* clusters IV and XIVa, which resulted in the release of extracellular fructose. Furthermore, the consumption of energy sources by these bacteria resulted in the production of different end-metabolites, whose molar ratios depended on the bacterial strain, the energy source used, and the prevailing environmental conditions. All this was demonstrated by the construction of an adapted stoichiometrically balanced pathway for butyrate production.

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