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Cross-feeding between *Bifidobacterium longum* BB536 and acetate-converting, butyrate-producing colon bacteria during growth on oligofructose

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Running title: Butyrate production through cross-feeding

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ABSTRACT

In vitro coculture fermentations of *Bifidobacterium longum* BB536 and two acetate-converting, butyrate-producing colon bacteria, *Anaerostipes caccae* DSM 14662 and *Roseburia intestinalis* DSM 14610, with oligofructose as the sole energy source, were performed to study interspecies interactions. Two clearly distinct types of cross-feeding were identified. *A. caccae* DSM 14662 was not able to degrade oligofructose, but could grow on the fructose released by *B. longum* BB536 during oligofructose breakdown. *R. intestinalis* DSM 14610 could degrade oligofructose, but only after acetate was added to the medium. Detailed kinetic analyses of oligofructose breakdown by the latter strain revealed simultaneous degradation of the different chain length fractions, in contrast with the preferential degradation of shorter fractions by *B. longum* BB536. In a coculture of both strains, initial oligofructose degradation and acetate production by *B. longum* BB536 took place, which in turn also allowed oligofructose breakdown by *R. intestinalis* DSM 14610. These and similar cross-feeding mechanisms could play a role in the colon ecosystem and contribute to the combined bifidogenic/butyrogenic effect observed after addition of inulin-type fructans to the diet.

Keywords: Gut microbiota, bifidobacteria, oligofructose, butyrate production, cross-feeding

INTRODUCTION

Since the introduction of the concept of prebiotics more than a decade ago (22), various food components have been screened for their ability to beneficially alter the composition and/or the activity of the colon microbiota (21). However, inulin-type fructans, such as oligofructose

and inulin, largely remain the most studied and well-established prebiotics (43). The vast amount of research performed on their health-promoting properties led to their acceptance as model prebiotics (44).

After their ingestion as a part of the human diet, inulin and oligofructose largely escape digestion in the upper gastro-intestinal tract and reach the colon virtually intact (20, 39), where they are fermented by some members of the resident microbiota (1, 39). Their stimulating effect on the colonic bifidobacterial population has been demonstrated extensively by *in vitro* and *in vivo* animal and human trials (21, 30, 45). Also, it has been shown that inulin-type fructans have a regulatory effect on bowel functions (9, 40), increase calcium (23, 51) and magnesium (50) absorption, and reduce triglyceridemia in slightly hyperlipidemic individuals (13, 32). The influence of oligofructose and inulin on bone health (12, 58), cholesterolemia (6, 13), the immune system (57), and the development of inflammatory bowel disease (24) and colon cancer (41) remains under investigation.

Many of the health-promoting effects attributed to oligofructose and inulin are at least partially due to their influence on the production of short-chain fatty acids (SCFA) by the colon microbiota (13, 24, 40, 41, 58). In humans, colonic fermentation of inulin-type fructans generally does not lead to a significant increase of fecal concentrations of SCFA, nor to a change in molar proportions of acetate, propionate, and butyrate (40), which can be explained by their very efficient colonic absorption (34, 35). However, *in vitro* and animal studies show an enhancement of SCFA production by inulin and oligofructose (14, 56). In particular, fermentation of inulin-type fructans by the colon microbiota seems to cause an increase in butyrate formation, the so-called butyrogenic effect (8, 31, 52). Butyrate is of key importance for gut health: it is not only the preferred energy source for the colonic epithelium (34), but it also has important effects on the development of and the gene expression in intestinal cells

(37, 47). In addition, it is generally thought to play a protective role against colorectal cancer and colitis (2, 10, 25).

The link between consumption of inulin-type fructans, the bifidogenic effect, and the increase of butyrate production in the colon remains unclear up to now. Bifidobacteria produce lactate, acetate, formate, ethanol, and even minor amounts of succinate (3, 53, 54, 59), but have never been reported to produce butyrate (L. Makras, G. Falony, R. Van der Meulen, and L. De Vuyst, Letter to the Editor, J. Appl. Microbiol. **100**:1388-1389, 2006). Furthermore, evidence of direct degradation of inulin-type fructans by butyrate-producing colon bacteria is scarce (19). Cross-feeding between different members of the colon microbiota has been suggested as a possible mechanism responsible for colonic butyrate production (5, 7, 18, 29, 42). However, research attempting to unravel the exact nature of this cross-feeding has until now been limited to small-scale experiments under uncontrolled conditions, not allowing kinetic analysis (5, 18, 29).

Recently, the use of more complex culture media has allowed the isolation of some previously unknown butyrate-producing colon bacteria (4), belonging to the clostridial cluster XIVa (11, 17, 48), one of the most abundant bacterial groups in human feces (26, 49). Species such as *Anaerostipes caccae* and *Roseburia intestinalis* have been shown to be efficient lactate and/or acetate convertors (16, 18). Bacteria related to these species have been reported to compose up to 3% of the colon microbiota (27). Cross-feeding between these microorganisms and lactate- and/or acetate-producing inulin degraders, such as bifidobacteria, might be a key aspect of the gut ecosystem, with important consequences towards human health. The aim of this work was to investigate kinetically some of the mechanisms behind this type of cross-feeding, using intermediate-scale, *in vitro*, mono- and coculture batch fermentation techniques under strictly controlled conditions.

MATERIALS AND METHODS

Microorganisms and media

The commercialized probiotic strain *Bifidobacterium longum* BB536 was kindly provided by Morinaga Industry Co., Ltd. (Tokyo, Japan). *Anaerostipes caccae* DSM 14662 (48) and *Roseburia intestinalis* DSM 14610 (17), two recently described butyrate-producing colon bacteria, were obtained from the Deutsche Sammlung von Mikro-Organsimen und Zellkulturen (DSM; Göttingen, Germany). All strains were stored at -80°C in Reinforced Clostridial Medium (RCM; Oxoid Ltd., Basingstoke, United Kingdom), supplemented with 25% (vol/vol) glycerol as a cryoprotectant.

Fermentation experiments were performed in a Medium for Colon Bacteria (MCB), developed by Van der Meulen *et al.* (55) to support growth of various human colon bacteria when supplemented with an adequate energy source. The medium was composed of (in g liter^{-1}): bacteriological peptone (Oxoid), 6.5; soy peptone (Oxoid), 5.0; yeast extract (VWR International, Darmstadt, Germany), 3.0; tryptone (Oxoid), 2.5; NaCl, 4.5; KCl, 2.0; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.45; cysteine-HCl.H_2O, 0.4; $NaHCO_3$, 0.2; $MnSO_4.H_2O$, 0.2; $FeSO_4.7H_2O$, 0.005; $ZnSO_4.7H_2O$, 0.005; hemin, 0.005; and menadione, 0.005. It also contained H_3PO_4, 0.5 ml liter^{-1}, and Tween 80, 2 ml liter^{-1}. When proven indispensable for bacterial growth, modified MCB containing 6.8 g liter^{-1} of $NaCH_3COOH.3H_2O$ was used. The pH was adjusted to 5.8 and the medium was autoclaved at 210 kPa and 120°C for 20 min. After sterilization, fructose (VWR International) or oligofructose (RaftiloseP95; Orafti NV, Tienen, Belgium) was added aseptically as sole energy source at a concentration of 9 g liter^{-1}. Fructose was autoclaved under the same conditions as the MCB; oligofructose was sterilized through membrane filtration using Minisart filters (pore size 0.2 µm; Sartorius AG, Göttingen, Germany). RaftiloseP95 is a commercial powder obtained through enzymatic

hydrolysis of chicory inulin. It consists mainly of oligofructose (≥93.2%, wt/wt), but contains also some minor amounts of glucose, fructose, and sucrose (<6.8%, wt/wt). The degree of polymerization (DP) of the oligofructose fractions varies between 2 and 8, with an average of 4.

Solid RCM was prepared by adding 1.5% (wt/vol) of agar (Oxoid) to RCM.

Fermentation experiments

Mono- and coculture fermentations were carried out in 1.5 liter Biostat B-DCU fermentors (Sartorius AG). Inocula were prepared as follows: strains were transferred from -80°C to RCM and incubated anaerobically at 37°C for 24 h in a Modular Atmosphere-Controlled System (MACS, MG Anaerobic Work Station; Don Withley Scientific, West Yorkshire, United Kingdom) that was continuously sparged with a mixture of 80% nitrogen, 10% carbon dioxide, and 10% hydrogen (Air Liquide, Paris, France). Subsequently, the strains were propagated twice in MCB with fructose (and acetate, when proven indispensable) as sole energy source(s) and finally added to the fermentor. During inoculum build-up, the transferred volume was always 5% (vol/vol). Anaerobic conditions were assured during fermentation experiments by continuously sparging the medium with a mixture of 90% nitrogen and 10% carbon dioxide (Air Liquide). Fermentation temperature was maintained constant at 37°C. A linear pH profile, mimicking colonic pH and transit by raising from pH 5.8 to pH 6.8 in 48 h, was imposed and controlled automatically, using 1.5 M solutions of NaOH and H_3PO_4. To keep the medium homogeneously, a gentle stirring (100 rpm) was applied. Temperature, pH, and agitation speed were controlled on-line (MFCS/win 2.1; Sartorious AG). Fermentations were followed up during 48 h; samples for further analysis were taken at regular time intervals. All fermentations were carried out in duplicate; the results and figures presented are representative for both fermentations.

143

144 **Analysis of growth**

145 Growth was followed throughout all fermentations by both plating and biomass
146 determination. Bacterial biomass was determined as cell dry mass (CDM) through membrane
147 filtration of 15 ml of sample using cellulose nitrate filters (pore size 0.45 µm; Sartorius AG).
148 The filters were dried at 105°C for 48 h and weighed. Samples were plated on RCM agar and
149 incubated for 24 h under anaerobic conditions (MACS). However, as *A. caccae* DSM 14662
150 and *R. intestinalis* DSM 14610 grew poorly on solid culture media, results of enumerations
151 are not reported.

152

153 **Analysis of fructose and oligofructose breakdown**

154 Residual concentrations of glucose, fructose and oligofructose (expressed as mM fructose
155 equivalents [FE]) were determined by high performance liquid chromatography (HPLC) with
156 a Waters chromatograph (Waters Corp., Milford, MA), equipped with a 2414 differential
157 refractometer, a 600S controller, a column oven, and a 717plus autosampler. An ICSep ICE
158 ORH-801 column (Interchim, Montluçon, France) was used with 10 mM of H_2SO_4 as mobile
159 phase at a flow rate of 0.4 ml min^{-1}. The column temperature was kept constant at 35°C.
160 Samples were centrifuged (21,036 x *g* for 20 min) and an equal volume of 20% (wt/vol) of
161 trichloroacetic acid (TCA) was added for protein removal. After centrifugation (21,036 x *g*,
162 20 min) the supernatant was filtered (pore size 0.2 µm; Minisart RC4 filters, Sartorius AG)
163 before injection. All samples were analyzed in triplicate.
164 Detailed analysis of the breakdown of the different oligofructose fractions of RaftiloseP95
165 was performed by gas chromatography (GC) using a HRGC 5300-HT Mega (Carlo Erba,
166 Rodina, Italy) as described previously (28, 54). The GC was equipped with a SGE Aluminium
167 Clad-5 capillary column (Achrom NV, Zulte, Belgium), a cool on-column auto-injector AS-

550, and a flame ionization detector (FID, detector temperature of 447°C). The oven temperature varied linearly from 105 to 440°C at 10°C min^{-1}. Samples were derivatizised following a procedure involving oximation and silylation (28). The oxime-trimethylsilyl sugar derivatives were extracted using iso-octane; the resulting iso-octane phase was injected into the GC. The same procedure was carried out for reference samples containing reference oligofructose (RaftiloseP95X, Orafti NV), glucose, fructose, and sucrose as external standards.

Analysis of metabolite production

The concentrations of lactate, acetate, butyrate, and formate were determined through HPLC as described above.

Succinate was determined using a 2695 HPLC (Waters) coupled to a Quattro Micro mass spectrometer (Waters). The column (Atlantis, Waters) was kept at 35°C. The mobile phase, at a flow rate of 0.2 ml min^{-1}, was composed of ultrapure water (eluent A), acetonitrile (eluent B), and 10 mM of ammonium acetate (pH 6.5; eluent C). The gradient (vol/vol) used was as follows: 0.0 min, 85% A, 5% B, and 10% C; 15.0 min, 40% A, 50% B, and 10% C; 15.1 min, 10% A, 80% B, and 10% C; 23.0 min, 10% A, 80% B, and 10% C; 23.1 min, 85% A, 5% B, and 10% C; 30.0 min, 85% A, 5% B, and 10% C. Samples were centrifuged (21,036 x *g* for 20 min) and 100 µl of internal standard (3,4-dihydroxybenzoic acid) was added to 500 µl of supernatant. Afterwards, 600 µl of acetonitrile was added and the samples were again centrifuged (21,036 x *g* for 20 min). The supernatant was filtered (Minisart RC4) and injected. All samples were analyzed in triplicate.

Ethanol was measured on a 6890 GC (Agilent Technologies, Palo Alto, CA) equipped with a programmable temperature vaporization (PTV) injector, coupled to a 5973N mass spectrometer (Agilent Technologies). A capillary column (DB-WAXetr; Agilent

193 Technologies) was used with the following oven temperature program: 0.0 min, 40°C; 5.0
194 min, 40°C; 9.29 min, 100°C; 10.37 min, 230°C; 15 min, 230°C. Helium (Air Liquide) was
195 used as carrier gas at a flow rate of 1.1 ml min^{-1}. The samples were centrifuged (21,036 x *g*
196 for 20 min) and 100 µl of methanol (0.5% [wt/vol] in ultrapure water) was added as an
197 internal standard to 500 µl of supernatant. After mixing for 15 s, 750 µl of chloroform was
198 added to the sample and mixed thoroughly (30 min). Subsequently, the organic phase was
199 transferred into a vial. The extraction procedure with chloroform was performed twice after
200 which the samples were injected. Analyses were performed in triplicate.
201
202 **Carbon recovery**
203 The carbon recovery (CR, in percentage) was calculated by dividing the total amount of
204 carbon recovered in the sugar metabolites by the total amount of carbon present in the added
205 energy source(s). In the case of fructose or oligofructose (RaftiloseP95) degradation by *A.*
206 *caccae* DSM 14662 or *R. intestinalis* DSM 14610, production of two moles of carbon dioxide
207 for every mole of fructose (or FE) consumed, as suggested by Duncan *et al.* (16, 18), was
208 taken into account.
209
210 **RESULTS**
211
212 **Growth of *B. longum* BB536, *A. caccae* DSM 14662 and *R. intestinalis* DSM 14610 in**
213 **MCB supplemented with fructose or fructose and acetate.** *B. longum* BB536 reached a
214 maximal biomass of 2.0 g CDM $liter^{-1}$ after 15 h of fermentation. Acetate, formate, ethanol,
215 and lactate, as well as minor concentrations of succinate were produced (Table 1). The
216 calculated CR was 99.4%.

217 *A. caccae* DSM 14662 was able to degrade fructose without the addition of acetate to the
218 fermentation medium. A maximal biomass of 1.4 g CDM liter^{-1} after 30 h and 1.6 g CDM
219 liter^{-1} after 48 h of fermentation was reached in the presence and absence of acetate,
220 respectively. In both cases, butyrate and gases were the only metabolites produced (Table 1).
221 The CR was calculated as 91.4% and 86.6%, respectively. In the presence of acetate, fructose
222 and acetate consumption took place simultaneously. After fructose depletion, acetate
223 consumption stopped.
224 Growth of *R. intestinalis* DSM 14610 was only detected in MCB supplemented with both
225 fructose and acetate. Biomass reached a maximum of 1.3 g CDM liter^{-1} after 24 h of
226 fermentation. Acetate was only consumed during fructose degradation. Again, butyrate and
227 gases were the only metabolites produced (Table 1). CR was calculated as 97.5%
228
229 **Growth of *B. longum* BB536, *A. caccae* DSM 14662 and *R. intestinalis* DSM 14610 in**
230 **MCB supplemented with oligofructose or oligofructose and acetate.** With *B. longum*
231 BB536, a slower fermentation took place as compared with growth on fructose. Maximal
232 biomass of 1.4 g CDM liter^{-1} was measured after 48 h. Again, acetate, formate, ethanol,
233 lactate, and succinate were produced (Table 2). The calculated CR was 94.6%.
234 Addition of acetate to the MCB had no influence on the ability of *A. caccae* DSM 14662 to
235 degrade oligofructose: when RaftiloseP95 was used as an energy source, only minor growth
236 on the monosaccharides present in the preparation was observed (results not shown). Again,
237 butyrate and gases were produced.
238 *R. intestinalis* DSM 14610 was not able to degrade oligofructose in MCB without acetate. In
239 MCB supplemented with acetate, butyrate and gases were the only metabolites produced
240 (Table 2). A maximal biomass of 1.6 g CDM liter^{-1} was measured after 48 h of fermentation.
241 CR was calculated as 98.5%.

Detailed analysis of fructan degradation by *B. longum* BB536 (Fig. 1) and *R. intestinalis* DSM 14610 (Fig. 2) revealed a remarkable difference between the oligofructose breakdown patterns of both strains. *B. longum* BB536 showed preferential degradation of short-chain oligofructose fractions (≤ DP 3), only degrading longer fractions after nearly complete depletion of the shorter ones. *R. intestinalis* DSM 14610 expressed simultaneous breakdown of different chain-length fractions (DP3 and DP4). In both cases, low concentrations of free fructose were detected in the medium during the entire course of the fermentations.

Coculture of *B. longum* BB536 and *A. caccae* DSM 14662 in MCB supplemented with oligofructose. In a coculture of *B. longum* BB536 and *A. caccae* DSM 14610, maximal biomass was 1.7 g CDM liter^{-1} after 48 h of fermentation,. Acetate, butyrate, formate, ethanol, and traces of succinate were produced (Fig. 3A). Gas production was observed.

Detailed analysis of fructan degradation (Fig. 3B) revealed a similar breakdown profile as in *B. longum* BB536 monoculture fermentations. However, almost no free fructose was detected in the medium during oligofructose breakdown, indicating fructose consumption by *A. caccae* DSM 14662. Considering that the metabolism of *A. caccae* DSM 14662 in the coculture was comparable with the one observed in the monoculture in MCB supplemented with fructose and acetate (see above), it can be calculated that the strain would have needed 19.8 mM of fructose to produce 22.5 mM of butyrate, which is nearly half of the total concentration of FE consumed during the fermentation. This clearly shows intense cross-feeding between both strains. The previous considerations allow the calculation of the CR in this coculture as 96.6%, taking into account CO_2 production by *A. caccae* DSM 14662.

Coculture of *B. longum* BB536 and *R. intestinalis* DSM 14610 in MCB supplemented with oligofructose. In a coculture of *B. longum* BB536 and *R. intestinalis* DSM 14610,

biomass reached a maximum of 2.2 g CDM liter^{-1} after 18 h of fermentation. Besides gases, butyrate, acetate, formate, ethanol, and traces of succinate were the metabolites produced (Fig. 4A).

Kinetic analysis of the degradation of the different oligofructose fractions showed preferential breakdown of shorter (≤ DP 3) fructan components (Fig. 4B). However, degradation of longer fractions started before depletion of the shorter ones and the overall consumption rate was higher than in *B. longum* BB536 monoculture fermentations. During oligofructose degradation, concentrations of free fructose varied between 0.57 and 0.59 mM.

Considering that *R. intestinalis* DSM 14610 as well as *B. longum* BB536 behaved similarly in mono- and coculture fermentations (see above), it can be calculated that *R. intestinalis* DSM 14610 needed 33.2 mM FE of oligofructose to produce 42.4 mM of butyrate. This estimation allows the calculation of the CR in the coculture as 102.9%, taking into account CO_2 production by *R. intestinalis* DSM 14610.

DISCUSSION

In vitro fermentation studies using monocultures and cocultures certainly have their limitations regarding their *in vivo* significance, as they take by no means into account the complexity of the human colon ecosystem. These inherent limitations make them, however, an excellent tool for the study of individual bacterial metabolism and interspecies interactions (5, 55). Hence, they can provide a valuable contribution to the further exploration of the gut ecosystem, together with *in vitro* studies using fecal slurry and *in vivo* animal or human trials.

Compared with the results of an earlier study with *B. longum* BB536 carried out by Van der Meulen *et al.* (55), the monoculture fermentations performed with this strain in the present study confirmed its preferential degradation of shorter oligofructose fractions, a characteristic

292 that seems to be common among bifidobacteria, and its mixed acid fermentation depending on
293 its growth rate (53-55). Growth on low concentrations of oligofructose led to an even more
294 marked metabolic shift towards mixed acid fermentation than reported earlier (55), indicating
295 that both sugar consumption rate and substrate concentration determine the metabolic profile
296 expressed by this strain. Indeed, when growing on RaftiloseP95, *B. longum* BB536 produced
297 more formate, ethanol, and acetate, at the expense of lactate, to generate more ATP.
298 The present kinetic study showed that *A. caccae* DSM 14662 was able to grow on fructose
299 without addition of acetate to the fermentation medium. This might imply a high flexibility in
300 the metabolism of this strain, allowing it to produce enough endogenous acetate to
301 compensate for the lack of exogenous acetate necessary for butyrate production (16, 18).
302 However, optimal growth only occurred after addition of acetate to the medium. Indeed,
303 growth of *A. caccae* DSM 14662 in a complex medium showed that it was a net convertor of
304 glucose or lactate in combination with acetate (5, 18). This led to the suggestion that it
305 produced butyrate through a butyryl-Coenzyme A (CoA):acetate-CoA transferase pathway,
306 with use of (partly) exogenous acetate. Louis *et al.* (33) showed that this pathway is most
307 common among butyrate-producing members of the colon microbiota, instead of the butyrate
308 kinase pathway, as was thought earlier (38). *A. caccae* DSM 14662 was not able to degrade
309 oligofructose, in spite of earlier reports of acid production on fructooligosaccharides (48),
310 stressing the necessity to report on the exact composition of the inulin-type fructans used and
311 of detailed kinetic analyses of substrate degradation.
312 Enzymatic studies showed that *R. intestinalis* DSM 14610 possessed acetate kinase and
313 butyryl-CoA:acetate-CoA transferase activity, but no butyrate kinase was detected (16). The
314 results of this study confirmed these findings by showing the absolute need for acetate of this
315 strain to grow on fructose and oligofructose. Regarding growth of *R. intestinalis* DSM 14610
316 on inulin-type fructans, conflicting results have been published in the past (17, 19). Moreover,

317 it has recently been shown that the related strain *Roseburia* sp. A2-183, belonging to the
318 newly proposed species *Roseburia hominis* (15), is not able to grow significantly on
319 oligofructose (5). The present study revealed for the first time the kinetics of the degradation
320 of oligofructose by *R. intestinalis* DSM 14610 in an acetate-containing fermentation medium.
321 Non-preferential degradation of the different oligofructose fractions was observed, a
322 phenomenon that has also been reported for other colon bacteria, belonging to the genera
323 *Bacteroides* (55) and *Lactobacillus* (36), but so far not for *Bifidobacterium* (54, 55). It has
324 been suggested that simultaneous breakdown of oligofructose fractions with different chain
325 lengths indicates extracellular degradation (55), which also explains the higher concentrations
326 of free fructose found in the fermentation medium in those cases. Most bifidobacteria degrade
327 oligofructose intracellularly (46), which might contribute to their selective stimulation by
328 inulin-type fructans in the gut (55).
329 The present study of coculture fermentations led to the identification of two clearly distinct
330 types of cross-feeding between *B. longum* BB536 and butyrate-producing colon bacteria. In
331 the case of the cocultures with *A. caccae* DSM 14662, butyrate production could only be
332 attributed to the latter strain. As this strain was not able to grow on oligofructose, its only
333 available energy sources were the fructose released and the acetate produced by *B. longum*
334 BB536 during oligofructose degradation. This was reflected by the fact that the breakdown
335 profile in the coculture matched with the one of the *B. longum* BB536 monoculture, except
336 for the fact that no free fructose was detected. Recently, a study focusing on cross-feeding
337 between *A. caccae* DSM 14662 and a *Bifidobacterium adolescentis* strain in a rich medium
338 containing starch as an energy source, pointed out that lactate was the key substrate in the
339 described cross-feeding mechanism (5). The results presented in the current paper reveal the
340 flexibility of cross-feeding in function of the bacterial partner and the available substrate.

341 Butyrate in the coculture of *B. longum* BB536 and *R. intestinalis* DSM 14610 could only be
342 produced by the latter strain. Detailed kinetic analyses of oligofructose degradation showed a
343 profile in between simultaneous and preferential degradation of different oligofructose
344 fractions (Fig. 4B). *R. intestinalis* DSM 14610, capable of simultaneous breakdown, had an
345 absolute requirement for acetate to degrade oligofructose. Initially, no acetate was present in
346 the fermentation medium; it only appeared as a metabolic end-product of bifidobacterial
347 growth. Subsequently, oligofructose was further degraded as the result of the combined
348 efforts of both strains. A recently published, small-scale cross-feeding study performed by
349 Belenguer *et al.* (5) on a coculture of *B. adolescentis* L2-32 and *R. hominis* A2-183 did not
350 reveal oligofructose degradation by the latter strain. The cross-feeding mechanism described
351 in the present study for *A. caccae* DSM 14662 is probably comparable to the one observed
352 previously for *R. hominis* A2-183, where butyrate production by this strain was attributed to
353 cross-feeding of the partially degraded carbohydrate substrate, although degradation kinetics
354 were not studied in detail (5). Finally, other *Bifidobacterium* strains, and to a lesser extent
355 *Lactobacillus* species, are reported to produce lactate as well as acetate on oligofructose, or on
356 starch (5, 30, 36, 53, 54). Therefore, the types of cross-feeding may be strain-dependent.
357 In conclusion, the results presented in this paper are, to our knowledge, the first that
358 kinetically describe cross-feeding between a *Bifidobacterium* sp. and butyrate-producing
359 colon bacteria, growing on a prebiotic substrate under controlled conditions. This study
360 confirms the earlier reported shift of *in vitro* bifidobacterial metabolism towards less lactate
361 production when growing on oligofructose (54, 55). However, during oligofructose
362 degradation, *B. longum* BB536 released substantial amounts of free fructose into the
363 extracellular environment, enough to support growth of *A. caccae* DSM 14662, a strain that
364 was not able to degrade the substrate itself. This study is also the first detailed kinetic analysis
365 of oligofructose degradation by *R. intestinalis* DSM 14610. To do so, the strain showed an

absolute requirement for acetate, which is sufficiently present in the gut too (34) and, as was shown in this study, can be provided by bifidobacteria. These and similar cross-feeding mechanisms could play a role in the colon ecosystem and contribute to the combined bifidogenic/butyrogenic effect observed after addition of inulin-type fructans to the diet. However, the physiological relevance of oligofructose degradation by *R. intestinalis* DSM 14610 has to be studied further through *in vitro* colon simulation models and *in vivo* trials. Also, additional *in vitro* studies of the metabolism of butyrate-producing colon bacteria, combined with *in vivo* trials with genetic tools to monitor the effect of addition of oligofructose to the human diet on the colonic population of butyrate producers (27) are needed.

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386 **Figure legends**

387

388 FIG. 1. Oligofructose degradation by *Bifidobacterium longum* BB536 in MCB supplemented
389 with 50 mM fructose equivalents (FE) of oligofructose (RaftiloseP95).

390

391 FIG. 2. Oligofructose degradation by *Roseburia intestinalis* DSM 14610 in MCB
392 supplemented with 50 mM fructose equivalents (FE) of oligofructose (RaftiloseP95) and 50
393 mM of acetate.

394

395 FIG. 3. Substrate consumption and metabolite production (A) and oligofructose degradation
396 (B) by a coculture of *Bifidobacterium longum* BB536 and *Anaerostipes caccae* DSM 14662
397 in MCB supplemented with 50 mM fructose equivalents (FE) of oligofructose (RaftiloseP95).
398 ○, oligofructose (FE); ■, acetate; ◒, butyrate; ◆, formate; ▲, ethanol; □, succinate; F,
399 Fructose; G, Glucose.

400

401 FIG. 4. Substrate consumption and metabolite production (A) and oligofructose degradation
402 (B) by a coculture of *Bifidobacterium longum* BB536 and *Roseburia intestinalis* DSM 14610
403 in MCB supplemented with 50 mM fructose equivalents (FE) of oligofructose (RaftiloseP95).
404 ○, oligofructose (FE); ■, acetate; ●, butyrate; ◆, formate; ▲, ethanol; □, succinate; F,
405 Fructose; G, Glucose.

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TABLE 1. Growth characteristics of *Bifidobacterium longum* BB536, *Anaerostipes caccae* DSM 14662 and *Roseburia intestinalis* DSM 14610 in MCB supplemented with 50 mM of fructose or 50 mM of fructose and 50 mM of acetate. Mean values ± standard deviations are represented.

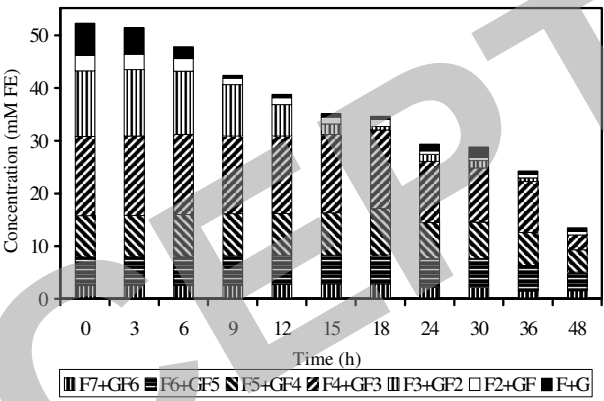
		B. longum BB536	*A. caccae* DSM 14662	*A. caccae* DSM 14662	*R. intestinalis* DSM 14610
Substrate consumed (mM)	Fructose	49.8 ± 0.4	50.2 ± 0.2	49.3 ± 0.1	47.9 ± 0.2
	Acetate			28.8 ± 0.3	19.7 ± 0.2
Metabolites produced (mM)	Lactate	10.7 ± 0.4			
	Acetate	93.8 ± 1.4			
	Butyrate		40.1 ± 0.5	56.1 ± 0.4	55.6 ± 0.4
	Formate	39.0 ± 0.5			
	Ethanol	18.2 ± 2.0			
	Succinate	0.60 ± 0.02			
	CO_2[a]		100.4	98.6	95.8
CR (%)		99.4	86.6	91.4	97.5
Fructose depletion time (h)		15	48	30	30

[a] Theoretical CO_2 production by *A. caccae* DSM 14662 and *R. intestinalis* DSM 14610, according to the pathway proposed by Duncan *et al.* (16, 18)

TABLE 2. Growth characteristics of *Bifidobacterium longum* BB536 and *Roseburia intestinalis* DSM 14610 in MCB supplemented with 50 mM fructose equivalents (FE) of oligofructose or 50 mM FE of oligofructose and 50 mM of acetate. Mean values ± standard deviations are represented.

		B. longum BB536	*R. intestinalis* DSM 14610
Substrate consumed (mM)	Oligofructose (FE)	38.8 ± 0.2	48.2 ± 0.2
	Acetate		29.3 ± 0.3
Metabolites produced (mM)	Lactate	2.6 ± 0.1	
	Acetate	78.9 ± 0.6	
	Butyrate		61.5 ± 0.5
	Formate	29.1 ± 0.2	
	Ethanol	12.8 ± 1.9	
	Succinate	0.80 ± 0.04	
	CO_2[a]		96.4
CR (%)		94.6	98.5
Fructose depletion time (h)		>48	>48
Free fructose in medium during fermentation (mM)		0.57 - 1.27	1.71 - 1.12

[a] Theoretical CO_2 production by *R. intestinalis* DSM 14610, according to the pathway proposed by Duncan *et al.* (16)


Concentration (mM FE)
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48
Time (h)
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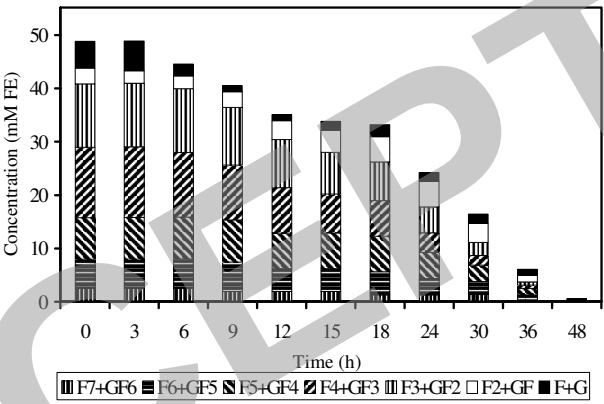
F6+GF5
F5+GF4
F4+GF3
F3+GF2
F2+GF
F+G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Time (h)
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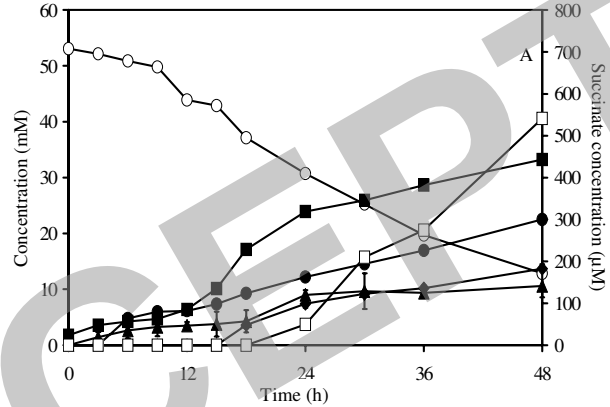
F4+GF3
F3+GF2
F2+GF
F+G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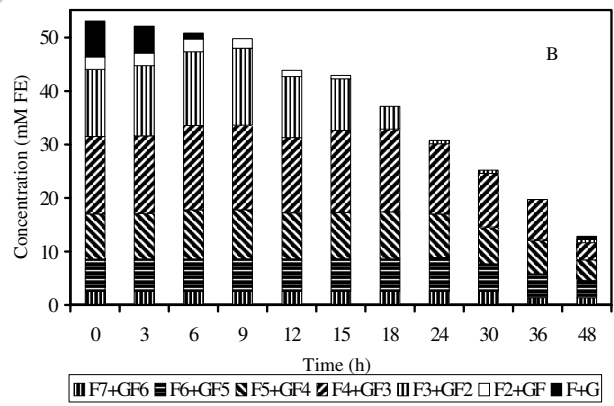
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Concentration (mM FE)
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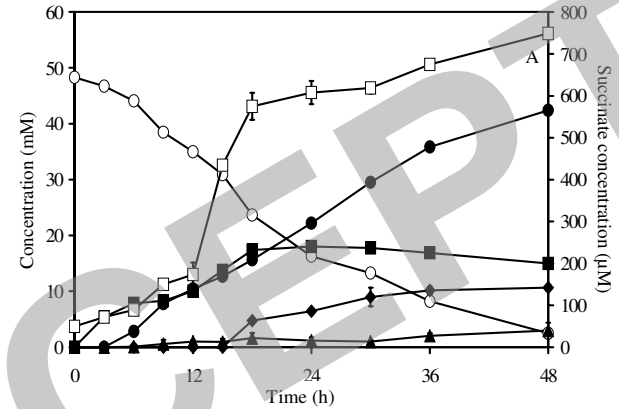
F2+GF
F+G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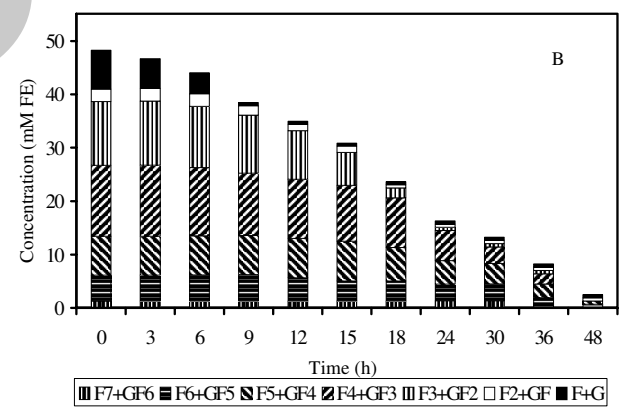
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Concentration (mM FE)
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