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PHYSIOLOGY AND BIOTECHNOLOGY

Mucin Cross-Feeding of Infant Bifidobacteria and *Eubacterium hallii*

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Abstract Mucus production is initiated before birth and provides mucin glycans to the infant gut microbiota. Bifidobacteria are the major bacterial group in the feces of vaginally delivered and breast milk-fed infants. Among the bifidobacteria, only *Bifidobacterium bifidum* is able to degrade mucin and to release monosaccharides which can be used by other gut microbes colonizing the infant gut. *Eubacterium hallii* is an early occurring commensal that produces butyrate and propionate from fermentation metabolites but that cannot degrade complex oligo- and polysaccharides. We aimed to demonstrate that mucin cross-feeding initiated by *B. bifidum* enables growth and metabolite formation of *E. hallii* leading to short-chain fatty acid (SCFA) formation. Growth and metabolite formation of co-cultures of *B. bifidum*, of *Bifidobacterium breve* or *Bifidobacterium infantis*, which use mucin-derived hexoses and fucose, and of *E. hallii* were determined. Growth of *E. hallii* in the presence of lactose and mucin monosaccharides was tested. In co-culture fermentations, the presence of *B. bifidum* enabled growth of the other strains. *B. bifidum*/*B. infantis* co-cultures yielded acetate, formate, and lactate while co-cultures of *B. bifidum* and *E. hallii* formed acetate, formate, and butyrate. In three-strain co-cultures, *B. bifidum*, *E. hallii*, and *B. breve* or *B. infantis* produced up to 16 mM acetate, 5 mM formate, and 4 mM butyrate. The formation of propionate (approximately 1 mM) indicated cross-feeding on fucose. Lactose, galactose, and GlcNAc were identified as substrates of *E. hallii.* This study shows that trophic interactions of bifidobacteria and *E. hallii* lead to the formation of acetate, butyrate, propionate, and formate, potentially contributing to intestinal SCFA formation with potential benefits for the host and for microbial colonization of the infant gut. The ratios of SCFA formed differed depending on the microbial species involved in mucin cross-feeding.

Keywords Mucin · *Eubacterium hallii* · Cross-feeding · *Bifidobacterium* · Propionate

Introduction

Mucin is produced by mucous and goblet cells of the gastrointestinal tract [1]. Production is initiated before birth, and a complete mucus layer has already developed several days after birth [2]. Human mucins are glycoproteins composed of a polypeptide backbone rich in tandem repeats of proline, serine, threonine, and *O*-glycosylated side chains [1]. Eight core structures of the mucin *O*-glycan chain have been identified [1], and the main monosaccharides are *N*-acetylgalactosamine, which *O*-links the glycan to the polypeptide, *N*-acetylglucosamine (GlcNAc), and galactose. The desoxyhexose L-fucose and *N*-acetylneuramic acid (sialic acid, NANA) are found at terminal positions. Mucins are constantly shed from the colon epithelium and are therefore a potential glycan source for the gut microbiota. However, mucin degradation requires several linkage-specific degradative enzymes; thus, only a low number of microbes are specialized to degrade mucin glycans [3].

It has been hypothesized that the presence of mucin-degrading specialists may play a role in early infant colonization,

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providing nutrients to other infant gut microbes before dietary fibers are introduced during weaning [4]. Bifidobacteria are the major bacterial group in feces of vaginally delivered and breast milk-fed infants [5]. Host-specific adaption in regard to carbohydrate degradation has been suggested [6, 7]. Infant species, such as *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) and *Bifidobacterium bifidum*, are adapted to utilize human milk oligosaccharides (HMOs), one of the major glycan sources supplied with breast milk [8–11]. The only *Bifidobacterium* species that can degrade and grow in the presence of mucin is *B. bifidum* [12–14]. *B. bifidum* degrades HMOs and mucin extracellularly through the activity of membrane-bound enzymes [15] enabling cross-feeding of other species. It was previously shown that *B. breve* grew using mono- and oligosaccharides released by *B. bifidum* from mucin glycans [16].

Bifidobacteria metabolize hexoses via the "bifid shunt" with fructose-6-phosphoketolase being the key enzyme to theoretically yield 1.5 mol acetate, 1 mol lactate, and 2.5 ATP from 1 mol glucose [17]. The ratios of lactate and acetate formed may vary with carbohydrate source and species, depending on whether the intermediate pyruvate is cleaved to acetyl phosphate and formate or reduced to lactate [18]. Strains of *B. infantis* and *B. breve* can metabolize L-fucose to acetate, formate, lactate, and 1,2-propanediol (1,2-PD) putatively using a pathway with non-phosphorylated intermediates as described for *Campylobacter* and *Xanthomonas* spp.: 1 mol of L-fucose thereby yields 1 mol of 1,2-PD [19, 20]. 1,2-PD is a precursor for bacterial propionate formation [21]. We could previously show that the commensal *Eubacterium hallii* uses 1,2-PD to form propionate [22].

E. hallii is a gut microbe that occurs in the first months after birth and reaches adult abundance levels at approximately 5–10 years of age independent of geographical donor origin [19]. *E. hallii* can use acetate and lactate, or glucose, to form butyrate. *E. hallii* is not able to utilize complex host- or diet-derived oligo- and polysaccharides [23], therefore relying on initial degraders for substrate supply. Accordingly, in co-cultures grown in the presence of the HMO fucosyllactose (2′- and 3′-fucosyllactose), *B. infantis* formed acetate, lactate, and 1,2-PD from fucosyllactose, which were used by *E. hallii* to produce butyrate and propionate [19]. The ability to cross-feed on HMOs could be one reason why *E. hallii* is present in the infant gut; however, this species could also profit from a mucin specialist releasing or fermenting mono- and disaccharides from mucin glycans. Mucin cross-feeding in the presence of *E. hallii* could finally lead to the formation of short-chain fatty acids (SCFA) butyrate and propionate [19]. Butyrate is a main energy source of colonocytes, impacts cell proliferation and differentiation, and lowers the risk of colitis and colorectal cancer [24]. Propionate acts as a precursor for gluconeogenesis in the liver and affects cell differentiation with potential health-promoting impact on intestinal inflammation and cancer development [21].

We therefore aimed to demonstrate that mucin-degrading *B. bifidum* enables growth of *E. hallii* and that *Bifidobacterium-E. hallii* cross-feeding on mucin monosaccharides and fermentation intermediates contributes to infant intestinal SCFA formation. We hypothesized that a mucus-degrading *B. bifidum* in co-culture with a fucose-utilizing *B. infantis*, or *B. breve* strain, and *E. hallii* would yield acetate, butyrate, and propionate. To verify this hypothesis, we incubated the strains in different combinations and monitored metabolite formation and growth. We also investigated whether *E. hallii* has the genomic potential to utilize lactose or mucin monosaccharides to ascertain to which extent the strain can cross-feed on sugars released by *B. bifidum*. Predictions made by genome analysis were confirmed in batch fermentations.

Methods

Strains and Culture Conditions

Bifidobacterium strains and *E. hallii* DSM 3353 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) or were previously isolated from stool samples of 6-month-old Kenyan infants (Table 1) [25].

To prepare the working cultures, bifidobacteria were streaked on Wilkins-Chalgren agar (Oxoid) supplied with soya peptone (5 g L^{-1}; Biolife), Tween 80 (1 mL L^{-1}, Sigma-Aldrich), and fresh filter-sterilized L-cysteine-HCl (0.5 g L^{-1}, Sigma-Aldrich) (WCSP). Single colonies were picked and were grown in liquid WCSP at 37 °C for 24 h. Stock cultures of bifidobacteria were prepared from overnight growth culture in phosphate buffer (1.2 g L^{-1} K_2HPO_4, 0.333 g L^{-1} KH_2PO_4, 0.5 g L^{-1} L-cysteine-HCl) and 60% glycerol in a ratio of 1:2 and were maintained at −80 °C.

Mucin-dependent growth of bifidobacteria was investigated in API 50 CHL Medium (10 g L^{-1} bovine/porcine origin polypeptone, 5 g L^{-1} yeast extract, 1 mL^{-1} Tween 80, 2 g L^{-1} dipotassium phosphate, 5 g L^{-1} sodium acetate, 2 g L^{-1} diammonium citrate, 0.2 g L^{-1} magnesium sulfate heptahydrate, 0.05 g L^{-1} manganese sulfate monohydrate, 0.17 g L^{-1} bromocresol purple) according to the manufacturer's composition (bioMérieux). Porcine mucin (type III; bound sialic acid 0.5–1.5%, Sigma-Aldrich) was added at 1 g L^{-1} final concentration. The pH was adjusted to 7.5 to give a final pH of 7 after autoclaving at 121 °C for 15 min. Glucose (Sigma-Aldrich) was used as a control carbohydrate source to verify that the strains grew under the assay conditions.

E. hallii DSM 3353 was routinely cultivated in modified YCFA medium (mYCFA_glc) containing 35 mM acetate and 35 mM glucose as described before (Table 2) [19, 26]. All components except L-cysteine-HCl were solubilized in deionized water, and the pH was adjusted to 7.6 with NaOH. The medium was flushed with CO_2 and boiled. When the color

Table 1 *Bifidobacterium* strains used in this study

Species	Strain code	Origin	Growth with mucin
B. bifidum	DSM 20456	Stool of breast-fed infant	+
	BRS26-2	Kenyan infant stool, 6 months old	+
	BSM2-3	Kenyan infant stool, 6 months old	+
	BRS-300	Kenyan infant stool, 6 months old	+
	BRS27-3	Kenyan infant stool, 6 months old	+
	BSM28-1	Kenyan infant stool, 6 months old	+
	TPY6-2	Kenyan infant stool, 6 months old	+
	DSM 20082	Intestine of adult	+
	DSM 20215	Intestine of adult	+
	DSM 20239	Stool of breast-fed infant	+
B. breve	DSM 20213	Intestine of infant	−
	TPY10-1	Kenyan infant stool, 6 months old	−
	TPY5-1	Kenyan infant stool, 6 months old	−
	BRS 26-2	Kenyan infant stool, 6 months old	−
B. infantis	DSM 20088	Intestine of infant	−
	BRS8-2	Kenyan infant stool, 6 months old	−
	TPY12-1	Kenyan infant stool, 6 months old	−
	BRS8-1	Kenyan infant stool, 6 months old	−
	TPY8-1	Kenyan infant stool, 6 months old	−
	BSM12-2x	Kenyan infant stool, 6 months old	−
B. longum subsp. *longum*	DSM 20219	Intestine of adult	−
B. longum subsp. *suis*	BSM11-5	Kenyan infant stool, 6 months old	−
B. pseudolongum subsp. *globosum*	DSM 20092	Rumen	−
	PV8-2	Kenyan infant stool, 6 months old	−
	BSM8-1	Kenyan infant stool, 6 months old	−
B. kashiwanohense	DSM 21854	Japanese infant stool, 1.5 years old	−
	PV20-2	Kenyan infant stool, 6 months old	−
	TPY11-1	Kenyan infant stool, 6 months old	−
	BSM11-1	Kenyan infant stool, 6 months old	−

Strains from Kenyan infants were isolated in a previous study [25]. Growth in the presence of mucin (1%) was determined using API medium

changed from blue to pink, L-cysteine-HCl was added. The medium (10 mL) was transferred to Hungate tubes flushed with CO_2, and the tubes were sealed and autoclaved. Stab cultures of *E. hallii* that were frozen at −20 °C in mYCFA agar (1.5% (*w*/*v*) agar) were used as stock cultures. For each experiment, a fresh agar stock was thawed; 1 mL of liquid YCFA_glc was added and thoroughly shaken before being transferred to 10 mL mYCFA_glc. After incubation at 37 °C for 24 h, the culture was transferred at least once to fresh mYCFA_glc before the experiment. For single and co-culture experiments in the presence of mucin, mYCFA contained 0.75% mucin instead of glucose (mYCFA_muc). For *E. hallii* growth studies in the presence of mucin monosaccharides, mYCFA_glc or mYCFA supplied with 49 mM galactose (mYCFA_gal), 50 mM lactose (mYCFA_lac), 33 mM GlcNAc (mYCFA_glcnac), or 40 mM NANA (mYCFA_nana) was prepared. All carbohydrates were obtained from Sigma-Aldrich with the exception of NANA, which was supplied by Glycom A/S.

Growth of Bifidobacteria in API Medium in the Presence of Mucin

Overnight cultures were centrifuged at 5000 rpm for 5 min, washed, and resuspended in equal volume of 50 mM phosphate buffer, pH 6.5, prior to inoculation of the glucose- or mucin-supplemented API medium. Growth was evaluated on 96-well microtiter plates in triplicate that were incubated in anaerobic jars. Strain suspensions (20 μL) were added to 180 μL API medium containing sterile-filtered L-cysteine-HCl (0.5 g L^{-1}) and were incubated at 37 °C for 48 h. API medium contained bromocresol purple as a pH indicator. A

Table 2 mYCFA medium composition

Component	Addition
Amicase	1% (*w*/*v*)
Yeast extract	0.25% (*w*/*v*)
Sodium bicarbonate	0.5% (*w*/*v*)
Glucose or mucin	1 or 0.75% (*w*/*v*)
Mineral solution (0.3% (*w*/*v*) potassium dihydrogen phosphate, 0.6% (*w*/*v*) sodium chloride, 0.06% (*w*/*v*) magnesium sulfate, 0.06% calcium chloride (*w*/*v*))	15% (*v*/*v*)
Vitamin solution (0.01% (*w*/*v*) biotin, 0.01 (*w*/*v*) cobalamin, 0.03% *p*-aminobenzoic acid (*w*/*v*), 0.05% folic acid (*w*/*v*), 0.15% pyridoxamine (*w*/*v*))	0.1% (*v*/*v*)
Volatile fatty acid mix (56.6% (*v*/*v*) acetic acid, 20% (*v*/*v*) butyric acid, 13.3% (*v*/*v*) propionic acid)	0.58% (*v*/*v*)
Hemin (50 g L^{-1})	0.02% (*v*/*v*)
Resazurin (1 mg L^{-1})	0.1% (*v*/*v*)
L-Cysteine hydrochloride monohydrate	0.1% (*w*/*v*)

Modified from [26]

decrease of pH due to the formation of acetate, formate, and lactate led to a color change from purple to yellow indicating growth and carbohydrate utilization. All strains grew in the presence of glucose, confirming the suitability of the assay.

Single and Co-culture Growth in the Presence of Mucin

For preparing co-culture experiments, 100–200 μL of either *B. infantis* or *B. breve* overnight culture grown liquid WCSP was added to 10 mL mYCFA_glc, while *B. bifidum* was subcultured in mYCFA_muc, and the cultures were incubated at 37 °C for 24 h. To initiate co-culture experiments, Hungate tubes containing 10 mL mYCFA_muc were inoculated with approximately log 7.7 cells mL^{-1} of *E. hallii* or *B. infantis*, approximately log 6.8 cells mL^{-1} of *B. breve*, or log 8.0 cells mL^{-1} of *B. bifidum*. Cell counts were determined using qPCR as described below. For comparison, strains were also grown in single cultures or in two-strain combinations. Samples were taken after 0, 4, 8, and 24 h of incubation for sugar and metabolite analyses and for DNA isolation. Growth was investigated in independent triplicates or quadruplicates.

Growth of *E. hallii* in mYCFA Supplied with Mucin Monosaccharides

Hungate tubes containing 10 mL mYCFA_glc, mYCFA_gal, mYCFA_lac, mYCFA_glcnac, or mYCFA_nana were inoculated with 100-μL overnight cultures of *E. hallii*. Optical density at 600 nm was monitored after 0, 3, 6, 8, and 24 h. Supernatants were collected after 24 h of incubation for substrate and metabolite analyses. Growth was investigated in independent quadruplicates.

Analysis of Substrate Utilization and Metabolite Formation

L-Fucose release and the formation of lactate and acetate were measured using high-performance liquid chromatography (Merck-Hitachi) equipped with an Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad) and a refractive index detector (HPLC-RI). Samples were centrifuged at 13,000×*g* for 5 min at 4 °C. Supernatants (40 μL injection volume) were eluted with 10 mM H_2SO_4 at a flow rate of 0.6 mL min^{-1} at 40 °C. Sugars, SCFAs, 1,2-PD, and lactate were quantified using external standards (all Sigma-Aldrich).

Propionate, butyrate, and formate were quantified by ion chromatography with suppressed conductivity detection on an ICS-5000$^+$ system (Thermo Scientific) using external standards. Analytes were separated on a IonPac AS11-HC 4 × 250-mm column supplemented with a guard column (Thermo Scientific) that was operated at 30 °C and at 1.5 mL min^{-1} using the following gradient conditions: 1.5 mM KOH, 0–6 min; 1.5–35 mM KOH, 6–21 min; 35–60 mM KOH, 21–26 min; 60 mM KOH, 26–27 min; and 60–1.5 mM KOH, 27–28 min, followed by appropriate reequilibration. The injection volume was 10 μL.

Gel Permeation Chromatography with RI Detection to Determine the Utilization of Mucin

The degradation of mucin of selected *B. bifidum* strains and one non-mucin-degrading *B. breve* BRS 26-2 during growth in API medium was analyzed using a Superdex 200 10/300 GL column (GE Healthcare Europe, GmbH) and 1 M NaCl as eluent at a flow rate of 0.4 mL min^{-1}. Polymers were detected with a RI detector. A dextran

analytical standard (dextran standard 12,000; 50,000; 150,000; 410,000; 670,000; and 2,000,000 Da from *Leuconostoc mesenteroides*, Sigma-Aldrich) was used to estimate mucin polymer size.

DNA Isolation and Quantification of *E. hallii* and Bifidobacteria in Co-culture Studies

Genomic DNA was isolated from 0.5 mL fermented mYCFA_muc using the FastDNA SPIN Kit for Soil (MP Biomedicals) according to the manufacturer's instructions. The abundance of *E. hallii* 16S ribosomal RNA (rRNA) genes was determined using primers EhalF (5′-GCGT AGGTGGCAGTGCAA-3′) and EhalR (5′-GCAC CGRAGCCTATACGG-3′) [27]. *B. bifidum* 16S rRNA genes were quantified using primers BiBIF-1 (5′-CCAC ATGATCGCATGTGATTG-3′) and BiBIF-2 (5′-CCGA AGGCTTGCTCCCAAA-3′). *B. breve* 16S rRNA genes were quantified using primers BiBRE-1 (5′-CCGGATGCTCCATC ACAC-3′) and BiBRE-2 (5′-ACAAAGTGCCTTGCTCCCT-3) [28], and a putative α-glucosidase gene of *B. infantis* (*glyc*) was quantified using primers BBMN68_650-F (5′-CGTA CGTCCGAAGTTCCCCG-3) and BBMN68_650-R (5′-CACGGTCAGGGAATGCTGGG-3′) [29]. Primer specificity was confirmed (Table S1). Reactions were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems) and the Kapa SYBR FAST qPCR Master Mix (Biolab Scientific Instruments, SA). Thermal cycling started with an initial denaturation step at 95 °C for 3 min, followed by 40 cycles consisting of denaturation at 95 °C for 3 s, annealing at 60 °C for 10 s, and elongation at 72 °C for 25 s. To verify the specificity of the amplification, melting curve analysis was performed. Standard curves were prepared from 10-fold dilutions of linearized plasmids harboring the target gene of interest [30]. Linear detection range was between log 2.3 and log 8.3 gene copies for *E. hallii* 16S rRNA genes, between log 4.0 and log 9.0 gene copies for *B. breve* 16S rRNA genes, between log 2.6 and log 8.6 gene copies for *B. bifidum* 16S rRNA genes, and between log 4.0 and log 9.0 gene copies for *B. infantis glyc*. Log gene copies were corrected for multiple copies of 16S rRNA genes (*Eubacterium* spp. (n = 5.5), *B. breve* (n = 2), and *B. bifidum* (n = 3) [31, 32]) to calculate the numbers of cells of each strain.

Genetic Capacity of *E. hallii* for Mucin Monosaccharide Utilization

To identify mucin monosaccharide utilization pathways, the genome of *E. hallii* DSM (SAMN02415618) was obtained from the NCBI database and functional assignment was automatically performed using the RAST [33].

Statistical Analysis

Student's paired t test with two-tailed distribution was used to identify significant differences in metabolite formation between treatments. A p value of <0.05 was considered significant.

Results

Bifidobacteria Growth and Mucin Degradation in API Medium

In the course of a previous study, several *Bifidobacterium* spp. were isolated (Table 1) [25]. We first assessed the growth capacity of these strains and of strains provided by microbial culture collections in the presence of mucin as a sole carbohydrate source in API medium. All *B. bifidum* isolates were able to grow in the presence of mucin while the remaining strains belonging to other species were not (Table 1). Mucin degradation by *B. bifidum* was confirmed by gel permeation chromatography (GPC). Mucin consisted of two polymer fractions (Fig. 1). The high molecular weight fraction was eluting at the upper limit of the column indicating a size of $>2.10^6$ Da; the lower molecular weight fraction consisted of polymers ranging from 10^4 to 10^5 Da. Strains of *B. bifidum* almost completely degraded the high molecular weight fraction (Fig. 1). For selected samples representing at least one isolate of the investigated species, metabolite formation was determined after 48-h growth in API medium with mucin

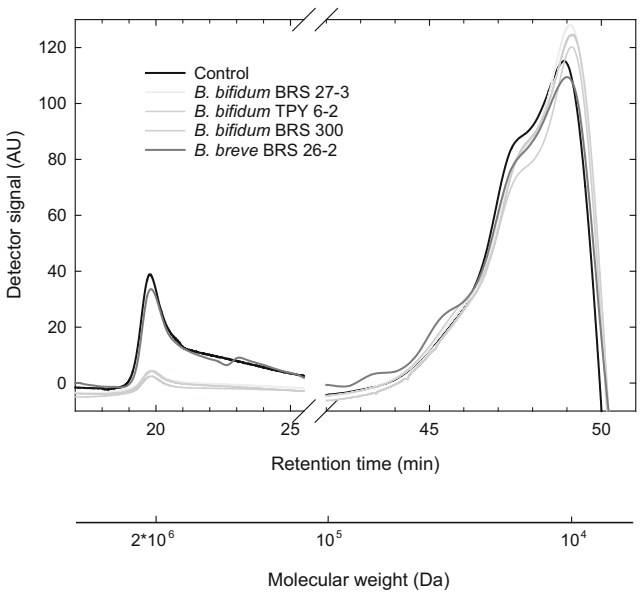

Fig. 1 Mucin utilization by *B. bifidum* and *B. breve* strains during growth in API medium. Shown are representative mucin degradation profiles during growth in API medium supplied with 1% mucin. Mucin profiles were determined using GPC-RI. Profiles looked similar for all *B. bifidum* strains investigated

(Table 3). *B. bifidum* isolates produced acetate (18.9–25.7 mM), formate (2.9–5.9 mM), and lactate (0–3.3 mM), while one strain of *B. breve* formed low amounts of acetate (2.8 mM). All *B. bifidum* strains released fucose (3.2–3.7 mM).

Metabolite production was not detected in cultures of *B. infantis*, *B. breve*, *B. longum* subsp. *longum*, *B. kashiwanohense*, and *B. pseudolongum* subsp. *globosum* which were not able to utilize mucin.

Single and Co-culture Fermentations

Single and co-culture fermentations were conducted in mYCFA_muc in Hungate tubes using *B. bifidum* BSM28-1, *B. infantis* DSM 20088, *B. breve* DSM 20213, and *E. hallii* DSM 3353.

In single culture, *B. infantis*, *B. breve*, and *E. hallii* did not grow, and no metabolites were detected after 24-h incubation. In contrast, *B. bifidum* increased by 1.5 log cells (Fig. 2a) and produced mainly acetate (14.0 ± 2.0 mM) and low amounts of lactate (1.3 ± 0.5 mM) and formate (3.8 ± 0.7 mM) and released 1.5 ± 0.5 mM fucose (Fig. 2d), confirming the results obtained using API medium.

We then performed two-strain co-cultures of *B. bifidum*, *B. infantis*, and *E. hallii* in different combinations. Growth and metabolite formation were only observed for co-cultures with *B. bifidum* (Fig. 2e, f), *B. infantis* and *E. hallii* increasing 12.6- and 10.7-fold, respectively (Fig. 2a, b). In addition, co-cultivation of *B. bifidum*/*B. infantis* produced significantly ($p < 0.05$) more acetate than *B. bifidum* alone (19.9 ± 2.0 versus 14.0 ± 2.0 mM) (Fig. 2d, e). These results suggest cross-feeding of mucin components as observed before [16]. Acetate formation was also significantly higher ($p < 0.05$) in *B. bifidum*/*B. infantis* co-cultures compared to *B. bifidum*/*E. hallii* co-cultures (12.6 ± 2.0 mM). Lactate was only present in *B. bifidum*/*B. infantis* co-cultures (1.2 ± 0.3 mM) while *B. bifidum*/*E. hallii* co-cultures produced butyrate (3.8 ± 0.5 mM) (Fig. 2e, f). On average, 5.6 ± 1.5 and 3.8 ± 1.5 mM formate was produced by *B. bifidum*/*B. infantis* and *B. bifidum*/*E. hallii* co-cultures, respectively. *B. infantis* utilized the fucose released by *B. bifidum*; 1,2-PD formation and propionate were not observed (data not shown).

To investigate whether co-cultures of infant bifidobacteria and *E. hallii* would yield propionate during mucin degradation, we then conducted three-strain fermentations: *B. bifidum* and *E. hallii* were inoculated together with *B. infantis* or with *B. breve*. All strains grew in three-strain fermentations (Fig. 3a, b). When inoculated together, *B. bifidum*, *E. hallii*, and *B. infantis* cell counts increased 8.7-, 4.2-, and 5.9-fold, respectively, while there was a 7.7-, 8.7-, and 2.6-fold increase when *B. bifidum*, *E. hallii*, and *B. breve* grew in co-culture, respectively. Formation of acetate (2.5–5.6 mM), lactate (1.3–1.8 mM), and the release of fucose (1.0–1.2 mM) was observed after 4 h of incubation (Fig. 3c, d). Lactate and fucose were not detected after 8 h of incubation when butyrate and formate appeared. Final acetate levels for both three-strain fermentations were similar (15.7 ± 1.5 and 15.6 ± 1.8 mM) and were significantly ($p < 0.05$) lower compared to acetate produced by *B. bifidum*/*B. infantis* co-cultures. Final butyrate and formate concentrations were 3.2–3.6 and 4.3–4.9 mM (Fig. 3c, d); propionate (0.8–1.4 mM) was formed by both three-strain co-cultures (Fig. 3c, d), indicating cross-feeding on fucose.

Table 3 Metabolite formation and release of lactate and acetate formation and release of fucose during growth of selected *Bifidobacterium* isolates in API medium supplied with 1% mucin

Species	Strains	Fucose released (mM)	Metabolite formed (mM)		
			Formate	Lactate	Acetate
B. bifidum	BSM2-3	3.2	3.9	0	19.9
	BRS27-3	3.5	5.6	3.3	25.7
	DSM 20082	3.7	5.4	1.5	22.3
	DSM 20215	3.5	2.9	2.5	18.9
B. breve	DSM 20213	0	0	0	2.8
	BSM1-2	0	0	0	0
B. infantis	DSM 20088	0	0	0	0
	BRS8-2	0	0	0	0
	TPY12-1	0	0	0	0
	BSM12-2x	0	0	0	0
B. longum subsp. *longum*	DSM 20213	0	0	0	0
B. kashiwanohense	DSM 21854	0	0	0	0
B. pseudolongum subsp. *globosum*	BSM8-1	0	0	0	0

Shown are mean values of two independent experiments

Genetic Capacity of *E. hallii* to Degrade Mucin Monosaccharides

To identify the genetic capacity of *E. hallii* to utilize monosaccharides present in mucin glycans, the genome of *E. hallii* DSM 3353 was obtained from the NCBI database and functional assignment was performed using the RAST platform. *E. hallii* harbor genes encoding enzymes of the Leloir pathway for galactose to glucose L-phosphate transformation [34]: galactokinase *galK*, galactose-L-phosphate uridylyltransferase *galT*, and UDP-glucose-epimerase *galE*, which were scattered across the genome. *E. hallii* also possess a gene encoding a β-galactosidase for hydrolysis of lactose. *E. hallii* harbor a gene potentially encoding a β-hexosaminidase and genes *nagA* and *nagB* encoding a GlcNAc-6P deacetylase and a GlcN-6P deaminase,

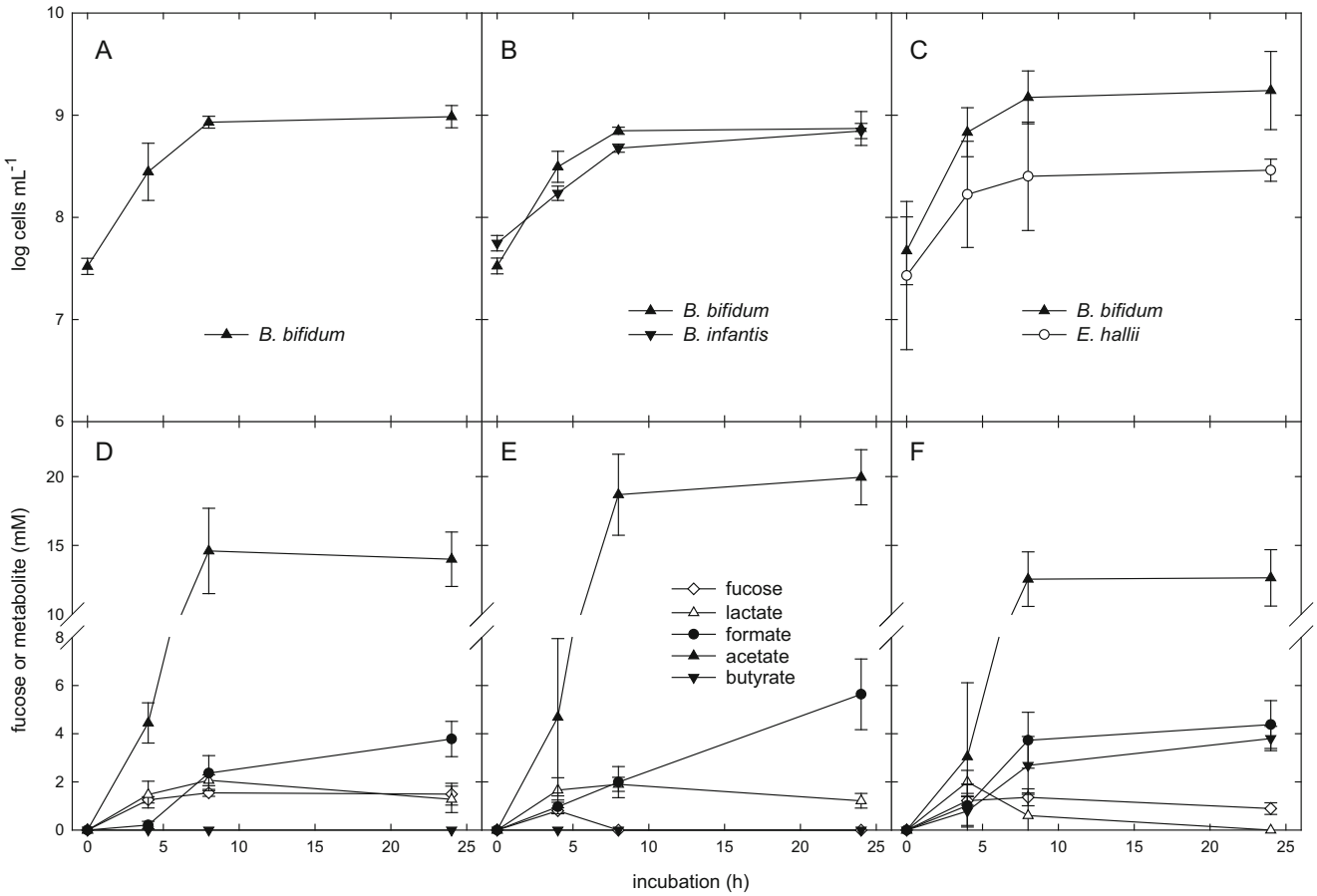

Fig. 2 Growth, release of fucose, and metabolite formation of *B. bifidum* or two-strain co-cultures. Cell counts and metabolite formation by *B. bifidum* (**a**, **d**), *B. bifidum*/*B. infantis* (**b**, **e**), and *B. bifidum*/*E. hallii* co-cultures (**c**, **f**) during growth in mYCFA_muc. Cell counts (**a**–**c**) were determined using qPCR; the release of fucose and the formation of metabolites (**d**–**f**) were monitored using HPLC-RI and IC-PAD. Metabolite production was not observed in the *B. infantis* co-culture/*E. hallii* co-culture (data not shown). Shown are results from three to four independent experiments

respectively, which yield fructose-6-phosphate from GlcNAc-6-phosphate [35]. *NagE* encoding *N*-acetylhexosamine L-kinase that would initially phosphorylate GlcNAc was not detected [35]. *E. hallii* does not possess genes encoding enzymes for *N*-acetylgalactosamine, NANA, and fucose utilization.

E. hallii Utilization of Mucin Monosaccharides

We then investigated whether *E. hallii* was indeed able to utilize monosaccharide components released by *B. bifidum* from mucin. *E. hallii* was incubated in mYCFA supplied with glucose, galactose, lactose, GlcNAc, and NANA, and growth was monitored by OD measurement for 24 h. As predicted by genome analysis, *E. hallii* grew in the presence of glucose, galactose, lactose, and GlcNAc, and growth was fastest with glucose (Fig. S1). There was no growth observed in the presence of NANA. With glucose, approximately 1.1 mol of butyrate and 0.7 mol formate were formed from 1 mol glucose and 0.4 mol acetate (Table 4). These proportions were slightly shifted when *E. hallii* was inoculated in mYCFA_gal; the utilization of 1 mol galactose and 0.4 mol acetate led to the formation of 0.3 mol formate and 0.7 mol butyrate. When grown in the presence of lactose, *E. hallii* consumed and produced equimolar amounts of acetate and formate, respectively, and released 25.8 ± 2.4 mM butyrate. GluNAc was completely utilized, yielding similar amounts of formate and butyrate as from glucose; however, acetate accumulated (13.7 ± 1.9 mM) (Table 4).

Discussion

Only selected taxa of intestinal microorganisms have been identified as mucin utilizers, including *Bacteroides* spp., *Akkermansia muciniphila*, *Ruminococcus torques*, and *Ruminococcus gnavus*, beside *B. bifidum* [3]. We confirmed here that growth on mucin is an intrinsic trait of the species *B. bifidum* [12, 13] and that, among the infant *Bifidobacterium* species tested, only the presence of *B. bifidum* enabled growth of other bifidobacteria or of *E. hallii* due to the release of monosaccharides and/or

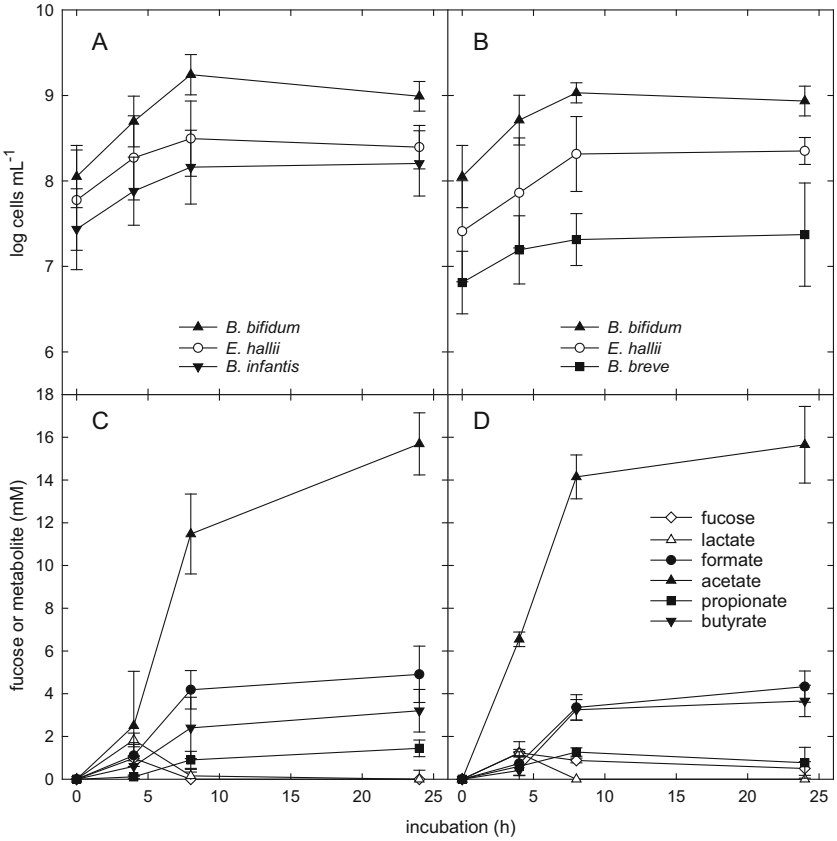

Fig. 3 Growth, release of fucose, and metabolite formation of three-strain co-cultures. Cell counts and metabolite formation by *B. bifidum*/*E. hallii*/*B. infantis* (**a**, **c**) and *B. bifidum*/*E. hallii*/*B. breve* co-cultures (**b**, **d**) during growth in mYCFA_muc. Cell counts (**a**, **b**) were determined using qPCR; the release of fucose and the formation of metabolites (**c**, **d**) were monitored using HPLC-RI and IC-PAD. Shown are results from three to four independent experiments

to the formation of lactate and acetate from mucin. The genomes of *B. bifidum* harbor several α-fucosidases, α-sialidases, endo-α-*N*-acetylgalactosaminidase, lacto-*N*-biosidase, β-galactosidases, and *N*-acetyl-β-hexosaminidases with varying copies between isolates [13, 36]. Transcriptomic and proteomic analyses have verified that several of these genes are expressed and the corresponding proteins produced during growth in the presence of mucin [13, 37].

Bifidobacteria are the major bacterial group in the infant gut [5] and provide, through their ability to degrade the two major glycan sources, HMOs and glycan, nutrients to other gut microbes such as *E. hallii*. *E. hallii* is one of the first butyrate producers colonizing the infant gut [19, 38]. *E. hallii* does not degrade complex glycans [23], but can use lactate or glucose, and acetate, to form butyrate and 1,2-PD to produce propionate. We confirmed our hypothesis that *E. hallii* cross-feeding on mucin components or mucin-derived metabolites yields butyrate and propionate. The amount of propionate formed (0.8–1.4 mM) was in the expected range as *B. bifidum* released between 1.1 and 2 mM fucose, enabling *B. bifidum* and *B. breve* to produce equimolar amounts of 1,2-PD [19]. From 1 mol 1,2-PD, *E. hallii* can form 1 mol propionate [19]. It is possible that the interaction of *E. hallii* with bifidobacteria communities first colonizing the infant gut could play a role in the transition of an infant's to an adult's gut microbiota. *E. hallii* and bifidobacteria cross-feeding improves environmental conditions for microbes such as *Faecalibacterium*, *Coprococcus*, and *Roseburia*, which rely on the presence of SCFAs for growth [39].

We also identified lactose, galactose, and GlcNAc as additional carbon sources of *E. hallii.* Amino sugars such as

Table 4 *E. hallii* utilization of mucin monosaccharides and metabolite formation

	Carbohydrate used (mM)	Metabolites used and formed (mM)		
		Formate	Acetate	Butyrate
Glucose	−31.8 ± 3.4	21.0 ± 0.9	−13.1 ± 0.6	34.7 ± 1.9
Galactose	−39.9 ± 3.2	13.1 ± 1.1	−17.1 ± 0.7	31.3 ± 0.8
Lactose	−17.2 ± 1.0	15.4 ± 1.3	−15.3 ± 2.8	25.8 ± 2.4
GlcNAc	−32.9 ± 0	25.0 ± 2.4	13.7 ± 1.9	32.9 ± 0.9

E. hallii was grown in mYCFA supplied with glucose, galactose, lactose, and GlcNAc ($n = 4$)

GlcNAc are abundant in the intestinal environment originating from mucin and from bacterial cell wall peptidoglycan. GlcNAc has also been linked to bacterial sensing and the regulation of virulence factors [40]. Pathways for the metabolism of amino sugars are conserved in bacteria, and it has been predicted that members of all major intestinal phyla (*Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, and *Proteobacteria*) can use GlcNAc as a carbon source [41]. During growth in the presence of GlcNAc, lactic acid bacteria release acetate and use the glucose moiety via the Embden-Meyerhof pathway [42]. Similarly, *E. hallii* released acetate while forming butyrate and formate from the glucose moiety.

In mucin-grown *B. bifidum* co-cultures, *E. hallii* used lactate and acetate to produce butyrate, but *E. hallii* could also have used galactose or GlcNAc released from the mucin glycans. Propionate was only produced if a fucose-utilizing, 1,2-PD-releasing strain (here, *B. infantis* or *B. breve*) was present. When grown with *B. infantis* in the presence of fucosyllactose, *E. hallii* produced butyrate from lactate, glucose or galactose, and acetate and propionate from 1,2-PD [19]. Further substrate- and co-species-dependent cross-feeding routes of *E. hallii* were observed before. In the presence of starch, *E. hallii* formed butyrate from lactate and acetate produced by *Bifidobacterium adolescentis* while with fructo-oligosaccharides or inulin, *E. hallii* also used mono- or disaccharides released by the bifidobacteria [43, 44]. *E. hallii* therefore possesses a versatile substrate spectrum, including mono- and disaccharides, as well as intestinal fermentation intermediates, which might explain its competitiveness and presence in infant and also in adult gut microbiota [19].

In adults, gut microbial dietary and host-derived glycan utilization mainly yields acetate, propionate, and butyrate. Acetate is a final fermentation metabolite for most gut microbes whereas butyrate and propionate are formed by only some species [45]. Until now, the contribution of mucin to gut microbial metabolism in infants has received little attention in contrast to the role of HMOs as glycan source. We observed here that acetate was the main metabolite derived from *Bifidobacterium* mucin degradation and cross-feeding and that butyrate and propionate were formed when *E. hallii* is present. The major proportion of acetate over the other SCFA is a feature generally observed in studies analyzing SCFAs in infant feces [38]. In a Swiss cohort study following infants from 2 weeks to 6 months, 35–55 mM acetate was detected on average and propionate and butyrate levels were initially low (<5 mM at 2 weeks) and increased with age (15 mM propionate and 5 mM butyrate at 6 months) [38]. As the strains investigated here are part of the infant microbiome [19], it is possible that microbial mucin fermentation already occurs at infant age, contributing to intestinal SCFA formation. The utilization of mucin as an alternative glycan source to HMOs might depend on infant diet, as a study in the early 1990s showed that the development of a mucin-degrading microbial community was significantly delayed in breast-fed compared to formula-fed infants [46].

Both bifidobacteria and *E. hallii* were able to produce formate during the degradation of mucin and fucosyllactose [19]; however, formate is rarely detected in infant feces [38]. Formate together with CO_2 can be used by acetogenic microbes, such as *Blautia* or *Marvinbryantia* spp. via the Wood-Ljungdahl pathway [47] or by methanogens [48]. To investigate whether *Blautia* and *Marvinbryantia* occur in infant gut microbiomes, we reanalyzed a previously generated dataset [19, 49] and found that *Blautia* and *Marvinbryantia* occurred during the first months of life (*Blautia* 0.01–0.1%, *Marvinbryantia* 0.001–0.00001% relative abundance) and reached adult levels within 1 year (*Blautia* 1–10%, *Marvinbryantia* 0.005–0.5%) in fecal 16S rRNA gene libraries from the USA, Venezuela, and Malawi (Fig. S2). In contrast, methanogens appear at child age [19, 30]. In general, little is known about intestinal formate cross-feeding [50].

In summary, we demonstrate that mucin degradation by *B. bifidum* enabled growth of the metabolic versatile *E. hallii.* Trophic interactions of bifidobacteria and *E. hallii* led to the formation of acetate, butyrate, propionate, and formate potentially contributing to intestinal SCFA formation with potential benefits for the host and microbial colonization for the infant gut. The ratios of SCFA formed differed depending on microbial species involved in mucin cross-feeding.

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