Selective induction of human gut-associated acetogenic/butyrogenic microbiota based on specific microbial colonization of indigestible starch granules

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INTRODUCTION
The intestinal microbiota contributes to many aspects of host health and diseases via bioactive metabolites including short-chain fatty acids (SCFAs). Diet is a rapid and powerful tool for modulating human microbiota and their metabolism [1–3]. A specific set of gut bacteria expands upon taking up each food, including indigestible polysaccharides, in some cases after degradation by host or microbial enzymes [4–7]. Direct utilization of each nutrient in the intestine by specific human gut-associated bacteria has been demonstrated using isolated strains and recently predicted from genomic data. However, it is still generally impossible to predict individualized responses to dietary intervention, and this will be the biggest limitation in the application of dietary intervention. To overcome this, it is necessary to thoroughly understand the nutritional ecology of gut bacteria, including specific competitions for each nutrient, and key behaviors that make bacteria more competitive in situ and enable them to take up nutrients before competitors consume them. A recent study showed a conceptual example of interspecies competition for a nutrient by using gnotobiotic mice [8], however, competitions and key behaviors are largely unidentified in vivo or in situ by analyzing normal human intestinal microbiota. To address this, we focused on the distribution of bacteria around food structures in human feces. We show specific bacterial colonization of a specific type of starch in human fecal sections, and also show the impact of the major colonizing species on the response of microbiota to a nutrient that is competed for by multiple gut bacterial species. We found that efficient utilization of starch granules was nearly monopolized by the major colonizing species Bifidobacterium adolescentis, although another colonizing species, Eubacterium rectale, also can bind and respond to the granules. The major gut fermentation product in each subject was determined by which of the two species responded and especially by the presence of B. adolescentis.

MATERIALS AND METHODS
Human studies and ethical approval
All human studies were conducted in accordance with the Declaration of Helsinki. The studies were scientifically and ethically reviewed and were approved by the Yakult Central Institute. Written informed consent was obtained from the participants after explanation of the study (aims, measurements, possible risks and consequences) before enrollment.

Experiment I. Five healthy male adults (age, 28–53 years) who had not taken antibiotics for at least one week prior to the day of the first sampling participated. Fecal specimens were collected twice with a 2-week interval. Subjects were asked to avoid overeating or overdrinking during the period. Contents of meals for 2 days before sampling were surveyed.

Experiment II. Ten healthy male adults (age, 29–56 years) were recruited. This experiment was designed according to a previous study [9]. After 10 days of pre-intake period, the participants were asked to consume raw...
potato starch (hereafter referred as pSt, unmodified potato starch, Bob's Red Mill, Milwaukee, OR, USA) as follows: on the first day, one serving of 12 g; on the second day, one serving of 24 g; and then 48 g/day divided into equal 2 servings for 12 days. Fecal samples were collected four times between the 3rd and 10th days of the pre-intake period and four times between the 4th and 14th days of the pSt intake period, with at least one-day interval between sampling in most cases. Intake of banana, any raw Japanese yam (other than that provided by the researcher), or raw Japanese potato starch was prohibited throughout the experiment to avoid introduction of large amounts of granular resistant starch to the intestine.

Experiment III. Ten healthy male adults (age, 28–54 years) were recruited. After 9 days of the pre-intake period, they were asked to take 40 g/day of raw Japanese yam (mix of Dioscorea japonica and D. polyestachya, contains approx. 14% [w/w] of granular starch, Maruko Foods, Saitama, Japan) at lunch time for 4 days, followed by 8 days of the post-intake period (starchy foods are rarely consumed raw, but Japanese yam is an exceptionally common raw starchy food in Japan; after examining meal logs, it was the only candidate source of B. adolescentis-colonized starch granules in Experiment I). Six fecal specimens were collected in total from each subject (two for each period). Intake of banana and additional raw Japanese yam (other than that provided by the researcher) was prohibited during all periods of this experiment. Two subjects (F and G) were recruited again after the experiment, another informed consent was obtained, and fecal material was collected once for isolation of dominant bacteria. For this sampling, food intake was not restricted.

Analysis of fecal sections

Fecal sections were prepared as described by Swidsinski et al. [10]. A fraction of fresh feces was sampled by subjects by puncturing it with a plastic straw (diameter 6 mm; length of the fecal specimen ~1 cm). The sample was kept cold using a cold pack (for up to 4 h) and plastic straw (diameter 6 mm; length of the fecal specimen ~1 cm). The ISME Journal (2022) 16:1502 – 1511

Microbial profiling

Microbial profiling based on 16 S rRNA gene sequences was performed as previously described [13]. DNA was extracted from 20 mg of feces by bead–phenol method [14] and dissolved in 1 ml of TE buffer. The V1–V2 region of the 16 S rRNA gene was amplified by PCR using DNA (1 µl) as a template, TB Green Premix Ex Taq II (Tli RNaseH Plus) (Takara Bio, Shiga, Japan), and primers (27Fmod2-MiSeq and 338R-MiSeq, Supplementary Table 1). To prevent erroneous amplification, the reaction was stopped when the TB Green signal was close to saturation. The products were purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA) and all samples were pooled to make a library. The library was sequenced using a MiSeq Reagent Kit v2 (500 cycles) and a MiSeq instrument (Illumina, San Diego, CA, USA). Raw sequence data were deposited in DDBJ DRA under accession number PRJDB11235.

Measurement of SCFAs

SCFAs were measured as previously described [6]. Fecal samples were suspended in a 9-fold volume of PBS, and an aliquot of the suspension was mixed with one-ninth volume of 10% perchloric acid. The mixture was tightly sealed and kept at 4 °C until analysis. On the day of analysis, the samples were filtered through Centricut Ultramini filters (WW-MO-045, Kumasawa, Osaka, Japan) and reagents (other than those purchased by HPLC with pure SCFAs (lithium lactate, sodium acetate, sodium propionate and sodium butyrate) at known concentrations as standards.

Measurement of total fecal bacterial count

Fecal samples were washed with PBS, resuspended in PBS containing 0.1% Tween-80, sonicated and centrifuged at 100 x g for 1 min to remove large debris. Aliquots of the supernatants were mounted using Vectashield with DAPI, and fluorescence of DAPI was imaged for ten independent fields of view using Leica DM6000B microscope. The cells in these images were counted using Image-Pro Plus 6.

Isolation of major gut bacterial species

Fresh fecal samples or those stored at –80 °C were used. A small fraction of each sample was diluted and plated onto TOS propionate agar (Yukult Pharmaceutical Industry, Tokyo, Japan) supplemented with 50 µg/ml mupirocin, or BL agar (Nissui, Tokyo, Japan) supplemented with 5% defibrinated horse blood. Plates were incubated for 1–2 days at 37 °C in an anaerobic glove box. Colonies were sorted into types by their morphology. For each type, several single colonies were isolated and subjected to taxonomic identification by 16 S rRNA gene sequencing (Supplementary Table 1). Sequences were analyzed by NCBI BLAST against the bacterial 16 S rRNA gene database; 97% identity was used as a threshold to assign strains to species. Bifidobacterial isolates were additionally typed by RAPD (analysis of random amplified polymorphic DNA) using three primers (Supplementary Table 1). An isolate was chosen per RAPD type as a representative and was evaluated in vitro. For species other than bifidobacteria, a single strain was randomly chosen per species per subject and evaluated.

In vitro evaluation of isolates

Binding to starch granules was analyzed as follows. Bacterial strains were grown anaerobically on modified GAM agar (Nissui, contains 0.5% soluble starch as the major carbon source). Approximately 1 µl of a colony was taken with an inoculating loop and suspended in 150 µl of PBS; then, 12 µl of this suspension, 80 µl of pSt suspension (1% w/v in PBS), and 88 µl of PBS were mixed by vortexing, and formation of bacteria–starch aggregates was examined. If aggregation occurred and the flocc fell immediately to the bottom of the tube making the supernatant transparent, the strain was judged positive. If no aggregation was observed, the strain was judged negative. In negative cases and in the negative control (no bacteria), visible precipitation of starch requires more than ~15 s. Strains that showed self-aggregation (i.e., could not be dispersed or aggregated without starch added) were categorized as undeterminable.

Starch-degrading ability was screened as follows. Strains were grown anaerobically on modified GAM agar, the culture plates were flooded with 1/2 diluted Luguol’s solution for about 30 s and then the solution was removed. Starch-degrading ability was judged from a clear zone around the colonies. When the diameter of this zone was <1 mm or staining around the colony was reduced but was clearly visible, the strain was judged as weakly positive.

Utilization of pSt was analyzed as follows. The powder of GAM Semisolde without Dextrose (Nissui; does not contain starch) was thoroughly

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dissolved in water, agar was removed by precipitation, and the solution was autoclaved at 115 °C for 15 min. Broths containing different carbon sources were prepared by adding 1% w/v glucose, fructose, or pSt before autoclaving. All broths were introduced into an anaerobic chamber at least 1 or 2 days before use. A separate broth was prepared by adding 1% w/v pSt into the autoclaved broth without any additional carbon source, in an anaerobic chamber just before starting culture; starch used in this broth had been UV-irradiated in advance for at least 16 h. Bacterial strains were grown anaerobically on modified GAM agar. Each colony (~1 μl) was suspended in 150 μl PBS, and 5 μl was inoculated into each broth and incubated at 37 °C in the anaerobic chamber for 3 days, and then pH of the culture was measured.

**In vitro competition assay**

*Bifidobacterium adolescentis* G202, *Ruminococcus bromii* YIT 6078 T, and *R. bromii* ATCC 51896 were grown anaerobically on modified GAM broth at 37 °C. Fully grown cultures of either of two *R. bromii* strains and G202 were inoculated into GAM broth supplemented with 0.5% pSt (pSt-GAM) at an approximate 2:1 ratio and incubated for 24 h. Alternatively, *R. bromii* YIT 6078 T was grown on pSt-GAM broth for one day, then G202 was inoculated, and the resultant culture was incubated for 48 h.

Culture was sampled at the indicated time points. "Whole" samples were obtained by centrifuging the culture at 13,000 rpm for 10 min. "pSt-associated" samples were obtained as follows: the culture was centrifuged at 100 × g for 10 min, the supernatant was removed, PBS was added to the pellet, and resuspended, the sample was centrifuged as above and the supernatant was discarded. The pellets were heated at 95 °C for 15 min in TE, centrifuged at 13,000 rpm for 1 min, and the supernatants were used as PCR template. PCR was performed using Phusion Ex Taq Hot Start Version (Takara) and equal amounts of three primers: 27Fmod2-notag, g-Bifid-R, and Cple866mn (Supplementary Table 1). We confirmed that when the two species were mixed in arbitrary ratios after measuring OD, the culture was measured.

**Data analysis and statistics**

Output fastq files were analyzed in QIIME2 software (version 2018.11) and its plugins [15]. Noise and chimeras were removed and the sequences were trimmed using the DADA2 plugin [16] with the following settings: denoised of 1 –p-trim-left-f 20 –p-trim-left-r 17 –p-trunc-len-f 220 –p-trunc-len-r 200. For Experiment II, a total of 3.1 M reads were obtained (6K N, P, S) separately.

For Experiment I, a total of 3.1 M reads were obtained (6K N, P, S) separately.

**RESULTS**

To explore the nutrient-harvesting strategies of human gut bacteria in vivo, we examined the microscale localization of bacteria around intestinal nutrients (Experiment I). This method has been employed in a murine study and proven useful for elucidating how specific bacteria acquire nutrients in the intestine [12]. Stool samples were collected from five subjects who were taking habitual diet, and fecal paraffin sections were analyzed by fluorescence in situ hybridization (FISH) with probes specific for major bacterial taxa. Plant-like structures and bacteria were found in every specimen. Particle density differed substantially among subjects (Supplementary Fig. 1a and b). Several types of bacterial localization were found (Fig. 1 and Supplementary Fig. 1c). Food structures colonized by bacteria in some subjects were often not found in the others, reflecting diversity in diet. In every case, local accumulation of bacteria of the same taxa or morphology was evident, which was distinct from distal diffusion (Supplementary Fig. 1d). The most eminent feature was colonization of bifidobacteria on undigested starch granules, which have been known as a type of resistant starch (resistant starch type 2: RS2) (Fig. 1a and Supplementary Fig. 1e). Granule surfaces were densely covered by bifidobacteria, which were identified as a starch-utilizing bacterium, *B. adolescentis* [27], by using a species-specific probe (Fig. 1a). We also found other features, for example, an unspecified clade of *Lachnospiraceae* localized along a plant exodermis–like cell layer (Fig. 1b), and some bifidobacteria and *Lachnospiraceae* bacteria enriched near the mucus layer (Fig. 1c).

Bacterial colonization of a nutrient suggests that the nutrient is utilized by or serves as an important habitat for the bacteria. Dense colonization of starch granules by *B. adolescentis* led us to speculate that the species is a key degrader, which breaks down a large fraction of starch granules, possibly providing oligosaccharides, or a key consumer, which takes up and metabolizes a large fraction of starch granules, and affects the overall response of the microbiota to the nutrient. To test this hypothesis, we next checked the response of *B. adolescentis* and whole-gut microbiota to intake of starch granules in ten healthy adults and evaluated how the presence or absence of the species affects the response of the microbiota (Experiment II; Fig. 2a). Commonly consumed purified potato starch (pSt) was used as a source of starch granules, as in a previous study [28]. Intake of 48 g/day pSt for 2 weeks did not considerably change total bacterial count (Supplementary Fig. 2), but it resulted in a massive increase in *Bifidobacteriaceae* including *B. adolescentis* in all *B. adolescentis* carriers (Subjects K, M, O, Q, R, T; Fig. 2b–d). Relative abundance of this species increased from 0.1%–28.9% to 32.0%–77.6% (Fig. 2c). Colonization of the species on starch granules was reproducible in every *B. adolescentis* carrier (Fig. 3a, Supplementary Fig. 3). In subjects without *B. adolescentis* (*B. adolescentis* non-carriers; Subjects L, N, P, S), the abundance of another known starch-utilizing species, *Eubacterium rectale* [29], increased substantially, from 2.7–15.2 to 12.6–45.9% (Fig. 2c). Yet another major known starch-utilizing species, *R. bromii* [30], also showed some increase in two subjects (Subjects P, S; Fig. 2c). A sporadic increase was also found in *Ruminococcus torques* (Supplementary Fig. 4).

Spearman’s rank correlation coefficient (Spearman’s ρ) and p value between the abundance of OTUs and SCFA concentrations were calculated using the R-package Hmisc [25] function corrcor. The GMPR-normalized OTU count data and SCFA concentration data from 80 samples were divided into 20 groups by subject and period (pre-intake or pSt intake), and averaged within each group. Spearman’s p values were also calculated between inferred OTU counts. A network plot was generated using the R-package visNetwork [26]. Excretion of starch granules (% area) was compared between *B. adolescentis* carriers and non-carriers using two-sided Welch’s t test.

**Table**

| Subject | pSt Intake | SCFA Concentration | Starch Utilization |
|---------|------------|-------------------|--------------------|
| L       | 0          | Low               | No                |
| N       | 0          | Low               | No                |
| P       | 0          | Low               | No                |
| S       | 0          | Low               | No                |
| K       | 128        | High              | Yes               |
| M       | 128        | High              | Yes               |
| O       | 128        | High              | Yes               |
| Q       | 128        | High              | Yes               |
| R       | 128        | High              | Yes               |
| T       | 128        | High              | Yes               |

**Figures**

Fig. 1. Analysis of starch granules by FISH. **a**. Bacterial colonization of starch granules by *B. adolescentis* was observed in every specimen. Particle density differed substantially among subjects (Supplementary Fig. 1a and b). Several types of bacterial localization were found (Fig. 1 and Supplementary Fig. 1c). Food structures colonized by bacteria in some subjects were often not found in the others, reflecting diversity in diet. In every case, local accumulation of bacteria of the same taxa or morphology was evident, which was distinct from distal diffusion (Supplementary Fig. 1d). The most eminent feature was colonization of bifidobacteria on undigested starch granules, which have been known as a type of resistant starch (resistant starch type 2: RS2) (Fig. 1a and Supplementary Fig. 1e). Granule surfaces were densely covered by bifidobacteria, which were identified as a starch-utilizing bacterium, *B. adolescentis* [27], by using a species-specific probe (Fig. 1a). We also found other features, for example, an unspecified clade of *Lachnospiraceae* localized along a plant exodermis–like cell layer (Fig. 1b), and some bifidobacteria and *Lachnospiraceae* bacteria enriched near the mucus layer (Fig. 1c).

Fig. 2. Analysis of starch granules by qPCR. **a**. *B. adolescentis* carriers (Subjects K, M, O, Q, R, T; Fig. 2b–d). Relative abundance of this species increased from 0.1%–28.9% to 32.0%–77.6% (Fig. 2c). Colonization of the species on starch granules was reproducible in every *B. adolescentis* carrier (Fig. 3a, Supplementary Fig. 3). In subjects without *B. adolescentis* (*B. adolescentis* non-carriers; Subjects L, N, P, S), the abundance of another known starch-utilizing species, *Eubacterium rectale* [29], increased substantially, from 2.7–15.2 to 12.6–45.9% (Fig. 2c). Yet another major known starch-utilizing species, *R. bromii* [30], also showed some increase in two subjects (Subjects P, S; Fig. 2c). A sporadic increase was also found in *Ruminococcus torques* (Supplementary Fig. 4).
**Fig. 1** Microscopic survey of bacterial localization around materials in human feces. 

**a** Colonization of *B. adolescentis* on starch granules (arrowheads). Upper: iodine staining; Lower: FISH (Green: *B. adolescentis*, red: *B. catenulatum* group, blue: genus *Bifidobacterium*); *B. adolescentis* appears cyan because its green signal is merged with the blue signal from genus *Bifidobacterium*. Images on the right are high-power images of those on the left. 

**b** Colonization of *Lachnospiraceae* on plant tissue (arrowheads). Upper: phase contrast; Lower: FISH (Green: *Lachnospiraceae*, red: total bacteria). *Lachnospiraceae* appears yellow because its green signal is merged with the red signal from total bacteria. 

**c** Localization of *Bifidobacterium* and *Lachnospiraceae* near mucus layer (between arrowheads). Upper: Alcian Blue PAS staining; Lower: Fluorescent image of a serial section corresponding to the rectangle in upper image (Green: genus *Bifidobacterium*, red: *Lachnospiraceae*, blue: total bacteria). Bars = 50 μm. *, Autofluorescence from plant-like structures.

**Fig. 2** Two-week intake of potato starch results in expansion of *B. adolescentis*. 

**a** Scheme of the pSt intake experiment. **b** Family-level 16S rRNA profiles before and during intake of pSt. Less frequent families (average relative abundance <1%) are shown as others. **c** Relative frequency of known major starch-utilizing species before and during intake of pSt. The presence (>0.05%; colored cells) or absence (gray cells) of the indicated species is shown beneath the graph. Note that *B. adolescentis* was responsible for a large fraction of the increase in *Bifidobacteriaceae*. **d** Response of OTUs to pSt. Only OTUs with >0.01% average relative abundance are plotted. Those that showed significant differences between the pre-intake and intake periods (s value < 0.01) are labeled by taxon name. The s value represents the “false sign or small” (FSOS) rate, where “small” denotes LogFC between −1 and 1, shown as dashed lines. Values following taxonomic name are % identity to the reference sequence. Three known starch-utilizing species are shown as colored dots.
Surprisingly, though all subjects had *E. rectale*, an increase in this species was observed in all four *B. adolescentis* non-carriers but not in any of the six carriers (Fig. 2c). Similarly, increases in *R. bromii* and *R. torques* were evident only in *B. adolescentis* non-carriers (Fig. 2c and Supplementary Fig. 4). However, since *B. adolescentis* and *E. rectale* were both highly abundant, their increase might lead to compositional problem: non-differential features might appear to be reduced due to the constant-sum...
constraint in relative abundance. To reduce these effects, we normalized the data by geometric mean of pairwise ratios (GMPR) method, and successfully confirmed that the same conclusion as above was obtained (Fig. 2d). Between B. adolescentis carriers and non-carriers, we found significant differences in gut microbiota metabolism. The amount of undigested pSt granules in fecal sections (Supplementary Fig. 5) was smaller in B. adolescentis carriers than in non-carriers (Fig. 3b). Furthermore, in B. adolescentis carriers but not in non-carriers, fecal concentrations of major bifidobacterial metabolites acetic and lactic acids increased and those of propionate decreased upon intake of pSt (Fig. 3c). Instead, a major metabolite of E. rectale, butyric acid, increased in B. adolescentis non-carriers but not in carriers (Fig. 3c). In Spearman's correlation analysis, B. adolescentis was the most abundant among operational taxonomic units (OTUs) that were significantly correlated with the concentrations of acetic and lactic acids, and E. rectale was the most abundant among OTUs that were significantly correlated with the concentration of butyrate, suggesting that they produced a major fraction of each SCFA from pSt (Fig. 3d). In another Spearman's correlation analysis, E. rectale was positively correlated with R. bromii and negatively with B. adolescentis and several butyrate-producing bacteria (Fig. 3e). For B. adolescentis, positive correlation was found with B. longum and Collinsella aerofaciens. Overall, these results strongly suggest that (i) among all bacterial species detected in this study, B. adolescentis most efficiently consumes intestinal pSt granules, which is facilitated by their colonization, to increase its abundance and generation of acetic and lactic acids, and that (ii) E. rectale can increase its abundance upon pSt intake, generating butyrate, but only in the absence of the dominant consumer B. adolescentis.

To better understand the cause of the hierarchy between B. adolescentis and other species, especially E. rectale, we analyzed fecal sections again to distinguish colonizing and non-colonizing species (Experiment III). For this purpose, we employed another cohort, who consumed lower amounts of starch granules, to identify colonization more easily (Fig. 4a, b). This experiment confirmed that colonization of B. adolescentis on starch granules was outstanding among major bifidobacterial species (Table 1, Fig. 4c, Supplementary Fig. 6 and Supplementary note 1). Colonization by B. adolescentis was detected in every carrier of the species, and half or more of starch granules were densely colonized by the species. However, unfortunately we could not adequately determine the localization of E. rectale by rRNA-targeted FISH. In most of the specimens analyzed, the signal from E. rectale was too weak to identify its location. Presumably, the rRNA of this anaerobic species had already been degraded before fixation, when it was present in the rectum, or after the cells were damaged by exposure to oxygen at the moment of sampling.

Therefore, instead, we checked the distribution of abilities to colonize and utilize pSt granules in major gut bacterial species by isolating them from two subjects who participated in Experiment III. A variety of dominant species including bifidobacteria were isolated, and pSt granule-binding ability and soluble starch-degrading ability were evaluated for at least one strain per species and subject (Supplementary Table 2). Strains with any positive results were included in further analysis of their ability to utilize pSt. Bifidobacterium adolescentis was able to bind to and utilize pSt granules (Table 2), as were most of other strains of the species from different origins (Supplementary Table 3 and Supplementary note 2). Among evaluated isolates other than B. adolescentis, the ability to utilize soluble (boiled) pSt was shared by many taxa; all evaluated Bacteroidaceae species and E. rectale. However, pSt granule–binding ability was shared only by E. rectale

Fig. 4 Colonization of bifidobacterial species on starch granules in feces. a Design of the raw yam intake experiment (ten subjects). b Starch granules were specifically detected during the raw yam intake period. Typical images of iodine-stained fecal sections are shown (specimens from subject B). Bars = 100 μm. Arrowheads: starch granules. c Colonization of bifidobacterial species on starch granules during the raw yam intake period. Bars = 50 μm. *, Autofluorescence.
and C. aerofaciens, and it was strain-dependent (Table 2 and Supplementary Table 2). Surprisingly, none of the examined species other than B. adolescentis, including E. rectale, were able to degrade pSt granules. These results suggest that, among major gut bacterial species, both B. adolescentis and E. rectale are rare starch granule–binding species, and that the ability to efficiently utilize starch granules is nearly unique to B. adolescentis, whereas soluble starch can be utilized by many taxa including Bacteroides.

Finally, since we were unable to obtain any isolates of the known RS2-degrading species R. bromii [30] in the above experiment, we compared the ability of commercially available R. bromii strains and the B. adolescentis strain obtained in this study to dominate the surface of pSt in vitro. When an R. bromii strain and a smaller

Table 1. Determination of starch granule-colonizing bifidobacterial species.

| Subject ID | B | C | D | G | E | H | A | F | I | J |
|------------|---|---|---|---|---|---|---|---|---|---|
| Bifidobacterium | + | + | + | + | + | - | - | - | - | - |
| B. adolescentis | +* | +* | +* | +* | - | - | - | - | - | - |
| B. longum | - | - | - | - | - | - | - | - | - | - |
| B. catenulatum gr. | - | - | - | - | - | - | - | - | - | - |
| B. pseudolongum | - | - | - | - | - | - | - | - | - | - |

Frequency of starch granules colonized by indicated(*) taxa

- high: >65%
- medium: 35–65%
- low: <35%

Table 2. Binding to and utilization of starch granules by major gut bacterial species.

| Family | Identification (sequence identity, if < 97%) | Strain | pH control | Delta pH Glc or Frc | Delta pH boiled potato starch | Delta pH raw potato starch | Binding to raw potato starch |
|--------|---------------------------------------------|--------|------------|---------------------|------------------------------|----------------------------|-----------------------------|
| Bifidobacteriaceae | Bifidobacterium adolescentis | G202 | 6.3 | -1.9 | -1.9 | -1.7 | ++ |
| | Bifidobacterium catenulatum gr. | F01 | 6.4 | -2.1 | 0.1 | 0.1 | - |
| | Bifidobacterium catenulatum gr. | G43 | 6.5 | -2.1 | -0.1 | -0.1 | - |
| | Bifidobacterium longum | F03 | 6.5 | -2.2 | -0.3 | -0.1 | - |
| Coriobacteriaceae | Collinsella aerofaciens | F47 | 6.8 | -2.2 | 0.0 | 0.0 | + |
| Bacteroidiaceae | Bacteroides faecis | G51 | 6.0 | -1.2 | -1.1 | 0.0 | - |
| | Bacteroides stercoris | G60 | 6.3 | -1.4 | -1.2 | 0.0 | - |
| | Bacteroides thetaiotaomicron | F57 | 5.9 | -1.1 | -0.9 | 0.0 | - |
| | Bacteroides uniformis | F65 | 6.4 | -1.4 | -1.4 | 0.0 | - |
| | Bacteroides uniformis | G58 | 6.5 | -1.5 | -0.9 | 0.0 | - |
| | Bacteroides vulgatus | F61 | 6.5 | -1.6 | -1.5 | 0.0 | - |
| | Bacteroides vulgatus | G52 | 6.6 | -1.7 | -1.6 | 0.0 | - |
| | Bacteroides xylanisolvens | F59 | 6.2 | -1.4 | -1.4 | 0.1 | - |
| Lachnospiraceae | Eubacterium hallii (95.9%) | F84 | 6.6 | -1.3 | 0.4 | 0.0 | - |
| | Eubacterium hallii | G74 | 6.8 | -1.4 | 0.2 | -0.1 | - |
| | Eubacterium rectale | F82 | 6.5 | -0.4 | -1.3 | 0.0 | - |
| | Eubacterium rectale | G76 | 6.7 | -0.3 | -1.5 | 0.0 | + |
| | Blautia luti (97.0%) | F22 | 6.2 | -1.6 | -0.1 | 0.0 | - |
| | Eubacterium ventriosum | F30 | 6.5 | -1.8 | -0.1 | 0.0 | - |
| | Dorea formicigenerans | G47 | 6.7 | -1.8 | 0.0 | 0.0 | - |
| Erysipelotrichaceae | Dielma fastidiosa | F60 | 6.8 | -0.9 | 0.0 | 0.0 | - |
| (No inocula) | | | 7.0 | -0.2 | -0.1 | 0.0 | 0.0 |

Delta pH Values indicate pH after 3-day incubation for culture with no carbon source (control), and the difference in pH between control and corresponding culture with 1% carbon source. As a positive control, either glucose (Glc) or fructose (Frc) was used. ++ strongly positive, + positive, – negative.
amount of \textit{B. adolescentis} G202 were inoculated together and incubated, the amounts of both strains in the whole culture became comparable, while G202 accounted for the majority of the population attached to pSt after 24 h of incubation (Fig. 5a). Even when \textit{B. adolescentis} G202 was inoculated after \textit{R. bromii} YIT 6078T had densely covered the granular surface after one-day culture, the pSt-associated population after coculture contained a large amount of \textit{B. adolescentis}, while \textit{R. bromii} continued to constitute the majority of the whole culture (Fig. 5b, c). These results suggest that \textit{B. adolescentis} is able to take over the surface of starch granules in the intestine, even in the presence of the known RS2-degrader \textit{R. bromii}.

**DISCUSSION**

Bacterial strategies and processes for nutrient acquisition have not been surveyed by focusing on microscopic spatial information, at least for normal human gut bacteria. To uncover bacterial life in our gut, we applied histological methods to human fecal material and showed that a specific human-associated dominant bacterial species colonizes starch granules in vivo and effectively responds to their intake by the host. Our results suggest that activity of this species restrained other species with a potential to respond, and thereby determines the major SCFA generated in the gut.

We searched for the locations of major gut bacteria and found prominent and frequent colonization of \textit{B. adolescentis} on starch granules in the human intestinal environment. Although adhesion of \textit{B. adolescentis} onto starch granules has been demonstrated in vitro \cite{31,32} and ex vivo \cite{33}, we revealed selective, dense and frequent colonization in the normal human intestinal environment. This colonization was present in every subject who had taken pSt or raw yam starch granules and carried \textit{B. adolescentis}, suggesting that the relationship between starch granules and this species is robust and important for adaptation of this species to human gut.

We found a remarkable increase in \textit{B. adolescentis} abundance after pSt intake in each carrier of the species; the second largest response was found in the butyrate-producing bacterium \textit{E. rectale}. Surprisingly, the response of \textit{E. rectale} to pSt was observed in all \textit{B. adolescentis} non-carriers but not in carriers, and a similar response was also observed in \textit{R. bromii}. This relationship was consistently detected using GMPR method for normalization, which reduces the compositional problem. Given that the total bacterial count was comparable between the pre-intake and intake periods, the increase in the relative abundance of \textit{B. adolescentis} should reflect an increase in its count. Despite the limited number of subjects, the difference between \textit{B. adolescentis}...
The advantage of B. adolescentis in utilization of starch granule is substantiated by in vitro activities of dominant species isolated from the participants. These results reinforce that B. adolescentis is highly adapted to raw granular starches and suggest that monopolization of the starch surface by adhesion promotes the selective response of the species as it limits expansion of other species, which can increase in abundance in the absence of B. adolescentis. The model is substantiated by the results of our in vitro competition assay, which suggest that B. adolescentis can take over the pSt surface from the known starch granule-degrading species, R. bromii. The acidic environment induced by B. adolescentis (especially the presence of lactate, pHa 3.86) may also contribute to the advantage of B. adolescentis over other species such as E. rectale [40]. We confirmed a previous result [30, 42] that E. rectale cannot utilize raw pSt in vitro, but it did respond to the intake of raw pSt. This may be explained by the activity of host amylase or amylases from other bacteria. Because E. rectale responded to pSt in the absence (or presence below the detection limit) of R. bromii (subjects L, N), there should be another mediator with activity similar to that of R. bromii.

Taken together, our results provide a good example of competition for a common but specific food ingredient and hierarchical relationship among major bacteria in the normal human intestine. Our findings could provide a basis for understanding similar interspecies relationship. In situ colonization analysis brought us mechanistic understanding of inter-species hierarchy in response to a nutrient and prompted us to propose future plans for a precise control of microbiota. A similar approach may reveal competition among other species for other nutrients and contribute to our understanding and ability to use the microbiota.

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