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Inulin Fermentation by Lactobacilli and Bifidobacteria from Dairy Calves

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ABSTRACT Prebiotics are increasingly examined for their ability to modulate the neonate gut microbiota of livestock, and products such as inulin are commonly added to milk replacer used in calving. However, the ability of specific members of the bovine neonate microbiota to respond to inulin remains to be determined, particularly among indigenous lactobacilli and bifidobacteria, beneficial genera commonly enriched by inulin. Screening of *Bifidobacterium* and *Lactobacillus* isolates obtained from fresh feces of dairy calves revealed that lactobacilli had a higher prevalence of inulin fermentation capacity (58%) than bifidobacteria (17%). Several *Ligilactobacillus agilis* (synonym *Lactobacillus agilis*) isolates exhibited vigorous growth on, and complete degradation of, inulin; however, the phenotype was strain specific. The most vigorous inulin-fermenting strain, *L. agilis* YZ050, readily degraded long-chain inulin not consumed by bifidobacterial isolates. Comparative genomic analysis of both *L. agilis* fermenter and nonfermenter strains indicated that strain YZ050 encodes an inulinase homolog, previously linked to extracellular degradation of long-chain inulin in *Lacticaseibacillus paracasei*, that was strongly induced during growth on inulin. Inulin catabolism by YZ050 also generates extracellular fructose, which can cross-feed other non-inulin-fermenting lactic acid bacteria isolated from the same bovine feces. The presence of specific inulin-responsive bacterial strains within calf gut microbiome provides a mechanistic rationale for enrichment of specific lactobacilli and creates a foundation for future synbiotic applications in dairy calves aimed at improving health in early life.

IMPORTANCE The gut microbiome plays an important role in animal health and is increasingly recognized as a target for diet-based manipulation. Inulin is a common prebiotic routinely added to animal feeds; however, the mechanism of inulin consumption by specific beneficial taxa in livestock is ill defined. In this study, we examined *Lactobacillus* and *Bifidobacterium* isolates from calves fed inulin-containing milk replacer and characterized specific strains that robustly consume long-chain inulin. In particular, novel *Ligilactobacillus agilis* strain YZ050 consumed inulin via an extracellular fructosidase, resulting in complete consumption of all long-chain inulin. Inulin catabolism resulted in temporal release of extracellular fructose, which can promote growth of other non-inulin-consuming strains of lactic acid bacteria. This work provides the mechanistic insight needed to purposely modulate the calf gut microbiome via the establishment of networks of beneficial microbes linked to specific prebiotics.

KEYWORDS *Ligilactobacillus agilis*, inulin, bovine, synbiotic, exo-inulinase

The gut microbiota plays a vital role in maintaining host health through means that include aiding in nutrient absorption, immunomodulation, and pathogen exclusion (1, 2), and its development in production animals contributes to optimal growth and performance (3). Dysbiosis of the neonatal gut microbiota has been linked to malnutrition, type 1 diabetes, and asthma in children (4–6) and to diarrhea

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in livestock (7). Subtherapeutic doses of antimicrobials were commonly used in animal diets to improve the gut health and lower risk of enteric infections (8); however, increasing prevalence of antimicrobial resistance has led to strict regulation limiting the use of prophylactic use of antibiotics (9), necessitating novel strategies to manipulate the gut microbiota.

Among the many factors influencing the microbiota, diet is a major driver in shaping the taxonomic composition of the gut microbial community in livestock (10, 11). In this regard, dietary prebiotics such as inulin-type fructans, a natural fiber widely distributed in nature (e.g., chicory roots) and represented by several forms, including larger-chain inulin or smaller-chain fructo-oligosaccharides (FOS), are increasingly employed in foods directed at food production animals, particularly in early life (e.g., dairy calves) (12). In previous studies, prebiotics, including FOS and mannan-oligosaccharides (MOS), were administered to nursing animals, targeting improvement of host performance (e.g., reducing the frequency of diarrhea), but the treatment effects remained mixed (13–17). This is likely a result of a lack of bacterial "responders" to the specific fiber provided (18, 19), and few studies extensively deciphered the interaction between dietary fibers and resident gut microbiota at the bacterial strain level. For example, while inulin is routinely used in the form of commercial prebiotics in nursing animals (e.g., added in milk replacer for dairy calves), there is limited knowledge on the capacity of the resident bacteria in livestock to consume inulin. This knowledge gap is true for *Bifidobacterium* and *Lactobacillus*, genera known to be associated with beneficial host effects (20, 21).

To explore this knowledge gap, we examined *Lactobacillus* and *Bifidobacterium* species from dairy calves fed milk replacer containing inulin and identified a number of isolates that grew well on inulin. One isolate, *Ligilactobacillus agilis* YZ050, possessed a strong inulin fermentation capacity and expressed a specific exo-inulinase activity associated with high-molecular-weight inulin degradation. This work identifies and characterizes specific inulin-fermenting strains derived from dairy calves which could be used in future applications with inulin in milk replacer to enhance neonatal colonization.

RESULTS

Isolation and identification of calf-borne bifidobacteria and lactobacilli. A previous study performed in this lab carried out metagenomic analysis of early life microbiota development in calves focused on antimicrobial resistance (10). Notably, the milk replacer provided to the calves in this cohort contained inulin, a well-known prebiotic. When the calves received this milk replacer, their fecal microbiota exhibited an increase in bacterial families *Lactobacillaceae* and *Bifidobacteriaceae*, reaching 8% relative abundance (Fig. 1). In addition, a pre-isolation PCR screen of fecal enrichments revealed that 13 and 30 fecal DNA samples (among 42 feces samples from 17 nursing calves) were positive for *Bifidobacterium* and *Lactobacillus* spp., respectively (see Table S1 in the supplemental material). Culturing of bifidobacterium- or lactobacillus-PCR-positive fecal samples resulted in a total of 88 *Bifidobacterium* isolates from 10 samples and 206 *Lactobacillus* isolates from 29 samples (Table S1).

Among the bifidobacterial isolates, *Bifidobacterium pseudolongum* subsp. *globosum* ($n = 32$), *Bifidobacterium animalis* subsp. *lactis* ($n = 25$), and *Bifidobacterium animalis* subsp. *animalis* ($n = 23$) were the most frequently observed species, representing approximately 89.7% (80 of 88) of all bifidobacterial isolates. *Bifidobacterium choerinum* ($n = 5$), *B. aerophilum* ($n = 2$), and *B. pseudolongum* ($n = 1$) were also found in this cohort. We were able to compile a larger collection of lactobacilli ($n = 206$) in which *Limosilactobacillus reuteri* comb. nov. ($n = 87$), *Ligilactobacillus salivarius* ($n = 46$), and *Limosilactobacillus mucosae* ($n = 39$) were the most common species, representing 83.4% of all isolates. We also isolated *Limosilactobacillus ingluviei* ($n = 11$), *Ligilactobacillus agilis* ($n = 6$), *Lactiplantibacillus plantarum* ($n = 4$), and *Limosilactobacillus fermentum* ($n = 12$) in this cohort. de Man-Rogosa-Sharpe (MRS) media also enabled enrich-

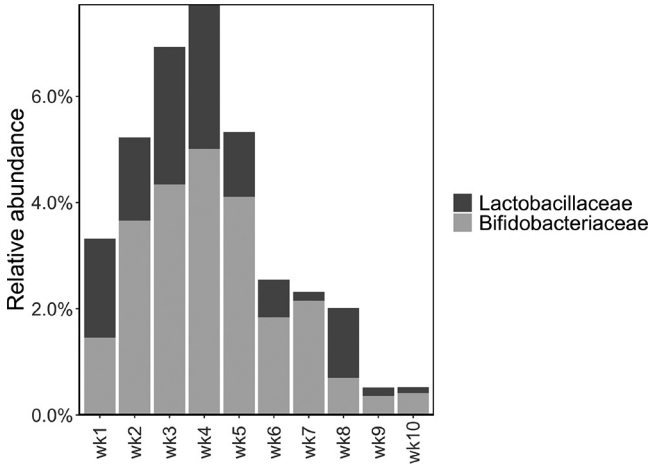

FIG 1 Relative abundances of members of the families *Lactobacillaceae* and *Bifidobacteriaceae* fecal microbiota in nursing calves in early life ($n = 22$) (16S amplicon sequence data were replotted from previously published data [10]).

ment of some pediococci, including *Pediococcus acidilactici* ($n = 26$) and *Pediococcus pentosaceus* ($n = 2$).

Inulin fermentation was more frequently observed among cultured lactobacilli than bifidobacteria. All of the *Lactobacillus* ($n = 206$) (Fig. 2a) and *Bifidobacterium* ($n = 88$) (Fig. 2b) isolates were initially screened for their capability to ferment inulin using MRS-inulin broth containing bromcresol purple (30 mg/liter), whereby fermentation lowered the culture pH, resulting in a color change from purple to yellow. From this screening, 15 *Bifidobacterium* isolates were shown to ferment inulin, among which *B. aerophilum* strain JL0026 showed the most significant color change (Fig. 2b; see also

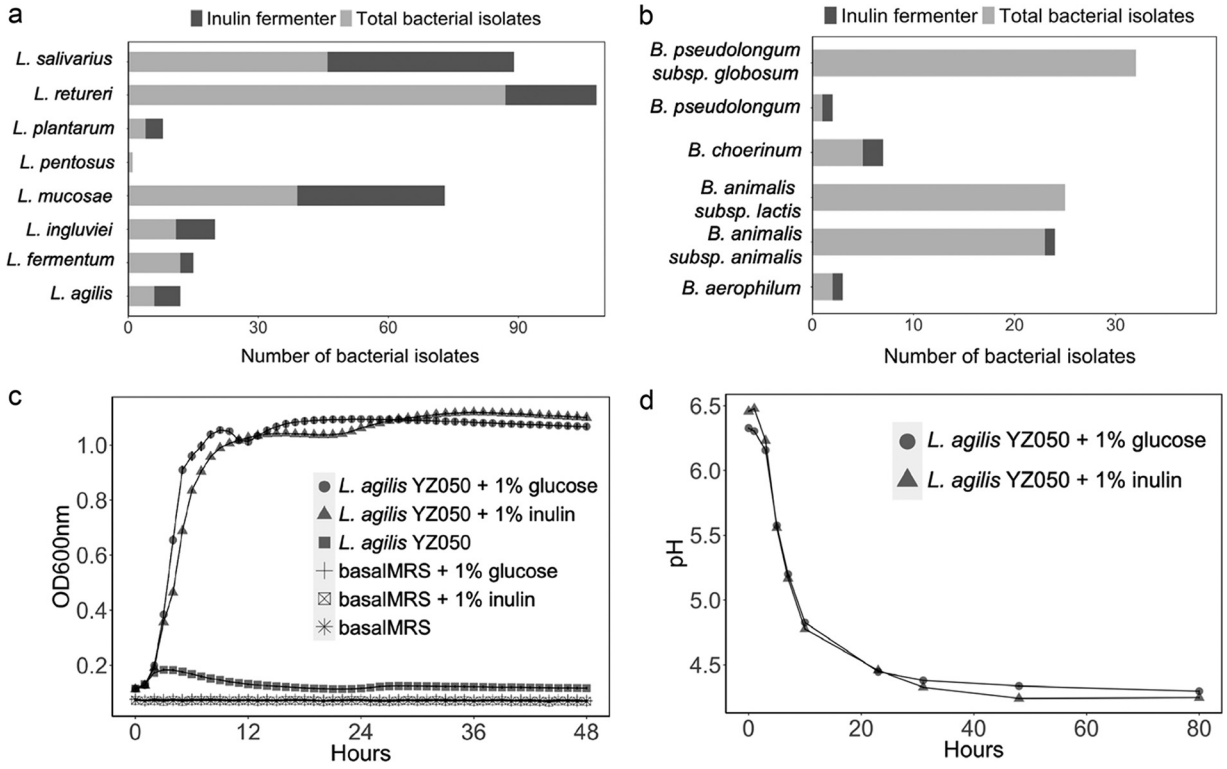

FIG 2 (a and b) Total bacterial isolates and inulin growth phenotypes of *Lactobacillus* ($n = 206$) (a) and *Bifidobacterium* ($n = 88$) (b) isolates from calf feces. (c and d) Growth curves of strain *L. agilis* YZ050 on inulin and glucose (c) and pH values of the corresponding media (d).

Table S1). Microtiter growth studies of these strains indicated moderate growth on inulin, with an ultimate cell density of 0.4 (see Fig. S1 in the supplemental material). Thin-layer chromatography (TLC) profiling of the fermentation supernatant indicated loss of the short-chain (oligofructose) portions of inulin but not of the longer-chain oligosaccharides (Fig. S2).

Inulin fermentation was found in over half of the *Lactobacillus* isolates (120 of 206), including *L. salivarius* ($n = 43$), *L. mucosae* ($n = 34$), *L. reuteri* ($n = 21$), *L. ingluviei* ($n = 9$), *L. agilis* ($n = 6$), *L. fermentum* ($n = 3$), and *L. plantarum* ($n = 4$) (Fig. 2b; see also Table S1). *L. agilis* YZ050 grew vigorously on inulin without a lag phase at a level (growth rate 0.0454 h^{-1}) comparable to its growth on glucose (0.0586 h^{-1}) (Fig. 2c) and similarly acidified the media to around pH 4.0 on either inulin or glucose over time (Fig. 2d). Consistent with this species phenotype (22), all *L. agilis* isolates collected were motile (data not shown).

Strain specificity of inulin consumption by *L. agilis*. Since inulin fermentation was not previously associated with *L. agilis*, we examined if this trait is common for the species. A total of six *L. agilis* isolates obtained in this study, together with a number of *L. agilis* strains obtained from other collections, were screened for inulin fermentation. Surprisingly, only *L. agilis* YZ050 and YZ058, which were isolated from the same calf at different time points, showed vigorous growth on inulin, while others demonstrated either limited growth (*L. agilis* YZ155 and YZ161) or no growth (Fig. 3a). Our TLC assay confirmed that all assessed isolates, except *L. agilis* YZ050, were able to deplete only the mono- and disaccharide parts available in commercial inulin and lacked the capability to utilize the longer chain length of fructo-oligosaccharides with degree of polymerization (DP) values of >3 (Fig. 3b). These results indicate that inulin consumption by *L. agilis* of calf origin is strain specific.

Previous studies indicated that glucose was able to induce catabolite repression of inulin fermentation in *L. paracasei* 1195 (23, 24). Grown on inulin and glucose, *L. agilis* YZ050 exhibited a typical diauxic growth pattern (Fig. 3c), likely a result of the initial depletion of glucose. Following this initial growth phase, *L. agilis* YZ050 entered a second growth phase, likely using inulin as the sole carbon source. The final cell densities of *L. agilis* YZ050 on glucose and inulin were at levels comparable to its growth solely on inulin (Fig. 3c).

Given the robust inulin fermentation by *L. agilis* YZ050, we also examined the strain for fermentation of other prebiotic oligosaccharides, including galactooligosaccharides (GOS), bovine milk oligosaccharides (BMOs), and human milk oligosaccharides (HMOs). *L. agilis* YZ050 was able to utilize GOS and BMOs, achieving moderate growth, with an ultimate cell optical density of 0.6 (Fig. S3). No growth was observed on HMOs.

Inulin consumption pattern by *L. agilis*. TLC revealed that shorter-chain oligofructoses (e.g., sucrose and trimers) were quickly consumed by *L. agilis* YZ050 (i.e., an inulin fermenter strain) during the first 3 h of fermentation whereas the longer-chain oligosaccharides with a degree of polymerization (DP) ranging from 4 to 7 disappeared around 7 h (Fig. 4a). Only oligosaccharides of moderate chain length (DP, 8 to 12) were present in the cell culture at 24 h of growth, while all inulin components had been completely consumed by *L. agilis* YZ050 by the end of 48 h of incubation (Fig. 4a). In contrast, nonfermenting strain *L. agilis* YZ054 consumed only oligosaccharides with a DP value of <3 over the entire period of incubation (Fig. 4b).

Cross-feeding of fructose from *L. agilis* YZ050 to coresident LAB strains. The inulin consumption phenotype exhibited by *L. agilis* YZ050 suggests extracellular degradation of high-molecular-weight oligosaccharides coincident with release of extracellular fructose followed by transport and metabolism by YZ050. However, this model also suggests potential release of fructose that could cross-feed other resident taxa. For instance, many of the *L. agilis* strains did not grow on inulin but grew well on monomer fructose (data not shown). Growth of *L. agilis* YZ050 on inulin resulted in fructose release into the media (Fig. 5a). *P. acidilactici* YZ049, an isolate which grew well on fructose but not on inulin (Fig. 5b), was able to grow on the conditioned media from

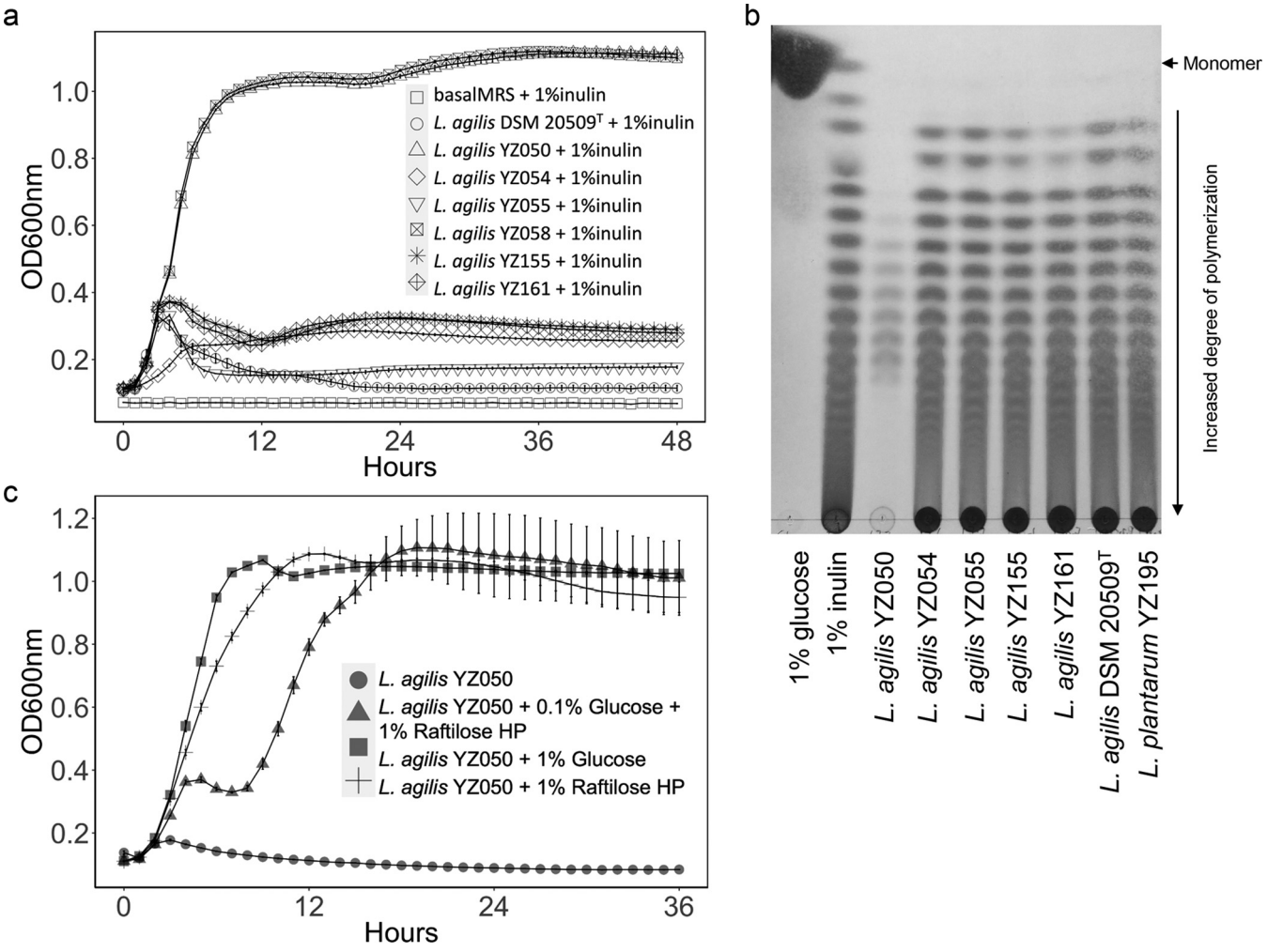

FIG 3 (a) Growth of seven *L. agilis* strains in basal MRS medium containing 1% inulin. (b) TLC profiling of supernatants after fermentation on 1% inulin for six *L. agilis* isolates (YZ050, YZ054, YZ055, YZ155, YZ161, and DSM 20509^T) using 1% glucose and 1% inulin as standards and for one nonfermenter *L. plantarum* YZ195 as the negative control. (c) Growth curves of *L. agilis* YZ050 in basal MRS medium containing both glucose (0.1%) and inulin (Raftilose HP inulin at 1%) using 1% glucose as the positive control and medium with no sugar added as the negative control. Raftilose HP inulin was used due to the more limited levels of monomer and dimer in this product (data not shown), which enabled better differentiation of the diauxic growth patterns presented in panel c.

growth of YZ050 inulin, suggesting clear cross-feeding on the released fructose (Fig. 5c). Notably, non-inulin-growing *L. agilis* strain YZ054 and *P. acidilactici* YZ049 were isolated from the same feces as inulin-consuming *L. agilis* YZ050, suggesting possible cross-feeding *in situ*.

Genetic analysis of inulin consumption by *L. agilis* YZ050. Inulin or FOS catabolism has been linked to specific operons in *L. paracasei* 1195 (23) and *L. plantarum* P14 (25) and to the levanase operon in *L. casei* ATCC 334 (25). To identify the genetic basis for inulin fermentation, the genomes of *L. agilis* YZ050 (inulin fermenter) and *L. agilis* YZ054 (nonfermenter of inulin isolated from the same feces) were sequenced. For YZ050, sequencing resulted in 29 contigs with an N_{50} value of 143,231 bp, while 30 contigs with an N_{50} value of 154,775 bp were generated for YZ054. The genome size and predicted gene numbers were 2.07 Mb and 2,109 for YZ050 and 2.24 Mb and 2,261 for YZ054, respectively.

Comparative genomic analysis of these two genomes identified a gene locus, *fruA*, encoding a putative beta-fructosidase (EC 3.2.1.80) present in the fermenter *L. agilis* YZ050 but not in YZ054 (Fig. 6). During growth of YZ050 on inulin, the *fruA* gene exhibited 51-fold (mean; interquartile range [IQR], 32.5) induction relative to the control, suggesting involvement of this extracellular glycosyl hydrolase in the catabo-

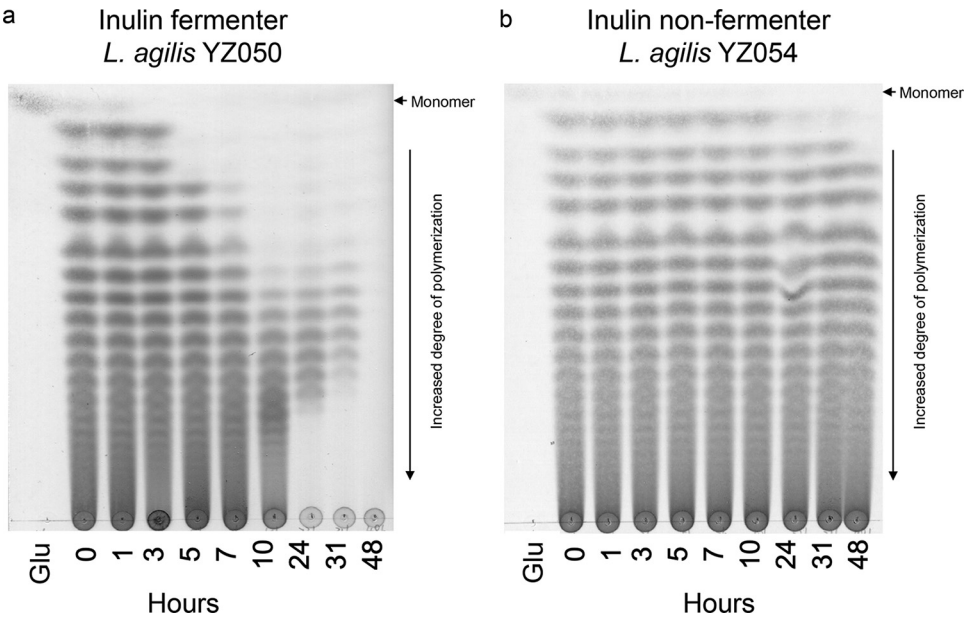

FIG 4 TLC results representing supernatants of fermented media at several sampling time points during fermentation in MRS broth containing1% inulin by (a) fermenter *L. agilis* YZ050 and (b) nonfermenter YZ054. Monomer sugars are marked with an arrow.

lism of inulin. The protein encoded by *fruA* (1,317 amino acids; 147.28 kDa) contains conserved domains common within glycoside-hydrolase family GH32 as well as an N-terminal KxYKxGKxW signal peptide sequence and an LPXTG-motif cell wall anchor domain in the C-terminal region and a similar cell wall-associated structure of *fosE* in *L. paracasei* 1195 (Fig. S4). Surprisingly, the homology of FruA from YZ050 was low (~14%

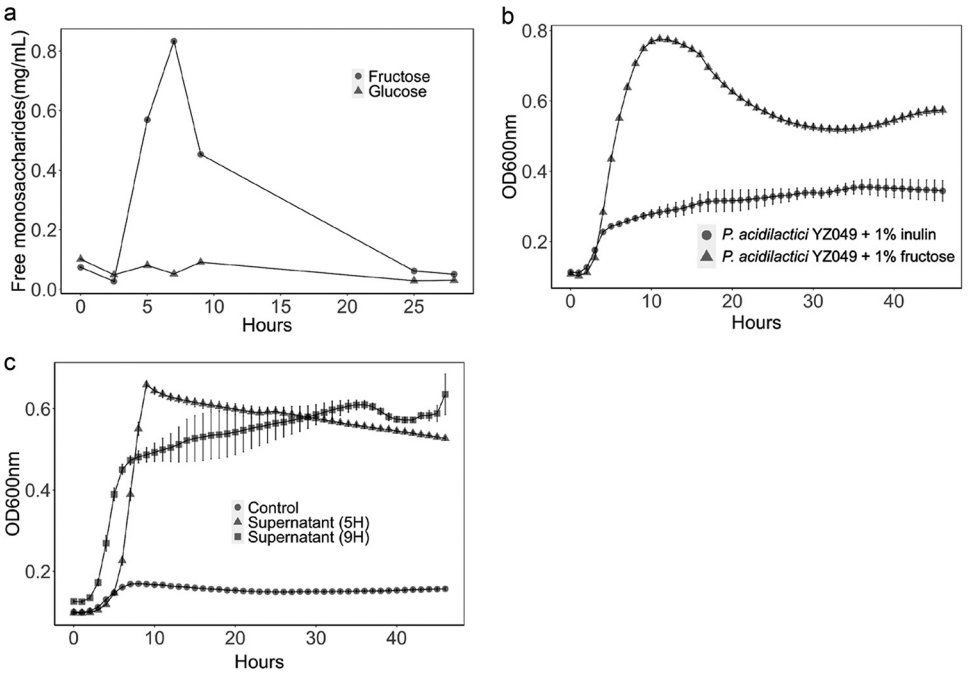

FIG 5 (a) Concentrations of free fructose and glucose in the supernatants taken at 0, 2.5, 5, 7, 9, 25, and 28 h during inulin fermentation by fermenter *L. agilis* YZ050. (b) Growth curves of *P. acidilactici* YZ049 in basal MRS medium containing inulin (1%) and fructose (1%) or glucose (1%). (c) Growth curves of *P. acidilactici* YZ049 grown on sterile supernatants taken at 5 h and 9 h (depicted in panel a).

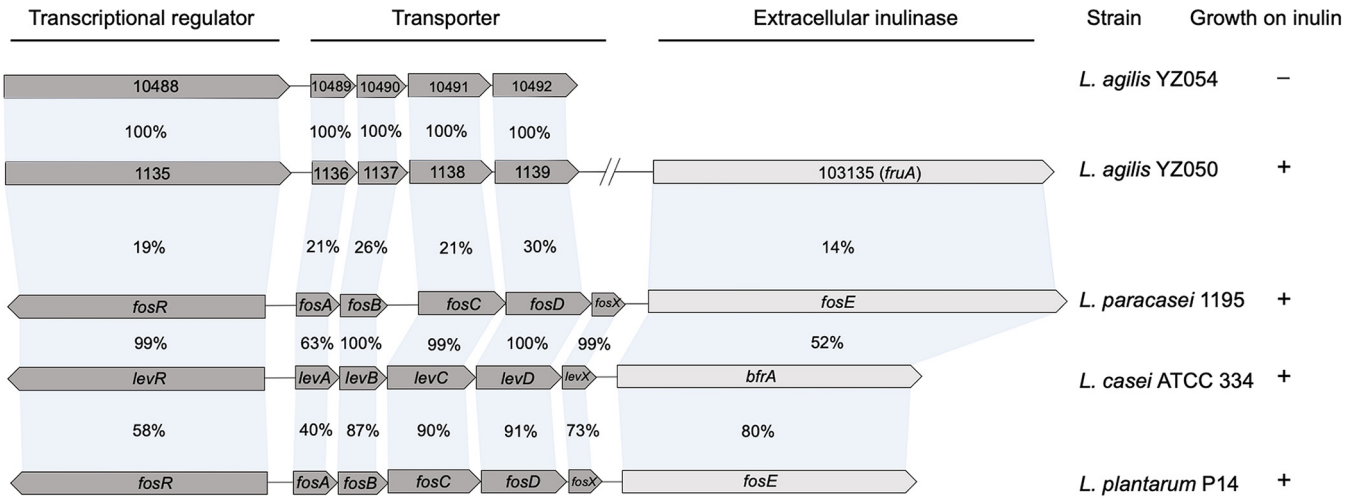

FIG 6 Comparative analysis of extracellular degrading inulin-gene cluster in *L. agilis* YZ050 and YZ054 and other *Lactobacillus* species. Ga0314164_103135 (*fruA*), predicted to be the fructan beta-fructosidase in YZ050, is absent in YZ054. Four genes involved in sugar transport, including Ga0314164_1136, Ga0314164_1137, Ga0314164_1138, and Ga0314164_1139, and the regulatory gene Ga0314164_1135 reside on a region of the YZ050 genome separate from *fruA*. Homologs from the *fos* operon in *L. paracasei* 1195 and *L. plantarum* P14 and the *lev* operon of *L. casei* ATCC 334 are included for comparison. Growth phenotypes on inulin are also indicated.

identity) in comparison to the homolog from *L. paracasei* 1195, suggesting a relatively novel beta-fructosidase in *L. agilis* (Fig. 6).

Other genes present in operons linked to exo-fructan beta-fructosidase in *L. paracasei* 1195, *L. plantarum* P14, and *L. casei* ATCC 334 were found at a distance from *fruA* on the *L. agilis* YZ050 genome (Fig. 6). Specifically, the components of a fructose-specific phosphotransferase system (PTS) comprising genes encoding a transcriptional regulator (Ga0314164_1135) and the transport components IIA (Ga0314164_1136), IIB (Ga0314164_1137), IIC (Ga0314164_1138), and IID (Ga0314164_1139) appear to form a separate operon in YZ050, and they share 19%, 21%, 26%, 21%, and 30% identity with the corresponding homologs in *L. paracasei* 1195, respectively (Fig. 6). Interestingly, the regulatory and transport gene cluster is present in non-inulin-growing strain *L. agilis* YZ054, a strain which readily consumed fructose (Fig. 3b). This suggests that a significant driver of inulin consumption in YZ050 is the presence of the *fruA* gene, with the remaining genes facilitating regulation and transport of extracellular fructose (Fig. 6). Notably, no homolog of *fosX* in *L. paracasei* 1195 and *L. plantarum* P14 or of *levX* in *L. casei* ATCC 334 was observed in the genome of YZ050 (Fig. 6).

DISCUSSION

In early life, the gut microbiome of mammals assembles in parallel with maturation of the immune system (26). This early development and microbial successions are increasingly recognized to associate with downstream health status (27, 28). An understanding of the dietary drivers of early life microbial successions associated with a healthy gut microbiome can result in dietary management tools aimed at the human gut microbiome with the goal of lowering disease risk and improving overall health. The situation is similar in livestock, where numerous studies have identified the successional nature of various microbial taxa in the development of chickens, pigs, and cows (11, 29, 30), among other livestock. The use of prebiotics in the milk replacer for rearing of dairy calves has the goal of enriching a more beneficial microbiota early, although few studies have defined a significant effect of this usage (31, 32).

Historically, the target of prebiotic supplementation is often LAB and bifidobacteria, microbes whose members are known to be responsive to these supplements and whose presence is linked to beneficial health effects. LAB are common members of the neonatal gut microbiota in dairy calves (33, 34). Using data from recent study of calf microbiota by our group, we note that *Lactobacillaceae* and *Bifidobacteriaceae* repre-

sent a significant, and increasing, population (up to 8%) in calf feces in the first several weeks of life during provision of milk replacer containing inulin (Fig. 1), before a change in diet to total mixed ration, a fiber-containing feed (10). These results are consistent with other studies that revealed bifidobacterial and LAB populations in the gastrointestinal tract of neonatal livestock, although their levels of diversity may differ across cohorts (10, 33, 34).

Given the prevalence of bifidobacteria and LAB in livestock and their associated benefits to the host, several groups characterized isolates of animal origin with a goal for future use as probiotics (35, 36). One study recovered 96 LAB spp. from young calves, among which *L. johnsonii*, *L. mucosae*, *L. murinus*, *L. salivarius*, and *L. amylovorus* exhibited some probiotic properties (35). In another study, Soto et al. successfully isolated *L. mucosae*, *L. salivarius*, *L. plantarum*, and *L. farciminis* from nursing calves, and *L. salivarius* appeared to be the dominant species (36). In addition to LAB, bifidobacteria are frequently observed in nursing calves. For example, *B. choerinum* AGR2158, *B. pseudolongum* subsp. *globosum* AGR2145, and *B. longum* subsp. *suis* AGR2137 were isolated from fresh feces of calves during the milk-feeding period (1 to 5 days old) (37).

In this work, specific *Lactobacillaceae* isolates obtained from feces of calves fed milk replacer exhibited a more robust catabolism of inulin relative to bifidobacterial isolates. This is consistent with a previous study which documented that, in a collection of 55 *Bifidobacterium* isolates representing 11 species (13 isolates of animal origin and 42 isolates of human origin), most were able to ferment FOS but only 8 were able to grow on inulin as the sole carbon source (38). Further, among the 13 isolates of animal origin, only *B. thermophilum* ATCC 25866 isolated from bovine rumen grew on inulin (38), with a delayed consumption of longer chains, a result consistent with our findings indicating that inulin fermentation is relatively rare in *Bifidobacterium*. While some lactobacilli have been shown to grow on fructo-oligosaccharides, only a few are able to degrade long-chain inulin (DP of 5 to 30), including specific strains of *L. plantarum*, *L. casei*, and *L. paracasei* (23, 25). In all cases, the presence and expression of an extracellular beta-fructosidase correlated with this long-chain inulin catabolism.

L. agilis YZ050 appears to catabolize long-chain inulin via an extracellular beta-fructosidase releasing monomer fructose. Importantly, extracellular release of a sugar monomer from a more complex glycan is the basis for various examples of cross-feeding among intestinal isolates. Extracellular degradation of mucin or human milk oligosaccharides by bacteroides or bifidobacteria has been shown to cross-feed various secondary consumers of the same genus (39) or different genera (40). A recent study demonstrated that *L. paracasei* W20, a strain expressing an extracellular exo-inulinase, was able to cross-feed fructose to *L. salivarius* W57, a non-inulin-growing strain, when cocultured on this prebiotic (41). In this work, non-inulin-consuming LAB strains isolated from the same feces that contained the inulin-consuming *L. agilis* YZ050 strain were able to grow on conditioned media from *L. agilis* YZ050 grown on inulin. This evidence suggests that both primary and secondary LAB consumer networks had assembled in calves fed specific inulin-containing milk replacer. Note that such cross-feeding networks are not always beneficial to the host as pathogens or more-problematic clades (i.e., *Enterobacteriaceae*) have been shown to be enriched in a similar fashion (42).

L. agilis, originally isolated from sewage (22), has been frequently isolated from avian intestinal tracts (43) and from the feces of mammals (e.g., pigs and humans) (44, 45). *L. agilis* has been examined as a probiotic in chickens (46), and a recent study implicated the mobility of *L. agilis* in colonization behavior in mice (47). While any probiotic abilities for *L. agilis* YZ050 remain to be determined, the unique capacity to consume inulin, a common presence in commercial milk replacers, might enable *L. agilis* YZ050 to achieve a higher level of colonization of the calf intestine *in situ*. Such glycan-specific enrichment of a targeted probiotic has been shown for other similar applications (48, 49) and has been linked to beneficial health outcomes in infants (50–52).

MATERIALS AND METHODS

Materials and reagents. Inulin (CAS no. 9005-80-5) was purchased from MP Biomedicals LLC (Santa Ana, CA), and Raftilose high-performance (HP) inulin was provided by Orafti Active Food Ingredients (Malvern, PA). Purimune galacto-oligosaccharides (GO-P 90) with high purity were purchased from GTC Nutrition (CO, USA). Human milk oligosaccharides (HMOs) and bovine milk oligosaccharides (BMOs) were purified and provided by Daniela Barile (Department of Food Science and Technology, University of California, Davis [UC Davis]). A QIAquick 96 PCR purification kit purchased from Qiagen was used to purify PCR product prior to Sanger sequencing. Bacterial genomic DNA extraction was carried out using a Qiagen DNeasy blood and tissue kit (Qiagen, Hilden, Germany). The presence of free fructose and glucose was determined using a D-fructose/D-glucose assay kit (Megazyme International Ltd., Wicklow, Ireland). RNAprotect bacterial reagent (Qiagen, Hilden, Germany), an RNeasy minikit (37) (Qiagen, Hilden, Germany), and a Turbo DNA-free kit (Ambion) were used for total RNA extraction and purification. A high-capacity cDNA reverse transcription kit (Applied Biosystems) and PowerUp SYBR green mix (Applied Biosystems) were used for cDNA synthesis and quantitative PCR (qPCR) analysis, respectively. All reagents used in this work were of analytical grade or higher.

Bacterial strains and culture media. *Bifidobacterium longum* subsp. *infantis* (referred to here as *B. infantis*) ATCC 15697, *Lacticaseibacillus paracasei* comb. nov. DSM23505, *Lacticaseibacillus rhamnosus* comb. nov. (*L. rhamnosus*) GG, *L. agilis* DSM 20508, *L. agilis* DSM 20509, and *L. agilis* DSM 20510 were obtained from the corresponding culture collections. Man-Rogosa-Sharpe (MRS) broth (Becton, Dickinson, Franklin Lakes, NJ) supplemented with 0.05% (wt/vol) L-cysteine was used for bacterial propagation or activation when appropriate. Basal MRS medium (500 ml) (i.e., MRS without carbon source) was prepared with 5 g peptone, 2.5 g yeast extract, 1 g ammonium citrate, 2.5 g sodium acetate, 50 mg magnesium sulfate, 17 mg monohydrate manganese sulfate, 1 g dipotassium phosphate, 500 μl Tween 80, and 250 mg L-cysteine hydrochloride. For bifidobacterial isolation, modified BSIM media (prepared by supplementing MRS broth with 13 g/liter agar, 500 mg/liter L-cysteine–HCl, 20 mg/liter of nalidixic acid, 50 mg/ml mupirocin, 50 mg/ml kanamycin, 50 mg/ml polymyxin B sulfate, 100 mg/liter Iodoacetate, and 100 mg/ml 2,3,5-triphenyltetrazolium) was used (53). LAMVAB medium (anaerobic MRS medium with vancomycin [20 mg/liter] and bromocresol green) was prepared following published protocols for isolation of lactobacilli (54).

Calf fecal samples. Fresh fecal samples ($n = 42$) were obtained from dairy calves raised at the UC Davis Teaching and Research Facility (Davis, CA) within 2 weeks after birth between April and September 2015, by swabbing the rectum with a sterile cotton swab (see Table S1 in the supplemental material). These dairy calves were fed colostrum within the first 12 h of birth and were then fed with milk replacer twice a day (Calva Products, Acampo, CA) and a commercial calf starter (oat starter) (Associated Feed & Supply Co., Turlock, CA) until weaning (10). The milk replacer was supplemented with inulin as dietary prebiotics.

Isolation and identification of *Bifidobacterium* and *Lactobacillus* species. Prior to the isolation of *Bifidobacterium* or *Lactobacillus*, we started by enrichment of fresh feces in MRS broth anaerobically overnight at 37°C, which was followed by a PCR prescreening. Specifically, primers F (5′-AGCAGTAGGG AATCTTCCA-3′) and R (5′-ATTYCACCGCTACACATG-3′) (IDT, Coralville, IA) were used to determine the presence of *Lactobacillus* spp. (55) and primers F (5′-GGGTGGTAATGCCGGATG-3′) and R (5′-CCACCGTT ACACCGGGAA-3′) (IDT, Coralville, IA) were used for probing *Bifidobacterium* spp. *B. infantis* ATCC 15697 and *L. rhamnosus* GG were used as positive controls for PCRs when appropriate. PCR amplification was examined via gel electrophoresis (1%), and samples with positive results were subjected to bacterial isolation.

Following the overnight enrichment, aliquots from bacterial culture were serially diluted (10-fold) in prereduced phosphate-buffered saline (PBS) in an anaerobic chamber. Dilutions (100 μl) were plated onto modified BSIM agar (53) and LAMVAB (54) followed by 48 h of incubation at 37°C under anaerobic conditions for bifidobacterium and lactobacillus isolations, respectively. Presumptive colonies with different morphologies (4 to 16 per sample) were picked and further streaked onto prereduced MRS-cysteine agar twice consecutively to obtain pure bacterial isolates. The bacterial identity was confirmed by examining the 16S rRNA gene using primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1391R (5′-GACGGGCGGTGTACA-3′) (IDT, Coralville, IA) via Sanger sequencing. The resulting isolates were stored at −80°C in 50% glycerol for further analysis.

Inulin fermentation capability of *Lactobacillus* and *Bifidobacterium* isolates. MRS medium containing 0.05% L-cysteine and bromcresol purple (30 mg/liter) supplemented with 1% inulin (MRS-inulin) was used to examine the bacterial fermentability of inulin. Specifically, activated bacterial cell culture (1% [vol/vol]) was inoculated into MRS-inulin (MRS medium containing 1% inulin), MRS-glucose (MRS medium containing 1% glucose; positive control), and MRS-NS (MRS medium without sugar; negative control). An MP Biomedicals inulin product was used for all experiments involving inulin growth, with the exception of the diauxic growth experiment profiled in Fig. 3c, where Raftilose high-performance (HP) inulin was used given that it contained lower levels of monomer and dimer constituents (data not shown). All incubations were carried out at 37°C anaerobically (Coy Laboratory Products, Grass Lake, MI) for 48 h. A color change from purple to yellow in the media as indicated by bromcresol purple results was considered to represent successful fermentation of inulin, and the corresponding isolate was designated a fermenter (56). *B. infantis* ATCC 15697 (57) and *L. paracasei* DSM 23505 (58) were used as positive controls (i.e., fermenters), and *L. rhamnosus* GG (23) was used as a negative control (i.e., nonfermenter).

Growth studies. Bacterial growth experiments were performed in triplicate in 96-well plates (Nalgene Nunc International, Rochester, NY) containing 200 μl of medium per well covered with 40 μl

sterilized mineral oil as described previously (59). Cultures were inoculated (1% [vol/vol]) into MRS medium containing 1% (wt/vol) inulin, BMOs, HMOs, GOS, glucose, or fructose or into no-sugar controls. Cell growth was measured via determination of the optical density at 600 nm (OD_{600}) every 30 min using an automated PowerWave microplate spectrophotometer (BioTek Instruments, Winooski, VT) inside an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). Growth rates were calculated from the generation times determined from the straight-line portions of plots of the logarithm of the optical densities versus time based on the growth curves as described previously (60). The effect of glucose on bacterial growth in the presence of inulin was investigated by inoculating the cell cultures into MRS medium containing 0.1% glucose and 1% inulin. The pH of the cell culture during bacterial fermentation was measured in 5 ml of fermentation culture consecutively at 0, 1, 3, 5, 7, 10, 23, 31, or 48 h with a pH meter (Thermo Scientific Orion Versa Star Pro meter).

Cross-feeding experiments. The presence and quantity of free fructose and glucose in the supernatants during inulin fermentation were determined using a D-fructose/D-glucose assay kit (Megazyme International Ltd., Wicklow, Ireland). The cross-feeding experiments were then carried out by inoculating *Pediococcus acidilactici* YZ049 (1%), which was isolated from the same fecal sample as *L. agilis* YZ050, into sterile filtered supernatants, during which the free fructose was released at the highest level following the growth of YZ050 on inulin.

Thin-layer chromatography. The dynamic changes of inulin composition during bacterial fermentation were analyzed using thin-layer chromatography (TLC). Briefly, cell culture samples (4 μl) were spotted twice onto TLC aluminum silica-gel sheets (St. Louis, MO, USA) using microhematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA, USA). Inulin, glucose, and fructose were used as controls. The TLC sheets were developed twice with a mixture of *n*-butanol, acetic acid, and water at a ratio of 2:1:1 (vol/vol). Bands were visualized by staining with a solvent consisting of 0.5% α-naphthol–5% H_2SO_4–ethanol, followed by air drying at 105°C.

Genome sequencing and analysis. Genomic DNA of *L. agilis* YZ050 and *L. agilis* YZ054 was prepared using a DNeasy blood and tissue kit (Qiagen, Hilden, Germany) and sequenced on an Illumina MiSeq platform (250-bp paired end) at the UC Davis Genome Center DNA Technologies core. Sequencing reads were assembled into contigs using SPAdes 3.10.1 (61), and the quality of assembly was assessed by QUAST 3.0 (62). Genome sequences (contigs) were submitted to the Integrated Microbial Genome Expert Review (IMG/ER) annotation platform (https://img.jgi.doe.gov) under GOLD Project identifier [ID] Ga0314164 (IMG genome IDs, 2799112279 for YZ050 and 2799112280 for YZ054) for annotation and comparative genomic analysis. Using the dbCAN meta server (automated CAZyme annotation) (63), glycosyl hydrolyses (GH) were identified and quantified across genomes to identify shared and unique GH families related to inulin fermentation. The genomic sequence of the best fermenter (*L. agilis* YZ050) was queried for acquired antibiotic resistance genes using ResFinder 3.1 with default settings (64).

Gene expression analysis. *L. agilis* YZ050 was grown on bMRS medium (MRS broth without carbohydrate) supplemented with either 1% glucose or 1% inulin. Cell cultures were collected at mid-log phase ($0.3 < OD_{600} < 0.7$) and were immediately pelleted at $12{,}000 \times g$ for 1 min, suspended in RNAprotect bacterial reagent (Qiagen, Hilden, Germany), and stored at −20°C. Thawed cell cultures were centrifuged at $10{,}000 \times g$ for 2 min, and the cell pellet was washed twice with RNase-free PBS buffer to remove residual reagent. Pellets were then resuspended in 250 μl lysozyme (15 mg/ml; Sigma) which had previously been subjected to filter sterilization in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.2). Following incubation at 37°C for 10 min, the lysate, including any precipitate, was transferred to an RNeasy mini spin column. Total RNA was extracted using an RNeasy minikit (37) (Qiagen, Hilden, Germany) and was eluted into 50 μl RNase-free water. RNA integrity was examined by checking a 1.2% agarose gel, and DNA contaminants were removed using a Turbo DNA-free kit (Ambion) with an extended incubation time (1 h) with DNase. RNA was converted to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems) and was then stored at −20°C.

Primers (F, 5′-GGGCGGCGTCATTAGTTTTT-3′; R, 5′-TCTTGTGTGGCTGCTATTTCCT-3′) (IDT, Coralville, IA) were used to quantify the abundance of *fruA*, and the *GAPD* gene (65) was used as a housekeeping gene for relative quantification calculations. Quantitative PCR (qPCR) was performed using a mixture of Applied Biosystems PowerUp SYBR green mix (Applied Biosystems), 10 pmol primers, cDNA, and DNase-free water. Reactions were performed on a QuantStudio 3 system (Applied Biosystems) as follows: an initial denaturation at 94°C for 2 min, and then 40 cycles of 94°C for 15 s followed by 60°C for 1 min. Threshold cycle ($\Delta\Delta C_T$) values were calculated and used to determine the fold change in expression of *fruA* in the presence of inulin relative to the *GAPD* gene.

Data availability. Assembled contigs from genomes of both *L. agilis* YZ050 and *L. agilis* YZ054 were submitted to the IMG Expert Review (IMG/ER) for annotation (IMG genome IDs, 2799112279 for YZ050 and 2799112280 for YZ054) and are publicly available.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.4 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.02 MB.

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