Stable Engraftment of Bifidobacterium longum AH1206 in the Human Gut Depends on Individualized Features of the Resident Microbiome

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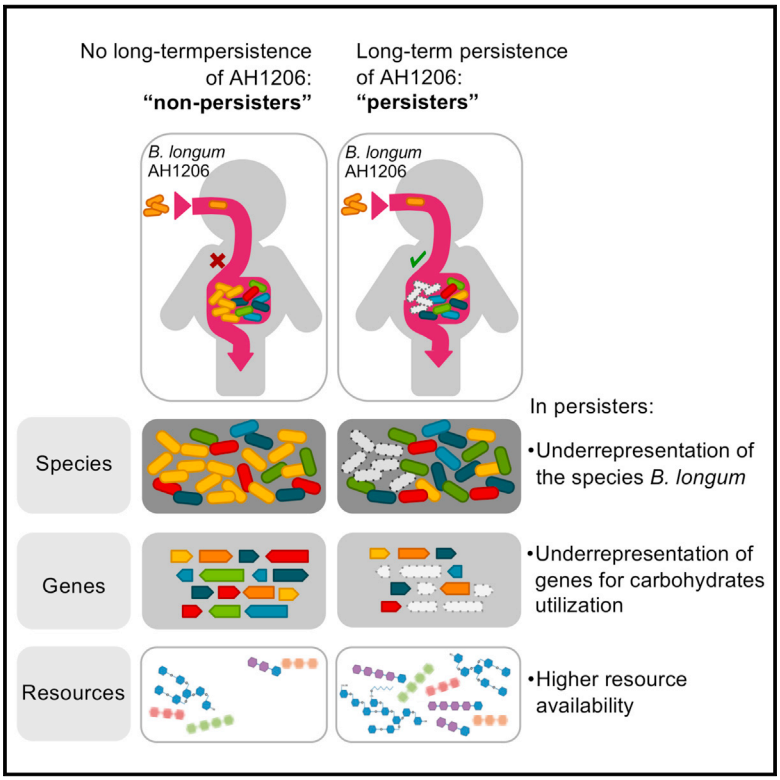
Stable Colonization of a Probiotic *Bifidobacterium* in the Human Gut Depends on Individualized Features of the Resident Microbiome

Graphical Abstract



Highlights

- Orally administered *B. longum* AH1206 persisted in the gut of 30% of humans for 6 months
- AH1206 engraftment did not alter resident microbiota composition or cause GI symptoms
- Lower levels of *B. longum* in the pre-treatment microbiome predict AH1206 persistence
- Underrepresentation of carbohydrate-utilization genes is linked to AH1206 persistence

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In Brief

Understanding the principles underlying long-term bacterial colonization in humans will be crucial to the success of microbiome-based therapies. Maldonado-Gómez et al. show that an orally administered bacterial strain persists long term in a subset of individuals. Engraftment depended on individualized features of the pre-treatment microbiome, likely representing a niche opportunity.

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Article

Stable Colonization of a Probiotic *Bifidobacterium* in the Human Gut Depends on Individualized Features of the Resident Microbiome

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SUMMARY

Live bacteria (such as probiotics) have long been used to modulate gut microbiota and human physiology, but their colonization is mostly transient. Conceptual understanding of the ecological principles as they apply to exogenously introduced microbes in gut ecosystems is lacking. We find that, when orally administered to humans, *Bifidobacterium longum* AH1206 stably persists in the gut of 30% of individuals for at least 6 months without causing gastrointestinal symptoms or impacting the composition of the resident gut microbiota. AH1206 engraftment was associated with low abundance of resident *B. longum* and underrepresentation of specific carbohydrate utilization genes in the pre-treatment microbiome. Thus, phylogenetic limiting and resource availability are two factors that control the niche opportunity for AH1206 colonization. These findings suggest that bacterial species and functional genes absent in the gut microbiome of individual humans can be reestablished, providing opportunities for precise and personal microbiome reconstitution.

INTRODUCTION

The human gut microbiota makes critical contributions to host health (Fukuda et al., 2011; Round and Mazmanian, 2009). However, it has also become increasingly clear that several systemic diseases are associated with aberrations in gut microbiota composition often referred to as "dysbiosis" (Walker and Lawley, 2013). Although few cause-and-effect relationships have been established in humans, strong associations between gut microbiota composition and various pathologies provide a compelling case for the development of strategies that target the gut microbiota and, ideally, reverse dysbiotic patterns (Olle, 2013).

One of the strategies used to modulate the gut microbiota is by dietary administration of live microorganisms, often referred to as probiotics (Hill et al., 2014) or live biotherapeutics (Olle, 2013). There is substantial literature published on the use of lactic acid bacteria in human clinical trials, many of which reported beneficial effects (Ceapa et al., 2013). However, the degree to which clinical improvements have been causally linked to alterations in the changes has not been established (Sanders et al., 2013). Moreover, most strains show high survival rates in the gastrointestinal tract, but are subsequently detectable for less than 2 weeks following cessation of intake (Alander et al., 2001; Charbonneau et al., 2013; Firmesse et al., 2008; Frese et al., 2012; Malinen et al., 2002; Rattanaprasert et al., 2014; Rochet et al., 2008). In most studies, the introduction of a live microbe did not result in significant alterations of the fecal microbiota (Kristensen et al., 2016). Overall, the persistence and ecological impacts of introducing live microbes in the human gut appear limited, and fundamental questions remain regarding the feasibility of establishing live microbes in the human gut and the modulatation of the microbiome through such an approach.

The gastrointestinal tract and its microbiota constitute an interactive ecosystem in which spatial, temporal, and functional characteristics are governed by the principles of community ecology (Walter and Ley, 2011). The introduction of a microorganism into the gut ecosystem can be considered as a biological invasion of a non-native microbe into a resident microbial community (Costello et al., 2012). Invasion ecology provides a framework to study and understand the incursion of a foreign species into an established community (Shea and Chesson, 2002). Several questions addressed in the field directly apply to the application of probiotics and live biotherapeutics. Which traits make a species a successful invader or colonizer? What features

of an ecosystem determine their susceptibility to invasions? What is the ecological impact? Although invasion ecology currently lacks a unifying theory that addresses these questions (Guo et al., 2015), it provides a useful framework for understanding probiotic functionality and impact and ultimately may advance their application. For example, according to theory, specific traits that overcome the environmental filters of the ecosystem are required for biological invasions (Mächler and Altermatt, 2012; Olyarnik et al., 2009). The inability of commercial probiotic strains to persist in the human gut might therefore stem from the absence of key adaptations necessary to successfully compete in this ecosystem. This might be because most probiotic strains currently used belong to bacterial taxa (e.g., *Lactobacillus* species, *Bifidobacterium animalis* subspecies [subp.] *lactis*, etc.) that are allochthonous to the human gastrointestinal tract (Biavati and Mattarelli, 2012; Walter, 2008). There is clearly a knowledge gap regarding how dominant autochthonous members of the gut microbiota would perform ecologically, both in terms of persistence as well as their impact on the resident microbiota, after being introduced into the human gut.

The goal of this study was to assess systematically the ecological role and impact of a bacterial species autochthonous to the human intestinal tract when administered to human individuals, applying a framework based on invasion ecology theory. We selected a strain of *Bifidobacterium longum*, a bacterial species known to be a core member of the human gastrointestinal microbiota that forms stable and numerically dominant populations in most individuals (Martínez et al., 2013; Schloissnig et al., 2013; Turroni et al., 2009). We conducted a double-blinded, crossover, placebo-controlled trial in healthy humans aimed at (1) assessing how long a highly adapted bacterium can be established in the human gastrointestinal tract, (2) determining the impact of this administration on the resident bacterial community, (3) determining the effect of this strain on gastrointestinal symptoms of the subjects, and (4) identifying the precise community features of the resident microbiome that determine colonization.

RESULTS

Introduction of *B. longum* AH1206 into the Digestive Tract of Humans

We performed a human crossover trial in which 23 subjects consumed a daily dose of 10^{10} viable cells of *B. longum* subsp. *longum* AH1206 or a placebo (maltodextrin) in random succession, with 38 or 46 days washout between treatments (Figure 1A). Fecal samples were collected at baseline, days 7 and 14 of the treatment period, and 4, 8, 15, and 28 days after completion of each treatment for ''test of persistence'' (TOP). From fresh fecal samples, total bifidobacteria were quantitatively cultured, and 30 randomly picked colonies per subject at baseline (pre-treatment) and 14 days of treatment were typed by strain-specific PCR. As shown in Figure 1B, this analysis revealed that the test strain was detectable at around 7.4 ± 1.2 $\log_{10}$ viable cells/gram of feces during AH1206 treatment period, but not during the baseline or placebo periods (samples that showed a carry-over effect were excluded), indicating survival of AH1206 during transit through the gastrointestinal tract. No adverse effects were detected during consumption of AH1206 as determined by a standardized questionnaire for. gastrointestinal symptoms (Figure S1).

B. longum AH1206 Colonizes the Gut of a Subset of Individuals for Extended Periods of Time

Three independent approaches were used to test for AH1206 persistence. Quantification of *B. longum* AH1206 in fecal samples via strain-specific quantitative real-time PCR revealed that the strain was undetectable ($<10^4$ cells per gram) prior to treatment in all but one subject (this subject was therefore removed from further analysis). The strain became detectable in all subjects during the treatment period and reached an average population of 7.7 ± 0.9 and 7.6 ± 0.8 $\log_{10}$ cells/gram of feces after 1 and 2 weeks of administration, respectively (Figure 1C). The average levels of AH1206 decreased after cessation of consumption, but remained significantly elevated 28 days after treatment compared to the pre-treatment baseline ($p < 0.05$) (Figure 1C). In addition, AH1206 remained detectable in three subjects during the baseline and placebo phases of the second test period, suggesting the strain could persist in a subset of subjects beyond 28 days after being consumed. To test for long-term persistence of AH1206, we collected fecal samples at 11 and 20 weeks after completion of the trial. This analysis revealed that AH1206 abundance was still significantly elevated in the 20 week follow-up samples compared to pre-treatment baseline levels (Figure 1C). When assessing AH1206 levels among all subjects in an individual basis, it became clear that there was a substantial degree of inter-personal variability in the levels of persistence (Figure 1D). In 14 subjects (64%), the strain became undetectable between day 4 and 28 of washout. However, in eight subjects (36%) the strain remained present at levels between 10^6 and 10^{10} (average 7.43 ± 1.33 $\log_{10}$) cells per gram of feces >88 days and in seven subjects (27%) for >166 days after consumption cessation.

To determine persistence of AH1206 using an approach not reliant on the detection of a single marker gene, we sequenced the genome of *B. longum* AH1206 and identified genes of this strain that were absent in the combined pre-treatment (baseline) microbiomes of all participants. This was done by mapping metagenomic reads against the AH1206 genome and considering only matches with 100% homology. This analysis identified 26 genes specific to AH1206. We then determined the abundance of these genes in samples collected pre-treatment, after 14 days of probiotic consumption, and around 200 days after consumption by mapping the metagenomics sequence reads against the AH1206-specific genes. Most of the genes became detectable during treatment ($p < 0.0001$ for all subjects) and remained significantly higher for persisters after treatment cessation ($p = 0.0313$ for all subjects baseline versus ~200 days TOP), while they disappeared in most non-persisters ($p = 0.2500$ baseline versus ~200 days TOP) (Figure 2A). These results demonstrate that a set of genes unique to AH1206 remained detectable in persisters for around 200 days after consumption of the strain, confirming long-term persistence.

To determine if AH1206 could be recovered after prolonged colonization periods, we selectively cultured bifidobacteria from fecal samples of persisters collected after 20 weeks and typed 30 random colonies per subject by strain-specific PCR. Isolates that typed as AH1206 were recovered from four

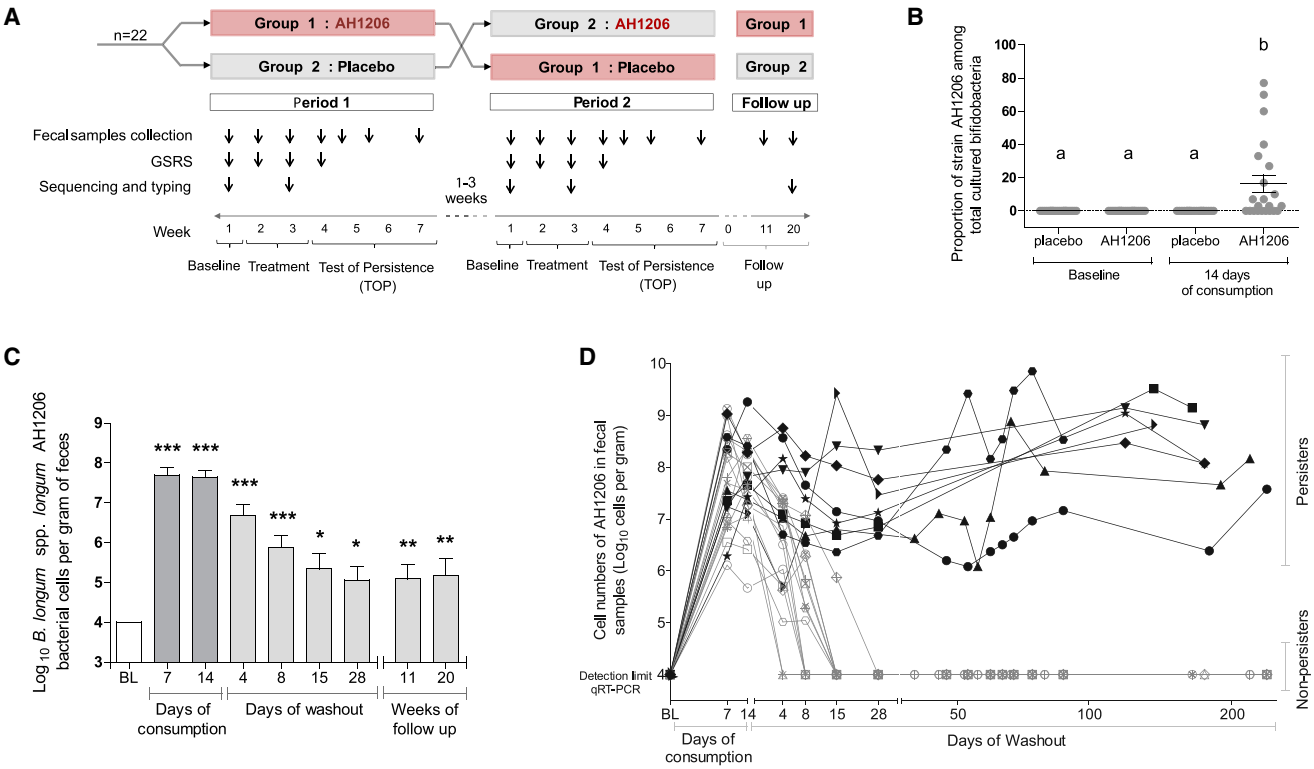


Figure 1. Experimental Design and Survival and Persistence of *B. longum* Subsp. *longum* AH1206 in the Human Gut

(A) Experimental design of the human trial. The arrows indicate the collection of fecal samples, completion of Gastrointestinal Symptom Rating Scale (GSRS) forms, and samples that were sequenced and used for typing of isolates.

(B) Proportions of strain AH1206 among the total cultured bifidobacteria during the two baseline periods, probiotic treatment, and placebo as determined by colony PCR with strain-specific primers. The groups labeled with different letters are significantly different (repeated-measures two-way ANOVA; $\alpha = 0.05$, $p < 0.0001$).

(C) Quantification of *B. longum* AH1206 in fecal samples by strain-specific qPCR. The data are represented as mean and SE. Baseline: BL. The significant difference between time points and baseline are indicated by asterisks. Repeated-measures two-way ANOVA with Dunnett's multiple comparison; ***$p < 0.001$; **$p \leq 0.01$; and *$p \leq 0.05$.

(D) Cell numbers of *B. longum* AH1206 in fecal samples determined by qPCR throughout the entire trial with subjects categorized as persisters (closed symbols) and non-persisters (open symbols).

subjects. Of those, five random isolates per individual were then typed by multilocus sequence analysis (MLSA). This analysis showed that all isolates belonged to the species *B. longum,* and for three subjects, the isolates displayed the same sequence type as AH1206 (i.e., the sequences of all seven housekeeping genes were identical) (Table S1). These findings demonstrated that, despite the difficulty to detect a particular strain by culture among the resident cultivable *Bifidobacterium* population of around 10^9 cells per gram, AH1206 could be recovered from three individuals around 200 days after being administered.

Overall, the results clearly demonstrate that AH1206 can colonize the human gut in high numbers for prolonged periods of time in a subset of individuals. We refer to this subset of individuals as ''persisters'', while those who did not permit colonization are named ''non-persisters''. Persistence was at least 166 days for seven subjects, but in one subject, (J), AH1206 became undetectable in the first follow up sample, which coincided with the use of a prescribed laxative product containing antimicrobial components. Since this subject completed the entire crossover trial and showed persistence of AH1206 for 88 days, which was substantially longer than persistence of in non-persisters (max. 28 days; Figure 1D), this subject was considered a persister. It was therefore included for analyses that compared microbiome at baseline and during treatments, but excluded from the analyses of the microbiome of the 20 week follow up sample.

Administration of AH1206 Does Not Alter the Taxonomic Composition of the Fecal Microbiota

Illumina sequencing of 16S rRNA gene tags (V5–V6 region) revealed that administration of AH1206 did not change the structure of the overall bacterial communities of the fecal microbiota (Adonis permutational multivariate ANOVA [PERMANOVA], $p = 0.999$) and proportions of specific members, including *Bifidobacterium* species (Figures S2A–S2E). In addition, α-diversity was not altered by either treatment (data not shown). Compositional analysis by whole-metagenome sequencing (WMS) confirmed the lack of impact of AH1206 on the taxonomical composition of the fecal microbiota (Figure S2A). However, the functional analysis revealed a significant increase of 29 genes during consumption of AH1206 (Figure 2B; Table S2).

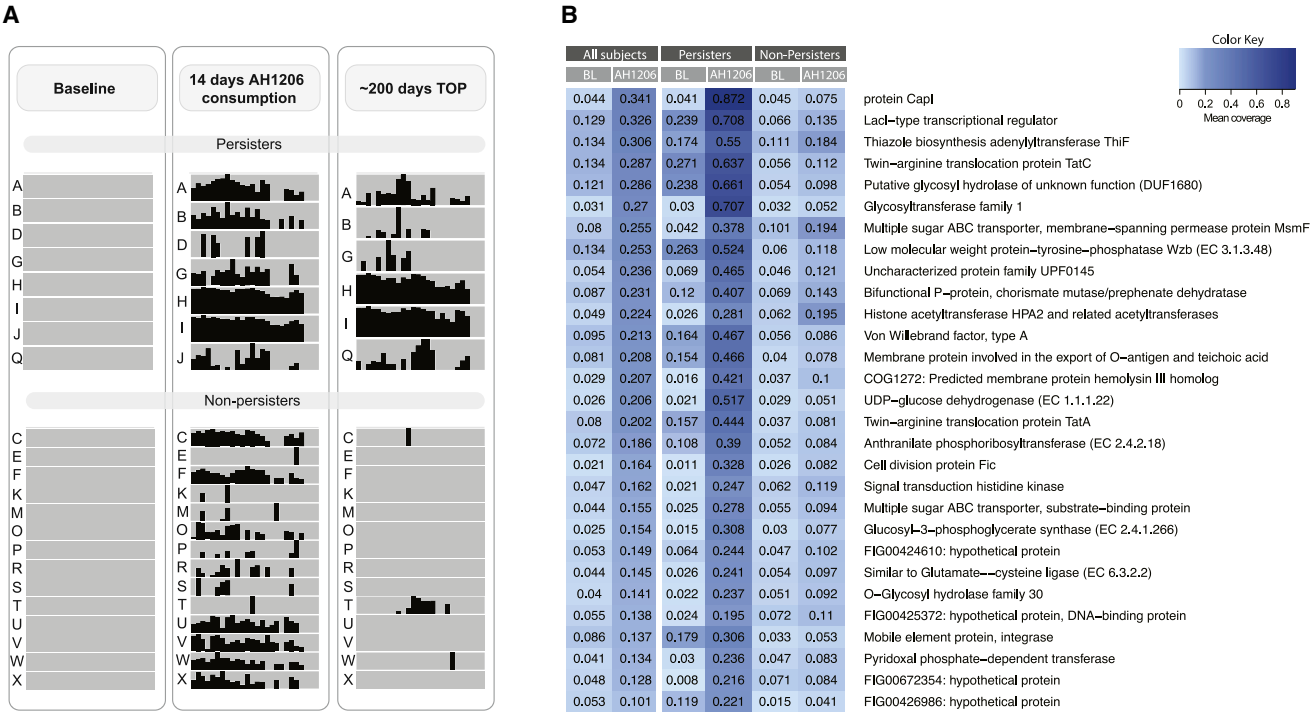


Figure 2. WMS Analysis Confirms Establishment and Persistence of AH1206

(A) Quantification of genes unique to *B. longum* AH1206 in fecal samples taken during pre-treatment baseline, consumption of the strain (14 days), and after around 200 days after consumption. Mean coverage for each AH1206-specific gene was obtained by mapping metagenomics sequence reads against AH1206 specific genes absent in the combined pre-treatment metagenome. The bars represent the relative abundance (y axis) of each of the 26 genes (x axis).

(B) Administration of AH1206 caused enrichment of a set of genes belonging to the species *B. longum* into the gut ecosystem. The relative abundances (mean coverage) before and during consumption are shown.

Remarkably, all these genes were assigned to the species *B. longum* and have more than 98% DNA identity to AH1206 genes (100% coverage). Therefore, although administration of AH1206 did not alter the taxonomic composition of the fecal microbiota, it enriched for functional genes belonging to the species *B. longum*.

Ecological Characteristics of the Resident Gut Microbiota Are Not Major Determinants of Long-Term Persistence

Comparison of overall community configurations in persisters and non-persisters at baseline using β-diversity values (Bray-Curtis distances), NMDS plots, and Adonis PERMANOVA revealed no differences ($p = 0.2488$) (Figure 3A). Higher diversity has been associated with a lower invasion susceptibility of a community, as higher species richness is likely to translate directly into higher diversity of functional traits, elevated resource depletion, as well as an increased probability of competitive species to be present (Díaz and Cabido, 2001; Kennedy et al., 2002; Mallon et al., 2015; Tilman, 2004). However, although trends were observed, neither total α-diversity ($p = 0.0759$ for total operational taxonomic units [OTUs] and $p = 0.2901$ for Shannon diversity index) (Figure 3B) nor functional richness ($p = 0.0759$ and $p = 0.0818$, for single and assembled reads, respectively, data not shown) were significantly higher in non-persisters. Evenness has also been described as a barrier to invasions (De Roy et al., 2013; Wittebolle et al., 2009), but no differences were detected ($p = 0.1423$) (Figure 3C). In addition, disturbances have been shown to facilitate invasion of non-native species (Elton, 1958; Kneitel and Perrault, 2007). However, although higher stability was associated with resistance to short-term colonization of food-born microbes in a rat model (Zhang et al., 2016), long-term colonization of AH1206 in humans was not linked to fecal microbiota stability ($p = 0.6087$ for Morisita-Horn) (Figure 3D).

Community Membership Differs in Persisters and Non-persisters

To determine possible competitive interactions of AH1206 with specific members of the gut microbiota, we compared the composition of fecal bacterial communities at baseline (i.e., pre-treatment) to identify and rank taxa that discriminate persisters from non-persisters using both Wilcoxon tests and random forest (RF) analysis (Figure 3E). Results revealed that Coriobacteriaceae family ($p = 0.0243$, mean importance 2.5), *Collinsella* genus ($p = 0.0169$, mean importance 2.57), and *Collinsella aerofaciens* (OTU 1, $p = 0.0357$, mean importance 1.52) were reduced in persisters. In contrast, *Eggerthella* ($p = 0.0229$, mean importance 3.30) and *Eggerthella lenta* (OTU 52, $p = 0.0497$, mean importance 1.96) were elevated in persisters (Figure S3B; Table S3). The most discriminative taxon, however, was the OTU that represented *B. longum* OTU 11, which showed a higher abundance in non-persisters (Wilcoxon test, $p = 0.0022$; random forest mean importance 6.12) (Figures 3A and S3C).

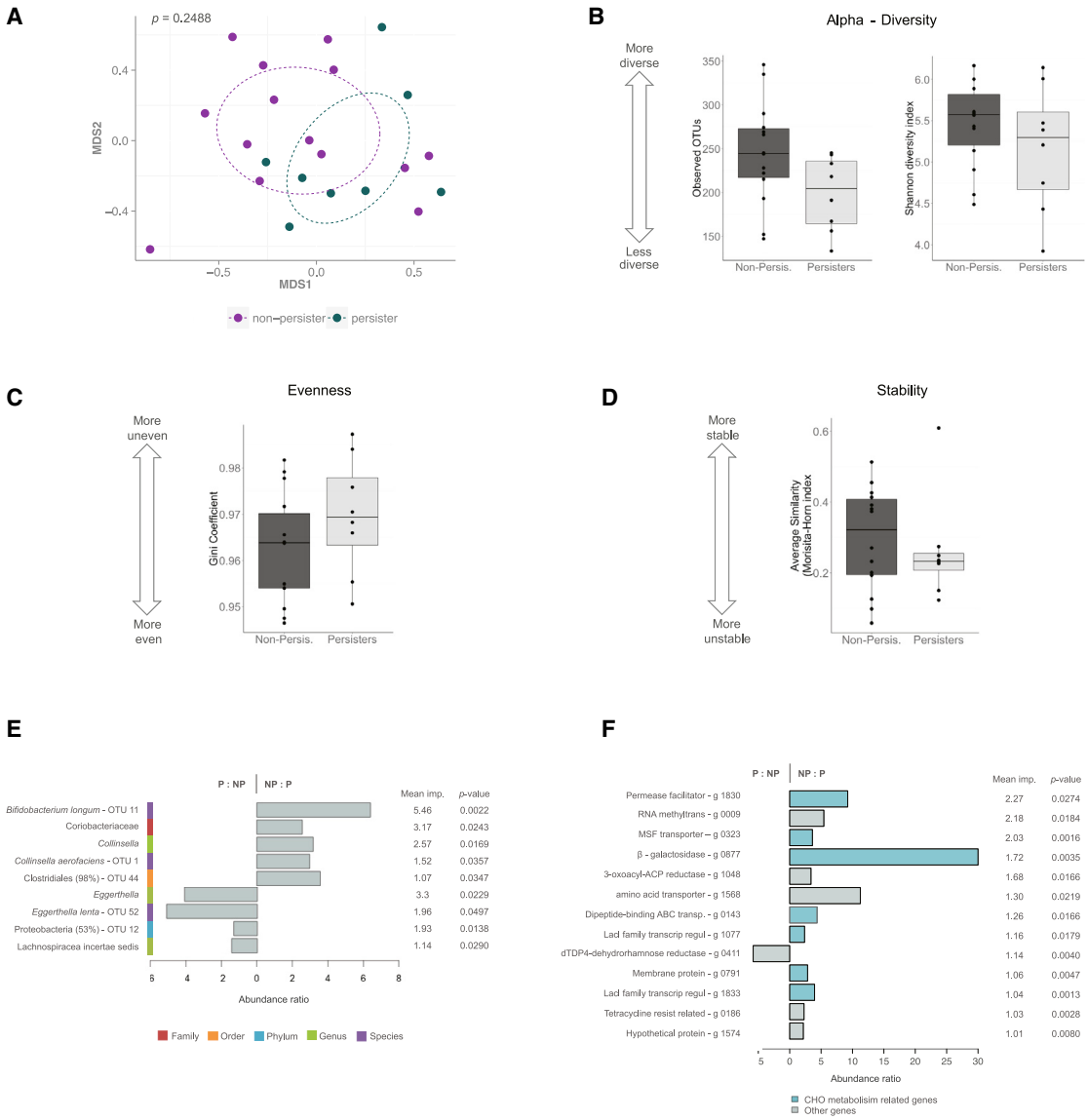


Figure 3. Influence of Ecological Factors on AH1206 Invasibility of Baseline Fecal Microbial Communities

(A) Overall comparison of community profiles between persisters and non-persisters revealed no significant differences (p = 0.2488; Adonis PERMANOVA). The data are shown by NMDS.

(B–D) α-diversity (B), evenness (C), and stability (D) of the fecal microbiota of persisters and non-persisters.

(E and F) Barplots of the average abundance ratio of highly discriminant taxonomic groups (E) and *B. longum* genes in pre-treatment samples in persisters and non-persisters (F). OTUs for which percentages of identity are not indicated have >99% identity to the type strain of the indicated bacterial species.

Mean importance and p values were determined by random forest analysis and Mann-Whitney-Wilcoxon test, respectively. Data in boxplots show the median, first, and third quartiles (hinges); whiskers are extended to the highest value within the 1.5 × IQR of the upper and lower hinges. Persister: P, Non-Persister: NP.

Consistent findings were obtained with minimum entropy decomposition (MED) (p = 0.004) and species-specific qPCR (p = 0.0367) (Figure S3C).

The significantly higher proportions of fecal *B. longum* at baseline in non-persisters suggest that colonization of AH1206 might be prevented by other strains within the same species. Ecology theory predicts that competition would be stronger between phylogenetically related organisms as they share similar traits and therefore display a larger degree of niche overlap. Accordingly, colonization would be prevented by competitive exclusion (Hardin, 1960), a process collectively referred to as phylogenetic limiting (Violle et al., 2011). To test for phylogenetic limiting, we quantified the proportion of 16S rRNA-gene tag sequences from fecal samples obtained during baseline and the placebo period that matched strain AH1206 with decreasing levels of sequence similarity. This analysis revealed significantly higher proportions in non-persisters at higher percent similarity (>97%), while at lower sequence similarities (93%–95%) differences became non-significant (Figure S3D), suggesting that AH1206 colonization is prevented through phylogenetic limiting.

The Microbiome of Persisters and Non-persisters Differs in the Abundance of *B. longum* Genes

The findings described above indicated that baseline *B. longum* abundance is inversely associated with persistence. However, *B. longum* levels did not explain the findings in all subjects, in that some persisters had relatively high levels of *B. longum*. Because traits that define niches and competitive interactions are not necessarily phylogenetically conserved in bacteria, and can differ substantially within the same bacterial species (Zhu et al., 2015), we used WMS data and compared the abundance of genes identified to belong to the species *B. longum*, at pre-treatment baseline between persisters and non-persisters. A Wilcoxon test resulted in 70 genes with differential abundance between persisters and non-persisters (Figure 3F; Table S4). These genes comprised functions involved in metabolism, cellular processes, and information storage and processing, with genes involved in carbohydrate metabolism accounting for many of these functions (38% of the genes with assigned functions). Even though multiple correction test (FDR) rejected all genes identified as significant, 93% of the discriminative genes and 100% of the genes involved in carbohydrate metabolism were enriched in non-persisters, suggesting a clear pattern in the discriminant features.

Given the limited ability of classical hypothesis testing methods in identifying significant features from highly complex and noisy data sets like microbiomes (Knights et al., 2011), we independently identified genes that discriminated persisters from non-persisters using a random forest classifier. This analysis revealed that a total of 60 (90%) of the previously identified genes had good discriminative power (mean importance $\geq$ 0.01) (Table S4). The 13 genes that best discriminate (mean importance > 1) between persisters and non-persisters are shown in Figure 3F. Consistent with the findings above, more than half of the genes were involved in carbohydrate metabolism and all but one were overrepresented in non-persisters.

To determine which taxonomic and gene-based microbiome signatures are the best predictors of *B. longum* AH1206 colonization, all predictive features, taxonomic groups (Table S3), and functional genes (Table S4) were ranked by random forest classifier based on AH1206 persistence. This analysis showed that, overall, *B. longum* genes were better predictors of AH1206 persistence than bacterial taxa (Table S5). However, OTU 11 ranked second, showing that this taxon has a strong predictive value. To test specifically if genes are better predictors of invasion than OTU 11 proportions, we compared independent linear models regressing persistence against the OTU and a gene, respectively, to a combined linear model, using ANOVA. This analysis revealed that genes, 1830 (permeases of the major facilitator superfamily) and 0877 (β-galactosidase ranked sixth), outperform *B. longum* levels at predicting persistence of AH1206 (p value < 0.0005 and p value < 0.001 for 1830 and 0877, respectively).

Genes that Predict Colonization Show Inter-individual Variation among the Resident *B. longum* Populations

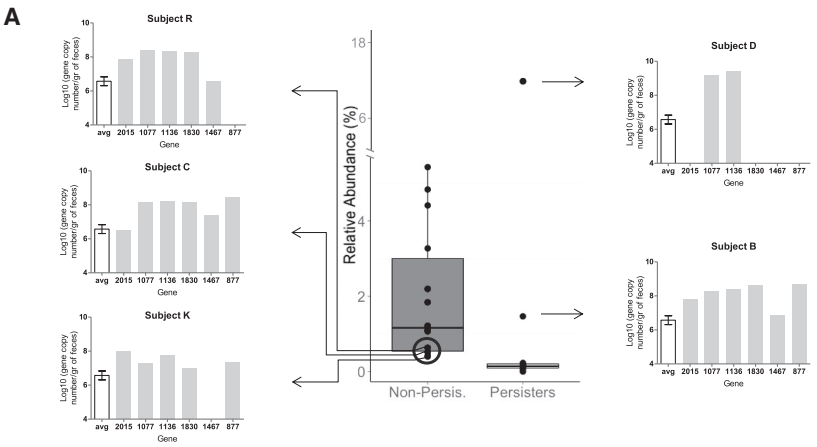
The analyses above showed that although *B. longum* abundance was identified as a significant predictor of invasibility, several *B. longum* genes scored higher. In addition, the subject with the highest population of *B. longum* (around 17% of total bacterial sequences at baseline pre-treatment) was a persister (subject D), while several non-persisters harbored low proportions of *B. longum* (Figure 4A). To determine whether this discrepancy could be explained by inter-personal variation in gene abundance, we selected six discriminant genes that were predicted to have a key role in carbohydrate utilization and quantified their abundance by qPCR. This analysis revealed that at baseline, non-persisters that had relative low levels of resident *B. longum* still harbored, for the most part, comparable levels of these genes as did non-persisters with higher levels of *B. longum* (Figure 4A). In contrast, four of the discriminant genes were not detectable in the outlier persister with high levels of native *B. longum* (subject D). This analysis suggests inter-individual variability of genes among the resident *B. longum* population, which would be consistent with recent findings in several dominant species present in the human gut (Zhu et al., 2015).

To address this variability from a genomic perspective, we analyzed gene coverage within the genomic regions (obtained after metagenome assembly) that included the discriminative genes in individual participants. Gene coverage of the entire gene-containing contigs revealed higher and consistent coverage for non-persisters, reflecting the higher average levels of *B. longum* (Figure 4B). In contrast, the discriminative genes were absent in most persisters, even those that showed a high degree of coverage in the flanking regions (e.g., subject D). Overall, these findings indicate that genes that predict persistence are not phylogenetically conserved, as they were present in non-persisters with low *B. longum* abundance and absent in most persisters with high *B. longum* levels, with only one single exception (subject B).

Genes Predictive of Colonization Encode Functional Traits that Constitute a Niche for AH1206

The underrepresentation of carbohydrate-utilization genes in persisters might result in the availability of resources that constitute a vacant niche for AH1206. For this to apply, two conditions would have to be met. First, strain AH1206 would have to possess the functional traits encoded by these genes to successfully occupy the niche, and second, the differences in the relative abundance of these genes in persisters and non-persisters should disappear after the establishment of AH1206 as a consequence of the strain occupying the niche.

To determine if both conditions apply, we first determined if the genes that were shown to discriminate persisters from non-persisters (Table S4) were present in AH1206. A total of 65 out of the 70 discriminate genes (93%) had homologs (amino-acid similarity >98%, 100% coverage) in the genome of AH1206, suggesting that this strain indeed possesses most of the traits necessary to occupy the vacant niche. Second, to test if the niche becomes occupied after AH1206 is established, we conducted an NMDS analysis using the mean coverage (based on WMS data) of the 70 discriminative genes of persisters and non-persisters at baseline. This analysis revealed that while baseline samples of persisters and non-persisters clustered separately (Figure 5A) (Adonis PERMANOVA, $p = 0.005$), the clustering disappeared during AH1206 consumption (Figure 5B) and ~200 days after consumption (Figure 5C) (Adonis PERMANOVA, $p = 0.2736$ and $p = 0.3383$), respectively. In



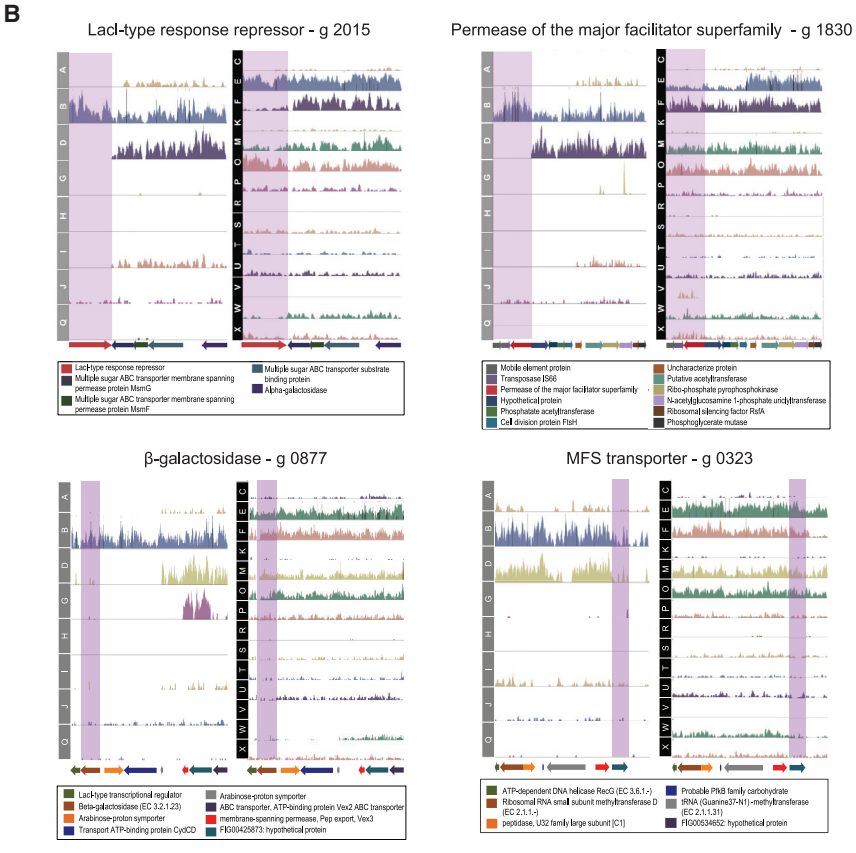


Figure 4. Abundance of Representative Discriminatory Genes in Individual Subjects

(A) Absolute abundance of representative genes that predict colonization as quantified by qPCR of persisters with high levels and non-persisters with low levels of *B. longum*. The average and SE of the absolute abundance of genes in all subjects is also shown.

(B) Relative abundance represented as the mean coverage of the WMS reads within representative genomic regions containing genes that predict persistence. The red-shaded areas display the gene of interest.

DISCUSSION

In this study, we applied a conceptual framework based on invasion ecology to reveal insights into how an autochthonous member of the human gut microbiota colonizes the human gastrointestinal tract, the ecological characteristics of the resident microbiome that influence colonization, and the implications that arise from colonization for both the microbiome and the host. By using a combination of complementary methods, we demonstrated stable persistence of *B. longum* AH1206 in a subset of individuals dependent on individualized features of the resident gut microbiome likely related to variations in the existence of an open niche. One central focus of invasion ecology is the determination of the ecological impact that invasive species have on an ecosystem (Simberloff et al., 2013). This is relevant for the application of probiotics and live biotherapeutics as their effect on the resident microbiota could range from beneficial to detrimental. Our study clearly established that administration of AH1206 neither resulted in changes to the resident microbiome nor detectable adverse effects (gastrointestinal symptoms) for the human hosts. Based on these findings, and in accordance to proposed invasion ecology nomenclature (Davis and Thompson, 2000), we argue that the long-term establishment of AH1206 qualifies this organism as a colonizer and not an invader. Here, we propose the term "engraftment", which refers in general terms to an "incorporation in a firm or permanent way" (Dictionaries, 2014), and in medical terms, to an "incorporation of grafted tissue into the body of the host" (Miller et al., 2005), as the term to refer to the stable establishment of a bacterial strain in the human gut.

The findings of long-term persistence of AH1206 contrast with those from a vast majority of studies with live microbes that show limited persistence. Interestingly, the only other study to our knowledge that established long-term persistence of an

addition, individual carbohydrate-utilization genes showed significant differences in abundance at baseline, yet these differences disappeared during establishment and persistence of AH1206 (Figures 5D–5I). We confirmed quantification of the six genes with key roles in carbohydrate utilization by qPCR. This analysis revealed that the significant differences in abundance of five out of the six genes detected at baseline between persisters and non-persisters disappeared after AH1206 was established (Figure S4). Overall, these findings support the hypothesis that the genes that are underrepresented in persisters encode traits that reflect an open niche, allowing AH1206 to colonize stably.

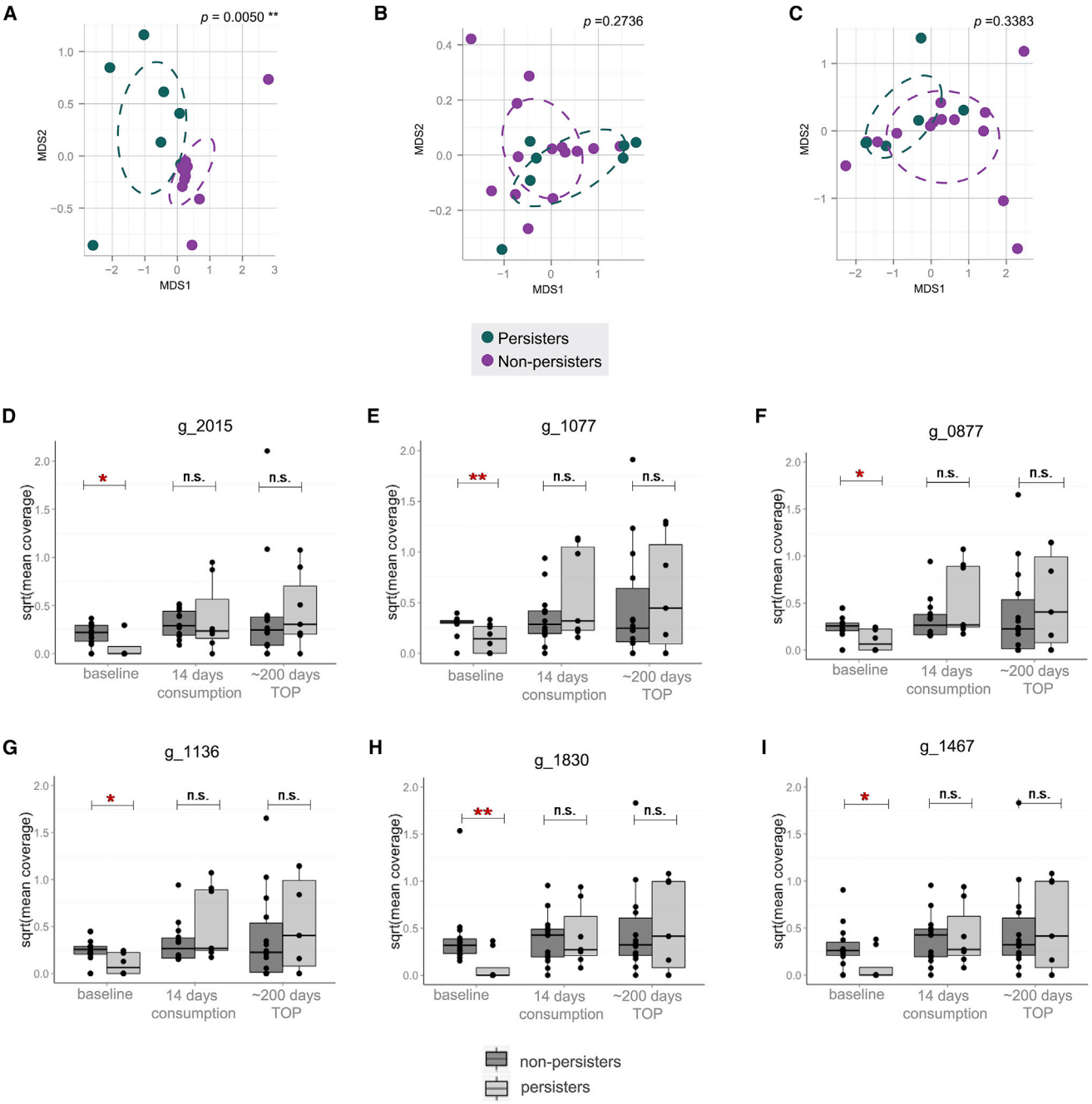


Figure 5. Differences in Gene Distribution in Persisters and Non-persisters Disappear after Engraftment of *B. longum* AH1206

NMDS plots based on Bray-Curtis distance calculated with the abundance of 70 genes that differ in persisters and non-persisters. Baseline (A), probiotic treatment (B), and around 200 days TOP (C). Boxplots showing average gene abundance for genes g 2015, LacI-type response repressor (D); g 1077, LacI-family transcriptional regulator (E); g 1467, (F), and g 1830, permeases of the major facilitator superfamily (COG0477) (G); g 1136, ABC-type sugar transport system permease component (H); and g 0877, β-galactosidase (I). Means and SEM are shown. Wilcoxon test; **p $\leq$ 0.01; *p $\leq$ 0.05; and not significant: n.s.

ingested strain in a subset of participants using a strictly strain-specific approach also utilized a strain of *B. longum* (Fujiwara et al., 2001). Together, these findings suggest that autochthonous core members of the human gut microbiome may display elevated levels of persistence when compared to allochthonous species currently used in commercial probiotics. However, it remains unclear whether the ability of stable engraftment is a more general attribute of autochthonous gut microbes or rather is a particular attribute of specific strains of *B. longum*. Next-generation probiotics and live biotherapeutics based on core members of the gut microbiome (e.g., those that are underrepresented in dysbioses) are currently being developed (Olle, 2013).

The findings obtained in this study are therefore directly relevant for the development of microbiome-targeted strategies as they suggest that an autochthonous bacterial strain may have a higher ecological fitness when administered to humans than allochthonous strains. Although it is not yet known if ecological performance relates to increased probiotic functionality, the findings warrant future research on the health effects on autochthonous gut microbes.

This study identified a strong and statistically significant phylogenetic signal associated with colonization of AH1206, showing that establishment was prevented, likely through competitive exclusion, in the presence of phylogenetically related organisms within the resident microbiome. Therefore, our findings support Darwin's naturalization hypothesis that states that introduced species are more successful at surviving in communities in which their close relatives are absent (Darwin, 1859). In contrast, Li et al. (2016) showed that bacterial strains introduced through fecal transplants are more likely to be established in the recipient human if the species are already present. Similarly, Stecher et al. (2010) suggested a "like will to like" rule in that colonization of both pathogens and commensals in the murine gut is favored by the presence of related species. Inconsistencies regarding the role of phylogenetic relatedness in colonization success have been observed in other ecosystems and have been attributed to the notion that phylogenetic patterns of community assembly are often scale dependent and confounded by extrinsic factors such as fluctuations in environmental conditions (Li et al., 2015; Proches et al., 2008; Thuiller et al., 2010). In this respect, it is important to recognize that environmental filtering (selection for phylogenetically related members that share traits that enable colonization) and competitive exclusion (selection for phylogenetically distinct members with different traits that result in niche differences) act together in ecosystems (Mayfield and Levine, 2010). The processes that determine which species co-exist and assemble within gut microbial ecosystems are therefore likely to be context, scale, and taxon dependent.

Although our findings indicate competitive exclusion of AH1206 by the resident *B. longum* population, exceptions existed where either persisters harbored high populations of *B. longum* or non-persisters had low levels. In this respect, it has to be noted that functional traits (including those that define niches) are not necessarily evolutionarily conserved in bacteria (Shafquat et al., 2014). This can directly influence the degree by which phylogenetic relatedness conveys competitive interactions among members of the human gut microbiome. Most bacterial species, including *B. longum*, possess pan-genomes with individual strains displaying variation in genomic content (Chaplin et al., 2015; O'Callaghan et al., 2015). Horizontal gene transfer can further lead to similar genetic information being shared among bacterial taxa that are not closely related, resulting in phylogenetically distant taxa with similar traits (Boto, 2010). Our trait-based analysis demonstrated that specific functional genes of the species *B. longum* are better predictors of engraftment when compared to *B. longum* itself. It appears that the niche of AH1206 is occupied by *B. longum* in most non-persisters, although exceptions exist in which it is either not occupied by *B. longum* in persisters (through inter-species genetic variability) or is occupied by other taxa in non-persisters (through HGT). There was only one exception in our study that did not concur with this explanation (subject B), a persister harboring both high levels of *B. longum* and discriminative genes (Figure 4). We speculate that persistence in this individual might have been caused by AH1206 being able to compete with the resident strain(s), which is supported by a metagenomics analysis of single nucleotide polymorphisms that suggests that AH1206 co-exists with native members of *B. longum* (data not shown).

The majority of the genes that discriminated the pre-treatment microbiome of non-persisters from persisters were underrepresented in persisters and involved in carbohydrate utilization. This finding suggested that the establishment of AH1206 was dependent on resource availability, or accordingly, a niche opportunity (Shea and Chesson, 2002). Some of the genes encode for β-galactosidases and LacI-type regulatory proteins, indicating that galactose-containing substrates, which have been shown to contribute to the nutritional niche of bifidobacteria in the human gut (Davis et al., 2011; O'Connell et al., 2013), contribute to the colonization success of AH1206. Accordingly, homologs of the discriminative genes g0877 and g2015 are present in gene clusters of AH1206 that are highly conserved in *Bifidobacterium breve* (Figure S5). These particular regions have been functionally characterized in *B. breve* to be involved in the utilization of the plant-derived carbohydrates galactan and melezitose, respectively (O'Connell Motherway et al., 2011; O'Connell et al., 2014). It is therefore possible that the variation of dietary habits (not assessed during this study) might have contributed to the individual differences in AH1206 colonization. Overall, the findings support the theoretical framework proposed by Mallon et al. (2015), which postulates that resistance of microbial communities to invasion is linked to resource depletion. However, engraftment of AH1206 appears to be less dependent on species richness and evenness and more on the presence of community members that are functionally similar to AH1206. Our findings are in complete agreement with mechanistic studies in mice showing that colonization of *Bacteroides* strains was prevented only by members of the same species and driven by competition for specific carbohydrates (Lee et al., 2013). Deletion of a polysaccharide utilization locus in *Bacteroides fragilis* reduced competitive exclusion and allowed colonization of a second strain. These findings provide an explanation for why the persisters with high baseline *B. longum* levels in our study may still allow engraftment of AH1206 if the resident strains lack specific carbohydrate-utilization genes.

Overall, the findings of this study suggest that specific core members of the gut microbiome and functional genes associated with them can be established in humans in which they are absent. This important discovery could provide a basis for precision microbiome reconstitution to redress specific dysbiotic patterns. In addition, this study provides an early demonstration for how engraftment of a bacterial strain can be predicted based on microbiome features. Such predictions could be applied to personalize the use of probiotics and live biotherapeutics to increase their beneficial value. More extensive human studies with autochthonous bacterial strains are warranted to establish their health benefits, develop and validate reliable predictive models, and test the potential to personalize such approaches based on these models. Moreover, the ability of a probiotic strain to persist when specific niche-defining resources are available reinforces the potential of the synbiotic concept (Kolida and

Gibson, 2011). If systematically developed based on ecological criteria (Krumbeck et al., 2015), synbiotics could ensure the provision of growth substrates (in the form of dietary carbohydrates or prebiotics) that enable long-term persistence of probiotic strains.

EXPERIMENTAL PROCEDURES

The human trial was approved by the Institutional Review Board of the University of Nebraska (IRB Approval Number: 222-12-FB) and registered in Clinical Trials web page (Identifier: NCT01650753). Written informed consent was obtained from all subjects.

Experimental Design

A double-blind, placebo-controlled, human crossover study with two 7-week test periods (including a baseline, a 14-day treatment, and a 28-day TOP) in 24 healthy human subjects (22 and 38 years of age) was conducted with the primary objective to determine acceptability and safety of *B. longum* subsp. *longum* AH1206 at a daily dose of 10^{10} cells (Figure 1A). In total, 16 fecal samples were collected from every subject for selective culture of bifidobacteria (for strain typing of bacterial isolates) and DNA isolation (for sequencing and qPCR). To evaluate tolerance of AH1206 intake, subjects provided weekly reports of gastrointestinal symptoms as described previously (Rattanaprasert et al., 2014). Detailed information about the experimental design, bacterial culture and strain typing, and DNA isolation is provided in the Supplemental Experimental Procedures.

Quantification of Strain AH1206 and the Species *B. longum* by qPCR

B. longum AH1206 was quantified in fecal DNA by qPCR using a strain-specific primers and probe system (probe #89; Roche, Universal ProbeLibrary). *Bifidobacterium longum* was quantified by qPCR with species-specific primers. Details about the PCR program, primers, and validation of the PCR system can be found in the Supplemental Experimental Procedures.

Sequencing of 16S rRNA Gene Tags and WMS

The fecal microbiota in samples collected during the baseline (of AH1206 treatment period), the last day of AH1206 consumption, the last day of placebo consumption, and 28 days of TOP was characterized by next-generation sequencing as described by Krumbeck et al. (2015). WMS was performed with fecal samples obtained during baseline, the last day of consumption of the probiotic, and the 20 week follow-up sample using the Illumina HiSeq 2500 platform. The accession number for the raw sequences reported in this paper is NCBI SRA: PRJNA324129. The software Anvi'o (Eren et al., 2015) was used to generate, visualize, and quantify genomic bins in the metagenomic data set. See Supplemental Experimental Procedures for details on sequencing and a description of the bioinformatics analysis.

Ecosystem Characteristics

Measurements of α and β diversity as well as community evenness were calculated using QIIME tools v 1.8.0 (Caporaso et al., 2011). β-diversity measurements were used to assess temporal stability of an individual's fecal microbiota as the average of the distance between all consecutive time points. Bray-Curtis distances between samples were calculated and visualized in by non-metric multidimensional scaling (NMDS). See Supplemental Experimental Procedures for details.

Absolute Quantification of Functional Genes by qPCR

To confirm findings obtained with the metagenomics analysis, qPCR systems were developed for representative genes discriminant between persisters and non-persisters (see Supplemental Experimental Procedures).

Statistical Methods

Repeated-measures two-way ANOVAs with Bonferroni correction, repeated-measures one-way ANOVAs, and Friedman tests were used to determine the effect of treatment on microbiota taxonomic and functional composition and diversity measurements. Mann-Whitney-Wilcoxon tests were used to conduct pairwise comparisons between time points and compare bacterial and gene abundances, α-diversity, stability, evenness, and functional richness between persisters and non-persisters. Permutational multivariate ANOVA (Adonis PERMANOVA with 200 permutations) based on sample distances (β-diversity) was used to test for changes in the community composition. In order to identify discriminative independent variables, generate predictive models, and compare their performance, and compare the importance of variables that predict the persistence of AH1206, random-forest analysis and ANOVA tests were used. See the Supplemental Experimental Procedures for detailed descriptions of the statistical methods.

ACCESSION NUMBERS

The accession number for the raw 16S rRNA, metagenomics, and MLST reported in this paper is NCBI SRA: PRJNA324129.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2016.09.001.

AUTHOR CONTRIBUTIONS

J.W. designed the project. M.X.M.-G. conducted the human trial and experimental work. M.X.M.-G. and I.M. performed gut microbiome analyses. M.X.M.-G., I.M., P.V., B.H., and D.K. performed statistical and machine learning analyses. F.B. designed and initially validated specificity of strain-specific primers. F.B., A.O'C., M.V., and D.v.S. sequenced and annotated AH1206 genome. F.B. and D.v.S. were responsible for comparative genomic analyses and gene interpretations. J.W., R.W.H., and I.M. supervised and oversaw research activities. M.X.M.-G. and J.W. wrote the manuscript, with I.M., D.v.S., D.K., and R.W.H. providing text and thorough editing. All authors helped with data interpretation and the discussion of the results.

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