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RESEARCH PAPER



## A novel probiotic, *Lactobacillus johnsonii* 456, resists acid and can persist in the human gut beyond the initial ingestion period

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### ABSTRACT

Probiotics are considered to have multiple beneficial effects on the human gastrointestinal tract, including immunomodulation, pathogen inhibition, and improved host nutrient metabolism. However, extensive characterization of these properties is needed to define suitable clinical applications for probiotic candidates. *Lactobacillus johnsonii* 456 (LBJ 456) was previously demonstrated to have anti-inflammatory and anti-genotoxic effects in a mouse model. Here, we characterize its resistance to gastric and bile acids as well as its ability to inhibit gut pathogens and adhere to host mucosa. While bile resistance and in vitro host attachment properties of LBJ 456 were comparable to other tested probiotics, LBJ 456 maintained higher viability at lower pH conditions compared to other tested strains. LBJ 456 also altered pathogen adhesion to LS 174T monolayers and demonstrated contact-dependent and independent inhibition of pathogen growth. Genome analyses further revealed possible genetic elements involved in host attachment and pathogen inhibition. Importantly, we show that ingestion of *Lactobacillus johnsonii* 456 over a one week yogurt course leads to persistent viable bacteria detectable even beyond the period of initial ingestion, unlike many other previously described probiotic species of lactic acid bacteria.

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Gut microbiota; probiotics; microbiology; bacteria; lactobacillus


The human gastrointestinal (GI) tract is home to over 500 species of bacteria in a given individual.<sup>1</sup> These microbes and their byproducts play an important function in our bodies as any other organ, and have been subject to co-adaptation with their hosts for at least 500 million years.<sup>2</sup> The microbiome has been demonstrated to have an impact on nearly every aspect of human health. Gut microbiota composition is a risk factor for inflammatory bowel diseases (IBDs) such as Crohn's Disease and ulcerative colitis.<sup>3</sup> Resident bacteria play a critical role in the development of healthy immune system function.<sup>4</sup> Microbial metabolic processes generate short-chain fatty acids (SCFAs) that provide a primary energy source for the cells of the gut,<sup>5,6</sup> as well as vitamins and amino acids necessary for systemic health.<sup>7</sup> Microbiome composition affects efficiency of nutrient metabolism, playing a role in obesity

risk and even cholesterol levels.<sup>8–10</sup> There is even evidence to suggest that the microbiome plays a role in normal CNS function and depression incidence.<sup>11</sup> The manipulation of the microbiome by purposefully seeding certain probiotic, or beneficial, strains for their properties may allow us to better control every one of these endpoints – with the right level of understanding. However, these multifactorial effects are often difficult to study in a well-controlled environment.

A probiotic with strong clinically demonstrated effects could be employed in a number of ways to induce a wide variety of health benefits, both as a treatment and as a health maintenance supplement. Probiotic *lactobacillus* bacteria have been demonstrated to reduce inflammation both in the gut itself and systemically,<sup>12</sup> making them a tempting therapy for researchers seeking effective treatments for inflammatory gut conditions, such as ulcerative colitis

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and Crohn's disease, although results as of yet have not been particularly strong.<sup>13–15</sup> Despite a global market value in the tens of billions of US dollars, growing by over 10% per year,<sup>16</sup> there is, as of the end of 2017, no probiotic that is clinically approved by the FDA. The popular probiotic *Lactobacillus rhamnosus* GG did not yield significant results in a clinical trial against vancomycin-resistant enterococcus (VRE),<sup>17</sup> and *L. johnsonii* NCC 533 failed in a clinical trial against Crohn's disease.<sup>18</sup>

Although the use of probiotics to treat chronic states like inflammation and provide subtle benefits to health is an attractive goal, especially due to the millions of people who suffer from some form of inflammatory disease,<sup>19</sup> our incomplete understanding of the multifactorial complexity of interactions between the microbiome and immunity makes it difficult to accurately predict which treatments will work and why.<sup>4</sup> Instead, we suggest that an interim application, with more clearly definable endpoints, be the primary focus of treatments in the nascent field of clinical probiotics. In particular, the use of probiotic strains to both shorten active and prophylactically prevent instances of pathogenically induced diarrhea should be a top priority – especially given that diarrheal disease led to 1.3 million deaths worldwide in 2015.<sup>20</sup>

In this regard, the most clearly demonstrable and valuable attribute of probiotic strains is their capability to reduce the adhesion and subsequent activity of pathogenic strains. Probiotic bacteria can perform this useful service via a number of mechanisms, including indirect competition for nutrients and binding sites in the host,<sup>21</sup> and directly through the production of bacteriocins, acids, and other compounds.<sup>22–24</sup> In animal models, *Lactobacillus* species have been broadly shown to inhibit gut pathogens. *L. johnsonii* NCC 533 (formerly referred to as La1) been shown to reduce gastritis induced by *H. pylori* and infection by the diplomonad *G. intestinalis* in gerbils, while *L. johnsonii* FI9785 inhibited *C. perfringens* colonization in chickens.<sup>25–27</sup> Multiple probiotic formulations, including *Lactobacillus* and *Bifidobacterium* strains, have been shown to reduce the duration of diarrhea and enterocolitis in children.<sup>28</sup>

Considering the strong evidence for anti-inflammatory and antipathogenic effects, it is clear that both the search for new probiotic strains

and the continued testing of existing ones will yield clinically effective treatment methods. For dedicated clinical application, strains will need to be characterized based on their effects on individual disease states. For example, a strain that induces a beneficial cytokine response under certain circumstances might exacerbate pathogen-induced disease in others by interfering with the immune response.<sup>29</sup> For this reason, it is imperative that every isolated strain of probiotic bacteria be individually tested and characterized in multiple models. A rationally designed set of experiments demonstrating survival, adhesion, and pathogen inhibition should be carried out with strains that show promising attributes.<sup>30</sup>

*Lactobacillus johnsonii* strain 456 (LBJ 456) was discovered by examining bacterial strains overrepresented in the microbiota of a cancer-resistant colony of DNA-repair deficient mice.<sup>12</sup> Considering that oral gavage with this strain over the course of 4 weeks was capable of significantly reducing systemic inflammation and genotoxicity in this mammalian model, LBJ 456 represents a strong candidate probiotic strain. As *Lactobacillus* bacteria, this strain is conducive to use not only in traditional manners of application such as supplement pills, but also in active foodstuff delivery methods such as yogurts and kombuchas. In this article, we further demonstrate this strain's potential for use in humans by characterizing its acid and bile resistance as well as its host adhesion, pathogen inhibition, and colonization properties. We also analyze the LBJ 456 genome to investigate the genetic basis underlying some of these properties.

## Results

### ***L. johnsonii* 456 shows exceptional resistance to gastric acid and moderate bile acid tolerance**

To assess LBJ 456's viability in the GI tract, we compared its relative tolerance to simulated gastric acid (SGA) and bile against a panel of type strains representing commonly used probiotic species, including the two commercially available strains *B. lactis* HN019 and *L. plantarum* 299V (Table 1). We also included *S. salivarius* subsp. *thermophilus*, which is not a *Lactobacillus* or *Bifidobacterium* species but is still considered

**Table 1.** Strains and cell lines used.

Probiotic Bacterial Strain	Source and Description
<i>Lactobacillus johnsonii</i> 456	Mouse gut isolate, UCLA
<i>Lactobacillus johnsonii</i> VPI 7960	ATCC 33200 (type strain)
<i>Lactobacillus casei</i> 03	ATCC 393 (type strain)
<i>Lactobacillus acidophilus</i> ATCC 4356	ATCC 4356 (type strain)
<i>Lactobacillus plantarum</i> 299V	Purified from commercial product
<i>Streptococcus salivarius thermophilus</i> NCD0 573	ATCC 19258 (type strain)
<i>Bifidobacterium lactis</i> HN019	Purified from commercial product
Pathogenic Bacterial Strain	<b>Source and Description</b>
<i>Escherichia coli</i> H10407	ATCC 35401 (enterotoxigenic)
<i>Enterococcus faecalis</i> NCTC775	ATCC 19433 (type strain)
<i>Salmonella enterica enterica</i> serovar typhimurium	ATCC 13311 (enteropathogenic)
Human Cancer Cell Line	<b>Source and Description</b>
Caco-2	ATCC HTB-37, human colorectal adenocarcinoma, enterocyte-like
LS 174T	ATCC CL-188, human colorectal adenocarcinoma, goblet cell-like

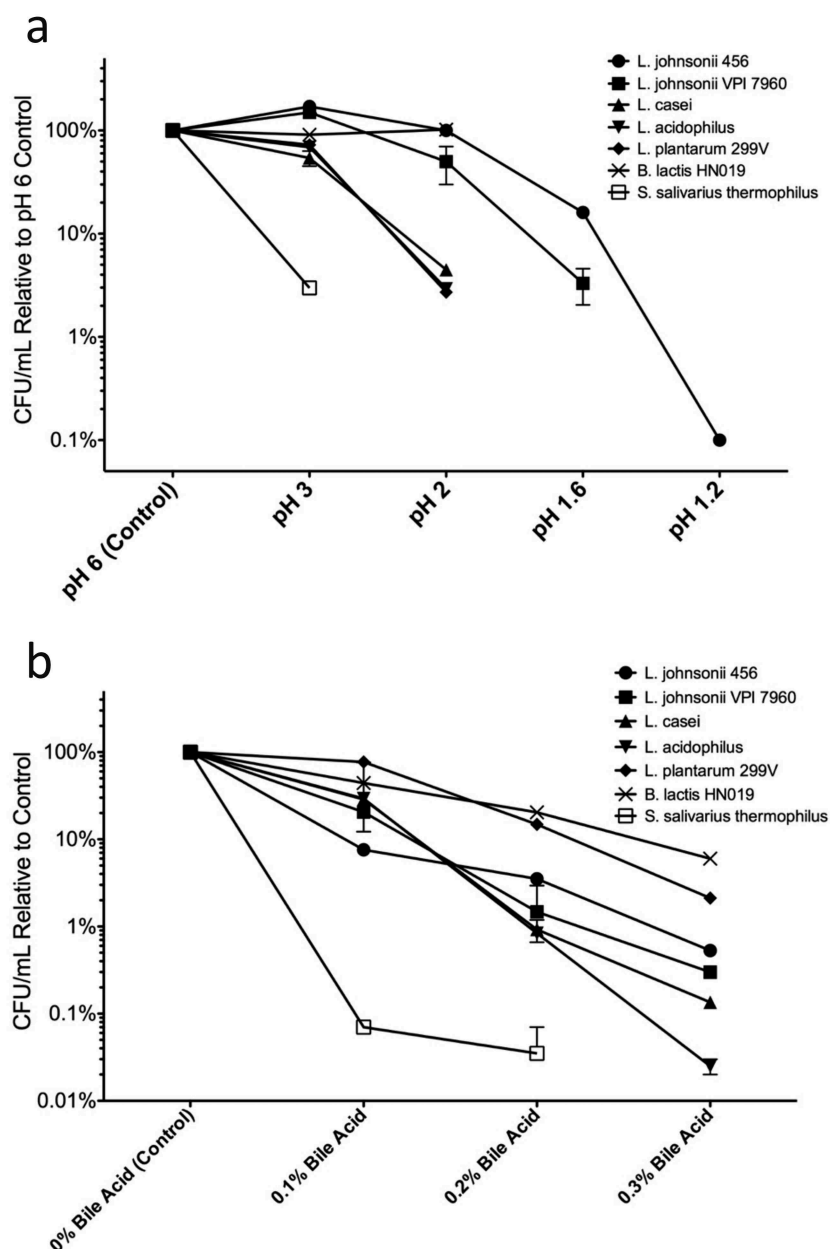
a “probiotic” by the European Food Safety Administration for its potential assistance in lactose digestion, and traditional role in yogurt preparation.<sup>31</sup> We measured the viable bacteria recovered for each strain after incubation for 2 hours in gastric conditions that ranged from pH 3 to pH 1.2 (Figure 1a, Fig. S1). While recoverable CFU from each strain generally decreased with lower pH conditions, the viability of the two *L. johnsonii* strains LBJ 456 and VPI 7960 was observed to increase beyond the input CFU at pH 3. Moreover, the viability of LBJ 456 in particular was consistently the highest at all pH conditions tested. Importantly, LBJ 456 was also the only strain to show viability at pH 1.2, albeit at a 1000-fold reduction compared to its viability in a control pH 6 incubation.

Next, we compared the growth of the probiotic strain panel in media under different physiologically relevant bile acid conditions (Figure 1b, Fig. S2). A relatively bile acid rich environment (0.3%/ ~ 6mM) impairs the growth of LBJ 456 to a certain extent, but it still reached concentrations of around  $4\text{--}5 \times 10^6$  cells/mL after 24 hours (as opposed to nearly  $1 \times 10^9$  cells in bile-free media control). Interestingly, bile acid resistance among strains allowed for clear delineation between genera, especially at 0.2 and 0.3%. *B. lactis* growth was only decreased to about 10% of control at 0.3% bile acid. *Lactobacillus* species as a whole had moderate resistance, but *L. plantarum* 299V’s growth was impaired the least of all *Lactobacillus* strains. *S. salivarius* was highly sensitive to acid and bile acid exposure and was unable to grow at

all in 0.3% bile acid, suggesting that this strain likely does not survive in the human GI tract.

#### ***L. johnsonii* 456 adheres most strongly to goblet cell-like monolayer forming line LS 174T**

Bacterial adhesion to the intestinal epithelium, as well as the associated mucus secretions, has long been considered an important probiotic criterion.<sup>32,33</sup> We evaluated the host attachment capabilities of our probiotic panel by measuring the percentage of CFU-forming cells that could adhere to two monolayer-forming human cancer cell lines, the enterocyte-like Caco-2 line and the goblet cell-like LS 174T line (Figure 2). All tested probiotics, including LBJ 456, adhered better to the secreted mucin-rich LS 174T cultures than to Caco-2 cultures. The relative adhesion of each strain to the two monolayer types did not directly correlate ( $r = -0.1429$ , Spearman’s rank correlation). For example, *L. casei* showed the lowest rate of adhesion to Caco-2, but was one of the most adherent strains on LS 174T. These data suggest potential specialization of different *Lactobacillus* strains to better adhere to different gut mucin phenotypes. Adhesion of LBJ 456 to LS 174T was observed to a greater extent than the commercially available probiotic strains *L. plantarum* 299V and *B. lactis* HN019. LBJ 456 also adhered to LS 174T an order of magnitude better than *L. acidophilus* ATCC 4356, which demonstrated strong adhesion in the 5 hour exposure model used by Jung et al.<sup>34</sup> Interestingly, *S. salivarius* adhered relatively well to both cell lines, despite the fact that its survival until that point in the



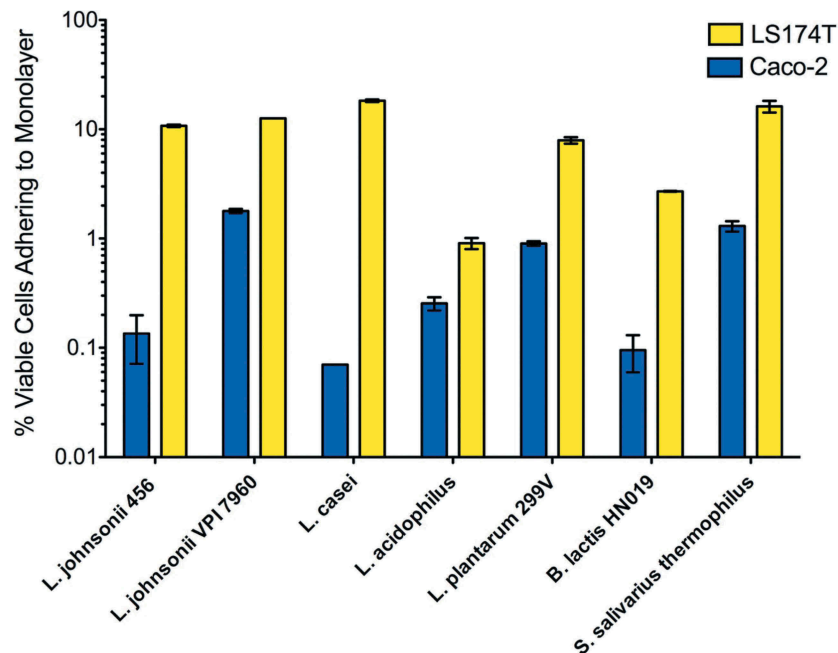
**Figure 1.** *L. johnsonii* 456 has exceptional resistance to simulated gastric conditions and moderate bile acid tolerance. (a) Survival of *Lactobacillus* and other probiotic associated strains after 2 hours in SGA. (b) Relative growth capability of test strains after growth in media supplemented with bile acids. Results are expressed as means and SEMs ( $n = 2$ ). Each experimental pH or bile acid concentration vs. control was run as a separate experiment, and all experiments were repeated at least twice. Specific representation at each pH reading by histogram is included in Supplemental Figures S1 and S2.

digestive tract would seem unlikely based on acid and bile sensitivity.

### ***L. johnsonii* 456 significantly alters pathogen adhesion to LS 174T, but not to caco-2 monolayers**

Using the LS 174T and Caco-2 monolayer models described above, we examined the capacity of

adherent LBJ 456 to inhibit the attachment of three pathogenic strains of gut bacteria: Enterotoxigenic *E. Coli* (ETEC), *E. faecalis*, and *S. enterica*. (Table 1). Pretreatment of Caco-2 monolayers with LBJ 456 did not lead to any significant difference in the level of pathogen adhesion, likely because of the LBJ 456's limited ability to adhere to this cell type (Figure 3a). However, LBJ 456 pretreatment of LS 174T monolayers led



**Figure 2.** *L. johnsonii* 456 adheres best to a goblet cell-like gut epithelial monolayer. Relative adhesion of viable cells shown as a percentage of viable cells plated ( $2\text{--}5 \times 10^8$  cells/well) ( $n = 2$ ). Data expressed as means and SEM. Experiment was repeated twice.

to significant changes in pathogen adhesion (Figure 3b). (ETEC) and *S. enterica* adhesion were reduced by about 30% ( $p = 0.0423$ ) and 40% ( $p = 0.0658$ ), respectively. However, *E. faecalis* adhesion increased slightly after pre-treatment with LBJ 456, indicating that the inhibitory capability of this strain is not universal.

#### ***L. johnsonii* 456 significantly inhibits pathogen growth in co-culture**

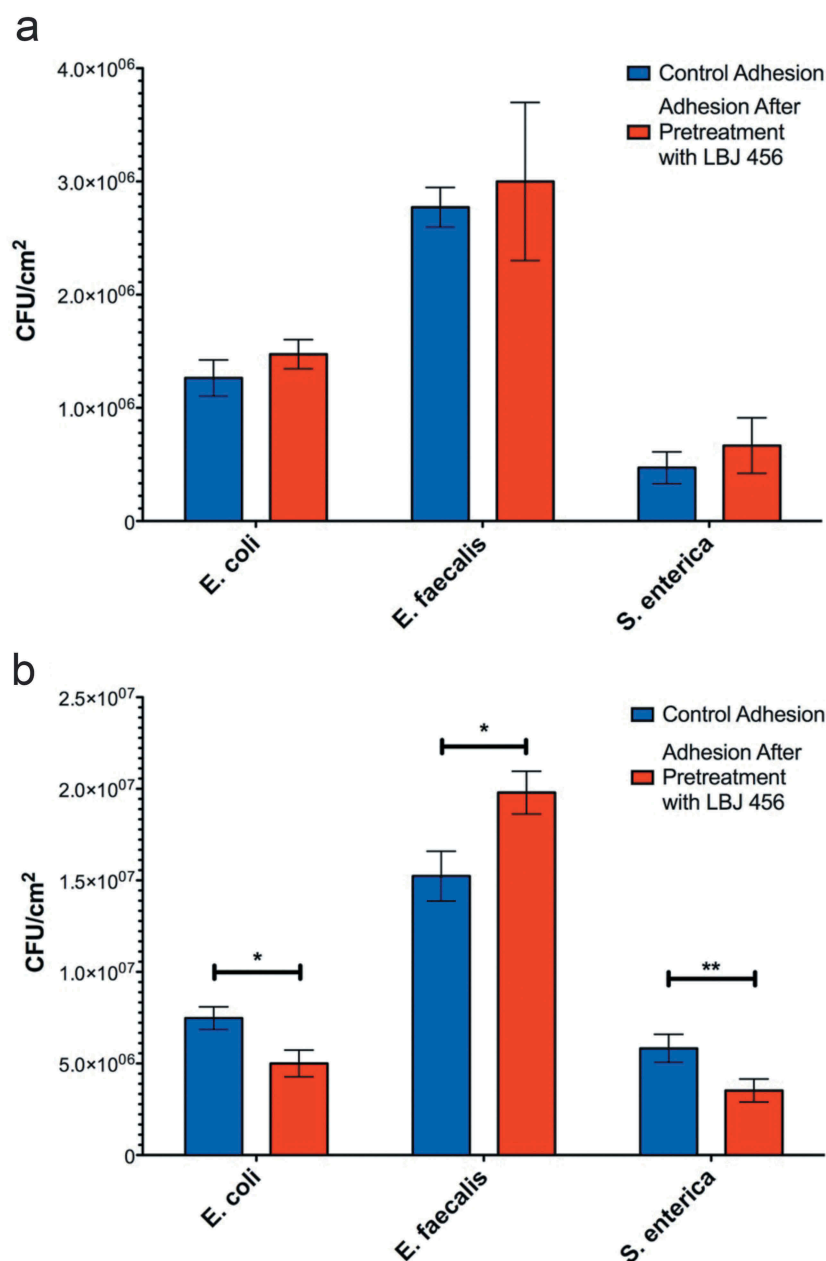
We determined whether LBJ 456 can directly inhibit the growth of pathogens by co-culturing it with equal CFU ratios of each pathogenic strain. The growth of all three pathogens was significantly reduced when they were co-cultured with LBJ 456 (Figure 4). ETEC growth was suppressed from a final concentration of  $5$  to  $2 \times 10^8$  cells/mL. Final *E. faecalis* concentrations were cut by more than half as well. The greatest effect was seen against *Salmonella*, with a full order of magnitude decrease in viable CFU detected from coincubation. ( $p < 0.0001$  for all comparisons) *Lactobacillus* was readily capable of growth in media other than its own, although colonies were petite under aerobic growth conditions (Fig. S3).

#### **Filter sterilized supernatant of *L. johnsonii* 456 significantly inhibits the survival of *S. enterica* and *E. faecalis*, but not of ETEC**

As shown in Table 2, ETEC was unaffected by filtered supernatant (FS) from any *Lactobacillus* strain except *L. plantarum*, which completely prevented its survival. LBJ 456 FS significantly inhibited *E. faecalis* survival by over half ( $p = 0.0427$ ), while *L. plantarum* FS killed off over 99% of this strain ( $p = 0.0051$ ). All tested *Lactobacillus* strains significantly decreased *S. enterica* survival, with all tested strains beside *L. casei* leading to a complete absence of viable CFU after 18–20 hours. Surprisingly, all tested FS led to significantly decreased *Bifidobacterium* viability as well, even though this strain was introduced as a non-pathogen control. As pH was controlled for, another acellular factor must be responsible for these differences.

#### **Detectable LBJ 456 can persist in the human gut long after initial ingestion**

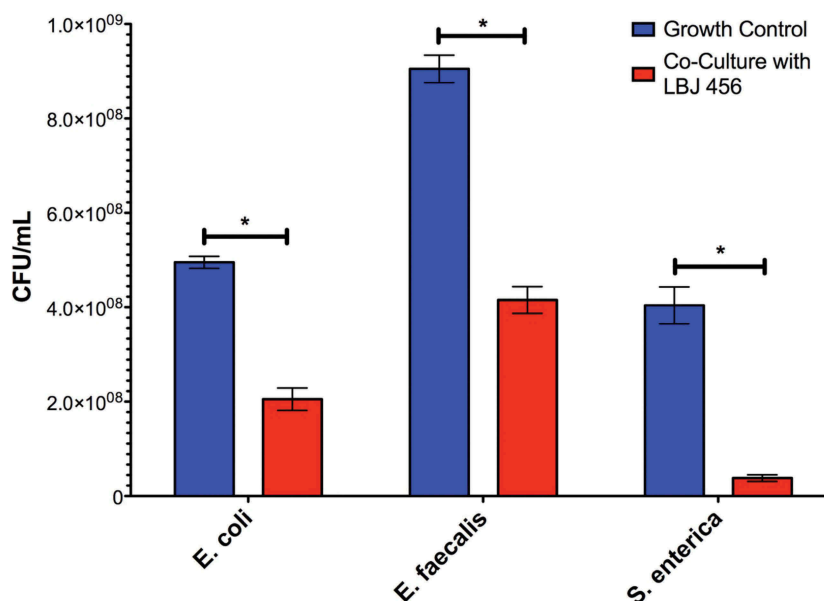
We determined whether LBJ 456 was capable of long-term survival in the human gut. 11 healthy adult individuals completed a 7 day LBJ yogurt trial and supplied fecal samples before yogurt



**Figure 3.** *L. johnsonii* 456 significantly inhibits pathogenic strain adhesion to goblet cell-like gut epithelial monolayers, but not enterocyte-like monolayers. (a) Adhesion of pathogenic bacteria to a Caco-2 monolayer after 1 hour pre-exposure to LBJ 456. (b) Adhesion of pathogenic bacteria to an LS 174T monolayer after 1 hour pre-exposure to LBJ 456. Data expressed as means and SEM. Relevant statistically significant differences are indicated [\* =  $p < 0.05$ , \*\* =  $p < 0.1$  (t test);  $n = 4$ ]. All experiments were performed twice.

consumption (day 0), immediately after (day 7) and at 30 and 60 days after initiation. Over the course of the study, no adverse side effects or diarrhea symptoms were reported for any of the volunteers. Background levels of live lactobacillus varied significantly between volunteers, from undetectable (6 subjects) to nearly 40 million viable CFU/gram in one subject. After sample

collection, the subset of volunteers that tested negative for background *Lactobacillus* before study initiation were grouped for secondary analysis. First, stool samples were analyzed for the presence of live lactic acid bacteria (LAB) (Figure 5a). In the full group of participants, recovered LAB differed significantly between all time points over the course of the 60 day period ( $p < 0.001$ ,



**Figure 4.** Co-culture with *L. johnsonii* 456 significantly inhibits growth of pathogenic strains. Data expressed as means and SEM. Relevant statistically significant differences are indicated [\* =  $p < 0.05$  (t test);  $n = 6$ ]. Experiment was repeated twice.

**Table 2.** Inhibition of growth by *Lactobacillus* filtered supernatant.

Bacterial Strain	Source of Supernatant	CFU/mL*	% Relative to Control*	p Value**
<i>E. coli</i> H10407	MRS Control	1.92e+ 008 ± 1.90e+ 007	-	-
	<i>L. johnsonii</i> 456	1.94e+ 008 ± 1.00e+ 007	101.0 ± 5.21	0.9485
	<i>L. johnsonii</i> VPI 7960	1.54e+ 008 ± 3.00e+ 007	80.21 ± 15.6	0.3247
	<i>L. casei</i> 03	2.22e+ 008 ± 1.80e+ 007	115.6 ± 9.38	0.3813
	<i>L. plantarum</i> 299V	0	0%	n/a
<i>E. faecalis</i> NCTC775	MRS Control	6.41e+ 008 ± 7.67e+ 007	-	-
	<i>L. johnsonii</i> 456	3.00e+ 008 ± 2.80e+ 007	46.80 ± 4.37	<b>0.0427</b>
	<i>L. johnsonii</i> VPI 7960	3.34e+ 008 ± 6.60e+ 007	52.11 ± 10.30	0.0654
	<i>L. casei</i> 03	3.48e+ 008 ± 2.80e+ 007	54.29 ± 4.37	0.0653
	<i>L. plantarum</i> 299V	1.34e+ 006 ± 4200	0.205 ± 0.065	<b>0.0051</b>
<i>S. enterica</i> typhimurium	MRS Control	2.35e+ 008 ± 2.73e+ 007	-	-
	<i>L. johnsonii</i> 456	0	0%	n/a
	<i>L. johnsonii</i> VPI 7960	0	0%	n/a
	<i>L. casei</i> 03	2.80e+ 007 ± 8.00e+ 006	11.92 ± 3.41	<b>0.0074</b>
	<i>L. plantarum</i> 299V	0	0%	n/a
<i>B. lactis</i> HN019	MRS Control	1.37e+ 009 ± 9.60e+ 007	-	-
	<i>L. johnsonii</i> 456	5.12e+ 008 ± 1.20e+ 007	37.32 ± 0.875	<b>0.0124</b>
	<i>L. johnsonii</i> VPI 7960	5.64e+ 008 ± 4.40e+ 007	41.11 ± 3.21	<b>0.0167</b>
	<i>L. casei</i> 03	5.40e+ 008 ± 8.00e+ 007	39.36 ± 5.83	<b>0.0218</b>
	<i>L. plantarum</i> 299V	8.40e+ 008 ± 4.80e+ 007	61.23 ± 3.50	<b>0.0384</b>

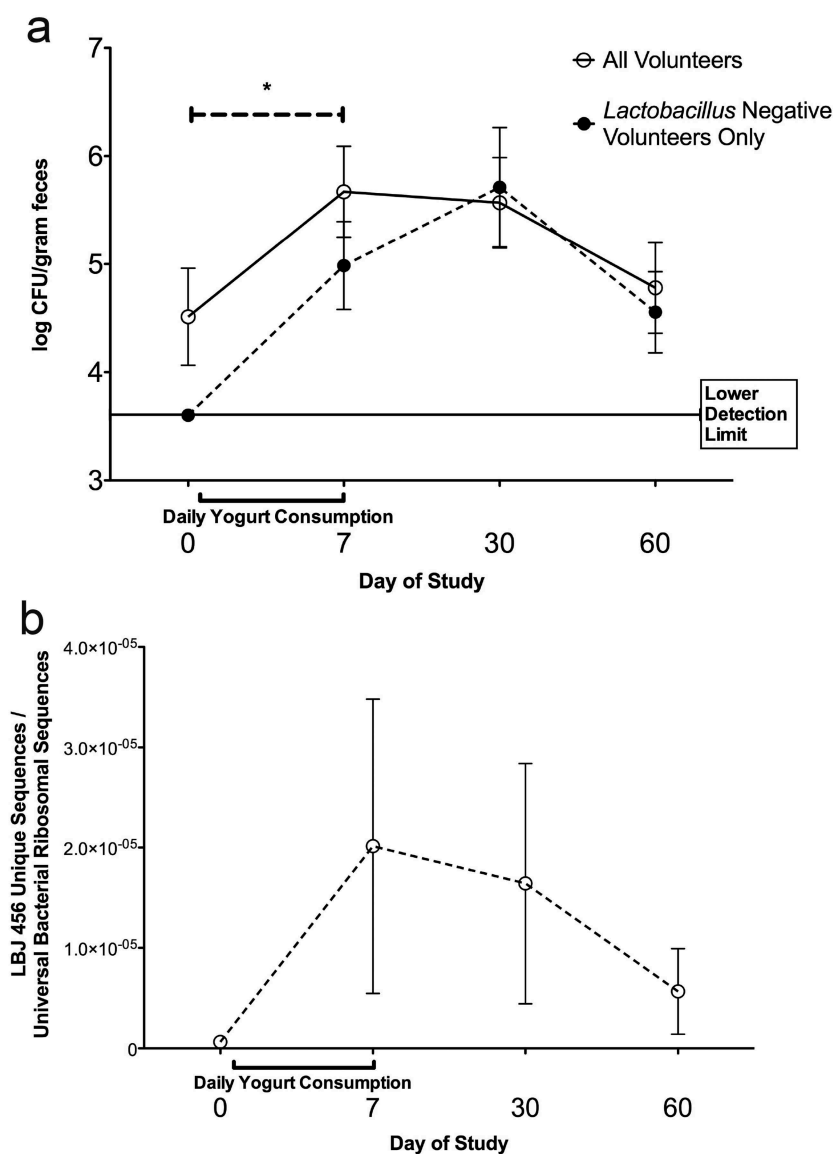
\* Numerical values are listed as the mean ± SEM

\*\* p values of experimental groups against MRS control were calculated by two-tailed unpaired t test, except for instances of repeated zero values. Bold entries indicate a p values under 0.05. N = 4

Friedman test). To ascertain differences between individual time points, post hoc analyses with Wilcoxon signed rank tests were conducted and a Holm-Bonferroni correction was applied. After correction, statistical significance was observed only between baseline and day 7 samples ( $p = 0.048$ ), indicating that the yogurt course successfully introduced live LAB even under the most stringent conditions of analysis. Individuals with

*Lactobacillus*-negative baseline fecal readings also showed detectable lactobacillus over the 60 day course ( $p < 0.001$ , Friedman test). After post hoc analysis with Wilcoxon signed rank tests and Holms-Bonferroni correction, no differences were significant in this group due to the low number of participants. Despite this, there was a clear upward trend in detectable live *Lactobacillus* counts in both the whole group and the LB-negative





**Figure 5.** A 7 day course of LBJ 456 yogurt leads to elevations in both total lactic acid bacteria (LAB) and LBJ 456 specific DNA. (a) Fecal load of viable LAB, as detectable by anaerobic growth on MRS agar, prior to and after 7 day course. Solid line: All volunteers that completed a full course and supplied all four fecal samples ( $n = 11$ ). Dashed Line: individuals with no detectable LAB at day 0, prior to initiation of yogurt course ( $n = 6$ ). All individuals with LAB negative backgrounds had detectable LAB at day 7. 5/6 still had detectable levels at 30 days, and half were still detectable at day 60. Lower detection limit of this assay = 4000 CFU/mL. \* = adjusted  $p < 0.05$  (Wilcoxon signed rank test with Holm-Bonferroni correction) for Day 0–7 (all volunteers). (b) qPCR of detectable DNA sequences in fecal samples, expressed as ratio of LBJ 456 specific DNA sequence to 16S gene as a universal bacterial ribosomal marker sequence. Ratio of LBJ 456 specific DNA to 16S DNA sequence is approximately 1:1 in cultured LBJ 456, and undetectable in LBJ VPI 7960 control (control data not shown). Data expressed as means and SEM.

background subset that lingered through at least one month after the weeklong course. Viable LAB increased by about an order of magnitude ( $\sim 10^{4.5}$  to  $10^{5.5}$  CFU/gram feces) at days 7 and 30 in the full group, and remained higher at day 60. Quantitative polymerase chain reaction (qPCR) targeting a LBJ 456-specific gene sequence in fecal DNA confirmed that gut abundance of LBJ 456 was specifically increased by the yogurt trial.

(Figure 5b, Fig. S4). Overall, the 16S-normalized abundance of this sequence followed a similar pattern as LAB CFU after ingestion. Background counts increased from LBJ 456-negative at day 0 ( $< 1$  copy/million copies of 16S RNA) to an average of roughly one copy per 50,000 from days 7–30. Although a clear trend towards increased LBJ 456 was observed over time, this difference did not reach significance due to the high variance

and low number of participants ( $p = 0.0547$  for Day 0 vs. Day 30, Wilcoxon signed rank test,  $n = 11$ ).

According to recent estimates, about  $3.8 \times 10^{13}$  bacteria make their home in the average human, with most of those in the gut.<sup>35</sup> Of these, approximately  $10^{11}$  cells are shed with every gram of feces. Based on our qPCR estimates, at least two million LBJ 456 genomes would be represented in the feces of individuals recently inoculated with the strain, assuming the average cell has one detectable copy of the 16sRNA gene (a conservative estimate, considering that many species encode multiple copies).<sup>36</sup> This number is relatively consistent with a viable CFU estimate on the order of  $10^{5.5}$  CFU/gram, especially considering cells rendered nonviable during fecal storage at freezing temperature.

### **Potential genetic basis of *L. johnsonii* 456 persistence and inhibition properties revealed by comparative genomics**

To better understand the genomic basis underlying the probiotic properties of LBJ 456, we sequenced its genome and compared it to 23 publicly available *L. johnsonii* genomes. The 24 *L. johnsonii* genomes share 1.2 Mb of conserved genomic sequence. Alignment of 101,088 SNPs in this core genome revealed two groups of highly similar *L. johnsonii* genomes, one consisting of 117a, 117c, 117d, 117k, 117q, and 117x, and the other consisting of UMNLJ21 and UMNLJ22 (Figure 6a). The isolates within these groups differ by less than 300 SNPs and may represent isolates of the same strain. Considering 117a and UMNLJ22 as representative isolates for these groups, we found that the *L. johnsonii* strains differ by an average of 29,943 core genome SNPs. In particular, LBJ 456 differs more from VPI 7960 (33,051 core SNPs), an isolate from human blood, than W1 (20,369 core SNPs), another isolate from mouse gut,<sup>37</sup> consistent with previous observations that genetic similarity among *L. johnsonii* strains is highest among strains from the same host organism.<sup>38</sup>

We then identified 550 genomic regions that are not shared among all 24 *L. johnsonii* strains. These non-core regions total 1,067,226 bp and include

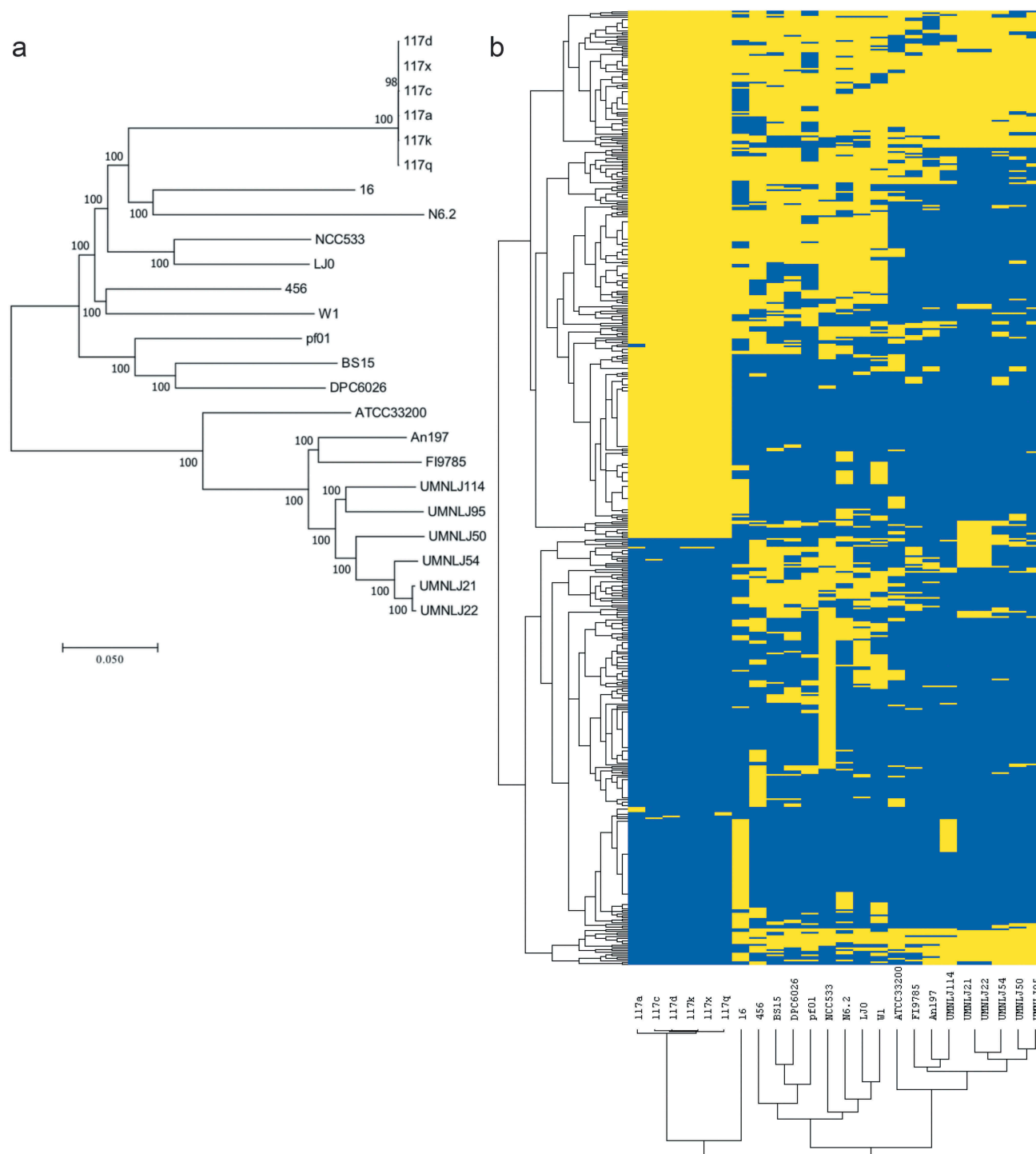
regions unique to each of LBJ 456, NCC 533, 117c, 117d, 117q, and 16 (Figure 6b). Within the LBJ 456 genome, we identified 11 non-core regions totaling 41,781 bp. As shown in Table 3, these regions encode proteins mostly involved in replication (e.g. FtsK/SpoIIIE family protein, plasmid replication protein), and antiviral defense (restriction-modification enzymes), suggesting that cell proliferation strategies and phage exposure may be important processes that determine the compatibility and viability of LBJ 456, and *L. johnsonii* strains in general, within particular hosts.

Moreover, we observed two mucus binding proteins (MBPs) encoded in the non-core regions unique to LBJ 456. A more comprehensive comparison of MBPs across the 24 *L. johnsonii* strains revealed between 3 and 18 MBPs (average 7) in each genome, with LBJ 456 containing the most. These MBPs represent a repertoire of 34 unique homologs based on clustering by 70% identity. 13 of these homologs are encoded only by single strains, including two that are uniquely encoded by LBJ 456 (PROKKA\_00690 and PROKKA\_00875).

We also identified two bacteriocin-encoding loci in the LBJ 456 genome (Figure S6, Table S2). The first locus contains three putative type II bacteriocins (PROKKA\_00793, PROKKA\_00800, and PROKKA\_00806) and two other genes (PROKKA\_00798 and PROKKA\_00799) predicted to encode proteins involved in bacteriocin processing and secretion. The second locus contains a single predicted bacteriocin gene, PROKKA\_00732, surrounded by genes with no clear bacteriocin processing or secretion function. However, as two transposase-related proteins, PROKKA\_00728 and PROKKA\_00729, were also detected in this locus, it is possible that this putative bacteriocin may be part of a mobile genetic element.

## **Discussion**

Immediately after ingestion, bacteria face the twin challenges of low pH and protease activity in the gastric environment. Resistance to gastric acid is one of most important selection criteria for any potential probiotic. Strong acid tolerance is not universal amongst LAB, even at the species level,



**Figure 6.** *L. johnsonii* 456 is genetically distinct from other described *L. johnsonii* strains. (a) Phylogenetic tree of 24 *L. johnsonii* isolates based on 101,088 SNPs in core genomic regions. Distances represent percentage of SNP differences out of total compared SNPs. (b) Frequency of non-core regions among the 24 *L. johnsonii* strains. Yellow cells indicate presence of a non-core region (along columns) in a strain (along rows). Dendrograms based on complete linkage hierarchical clustering of strains or regions based on Euclidean distances. Non-core region lengths are not represented here.

and must be tested on a strain-by-strain basis.<sup>39</sup> Survival at pH 3 is generally considered to be the absolute minimum necessary for a probiotic strain to remain viable *in vivo*, although pH 2 is

a much more commonly encountered level of acidity.<sup>40</sup> In a fasting stomach, pH can drop as far as 1.2–1.5<sup>41</sup> The results of our viability assays are consistent with literature values for

**Table 3.** Genes within noncore regions unique to *L. johnsonii* 456.

Start	End	Strand	Function
125191	125331	-	hypothetical protein
125331	126032	-	Transposase DDE domain protein
126112	126417	-	hypothetical protein
234405	235364	-	hypothetical protein
235812	236573	+	FtsK/SpoIIIE family protein
237107	237385	+	hypothetical protein
237570	237938	+	Plasmid replication protein
238066	238638	+	RNA helicase
238731	238922	+	hypothetical protein
238985	239779	+	hypothetical protein
239776	240219	+	hypothetical protein
255022	256620	+	Lipid A export ATP-binding/permease
605765	609664	+	YSIRK type signal peptide
734735	751894	+	Serine-aspartate repeat-containing
939056	939733	+	Sortase family protein
940040	951076	+	MucBP domain protein
951317	952744	+	MucBP domain protein
1292897	1305865	-	Extracellular matrix-binding protein
1336160	1337077	-	Mrr restriction system protein
1337141	1338232	+	Type I restriction modification DNA
1338298	1339023	-	Type I restriction modification DNA
1386125	1386856	-	UvrD/REP helicase
1782742	1783119	+	hypothetical protein
1783116	1784315	-	Type I restriction modification DNA
1784293	1785915	-	Type I restriction enzyme EcoKI M
1785915	1788983	-	Type I restriction enzyme EcoR124II

well-researched strains, including the high tolerance of *B. lactis* HN019 to pH 2, but relatively poor tolerance of *L. acidophilus* 4356 to the same level of acidity.<sup>42,43</sup>

LAB isolated from both probiotic foods and human samples have shown large decreases in viability when exposed to pH values between 1.5 and 2, though some strains with higher resistance to acid have been documented.<sup>44–46</sup> For example, Aiba et al identify an *L. johnsonii* and an *L. gasseri* strain that maintain over 10% viability in growth media acidified to pH. 1<sup>47</sup> However, it is still unclear how well resistance to simulated gastric conditions translates to tolerance of the gastric environment *in vivo*. The SGA used in our study simulates a scenario in which probiotic strains are introduced, with minimal protective adjuvants, into a gastric environment consisting of low pH, few nutrients, and proteolytic activity from added pepsin enzyme, as would be expected in an empty stomach. Based on the detectable viability maintained by LBJ 456 at all tested pH levels in SGA, we predict robust survival of this probiotic even under the most restrictive gastric conditions, although the mechanism of this strong resistance has yet to be determined. As the presence of milk

and metabolizable sugar have both been found to afford some protection from low pH over buffered saline of the same acidity, the growth of LBJ 456 should be further enhanced when delivered in most conventional formats, such as dairy products.<sup>48,49</sup>

Bile acids in the small intestine inhibit growth via their detergent effects on bacterial cell membranes. Some probiotic species can hydrolyze these bile acids directly.<sup>50,51</sup> They are very rarely lethal for LAB at the lower end of physiological concentrations, which can vary from up to 10mM in the upper ileum to 2mM in the lower ileum after a meal.<sup>52,53</sup> We observed moderately retarded growth rates in all LAB strains exposed to physiologically relevant bile acid concentrations. Literature values generally agree with our observations, including our assessment of *L. acidophilus* 4356 being particularly sensitive, and *B. lactis* being relatively bile acid tolerant.<sup>42,43</sup> Although LBJ 456 is only moderately bile tolerant relative to other strains, it remains viable and capable of slower growth at bile acid concentrations between 0.1 and 0.3% (~ 2–6 mM).

Even in light of the strain's *in vitro* survival through simulated GI tract barriers, we find that LBJ 456 demonstrates exceptional persistence in the human gut. A weeklong course of a small, daily amount of live culture led to elevated fecal abundance through at least a month, as determined by both culture-based and qPCR analyses. It is important to note that tests included here, especially detection of live CFU, revealed heavy variation in lactic acid bacterial load between individuals, and even in the same individual over time. As previously mentioned, background levels varied from under 4000 CFU/gram feces to over 40 million. This heavy interpersonal variability is consistent with that found in other studies. Tannock et al. found CFU counts ranging from 100 to 4 billion per gram, and Goossens et al found between 2500 and 80 million CFU/gram in human fecal samples.<sup>54,55</sup> Dietary intake of lactic acid bacteria likely explains a great deal of this variation, as countless strains and varieties of *Lactobacillus* are naturally found in common fruits and vegetables.<sup>56</sup> Despite this near constant incidental intake, probiotic genera like *Lactobacillus* and *Bifidobacterium*, are generally transient and

do not colonize the human gut for long periods.<sup>57</sup> Most clinically tested strains, including *L. plantarum* 299V, *L. rhamnosus* GG, and *L. casei* Shirota, are not recoverable in host feces after more than a week or so post ingestion.<sup>55,58,59</sup> Of *Lactobacillus* species investigated so far, *L. reuteri* ATCC 55730 seems to have one of the longest reported persistence records, with live bacteria detectable by biopsy (though not in the feces) up to four weeks after the cessation of a monthlong course of ingestion.<sup>60</sup> This persistence has not been fully reproduced consistently, though, with shorter courses of inoculation or by standard fecal assay, and other authors have concluded that it does not effectively establish long term colonization.<sup>61</sup> Despite the fact that the elevated gut counts observed beyond the week of inoculation were not significant (likely due to the small pilot nature of the trial) LBJ 456's long duration of detectability warrants further investigation, particularly of the factors that promote survival and host attachment. Future clinical studies in humans (particularly those that would measure a specific effect on other bacteria or levels of inflammation) should include the use of a placebo control group and multiple benchmark samples to control for natural variation within an individual.

Host mucins provide a common binding site for both beneficial and pathogenic bacterial strains.<sup>62</sup> We observed a broad array of MBPs encoded by *L. johnsonii* strains, some of which appeared to be unique to LBJ 456. Different combinations of these MBPs may confer unique binding properties and contribute to host specificities previously observed among *L. johnsonii* subtypes.<sup>38</sup> The secreted mucin Muc2 constitutes the main mucus glycoprotein in the mouse small intestine, from which LBJ 456 was derived.<sup>63</sup> Its ortholog, MUC2, is the main secreted mucin in the human gut as well, comprising most of the upper, gel-like layer of mucus.<sup>64</sup> The mucin profile of the enterocyte-like cell line Caco-2 is almost purely limited to the expression of membrane-bound mucins like MUC1. The goblet cell-like line LS 174T, however, has much higher expression of secretory mucins, including MUC.2<sup>65</sup> As the secreted mucus layer is the major site of microbe-host interaction, increased adhesion of LBJ 456 to LS 174T could explain this

strain's persistence *in vivo*, especially if MUC2 or another secreted mucin is assumed to be a putative binding target.<sup>66</sup>

Although adhesion to mucins and other mucosal proteins is difficult to study in the human intestine proper, cell monolayer assays correlate reasonably well with *in vivo* persistence data and provide a method for the investigation of both relative adhesion and adhesion inhibition between microbes.<sup>67,68</sup> The adhesion of LAB to monolayer models such as Caco-2 and LS 174T is highly variable between strains. Many probiotic *Lactobacillus* species barely adhere to enterocyte-like Caco-2 cells at all, while others adhere reasonably well.<sup>69</sup> Interestingly, while none of the strains tested here were especially adherent to Caco-2 monolayers, LBJ 456's adhesion was particularly low, roughly 1/20<sup>th</sup> that of its type strain LBJ VPI 7960. This intraspecies difference may reflect adaptations to the different natural hosts or environments from which the substrains were derived. Todoriki et al report that the likewise murine-derived *L. johnsonii* strain JCM 8792 exhibits very low adhesion to Caco-2 cells relative to such strains as the chicken derived *L. reuteri* JCM 1081,<sup>70</sup> strengthening our suggestion that *Lactobacillus* of murine origin may be less specialized to adhere to secreted mucin-poor culture.

The pathogenic activity of many diarrheagenic bacteria, such as ETEC and *Salmonella*, is dependent upon adhesion to the gut mucosa and can therefore also be modeled with monolayers *in vitro*.<sup>71,72</sup> *Salmonella enterica* typhimurium and ETEC strain H10407 are both prototypical diarrhea inducers that require close adhesion to the host cell in order to cause disease.<sup>73-75</sup> *E. faecalis*, although normally considered a commensal, can also become an opportunistic pathogen and diarrhea inducer in immunocompromised individuals, with multidrug resistant strains causing particularly stubborn nosocomial infections.<sup>76</sup> A number of studies have demonstrated that adherent *Lactobacillus* species can inhibit subsequent pathogen adhesion. Todoriki showed that *L. crispatus* JCM 8779 itself reduced *E. faecalis* adhesion by 99% in a Caco-2 model, as well as *Salmonella* and ETEC adhesion by 28 and 47% respectively. Filtered supernatant from *L. crispatus* inhibited *E. faecalis* growth, but not *Salmonella* or ETEC.<sup>70</sup>

Maragkoudakis et al showed that Caco-2 adherent strains could reduce *E. coli* and *Salmonella* adhesion by 10–50%, although they noted no inhibition from supernatant-localized factors.<sup>51</sup> *L. johnsonii* 456's capacity to inhibit pathogen adhesion appears to depend on its own ability to adhere to the monolayer in question. Pre-treatment with one hour of LBJ 456 led to no significant change in pathogen adhesion on Caco-2 cells. On LS 174T monolayers, both ETEC and *Salmonella* adhesion were cut by about 33 and 40%, respectively. Unexpectedly, *E. faecalis* adhesion increased slightly with LBJ 456 incubation, suggesting that LBJ 456 would not necessarily displace other commensals from its milieu, but may potentially promote the attachment of certain gut flora, perhaps through direct binding to the cell surface or via substrates secreted, induced, or modified by it.

In co-culture conditions, LBJ 456 drastically reduced the growth of all three pathogenic strains, particularly *S. enterica*. This inhibition appears to occur through different mechanisms. FS from LBJ 456 grown to the beginning of stationary phase was capable of inhibiting *S. enterica* and *E. faecalis* growth but not ETEC. As *L. johnsonii* VP 7960 FS produced a very similar pattern of inhibition, a common *L. johnsonii* factor may be responsible. Interestingly, *L. plantarum* 299V FS inhibited all three pathogenic strains. *L. plantarum* strains are known to produce a class of two-peptide, class IIB bacteriocins called plantaricins.<sup>77,78</sup> The 299V genome is specifically known to encode a number of predicted bacteriocins including plantaricin components (BT929\_RS02485, BT929\_RS02490, BT929\_RS02515, BT929\_RS02535, BT929\_RS02545, and BT929\_RS02550). Most plantaricins are effective against other Gram-positive bacteria, like *Listeria* and *Enterococcus*, so this particular class of peptide antimicrobial likely does not explain the strong inhibition of ETEC. However, FS inhibition of *E. faecalis* growth could be due to a bacteriocin. *L. johnsonii* strains have been shown to produce a bacteriocin, lactacin F, that inhibits the growth of both *E. faecalis* and other LAB through membrane-disrupting pore formation.<sup>79</sup> Based on our genomic analysis, it is likely that LBJ 456 does in fact produce

functional bacteriocins, some of which may be responsible for this cell-independent inhibitory effect.

At least two proposed mechanisms exist regarding probiotic-mucin interaction and pathogen binding inhibition. Through competitive adhesion, *Lactobacillus* or other beneficial species could compete directly for mucin binding sites with pathogens, preventing them from having a chance to interact with host cells.<sup>80</sup> Alternatively, it has been suggested that *Lactobacillus* binding to host mucins can lead to the secretion of even more mucins, essentially flushing pathogens from the lumen.<sup>81,82</sup> This increased mucin production could potentially counteract the mucin degradation induced by pathogens like ETEC.<sup>83</sup> Regardless of the precise mechanism, secreted mucins are critical for gut homeostasis. Lower levels of Muc2 expression are associated with increased inflammation, colitis, and even rates of colon cancer in mice.<sup>84,85</sup> These conditions have all been associated with gut pathogen infection.<sup>86,87</sup>

Our laboratory previously demonstrated LBJ 456's anti-inflammatory properties in a mouse model.<sup>12</sup> However, the specific mechanisms of this effect remain unclear. *Lactobacillus* species have been suggested to induce regulatory T cells and modulate host inflammatory factors.<sup>88,89</sup> It is possible that the adhesion inhibition shown by LBJ 456 in our study may act in concert with direct host immunomodulation to reduce pathogen-associated inflammation, as has been observed with other probiotics.<sup>90</sup>

Probiotic bacteria represent a potential method for both prevention and treatment of diarrheal diseases.<sup>91,92</sup> Diarrheal infections are a major complication in hospital patients. Gao et al showed that prophylactic administration of a blend of two *Lactobacillus* species cut antibiotic and *C. difficile* associated diarrhea by over half in a clinical environment.<sup>93</sup> Intervention is even more important in children. Diarrheal diseases, including those induced by ETEC and salmonella infection, are responsible for an eighth of childhood deaths below the age of 5 worldwide.<sup>20</sup> Probiotics are effective here, too; vigilant and repeated *L. rhamnosus* GG and *B. lactis* BB-12 supplementation have been demonstrated to reduce the duration of acute diarrhea in a number of studies.<sup>94–97</sup> Unfortunately, the majority of this burden occurs in developing nations

with lower rates of regular access to healthcare. Large outbreaks of diarrheal disease are also common after disasters, like floods, that disrupt stable access to clean water and services.<sup>98</sup> An inexpensive probiotic supplement with a relatively wide “useful prophylactic duration” could be of great use in situations where repeated supplementation is difficult.

### **L. johnsonii 456 represents a promising probiotic lactobacillus strain**

Unique attributes include exceptional acid resistance and well-documented, inoculation-inducible anti-inflammatory effect in mice. The strain is capable of inhibiting the growth and adhesion of multiple types of pathogens *in vitro*. Importantly, the human pilot study described here suggests that *L. johnsonii* 456 may be persistent in the human gut for longer than many other documented strains of probiotic bacteria. Although larger scale clinical studies are needed, the combination of attributes demonstrated here suggest future use as part of an antidiarrheal regimen, or even in the treatment of gut inflammation.

## **Materials and methods**

### **Bacterial strains and growth culture conditions used**

Bacterial strains used are detailed in Table 1. *Lactobacillus johnsonii* 456 was isolated from wild-type mice with restricted gut microflora, housed under specific pathogen free (SPF) conditions at UCLA, by Yamamoto et al.<sup>12</sup> The samples used in this study were derived from frozen stock stored by the Schiestl laboratory. *Lactobacillus plantarum* 299V (NCBI Refseq genome accession NZ LEAV000000000.1) was isolated from Goodbelly Probiotic Juice Drink (NextFoods; Boulder, CO). *Bifidobacterium lactis* HN019 was isolated from Tropicana Essentials Probiotic Juice (Tropicana Products; Chicago, IL). *Lactobacillus johnsonii* VPI 7960, *Lactobacillus casei* 03, *Lactobacillus acidophilus* ATCC 4356, *Streptococcus salivarius* subsp. *thermophilus* NCDO 573, *Escherichia coli* H10407, *Enterococcus faecalis* NCTC775, and *Salmonella enterica* subsp. *enterica* serovar

*typhimurium* were obtained from the American Type Culture Collection (ATCC; Manassas, VA).

All *Lactobacillus* species were cultured in MRS (De Man, Rogosa, and Sharpe) broth (Sigma-Aldrich; St. Louis, MO) for 18–20 hours at 37° C under microaerophilic conditions with sealed test tubes. Colony-forming units (CFU) of *Lactobacillus* were enumerated after 48 hours of growth at 37° C on MRS agar (Sigma) incubated in chambers with anaerobic sachets (Sigma). *L. johnsonii* 456 colonies are distinguishable as smooth bordered, white colonies (Supp. Fig S5) *B. lactis* was cultured and enumerated similarly, except that MRS broth and agar were supplemented with 0.5g/L Cysteine-HCl. *S. salivarius* was cultured in tryptic soy (TS) broth (Sigma) for 18–20 hours at 37° C with no special anaerobic considerations (aerobically), and enumerated on TS agar plates after 48 hours aerobically at 37° C. ETEC and *S. enterica* were cultured in TS broth for 18–20 hours at 37° C aerobically, and enumerated on TS agar plates after 24 hours at 37° C aerobically. *E. faecalis* was cultured in Brain-Heart (BH) broth (Sigma) for 18–20 hours at 37° C aerobically, and enumerated on BH agar plates after 24 hours at 37° C aerobically.

### **Acid resistance during simulated gastric transit**

SGA was prepared by dissolving 3.3ppm pepsin (Sigma) and 0.2% NaCl w/v in 0.1% peptone water (Becton Dickinson; Franklin Lakes, NJ). The pH of this solution was then brought to 1.2 with the addition of 11.65M hydrochloric acid to recapitulate concentrated gastric fluid in an otherwise empty human stomach. This solution was diluted using additional 0.1% peptone water to pHs of 1.6, 2.0, and 3.0. The probiotic strains (*L. johnsonii* 456, *L. johnsonii* VPI 7960, *L. casei*, *L. acidophilus*, *L. plantarum*, *B. lactis*, and *S. salivarius*) were grown to a concentration of roughly  $1 \times 10^8$  CFU/mL by the methods described above.  $10^6$  mid-log phase cells were inoculated into 10mL SGA or 0.1% peptone water control (pH 6) and incubated for 2 hours at 37° C to simulate gastric transit. After incubation, samples were diluted in 0.1% peptone water and plated on agar for enumeration.

### **Bile acid tolerance**

Bile acid tolerance was evaluated using a modified version of the method of Gilliland and Walker.<sup>99</sup> Each probiotic strain was evaluated based on addition of bile salts to their standard growth conditions. Freshly inoculated culture media was vortexed heavily and then split evenly into either a fresh vial or one containing ox gall extract (Sigma) to 0.1, 0.2, or 0.3% of final solution by weight (2.12, 4.24, and 6.36 mM based on rough Sigma ox gall extract bile acid salt composition: 10% glycocholic acid, 15% glycodeoxycholic acid, 30% taurocholic acid, 55% cholic acid) Culture media was then incubated anaerobically for 18–20 hours at 37° C, and samples were plated and enumerated.

### **Human gut monolayer culture conditions**

Caco-2 and LS 174T cell lines were initially obtained from ATCC. Cells were cultured in Eagle's Minimal Essential Media with 4mM glutamine (Caisson Labs; Smithfield, UT) supplemented with the following: 20% fetal Bovine Serum by volume (Corning Cellgro; Manassas, VA), non-essential amino acids (from 100x, Corning), sodium pyruvate (from 100x, Lonza; Walkersville, MD), and 100u/mL PEN-STREP (penicillin-streptomycin mixture, from 100x, Corning). Cells were grown at up to 50% confluence, trypsinized, and subcultured at a 1:4 ratio roughly every 3 days. Conditions were maintained at 37° C in a 5% CO<sub>2</sub> atmosphere.

Caco-2 monolayers were prepared with small modifications to the method described by Natoli et al.<sup>100</sup> Approximately  $3 \times 10^5$  Caco-2 cells/cm<sup>2</sup> were seeded into a 12 or 24 well plate (BD). Cells were maintained in these plates while growing to confluence under the same controlled conditions as previous. The nascent monolayers were rinsed with warmed PBS and given fresh EMEM media containing all previous additives except antibiotics three times a week. After 15 days of culture, the Caco-2 monolayer was considered to be “mature” for adhesion experimental purposes.

LS174 T monolayers were prepared similarly to Caco-2 cells, with a few modifications to the conditions used by Jung et al.<sup>34</sup> After approaching

50% confluence in growth culture, cells were trypsinized and resuspended in fresh EMEM without antibiotics, then seeded into a 12 or 24 well plate at approximately  $3 \times 10^5$  cells/cm<sup>2</sup>. After 3 days, the LS174T monolayer was inspected and rinsed in warmed PBS. Small areas of the growing monolayer that became detached from the substrate were carefully removed during this rinse step, then the media was replaced. Fresh media without antibiotics was then added after PBS rinsing 3 times a week. After roughly 10 days, the LS 174T monolayer was fully confluent and considered “mature” for adhesion experimental purposes.

### **Monolayer adhesion assay**

Bacterial adhesion to monolayers was assayed with small modifications to previously described methods.<sup>69,70</sup> Overnight cultures of probiotic associated test strains (*L. johnsonii* 456, *L. johnsonii* VPI 7960, *L. casei*, *L. acidophilus*, *L. plantarum*, *B. lactis*, and *S. salivarius*) were pelleted via centrifuge (10 minutes, 3K RPM) (Allegra 6R, Beckmann-Coulter), rinsed, and resuspended in antibiotic-free EMEM and an equal amount of their bacterial culture media to a concentration of  $2\text{--}5 \times 10^8$  cells/mL. Mature Caco-2 or LS 174T monolayers were rinsed twice with warmed PBS. A 1 mL volume of resuspended bacterial sample was then applied to each well of the tissue culture plate. Plates were incubated at 37° C in a 5% CO<sub>2</sub> atmosphere for 2 hours with gentle intermittent rocking. After incubation, supernatant was removed and monolayers were rinsed 3 times with warmed PBS. The monolayers were then covered in 1 mL fresh PBS per well and vigorously agitated with micropipette until disrupted and fully resuspended. 10-fold serial dilutions were then plated on strain specific agar media to enumerate adherent cells.

### **Pathogen adhesion inhibition assay**

Pathogen adhesion was determined by the method of Todoriki et al.<sup>70</sup> Overnight cultures of 3 potentially pathogenic strains (ETEC, *E. faecalis*, and *S. enterica*) and *Lactobacillus johnsonii* 456 were pelleted via centrifuge, rinsed, and resuspended in antibiotic-free EMEM to a concentration of



$2 \times 10^8$  cells/mL, as determined by microscope count. Samples of this media were taken aside, diluted, and plated on specific agar media to control precisely for viable CFU plated. Mature Caco-2 or LS 174T monolayers were rinsed twice with warmed PBS. 0.5 mL of *L. johnsonii* in EMEM was added to each well. Monolayers were then incubated at 37° C in a 5% CO<sub>2</sub> atmosphere for an hour with gentle intermittent rocking. After initial incubation, wells were rinsed twice with warmed PBS to remove nonadherent LBJ 456. 0.5 mL of pathogen suspensions were then applied to experimental and control (no LBJ pretreatment) wells. Plates were returned to the incubator for an additional hour. After incubation, supernatant was removed and monolayers were rinsed 3 more times. The monolayers were then covered in 1 mL fresh PBS per well and vigorously agitated with micropipette until disrupted and fully resuspended. 10-fold serial dilutions were then plated on specific agar media to enumerate colonies from adherent bacteria of the test strain.

### Co-culture inhibition assay

Cultures of the 3 potentially pathogenic strains (ETEC, *E. faecalis*, and *S. enterica*) and LBJ 456 were grown overnight as described earlier to concentrations of  $2\text{--}10 \times 10^8$  cells/mL, by the method of Hsieh et al.<sup>101</sup> Cells were rinsed and pelleted via centrifuge, then  $1 \times 10^8$  cells of each pathogenic strain were co-inoculated with  $1 \times 10^8$  cells of LBJ 456 into TS (ETEC and *S. enterica*) or BH (*E. faecalis*).  $1 \times 10^8$  cells of each test strain were also resuspended alone in their respective media as a control. Co-cultures were incubated for 18–20 hours at 37° C, then plated for enumeration. After 24 hours at 37° C, colonies of the pathogenic strain were enumerated, and easy to distinguish morphologically from the inhibited growth of *Lactobacillus* colonies under aerobic conditions. LBJ 456 controls were enumerated after 48 hours at 37° C on MRS agar.

### Supernatant inhibition assay

A slight modification on the method used by Sgouras et al was used to determine whether an acellular factor could inhibit pathogen growth.<sup>102</sup>

Overnight cultures of *Lactobacillus* strains *L. johnsonii* 456, *L. johnsonii* VPI 7960, *L. casei*, and *L. plantarum* were cultured in MRS broth for 18–20 hours at 37° C under microaerophilic conditions. The test strains *B. lactis*, ETEC, *E. faecalis*, and *S. enterica* were also inoculated into their respective growth media, and cultured 18–20 hours at 37° C. Spent supernatant from the *Lactobacillus* cultures was vacuum filtered via a 0.45 micron filter (Nalge Nunc; Rochester NY) to exclude cellular components. *B. lactis*, ETEC, *E. faecalis*, and *S. enterica* overnight growth cultures were rinsed and pelleted via centrifuge, then  $1 \times 10^8$  CFU of each test strain were inoculated into 5 mL of their fresh respective growth media, and either 5 mL of filter sterilized supernatant (FS) from one of the *Lactobacillus* cultures or fresh MRS adjusted to pH 4 with lactic acid as a control. Cultures were grown for 18–20 hours at 37° C, then serially diluted and plated on each strain's respective growth agar for enumeration. All plates were incubated at 37° C under their respective growth conditions described above. *B. lactis* was grown for 48 hours under anaerobic conditions while ETEC, *S. enterica*, and *E. faecalis* were incubated for 24 hours aerobically.

### Human gut survival trial and enumeration of viable lactic acid bacteria

Yogurt containing a starter culture of LBJ 456 was generated using commercially available whole fat milk. The yogurt was kept fermenting at room temperature until fully solidified, then refrigerated at 4° C. 11 mixed gender individuals in good health (no inflammatory gut conditions or known disease states) received a 7 day course of this yogurt, and consumed 100mL, or roughly  $1 \times 10^{10}$  CFU, every morning over the course of the trial week. A baseline fecal sample was taken prior to first yogurt consumption, and then at 7, 30, and 60 days after study initiation. Fecal samples were stored at –20° C. 11 individuals supplied fecal samples for each time point, and the others were excluded. To determine viable LAB load at each timepoint, 0.1g of fecal matter was thawed and serially diluted in PBS, then plated on MRS agar to select for LAB. Plates were

enumerated after 48 hours at 37° C. Volunteers were asked to refrain from consuming other probiotics for the duration of the study, but otherwise maintain a normal diet. Researchers were blinded to the identity of volunteers. Study design was reviewed and volunteer consent was obtained by MicroBio Pharma, Inc., and determined to be ethically and clinically sound based on previous demonstrations of *Lactobacillus johnsonii* safety in the literature.

### **LBJ 456 DNA detection in human fecal samples by rt-qpcr**

Fecal bacterial DNA was purified using a Zymo QuickDNA Fecal/Soil Microbe Miniprep Kit (Zymo; Irvine, CA) according to manufacturer instructions. DNA content was verified using a Nanodrop (Thermo Fischer; Canoga Park, CA). Real-Time quantitative Polymerase Chain Reaction (RT-qPCR) was performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using PerfeCTa SYBR Green SuperMix Low ROX reagent (Quantabio; Beverly, MA). DNA was diluted to a working solution of 10 ng/μL and 1 μL was used per replicate of each sample. Samples were analyzed in technical triplicates. Presence of LBJ in each sample was normalized to 16S levels. Primer sequences used are listed in **Table S1**. Optimal qPCR temperature of 55° C was determined by temperature gradient. Specificity of primers was determined by melt curve analysis and comparison of strains (**Supp. Sig. S4**).

### **Genome sequencing, assembly and annotation**

Whole genome sequencing of *Lactobacillus johnsonii* 456 was performed by Genewiz (South Plainfield, NJ). In brief,  $6 \times 10^9$  CFU of LBJ 456 bacteria were shipped to Genewiz. Cells were lysed and total DNA was purified. Sequencing was performed using PacBio Sequel to an average coverage of 100X per sample. Read assembly was performed using Canu<sup>103</sup>. Coding and tRNA genes were annotated using Prodigal 2.6<sup>104</sup> and Aragorn 1.2<sup>105</sup>, respectively, via the PROKKA pipeline.<sup>106</sup> Genome uploaded to NCBI database under accession code QGQW00000000.

### **Core genome comparison**

We compared the genome of *Lactobacillus johnsonii* 456 (LBJ 456) with 23 other *L. johnsonii* genomes available from NCBI on September 7, 2017. We first calculated the core genomic regions shared by all 24 *L. johnsonii* strains, as described by Tomida et al.<sup>107</sup> Briefly, Nucmer was used to identify homologous regions between the genome of NCC 533 and each of the other 23 genomes. The set of core genomic regions was determined to be the regions homologous to NCC 533 that were present in the genomes of the 23 other strains. Single nucleotide polymorphisms (SNPs) were identified within core regions using Nucmer and were used to construct a phylogenetic tree in MEGA 7 using the Neighbor-Joining method on p-distances.<sup>108</sup> Bootstrapping was performed using 500 replicates.

### **Non-core genome comparison**

Non-core genomic regions among the *L. johnsonii* genomes were also identified as described by Tomida et al.<sup>107</sup> Briefly, a pan-genome across all 24 *L. johnsonii* strains was constructed by first using Nucmer to compare the NCC 533 genome with one of the 23 other genomes. Regions in this genome without homology to NCC 533 were concatenated to the NCC 533 genome sequence. This concatenated sequence was then iteratively compared using the same method to each of the remaining genomes to construct the pan-genome. Finally, the pan-genome was compared to each of the 24 genomes individually to identify non-core regions  $\geq 500$  bp that were absent in at least one of the genomes.

### **Genome analysis**

To determine whether the 24 *L. johnsonii* isolates, and LBJ 456 in particular, may possess distinct host-binding properties, we identified putative MBPs within the isolate genomes. A set of reference MBPs was compiled from amino acid sequences matching the search term “((((mucus [Title] OR mucin[Title])) AND binding[Title])) OR ((mucus-binding[Title] OR mucin-binding [Title]))”, downloaded from the NCBI protein

database as of September 9, 2017. A non-redundant set of reference MBPs was obtained by clustering sequences with  $\geq 97\%$  identity using CD-HIT,<sup>109</sup> and all proteins from all 24 *L. johnsonii* strains were aligned to this reference set using BLASTP. Putative *L. johnsonii* MBPs were identified as sequences showing at least 60% identity to at least one reference MBP. *L. johnsonii* MBPs were further clustered by 70% identity using CD-HIT. Bacteriocin-encoding loci in the LBJ 456 genome were detected using BAGEL.4<sup>110</sup>

### Statistical analyses

Statistical analyses were carried out using Graphpad Prism 5 software and Microsoft Excel. T tests were used to assess significance of differences in bacterial survival and adhesion assays. Changes within fecal CFU were carried out using the non-parametric Friedman test and Wilcoxon signed rank post-hoc analysis; *P* values were adjusted with a Bonferroni correction.

### Significance

Bacterially derived diarrheal disease is a major contributor to worldwide deaths for children under the age of five. On the other end of the spectrum, chronic inflammation contributes to cancer, which claims more lives in developed countries than any other illness beside cardiovascular disease. Probiotic bacteria offer a method of intervention that could reduce fatalities from both of these seemingly disparate diseases. It is imperative that new strains of probiotic bacteria be characterized while we simultaneously develop our understanding of their mechanisms of action. *Lactobacillus johnsonii* 456 is associated with reduced inflammation and genotoxicity in vertebrate models, pathogen inhibition *in vitro*, and long persistence in human trials, making it a powerful option for populations without consistent access to resources.

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### Author contributions

R.H.S. designed research and prepared manuscript, M.J.D. and J.L. designed research, performed research, analyzed data, and prepared manuscript; J.C. and N.I.R.M. performed research, analyzed data, and prepared manuscript. All authors read and approved the final manuscript.


### Conflict of Interest Statement

R.H.S. has a financial interest in MicroBio Pharma, Inc, which might benefit from the commercialization of the results of this research.

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