Resilience mechanisms of small intestinal lactobacilli to the toxicity of soybean oil fatty acids

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Summary:

Over the past century, soybean oil (SBO) consumption in the United States increased 100,000%. The main SBO fatty acid, linoleic acid (18:2), inhibits the growth of lactobacilli *in vitro*. Human-associated lactobacilli have declined in prevalence in Western microbiomes, but how dietary changes may have impacted their ecology is unclear. Here, we compared the *in vitro* and *in vivo* effects of 18:2 on *Lactobacillus reuteri* and *L. johnsonii*. *In vitro*, directed evolution led to strong 18:2 resistance with mutations in genes for lipid biosynthesis, acid stress, and the cell membrane or wall. *In murine*, microbiomes conditioned on a high-18:2 diet yielded *L. reuteri* isolates with improved resistance. Small-intestinal *Lactobacillus* populations *in vivo* were unaffected by chronic and acute 18:2 exposure, yet harbored 18:2- sensitive strains. This work shows that small intestinal beneficial microbes are protected from toxic dietary components via the gut environment and their own capacity to evolve resistance.

Introduction

During the 20th century, the greatest dietary change in the United States was in the consumption of soybean oil (SBO), which increased over 100,000%, from less than 0.001 kg/person/year to 12 kg/person/year (Blasbalg et al., 2011). Soybean oil, frequently labeled as 'vegetable oil', is a mixture of triglycerides composed of five long chain fatty acids (FAs), with linoleic acid (18:2) comprising over 50% of the FAs. When triglycerides are hydrolyzed by lipases active in the saliva, stomach, and upper duodenum, free FAs and monoglycerides are absorbed in the small intestine (Mansbach et al., 2000). The microbiota of the human small intestine is exposed to FAs during transit (El Aidy et al., 2015). Thus, a change in the concentration of specific dietary FAs will impact the small intestinal microbiota.

Linoleic acid and the other two major unsaturated FAs in SBO, oleic acid (18:1), and alpha-linolenic acid (18:3), are bacteriostatic and/or bactericidal to small intestinal bacteria as non-esterified (free) fatty acids *in vitro* at concentrations found in the small intestine (Kabara et al., 1972; Kankaanpaa et al., 2001; Kodicek, 1945; Nieman, 1954). The primary modes of killing include permeabilization of cell membranes (Greenway and Dyke, 1979) and interference with FA metabolism (Zheng et al., 2005). Affected microbes are predominantly Gram-positive bacteria including the genus *Lactobacillus* (Nieman, 1954). Lactobacilli are particularly important as they are considered beneficial members of the human small intestine (Walsh et al., 2008; Walter et al., 2011; Walter et al., 2007). They have been shown to be growth inhibited by the specific FAs present in SBO (Boyaval et al., 1995; De Weirdt et al., 2013; Jenkins and Courtney, 2003; Jiang et al., 1998; Kabara et al., 1972; Kankaanpaa et al., 2001; Kodicek, 1945; Raychowdhury et al., 1985). It is interesting to note that the human-associated *L. reuteri* underwent a population bottleneck that coincides with the increase in SBO consumption in the

U.S. and is far less prevalent than it was in the past (Walter et al., 2011). Despite its decline, *L. reuteri* and other lactobacilli persist in the small intestine of Western individuals, suggesting mechanisms to counter the inhibitory effects of FAs *in vivo*.

While antibiotics can cause lasting alterations to the microbiome (David et al., 2014a; Dethlefsen et al., 2008; Dethlefsen and Relman, 2011; Jakobsson et al., 2010), dietary perturbations rarely do so (Sonnenburg et al., 2016). In humans and in mice, the gut microbiome can be quickly altered by diet but community composition generally recovers within days (Carmody et al., 2015; David et al., 2014a; David et al., 2014b; Zhang et al., 2012). Resilience to dietary perturbation may be direct, as gut microbes functionally adapt to diet, or indirect through buffering by the gut environment.

Here, we explored mechanisms of microbiome resistance to toxic dietary components with a focus on linoleic acid (18:2) toxicity to *L. reuteri* that was either chronic or acute *in vivo* and under laboratory conditions. Using an *in vitro* evolution assay, we observed that *L. reuteri* can develop 18:2 resistance upon exposure to increasing concentrations of 18:2. In mice, *L. reuteri* populations were unaffected by chronic dosing of 18:2 from diet and acute dosing via gavage; however, once isolated and tested *in vitro*, many strains were nevertheless 18:2-sensitive. The other predominant *Lactobacillus* in the mouse gut, *L. johnsonii*, behaved similarly. Together, these results show that although lactobacilli are capable of functional adaptation to chronic and acute 18:2, it is not required in the mouse gut to survive 18:2 exposure. Thus, although dietary FA toxicity is buffered in the small intestine, the ability of lactobacilli to evolve resistance constitutes an additional insurance against this potential stressor and may explain the variation in 18:2 resistance observed across strains and within human hosts.

Results

Strains of *L. reuteri* show variable resistance to 18:2 *in vitro*

To confirm the previously reported toxicity of long chain FAs towards *L. reuteri*, we performed disc diffusion assays with the individual free FAs of soybean oil (SBO) using the commonly studied *L. reuteri* ATCC 53608. We observed growth inhibition of this strain by free 18:1, 18:2, and 18:3 (**Fig. 1A**), and this inhibition occurred in the presence of completely hydrolyzed SBO (**Fig. S1**). The two saturated free FAs 16:0 and 18:0 and glycerol did not interfere with growth. To determine if the inhibitory concentration of 18:2 was comparable to concentrations in the mammalian digestive tract, we performed a cell permeability assay using propidium iodide with *L. reuteri* ATCC 53608 over a 10-fold dilution range from 0.01 to 1000 μg/ml of 18:2. We observed that 18:2 permeabilized the cells with an estimated inhibitory concentration 50 (IC50) of 20 μg/ml ($p<0.001$) (**Fig. 1B**). This IC50 concurs with our estimates of the concentration of 18:2 present in a mouse consuming a SBO diet (11 to 28 μg/ml for a mouse on a 7% by weight SBO diet) and with previous estimates of mammalian physiological relevant concentrations of unsaturated FAs (Kankaanpaa et al., 2001; Kodicek, 1945).

We next assessed 40 strains of *L. reuteri* for 18:2 resistance in liquid culture. These 40 strains were previously isolated from different sources - humans, pigs, rodents (mice, rats), birds (chicken, turkey), and sourdough, and stemmed from six different continents (**Table S1**) (Böcker, 1995; Oh et al., 2010). We quantified how the strains grew in 18:2 by taking the mean of the ratios for cells growing in 18:2 to cells growing in medium alone for each of the last three OD600 measurements at hours ~4, 6, and 8 during the growth assay. *L. reuteri* strains have been shown to be host-specific and form host specific clades (Walter et al., 2011). The phylogenetic

relationships of these strains indicate that the rodent-associated strains form clades that are basal to the other clades (Duar et al., 2017; Oh et al., 2010); therefore, if 18:2 resistance is vertically transmitted and had evolved in the human-associated strains, the rodent strains should display less 18:2 resistance than the human strains. In accord, we observed that the rodent-associated strains on average were inhibited by 18:2 more strongly than the other strains (t-test, $p<0.001$) (**Fig. 2**). However, we observed variation in *L. reuteri* 18:2 resistance regardless of source. For instance, the human-associated strains were not more resistant to 18:2 than the strains derived from pig, poultry, or sourdough. Moreover, within human strains, 18:2 resistance did not relate to *L. reuteri* isolation site (**Fig. S2A**). There also did not appear to be a clear relationship of 18:2 resistance with *L. reuteri* clades as defined by Oh et al. (2010) (**Fig. S2B**).

Evolved 18:2 resistance is associated with mutations in lipid-related, acid stress, and cell membrane/wall genes

To directly test if 18:2 resistance could evolve in *L. reuteri* through exposure to 18:2, we isolated an 18:2-sensitive *L. reuteri* strain (LR0) from the jejunum of a conventionally-raised mouse fed a 16% by kcal fat (low fat) SBO diet (see Methods). We seeded five cultures with LR0 and passaged them twice daily from a growth-dampening concentration of 18:2 up to a growth-inhibitory concentration over a period of 6 weeks (**Fig. 3A**). We also evolved five cultures of *L. johnsonii* strain (LJ0) obtained from the same mouse. We selected *L. johnsonii* based on its high abundance in mouse small intestinal microbiota (see below). At the end of the passaging regime, all of the evolved lactobacilli populations showed smaller zones of inhibition around 18:2 and 18:3 in the disc diffusion assay compared to their respective starting strains (**Fig. 3B** and **Fig. S3**). Notably, populations LR2 and LJ4 had very small to no zones of

inhibition around 18:2 and 18:3. We picked an isolate from each of these populations (LR2-1 from LR2 and LJ41072 from LJ4) for testing in liquid culture supplemented with 18:2 to confirm their 18:2 resistance. We observed that LR2-1 was resistant to the maximal concentration of 18:2 used in the evolution assay, whereas LJ41072 was somewhat inhibited by this concentration (**Fig. 3C and 3D**). We also observed that both isolates had increased growth rates compared to their parental strains.

To characterize the mutations these populations acquired, we sequenced all five of the *L. reuteri* populations at the population level, four of the five *L. johnsonii* populations (the fifth was lost), the evolved isolates LR2-1 and LJ4107, and the starting strains LR0 and LJ0, using 300 bp paired end sequencing on an Illumina MiSeq. For the populations, we achieved approximately 500X coverage, and for the isolates, 50X coverage (**Table S2**). Mutations were called in the populations and isolates by aligning sequencing reads derived from the populations or isolates to the assembled genome for the respective starting strain (LR0 or LJ0). After filtering mutations appearing at a minimum frequency of 10% in a population and confirming all mutations were not due to potential mismapping in repeated regions, we observed 30 mutational events in 15 genes across the five *L. reuteri* populations and 35 mutational events in 21 genes in the four *L. johnsonii* populations. On average, each population had 6 mutations in 6 genes (**Table S3**, **S4**); exceptionally, LJ5 had 17 mutations in 17 genes. The isolates LR2-1 and LJ41074 had three and five mutations, respectively. Overall, the total number of mutations acquired by all lactobacilli populations were similar.

In each population, a few mutations had swept the entire population (**Tables 1**, **2, S3, and S4**). Both the *L. reuteri* and *L. johnsonii* populations bore high frequency variants (>60%) in genes relating to FA metabolism, ion transport, and the cell membrane/wall. In the *L. reuteri*

152 populations, we found high frequency variants in (i) FA biosynthesis transcriptional regulator
153 *FabT* (Eckhardt et al., 2013), (ii) two related tyrosine-protein kinases involved in
154 exopolysaccharide synthesis, *EpsD*, and *EpsC* (Minic et al., 2007), (iii) an HD family hydrolase,
155 (iv) a hypothetical protein, and (v) in the region upstream of an ammonium transporter that may
156 respond to acid stress (Wall et al., 2007). In the *L. johnsonii* populations, high frequency
157 mutations were present in (i) two distinct intracellular lipases, (ii) a putative membrane protein
158 gene, (iii) the potassium efflux system *KefA*/small-conductance mechanosensitive channel,
159 which protects against growth defects in acidic conditions (Cui and Adler, 1996; McLaggan et
160 al., 2002), (iv) the glycosyltransferase *LafA*, which affects the lipid content of the cell wall and
161 membrane (Webb et al., 2009), (v) a *TetR* family transcriptional regulator, and (vi) the
162 ribonucleotide reduction protein *NrdI*. All but two of the above mutations are non-synonymous
163 or cause protein truncations. The other two mutations are in intergenic regions and thus may alter
164 the expression of the downstream gene. Although the specific genes mutated differed between *L.*
165 *reuteri* and *L. johnsonii*, they are associated with similar functions, suggesting that *Lactobacillus*
166 species can evolve 18:2 resistance through changes relating to lipid metabolism, acid stress, and
167 the cell wall/membrane.
168
169 **_L. reuteri_ can survive chronic and acute 18:2 exposure in the mouse**
170 Given that 18:2 resistance can evolve *in vitro*, we asked if *L. reuteri* and *L. johnsonii* could
171 survive 18:2 exposure *in vivo*. To this end, we assessed the response of *L. reuteri* to both a
172 chronic and an acute exposure to 18:2 *in vivo*. For the chronic exposure, weaned (3 week old)
173 male C57BL/6J mice from Jackson Laboratories were fed *ad libitum* for 10 weeks one of two
174 diets, wherein all of the fat was derived from SBO. The diets differed in the percentage of

calories derived from fat: 16% for the "low fat" (LF) diet and 44% for the "high fat" (HF) diet (**Table S5**). For the acute exposure, at the end of the 10 weeks, we gavaged (delivered to the stomach) 6 mg 18:2 per gram mouse weight (e.g., double the 18:2 consumed by mice daily on the LF diet) or vehicle control (saline). At 1.5 hours post-gavage, the time at which we observed gavaged 18:2 in the blood stream (**Fig. S4A**), mice were sacrificed, and the small intestine contents were collected (**Fig. 4A**).

To assess how the gavage impacted the microbiome of the jejunum, where the bulk of fat absorption occurs (Mariadason et al., 2005), we sequenced the V4 region of 16S rRNA gene amplicons derived from one half of each jejunal luminal sample. In addition to live cells, the "total biomass" assessed in this way included cells permeabilized by 18:2 and dead cells, all of which may contain DNA that will be sequenced in this analysis. To assess the diversity of "live-only" biomass, we removed the DNA of cells with compromised membranes with the use of propidium monoazide (PMA) (Chiao et al., 2014; Exterkate et al., 2015; Nocker et al., 2006; Rogers et al., 2013) applied to the other half of each sample. This approach allowed us to gauge which taxa were still alive after the 18:2 treatment.

The effect 18:2 treatment on the microbial community was evident from analysis of the live cells but not the total biomass: microbiomes within a diet-group (HF or LF) could be distinguished by gavage treatment (18:2 or saline) only when live-only biomass was analyzed (live-only; weighted UniFrac, n = 22 for LF diet: adonis, pseudo-F=4.78, 15% of variance explained, $p<0.05$; n = 24 for HF diet: adonis, pseudo-F= 7.84; 28% of variance explained, $p<0.005$; also see **Figs. S4B and S4C**). Furthermore, we observed that for both diets, although the control gavage did impact the difference between live-only and total-biomass diversity, this difference was greater when 18:2 was administered (**Fig. 4B**). We note that this difference (beta-

198 diversity distance) was greater for the HF than the LF diet samples (**Fig. 4B**), suggesting that the
199 HF-diet conditioned microbiome was disrupted to a greater extent by 18:2 than the LF-diet
200 microbiome.

201 In the total biomass, OTUs 692154 and 592160, taxonomically assigned to *L. reuteri* and
202 *L. johnsonii*, respectively, were the two most abundant lactobacilli OTUs. These OTUs were at
203 comparable relative abundances in the two diets (total biomass; t-test and ANOVA on a linear
204 mixed model to include cage effects, p values>0.05, **Fig. 4C and D**). Note that when we detected
205 *L. reuteri* OTU 692154, it was at very low levels in the microbiota of mice housed in three out of
206 six LF diet cages and in two out of six HF diet cages (**Fig. S4D**). Additionally, these *L. reuteri*
207 and *L. johnsonii* OTUs were present in the 18:2, live-only microbiota in both sets of mice (**Fig.**
208 **4E and F**), suggesting these taxa survived the 18:2 acute treatment regardless of the dietary fat
209 content. Comparison of the relative abundance of these two OTUs in the total and live-only
210 microbiota revealed these lactobacilli (with the exception of *L. johnsonii* in the LF diet) as
211 enriched (2- to 4-fold, t-test, p values <0.01) in the total population after 18:2 gavage.
212 Furthermore, the live-only microbiota of HF diet mice had an enrichment of 11 lactobacilli
213 OTUs after 18:2 gavage (5- to 9-fold enrichment compared to control gavage, Kruskal-Wallis,
214 FDRs <0.1 **Fig. S4E**). Similar enrichment of live lactobacilli after the 18:2 gavage was observed
215 for the LF diet, although no OTU passed our significance threshold. These observations suggest
216 that lactobacilli resist acute 18:2 exposure, particularly if they are conditioned to a high-18:2
217 diet.

218 To confirm that the *Lactobacillus* population was not reduced by the 18:2 gavage and that
219 any changes in their relative abundances were due to die-offs of other susceptible bacteria, we
220 quantified their population levels in total and live cell fractions by qPCR (see Methods). We

221 determined the difference in the copy number of *Lactobacillus* 16S rRNA sequences in the total
222 and live-only samples. This difference was normalized to the equivalent difference for total
223 Eubacteria. We observed no difference between the saline (control) and 18:2 gavage samples for
224 either diet (**Fig. S4F**, t-test, p values > 0.1). All live-only to total relative copy numbers were
225 close to 1, as expected if the *Lactobacillus* population was not reduced by 18:2 exposure.
226 We repeated the chronic 18:2 exposure with two additional sets of mice originating from
227 Taconic Biosciences and an F2 generation of mice from Jackson Laboratories (see Methods). In
228 these two additional sets of mice, 16S rRNA gene sequence diversity analysis of jejunal contents
229 showed that the same two OTUs annotated as *L. reuteri* and *L. johnsonii* were again the
230 predominant lactobacilli. In mice from Taconic, *L. reuteri* and *L. johnsonii* were detected in the
231 jejunum after 10 weeks on both HF and LF diets (**Fig. S5A and B**). In F2 Jackson mice, *L.*
232 *johnsonii* alone was detected after 10 weeks on both HF and LF diets (**Fig. S5C**), whereas *L.*
233 *reuteri* was only present in the mice on the LF diet (**Figs. S5D**). *L. reuteri*, however, was not
234 observed in the fecal samples of any mouse at time 0 (**Figs. S5E**). We detected *L. reuteri* in LF
235 diet-fed mouse fecal samples from weeks 5 and 10 (**Figs. S5E**). While it appears in the F2
236 Jackson mice that *L. reuteri* may have invaded the mice on the LF diet at a later time point, we
237 cannot rule out the possibility of *L. reuteri* existing below detection. It seems unlikely that only
238 the LF mice were exposed to *L. reuteri* as the mice were handled in the same manner, the diets
239 were sterilized, and the mice were all bred in the same facility. Hence, if all cages of mice were
240 exposed to *L. reuteri* then only the mice on the LF diet were capable of being invaded.
241 Previously, Kanaanpaa et al. (2001) observed that 18:2 decreased the ability of *Lactobacillus* GG
242 to adhere to Caco-2 cells, and 18:2 inhibited *L. casei* Shirota adhesion to human infant mucus *in*
243 *vitro*. Hence, it is possible that a high soybean oil diet prevents *L. reuteri* from stably associating

in a mouse, potentially preventing adhesion to the forestomach epithelium (see Discussion), but overall the results from these mice support the notion that *L. reuteri* and *L. johnsonii* populations are minimally impacted by chronic dosing of 18:2.

L. reuteri isolated from SBO diet mice are sensitive to 18:2, but HF diet isolates show increased 18:2 resistance

Our results in mice suggested that *L. reuteri* and *L. johnsonii* survived chronic and acute exposure to 18:2 either directly, by 18:2 resistance, or indirectly, through buffering by the mouse gut environment. To assess the resistance of these lactobacilli to 18:2 *in vitro*, we established a collection of *L. reuteri* and *L. johnsonii* isolates derived from the upper ileum of mice on both HF and LF diets. Specifically, we determined the ability of these isolates to grow in liquid culture with a concentration of 18:2 that weakly inhibited their growth. After excluding 7/120 isolates that failed grow in medium lacking 18:2, we measured growth in 18:2 for 113 isolates of *L. reuteri* derived from 15 mice housed in 8 of 12 cages (5 HF diet cages and 3 LF diet cages; note that *L. reuteri* was not detected by 16S rRNA gene amplicon sequencing in several of the cages, **Fig. S4D**). While most isolates were sensitive to 18:2, we observed that *L. reuteri* isolates recovered from the HF-diet fed mice were on average more resistant to 18:2 than *L. reuteri* isolated from the LF-diet fed mice (t-test, $p < 0.05$, **Fig. 4G** and **S6A**). This observation is consistent with the hypothesis that chronic exposure to a diet high in 18:2 promotes resistance in the resident *L. reuteri* population.

Lactobacilli population-level 18:2 resistance _in vivo_ does not predict the resistance of isolates _in vitro_

Next, we sought to relate the *in vitro* resistance of the *L. reuteri* isolates to the *in vivo* changes in *L. reuteri* populations before and after acute 18:2 exposure. To do so, we assessed the enrichment of *L. reuteri* OTU 692154 in the live jejunal biomass post 18:2 gavage. We compared the sequence counts for this OTU in the live-only biomass (i.e., in PMA-treated samples) in mice gavaged with 18:2 to those obtained for the same OTU in the saline gavage group (see Methods). A resulting "18:2 gavage ratio" greater than 0 indicates that live *L. reuteri* OTU 692154 had greater relative abundance counts in mice gavaged with 18:2 compared to same-cage controls gavaged with saline, signifying that other OTUs had been depleted. There was no correlation between the ability of these strains to grow *in vitro* in 18:2 and their abundance in mice gavaged with 18:2 for mice on either diet (**Figs. S6B** and **S6C**). Note, however, that we cannot exclude the possibility that the isolation procedure (growth in MRS, which contains a very small amount, 0.1 μg/ml, of 18:2) favored susceptible strains over resistant strains, such that the *in vitro* resistance patterns of the isolates is not representative of the whole population *in vivo*. With this caveat in mind, these results indicate that while chronic exposure to 18:2 can result in *L. reuteri* strains with higher 18:2 resistance, the mouse gut environment also buffers its toxicity, allowing susceptible strains to remain in the population.

We partially replicated these findings with *L. johnsonii*: as with *L. reuteri*, we isolated and tested the resistance of *L. johnsonii* strains from the ileum, where it was quite abundant (~10% relative abundance from 16S rRNA amplicon data in all mice). Again, all isolates were sensitive to 18:2, but *L. johnsonii* from the HF diet-fed mice were more strongly inhibited by 18:2 than those isolated from the LF-fed mice (159 isolates from 22 mice in 12 cages; t-test, $p<0.01$, **Fig. 4G**). The results for *L. johnsonii* are similar to those of *L. reuteri*, with a lack of congruence between the response of the population *in vivo* and the resistance of isolates *in vitro*.

290

291 **Putative fatty acid responsive genes are mutated in HF diet isolated *L. reuteri***

292 We sequenced to 50x coverage, an isolate of *L. reuteri* resistant to 18:2 and derived from a
293 HF diet mouse (strain LRHF, **Table S2, Fig. S7**). Although we cannot be certain that *L. reuteri*
294 from the HF diet mice shared a common ancestor with those present in the LF diet mice, we
295 compared LRHF to LR0, the 18:2-susceptible isolate derived from a LF diet mouse and used in
296 the *in vitro* evolution assay. The comparison revealed 71 mutations in 60 genes present in LRHF
297 and absent in LR0 with functions in DNA metabolism, energy metabolism, and stress response
298 (**Table 3**, **Table S3**). None of the genes mutated in the *in vitro* evolution assay were recovered as
299 mutated in LRHF. However, relative to LR0, LRHF exhibited mutations in a sodium-hydrogen
300 antiporter gene and a peroxide stress (*PerF*) gene, both of which may represent adaptation to an
301 acidic environment caused by exposure to FAs. Of potential relevance to FA exposure, we
302 observed mutations in a membrane-bound lytic murein transglycosylase D precursor involved in
303 the production of the peptidoglycan layer (Vollmer et al., 2008), and the fructosyltransferase *Ftf*
304 involved in the production of exopolysaccharide (Sims et al., 2011). These results suggest that,
305 evolution in response to 18:2 exposure *in vivo* involves genes for acid stress and the cell
306 wall/membrane.

307

308 **Discussion**

309 A drastic change in dietary macronutrient composition has the capacity to restructure the
310 microbiome within a day (David et al., 2014b; Faith et al., 2011; Turnbaugh et al., 2009) and is
311 one of the most influential contributors to microbiome composition (Carmody et al., 2015). Here,
312 we present the first study to consider how the gut microbiome is influenced by diet from the

313 perspective of a single FA known to be toxic to gut microbes. In this work, we investigated the
314 interaction between lactobacilli and linoleic acid (18:2). As previously observed, we found 18:2
315 to inhibit the growth of most natural-derived lactobacilli *in vitro*. However, in the murine gut, *L.*
316 *reuteri* and *L. johnsonii* persisted through both chronic and acute exposures to 18:2. *L. reuteri*
317 isolates derived from mice on a diet high in 18:2 included some that were more resistant to 18:2.
318 This observation suggests that 18:2 resistance has the potential to be selected in a host. *In vitro*,
319 we showed that *L. reuteri* and *L. johnsonii* both evolve 18:2 resistance through mutations in the
320 cell wall/membrane and fat metabolism genes. Collectively, these data suggest that the host-
321 specific environment protects gut microbes from the inhibitory effects of FAs, but that these
322 microbes can also evolve resistance, providing additional resilience.
323 In addition to being anionic surfactants, previous research demonstrated that long chain
324 unsaturated FAs are both bacteriostatic and bactericidal (Kabara et al., 1972; Kankaanpaa et al.,
325 2001; Kodicek, 1945; Nieman, 1954), by increasing membrane fluidity and permeability
326 (Greenway and Dyke, 1979) potentially leading to cell lysis or leakage (Galbraith and Miller,
327 1973b; Parsons et al., 2012), blocking absorption of essential nutrients (Nieman, 1954), and by
328 inhibiting FA synthesis (Zheng et al., 2005) and oxidative phosphorylation (Galbraith and Miller,
329 1973a). We showed through directed evolution that 18:2 susceptible lactobacilli could evolve
330 resistance. The mutations we recovered from our 18:2 *in vitro* adapted lactobacilli strains are
331 consistent with these killing mechanisms: over 60% of each of our 18:2 adapted populations had
332 mutations affecting FA metabolism and/or the bacterial membrane/wall structure. Additionally,
333 genes contributing to the bacterial membrane/wall structure were mutated in an 18:2 resistant *L.*
334 *reuteri* isolate recovered from a mouse on the HF SBO diet.

Despite the toxicity of 18:2 towards lactobacilli, mouse-associated *L. reuteri* and *L. johnsonii* were present at equivalent relative abundances in mice fed diets high or low in 18:2. Moreover, these microbes survived a gavage of 18:2 equal to double what mice normally encounter in their daily diet. Our results are consistent with the findings of Holmes and colleagues, who analyzed the fecal microbiomes of mice on 25 different SBO diets varying in their macronutrient (fat, protein, carbohydrate) composition. Their results demonstrate that fat has only a minor effect on microbiome structure (Holmes et al., 2017). In contrast, in engineered microbial systems for waste processing, concentrations of linoleic acid exceeding its IC50 but within the range predicted to be consumed by animals can cause failure of the desired microbial biodegradation processes (Lalman and Bagley, 2000). Thus, the resilience of the murine small intestinal microbiome to 18:2 points to mechanisms of resistance.

How then do lactobacilli survive 18:2 in the gut? In mice, lactobacilli colonize both the small intestine and forestomach (Walter et al., 2007). While lingual lipases exist in mice (DeNigris et al., 1988), fat digestion occurs primarily the small intestine. As a result, forestomach microbes should not be exposed to a high concentration free FAs, and SBO itself is not toxic. A gavage of 18:2, on the other hand, should expose forestomach microbes to free 18:2. However, in the rodent forestomach, lactobacilli form a dense biofilm on non-mucus secreting stratified epithelial cells (Frese et al., 2013), which may protect them from free FAs.

The decline of *L. reuteri* in Western populations may never be fully explained. Prior to the emergence of SBO as a major dietary fat source, *L. reuteri* was readily isolated from human stool. Today, however, it is found in less than 10% of humans in the USA and Europe (Walter et al., 2011). Moreover, *L. reuteri* appears to have undergone a population bottleneck that can be dated to approximately when SBO consumption increased (Walter et al., 2011). These

358 observations raise the question of whether a change in dietary habits drove the decline in the
359 prevalence of *L. reuteri* in Western populations. In humans, *L. reuteri* forms neither high gastric
360 populations nor biofilms (Frese et al., 2011; Walter, 2008), thus human *L. reuteri* strains may
361 have survived increased exposure to 18:2 by developing resistance. Indeed, we did observe that
362 some human *L. reuteri* strains are resistant to 18:2, but not all. While the increase in SBO
363 consumption may have conspired with other facets of modernization to reduce the prevalence of
364 *L. reuteri* in Western populations, it did not appear to have resulted in a selective sweep of 18:2
365 resistant *L. reuteri*.

366 The mechanistic underpinnings of how dietary components shape the composition of the
367 gut microbiome need to be further elucidated if manipulation of the microbiome for therapeutic
368 applications is to succeed. Dietary components have the potential to inhibit microbes directly
369 through their toxicity, or indirectly by promoting the growth of other, more fit, microbes. While
370 FAs are generally toxic to many lactobacilli, this work suggests that toxicity is greatly reduced
371 when lactobacilli are host-associated. Future work in this area will elucidate how the host
372 environment protects gut microbes from otherwise toxic dietary components, and the ways
373 specific strains within the microbiome can be resilient to such stresses.

374

375 **Abbreviations**: low fat (LF), high fat (HF), soybean oil (SBO), fatty acid (FA)

376

377 **Author Contributions:** Conception/design of study (SD, RL); acquisition, analysis, and
378 interpretation of data (SD, RL, JJ, EK, MB, JLW, QS, PL); contributed resources (JW, JB);
379 writing and editing manuscript (SD, RL, JW, JB).

380

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603

Figure Legends

Figure 1. ***L. reuteri* is inhibited by 18:2. A**) Disc diffusion of *L. reuteri* plated with the FAs of SBO. FAs were dissolved in DMSO to a concentration of 50 mg/ml, except for palmitic acid (16:0), which was dissolved to a concentration of 5 mg/ml. Clearings around the discs indicate growth inhibition. **B**) Dose response curve of 18:2 with *L. reuteri*. IC50 is estimated at 20 μg/ml ($p<0.001$). See also **Figure S1**.

Figure 2. **Variation in natural *L. reuteri* strains' response to 18:2.** Fourteen rodent (RD), six porcine (PR), nine human (HM), seven poultry (PL), and four sourdough (SD) strains were tested. Standard deviations in 18:2 growth ratios are shown. The significance between the 18:2 growth ratios was determined using a t-test. See also **Figure S2** and **Table S1**.

Figure 3. *In vitro* evolution of 18:2 resistance in lactobacilli. A) *L. reuteri* LR0 and *L. johnsonii* LJ0 were passaged in liquid culture supplemented with a fixed concentration of 18:2 for one week. That weekly concentration of 18:2 was increased by 1 mg/ml from 5 to 10 mg/ml over 6 weeks. Five cultures for each strain were passaged twice daily via a 100x dilution. **B**) Disc diffusion (as in **Figure 1**) of *L. reuteri* and *L. johnsonii* starting strains LR0 and LJ0 and evolved populations LR2 and LJ4. Tested compounds: A. SBO, B. Saline, C. DMSO, D. 16:0, E. 18:0, F. 18:1, G. 18:2, H. 18:3. Growth curve of **C**) *L. reuteri* starting strain LR0, evolved isolate LR2-1 (from population LR2), **D**) *L. johnsonii* starting strain LJ0, and evolved isolate LJ41072 (from population LJ4) in liquid medium with and without 18:2. Each point represents triplicate

cultures and standard deviations are shown. See also **Figure S3, Table S2, S3, S4, File S1,** and **File S2.**

Figure 4. *L. reuteri* and *L. johnsonii* can survive 18:2 *in vivo* without 18:2 resistance. A) Schematic of the SBO diet experiment. After 10 weeks on either the LF or HF SBO diet, mice were gavaged with 18:2 or saline. 1.5 hours post gavage, mice were sacrificed, jejunal contents collected and split into two. One aliquot was PMA treated (live-only biomass) and the other was not (total biomass). 16S rRNA gene sequencing was performed on both aliquots. **B**) Weighted UniFrac distances between the live-only and total biomass aliquots for each mouse sample. **C**) Relative abundance of *L. reuteri* OTU 692154 and **D**) *L. johnsonii* OTU 592160 (post rarefication, N= 22 for LF diet; N= 24 for HF diet). **E**) Relative abundance in the 18:2 live-only biomass aliquots for *L. reuteri* OTU 692154 and **F**) *L. johnsonii* OTU 592160 (post rarefication, N= 11 for LF diet; N= 10 for HF diet). For **C**) through **F**), dark lines indicate the 50% quartile, and the two thinner lines show the 25% and 75% quartiles. **G**) 18:2 growth ratio of lactobacilli isolated from mice on the low or high SBO diets. Higher growth ratios indicate cells were less inhibited by 18:2. A value of one means cells were not inhibited by 18:2. Black lines indicate the mean and standard deviations of the entire set of colonies. The colored lines show the standard deviations for replicate tested colonies. Significance values were determined by t-tests. See also **Figures S4, S5, S6, S7,** and **Table S5**.

Tables

Table 1. High frequency mutations in *L. reuteri in vitro* evolved populations.

Gene	Function	LR1	LR2	LR3	LR4	LR5
FabT (5)*	Fatty acid biosynthesis	71% NS	99% IT	98% NS	76% U	81% NS
EpsD (2)*	Exopolysaccharide synthesis	76% FS		99% NS		
EpsC	Exopolysaccharide synthesis					86% NS
FIG005986 HD family hydrolase	Hydrolase					77% NS
FIG00745602 hypothetical protein	Transmembrane protein		99% PS			
Ammonium transporter	Ammonium transporter			67% U		

*(#) indicates number of distinct mutations across the populations. The percent of the population with a mutation in the named gene is shown. Variants at frequency greater than 60% are shown. NS = nonsynonymous; IT = internal truncation; U = intergenic upstream; PS = premature stop; FS = frameshift. See also **Figure S3, Table S3,** and **File S1.**

656 **Table 2**. High frequency mutations in *L. johnsonii in vitro* evolved populations.

Gene	Function	LJ2	LJ3	LJ4	LJ5
Esterase/lipase	Intracellular esterase/lipase	88% NS			
Putative membrane protein (2)*	Transmembrane protein	100% NS	100% FS	100% NS	
Lipase/esterase	Intracellular esterase/lipase		99% NS		93% NS
KefA	Small-conductance mechanosensitive channel			62% DEL	
LafA	Glycosyltransferase		100% NS		
NrdI (2)*	Ribonucleotide reduction	100% NS		100% NS	
TetR family transcriptional regulator	Membrane structure			>60% PS	

657 Data are presented as in **Table 1**. NS = nonsynonymous; FS = frameshift; DEL = in-frame

658 deletion; PS = premature stop. The insertion in *TetR* in LR4 was not properly called by GATK;

659 the frequency is estimated. See also **Figure S3, Table S4,** and **File S2.**

660 **Table 3**. Nonsynonymous mutations in genes with known function in LRHF.

Gene	Functional group	Mutation type
helicase	DNA metabolism	NS
N-acetyl-L,L-diaminopimelate aminotransferase	energy metabolism	NS
Mrr restriction system protein	DNA metabolism	NS
Putative NADPH-quinone reductase	energy metabolism	NS
Accessory gene regulator C (sensor histidine kinase)	environmental response	NS
Transcriptional regulator, XRE family	environmental response	FS
ATPase component BioM of energizing module of biotin ECF transporter	energy metabolism	NS
CRISPR-associated protein, Csn1 family	DNA metabolism	NS
Transcriptional regulator, XRE family	environmental response	NS
Exodeoxyribonuclease VII small subunit	DNA metabolism	NS
Type I restriction-modification system, specificity subunit S	DNA metabolism	NS
ABC1 family protein	energy metabolism	NS
Protein serine/threonine phosphatase PrpC, regulation of stationary phase	energy metabolism	FS
Nucleotide sugar synthetase-like protein	DNA metabolism	NS
DNA repair protein RecN	DNA metabolism	NS
ABC transporter substrate-binding protein	energy metabolism	FS
Fructosyltransferase Ftf	membrane	PS
Oxidoreductase	energy metabolism	NS
Ribonuclease M5	DNA metabolism	NS
Zinc-containing alcohol dehydrogenase; quinone oxidoreductase	energy metabolism	FS
DinG family ATP-dependent helicase YoaA	DNA metabolism	NS
Aromatic amino acid aminotransferase gamma	energy metabolism	NS

661 Mutations: NS = nonsynonymous; FS = frameshift; PS = premature stop. See also **Figure S7,**

662 **Table S3,** and **File S1.**

Materials and Methods

Strains

Table S1 details the naturally derived *L. reuteri* strains from various hosts and countries. The *L. reuteri* strain (LR0) and *L. johnsonii* strain (LJ0) used in the *in vitro* 18:2 evolution assay were isolated from the jejunum contents of a mouse originally purchased from Taconic Biosciences (Hudson, NY, USA) and maintained on the low fat soybean oil diet for 6 weeks since weaning, and strain LRHF was isolated from a parallel mouse on the high fat soybean oil diet for 6 weeks since weaning (see Mouse care section for further details).

Media and culturing

Lactobacilli were cultured in MRS liquid medium (Criterion, Hardy Diagnostics, Santa Maria, CA, USA) or on MRS agar plates (Difco, BD, Sparks, MD, USA), pH-adjusted to 5.55 using glacial acetic acid. All liquid cultures and plates were incubated at 37°C in an anoxic chamber (Coy Lab Products, Grass Lake, MI, USA) supplied a gas mix of 5% H_2, 20% CO_2, and 75% N_2.

Disc diffusions

We plated 100 µl of a dense, overnight culture of *L. reuteri* strain ATCC 53608 on an agar plate and applied sterile Whatman paper (Buckinghamshire, UK) discs to the surface of the culture plate. To each disc, we added 10 µl of each test compound or control. Compounds tested were alpha-linolenic acid (18:3) (≥ 99%, L2376, Sigma Aldrich, St. Louis, MO, USA), linoleic acid (18:2) (≥ 99%, L1376, Sigma Aldrich), oleic acid (18:1) (≥ 99%, O1008, Sigma Aldrich), stearic acid (18:0) (≥ 98.5%, S4751, Sigma Aldrich), palmitic acid (16:0) (≥ 99%, P0500, Sigma

Aldrich), 0.85% NaCl (saline), DMSO, glycerol, all afore mentioned FAs mixed (FA mix), the FA mix with glycerol, and soybean oil (Wegmans, NY, USA). FAs were dissolved in DMSO to a concentration of 50 mg/ml, except for stearic acid, which was dissolved to a concentration of 5 mg/mL due to its lower solubility. For the FA mix, the five FAs were mixed in the ratio that these FAs are present in soybean oil: 14% 16:0, 4% 18:0, 23% 18:1, 52% 18:2, 6% 18:3. For the FA mix with glycerol, glycerol was mixed with the FA mix to a molar mass ratio of 0.1 (e.g., the molar mass ratio of glycerol in the total molar mass of soybean oil). For testing glycerol alone, the same amount of glycerol used in the FA mix with glycerol was used, and the total volume was brought up to 10 µl with DMSO. Plates were dried for 20 min at 37°C before being turned agar side up and incubated overnight.

Live/dead assay

First, we centrifuged 5 mL of an overnight culture of *L. reuteri* ATCC 53608 at 10,000 rcf for 10 min and resuspended the pellets in 30 mL of 0.85% NaCl solution. Then we centrifuged 1 mL aliquots of the resuspended culture at 15,000 rcf for 5 min. The resulting pellets were resuspended in 0.85% NaCl solution to a total volume of 1 mL in the presence of 18:2, 18:3, 0.85% NaCl, or ethanol. We diluted FAs in 100% ethanol in a ten-fold dilution series ranging from 0.01 to 1000 µg/ml. We incubated samples at room temperature for 90 min on a rocking platform (setting 6; VWR, Radnor, PA, USA) and inverted the samples by hand every 20 min to ensure adequate mixing. After exposure to the FA, we washed the cells by centrifuging at 15,000 rcf for 5 min, and resuspending the pellets in 1 mL 0.85% NaCl; we repeated this wash a second time. To measure the permeability of the cells, we stained samples using the Live/Dead BacLight Bacterial Viability Kit (L7007, Invitrogen, Life Technologies, Grand Island, NY,

USA) according to the manufacturer's instructions. We measured fluorescence from propidium iodide and SYTO9 on a BioTek Synergy H1 Hybrid Reader (BioTek Instruments, Inc., VT, USA). We used the drc package (Ritz et al., 2015) in R (Team, 2016) for dose-response modeling and statistical analyses.

Linoleic acid liquid growth assay

We inoculated a *Lactobacillus reuteri* or *L. johnsonii* colony grown 1 to 2 days on an MRS agar plate into a well containing 300 µl MRS liquid medium on a sterile 2 ml 96 well polypropylene plate (PlateOne, USA Scientific, FL, USA). We covered the plate with Breathe-Easy polyurethane film (USA Scientific, FL, USA) and incubated the plate overnight at 37°C in an anoxic chamber (Coy Lab Products, Grass Lake, MI, USA) supplied a gas mix of 5% H_2, 20% CO_2, and 75% N_2. Following overnight growth, we split the cultures 100-fold into a new 96 well plate, whereby each overnight culture was diluted into a well containing MRS medium and to a well containing MRS medium plus 1 mg/ml linoleic acid. To emulsify the FA in solution, prior to and following inoculation, we vortexed the 2 ml plate on a Multi-Tube Vortexer (VWR, PA, USA) for 30 seconds at setting 3.5. We then transferred the entire plate to a 300 µl Microtest Flat Bottom non-tissue treated culture plate (Falcon, Corning, NY, USA). We measured the OD600 of the plate on a BioTek Synergy H1 Hybrid Reader (BioTek Instruments, Inc., VT, USA) at approximately 0, 2, 4, 6, and 8 hours.

We quantified how well the strain grew in 18:2 compared to the without 18:2 control by analyzing the last three time points of the growth assay. We used this approach over fitting a doubling time because, in the first few points of the growth curve, the OD values in wells with cells and 18:2 were lower than those in inoculation-control wells (e.g., with 18:2, but lacking

cells). Hence, for the first few time points when subtracting the OD600 of medium with 18:2, without cells from the OD600 of medium with 18:2, with cells, we obtained negative OD600 values. As well, the time spent in log phase varied among the strains and proper modeling of log to late-log phase could not be achieved without significant trimming and manipulation of the data. At these final three time points, we determined the ratio of the "blanked OD600s" for the strain growing in MRS medium with linoleic acid to the strain growing in MRS medium alone:

$$\frac{OD600_{MRS\ with\ 18:2}}{OD600_{MRS}}$$

We excluded time points in which the OD600 in MRS medium alone was less than 0.1 (i.e. strain did not grow). We determined the mean of the above ratios for the last three time points and refer to this mean as the "18:2 growth ratio". All negative 18:2 growth ratios were confirmed to result from negative values in the OD600 of cells growing in 18:2.

For the naturally derived *L. reuteri* strains, we tested strains in triplicate to sextuplet and averaged replicate 18:2 growth ratios. For each *L. reuteri* and *L. johnsonii* strain isolated from mice on the SBO diet, we tested eight isolates from two mice per cage. Sixty-two isolates were tested between 2 and 5 times and 18:2 growth ratios were averaged across replicates. Statistical analyses were completed using the aov and t.test functions in the R stats package (Team, 2016).

In vitro evolution of 18:2 resistant lactobacilli

For *L. reuteri* strain LR0 and *L. johnsonii* strain LJ0, both originating from a mouse on the LF SBO diet for 6 weeks, we inoculated a single colony into 5 ml MRS and grew the cultures overnight. The following day, we diluted the overnight cultures for LR0 and LJ0 100-fold, separately, into five 5 ml MRS medium supplemented with 5 mg/ml 18:2. These five cultures became the five populations evolved for *L. reuteri* or *L. johnsonii* and we refer to them as LR1-5

and LJ1-5, respectively. We passaged these cultures twice daily using a 100-fold dilution. We omitted an emulsifier (DMSO or ethanol) from this assay to avoid the possibility of the lactobacilli adapting to the emulsifiers rather than to 18:2. As a result we needed to use a relatively high concentration of 18:2. To promote and maintain emulsification of the FA, we rigorously vortexed the tubes every few hours throughout the day. After seven days, we increased the concentration of 18:2 to 6 mg/ml. Each subsequent week, we increased the concentration by 1 mg/ml until reaching a final concentration of 10 mg/ml. Each week, we froze a 20% glycerol stock of each population at -80°C. We excluded *L. johnsonii* population #1, LJ1, from further study due to contamination.

Whole genome sequencing of *Lactobacillus* populations and isolates

We isolated genomic DNA from approximately 30 µl cell pellets frozen at -20°C using the Gentra Puregene Yeast/Bact. Kit (Qiagen, MD, USA). For isolates, we grew a single 50 ml log to late-log phase culture from a single colony. For populations, we inoculated five 10 ml cultures directly from glycerol stock, grew the cultures to log to late-log phase, and thoroughly mixed the replicate cultures together before pelleting to aid in representing the diversity of original population structure. We grew 18:2-adapted isolates and populations in MRS medium with 10 mg/ml 18:2, and non-adapted isolates in MRS medium. We used the Gentra Puregene Yeast/Bact. kit following the optional protocol adjustments: a 5 min incubation at 80°C following addition of the Cell Lysis Solution, a 45 min to 60 min incubation at 37°C following RNase A Solution addition, and a 60 min incubation on ice following addition of Protein Precipitation Solution. DNA was resuspended in Tris-EDTA and further purified using the Genomic DNA Clean & Concentrator™-25 (Zymo Research, CA, USA). We quantified isolated

DNA using the Quant-it PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific MA, USA). Lastly, to ensure we had obtained large molecular weight DNA, we ran the DNA on a 1% sodium borate agarose gel (Agarose I, Amresco, OH, USA).

We prepared barcoded, 350 bp insert libraries using the TruSeq DNA PCR-Free Library Preparation Kit (Illumina, CA, USA). We fragmented starting genomic DNA (1.4 μg) using the recommended settings on a Covaris model S2 (Covaris, MA, USA). The barcodes used for each library are indicated in **Table S2**. We submitted these barcoded libraries to the Cornell University Institute of Biotechnology Resource Center Genomics Facility where they were quantified by digital PCR using a QX100 Droplet Reader (Bio-Rad Laboratories, CA, USA), pooled (**Table S2**), and pair-end sequenced on an Illumina MiSeq 2x300 bp platform using reagent kit V3 (Illumina, CA, USA). Resulting reads from libraries sequenced on multiple MiSeq runs were merged for further analyses.

Genome assembly of *Lactobacillus* populations and isolates

To generate reference genomes for the ancestor strains used in the *in vitro* evolution assay, we assembled paired-end sequences for *L. reuteri* LR0 and *L. johnsonii* LJ0 using SPAdes v3.7.1 (Nurk et al., 2013; Prjibelski et al., 2014) with k-mers 21, 33, 55, 77, 99, and 127 using the "careful" option to reduce mismatches and indels. To select and order contigs, we aligned the assembled genomes against the closest complete genome available: NCC 533 for *L. johnsonii* and TD1 for *L. reuteri* as determined by a whole genome alignment using nucmer in MUMmer (Kurtz et al., 2004). The assembled genomes we aligned against the NCC 533 or TD1 genome using ABACAS.1.3.1 (Assefa et al., 2009) with the "nucmer" program. Next, we aligned previously unaligned contigs using promer. We merged these sets of aligned contigs into one file

and contigs with low coverage, less than 20, were removed. Finally, we ordered these filtered contigs using promer without the maxmatch option (-d) to prevent multiple reference-subject hits. For the LR0 genome, we identified a contig representing a plasmid from the assembly and included it in the set of assembled contigs. We uploaded these assembled genomes to RAST (Aziz et al., 2008; Brettin et al., 2015; Overbeek et al., 2014) for annotation (see **Table S2** for details on the assembled genomes).

Variant allele detection in 18:2 resistant lactobacilli

First, we manually identified variant alleles in an isolate from *L. reuteri* population LR2, LR2-1, and an isolate from *L. johnsonii* population LJ4, LJ41072, using the Integrative Genomics Viewer (Robinson et al., 2011; Thorvaldsdottir et al., 2013). We used the variants in these isolates to calibrate the allele detection methods applied to the whole populations. Next, we identified variant alleles in the populations by aligning the paired-end sequence reads to the ancestor genome (LR0 or LJ0) using BWA-MEM (Li and Durbin, 2009). We marked duplicate sequences using Picard 2.1.1 (http://broadinstitute.github.io/picard) and utilized Genome Analysis Toolkit (GATK) (McKenna et al., 2010), and the GATK Best Practices recommendations (DePristo et al., 2011; Van der Auwera et al., 2013) to accurately select true variants. This pipeline realigns indels and recalibrates and filters base calls using the known alleles identified in the isolates using a BQSR BAQ gap open penalty of 30. We used the GATK HaplotypeCaller to call alleles with the maxReadsInRegionPerSample option set utilizing the observed coverage binned across the genome by the GATK DepthOfCoverage script. We applied the following options for populations and isolates: pcr_indel_model was set to “NONE”, stand_call_conf was set at “10”, stand_emit_conf at “4”. For populations only, we set

sample_ploidy at “10” and for isolates, “1”. After we had separately processed all populations and isolates, we jointly called alleles across the entire set of populations and isolates using GenotypeGVCFs with sample_ploidy at “10”, stand_call_conf at “10”, and stand_emit_conf at “4”.

We filtered these results to remove alleles with frequencies less than 10% and to remove alleles in genes annotated with “mobile element protein”, “transposase”, “phage”, or “RNA”. In addition, the ancestor genomic reads were mapped onto the ancestor genome to aid in the removal of poorly mapping reads. We removed alleles discovered in the evolved isolates and populations that were also present at frequencies greater than 0.5 in the aligned ancestor reads against the reference. The remaining alleles we manually checked using IGV to remove any alleles in regions of the genome with abnormally high coverage, compared to the directly adjacent regions, likely representing genomic repeat regions. Filtered and unfiltered reads are presented in **Tables S3** and **S4**.

Analysis of mutated genes

We used PredictProtein (Yachdav et al., 2014) to predict the cellular location and structure of hypothetical and putative proteins and SignalP 4.0 (Petersen et al., 2011) to predict signal peptides sequences.

Mouse experiments

All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Usage Committee of Cornell University protocol 2010-0065.

Mouse soybean oil diets

The 16% and 44% SBO diets were custom designed by and purchased pelleted, irradiated, and vacuum packed from Envigo (formerly Harlan Laboratories, Inc., Madison, WI, USA, www.envigo.com). We stored open, in-use diet bags at 4°C and unopened, bags at -20°C. See **Table S5** for the diet compositions.

Determination of transit time of fatty acids to the bloodstream

We gavaged nine mice with 6 mg per gram mouse weight 18:2. Every half hour following gavage, we euthanized a mouse by CO_2 asphyxiation and collected blood by cardiac puncture. Blood was collected into EDTA coated tubes and stored on ice. Tubes were spun at 900 rcf at 4ºC for 10 min, plasma was collected and stored at -80°C. We extracted lipids using the Bligh and Dyer method (Bligh and Dyer, 1959) and quantified FA methyl esters on a Hewlett-Packard 5890 series II gas chromatograph with a flame ionization detector (GC-FID) using H_2 as the carrier. See Su et al. (1999) for further details.

Mouse care

In this study, we used three sets of male C57BL/6 mice bred in three different facilities: Jackson Laboratories (Bar Harbor, ME, USA), Taconic (Hudson, NY, USA), and an F2 generation of mice originally purchased from Jackson Laboratories. At weaning (3 weeks of age), we split littermates into cages housing up to four mice and provided the mice either the LF (16% kcal SBO) or HF (44% kcal SBO) diet (**Table S5**). Littermates were split so to balance mouse weights within a cage and between the two diets. All mice were housed in the Accepted Pathogen Facility for Mice at Cornell University.

In total, 24 mice were purchased directly from Jackson Laboratories and maintained in 6 cages on the LF diet and 24 mice in 6 cages on the HF diet; from Taconic, 12 mice in 3 cages on the LF diet and 12 mice in 3 cages on the HF diet; and the F2 mice from Jackson Laboratories were comprised of 11 mice in 5 cages on the LF diet and 15 mice in 5 cages on the HF diet. Up to four mice were co-caged. We stocked cages with Pure-o-cel (The Andersons, Maumee, Ohio, USA), cotton nestlets, and plastic igloos so to avoid the introduction of exogenous fat. Food was placed in the cages and not on the wire racks to minimize loss and crumb buildup of the diets as the HF SBO diet does not maintain pelleted form. Twice weekly, we completely replaced cages and food. We weighed the amount of new food provided. To obtain mouse weights, we weighed mice in plastic beakers at the same approximate time of day twice weekly. We collected fresh fecal samples once weekly from the beakers into tubes on dry ice, which were later stored at -80°C. Mice were handled exclusively inside of a biosafety cabinet. We changed personal protective equipment and wiped all surfaces with a sterilant between cages to prevent cross-contamination. To measure food consumption, we filtered food crumbs out of the used bedding using a large hole colander followed by a fine mesh sieve, weighed the recovered food, and subtracted this amount from the known amount of food provided.

After 10 weeks on the SBO diets, we gavaged the Jackson Laboratory mice with saline (0.85% NaCl) or 18:2. The Taconic mice were gavaged with phosphate buffered saline (PBS) or 18:2, and the F2 mice from Jackson Laboratories with PBS, 18:2, or 18:3. The volume gavaged was 6 mg per gram mouse weight. The amount of FA gavaged is roughly double the amount of 18:2 consumed by mice on the LF diet each day, and more than half of the 18:2 consumed per day by mice on the HF diet. Within a cage, we gavaged half of the mice with a FA and the other half with saline/PBS, selecting which mouse received which gavage so to balance mouse weights

between gavage groups. Following gavage, we moved mice to a fresh cage supplied with water, but lacking food. After 1.5 hours, we euthanized mice by decapitation and harvested small intestines contents (see below).

Processing of small intestine contents

To harvest the jejunal contents, we divided mouse small intestines into three equivalent pieces. For Jackson Laboratory mice, we flushed the middle segment, the jejunum, with 10 ml anoxic 0.85% NaCl using a blunt, 18G, 1.5 inch needle into a 15 ml conical tube that we immediately placed on ice. After flushing, we quickly shook the tube and split its contents roughly equally into a second 15 ml conical tube. One of the tubes we covered with foil to which we added 12.5 µl of propidium monoazide (PMA) (Biotium, Fremont, CA, USA; f.c. 50 µM from a 2 mM stock dissolved in DMSO). Which tube received PMA, the original or the second, we alternated between mice. To the other tube, we added 12.5 µl DMSO. To allow the PMA time to enter permeabilized cells, we placed all tubes on ice on a rocking platform for 5 min. To activate the azido group in PMA and cause DNA damage, we removed the foil from the tubes, placed the tubes horizontally on ice, and exposed the tubes for 5 min to a 650W halogen bulb (Osram 64553 C318, Danvers, MA, USA) positioned 20 cm from the samples. We frequently rotated the tubes during these 5 min to ensure equal light exposure across the whole sample. We immediately spun these tubes at 4,500 rcf for 5 min at 4°C. After we discarded the supernatant, we flash froze the tubes on liquid N_2, placed them on dry ice, and later stored the tubes at -80°C. We also flushed the upper half of the last segment of the small intestine, the ileum, with MRS medium and 20% glycerol, immediately placed the glycerol stock on dry ice, which we later stored at -80°C. For the other mice, we flushed the jejunum with 10 ml anoxic PBS (pH 7.4) and

914 did not use a PMA treatment. The small intestine contents for these mice were pelleted as
915 described above.
916
917 **DNA isolation and 16S rRNA gene sequencing**
918 We used the PowerSoil DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA) to
919 extract DNA from these jejunum pellets frozen in 2 ml tubes containing 0.1 mm glass beads (Mo
920 Bio Laboratories, Carlsbad, CA, USA). We eluted the DNA on the spin filter using 50 µl
921 Solution C6 and stored the DNA at -20°C. We conducted blank extractions in parallel. We
922 processed mouse fecal pellets in a similar manner.
923 We quantified DNA samples and blank extractions using the Quant-it PicoGreen dsDNA
924 Assay Kit. For each sample, we performed two 50 µl PCRs to amplify the V4 region of the 16S
925 rRNA gene using primers 515F (f.c. 100 nM), Golay barcoded 806R (f.c. 100 nM) (Caporaso et
926 al., 2012), 5 Prime Mix (Quanta Biosciences, CA, USA) or Classic++™ Taq DNA Polymerase
927 Master Mix (TONBO biosciences, CA, USA), and 25 ng of DNA. PCR conditions were 94°C for
928 3 min, 30 cycles of 94°C for 45 seconds, 50°C for 1 min, and 72°C for 1.5 min, followed by a
929 final extension at 72°C for 10 min. Reactions were held at 4°C and stored at -20°C.
930 We combined the two 50 µl PCRs and purified DNA using Mag-Bind® E-Z Pure
931 (OMEGA Bio-tek, GA, USA) following the manufacturer's instructions and eluting with 35 µl
932 TE. We measured DNA concentrations using PicoGreen. We pooled 100 ng of amplicon DNA
933 from each sample together and sequenced the pool using the Illumina MiSeq 2x250 bp platform
934 at the Cornell Biotechnology Resource Center Genomics Facility.
935
936 **16S rRNA gene amplicon analysis**

We processed, filtered, and analyzed the 16S rRNA gene amplicon data from all studies using QIIME 1.9.0 (Caporaso et al., 2010). Paired-end reads were joined using join_paired_ends.py running the fastq-join method and requiring at least 200 bp of sequence overlap. Joined reads were demultiplexed using split_libraries_fastq.py requiring a Phred quality cutoff of 25 to remove ambiguous barcodes and low quality reads. Reads were clustered into operational taxonomic units (OTUs) using open-reference OTU picking at 97% sequence identity to the Greengenes database version 13.8 (DeSantis et al., 2006). We focused our analyses on the two most abundant lactobacilli OTUs: OTU 692154 identified as *L. reuteri* and OTU 592160 as *L. johnsonii* as denoted by the Greengenes assignment. We confirmed these assignments by sequencing the full 16S rRNA gene of lactobacilli isolates (see below).

Except where noted, for all subsequent analyses, we rarified data to 40,000 sequences per sample. We calculated beta-diversity using the weighted UniFrac metric implemented in QIIME 1.9.0. We performed adonis (PERMANOVA) with 10,000 iterations in QIIME using the compare_categories.py script. Beta-diversity plots were made using the phyloseq package (McMurdie and Holmes, 2013) with the ordplot function using a t-distribution. We identified OTUs differentiating samples by first filtering OTU tables to only include those OTUs present in at least 25% of samples and with at least one sample having at least 100 counts of that OTU. To the filtered OTU tables, we applied a Kruskal-Wallis test with an FDR cutoff of 10% using the group_significance.py script in QIIME. We created heatmaps of OTUs passing with FDR < 0.1 using the make_otu_heatmap.py script in QIIME. To detect *L. reuteri* in the fecal pellets of F2 mice from Jackson Laboratories, samples with at least 10,000 sequences were used (sequencing depth was lower for the fecal pellets), and data were not rarefied so to maximize detection of *L. reuteri*.

960

961 **qPCR analysis of lactobacilli copy number altered by PMA treatment**

962 We determined the copy numbers of the lactobacilli 16S rRNA gene and total Eubacterial
963 16S rRNA gene in the PMA and non-PMA treated jejunal aliquots by quantitative real-time PCR
964 (qPCR) using the LightCycler 480 platform and the SYBR Green I Master kit (Roche
965 Diagnostics Corporation, Indianapolis, IN, USA). We utilized the lactobacilli and Eubacterial
966 primers described by Oh et al. (2012). PMA treatment reduces the total amount of DNA
967 extracted by removing DNA from any dead cells. Thus, using the same mass of DNA for the
968 PMA and non-PMA aliquots would result in quantifying copy numbers relative to the total
969 amount of DNA assayed, similar to the relative abundances determined from the 16S rRNA gene
970 sequencing. Therefore, we fixed the amount of DNA used for all non-PMA samples to 10 ng.
971 Thus 10 µl qPCRs consisted of 10 ng of DNA for the non-PMA aliquots and equal volume for
972 the PMA aliquot, each qPCR primer at 500 nM, and 5 µl of SYBR Green I Master mix. Cycling
973 conditions were 5 min at 95°C followed by 45 cycles consisting of 10 s at 95°C, 20 s at 56°C for
974 the Eubacterial primers and 61°C for the lactobacilli primers, and 30 s at 72°C after which
975 fluorescence from SYBR Green was read. Melting curve analysis was used to determine whether
976 each qPCR reaction generated a unique product. Cycle threshold (C_t) values were calculated
977 using the absolute quantification/2nd derivative max function available on the LightCycler 480
978 software. All reactions were run in triplicate, and the mean C_t values were used in subsequent
979 calculations.

980 To determine if the *Lactobacillus* population decreased due to the 18:2 gavage, we
981 calculated the difference in lactobacilli copy number between the PMA (live-only biomass) and
982 non-PMA (total biomass) aliquots relative to that for Eubacteria. That is,

$$\frac{2^{\Delta C_t\ Lacto(PMA-non.PMA)}}{2^{\Delta C_t\ Eubac(PMA-non.PMA)}}$$

If the *Lactobacillus* population is not affected by the 18:2 gavage, no difference should be observed between the saline and 18:2 gavage samples. Moreover, this ratio is expected to be close to one lactobacilli were not specifically killed by the 18:2 gavage.

Gavage ratio calculations

For each cage, we split the mice according to which gavage they received (18:2 or saline) and we took the mean of the rarefied sequence counts for OTU 692154 (*L. reuteri*). Then we calculated the $\log_{10}$ of the ratio of the 18:2 mean rarefied sequence counts to the mean saline relative abundance sequence counts. We refer to this ratio as the 18:2 gavage ratio:

$$log_{10}\frac{mean\ 18{:}2\ counts\ per\ cage\ for\ OTU}{mean\ saline\ counts\ per\ cage\ for\ OTU}$$

***L. reuteri* and *L. johnsonii* isolation from small intestine contents**

We streaked the glycerol stocks of mouse ileum contents onto MRS agar plates. One or two colony morphologies were present on nearly all plates: lowly abundant bright cream, round colonies present on most plates, and abundant flatter, dull white colonies present on all plates. We determined the species identity of these colony morphologies by full length 16S rRNA gene sequencing using primers 27F (f.c. 1 nM) and 1391R (f.c. 1 nM) (Turner et al., 1999), 10 µl of Classic++™ Hot Start Taq DNA Polymerase Master Mix (Tonbo Biosciences, CA, USA), and a small amount of a single bacterial colony in a 25 µl reaction. PCR conditions were 94°C for 3 min, 38 cycles of 94°C for 45 s, 50°C for 1 min, and 72°C for 1.5 min, followed by a final extension at 72°C for 10 min. We purified PCRs using Zymo DNA Clean & Concentrator™-5 (Zymo Research, CA, USA) and submitted samples to Cornell University Institute of

Biotechnology Sanger sequencing facility. Returned sequences were assembled using Sequencher® version 5.4.6 (DNA sequence analysis software, Gene Codes Corporation, Ann Arbor, MI, USA, http://www.genecodes.com) and aligned against National Center for Biotechnology Institute's nr database.

Statistical analyses

We conducted all other statistical analyses in R (Team, 2016). All t-tests are two-tailed two-sample t-tests.

Data deposition

The lactobacilli raw sequencing reads and the assembled genomes for strains LR0 and LJ0 are available under BioProject accession PRJNA376205 at National Center for Biotechnology Institute. The RAST genome annotations for these genomes are available in Supplement Files S1 and S2. The 16S rRNA gene amplicon data are available under the study accession PRJEB19690 at European Nucleotide Archive.

Table S1. ***L. reuteri*** **strains isolated from various hosts. (Related to Figure 2).** Host, strain name, country of origin, clade, and site of isolation on the human body (if applicable) are given as determined by Oh et al. (2010) and Zheng et al. (2015).

Table S2. ***Lactobacillus in vitro*** **population sequencing. (Related to Figure 3).** Tab "SequencingCoverage" gives information on the sequencing run, barcode, number of sequences obtained, and estimated genomic coverage. Tab "AncestorGenomes" gives information on the assembled LJ0 and LR0 genomes.

Tables S3. **Filtered and unfiltered mutations in the *L. reuteri in vitro* population and HF diet isolate sequencing data. (Related to Figure 3, Table 1, and Table 3).** Tab "Key" describes the information in the subsequent tabs. Tab "All" shows all variants passing filtering by GATK. Tab "Filtered" shows variants filtered to exclude alleles in genes annotated with "mobile element protein", "transposase", "phage", or "RNA", alleles at less than 10% frequency, and alleles at frequency greater than 0.5 in the aligned ancestor reads against the reference genome. Tab "Handchecked" shows variants passing previous filtering and confirmed manually in IGV. For *L. reuteri* two additional tabs are included: "LRHF only" shows variants only found in the *L. reuteri* isolate from a mouse on the HF SBO diet. "Populations only" tab shows variants only found in the *in vitro* evolution assay. Genomic details are taken from the RAST annotation of the ancestor genome. Other columns are taken from the GATK vcf file. For allele variants falling in intergenic regions, the surrounding genes are listed in HitGene and HitChrom, HitStrand, HitStart, HitEnd, HitDNA, and HitProtein are listed as "NA".

Table S4. **Filtered and unfiltered mutations in the *L. johnsonii in vitro* population sequencing data. (Related to Figure 3 and Table 2).** Tabs and details are the same as for **Table S3**.

File S1. **RAST annotation for the assembled LR0 genome. (Related to Figure 3, Table 1, and Table 3).**

File S2. **RAST annotation for the assembled LJ0 genome. (Related to Figure 3 and Table 2).**

Figure 1

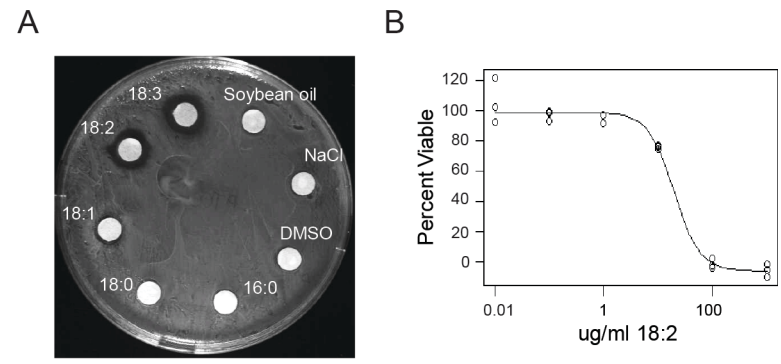

Figure 2

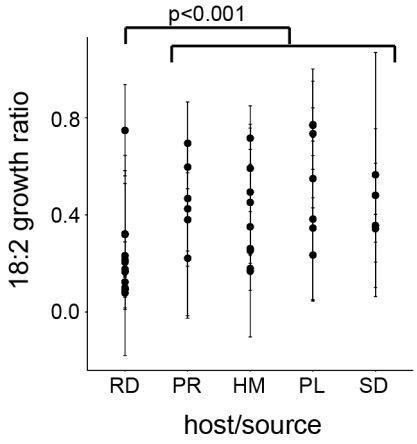

Figure 3

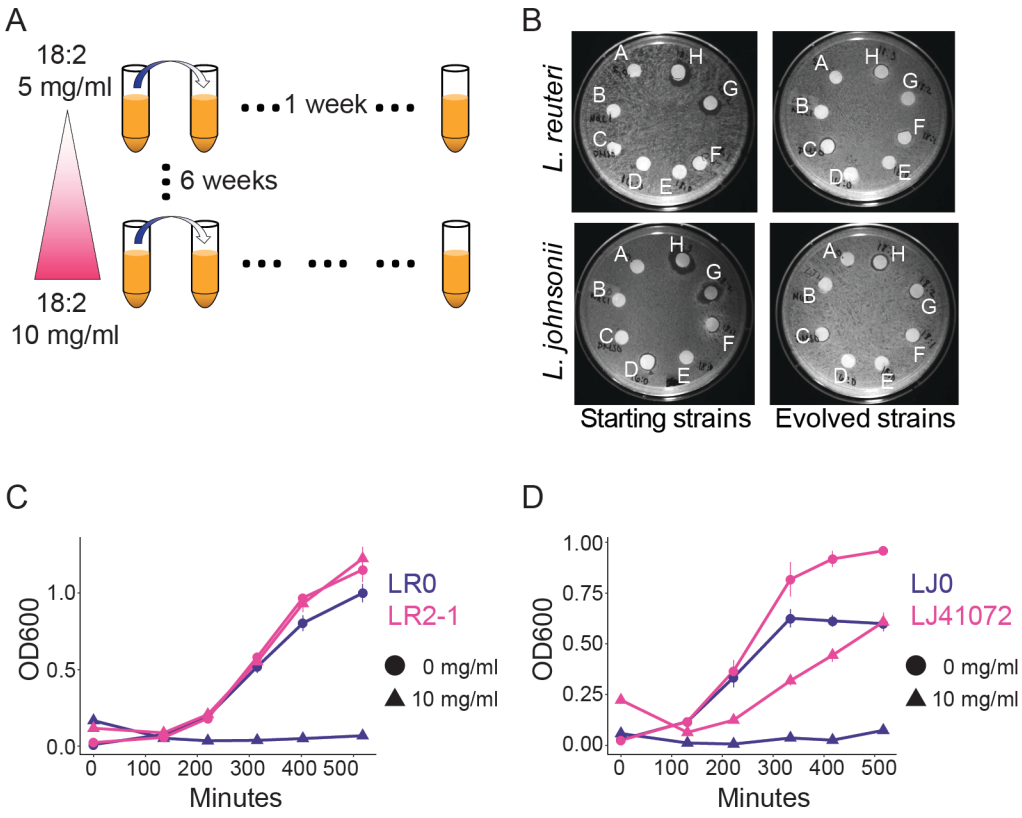

Figure 4