Lactobacillus spp. decrease the susceptibility of *Salmonella* Typhimurium to the last resort antibiotic azithromycin

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RUNNING TITLE Azithromycin tolerance of Salmonella by Lactobacillus

KEYWORDS *Lactobacillus spp.*, *Salmonella* Typhimurium, antibiotic tolerance, azithromycin, genetic screen

Abstract

Bacteria are involved in numerous interactions during infection and among host-associated microbial populations. *Salmonella enterica* serovar Typhimurium is a foodborne pathogen of great importance as well as a model organism to study interactions within a microbial community. In this

study, we found that *S*. Typhimurium becomes tolerant to azithromycin when co-cultured with strains of *Lactobacillus*. Similarly, acidified media, from cell-free supernatant of *Lactobacillus* cultures for instance, also induced the tolerance of *S*. Typhimurium to azithromycin. The addition of membrane disruptors restored the normal sensitivity to azithromycin in acidified media, but not when *Lactobacillus* was present. These results suggested that the acidification of the media led to modification in envelope homeostasis, but that a different mechanism promoted the tolerance to azithromycin in the presence of *Lactobacillus* strains. To further understand how *Lactobacillus* strains modify the sensitivity of *S*. Typhimurium to azithromycin, a high-throughput assay was carried using the single gene deletion collection of the *S.* Typhimurium (1) in coculture with *L. rhamnosus* and (2) in sterile acidic conditions (pH 5.5 media only). As expected, both screens identified genes involved in envelope homeostasis and membrane permeability. Our results also suggest that changes in the metabolism of *S.* Typhimurium induce the tolerance observed in the presence of *L. rhamnosus*. Our results thus highlight two different mechanisms by which *Lactobacillus* strains induce tolerance of *S.* Typhimurium to antibiotics.

Importance

This study provides valuable insights into the intricate interactions between bacteria during infections and within host-associated microbial communities. Specifically, it sheds light on the significant role of *Lactobacillus* strains in inducing antibiotic tolerance in *Salmonella enterica* serovar Typhimurium, a critical foodborne pathogen and model organism for microbial community studies. The findings not only uncover the mechanisms underlying this antibiotic tolerance but also reveal two distinct pathways through which *Lactobacillus* strains might influence *Salmonella*'s response to antibiotics. Understanding these mechanisms has the potential to enhance our knowledge of bacterial infections and may have implications for the development of strategies to combat antibiotic resistance in pathogens like *Salmonella*. Furthermore, our results underscore the necessity to explore beyond the direct antimicrobial effects of antibiotics, emphasizing the broader microbial community context.

66 Bacterial pathogens are often found within polymicrobial communities and thus must interact with
67 other microbes present in the environment. For instance, members of host-associated communities
68 (*i.e.*, microbiota) compete with pathogens entering a host and promote resistance to colonization
69 by foreign microbes (1). Not surprisingly, pathogens have adapted to this polymicrobial
70 environment and, for instance, overcome colonization resistance through the use of unique
71 nutrients to persist in their ecological niche (2). There are many examples of the intricate
72 relationships between pathogens and host-associated communities, but we are only beginning to
73 understand the consequences of these interactions. A notorious example of interbacterial
74 relationship is between *Pseudomonas aeruginosa* and *Staphylococcus aureus*, where *P. aeruginosa*
75 profoundly alters the physiology of *S. aureus* and, accordingly, its susceptibility to antimicrobial
76 agents (3–5). Therefore, investigating microbe-microbe interactions could provide new details
77 about bacterial cells found in polymicrobial contexts. Herein, we probed the physiological state of
78 the model intestinal pathogen *Salmonella enterica* serovar Typhimurium by determining its
79 susceptibility against various antibiotics in the presence of *Lactobacillus* strains and found that *S.*
80 Typhimurium becomes tolerant to azithromycin in presence of *Lactobacillus* isolates.

81 To explore the impact of *Lactobacillus* spp. on *S.* Typhimurium, we assessed the susceptibility of
82 *S.* Typhimurium against various antibiotics during co-incubation experiments. Changes in
83 antibiotic sensitivity are a powerful tool to assess the physiological state of the cell (6,7). We used
84 selective media to determine the minimal inhibitory concentration (MIC) of antibiotics against
85 *S.* Typhimurium co-incubated with one of two *Lactobacillus* strains, *L. rhamnosus* LMS2-1 or
86 *L. reuteri* CF48-3A (Fig.S1A). A proof-of-concept experiments showed that, as expected, a beta-
87 lactamase-producing *S. aureus* decreased the sensitivity of *S.* Typhimurium against ampicillin
88 (Fig.S1B). We then assessed the susceptibility of *S.* Typhimurium against various antibiotics,
89 including relevant antibiotics for treating salmonellosis such as ciprofloxacin, ceftriaxone and
90 azithromycin (8,9), in the presence of either of the two *Lactobacillus* strains (Fig.1A). While we
91 saw some isolate-specific effects, co-incubation with both *Lactobacillus* isolates resulted in a 16-
92 fold increase in azithromycin MIC against *S.* Typhimurium (from 2 to 32 µg/mL; Fig.1A and
93 Fig.S1C). Interestingly, *S.* Typhimurium seemed tolerant rather than resistant to azithromycin in
94 the presence of the *Lactobacilli*, as cells recovered from the co-cultures where reverting back to
95 their initial susceptibility to azithromycin (MIC of 2 µg/mL). In addition, heat-killed *Lactobacillus*
96 cells did not increase the MIC to azithromycin against *S.* Typhimurium (Table S1).

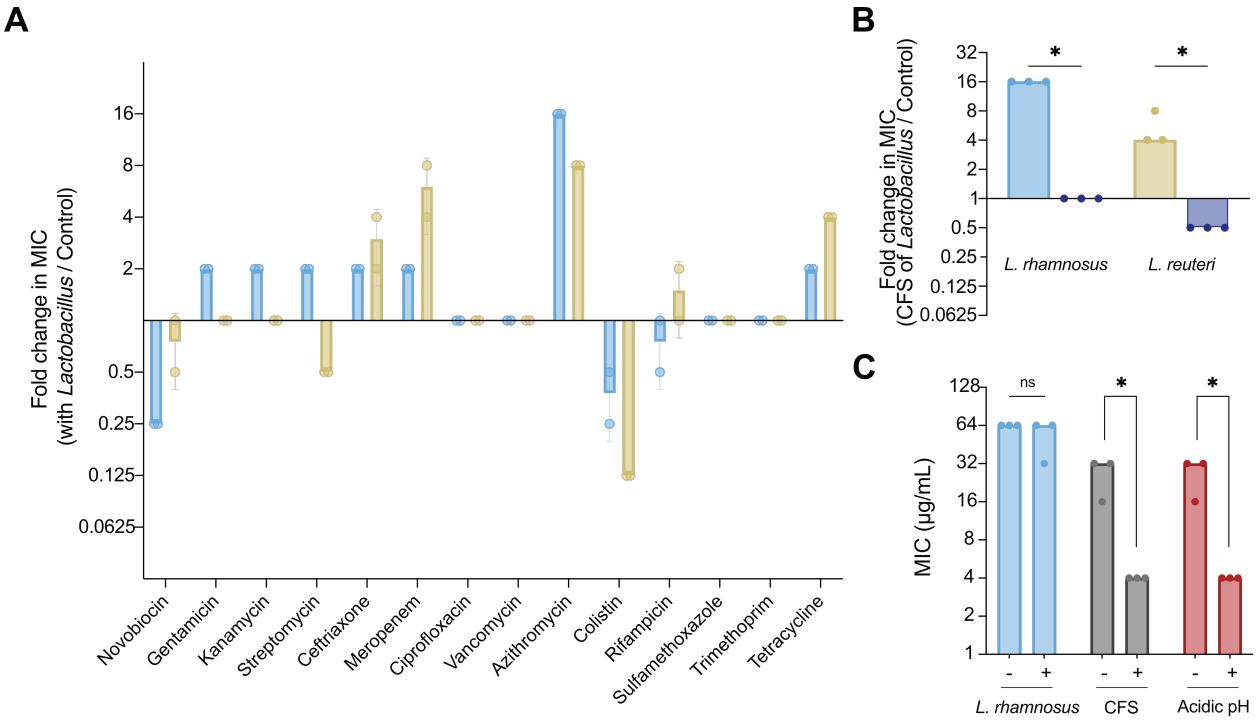

Figure 1. *Salmonella* Typhimurium becomes tolerant to azithromycin when cocultured with strains of *Lactobacillus*. (A) Fold change in the MIC of several antibiotics against *S.* Typhimuirum in the presence of *Lactobacillus* spp. Fold change was determined by producing a ratio of the MIC of *S.* Typhimurium in the presence of *L. rhamnosus* (blue) or *L. reuteri* (yellow) and the MIC of *S.* Typhimurium grown by itself against a selected panel of antibiotics. n=2 (B) Fold change in the MIC of azithromycin against *S.* Typhimurium in the presence of cell-free supernatant (CFS) (blue/yellow) or neutralized CFS (purple) of *Lactobacillus* spp. compared to the MIC of azithromycin against *S.* Typhimurium in monoculture. Initial pHs were 5.66 and 5.34 respectively for of *L. rhamnosus* and *L. reuteri* CFS. CFS was neutralized for both *Lactobacillus* to a pH of 7.00. Wilcoxon-Mann-Whitney U test, * indicates a p-value ≤ 0.05. n=3 (C) Impact of the membrane-active compound pentamidine on the MIC of azithromycin for *S.* Typhimurium in presence of *L. rhamnosus*, CFS or acidified media. Pentamidine was added at a concentration of 64 µg/mL. Wilcoxon-Mann-Whitney U test, * indicates a p-value ≤ 0.05. n=3.

Cell-free supernatants (CFS) from both *Lactobacillus* strains also induced azithromycin tolerance in *S.* Typhimurium (Fig.1B). As expected, CFS from the *Lactobacillus* strains were acidified compared to the media used for the experiment (pH of 5.66 for *L. rhamnosus* and 5.34 for *L. reuteri*). When neutralized CFS were used, the MICs went back to normal (Fig.1B). In addition, replacement of CFS by acidified media (BHI at pH 5.5) in combination with *S.* Typhimurium

116 showed a similar increased in MIC (Table S1), suggesting that the decrease in pH caused the
117 observed tolerance against azithromycin. In contrast, lactate, one of the main acidic compound
118 produced by *Lactobacilli* (10), did not increase the MIC by itself (Table S1). It is important to note
119 that azithromycin is stable in acidic conditions (9), and thus the tolerance to the antibiotic is not
120 likely due to compound break down.

121 Azithromycin is dependent on envelope homeostasis to enter bacterial cells. Consequently,
122 modification of cell permeability is a prevalent resistance mechanism (11,12). To investigate the
123 hypothesis that the azithromycin tolerance was due to modified cell permeability and reduced
124 azithromycin uptake, we conducted co-incubation assays in the presence of two membrane
125 destabilizing agents: pentamidine and EDTA (13,14). The addition of pentamidine (Fig.1C) or
126 EDTA (Fig.S2) still resulted in an increased MIC of azithromycin against *S.* Typhimurium when
127 co-cultured with *L. rhamnosus*. However, when CFS of *L. rhamnosus* or acidified media were
128 used, pentamidine and EDTA decreased the MIC of azithromycin compared to values observed for
129 monocultures of *S.* Typhimurium. These results demonstrate the existence of two discrete and
130 sperate mechanisms contributing to the observed phenotype. First, as expected, the acidification of
131 the media most likely induced alterations in envelope permeability, decreasing the entry of
132 azithromycin (15). Second, in the presence of *Lactobacillus*, a distinct mechanism emerges as a
133 strong driver of the tolerance of *S.* Typhimurium to azithromycin.

134 To investigate the response of *S.* Typhimurium to the presence of *Lactobacillus* isolates or to
135 acidified media, we reproduced the co-culture experiments to a larger scale, using a genome-wide
136 single-gene deletion (SGD) collection of *S.* Typhimurium (16) and either high-density cultures of
137 *L. rhamnosus* or acidified media, both in presence or absence of azithromycin. In our high-
138 throughput experiments, the MIC of azithromycin against *S.* Typhimurium alone was 8 µg/mL.
139 Therefore, we used a concentration of 128 µg/mL of azithromycin in our high-throughput assay to
140 be representative of our initial observation (16-fold increase in MIC). At this concentration,
141 *S.* Typhimurium only grows when *L. rhamnosus* is present or in acidified media, but not in
142 monoculture. Mutants showing a growth defect specifically when treated with high concentrations
143 of azithromycin indicate that their deleted gene is essential for the tolerance phenotype. We
144 compared the growth of the SGD library with and without azithromycin in the presence of either
145 (i) *L. rhamnosus* or (ii) acidified media. The two conditions showed different responses to the

146 presence of azithromycin, with the presence of *L. rhamnosus* having a bigger impact on
147 *S.* Typhimurium (Fig.2A), corroborating our hypothesis that different mechanisms are involved in
148 the increased tolerance. We transformed screening values into Z-scores and selected gene deletions
149 that were significatively different than the population (p-value ≤ 0.05) (Fig.2B and Table S3).
150 While we did not necessarily observe the same hits, both screens were enriched for gene deletions
151 known to disrupt the permeability of the outer membrane in *Escherichia coli* (*ldcA*, *mdoG*, *pal*,
152 *pgm*, *prc, rfaP*, *rfaY*, *wecC*, *wecG*, *yceG* for instance) (17). In addition, in the presence of *L.*
153 *rhamnosus*, the growth of both gene deletions from the two-component systems *envZ/ompR* and
154 *phoP/phoQ* was impaired in the presence of azithromycin. The prevalence in genes affecting
155 membrane homeostasis supports the effect of membrane disruptors in acidified media. In the
156 presence of *L. rhamnosus*, additional gene deletions affecting core metabolic processes also
157 impaired the tolerance of *S.* Typhimurium to azithromycin (*nuo* genes, *sdhAB*, *sucB*, *arcA* for
158 instance). Our results suggests that, in the presence of *Lactobacillus*, *S.* Typhimurium undergoes
159 changes in its metabolism leading to tolerance to azithromycin that prevails the modification in
160 envelope homeostasis observed with acidified media.

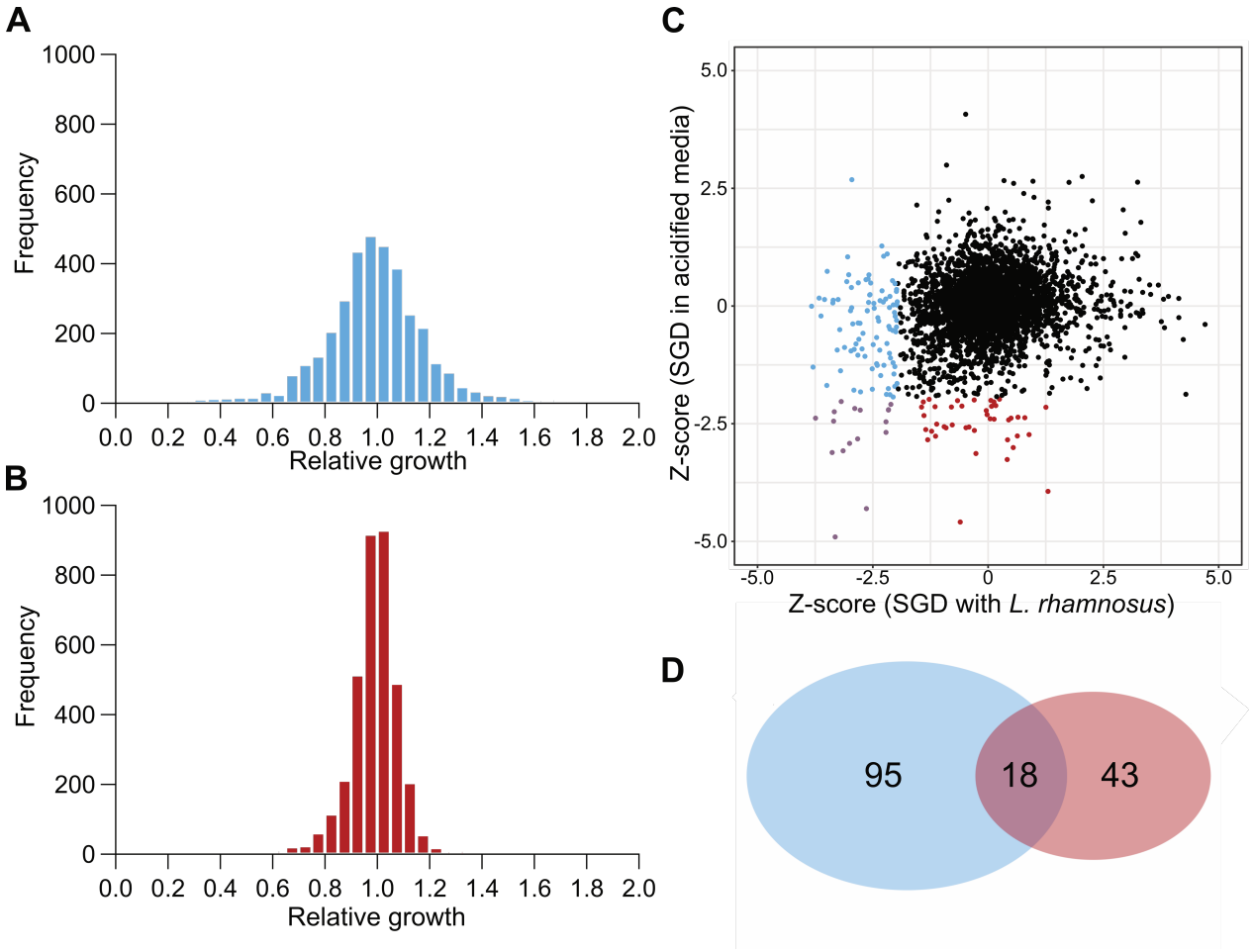

Figure 2. Growth of the SGD library at high azithromycin concentrations in the presence of *L. rhamnosus* or acidified pH. The SDG library was grown in the presence of (A) *L. rhamnosus* (blue) or (B) acidified media (red) and with 128 µg/mL of azithromycin (16X MIC in tested conditions). The growth of each mutant in the presence of azithromycin was compared to controls without azithromycin to yield a relative growth (a.u.) value, where a value of 1 means that azithromycin did not affect the growth of the mutant. (C) Relative growth values were converted to Z-scores. We selected hits corresponding to gene that were significatively different from the population (Z-score < -1.96, p-value ≤ 0.05) only the presence of *L. rhamnosus* (blue), acidified media (red) or in both conditions (purple). (D) Venn diagram of selected hits.

Further investigations of the interaction between *S.* Typhimurium and strains of *Lactobacillus* spp. will be necessary to fully understand the molecular basis behind how *Lactobacillus* isolates are

able to induce a change in the susceptibility of the pathogen to azithromycin. If the enhanced tolerance of *S.* Typhimurium against azithromycin under acidic conditions is linked to modified membrane permeability, a distinct mechanism of action appears to be responsible for the same phenotype in presence of *L. rhamnosus*. In light of the escalating antibiotic resistance worldwide and the considerable obstacles to developing novel antibiotic molecules, our results underscore the urgency of delving deeper into the intricate dynamics between pathogens like *S.* Typhimurium and common human-associated bacteria like *Lactobacillus* spp., as they have the potential to significantly impact the effectiveness of antibiotic therapies.

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251

252 **Supplemental Material**

253 ***Materials and Methods***

254 Bacterial strains and growth conditions

255 All experiments in this study were performed with *Salmonella enterica* serovar Typhimurium strain
256 14028s or the described single-gene deletion library (SGD library) of *S.* Typhimurium 14028s (16).
257 *Lactobacillus reuteri* CF48-3A, *Lactobacillus rhamnosus* LMS2-1 and *Staphylococcus aureus* 130
258 were obtained from the Human Microbiome Project (18) and were cultured in Brain-Heart infusion
259 (BHI) broth prepared according to Lau *et al* (19). All strains were grown at 37°C. The SGD library
260 was maintained in BHI broth supplemented with kanamycin at a concentration of 50 mg/ml.

261 Products and solutions

262 A selective panel of antibiotic compounds was selected to cover various antibiotic classes.
263 Azithromycin, meropenem and sulfamethoxazole were obtained from Sigma Aldrich and diluted
264 in dimethyl sulfoxide (DMSO). Tetracycline, novobiocin, cefuroxime, ceftriaxone and colistin
265 were also obtained from Sigma Aldrich but were diluted in water. Rifampicin, erythromycin,
266 nalidixic acid and trimethoprim were obtained from Bio Basic and diluted in DMSO. Streptomycin,
267 gentamicin, ampicillin, kanamycin, ciprofloxacin and vancomycin were also obtained from Bio
268 Basic but were diluted in water. Lastly, apramycin was obtained from Alfa Aesar and was diluted
269 in water. Ethylenediamine tetraacetic acid (EDTA) was obtained from Fisher Bioreagents and
270 diluted in water. Pentamidine isethionate salt (Sigma Aldrich) was diluted in DMSO. All antibiotics
271 and pentamidine were solubilized initially at 25mg/mL. Sodium L-lactate was also obtained from
272 Sigma Aldrich and diluted in BHI.

273 Minimal inhibitory concentration assay

274 Determination of the minimal inhibitory concentration (MIC) was adapted from the Clinical and
275 Laboratory Standards Institute (CLSI) guidelines (20). Briefly, each strain was grown overnight,

276 then diluted 1:1000 in BHI. 2-fold dilution series of the antibiotics were added to the diluted culture
277 in a 96-well plate starting at 256 µg/mL. The MIC, defined as the lowest concentration to inhibit
278 visible growth, was determined at 18h incubation at 37°C. When indicated, EDTA or pentamidine
279 was supplemented at a final concentration of 2 mM and 64 µg/mL, respectively. The MIC
280 determination assay was also performed in acidic conditions as previously stated. Acidified BHI
281 (pH of 5.5) was prepared with a solution of HCl 1N and used to dilute the different antibiotics to
282 be tested in serial dilutions in 96-well plate.

283 Coculture minimal inhibitory concentration assay

284 The MIC of *S.* Typhimurium against various antibiotics was determined in the presence of
285 *L. reuteri* CF48-3A or *L. rhamnosus* LMS2-1. The method for these combination assays was also
286 adapted from the CLSI guidelines (20). Overnight cultures of *Lactobacillus* strains were grown for
287 18h at 37°C with agitation. *S.* Typhimurium was also grown overnight, then diluted 1:1000 in BHI.
288 The co-culture experiments were performed by mixing the diluted culture of *Salmonella* with
289 overnight cultures of the *Lactobacillus* strains in 96-well plates in a 1:1 ratio. Antibiotics were
290 serially diluted as described for the MIC assays. The coculture plates were then incubated for 18h
291 at 37°C. After the incubation, 5 µL of each well was plated on *Salmonella Shigella* (SS) agar, which
292 is selective for Gram-negative bacteria. The agar plates were incubated overnight at 37°C, after
293 which the MIC of *S.* Typhimurium was determined visually. The method is shown in
294 Supplementary Figure S1A. When needed, EDTA or pentamidine were also added to the wells as
295 described in the MIC assays.

296 For cocultures with the cell free supernatant (CFS) of *Lactobacillus* strains, *Lactobacillus* cultures
297 were grown for 18h at 37°C with agitation. The overnight cultures were then centrifugated at 4,000
298 rpm for 10 min. The resulting supernatant was collected and filtered with a sterile 0.22 µm syringe
299 filter. The sterile supernatants were then added to the 96-well plates containing the diluted
300 *S.* Typhimurium and the antibiotics, as described above. For cocultures with heat-killed strains,
301 cultures were grown for 18h at 37°C with agitation. After the incubation, strains were subjected to
302 a heat treatment of 20 min at 70°C, followed by two rounds of 5 min centrifugation at 13,000 rpm
303 and washing in 1X PBS. Washed pellet was resuspended in BHI before being added to the 96-well
304 plates containing the diluted *S.* Typhimurium and the antibiotics, as described above. For the lactate

305 assay, strains were grown as described above, and the lactate solution was added at a final
306 concentration of 80 mM (21) in the 96-well plate containing the diluted *S.* Typhimurium and the
307 antibiotics. It is important to note that the addition of the compound did not affect the initial pH of
308 BHI, therefore the assay was conducted under neutral pH conditions.

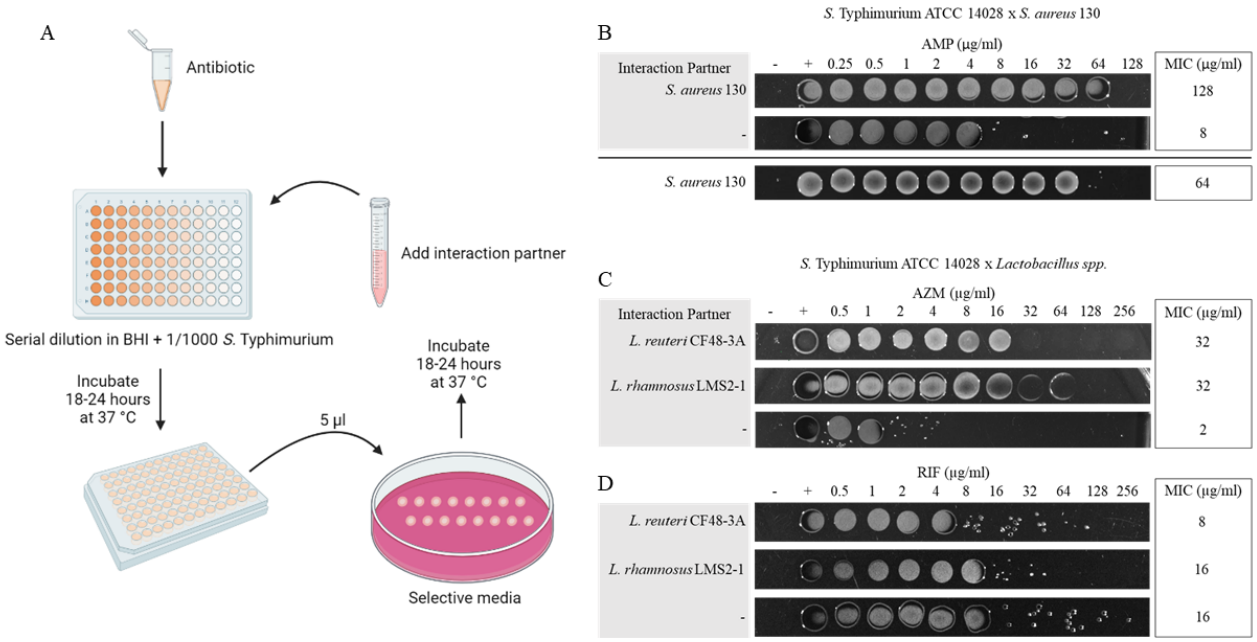
309 High-throughput co-culture assay using the SGD collection

310 *S*. Typhimurium SGD collection was grown for 18h at 37°C in 384-well plates filled with 50 µL
311 of BHI. *L. rhamnosus* LMS2-1 was grown separately in 25 ml of BHI at 37°C for 18h. The
312 coculture plates were then prepared by adding 25 µL of overnight *L. rhamnosus* culture and 25 µL
313 of BHI supplemented with 256 µg/mL of azithromycin in 384-well plates (final concentration of
314 128 µg/mL in 50 µl). The SGD collection was finally inoculated to the 384-well coculture plates
315 with the Rotor HDA (Singer Instruments, UK). This setup transfers ~0.5 µl of the SGD culture into
316 the new 384-well plates. The coculture plates were incubated for 18h at 37°C. The viability of
317 *S*. Typhimurium was then determined by pinning from the 384-well coculture plates onto SS agar,
318 which were incubated overnight at 37°C. Images of each plate were taken with the Phenobooth
319 (Singer Instruments, UK) for analysis. This high-throughput method was also performed under
320 acidic pH conditions. In this case, the 25 µL of the overnight culture of *L. rhamnosus* was replaced
321 by 25 µL of BHI pH 5.5.

322 Images of the bacterial growth on SS agar plates were analyzed by an in-house analysis pipeline,
323 as described previously (22). Briefly, imaged plates were divided into 384 region-of-interests (roi)
324 to isolate each colony and the density within each roi was measured using Fiji (23). Density values
325 were then normalized with the *lowess* in R (24). We determined to relative growth of every mutant
326 of the collection for a given treatment (the presence of *L. rhamnosus* or acidified media) by
327 calculation the ratio of the normalized density value with azithromycin and without azithromycin.
328 A relative growth around 1 signifies that the gene deletion growth is similar with or without
329 azithromycin, while a relative growth < 1 means that the growth of gene deletion is impaired in the
330 presence of azithromycin. Relative growth values were then converted to Z-scores, Z score =
331 (Conjugation score – screen mean)/screen sd. Based on the statistical distribution obtained from

332 the Z-scores, we selected the gene deletions that were the most distant from the dataset average (p-
333 values ≤0.05).

334 **Figures & Tables**



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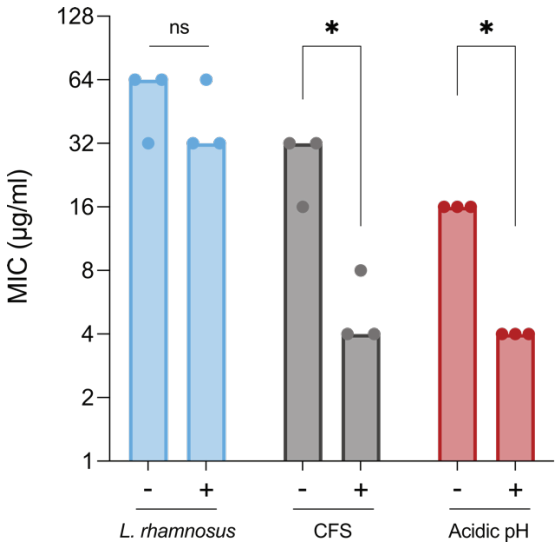
336 **Figure S1. Coculture minimal inhibitory concentration assay.** (A) Summary of the method used
337 to determine the susceptibility of *S*. Typhimurium to different antibiotics following a co-culture.
338 Co-cultures were performed in BHI media. After incubation, *S*. Typhimurium is selected by
339 spotting 5 µL of the co-cultures on SS agar. (B) Proof-of-concept interaction between
340 *S*. Typhimurium and *S. aureus* 130. The combination was done in the presence of increasing
341 concentrations of ampicillin. MIC against *S.* Typhimurium is shown on SS agar. MIC against *S.*
342 *aureus* is shown on LB agar. (C) Changes in the susceptibility of *S*. Typhimurium to azithromycin
343 when cocultured with *Lactobacillus* spp. MIC against *S*. Typhimurium is shown on SS agar either
344 alone with the antibiotic or in combination with *Lactobacillus*. (D) Strain combinations performed
345 with rifampicin which is not impacted by the co-culture.



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347 **Figure S2. Addition of membrane-destabilizing agents in coculture MIC assays.** Impact of
348 EDTA on the MIC of azithromycin against *S.* Typhimurium in presence of *L. rhamnosus* culture,
349 *L. rhamnosus* derived CFS or an acidified media. EDTA was added at a concentration of 2 mM.
350 Wilcoxon-Mann-Whitney U test, * indicates a p-value ≤ 0.05.

351 **Table S1. MIC of azithromycin against *S.* Typhimurium in various conditions.**

Strain	Combination	MIC of azithromycin (µg/mL)
S. Typhimurium	-	2-4
	L. rhamnosus	32-64
	L. rhamnosus CFS	32-64
	L. rhamnosus neutralized CFS	4-8
	L. reuteri	32-64
	L. reuteri CFS	32-64
	L. reuteri neutralized CFS	2-4
	BHI (pH 5.5)	32-64
	80mM lactate	2
	Heat-killed *L. rhamnosus*	2

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356 **Table S2. MIC of azithromycin against *S.* Typhimurium in different combination with the**
357 **addition of membrane-destabilizing agents**
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Strain	Combination	MIC of azithromycin (µg/mL)		
			+ EDTA	+ pentamidine
S. Typhimurium	-	2	0.5	0.5
	L. rhamnosus	32-64	32-64	32-64
	L. reuteri	16-64	32-64	32-64
	BHI (pH 5.5)	16-32	4	4
	L. rhamnosus CFS	16-32	4-8	4

359 Note: EDTA was used at 2 mM and pentamidine at 64 µg/mL.
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361 **Table S3. Relative growth and Z-score values for the complete SGD collection in the presence**
362 **of *L. rhamnosus* or in acidified conditions as well as for selected hits.** See Excel file.