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

Authors: Lya Blais[1]*, Laurence Couture[1]* & Jean-Philippe Côté[1#]

*The authors contributed equally to this work.

Affiliations:

[1]Département de biologie, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1K 2R1

Correspondence may be addressed to:

Jean-Philippe Côté
Tel :1-819-821-8000 ext. 65280
Email: jp.cote@usherbrooke.ca

RUNNING TITLE Lactobacillus-mediated azithromycin tolerance of Salmonella

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

Abstract

Bacteria are involved in many interactions during events of co-infection and among microbial populations such as the gut microbiota. *Salmonella enterica* serovar Typhimurium is a foodborne pathogen of great importance as well as a great model to study interactions that may occur in a microbial community. In the present study, we found that when *S*. Typhimurium is co-cultured with probiotic strains of *Lactobacillus* spp., it becomes tolerant to azithromycin. A similar observation was made when *S*. Typhimurium was cultured in the presence of the acidic filtered spent media from *Lactobacillus* spp. Using membrane destabilizers, we determined that acidic pH triggers a modified membrane permeability leading to the tolerance to azithromycin, whereas a distinct mechanism is responsible for the increased tolerance in the presence *Lactobacilli*. We next used a high-throughput assay using the single gene deletion mutants of the *S.* Typhimurium collection (SGD) and the probiotic *L. rhamnosus* or pH 5.5 media to understand how *S.* Typhimurium was impacted in these conditions. Genes of importance in these two conditions were either for cellular respiration or cell surface and transmembrane transport, which indicates a possible mechanism for the observed tolerance of *S.* Typhimurium induced by the presence of *Lactobacillus* spp. that is different from a simple acidification of the media.

Bacterial pathogens are rarely found alone and thus have to interact with other microbes that are present in their environment. This is especially true in the gut where the microbiota present competes with intestinal pathogens and often promotes resistance to colonization by pathogens (1). Not surprisingly, pathogens have also adapted to the microbiota to overcome the mechanisms of colonization resistance or to utilize unique nutrients that are present because in this environment (2). There are many examples of the intricate relationship between bacterial pathogens and the microbiota, but we are only beginning to understand how resident microbes affect pathogens. The most studied interbacterial relationship is between *Pseudomonas aeruginosa* and *Staphylococcus aureus*, where *P. aeruginosa* profoundly alters the physiology of *S. aureus* which results in modifications of its susceptibility to various antimicrobial agents (3,4). Therefore, determining how resident microbes found at the infection site affect pathogens could reveal important details about the physiological state of the pathogen cell. Herein, we probed the

physiological state of model intestinal pathogen *Salmonella enterica* serovar Typhimurium by determining its susceptibility against various antibiotics in the presence of probiotic *Lactobacillus* strains, and found that *S.* Typhimurium becomes tolerant to azithromycin when *Lactobacillus* sp. is present.

To probe the impact of *Lactobacillus* sp. on *S.* Typhimurium, we decided to look at the susceptibility of *S.* Typhimurium against various antibiotics, as antibiotics are also probes of the physiological state of the cell (5,6). A shift in antibiotic susceptibility therefore correlates with pathways that become more or less essential in the condition tested. We used selective media to determine the minimal inhibitory concentration (MIC) of antibiotics against *S.* Typhimurium co-incubated with two probiotic strains of *Lactobacilli*, *L. rhamnosus* LMS2-1 or *L. reuteri* CF48-3A (Supp. Fig.1A). As a proof-of-concept, we first tested how a beta-lactamase producing *S. aureus* strains would modify *S.* Typhimurium MIC against ampicillin and observed that, in the co-incubation mixture, *S.* Typhimurium was also resistant to ampicillin (Supp. Fig.1B). We then tested the susceptibility of *S.* Typhimurium against various antibiotics, including relevant antibiotics for treatment of salmonellosis, such as ciprofloxacin, ceftriaxone and azithromycin (7,8), in the presence of the two *Lactobacilli* (Fig.1A) . Co-incubation with both probiotic strains resulted in a significant increase in azithromycin MIC against *S.* Typhimurium, from 2 to 32 µg/mL (Fig.1A and Supp. Fig. 1C). *S.* Typhimurium was tolerant rather resistant to azithromycin in the presence of the *Lactobacilli*, as cells recovered from the *Salmonella* selective media where susceptible to azithromycin (MIC of 2 µg/mL). In addition, heat-killed *Lactobacillus* cells did not increase the MIC to azithromycin against *S.* Typhimurium (Supp.Fig2).

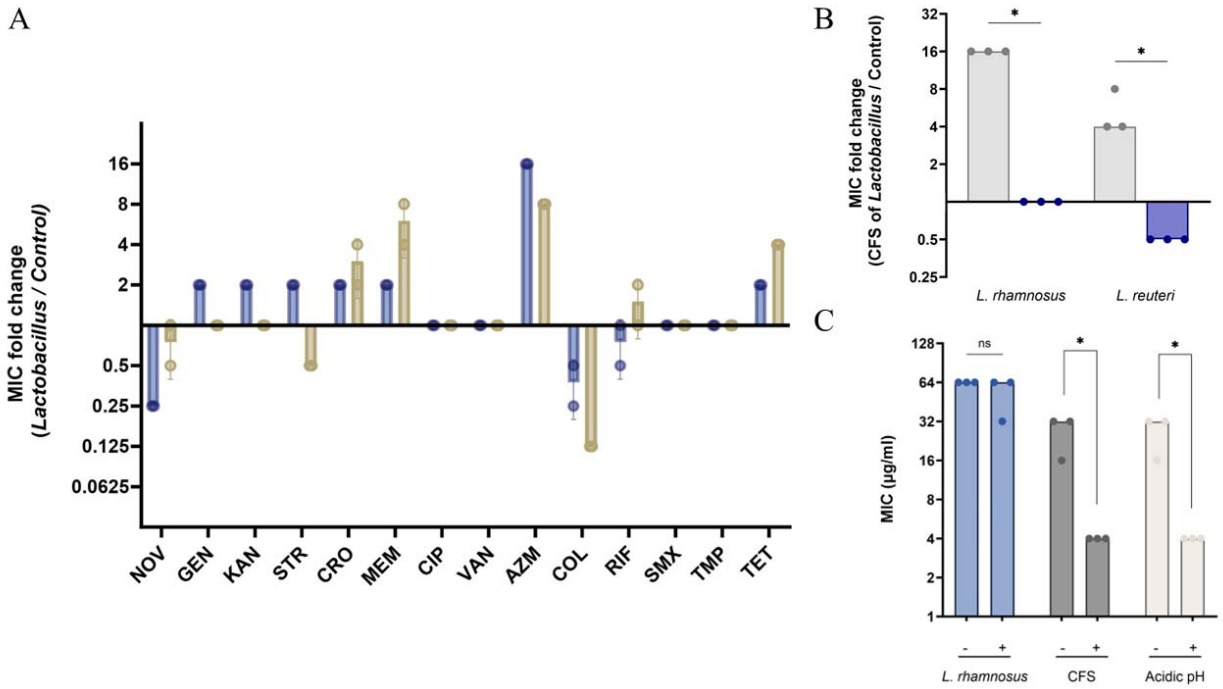

Figure 1. *Salmonella* Typhimurium becomes tolerant to azithromycin when cocultured with strains of the *Lactobacillus* genus. (A) Fold change in the MIC of *S.* Typhimurium against several antibiotics in the presence of *Lactobacillus* spp. Fold change was determined by producing a ratio of the MIC of *S.* Typhimurium in the presence of *L. rhamnosus* (blue) or *L. reuteri* (beige) and the MIC of *S.* Typhimurium grown by itself against a selected panel of antibiotics. (B) MIC fold change of azithromycin for *S.* Typhimurium in the presence of CFS (grey) or neutralized CFS (purple) of *Lactobacillus* spp. Fold change was determined using the MIC of *S.* Typhimurium alone with azithromycin, which is 4 µg/ml. Initial pH of *L. rhamnosus* CFS was of 5.66 and 5.34 for *L. reuteri* (grey). CFS was neutralized for both *Lactobacillus* to a pH of 7.00 (purple). Wilcoxon-Mann-Whitney U test, * indicates a p value ≤ 0.05. (C) Impact of 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.

Cell-free supernatants (CFS) from both *Lactobacillus* strains were also capable of inducing the observed tolerance phenotype (Fig.1B). As expected, CFS from the probiotic 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 showed a similar increased in MIC, suggesting that the decrease in pH triggered the observed tolerance against azithromycin. On the other hand, lactate, one of the main acidic compound produced by *Lactobacilli* (9), did not increase the MIC by itself (Supp Table S2). It is important to note that azithromycin is stable in acidic conditions (8), and thus the tolerance to the antibiotic is not likely due to compound break down.

Azithromycin is notably dependant on envelope homeostasis for its entry inside the bacterial cell and modifications of cell permeability are important resistance mechanisms (10–13). To test the hypothesis that the increase in MIC is caused by changes in the bacterial envelope resulting in modified cell permeability and decreased entry of azithromycin inside the cell, co-incubation assays were repeated in presence of membrane destabilizers such as pentamidine and EDTA (14,15). The addition of pentamidine (Fig. 1C) or EDTA (Supp. Fig.2) still resulted in an increased MIC (64 µg/mL) of azithromycin against *S.* Typhimurium when combined with *L. rhamnosus*. However, when CFS or acidified media were used, the MIC of azithromycin decreased to the value observed when *S.* Typhimurium was grown by itself. These results suggests that two different mechanisms can result in the observed increase in MIC of azithromycin against *S.* Typhimurium. Unsurprisingly, the acidification of the media resulted in changes in envelope permeability resulting in a decreased entry of azithromycin inside the cells (16). However, when *Lactobacillus* are present, a second mechanism possibly independent of envelope permeability increases the tolerance of *S.* Typhimurium to azithromycin.

To better understand the difference in the response of *S.* Typhimurium to the presence of *Lactobacillus* sp. or to acidified media, we reproduced the co-culture experiments to a larger scale, using a genome-wide single-gene deletion collection of *S.* Typhimurium (SGD) (17) and either high-density cultures of *L. rhamnosus* or BHI at pH 5.5, both with or without azithromycin. In our high-throughput experiments, the MIC of azithromycin against *S.* Typhimurium alone was 8 µg/mL. Therefore, we used a concentration of 128 µg/mL of azithromycin in our high-throughput assay to be representative of our initial observation (16-fold increase in MIC). At 128 µg/mL, *S.* Typhimurium grows when *L. rhamnosus* is present or in acidified media, but not by

itself in traditional media. Mutants showing a growth defect specifically when treated with high concentrations of azithromycin indicated that their interrupted gene was essential for the observed tolerance of *S.* Typhimurium to the antibiotic. We thus compared the growth of the SGD library in the presence of azithromycin and either *Lactobacillus* or acidified media to controls without azithromycin. The complete list of hits can be found in Supplementary Table S3. Both conditions showed different responses to the presence of azithromycin, with the acidified media having a smaller impact on *S.* Typhimurium (Fig. 2A). We transformed the values into Z-scores to select gene deletions that were significatively different than the population (p-value < 0.05) (Fig.2B). While only a few hits were shared between both screens, the screens highlighted similar processes including many genes involved in envelope homeostasis. For instance, in the presence of *L. rhamnosus*, the growth of both genes from the two-component systems *envZ/ompR* and *phoP/phoQ* was impaired, as well as for genes known to disrupt the permeability of the outer membrane (*mdoG*, *pal*, *prc*, *rfaP*, *rfaY*, *wecC*, *wecG*, *yceG* for instance) in *E. coli* (18). In acidified media, we did not necessarily observe the same hits, but genes with similar functions had impaired growth (*pgm*, *ldcA*, *pal*, *prc*, *yceG*, *wecG* for instance). The prevalence in genes affecting membrane homeostasis explains the effect of membrane disruptors in acidified media. In the presence of *L. rhamnosus*, gene deletions affecting core metabolic processes also impaired the tolerance of *S.* Typhimurium to azithromycin (*nuo* genes, *sdhAB*, *sucB*, for instance).

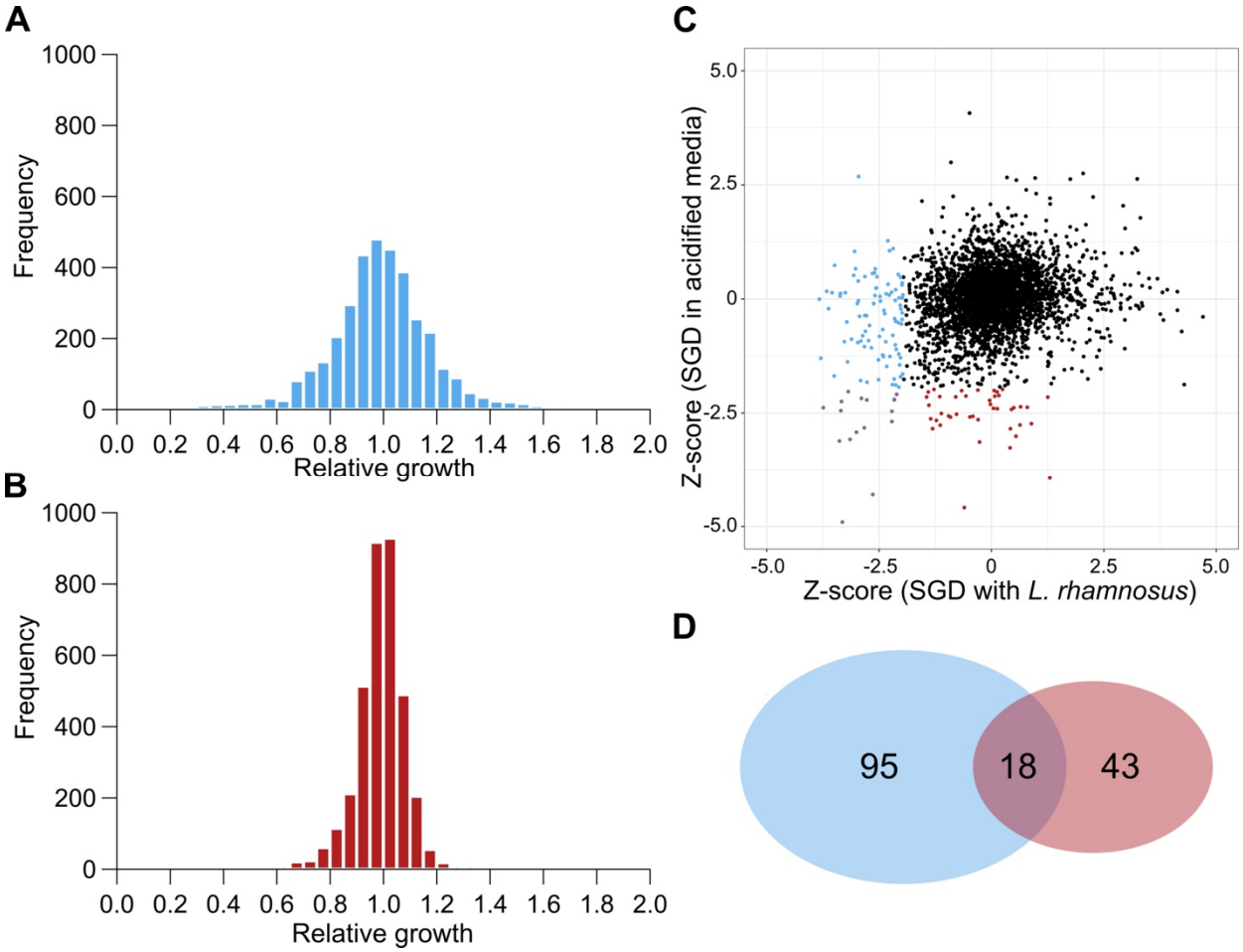

Figure 2. Growth of the SGD library at high azithromycin concentrations in the presence of *L. rhamnosus* or acidified ph. The SDG library was grown in the presence of (A) *L. rhamnosus* (blue) or (B) acidified (red) and with 128 µg/mL of azithromycin (16 x MIC in tested conditions). The growth of each 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 study of this interaction between *S.* Typhimurium and strains of *Lactobacillus* spp. will be necessary to fully understand the molecular basis behind how the probiotic partners 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 different mechanism of action is responsible for the same phenotype in presence of *L. rhamnosus*. Overall, the finding of this interaction between a well-known pathogen and probiotic strains from the human gut microbiota support the fact that bacterial interactions can take place in complex microbial communities and could possibly affect the efficacy of different antibiotic treatments.

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Supplemental Material

Materials and Methods

Bacterial strains and growth conditions

All experiments in this study were performed with *Salmonella enterica* serovar Typhimurium strain 14028 or the described single-gene deletion library (SGD library) (17). *Lactobacillus reuteri* CF48-3A, *Lactobacillus rhamnosus* LMS2-1 and *Staphylococcus aureus* 130 were obtained from the Human Microbiome Project (19) and were cultured in Brain-Heart infusion broth (BHI) or supplemented BHI broth (BHIS) prepared according to Lau *et al* (20). All strains were grown at 37 □. The SGD library was maintained in BHI broth supplemented with kanamycin (KAN) at a concentration of 50 mg/ml.

Products and solutions

A selective panel of antibiotic compounds was selected to cover the most important antibiotic classes. Azithromycin (AZM), meropenem (MEM) and sulfamethoxazole (SMX) were obtained from Sigma Aldrich and diluted in dimethyl sulfoxide (DMSO). Tetracycline (TET), novobiocin (NOV), cefuroxime (CXM), ceftriaxone (CRO) and colistin (COL) were also obtained from Sigma Aldrich but were diluted in water. Rifampicin (RIF), erythromycin (ERY), nalidixic acid (NAL) and trimethoprim (TMP) were obtained from Bio Basic and diluted in DMSO. Streptomycin (STR), gentamycin (GEN), ampicillin (AMP), kanamycin (KAN), ciprofloxacin (CIP) and vancomycin (VAN) were also obtained from Bio Basic but were diluted in water. Lastly, apramycin (APR) was obtained from Alfa Aesar and was diluted in water. Membrane-active sensitizing adjuvants were used to permeabilize the membrane of the pathogen cell. Ethylenediamine tetraacetic acid (EDTA) was obtained from Fisher Bioreagents and diluted in water. Pentamidine isethionate salt was obtained from Sigma Aldrich and diluted in DMSO. Sodium L-lactate was also obtained from Sigma Aldrich.

Minimal inhibitory concentration assay

Determination of the minimal inhibitory concentration (MIC) for the strains against AZM and RIF was adapted from the Clinical and Laboratory Standards Institute (CLSI) guidelines (21). Briefly, each strain was grown until a dense culture was obtained, then diluted by a factor of 1000 in BHI. Diluted cultures were then put in the presence of 2-fold dilution series of the antibiotics to be tested in a 96-well plate starting at 256 µg/ml. The MIC, corresponding to the lowest concentration to inhibit visible growth, was determined after 18 h incubation at 37 ˚C. When indicated, EDTA or pentamidine was added to the media before being added to the plate at a final concentration of 2 mM and 64 µg/ml, respectively. The MIC determination assay was also performed in acidic conditions. For this, strains were grown until a dense culture was obtained, then diluted by a factor of 1000. An appropriate volume of BHI was then acidified to a pH of 5.5 with a solution of HCl 1N. This acidified BHI solution was used to dilute the different antibiotics to be tested in serial dilutions in 96-well plate.

Coculture minimal inhibitory concentration assay

The MIC of *S.* Typhimurium ATCC 14028 against different antibiotics was also determined in the presence of companion strains from the HMP, notably with *L. reuteri* CF48-3A and *L. rhamnosus* LMS2-1. The method for these combination assays was also adapted from the CLSI guidelines (21).

Overnight cultures of companion strains were grown for 18 h at 37 □ with agitation. *S.* Typhimurium was also grown until a dense culture was obtained, then diluted by a factor of 1000 in BHI. The co-culture experiments were performed by mixing the diluted culture of *Salmonella* with overnight cultures of the partner strains in 96-well plates in a 1:1 ratio. Antibiotics were serially diluted as described for the MIC assays. The coculture plates were then incubated for 18 to 20 h at 37 □. After the incubation, 5 µl of each well was plated on *Salmonella Shigella* (SS) agar, which is selective for Gram-negative bacteria. The agar plates were incubated 18 to 20 h at 37 □, after which the MIC of *S.* Typhimurium was determined visually. The method is shown in figure 1A. When needed, EDTA or pentamidine were also added to the wells as described in the MIC assays.

For cocultures with the supernatant of companion strains, the desired strains were grown for 18 h at 37 □ with agitation. The overnight cultures were then centrifugated at 4000 rpm for 10 min. The resulting supernatant was collected and filtered with a sterile 0.22 µm syringe filter. The sterile supernatants were then added to the 96-well plates containing the diluted *S*. Typhimurium and the antibiotics, as described above. For cocultures with non-viable companion strains, the desired strains were grown for 18 h at 37 °C with agitation. After the incubation, strains were subjected to a heat treatment of 20 min at 70 °C, followed by two rounds of 5 min centrifugation at 13 000 rpm and washing in 1X PBS. Washed pellet was resuspended in BHI before being added to the 96-well plates containing the diluted *S.* Typhimurium and the antibiotics, as described above. For the lactate assay, sodium L-lactate was diluted in an appropriate volume of BHI. Strains were grown as described above, and the lactate solution was added at a final concentration of 80 mM (22). in the 96-well plate containing the diluted *S.* Typhimurium and the antibiotics. It is important to note that the addition of the compound did not affect the initial pH of BHI, therefore the assay was conducted under neutral pH conditions.

<u>Upscaled co-culture assay with the SGD collection</u>

S. Typhimurium SGD collection was grown for 18 h at 37 □ in 384-well plates filled with 50 µl of BHI. The companion strain *L. rhamnosus* was grown separately in 25 ml of BHI for the screen in the bacterial interaction context at 37 □ for 18 h. The co-culture plates were then prepared by adding 25 µl of overnight *L. rhamnosus* culture and 25 µl of BHI with 256 µg/mL of azithromycin in 384-well plates (final concentration of 128 ug/mL in 50 µl). The SGD collection was finally inoculated to the 384-well coculture plate with the Singer Rotor had using long-pins. This setup transfers ~0.5 µl of the SGD culture into the new 384-well plates. The co-culture plates were incubated for 18 h at 37 □. The viability of *S*. Typhimurium was then determined by pinning from the 384-well co-culture plates onto SS agar, which were incubated for 16 h at 37 □. Following the incubation, images of each plate were taken with the Phenobooth for analysis. This high-throughput method was also performed under acidic pH conditions. In this case, the 25 µl of the overnight culture of *L. rhamnosus* was replaced by 25 µl of BHI pH 5.5.

Images of the bacterial growth on SS agar plates were analyzed by an in-house analysis pipeline, as described previously (23). Briefly, imaged plates were divided into 384 region-of-interests (roi) to isolate each colony and the density within each roi was measured using Fiji (24). Density values were then normalized with the *lowess* function in R.

Figures & Tables

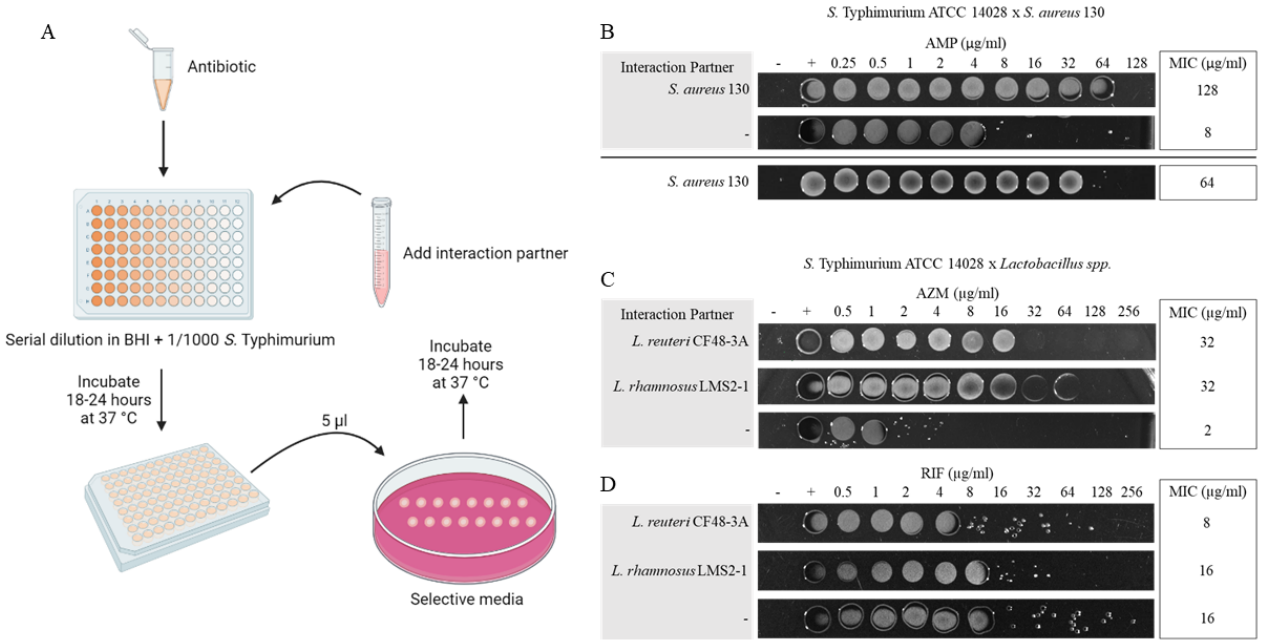
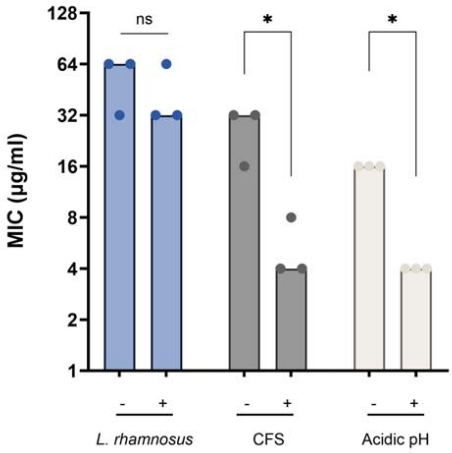


Figure S1. Coculture minimal inhibitory concentration assay (A) Summary of the method used to determine the susceptibility of *S*. Typhimurium to different antibiotics following a co-culture. Co-cultures were performed in BHI media. After incubation, *S*. Typhimurium is selected by spotting 5 µL of the co-cultures on SS agar. (B) Demonstration of the results that can be obtained from the method with the combination of *S*. Typhimurium and *S. aureus* 130. The combination was done in the presence of increasing concentrations of ampicillin (AMP). *S*. Typhimurium's MIC is shown on SS agar. *S. aureus*'s MIC is shown on LB agar. (C) Changes in *S*. Typhimurium's susceptibility to azithromycin (AZM) when cocultured with *Lactobacillus* sp. *S*. Typhimurium's MIC is shown on SS agar either alone with the antibiotic or in combination with *Lactobacillus*. (D) The strain combination was also performed with the control antibiotic rifampicin (RIF).

A



B

Combination with *S.* Typhimurium	MIC of *S.* Typhimurium (µg/ml)		
	AZM	AZM + EDTA	AZM + pentamidine
	2	0.5	< 0.5
L. rhamnosus	32 - 64	32 - 64	32 - 64
L. reuteri	16 - 64	32 - 64	32 - 64
Acidic pH	16 - 32	4	4
L. rhamnosus CFS	16 - 32	4 - 8	4

Figure S2. Addition of membrane destabilizers in coculture minimal inhibitory concentration assay (A) Impact of EDTA on the MIC of azithromycin for *S.* Typhimurium in presence of *L. rhamnosus*, its CFS or an acidified media. EDTA was added at a concentration of 2 mM. Wilcoxon-Mann-Whitney U test, * indicates a p value ≤ 0.05. (B) MIC of azithromycin for *S.* Typhimurium in different combination with addition of membrane-destabilizing chemicals.

Strains tested	MIC of *S.* Typhimurium (µg/ml)	
	AZM	RIF
S. Typhimurium ATCC 14028	4	8
S. Typhimurium ATCC 14028 in pH 5.5	32 - 64	8

Table S1. MIC of azithromycin for *S.* Typhimurium in an acidic pH.

Table S2. MIC of azithromycin for S. Typhimurium in combination with cell-free supernatant of *Lactobacillus* spp.

Combination with *S.* Typhimurium	MIC of *S.* Typhimurium (µg/ml)	
	AZM	RIF
	4	8
L. rhamnosus CFS	64	4
L. rhamnosus neutralized CFS	4 - 8	8
L. reuteri CFS	32	4
L. reuteri neutralized CFS	2 - 4	8
Lactate 80 mM	2	-
Heat-treated *L. rhamnosus*	2	-

Table S3. Complete hits genes and shared hits that were obtained from high-throughput experiments, their relative growth value and physiological GO Terms. See Excel file.