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In vitro horizontal transfer of ampicillin-resistance gene between bacterial species with implications in antibiotic-resistance problem in human microbiome

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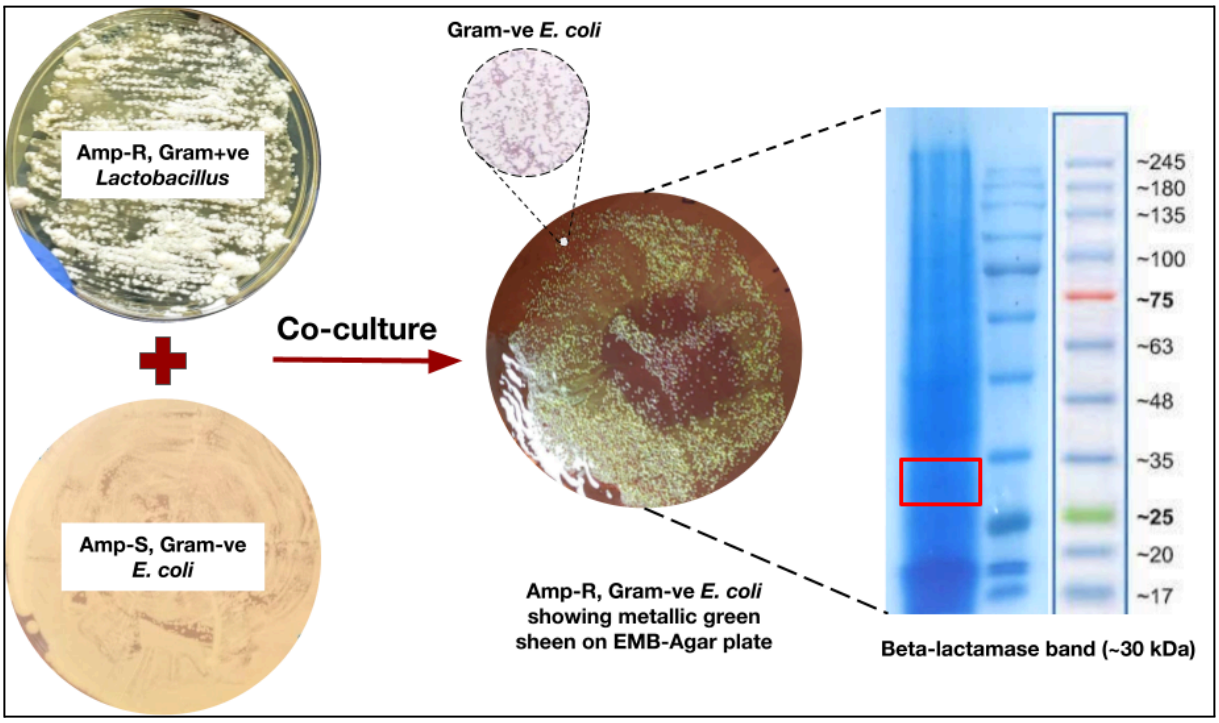
Multidrug-resistance (MDR) among various bacterial species through horizontal transfer of MDR among the bacterial species in the gut microbiome has become a global medical issue in recent years. Consumption of yogurt is very common in India and is usually prepared using *Lactobacillus spp*. In this study we evaluated the feasibility for the horizontal transfer of ampicillin-resistance (Amp^r) gene from *Lactobacillus spp*. to *E. coli* by using an inhouse patented transformation protocol for *Lactobacillus spp*. isolated from yogurt with Amp^r gene containing plasmid. As expected, the Amp^r gene was indeed transferred to *E. coli* through bacterial conjugation. Our results demonstrate that not all commercially available yogurt varieties are safe to consume due to the concern that they might contain *Lactobacillus spp*. harboring MDR genes that can be horizontally transferred to other bacterial species in the gut microbiome. These results have implications in understanding antibiotic-resistance in the human microbiome.



Keywords: Ampicillin-resistance, *Lactobacillus*, *E. coli*, horizontal gene transfer, beta-lactamase, EMB-Agar.

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Bacterial conjugation or horizontal gene transfer (HGT) is the movement of genetic material between organisms other than by the vertical transmission of DNA from parent to offspring (1, 2). HGT is the primary process for the spread of antibiotic-resistance in bacteria and plays an important role in the evolution, maintenance, and transmission of virulence (3-6). Genes responsible for antibiotic resistance in one species of bacteria can be transferred to another species of bacteria through such mechanisms of HGT subsequently arming for the antibiotic-resistant genes and it helps in the evolution of species of different kinds. The spread of these antibiotic- resistant genes in this manner is becoming a challenge in research (7). It is thought that during the HGT only 2% of the prokaryotic genome will be transferred (8). HGT-Based virulence and antibiotic-resistance genes contribute to the emergence of novel "superbugs" (9). Superbugs are referred to the strains of bacteria which are resistant to most of the antibiotics. For example, the common superbug which is increasingly seen in hospitals is methicillin-resistant bacteria known as *Staphylococcus aureus* (MRSA) (10).

In 2011 Popa and Dogan reported that almost 75% of the total genes in the microbial genomes are the result of horizontal gene transfer events (11). Bacterial and viral vectors are often used as the most likely mechanism for eukaryotic gene transfers because they frequently exchange the genes through their infection cycle (12, 13). The gene transfer can also happen with the help of transposable element (also called as the transposon or the jumping gene) which is a mobile segment of the DNA that can sometime pick up a resistance gene and insert it into a plasmid or a chromosome, thereby inducing lateral gene transfer of antibiotic-resistance (14). Similarly for the TetO gene conferring resistance to tetracycline among *Campylobacter jejuni* (15).

In this study, we demonstrated the process of bacterial conjugation-mediated transfer of ampicillin-resistance (Amp^r) gene from *Lactobacillus spp*. into the *E. coli*. The *Lactobacillus spp*. were isolated from natural yogurt, transformed with Amp^r gene using heat-shock method and were co-cultured with the common laboratory strains of *E. coli*.

Materials & Methods:

Bacterial strains and plasmids: Escherichia coli, DH5α strain cells and pUC19 plasmid containing Amp^r gene were purchased from HiMedia. *Lactobacillus spp*. were isolated from natural homemade yogurt. Competent cells were prepared using these *Lactobacillus spp*. Both natural homemade and commercially sold yogurt samples were tested and the homemade natural yogurt gave higher yield.

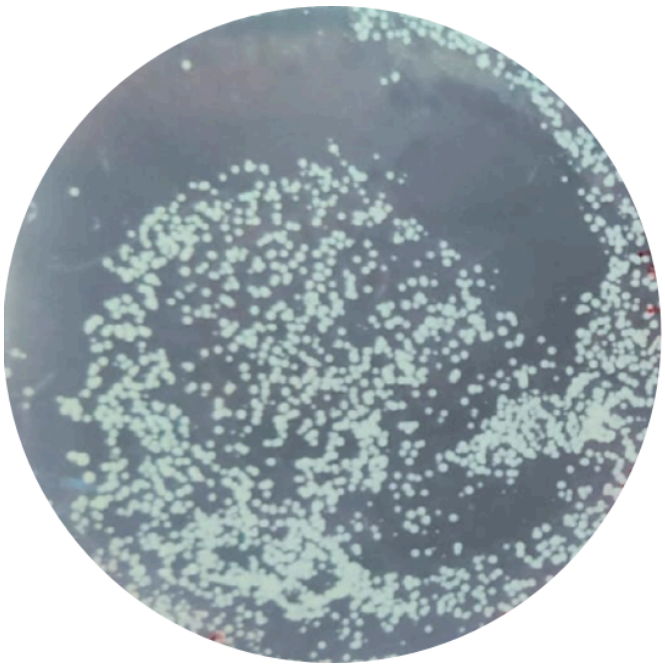

Figure 1. *Lactobacillus* transformants on MRS agar plate with ampicillin.

Media preparation and bacterial cell culture: Luria Bertani (LB) broth and LB Agar plates were used for culturing the *E. coli*, DH5α cells. The de Man Rogosa Sharpe (MRS) broth and MRS agar plates were used for *Lactobacillus spp*. Eosin-Methylene blue (EMB) broth and EMB agar plates were used for selective growth of Gram negative cells. All the liquid broth and agar plates were prepared as described previously (16). Bacterial cells were initially streaked on media plates and incubated at 37 °C overnight. The following day, a loopful of colonies were inoculated into 5 ml of media broth with ampicillin and incubated at 37 °C overnight.

Preparation of competent cells: The *Lactobacillus* cells from a 5 ml MRS broth (without ampicillin) were cooled to 4 ℃ and were harvested by centrifugation at 5,000 rpm for 10 min. The cell pellet was resuspended in 0.1 M Calcium chloride solution that was prechilled. Following an incubation on ice for 30 min, the cells were harvested by centrifugation at 5,000 rpm for 10 min. The pellet was resuspended in prechilled 0.1 M CaCl2 solution and were aliquoted for immediate usage.

Bacterial transformation: A vial containing 100 µl of competent cells prepared above was taken to which 5 µl of pUC19 plasmid was added and the mixture was incubated on ice for 30 min. The contents of the vial were then taken through the heat-shock (42 ℃ for 45 sec and back to the ice).

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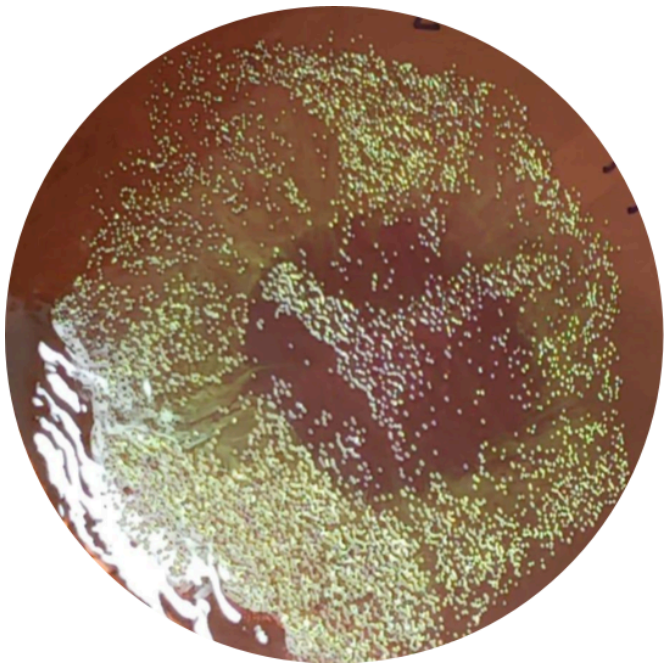

Figure 2. *E. coli* cells showing a metallic green sheen on EMB agar plate containing 50 µg/ml ampicillin, post HGT.

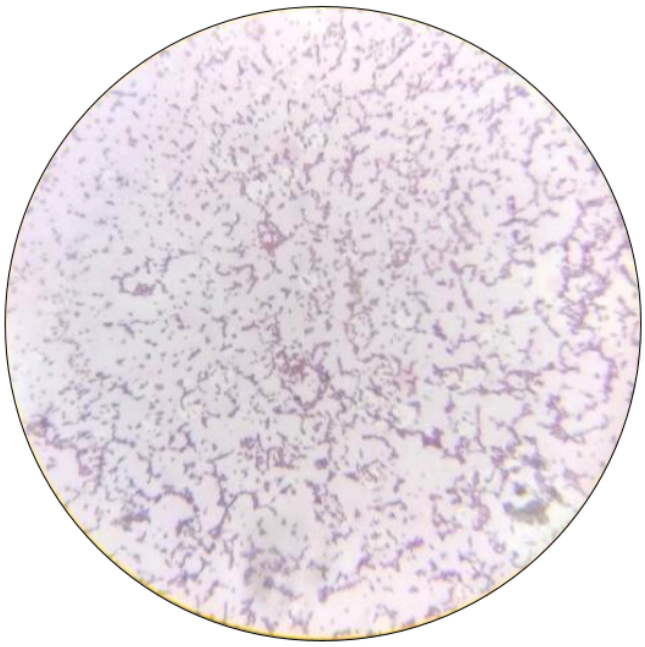

Figure 3. Gram staining results of recipient cells from EMB agar plate confirms pink color Gram negative cells.

Followed by 5 min. incubation on ice, 1 ml of sterile SOC medium was added to the vial for the recovery of cells post heat-shock and cells were incubated at 37 ℃ for 1 hour. The cells (200 µl) were then spread on an MRS agar plate containing 50 µg/ml ampicillin and the plates were incubated at 37 ℃ overnight.

HGT co-culture: Overnight cultures (5 ml each) of donor (*Lactobacillus* transformants) and recipient (*E. coli*, DH5α cells) were prepared in MRS broth containing 50 µg/ml ampicillin and LB broth without ampicillin, respectively. To these minicultures, 5 ml of fresh media was added and incubated for 3 hours at 37 ℃. To a sterile 15 ml tube, 200 µl of donor cells and 200 µl of recipient cells were added from their respective cultures and the co-culture was incubated for 1.5 hours at 37 ℃. Followed by the addition of 2 ml of LB broth, the co-culture was incubated for 1.5 hours at 37 ℃. The co-culture was then plated @ 200 µl on EMB agar plate containing 50 µg/ml ampicillin and the plates were incubated at 37 ℃ overnight.

Gram staining: Gram staining kit was purchased from HiMedia (Cat. No. K001L). Staining was performed according to the manufacturer's recommended protocol that was provided along with the kit. Followed by the staining procedure, the cells were visualized under a microscope and the images were taken. Gram staining was performed only for colonies obtained on the EMB agar plate post co-culture.

SDS-PAGE: The SDS-PAGE was performed using a 12% acrylamide-*bis* acrylamide gel in Tris-Glycine buffer as described previously (17). The cells from the EMB agar plate shown in Figure 2 were boiled with the laemmli buffer followed by centrifugation at 14,000 rpm for 15 min. The supernatant was loaded into the well. Prestained protein ladder was loaded for molecular weight comparison.

Results and Discussion:

Lactobacillus transformants: The pUC19 plasmid carrying Amp^r gene was used to transform the *Lactobacillus* competent cells. As shown in Figure 1, multiple colonies of the transformants were obtained on MRS agar plate containing 50 µg/ml ampicillin suggesting that the cells were able to take in the plasmid successfully. The control plate with competent cells without pUC19 in the presence of 50 µg/ml ampicillin did not show any colonies.

Amp^r E. coli cells on EMB agar plates: The EMB agar plate containing 50 µg/ml ampicillin showed multiple colonies with green color metallic sheen (Figure 2). In this study the EMB medium was particularly chosen to plate the co-culture because EMB inhibits the growth of Gram positive bacteria, i.e., Gram positive donor cells in this study. With this assumption, it is safe to conclude that the colonies on EMB agar plates are of the recipient *E. coli* cells. Additionally, the presence of ampicillin in EMB agar plates also confirms that

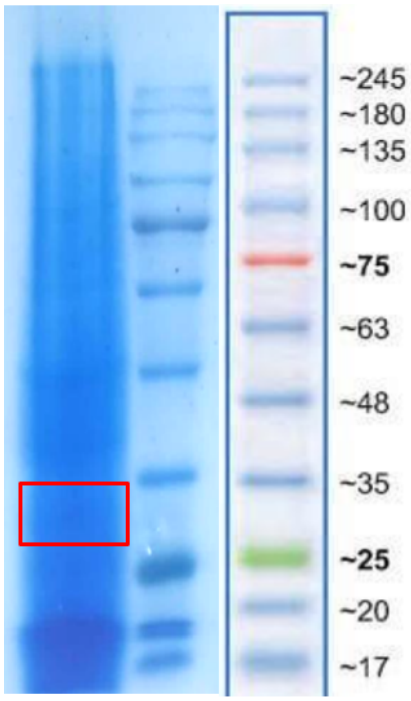

Figure 4. Recipient cell extract showing beta-lactamase band highlighted with a red box (~30 kDa).

the recipient cells gained the Ampr gene through HGT during the co-culture with the donor cells. In order to confirm the identity of the colonies on EMB agar plates, we performed Gram staining.

Gram staining confirms E. coli: Colonies from the EMB agar plates containing 50 µg/ml ampicillin were used to perform Gram staining. As shown in Figure 3 pink color cells indicate the presence of Gram negative bacterial cells. In this study we used Gram positive donor cells that are resistant to ampicillin and Gram negative recipient cells that are sensitive to ampicillin. Moreover, the co-culture containing a mixture of both donor and recipient cells was spread on the EMB agar plates that selectively inhibit the growth of Gram positive cells. Additionally, the presence of 50 µg/ml ampicillin in the EMB agar plates will only allow cells that contain Amp^r gene. Taken together, these results suggest that the colonies with metallic green color sheen on EMB agar plates were Gram negative *E. coli* cells thus confirming the HGT of Amp^r gene from the Gram positive donor cells.

SDS-PAGE shows beta-lactamase band: The whole cell extracts of *E. coli* recipient cells were qualitatively analyzed usIng SDS-PAGE in order to verify the presence of the 30 kDa protein, beta-lactamase that is responsible for causing the ampicillin-resistance and is coded by the Amp^r gene from the pUC19 plasmid. As shown in Figure 4, a thick band was seen around 30 kDa molecular weight as per the ladder. This band can be considered as the beta-lactamase. One should confirm this band as beta-lactamase by using either mass spectrometry or western blotting. However, in this study, the ampicillin-sensitive recipient cells became ampicillin-resistant after the co-culture suggesting that the recipient cells indeed acquired the pUC19 plasmid through HGT. Considering the colonies in Figure 2, the thick band highlighted in red box in Figure 4 can be concluded as the beta-lactamase.

Conclusion and Future directions:

The current study confirms the HGT of Amp^r gene from *Lactobacillus* to *E. coli* via *in vitro* co-culture and bacterial conjugation. This study not only demonstrates the HGT process that occurs in the human microbiome but also offers a versatile technique to test the HGT-mediated gene transfer between different species of bacteria *in vitro*. In future, a similar study will be performed on a large scale using human microbiome samples to test whether a similar HGT of antibiotic-resistance gene is feasible. The future studies will also cover the HGT of all the currently available antibiotics and their resistance related genes with a focus on the drug efflux pumps.

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Conflict of interest: The authors declare no conflict of interest in this study.

Author contributions: P.B., N.E., J.P., K.M. and R.M. performed all the web lab. S.A., M.S. and M.V. supervised them. R.S.Y. is the principal investigator who designed the project, trained all co-authors, secured required material for the project, provided the laboratory space and facilities needed. R.S.Y. edited and finalized the manuscript.

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Full figure legends:

Figure 1. *Lactobacillus* transformants on MRS agar plate with ampicillin. Competent cells prepared from the natural homemade yogurt samples were transformed with pUC19 plasmid containing Amp^r gene and were spread on MRS agar plate containing 50 µg/ml ampicillin.

Figure 2. *E. coli* cells showing a metallic green sheen on EMB agar plate containing 50 µg/ml ampicillin, post HGT. EMB medium inhibits the growth of Gram positive bacteria such as *Lactobacillus* that was used as the donor of pUC19 plasmid containing Amp^r gene. Hence the colonies seen here are the Gram negative recipient *E. coli* cells that gained ampicillin-resistance in the co-culture.

Figure 3. Gram staining results of recipient cells from EMB agar plate confirms pink color Gram negative cells.

Figure 4. Recipient cell extract showing beta-lactamase band highlighted with a red box (~30 kDa). Typically the molecular weight of beta-lactamase can range between 27 kDa and 30 kDa. Accordingly, the band was seen between 25 kDa and 35 kDa bands from the protein ladder.