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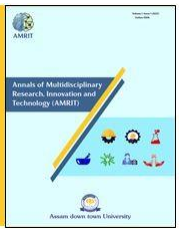

RESEARCH ARTICLE

CROSS KINGDOM ENDOPHYTE

Influence of Animal Gut Microbiota on Rice Seedling Growth and Endophytic Bacterial Populations

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Abstract

Our previous study demonstrated that indigenous rice genotypes from northeast India harbor a unique blend of endophytic bacteria within their mature seeds. Significant variations were observed among plants of the same genotype, but genotypes cultivated under completely different environmental conditions contained similar endophytic bacteria. The present study aims to investigate the effect of inoculation of rice seedlings with microbiota of animal gut origin. A scented rice variety from Assam, Kalajoha, was inoculated with either *Lactobacillus casei, Lactobacillus rhamnosus*, or the endophytic *Micrococcus luteus.* Significant differences in the population of endophytic bacteria were observed after seven days of inoculation, across six different growth media. Four growth parameters i.e. shoot length, root length, shoot weight, and root weight were measured. Seedlings inoculated with *Lactobacillus rhamnosus* had significantly longer primary roots compared to uninoculated controls ($p<0.05$). Additionally, seedlings inoculated with *Micrococcus luteus* exhibited higher biomass than those inoculated with other bacteria. The inoculation of *Kalajoha* seedlings with the *Lactobacillus* species resulted in changes to the population structure of endophytic bacteria in both roots and shoots, suggesting that both species may have successfully entered the plant system. Importantly, neither of the two *Lactobacillus* species demonstrated any harmful effects on plant growth, rather *Lactobacillus rhamnosus* notably promoted longer root growth compared to uninoculated seedlings. The study shows that the inoculation of *Kalajoha* rice seedlings with *Lactobacillus* species results in altered endophytic bacterial populations and promotes certain growth parameters without causing harm to the plants. If these lactic acid bacteria (LAB) confer beneficial effects on their host plants, they could have significant applications, demonstrating their ability to thrive in hosts across different kingdoms.

Keywords: *Rice; Animal gut microbiota; Plant seedling; Endophytic bacteria; Plant growth*

Abbreviations used: CFU - Colony Forming Units, CLSM - Confocal Laser Scanning Microscope, DDW - Double-Distilled Water, DNA - Deoxyribonucleic Acid, EB - Endophyte Bacteria, *E. coli - Escherichia coli,* FP - Fluorescent Protein, FW - Fresh Weight, LB - Luria-Bertani, NA - Nutrient Agar, OD - Optical Density, PEG - Polyethylene Glycol, PKA - Pikovskaya's Agar, RT - Room Temperature, SOC - Super Optimal broth with Catabolite repression

1 Introduction

Microbial cells have developed various strategies to exit their hosts and spread to new environments, either actively or passively [1]. Evolutionarily, natural selection has likely favored microbes that enhance their transmission, given the survival advantage this confers. Moreover, the exchange of microbes among conspecifics through intimate contact can be beneficial for the host organisms. This mutual benefit has led some researchers to propose that social behaviors in animals may have evolved, at least partially, to facilitate the transmission of beneficial microbes [2]. This exchange of symbionts can help recipients increase their resistance to pathogens and toxins, or improve their ability to digest and absorb a broader range of nutrients. Lombardo has referred to this phenomenon as "access to mutualistic endosymbiotic microbes," suggesting it as a driving force behind the evolution of sociality in animals. Interestingly, this concept is also applicable to plants [3]. Rice, for instance, was domesticated from its wild ancestor *O. rufipogon* around 10,000 years ago [4]. There are notable differences in the endophytic flora between wild rice genotypes and cultivated varieties, indicating that domestication and human activities have significantly influenced the plant's microbiome [5]. An example of inter-kingdom horizontal transfer involves the human pathogen *Propionibacterium acnes*, which has been found in grapevines. *P. acnes* was identified in the bark, xylem fibers, and pith tissues of grapevines, with phylogenetic analyses suggesting it has become an obligate endophyte of this plant. While some non-plant pathogenic bacteria within plants can be beneficial, others can be pathogenic to humans [6]. For example, *Achromobacter xylosoxidans*, a plant growth-promoting bacterium from wheat, is also an opportunistic pathogen in humans [7].

Human pathogens can encounter plants through various means, such as contaminated irrigation water, livestock, human feces, and insects. Some pathogens exploit plants as vectors, adopting the lifecycle of plant-associated bacteria and reinfecting humans upon ingestion [8]. Diet plays a crucial role in shaping the human gut flora, with plant-based foods like fruits, vegetables, and cereals being particularly influential. There are significant similarities in the diversity of beneficial microbiota for both plants and humans [9]. Domestication and human activities have led to plants hosting or vectoring human-associated bacteria. For instance, human-beneficial gut bacteria (HGBB) like *Bifidobacterium* and *Faecalibacterium* have been found in the rhizosphere of Antarctic plants and as dominant members of rice seed microflora. Species of *Lactobacillus*, which are important probiotics and dominant members of the human gut flora, have been found in various plants such as sorghum, corn, papaya, and sugarcane. Some *Lactobacilli* are endophytic, residing within plant tissues, while others are found on plant surfaces [9].

Given the association between plant and human-beneficial bacteria, this research aims to investigate whether HGBB, specifically *Lactobacilli*, can colonize the interior of rice plants upon inoculation [9]. Since plants naturally harbor their endophytic flora, introducing a non-plant-associated bacterium may impact the existing endophytic community structure. Therefore, the present study aims to examine the effect of inoculating HGBB into the plant system and compare its effects with those of the existing endophytic bacteria (EB).

2 Materials and Methods

2.1 Bacterial strains, plasmids, and growth conditions

Escherichia coli JM109 and *Escherichia coli* 5-α were used as hosts to propagate seven different fluorescent protein (FP) gene-containing plasmids (obtained from Clontech, Takara) i.e., pGFPuv, pGFP, pAmCyan, pE2-Crimson, pZsYellow, pmCherry, and pDsRed2 and the recipient plasmid pTRKH2 (6.712 kb). pTRKH2 is a theta replicating shuttle vector for *E.Coli* and gram-positive bacteria [10]. All other bacterial strains were recipients of constructed pTRKH2-FP plasmid. Strains of *E.coli* were grown on Luria-Bertani (LB) broth or agar at 37° C. All strains of *Lactobacillus* were cultivated in *Lactobacillus* MRS media (broth or agar) aerobically at 37° C to allow necessary for chromophore formation by the fluorescent proteins [11]. For *E.Coli* JM109 and NEB 5-α hosting FP gene-containing plasmids, ampicillin was added at a concentration of 50 µg/ml. Erythromycin at the strength of 150 µg/ml was added to growth media with *E.Coli* JM109 and *Lactobacillus* strains hosting pTRKH2/pTRKH2-FP plasmid.

2.2 Preparation of pTRKH2-FP plasmid vector and inserts by restriction digestion

To construct the plasmid vector for expression of fluorescent protein in gram-positive *Lactobacillus*, shuttle vector pTRKH2 was used as the recipient, and pGFPuv, pGFP, pE2-Crimson, pZsYellow, pmCherry, and pDsRed2 were used as a donor of FP gene inserts [9, 12]. All plasmids were digested with two different restriction enzymes to avoid self-ligation and generate compatible ends. pTRKH2 was digested with BglII (5'-A▼GATCT-3'/ 3'-TCTAG▼A-5') or XbaI (5'-T▼CTAGA-3'/3'-AGATC▼T-5') as necessary and BanII (5'-GRGCY▼C-3'/3'-C▼YCGRG- 5'). Donor plasmid pGFPuv was digested with XbaI (5'-T▼CTAGA-3'/3'-AGATC▼T-5') and ApaI (5'-GGGCC▼C-3'/3'-C▼CCGGG-5'). All remaining donors were digested with BamHI (5'-G▼GATCC-3'/3'-CCTAG▼G-5') and ApaI producing compatible ends with BglIIand BanII, respectively.

For restriction enzyme double digestion of recipient plasmid with BanII and BglII/XbaI, 1µg of DNA was mixed with 5 µl of 10X NEBuffer 3.1 followed by 1 µl (10 units enzyme per µg of DNA) of BanII and 1 µl of BglII, or 1 µl of XbaI and 1 µl of BanII (enzymes were added strictly in this order) [13]. Volume was made up to 50 µl with nuclease-free water. The reaction was incubated at 37°C for 1 hour. To prevent recircularization of recipient plasmid,5 units of thermolabile recombinant shrimp alkaline phosphatase (rSAP) was added after digestion and the reaction was incubated at 37°C for 30 minutes. The reaction was stopped by heat-inactivation at 65°C for 5 minutes in a water bath. Vector DNA treated with the combination of XbaI and BanII was incubated at 80°C for 20 minutes after dephosphorylation to heat inactivate all enzymes.

Due to the incompatibility of BamHI and ApaI buffers, donor plasmids were digested sequentially as follows: 1 µg of DNA was mixed with 5 µl 10X NE Buffer 3.1 followed by 1 µl of BamHI, volume was adjusted to 50 µl with nuclease-free water and mixture was incubated for 15 minutes at 37°C. Donor DNA was purified with Sigma PCR Cleanup Kit to remove enzymes from the first digestion. DNA was eluted in a minimum volume of nuclease-free water. For the second digestion, eluted DNA was mixed with 5 µl of 10X Cut Smart Buffer, 1µl (50 units) of ApaI, and the volume was adjusted to 50 µl with nuclease-free water. The mixture was incubated at 25°C for 15 minutes. Digest was incubated at 65°C for 5 minutes to heat inactivated enzyme. Donor plasmid pGFPuv was digested using XbaI and ApaI by mixing 1 µg of DNA with 5 µl 10X CutSmart buffer and 1 µl (50 units) of ApaI, volume was made up to 50 µl with nuclease-free water and the mixture was incubated at 25°C for15 minutes. The enzyme was heat inactivated by incubating at 65°C for 20 minutes. Then 1µl of XbaI was added to the reaction mixture and incubated at 37°C for 15 minutes. Heat inactivation was done by incubating at 65°C for 5 minutes.

2.3 Cloning and ligation of FP genes in shuttle vector pTRKH2

After obtaining the fragments of interest, a ligation reaction was performed using the NEB Quick Ligation Kit [14]. For ligation, a molar ratio of insert to vector of 3:1 was used. The molar concentration of both inserts and vector was calculated using the standard formulae. For a 20 µl reaction, 10 µl of 2X quick ligase reaction buffer was mixed with 50 ng (24.11 fmol) vector DNA (6.7 kb) and 2.97 ng (72.24 fmol) insert DNA (133 bp). Volume was made up to 20 µl with nuclease-free water, followed by the addition of 1 µl quick ligase (NEB) at the end. All components were mixed gently by pipetting and settled by microtubing briefly. The reaction mixture was incubated at 25°C for 5 minutes and stored at -20°C until further use.

2.4. Preparation of electrocompetent *Lactobacillus* sp.

All *Lactobacillus* cultures obtained from Microbiologics were revived on *Lactobacillus* MRS agar (HiMedia) and incubated at 37°C until growth appeared [9,15]. For *Lactobacillus rhamnosus*, electro-competent cells were prepared following Keersmaecker *et al* [17]. An overnight culture was subcultured in prewarmed MRS broth with 2% glycine and incubated at 37°C without agitation. At the exponential growth phase (OD600 = 0.8-1.0), 5 ml of this culture was reinoculated into 100 ml of prewarmed MRS broth with 2% glycine and incubated until OD600 reached 0.4-0.5. The culture was centrifuged at room temperature (RT) for 15 minutes at 6000g, the supernatant discarded, and the pellet washed twice with electroporation buffer (EB) containing 0.5 M sucrose, 7 mM potassium phosphate (pH 7.4), and 1 mM magnesium chloride. The pellet was then resuspended in 1 ml of EB and placed on ice for immediate electroporation. For *Lactobacillus acidophilus*, according to Kim *et al.* [16], an overnight culture at 10^6 cfu/ml was inoculated in 100 ml MRS broth with 1% glycine and incubated at 37°C without agitation [17]. Cells were harvested at early log phase (OD660 = 0.2-0.3) by centrifuging at 6000g, 4°C for 3 minutes, then chilled on ice for 10 minutes, washed twice with cold washing buffer (5 mM sodium phosphate, 1 mM magnesium chloride, pH 7.4), resuspended in cold electroporation buffer (1 M sucrose, 3 mM magnesium chloride, pH 7.4), and used within 30 minutes. For *Lactobacillus casei*, following Welker *et al* [18], cells were grown in MRS broth until OD660 reached 0.1, sub-cultured into 200 ml prewarmed MRS broth with 1% glycine, and incubated until OD660 reached 0.8 [18]. Cells were harvested by centrifugation at 4°C, 7000 rpm for 10 minutes, washed with cold sterile distilled water, then with 30% PEG-800, and resuspended in 0.5-0.6 ml cold 30% PEG-800 for storage at -80°C or immediate use. For *Lactobacillus gasseri*, the process was followed as per Welker *et al* [18] with certain modifications. An overnight culture was inoculated in MRS broth with 1% glycine and incubated at 37°C without agitation until OD660 reached 0.65, then a 1% inoculum was made into 200 ml fresh MRS broth with 1% glycine and incubated to an OD590 of 0.25-0.30. Cells were placed on ice, centrifuged at 7000 rpm, 4°C for 10 minutes, washed four times with ice-cold EB (0.1 mM HEPES, 0.5 M sucrose, pH 7.0), resuspended in 1.6 ml cold EB, and divided into 800 µl aliquots for immediate use.

2.5. Transformation of *Lactobacillus* sp.

Competent cells of *E. Coli* 5-α (NEB Inc., #C2987I) and *E. Coli* JM109 (Promega, #L2001) were transformed using the manufacturers' standard protocols. For *E. Coli* 5-α, a tube of competent cells was thawed on ice, mixed gently, and 50 µl was pipetted out for transformation [9,19]. After adding 1-5 µl of plasmid DNA (1 pg-100 ng), the mixture was flicked 4-5 times, placed on ice for 30 minutes, heat shocked at 42°C for 30 seconds, and immediately placed back on ice for 5 minutes. Then, 950 µl of room temperature SOC medium was added, and the cells were incubated at 37°C with vigorous shaking at 250 rpm for 60 minutes. Selected plates (LB with 50 µg/ml ampicillin) were pre-warmed to 37°C. The cell mixture was diluted 1:10 and 1:100 in SOC, and 100 µl from each dilution was plated in triplicates and incubated at 37°C until growth appeared. Transformants were stored in 15% glycerol at -80°C. For *E. Coli* JM109, competent cells were thawed on ice and 50 µl aliquots were prepared. After adding 50 ng of plasmid DNA and flicking the tubes several times, they were placed on ice for 30 minutes, heat shocked at 42°C for 15-20 seconds, and placed on ice for 2 minutes. Then, 450 µl of SOC medium was added, and the cells were incubated at 37°C with vigorous shaking for 60 minutes. The cells were diluted 1:10 and 1:100 with SOC, and 100 µl from each dilution was plated in triplicates on LB agar with 50 µg/ml ampicillin and incubated at 37°C until growth appeared. Transformants were stored in 15% glycerol at -80°C. For electroporation of *Lactobacillus* species, 100 µl of electro-competent cells of *L. rhamnosus* was mixed with 1 µg of plasmid DNA and electroporated using the Bio-Rad Gene Pulser Xcell system with settings: 1.7 kV/cm, 25 µF, 200 Ω, and a 0.2 cm cuvette gap. The

cells were immediately diluted with 5 ml MRS broth supplemented with 2 mM $CaCl_2$ and 20 mM $MgCl_2$ and incubated at 37°C for 3 hours without agitation. 100 µl was plated on MRS agar with 150 µg/ml erythromycin and incubated at 37°C until growth appeared. *L. acidophilus* cells were prepared similarly, but after adding 1 µg of plasmid DNA, the mixture was held on ice for 5 minutes before electroporation at 12.5 kV/cm, 10 pulses, 500 ms pulse interval, and 150 µs pulse length. Post-electroporation, cells were diluted with 1 ml MRS broth and incubated at 37°C for 3 hours before plating. For *L. casei*, after adding 1 µg of plasmid DNA, cells were electroporated at 10.0 kV/cm, 25 µF, and 400 Ω. The cells were then diluted with 900 µl MRS with 0.5 M sucrose and incubated at 37°C for 4 hours before plating. *L. gasseri* cells were prepared with 1 µg of plasmid DNA and electroporated at 2.45 kV/cm, 25 µF, and 200 Ω. After electroporation, cells were placed on ice for 5 minutes, diluted with 8 ml MRS containing 10 mM $CaCl_2$ and 0.5 M sucrose, and incubated at 37°C for 3 hours before plating on MRS agar with 10 mM $CaCl_2$ and 150 µg/ml erythromycin. All *Lactobacillus* transformants were incubated at 37°C until growth appeared.

2.6. Inoculation of transformed *E. coli* in rice plant

Approximately 60 rice seeds of the *Maguri bao* genotype were surface sterilized following a modified version of the method by Hardoim *et al.* [20, 21]. The sterilization cocktail included 1% sodium hypochlorite for surface sterilization of the seeds with husk. After rehydrating the seeds for 1 hour, the surface-sterilized seeds were placed on 0.4% water agar. A single colony of transformed *E. coli* was picked and inoculated into 150 µl of autoclaved water. Subsequently, 100 µl of this suspension was spread at the bottom of Hoagland media plates, keeping a distance from the roots of four germinated rice seedlings. These plates were incubated in the dark at 25°C.

Seven-day-old rice seeds, germinated on water agar, were then transferred to Hoagland media plates and co-cultivated with the transformed bacteria. The plates were maintained under the following plant growth conditions: a temperature of 25°C, a 16-hour light/8-hour dark cycle, and a light intensity of 4700 lux. To detect the presence of transformed *E. coli* inside the plant tissue, the roots were thoroughly washed and aseptically cleaned with double-distilled water (DDW). The outer surface of the roots was wiped to remove excess water, and transverse sections of the roots were cut and observed under a confocal laser scanning microscope (CLSM) (Leica DMi8) at specific wavelengths (nm) to check for *E. Coli* colonization and the visibility of fluorescent proteins in the plant samples (Fig. 1). Rice roots without bacterial inoculation were used as negative controls to account for auto-fluorescence.

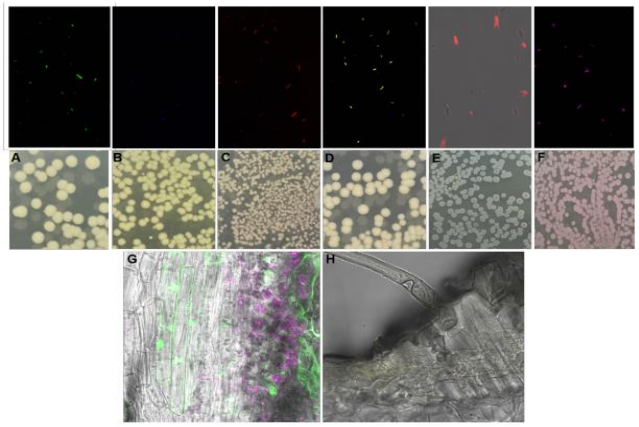

Figure 1: A. Colonies of *Escherichia coli* strains JM109 and 5-alpha transformed with fluorescent protein-expressing vectors, A-pGFP, B- pAmCyan, C- pDsRed2, D- pZsYellow, E-pE2Crimson, F-pmCherry and their corresponding cells visualized by confocal microscopy. Successful colonization of *E. coli* JM109 and 5-alpha expressing, G- Cherryand Green, and H- Yellow fluorescent protein in roots of seven-day-old *Maguri bao* seedlings.

2.7. Preparation of rice seedlings and inoculation with *L. casei, L. rhamnosus and M. luteus*

One gram of *Kalajoha* seeds was surface sterilized using 1% sodium hypochlorite, then rehydrated and placed on 0.4% water-agar plates at 25°C in the dark until germination [9,21]. Post germination, seeds were transferred to 2 L glass flasks containing 1 kg of sterile garden soil, incubated at 25°C with a 16-hour light/8-hour dark cycle at 4700 lux. The soil was autoclaved twice at 24-hour intervals to eliminate microbial colonies. After the first leaf emerged (4 days), seedlings were inoculated with *L. casei, L. rhamnosus, or M. luteus*, prepared by culturing in MRS broth or NB, incubating at 37°C (28°C for *M. luteus*) until OD 600 reached 0.8, centrifuging at 10,000 rpm for 10 minutes, washing with 0.9% NaCl, and resuspending to 10^8 cfu/ml. For inoculation, 6 ml of bacterial suspension was added to the soil; control plants received 6 ml of normal saline. Seedlings were grown for 7 days under the same conditions, and watered with distilled water. After 7 days, plants were harvested, and phenotypic traits such as shoot and root length, and fresh weight of roots and shoots were measured. Four seedlings were grouped for isolating culturable endophytes.

2.8. Quantification of culturable EB post inoculation and measurement of host phenotypic attributes

Harvested shoots and roots were surface sterilized as per Hardoim *et al.* [21], with sterilization confirmed by plating portions on NA plates and incubating at 28°C for five days. Post-rehydration, 1 gram of each plant sample was homogenized with normal saline using a mortar and pestle, then serially diluted with normal saline up to a 10^{-4} dilution. 100 µl of homogenate from each dilution was

plated in triplicate on six different culture media: nutrient agar (general purpose), Pikovskaya's agar (PKA), Jensen's agar (JA), cellulose congo red (CCR) with 0.2% cellulose, R2A-PEC with 0.2% (w/v) citrus pectin/0.1% triton X-100, and deManRogosa Sharpe (MRS). Plates were incubated at 28°C and observed for bacterial growth over four days. Colony forming units (cfu) were counted using a colony counter and reported in log cfu/g FW sample, with each replicate value multiplied by the dilution factor and calculated for 1 gram of sample. After converting to the log10 scale, means were calculated, standard errors determined, and results plotted in a bar graph using MS Excel. Unique isolates based on colony morphology (color, shape, size) were further cultured for pure colonies. Growth in selective media (PKA, JA, R2A-PEC, CCR) indicated functional traits: PKA for phosphate solubilization, JA for nitrogen fixation, CCR for cellulolytic activity, and R2A-PEC for pectinolytic activity.

3 Results

3.1. Plasmid extraction and preparation of insert

Plasmids extracted for vector and insert preparation, shown in Figure 2A-B, displayed unexpected sizes: pTRKH2 plasmid extracted using both a kit and manual methods resulted in bands near 10.0 kb and 2.0 kb instead of the expected 6.7 kb. Diagnostic digest with BamHI did not yield the anticipated plasmid size, but the insert from pE2crimson and pDsRed2 double—

Fig. 2

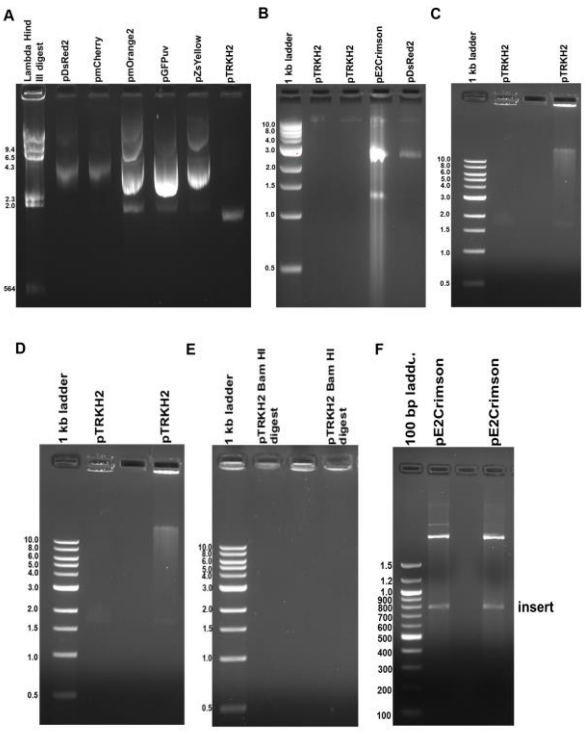

Figure 2: (A, B)-Extraction of plasmid vectors from *E. coli* hosts NEB 5-alpha, JM109 and JM110 using Sigma Mini prep plasmid extraction kit and C- manually by alkaline lysis method of recipient plasmid. D - Diagnostic digest of pTRKH2 with BamHI to reveal the plasmid at its expected size of 6.7 kb and E- restriction double digest of donor vectors pE2Crimson and pDSREd2 with BamHI/ApaI for preparation of fluorescent protein gene insert of size800 bp.

digested with BamHI/ApaI was correctly identified at 800 bp. Due to the unsuccessful isolation of the recipient vector pTRKH2, rice seedlings were inoculated with non-transformed *Lactobacillus casei*. Aerobic EB population (log cfu/g FW) in roots and shoots across six media varied between uninoculated and inoculated seedlings. In uninoculated shoots of *Kalajoha*, EB population ranged from 7.19-7.95 in P1, 7.91-7.94 in P2, and 7.80-7.94 in P3; in roots, it ranged from 7.76-7.94 in P1, 7.90-7.96 in P2, and 7.83-7.94 in P3. For *L. casei* inoculated seedlings (LCIS), EB in shoots ranged from 7.79-7.96 in P1, 6.36-7.94 in P2, and 6.15-7.95 in P3; in roots, it ranged from 7.79-7.96 in P1, 7.67-7.94 in P2, and 7.73-7.93 in P3. Phosphate solubilizers were more prevalent in uninoculated shoots compared to inoculated ones and similar in roots of both sets, with a tenfold decrease in cfu numbers on R2A-PEC media in inoculated P2 and P3 seedlings. Morphological diversity of endophytes was observed to be higher in roots and shoots of inoculated seedlings compared to uninoculated ones (Fig. 3A-B), with a greater diversity of phosphate solubilizing bacteria in roots of inoculated *Kalajoha*. In *Lactobacillus rhamnosus* inoculated seedlings, EB in uninoculated shoots ranged from 7.19-7.95 in P1, 7.91-7.94 in P2, and 7.80-7.94 in P3; in roots, it ranged from 7.76-7.94 in P1, 7.90-7.96 in P2, and 7.83-7.94 in P3. For inoculated seedlings, EB in shoots ranged from 6.63-8.88 in P1, 6.52-8.91 in P2, and 6.42-8.84 in P3; in roots, it ranged from 7.81-8.14 in P1, 7.89-8.15 in P2, and 7.82-8.19 in P3.

Fig. 3

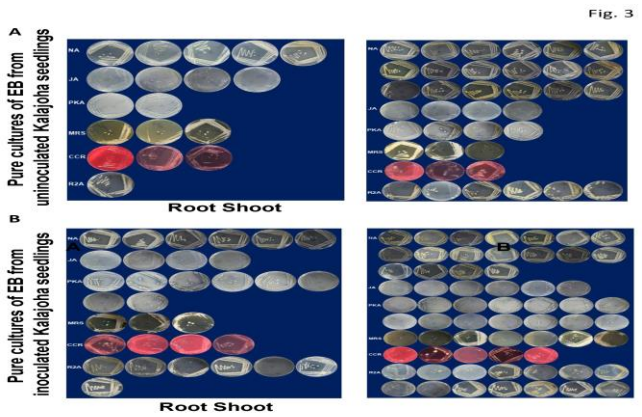

Figure 3**:** (A) Morphological diversity of culturable EB isolated from roots and shoots of uninoculated Kalajoha seedlings on six different growth media; NA- nutrient agar, as general-purpose media, JA- jensen's agar for nitrogen fixers, PKA- pikovskaya's agar for phosphate solubilizers, MRS- deMan, Rogosa and Sharpe agar, for *Lactobacillus*, CCR- cellulose congo red, for cellulolytic bacteria, R2A/PEC- reasoner's 2 agar/pectin for pectinolytic bacteria. (B) Morphological diversity of culturable EB isolated

from roots and shoots of Kalajoha seedlings inoculated with *Lactobacillus casei* on six different growth media; NA- nutrient agar, as general-purpose media, JA- jensen's agar for nitrogen fixers, PKApikovskaya's agar for phosphate solubilizers, MRS- deMan Rogosa Sharpe agar, for *Lactobacillus*, CCR- cellulose congo red, for cellulolytic bacteria, R2A/PEC- reasoner's 2 agar/pectin for pectinolytic bacteria.

The mean population of nitrogen-fixing bacteria was tenfold higher in inoculated seedlings compared to uninoculated ones, with phosphate solubilizer populations higher in uninoculated shoots but similar in roots. A decrease in EB on MRS and CCR and an increase in pectinolytic EB in inoculated *Kalajoha* shoots were observed, along with a significantly higher number of EB on NA in inoculated roots. Only one morphologically unique colony was isolated from inoculated seedlings on NA, compared to five from uninoculated, with lower morphological diversity of culturable EB in *L. rhamnosus* inoculated seedlings (LRIS) compared to uninoculated (Fig. 4A-D).

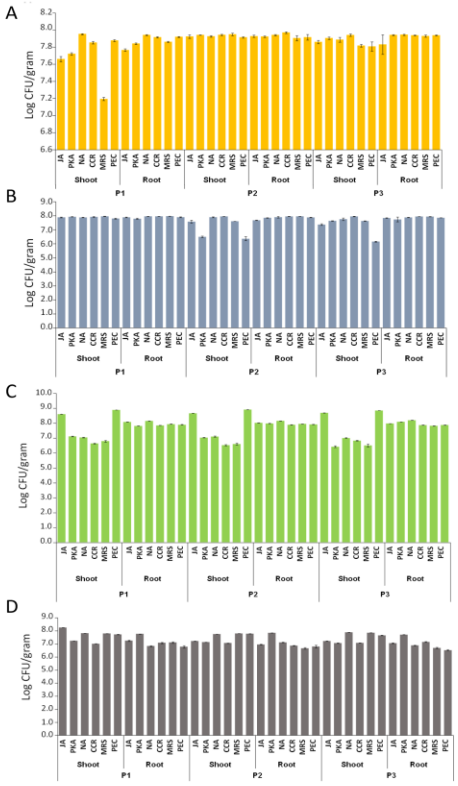

Fig. 4

Figure 4: Population of (A) endophytic bacteria isolated from roots and shoots of uninoculated Kalajoha seedlings (B) endophytic bacteria isolated from roots and shoots of Kalajoha seedlings inoculated with *Lactobacillus casei* (C) endophytic bacteria isolated from roots and shoots of Kalajoha seedlings inoculated with *Lactobacillus rhamnosus* (D) endophytic bacteria isolated from roots and shoots of Kalajoha seedlings inoculated with *Micrococcus luteus*, on six different growth media; NA- nutrient agar, as general purpose media, JA- jensen's agar for nitrogen fixers, PKA- pikovskaya's agar for phosphate solubilizers, MRS- deMan Rogosa Sharpe agar, for *Lactobacillus*, CCR- cellulose congo red, for cellulolytic bacteria, R2A/PEC- reasoner's 2 agar/pectin for pectinolytic bacteria. The error bar represents log ± S.E. The error bar represents log ± S.E.

For Micrococcus luteus inoculated seedlings (MLIS), EB in uninoculated shoots ranged from 7.19-7.95 in P1, 7.91-7.94 in P2, and 7.80-7.94 in P3; in roots, it ranged from 7.76-7.94 in P1, 7.90-7.96 in P2, and 7.83-7.94 in P3. In inoculated shoots, EB ranged from 7.01-8.25 in P1, 7.07-7.80 in P2, and 7.06-7.89 in P3; in roots, it ranged from 6.78-7.77 in P1, 6.66-7.85 in P2, and 6.52-7.71 in P3. A tenfold lower abundance of EB on NA and PEC was noted in inoculated roots compared to uninoculated, with similar EB populations in shoots of both groups. Pure culture images of EB isolated on the 6 growth media were also checked.

3.2 Analogy between *M. luteus* and *Lactobacillus* inoculated seedlings

Seven days after inoculating *Kalajoha* seedlings with *M. luteus*, *L. casei*, and *L. rhamnosus*, a tenfold increase was observed in the mean abundance of nitrogen fixers in the roots and shoots of LRIS compared to MLIS. There was no significant difference in the population of nitrogen fixers between LCIS and MLIS. On PKA media, the number of CFUs was lower in LRIS shoots and higher in LCIS shoots relative to MLIS shoots. However, the population of phosphate solubilizers was identical in LCIS and MLIS but higher in LRIS roots. The abundance of EB of LRIS root isolated on NA was highest in LRIS shoots, while it was similar in LCIS and MLIS shoots. Cellulolytic endophytes of LRIS shoots isolated on CCR were the lowest. Mean population of EB of MLIS roots obtained on MRS was lowest but was similar in case of LRIS and LCIS roots. However, the mean population of EB of MLIS shoots was highest followed by those of LCIS and LRIS. The population of pectinolytic bacteria was tenfold higher in LRIS shoots compared to MLIS and a hundredfold higher in LCIS shoots. The population of ML obtained on R2A-PEC from roots was the lowest. Overall, the morphological diversity of EB showed that MLIS shoots had more unique colonies compared to LRIS shoots, but LCIS obtained both from the root and shoots had the highest morphologically diverse CFUs.

3.3 Effect of HGBB and EB on phenotypic traits of host plants

Seven days after inoculation, the growth characteristics of *Kalajoha* seedlings were measured and compared with uninoculated seedlings. No significant difference in shoot length was observed between MLIS, LCIS, LRIS, and uninoculated plants ($p > 0.05$), suggesting that the inoculants did not negatively affect shoot length. Seedlings inoculated with ML showed significantly longer primary roots compared to LCIS ($p < 0.017$) and uninoculated seedlings ($p = 0.014$). The root length of LRIS was also higher than uninoculated seedlings ($p < 0.03$), while it was similar between LRIS and MLIS (Fig. 5).

Fig. 5

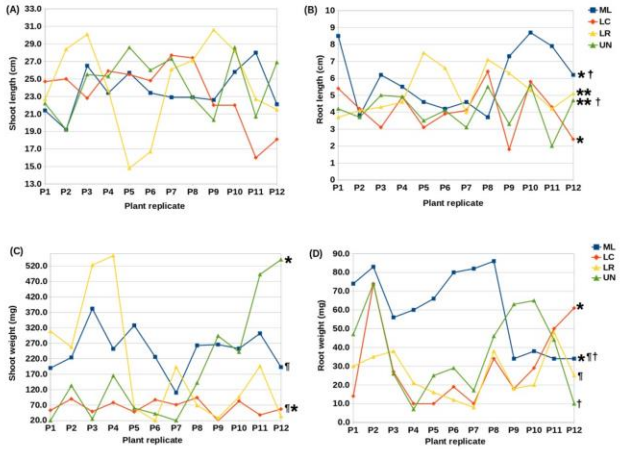

Figure 5: The effect of *M. luteus*, *L. casei* and *L. rhamnosus* on shoot length, root length, shoot weight and root weight in Kalajoha seedlings. Significant differences between the two groups are shown in the figure. ∗, ∗∗, †, significant at 5 % level; ¶, significant at 0.1 % level.

A comparison of root weight showed that MLIS had significantly higher fresh root weight (FRW) compared to LCIS ($p < 0.05$), LRIS ($p < 0.001$), and uninoculated seedlings ($p < 0.05$). The mean FRW of LCIS roots (29.7±6.2) was higher than that of LRIS roots (25.7±3.5) but lower than that of uninoculated roots (37.7±6.4). The weight of fresh shoots (FSW) was significantly higher in MLIS compared to LCIS ($p < 0.001$), and LCIS had a lower FSW than uninoculated seedlings ($p = 0.05$). Browning of shoot tips was observed, possibly due to potassium deficiency. Browning was highest in the shoot tips of LCIS, followed by LRIS, uninoculated, and lowest in MLIS (Fig. 6).

Fig. 6

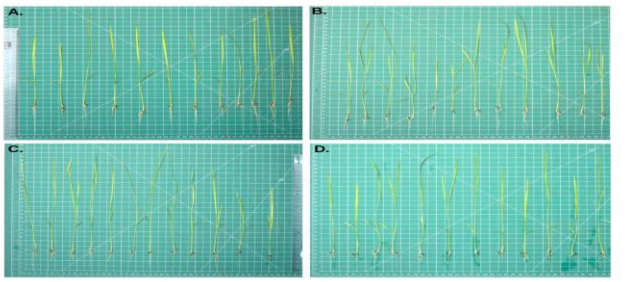

Figure 6: Images of seedlings seven days post inoculation with A- *M. luteus*, B- *L. rhamnosus*, C- *L. casei* and D- uninoculated. Seeds were allowed to germinate on 0.4 % water-agar. 12 seedlings as biological replicates were transferred to four 2 L flasks each, containing 1 kg sterile garden soil. Four days after transplanting 6 ml of 108 cfu/ml of inoculant in normal saline was added directly near the roots in the soil and seedlings were grown for another 7 days. Flasks were watered regularly with distilled water. Seedlings were maintained at 16 hours a day/ 8 hours night cycle, 25◦ C and 4700 lux. After seven days the seedlings were used to measure shoot and root length, shoot and root weight. After measurement, culturable EB was isolated separately from surface sterilized shoots and roots. Four seedlings were clubbed together to make one replicate for isolating culturable endophytes.

4 Discussion

Plant scientists have yet to fully understand the role of the seed microbiome in plant growth and development. Previous studies have reported that water in deep-water paddy fields has a low concentration of major metals [22]. This suggests that the low nutrient concentration in these deep-water systems may necessitate a higher population of seed endophytic bacteria (EB) to ensure optimal mineral absorption. In Assam, the acidic soil facilitates the reduction of Fe3+ to Fe2+, leading to easier and higher iron uptake. Conversely, the aerobic soil in upland fields retains iron as Fe3+, making it harder for plants to absorb. Predictive functional analysis of culturable isolates has shown that landraces from upland agroecosystems possess abundant pathways for synthesizing iron-chelating compounds, more so than midland and deep-water agroecosystems. This explains why rice seeds from upland areas have EB with multiple and dominant siderophore synthesis pathways.

Rice, a major food crop in northeast India, is highly nutritious and beneficial to the population. In addition to plant-beneficial traits, pathways for synthesizing many vitamins such as K_2, B_2, B_6, B_9, B_{12}, biotin, and folic acid, as well as selenium reduction were found. While these vitamins are known to be present in rice, vitamin B_{12} is generally not found in grains, vegetables, or fruits. However, significant amounts of vitamin B_{12} have been detected in fermented foods like tempeh (0.7-8.0 µg/100 g) and the

Japanese beverage bata bata-cha (0.1-1.2 µg/100 g) [23]. Although plants do not require vitamin B_{12}, six pathways for its synthesis were observed in traditional aromatic landrace *Kalajoha* and upland genotype Idaw, with the adenosylcobalamin salvage pathway present in all genotypes. During the ungerminated stage, rice seed EB may not actively divide, potentially resulting in undetectable or absent vitamin B_{12}. However, the detection of this vitamin in fermented foods suggests that fermented rice landraces might also contain this essential nutrient.

The primary aim of this hypothesis was to explore differences and similarities in the cultivable population of rice EB and the response of rice plants to inoculation with *Lactobacillus* sp., compared to inoculation with EB isolated from the rice genotype. The study revealed significant differences in the overall population of cultivable EB across six growth media in both roots and shoots of *Kalajoha* seven days post-inoculation with both HGBB and EB. Differences were also observed in the cultivable EB population across some growth media when compared with uninoculated seedlings. *L. casei*, known for its remarkable adaptability to diverse habitats, including dynamic and nutritionally variable plant- and vertebrate-associated niches [24], has been reported in various plant materials [24-27]. Thus, it may be capable of living as an endophyte. Our results clearly show changes in the population of endophytic flora after inoculation with *Lactobacillus*, compared to both uninoculated and MLIS plants. This might be due to altered microbe-microbe interactions in the roots and shoots [27-28].

The *L. rhamnosus* ATCC 53103, originally isolated from healthy human adult fecal samples, is widely studied for its probiotic benefits and is used in many commercial probiotic products [29]. It has also been found to ferment plant material [30]. The human gut microbiota is influenced by different foods consumed, and similarly, plants show the presence of many animal-associated bacteria, with some becoming endophytic. Given humans' close association with rice cultivation, only a few *Lactobacillus* species have been isolated from plants. This study aimed to determine if the dairy-fermenting, strictly animal-associated *L. rhamnosus* could enter rice seedlings, affect native microbiota structure, and confer benefits to the plants. Post-inoculation, a colony with morphological similarities to *L. rhamnosus* ATCC 53103 was isolated on MRS media, with sequencing needed to confirm our observations. Phenotypically, LRIS seedlings had longer average root lengths than uninoculated seedlings ($p < 0.03$), similar to MLIS, indicating that *L. rhamnosus* might contribute to root growth comparable to the known plant growth-promoting endobacteria *M. luteus*. Browning of shoot tips, typically indicative of potassium deficiency, was most pronounced in LCIS shoot tips, followed by LRIS. *L. casei*'s high potassium requirement for maximum growth [31] may have reduced the nutrient's availability to the seedlings, necessitating further investigation to confirm this observation.

5 Conclusion

This study highlights the significant role of seed endophytic bacteria (EB) and plant-associated Lactobacillus strains in enhancing rice plant growth and nutrient uptake, particularly within Assam's varied agroecosystems. The acidic soils in upland areas enable better iron absorption due to the prevalence of Fe2+, contrasting with the limited Fe3+ availability in aerobic soils. Upland rice landraces, adapted to these nutrient-scarce environments, show abundant iron-chelation pathways, possibly improving plant nutrition.

Interestingly, animal-associated Lactobacillus strains like *L. casei* and *L. rhamnosus* were found to integrate into rice tissues, modulating root growth and altering native microbiota. Inoculation with *L. rhamnosus* promoted root elongation similar to the growth-enhancing effects of *Micrococcus luteus*, while potassium deficiency symptoms in *L. casei* inoculated seedlings point to microbe-induced nutrient shifts.

The detection of vitamin B_{12} synthesis pathways in rice landraces and endophytic bacteria also hints at previously unrecognized nutritional potential, especially in fermented rice. Collectively, these findings reveal that rice seed microbiomes and *Lactobacillus* strains offer promising benefits for plant development, suggesting a path forward in leveraging microbial communities for sustainable agriculture.

Conflict of Interest

The authors declare that there is no conflict of interest in the work done and regarding the publication of this study

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