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Novel method to recover *Salmonella enterica* cells for Tn-Seq approaches from lettuce leaves and agricultural environments using combination of sonication, filtration, and dialysis membrane

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ABSTRACT

Salmonella enterica in agricultural environments has become an important concern, due to its potential transmission to humans and the associated public health risks. To identify genes contributing to *Salmonella* adaptation to such environments, transposon sequencing has been used in recent years. However, isolating *Salmonella* from atypical hosts, such as plant leaves, can pose technical challenges due to low bacterial content and the difficulty to separate an adequate number of bacteria from host tissues. In this study, we describe a modified methodology using a combination of sonication and filtration to recover *S. enterica* cells from lettuce leaves. We successfully recovered over a total of 3.5×10^6 *Salmonella* cells in each biological replicate from two six-week old lettuce leaves, 7 days after infiltration with a *Salmonella* suspension of 5×10^7 colony forming units (CFU)/mL. Moreover, we have developed a dialysis membrane system as an alternative method for recovering bacteria from culture medium, mimicking a natural environment. Inoculating 10^7 CFU/mL of *Salmonella* into the media based on plant (lettuce and tomato) leaf and diluvial sand soil, a final concentration of $10^{9.5}$ and $10^{8.5}$ CFU/mL was obtained, respectively. One millilitre of the bacterial suspension after 24 h incubation at 28 °C using 60 rpm agitation was pelleted, corresponding to $10^{9.5}$ and $10^{8.5}$ cells from leaf- or soil-based media. The recovered bacterial population, from both lettuce leaves and environment-mimicking media, can adequately cover a presumptive library density of 10^6 mutants. In conclusion, this protocol provides an effective method to recover a *Salmonella* transposon sequencing library from *in planta* and *in vitro* systems. We expect this novel technique to foster the study of *Salmonella* in atypical hosts and environments, as well as other comparable scenarios.

1. Introduction

Salmonellosis caused by *Salmonella enterica* subspecies *enterica* is one of the most important diarrheal diseases (EFSA, 2022). Agricultural environments, especially leafy vegetables contaminated by *Salmonella* are constantly endangering human health, either directly or indirectly (Gajraj et al., 2012; Sallach et al., 2015; Pornsukarom and Thakur, 2016; Koukkidis and Freestone, 2018). *Salmonella* can survive for up to several months in plant production environments (Schikora et al., 2008; Jechalke et al., 2019; Zarkani et al., 2019; Schierstaedt et al., 2020). However, the mechanisms employed by *Salmonella* for survival in such environments, especially the genes involved, remain unknown.

Although multi-omics have been widely employed to understand *Salmonella* behavior under various conditions considering different levels including transcription, translation, or metabolism (Schikora et al., 2011; Schlekera et al., 2012; Elpers et al., 2022), mutation remains a most direct way to link phenotypes to genes. Notably, a novel technique known as transposon sequencing (Tn-Seq), one of the transposon-insertion sequencing (TIS) methods, combines high-throughput sequencing with mutagenesis, making it possible to screen millions of mutants in a given condition in a single trial (van Opijnen et al., 2009; Cain et al., 2020). Briefly, a Tn-Seq library is introduced into the tested

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environment. The bacterial input and recovered cells (output) are sampled, and the DNA is extracted, processed, and sequenced. Eventually, the fitness change of each mutant is calculated to determine if its adaption is better or worse compared to overall mutants (van Opijnen et al., 2009). Tn-Seq requires a sufficient number of bacteria in both input and output samples to i) avoid bottleneck effects caused by the insufficient number of bacteria in the samples being lower than that in the library, ii) obtain adequate amount of material for subsequent processes, and iii) generate enough resolution to detect the difference between individual mutants.

Tn-Seq has been widely used to investigate previously uncharacterized *Salmonella* genes or genes relevant in various stress-causing conditions (Khatiwara et al., 2012; Karash et al., 2017; Karash and Kwon, 2018; Gu et al., 2021; Mandal et al., 2021). Sampling in such *in vitro* experiments appears to be straightforward. Only containers such as multi-well microplates, glass tubes, flasks, containing different test media, nutrients or inhibitory substances such as H_2O_2, acids, NaCl seem required (Wetmore et al., 2015; Karash et al., 2017; Price et al., 2018b; Gu et al., 2021; Mandal et al., 2021). Generally, experiments are set to inhibit but not completely suppress bacterial growth. A well-defined initial population, which would represent all the insertions throughout the genome, is applied and bacteria are allowed to reproduce under given conditions (Liu et al., 2018; Helmann et al., 2019; Royet et al., 2019). Subsequently, bacterial samples can be easily pelleted by centrifugation for further processing.

Tn-Seq libraries have been used also *in vivo* in order to better understand the interactions between *Salmonella* and animal hosts on many occasions (Chaudhuri et al., 2013; Ali, 2014; Vohra et al., 2019; Nguyen et al., 2020). However, unlike *in vitro*, *in vivo* sampling faces many challenges, including technical bottlenecks on different levels. Depending on the inoculation manner and on the experimental model, for example, oral, intraperitoneal, organoid, or cell culture infection, there can be varying degrees of bottleneck effects (Abel et al., 2015). Additionally, extracting and purifying bacteria from hosts can also be challenging. Generally, the output is obtained by homogenizing inoculated tissues. In preferable hosts for *Salmonella* such as mice, chickens, rabbits, or cattle, the amount of *Salmonella* recovered from these systems may exceed the input by even million times (Bowe et al., 1998; Tsolis et al., 1999). As a result, only as little as one gram of colonized tissue is needed (Vohra et al., 2019). Alternatively, output samples may be re-incubated on rich solid or in liquid media to obtain the satisfactory quantity, after verifying that this multiplication does not have an impact on the ratio of the different mutants (Fu et al., 2013; Subashchandrabose et al., 2016; Shames et al., 2017; Ireland et al., 2019).

Differently from typical *Salmonella* hosts or environments, agricultural production systems or produces may restrict the survival and growth of *Salmonella* (Jechalke et al., 2019; Zarkani et al., 2019; Schierstaedt et al., 2020). Such situation necessitates bigger amounts of colonized materials to ensure an adequate bacterial recovery rate. The difficulties of sampling vary between different scenarios. An efficient sampling of *S. enterica* serovar Typhimurium strain 14028s (*S.* Typhimurium 14028s) and *S.* Newport C4.2 Tn-Seq libraries from only one gram of tomato fruits was already reported (de Moraes et al., 2017; de Moraes et al., 2018). Until now, reports on a plant model are very sporadic. Nonetheless, some applications of plant pathogenic or beneficial bacteria TIS libraries are available. Multiple methods for harvesting bacteria from roots or xylem have been reported. For example, *Pseudomonas simiae* inoculated to Arabidopsis roots were liberated by brief homogenization using TissueLyser (Cole et al., 2017). Similarly, *Pseudomonas aeruginosa* introduced to corn roots were released by vortexing with the help of glass beads (Sivakumar et al., 2019). The *Rhizobium leguminosarum* TIS library in pea seedlings was recovered by grounding using pestle and mortar (Wheatley et al., 2020). However, while using a gentle method, longer extraction time is required. The *Pantoea stewartii* TIS library inoculated to corn xylem was released by cutting the plants into 1 mm pieces and immersing them in PBS for 2 h (Duong et al., 2018). *Azoarcus olearius* and *Herbaspirillum seropedicae* inoculated to *Setaria viridis* roots were recovered by immersing the roots in BRM medium with agitation for 6 h (do Amaral et al., 2020). However, in leaves with delicate architecture, another gentle way of sonication in water bath was preferably used (Helmann et al., 2019; Helmann et al., 2020; Morinière et al., 2022). Despite the multiplication of 7–10 generations of plant pathogens after inoculation, tens to even thousands pots of plants were still needed (Helmann et al., 2020; Wheatley et al., 2020; Morinière et al., 2022). Given the situations described above, determining which strategy would work, is time and material intensive. In this protocol, a modified small-scale method derived from Helmann's laboratory is proposed as an alternative (Helmann et al., 2019). Our main objective was to provide a protocol allowing (i) to sample Tn-Seq libraries *in planta* and (ii) to obtain sufficient amount of DNA (about 2 μg) from the output samples for the subsequent sequencing library preparation. Two main improvements were developed: the reduction of the sampling labor since only two lettuce leaves were sufficient and bacterial recovery was improved on a smaller scale in syringes rather than in beakers or bunks. Despite this minimization, the bacterial population density of the output was high enough to cover all insertions of a presumptive *Salmonella* Tn-Seq library.

Although a protocol for a small-scale leaf experiment is readily available in our lettuce model described above and easily modified, establishing a functional methodology for roots, soil or rhizosphere can be still difficult. Surrounding bacterial community and plant tissues can affect the extraction of DNA for NGS approaches, which can result in a high yield of total DNA but a poor yield of DNA from the Tn-Seq library and the presence of inhibitors hampering the subsequent steps (Pereira-Marques et al., 2019; Lazarevic et al., 2022). In order to overcome this restriction, we used a system based on dialysis membrane. The dialysis membrane system presented here proposes a method to simplify complicated *in vivo* experiments and converts them into *in vitro* system. Dialysis membrane is commonly used to filter molecules of various molecular weights (Himmelfarb et al., 1998; Vienken and Bowry, 2008). Furthermore, it was used to culture symbiotic bacteria with the assistance of associate microorganisms or to evaluate tumor growth affected by mechanical stress by positioning spheroid in dialysis membrane bags (Ueda et al., 2002; Montel et al., 2011). We used dialysis membrane bags to incubate *Salmonella* in plant- and in soil-based media without contaminations originating from the plant tissue or the soil. In our earlier publications, we have successfully sampled concentrated and pure *Salmonella* samples exposed to leaf extract media, root exudate media, and agricultural soil suspension in order to sequence the transcriptome (Jechalke et al., 2019; Zarkani et al., 2019; Schierstaedt et al., 2020). The membrane used, with a cut-off of 100 kDa, allows glucose, glycerol, lactate, pyruvate, and other substances to flow through (D'Souza et al., 2014). In this study, dialysis membrane was employed to incubate *S.* Typhimurium 14028s exposed to tomato leaf medium (TM), lettuce leaf medium (LM), and diluvial sand (DS) soil suspension. Using this method makes it possible to recover bacteria from complex environments to study interactions in genetic or metabolic approaches.

In summary, we demonstrated a viable approach for harvesting *Salmonella* from atypical host, namely lettuce leaves for *in planta* studies. As an alternative to difficult-to-perform *in vivo* experiments, a dialysis membrane system was proposed, allowing to sample bacteria from a simplified *in vitro* model. Both, the *in planta* sampling improvement and the *in vitro* sampling innovation, will assist in Tn-Seq based studies of *Salmonella* behavior in agricultural environments and produces.

2. Materials and equipment

2.1. Materials and reagents

1. Plant material: six-week old lettuce (*Lactuca sativa* L. cultivar Magician; Syngenta)

2. Potting substrate: Substrate 1 (Klasmann-Deilmann GmbH)
3. Bacterial material: *Salmonella enterica* serovar Typhimurium 14028s (*S.* Typhimurium 14028s), rifampicin resistant strain (50 μg/mL) was used in this study to imitate the real Tn-Seq library
4. Antibiotics: rifampicin (Carl Roth GmbH + Co. KG, catalog number: 4163.2)
5. $MgCl_2{\cdot}6H_2O$ (Carl Roth GmbH + Co. KG, catalog number: 2189.1)
6. Sterile distilled H_2O, filtered by pressure-resistant SG water de-ionizers (Wasseraufbereitung und Regenerierstation GmbH)
7. M9 minimal salts, 5× (Sigma-Aldrich, catalog number: M6030-1KG)

2.2. Medium recipes

1. Lettuce leaf medium (LM) (Fornefeld et al., 2017)
   a. 16.5 mL sterile dH_2O
   b. 7.5 mL lettuce leaf extract
   c. 6 mL M9 minimal salts, 5×
2. Tomato leaf medium (TM) (Zarkani et al., 2019)
   a. 16.5 mL sterile dH_2O
   b. 7.5 mL tomato leaf extract
   c. 6 mL M9 minimal salts, 5×
3. Diluvial sand (DS) soil suspension (Schierstaedt et al., 2020)
   a. 10 g sieved DS soil in 25 mL sterile dH_2O
4. LB medium (Luria/Miller; Carl Roth GmbH + Co. KG, catalog number: X968.1)
5. XLD agar (Carl Roth GmbH + Co. KG, catalog number: X941.2)

2.3. Equipment

1. 50 mL centrifuge tubes (SARSTEDT AG & Co. KG, catalog number: 62.547.254)
2. 1.5 mL and 2 mL centrifuge tubes (SARSTEDT AG & Co. KG, catalog number: 72.690.001 and 72.691)
3. Multi- and single-channel pipettes (Gilson), and pipette tips (SARSTEDT AG & Co. KG)
4. Shaking incubator (Shanghai ZHICHENG Analytical Instruments Manufacturing Co., Ltd., catalog number: S18YA080BY022E)
5. BioPhotometer plus (Eppendorf AG)
6. Cuvettes (SARSTEDT AG & Co. KG, catalog number: 67.742)
7. Cellulose ester (CE) dialysis membrane: MWCO 100000, 16 mm (Spectra/Por Biotech, catalog number: 131414)
8. Hair straightener (Povos, catalog number: PR2031)
9. 580 mL tumbler with glass lid (WECK, catalog number: 10013626)
10. Syringe needle (B. Braun Melsungen AG, Sterican, REF: 4657705)
11. 1 mL Syringe (B. Braun Melsungen AG, Injekt-F Luer Solo, REF: 9166017 V)
12. 10 mL Syringe (B. Braun Melsungen AG, Omnifix Luer Duo, REF: 4616103 V)
13. 20 mL Syringe (B. Braun Melsungen AG, Injekt, REF: 4606205 V)
14. Biopsy punchers (Ø = 5 mm) (Stiefel, catalog number: 270037)
15. Biopsy punchers (Ø = 3 mm) (Pfm, catalog number: X81238.92)
16. QSONICA sonicator (Qsonica LLC, catalog number: 89191C-02-16)
17. Filter paper circles (Whatman, catalog number: 1001–020 and 10311862)
18. Syringe filters (Sartorius, Minisart NML, catalog number: 512–3118)
19. Motor handpiece MHX-E (Xenox, catalog number: DE 47896957)

3. Methods

The workflow of the methods is shown in Fig. 1.

3.1. Plant cultivation

Lettuce seeds were sown in standard bedding substrate and cultivated in a greenhouse chamber set at 20 °C with 18 h of day light. After three weeks, seedlings were transplanted into 9 cm diameter pots. Plant leaves were ready for inoculation three weeks post transplantation.

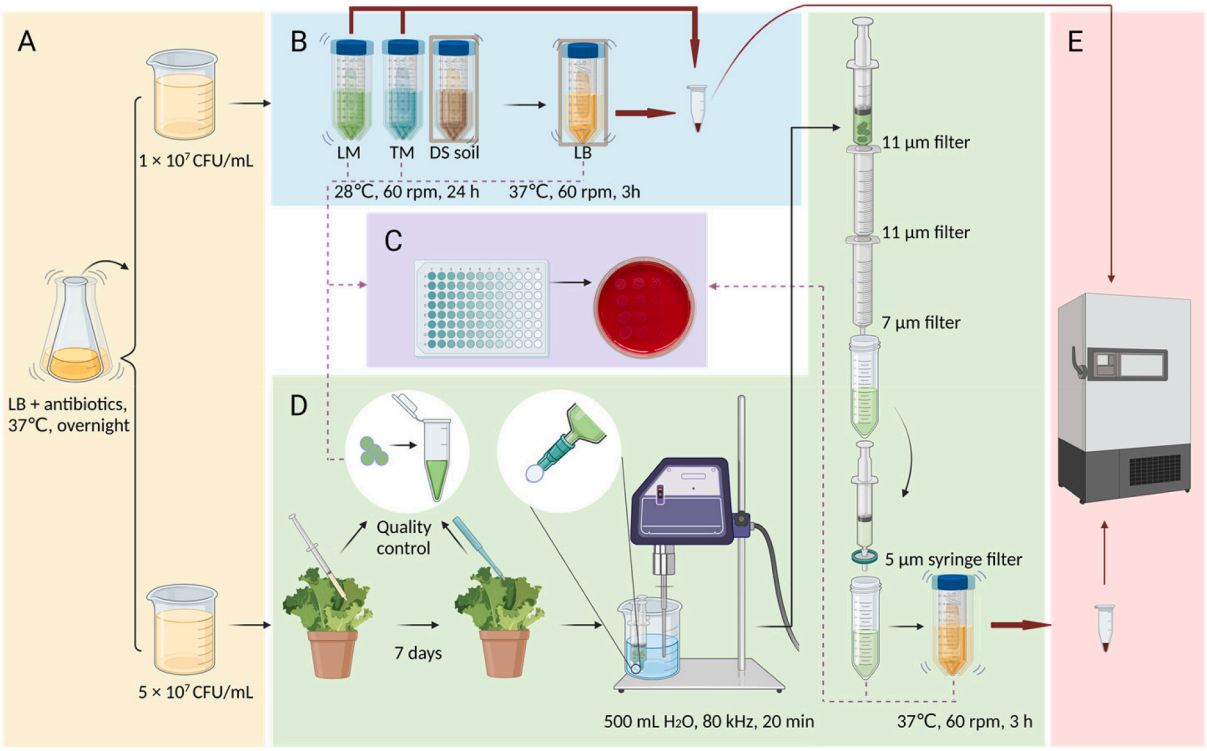

Fig. 1. Flowchart of the methods.
A. Preparation of bacterial inoculum. B. Dialysis membrane system. C. Bacterial enumeration. D. Plant system. E. Storage of pelleted samples.

3.2. Bacterial inoculum preparation

To prepare the bacterial inoculum for subsequent experiments, a single colony of *S.* Typhimurium 14028s from an agar plate was inoculated into LB medium supplemented with rifampicin (50 μg/mL) and incubated overnight in a shaker at 150 rpm at 37 °C. The resulting bacterial culture was then centrifuged at 6000 ×*g* for 10 min. The supernatant was removed, and pellets were washed with 10 mM $MgCl_2$. Afterwards, the bacteria were centrifuged again and the pellets were resuspended in 10 mM $MgCl_2$. The concentration was adjusted with 10 mM $MgCl_2$ to either 1×10^7 colony forming units (CFU)/mL for dialysis membrane bag inoculation, or 5×10^7 CFU/mL for lettuce leaf inoculation.

3.3. Bacterial enumeration

To quantify the bacterial number in lettuce leaf samples and dialysis membrane system originated samples, different procedures were employed. For lettuce leaf samples, four 5 mm-diameter leaf discs were sampled and homogenized using motor handpiece in 200 μL of sterile 10 mM $MgCl_2$, and then the leaf slurry was replenished to 1 mL. For dialysis membrane system originated samples, bacterial suspensions were pipetted straight for the next step. Subsequently, two technical replicates of 20 μL either homogenates or bacterial suspensions were transferred into 96-well plates, serially diluted, and plated on XLD agar plates with rifampicin for bacterial numeration.

3.4. Plant system

Six-week old lettuce leaves were infiltrated entirely with a suspension of *S.* Typhimurium 14028s (5×10^7 CFU/mL) (Fig. 2A). When sampling, at least two entire leaves from one plant should be pooled as one biological replicate. Three independent plants were prepared for output sampling ("output plants") and the same number of plants were prepared for bacterial enumeration of the input plants ("input plants"). After the leaf surfaces had recovered from their water-stained appearance (about 3 h post inoculation), *Salmonella* from the "input plants" were numerated, while the inoculated "output plants" were kept for 7 days in the greenhouse. On the 7th day post inoculation (dpi), *Salmonella* from "output plants" were numerated prior to harvesting. Thereafter, leaf discs were cut from twice-folded lettuce leaves with 3 mm-diameter punchers (Fig. 2B) and the remaining leaf parts were also briefly chopped. At least 500 leaf discs and irregularly shaped pieces were collected in a 20 mL syringe with 6 mL of 10 mM $MgCl_2$ (Fig. 2C). The capped syringes were fixed to a 1 L beaker filled with 500 mL H_2O and the samples were sonicated for 20 min at 80 kHz in order to free bacteria from the leaves (Fig. 2D). To eliminate the majority of the plant cells, the leaf slurry was filtered through filter circles with pore sizes of 11 μm twice and 7 μm successively (Fig. 2E), and subsequently through 5 μm syringe filters (Fig. 2F). *Salmonella* in the filtrate was numerated before the population enrichment. The filtrate was then transferred into dialysis membrane bags immersed in 30 mL of LB medium plus 50 μg/mL rifampicin and incubated at 37 °C for 3 h at 60 rpm to enrich the bacterial population (Fig. 2G). The bacteria were numerated, and the

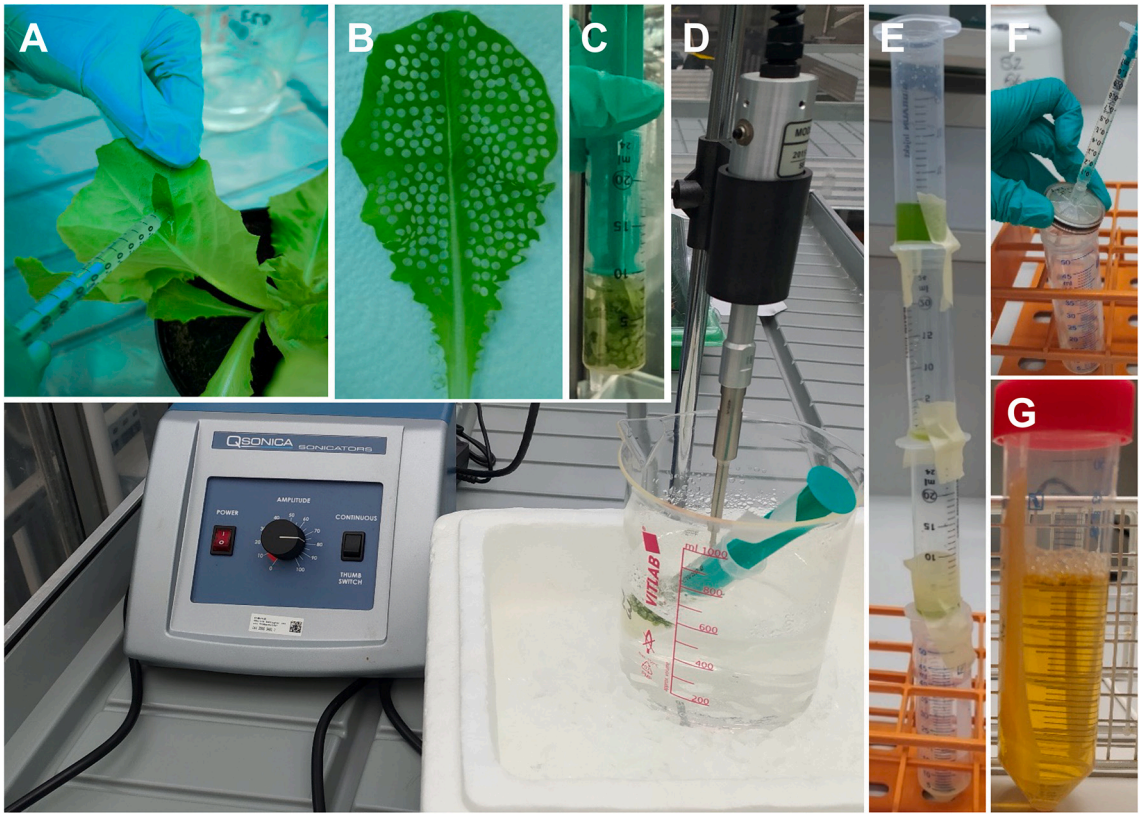

Fig. 2. Lettuce sample preparation.
A. Fully stretched lettuce leaves were infiltrated with bacterial suspension using a 1 mL syringe. The figure shows the difference in leaf appearance between infiltrated and non-infiltrated parts. B. The infiltrated leaves were cut into leaf discs using a 3 mm Biopsy puncher. C. Leaf discs and any leftovers were transferred into a 20 mL syringe and immersed in 6 mL of 10 mM $MgCl_2$ solution. The syringe opening was tightly capped with a matching lid that had been needle removed and adhesive sealed in advance. D. The loaded syringe was immersed in water and fixed with tape. The syringe system was sonicated at 80 kHz for 20 min to fully dissociate the bacteria from lettuce tissues. E. The slurry was filtered hierarchically using twice 11 μm and 7 μm pore size filter circles. F. The filtrate was continuously filtrated through a 5 μm syringe filter to remove the remaining plant debris mixed with bacteria. G. The final filtrate was transferred into dialysis membrane bags immersed in LB medium and incubated for 3 h to enrich the bacterial population.

remaining cells were pelleted (17,000 ×*g*, 5 min) in a 1.5 mL centrifuge tube and stored at −80 °C until DNA extraction.

3.5. Dialysis membrane system

In 50 mL centrifuge tubes, 30 mL of pre-prepared TM, LM, or DS soil suspension was added. At least three independent biological replicates were prepared for each treatment. To keep the dialysis membrane tubes moist and to remove any remaining storage solution, half of a sterile WECK jar was filled with sterile distilled water (dH_2O). Scissors and tweezers were flame-disinfected for cutting and holding dialysis membrane tubes. The dialysis membrane tubes were cut into 7.5 cm-long segments and were immersed in prepared sterile H_2O. The water in the jar was refreshed once more for a better scour. Tweezers were used to hold the dialysis membrane tube, which was then sealed on one side using heat from a hair straightener. Thereafter, the bag was filled with 2.5 mL bacterial suspension and the other side was sealed. The bags were submerged in the appropriate medium in 50 mL centrifuge tubes, which were then kept in an incubator at 28 °C for 24 h at 60 rpm. After incubation, the number of *Salmonella* incubated in TM and LM was evaluated as described in Bacterial enumeration. For sample collection, 1 mL of the remaining bacteria was pelleted (17,000 ×*g*, 5 min) in a 1.5 mL centrifuge tube and stored at −80 °C until DNA extraction. The bags incubated in DS soil suspension were washed briefly with sterile water to remove soil from the bag surface before being transferred into LB medium containing 50 μg/mL rifampicin. The samples were incubated for another 3 h at 37 °C to enrich the bacterial population. Then, the bacterial number was determined and the samples were harvested as described above.

4. Results

4.1. Enough bacteria can be yielded from two lettuce leaves after infiltration with a 5×10^7 CFU/mL solution

In order to determine the concentration for lettuce inoculation, corresponding harvests were tested 0 (3 h) and 7 days after infiltration with *S.* Typhimurium 14028s solution at different concentrations (1×10^7, 5×10^7, 1×10^8, and 5×10^8 CFU/mL). The highest recovery was obtained when leaves were infiltrated with the solution containing 5×10^7 CFU/mL. An increase in the inoculum concentration did not result in higher bacterial recovery at 7 dpi (Fig. 3A). In consequence, the inoculum concentration was set to 5×10^7 CFU/mL.

To track the persistence of *Salmonella* infiltrated into lettuce leaves, leaf discs were sampled and *Salmonella* was numerated on 0 dpi and 7 dpi as a quality control compared to previous results to exclude unexpected influences. *S.* Typhimurium 14028s cell counts decreased in our assays (Fig. 3B), consistent with the findings shown in Fig. 3A. To verify whether a bottleneck effect occurred during sampling owing to insufficient material amount, CFU counts were determined. The enumeration revealed that after filtration the recovered cell number met the mutant number criterion in the library (estimated to 1.3×10^6, unpublished data) (Fig. 3C). Nonetheless, since we expected a loss of material in the following steps, a non-selective incubation in LB was carried out in order to enrich the population. Usually, four to eight generations were used in other studies without causing a detectable bias (Price et al., 2018a). We performed a three-hour culture at 37 °C that corresponds to four to five generation cycles. About one $\log_{10}$ more *Salmonella* cells were recovered after the amplification in LB (Fig. 3C).

4.2. Enough bacteria can be yielded from dialysis membrane system

Since working directly with plant tissues is not always practicable, the Tn-Seq library could be recovered from an *in vitro* system using a mimicking medium. Here, we used *S.* Typhimurium 14028s as a model to test the proposed system. Prior to the sampling, the growth of *S.*

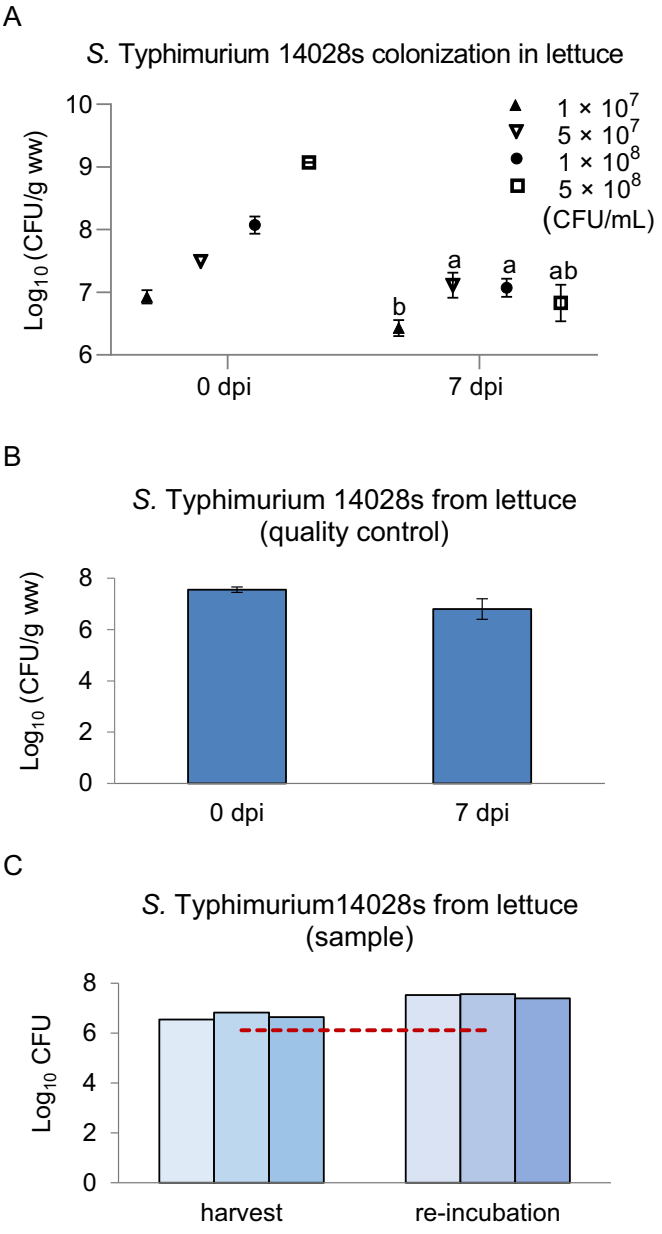

Fig. 3. Bacterial numbers in samples from the plant system.
A. Recovered bacterial numbers from input and output samples when inoculated with *S.* Typhimurium 14028s at different densities: 1×10^7, 5×10^7, 1×10^8, 5×10^8 CFU/mL. One-way ANOVA with Tukey HSD test was used. Significant differences ($p < 0.05$) are indicated by different letters above the bars. B. Bacterial numbers in plant system on 0 dpi and 7 dpi tested as quality control to exclude unexpected affects by environmental or management factors. C. Bacterial numbers in samples harvested from lettuce leaves on 7 dpi or after a second round of incubation in LB medium. The red dotted line represents the mutant number in presumptive *S.* Typhimurium 14028s Tn-Seq library (unpublished data). Biological replicates are represented by varying degrees of color, $n = 3$. ww: wet weight; dpi: days post inoculation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Typhimurium 14028s in dialysis membrane bags exposed to TM, LM, and DS soil suspension was assessed, since a sufficient bacterial growth is needed to detect the difference between input and output samples (Fig. 4A). Sigmoidal growth curves were observed when *Salmonella* was incubated into both TM and LM with an initial input of 10^7 CFU/mL. Already at 22 h post inoculation (hpi), bacteria entered the stationary

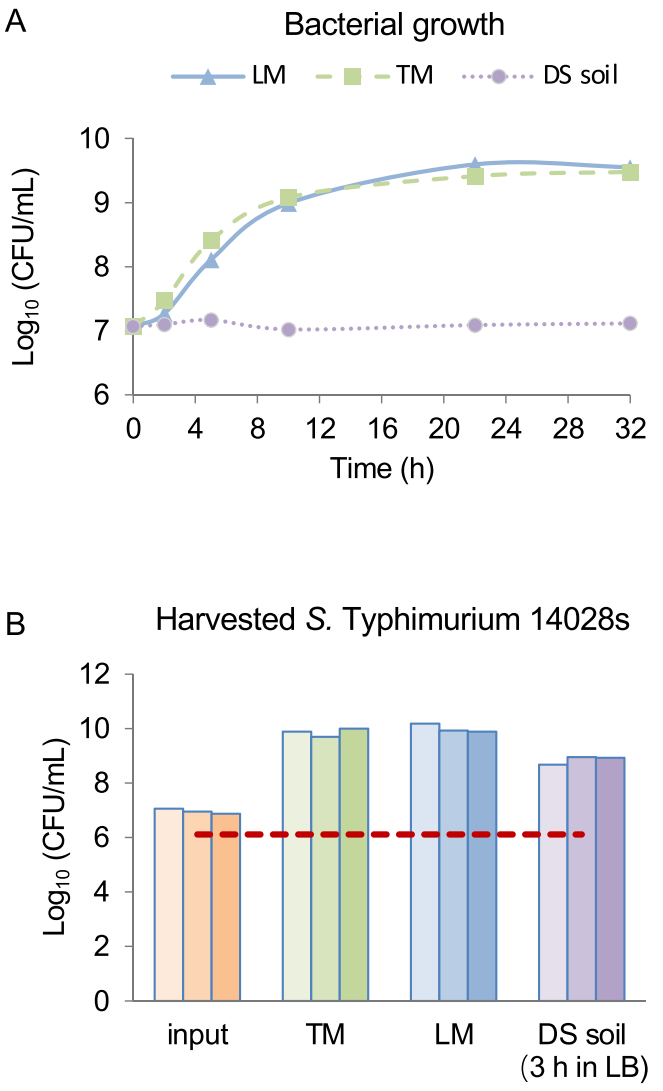

Fig. 4. Growth and bacterial numbers in samples from the dialysis membrane system.
A. *S.* Typhimurium 14028s growth in TM, LM, and DS soil in the dialysis membrane system. B. Bacterial numbers of *S.* Typhimurium 14028s input and output samples in the dialysis membrane system prior and after exposure to TM, LM, or DS soil (another 3 h in LB). The red dotted line represents the presumptive mutant number in *S.* Typhimurium 14028s Tn-Seq library (unpublished data). Biological replicates are represented by varying shades of color, $n = 3$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

phase with a concentration of $10^{9.5}$ CFU/mL. This phase lasted until at least 32 hpi. Such growth pattern suggests eight to nine proliferation cycles (Fig. 4B), and this cycle number is commonly used to distinguish possible changes in mutant proportions (van Opijnen et al., 2009; Price et al., 2016; Price et al., 2018a; Helmann et al., 2019). Situation in the DS soil-based suspension was different. No *S.* Typhimurium 14028s growth was observed, however, the number of bacteria did not decline thus reflecting a persistence of *S.* Typhimurium in DS soil suspension. Hence, we re-incubated bacteria harvested from DS soil suspension into LB medium. The additional duplication rounds allowed us to increase the total number of bacteria. After 3 h of incubation in LB, the bacterial population reached $10^{8.5}$ CFU/mL (Fig. 4B), representing five times of reproductions, if compared to the input, and a sufficient number of bacteria for DNA extraction.

5. Discussion

Due to the widespread use of Tn-Seq, understanding the function of previously uncharacterized genes or identifying conditionally essential genes has made great progress in recent years (Cain et al., 2020; Fabian et al., 2020; Liu et al., 2021). Since, agricultural environments and produces serve as a reservoir for *Salmonella* (Schierstaedt et al., 2019), it is necessary to comprehend how *Salmonella* behaves in such environments. This can be achieved by using an efficient and comprehensive method, such as Tn-Seq. Nonetheless, sampling *Salmonella* Tn-Seq libraries from *in planta* systems is challenging. The main issues are the lack of unified systems in plants, the vast amount of plant material required, as well as the low rate of bacterial reproduction observed in such conditions. Although several strategies to recover and concentrate microbes from complex matrices were already reviewed (Stevens and Jaykus, 2004; Wu, 2008), they are not ideal for Tn-Seq samples. Many studies focus rather on presence or absence, than a specific number of cells, which is necessary in Tn-Seq experiments.

In this study, we modified Helmann's laboratory approach to recover *Pseudomonas* RB-TnSeq libraries from leaves of 100 pot-grown bean plants (Helmann et al., 2019). This method was established for plant pathogens and its epiphytic application was adapted here to recover human pathogen from plant leaves. Only two lettuce leaves were needed to recover *Salmonella* with a cell number covering the density of a presumptive Tn-Seq library and to obtain the amount of DNA required for subsequent sequencing. Infiltration rather than surface dipping or spray inoculation contributed to a higher bacterial recovery (Zarkani et al., 2019). Because of the human pathogenic nature of *Salmonella*, we paid much attention to avoid any contaminations. In order to minimalize the risk of splashing bacterial solution on the working space, syringe infiltration was executed instead of vacuum inoculation. Punchers rather than blenders were used to carefully fragged leaves to avoid contaminating samples with too many tiny leaf pieces and blocking the filters in the following steps. Sonication in a water bath in the proposed conditions did not kill bacteria (Supplementary Fig. S1) and had a high efficacy in releasing them from lettuce leaves. In comparison to the 5 L to 10 L leaf slurry recovered from 10^8 CFU/g colonized leaves in Helmann et al. (2019), the presented here 6 mL system from $10^{6.8}$ CFU/g colonized leaves (Fig. 3B) made the filtration in syringes easier, faster, and more efficient. The use of syringe filters eliminated the problem of plant material passing through the gaps between filter circles and syringe inner walls. Because the amount of *Salmonella* decreased in lettuce leaves during the seven-day experimental period, a 3 h incubation in LB broth with antibiotics was executed to enlarge the difference between lethal, sublethal, and non-lethal mutants (Ray, 1979) and to recover enough DNA for library sequencing. The re-incubation in dialysis membrane bags shortened the time from overnight, which had been used in most previous experiments, to 3 h, saving even more time. The small volume maintained by the bags concentrated the population, making it easier for pelleting. Our lettuce leaf protocol can be easily altered and adjusted to other plant leaves or experimental settings.

When screening a Tn-Seq library, it is always preferable to operate in a system that is as close to the true environment, encountered by bacteria, as possible, as we did with lettuce leaves. However, this is not always feasible, for example, in other agricultural environments prone to severe microbiome contamination, such as water, roots or plant rhizosphere. One appropriate solution for Tn-Seq screening in such situations is to conduct the experiments in sterile conditions. *Pseudomonas* spp., *Azoarcus olearіusand*, *Herbaspirillum seropedicae*, and *Rhizobium leguminosarum* TIS libraries, for example, were successfully applied to roots or rhizosphere of Arabidopsis and corn grown in either sterile media or sterile clay (Cole et al., 2017; Liu et al., 2018; Sivakumar et al., 2019; do Amaral et al., 2020; Wheatley et al., 2020). Libraries of *Salmonella enterica, Streptococcus pyogenes*, and *Caulobacter crescentus* were incubated in filtered wastewater or lake water to identify conditionally essential genes (Kingsley et al., 2018; Hentchel et al., 2019). Sometimes,

key ingredients are isolated sterilely and then introduced to *in vitro* experiments to imitate certain circumstances. For example, ox bile was added to mimic the conditions in ox gall bladders and exudates from *Arabidopsis thaliana* roots were collected to mimic the rhizosphere environments (Langridge et al., 2009; Getzen, 2019). However, sterilization or pure substances may occasionally eliminate some relevant biological factors, for example, water or rhizosphere microbiome. This may result in the change or annihilation of the dynamic balance between the tested bacteria and natural microbiome.

Inspired by the use of dialysis membrane in medical research, we used a dialysis membrane system to maintain the purity of the *Salmonella* samples, avoiding the complicated steps of analyzing or extracting particular environmental ingredients, while keeping the surroundings as close as possible to the real environment, including the microbiome/metabolome. In our research, for example, soil particles were suspended in a soil slurry using $MgCl_2$, preserving the dynamic strategies between soil microbiome and *Salmonella*. We anticipate a better emulation of the dialysis membrane system for genes expressed without the requirement of surface contact, for example, nutrient and energy utilization. Genes involved in chemotaxis, biofilm formation, adhesion, surface recognition, and infection might be under-represented in this setup. However, *Salmonella* genes and mechanisms relevant for attachment have been extensively studied and reviewed in past decades (Baker et al., 2006; Valdez et al., 2009; Wagner and Hensel, 2011; Steenackers et al., 2012; Peng, 2016). In conclusion, for difficult-to-perform or time-consuming *in vivo* experiments, the dialysis membrane provides an alternative. Furthermore, it can be expanded to collect samples for transcriptomic (Jechalke et al., 2019; Zarkani et al., 2019; Schierstaedt et al., 2020) and metabolomics analysis or even other investigation methods, helping to study bacterial behavior at various levels.

Understanding the genes involved in *Salmonella* persistence in various agricultural environments will provide further insight into the dynamic nature of interactions between the pathogen and atypical hosts or ecological niches. By combining the plant system and dialysis membrane system, we propose here a practical methodology for harvesting *Salmonella* Tn-Seq library samples from both *in planta* and *in vitro* environments. The methods have been applied in *S.* Typhimurium 14028s Tn-Seq library inoculated to lettuce leaf, DS soil, TM, and LM. By analyzing the sequencing data, we obtained (6.8–13.3) $\times 10^6$ reads from each biological replicate, of which >74% were aligned to the *S.* Typhimurium 14028s genome (Supplementary Table S1). This sequencing depth and alignment is identical to published data of (0.5–23) $\times 10^6$ reads and 47% to 89% alignment using Tn-Seq *in vitro* or *in vivo* (van Opijnen et al., 2009; Khatiwara et al., 2012; Fu et al., 2013; Mandal and Kwon, 2017; Duong et al., 2018; Canals et al., 2019). In addition, in our results obtained from *Salmonella* recovered from lettuce leaves and LM, 4/5 of the identified essential genes are overlapping (unpublished data). All those results indicated the reliability of the methods to recover *Salmonella* for Tn-Seq. We assume that the results may represent better the molecular *Salmonella*-plant interactions when the samples are collected *in situ*. The leaf system can be also extended to sample *Salmonella,* and other human pathogens, from other raw eaten leafy green vegetables, addressing different issues, from low recovery rate to host contamination. Details of the protocol can be adjusted rather easily on this small scale, if other settings should be used. The dialysis membrane system may provide also insights on the chemical and metabolite level since bacteria and host or the environmental matrix is separated. This system provides therefore an alternative *in vitro* method for rather challenging *in vivo* Tn-Seq experiments.

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Author contributions

M.H., J.S., and A.S. conceptualized this study. J.T. and I.VP. provided information and resources on the *S.* Typhimurium 14028s Tn-Seq library. M.H. conducted the investigations. J.S. developed the dialysis membrane system in agricultural environments and M.H. improved it. M.H. developed the plant system and Y.D. assisted on the method exploration. M.H. analyzed and visualized the data. M.H. wrote the original draft of the manuscript and J.S., Y.D., J.T., I.VP., and A.S. contributed to the manuscript revision.

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Data availability

All data are included in the manuscript.

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