Curriculum

Laboratory Activity to Teach about the Proliferation of *Salmonella* in Vegetables [†]

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We designed a three-week laboratory experience that can complement any microbiology teaching laboratory to expand students' knowledge of the ecology of human enteric pathogens outside of their animal hosts. Through their participation in this laboratory activity, students learned that vegetative and reproductive plant parts could be a natural habitat for enteric bacteria such as non-typhoidal strains of *Salmonella enterica*. This field was recently brought to the forefront of the scientific community and public interest by outbreaks of human illness linked to the consumption of fresh fruits and vegetables. Students were encouraged to develop their own testable hypotheses to compare proliferation of *Salmonella enterica* sv Typhimurium LT2 in different vegetables: cherry and regular-size tomatoes, onions, lettuce, and yellow and red bell peppers (*Escherichia coli* can be substituted for BSL1 laboratories). Upon completion of the laboratory experience, students were able to: 1) Develop testable hypotheses addressing the ability of a human pathogen, *Salmonella enterica*, to colonize and proliferate in vegetables; 2) Determine that different vegetables support the growth of *Salmonella* to different extents; 3) Conduct statistical analysis and identify any significant differences. The teaching-learning process was assessed with a pre-/posttest, with an average increase in content understanding from ~15% to 85%. We also measured students' proficiency while conducting specific technical tasks, revealing no major difficulties while conducting the experiments. Students indicated satisfaction with the organization and content of the practices. All of the students (100%) agreed that the exercises improved their knowledge of this subject.

INTRODUCTION

Even though *Salmonella* has long been considered a zoonotic pathogen (i.e., communicable from animals to humans), it is clear that human salmonellosis is also likely to result from the consumption of plant-based (mostly raw) foods. Still, despite recurring outbreaks of produce-linked gastroenteritis, relatively little is known about the ecology of *Salmonella* and enterovirulent *E. coli* and their persistence on plants.

It is clear that these human pathogens can contaminate fruits, vegetables, nuts, and sprouts, pre- and/or post-harvest (1–3, 6, 7). At least in part, the lack of food-safety solutions to this problem is due to the limited understanding of the mechanisms of interaction between enteric bacterial strains and plants. *Salmonella* reaches high cell numbers inside fruits and vegetables, and its proliferation depends on the kind of plant and the genotype of a particular species (8, 9). Molecular mechanisms behind these differences are not yet fully understood; nevertheless, addressing various mechanisms of interaction between enterics and plants naturally lends itself as a potential exercise for an undergraduate microbiology lab. For example, plant genotypes can differ in recognition of the Microbe-Associated Molecular Patterns (MAMP) (such as flagella) (13), and differences in production of small molecule signals by bacteria and their recognition by plants may exist. Plant secondary metabolites and fruit maturity can also contribute to *Salmonella* proliferation in vegetables (10). These differences in the interactions of plant genotypes with *Salmonella* naturally open up the opportunity to broaden students' understanding of the ecology of enteric bacteria.

To that end, we developed this laboratory experience, which combines elements of expository and open-ended enquiry-driven activities. Furthermore, this experience showed students that *Salmonella* can infect different foods, including vegetables, in addition to meat and eggs. This three-week laboratory experience has been developed for

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first-year microbiology students to test how a variety of plants differ in supporting growth of *Salmonella*. The ecological consequences and food-safety implications of this *Salmonella*-plant interaction laboratory experience were also discussed with the students.

Intended audience

This activity was designed for undergraduate microbiology and biology majors during the first-year laboratory of the Microbiology section. However, it is also suitable for more advanced microbiology courses.

Prerequisite knowledge

The laboratory was carried out during the final part of the semester. Some of the required skills include: basic culturing and aseptic technique, utilization of pipettes, and dilution plating. To successfully complete the exercise, students need to understand basic microbiology concepts and the basics of plant-bacterial interactions. This laboratory could help reinforce lecture topics such as plant defenses, ecology and pathogenesis of *Salmonella* in the animal model, basic math and statistical concepts, such as the logarithmic function, making serial dilutions, and ANOVA and Tukey means separations. All these concepts were reinforced during the laboratory as part of the regular syllabus. General safety guidelines were regularly discussed in all microbiology labs in order to conduct the experiments.

Learning time

A three-week laboratory practice, consisting of two and a half hours on a weekly basis, is sufficient to effectively teach and demonstrate the concepts. As shown in Figure 1, during the first week, students are asked to work individually, and each person is given three fruits of two different vegetables (for example three cherry tomatoes and three large tomatoes, to obtain sufficient replicates for statistical analysis). Students are encouraged to prepare serial dilutions in phosphate-buffered saline (PBS) from an overnight culture of *S. enterica* sv Typhimurium LT2, and to inoculate a 10^{-5} dilution under the fruit's epidermis. The initial inoculum is also plated onto xylose lysine deoxycholate agar (XLD). During the following week, students harvest the vegetables. To do this, they record masses of fruits and then homogenize fruits in PBS using a stomacher. Students are asked to plate an aliquot on XLD. Finally, during the third week, students count the black colonies (*Salmonella*) on XLD plates. The instructor, in front of the class, performs the statistical analysis by collecting the data from the students. One Excel spreadsheet containing the students' results is used to infer ANOVA and Tukey means separations. At the beginning of each laboratory, the instructor reinforces the main concepts through a short discussion of the results and/or with the help of a slide presentation.

Learning objectives

Upon completion of this three-week laboratory, students will be able to:

1. Develop testable hypotheses addressing the ability of a human pathogen, *Salmonella enterica,* to colonize and proliferate in vegetables.
2. Determine that different vegetables support the growth of *Salmonella* to different extents.
3. Conduct statistical analysis and identify any significant differences.

PROCEDURE

Materials

To accomplish the three-week laboratory, each student requires the following materials (a complete and detailed list is available in Appendix 1):

- xylose lysine deoxycholate agar (XLD) Petri plates
- *Salmonella enterica* sv Typhimurium strain LT2
- Luria-Bertani broth
- the following vegetables: cherry tomatoes (small tomatoes), "beefsteak" tomatoes, yellow and red bell peppers, lettuce, onions
- gloves and other personal protective equipment
- stomacher bags
- 1-mL, 200-µL, 20-µL pipettes and sterile tips
- 1 beaker (200 mL)

The following equipment is also required: a safety biological hood (BSL level 2), a stomacher, and an incubator at 37°C.

Student instructions

After a quick introduction to the safety rules, students received a laboratory handout with a table to register their findings (please refer to Appendices 2 and 3 for a complete description). Students were asked to carefully read the protocol.

Students were asked to formulate their own hypothesis on why they expect that different vegetables support different populations of *Salmonella*. Examples of hypotheses were shared with the students on the second page of the student handout (Appendix 2).

Each student received two different kinds of vegetables, with three replicates of each (for example, three bell peppers and three tomatoes). Students were asked to carry out the serial dilutions and plate the initial inoculum obtained from the 10^{-5} dilution. Students were asked to work alone. Each student was responsible for the infection of the assigned vegetables (Fig. 2, panel A). The vegetables were incubated for one week at 22°C (room temperature) to simulate the shelf life during storage of contaminated
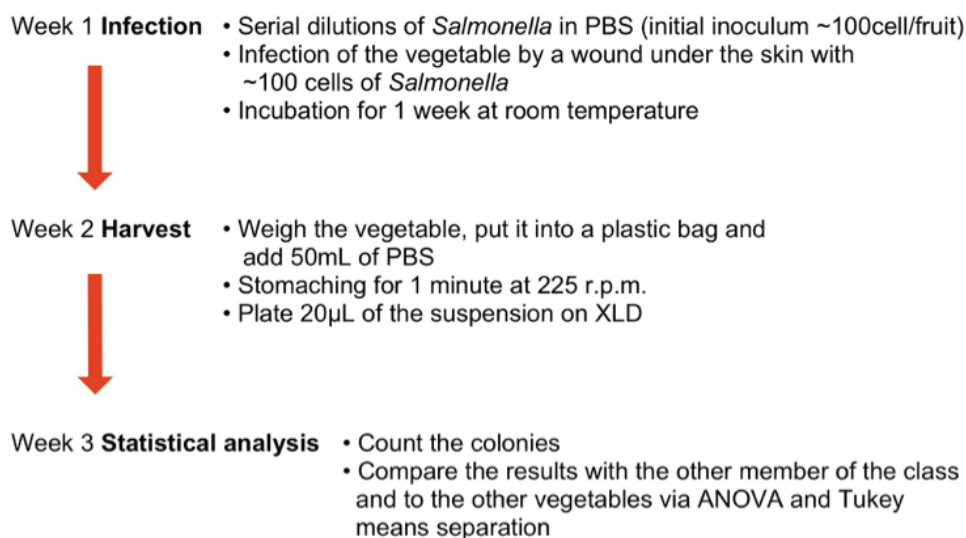
Workflow of the experiment

Week 1 **Infection**
- Serial dilutions of *Salmonella* in PBS (initial inoculum ~100cell/fruit)
- Infection of the vegetable by a wound under the skin with ~100 cells of *Salmonella*
- Incubation for 1 week at room temperature

Week 2 **Harvest**
- Weigh the vegetable, put it into a plastic bag and add 50mL of PBS
- Stomaching for 1 minute at 225 r.p.m.
- Plate 20μL of the suspension on XLD

Week 3 **Statistical analysis**
- Count the colonies
- Compare the results with the other member of the class and to the other vegetables via ANOVA and Tukey means separation

FIGURE 1. Example of workflow of the experiments conducted over the three-week period.

vegetables. Vegetables were kept in a plastic box container labeled with the biohazard sign.

During the second week, students were instructed how to sample the vegetables and use the stomacher. Once the concepts were understood, the students started the sampling process following the protocol provided to them. After stomaching, an aliquot of the smashed vegetable suspension was plated on XLD plates that were further incubated overnight at 37°C. In addition, students were asked to count the plate containing the initial inoculum from week 1.

During the third week, students were asked to count colonies on their plates (Fig. 2, panel B) and report the results on their cards (Appendix 3). At the end of the data collection, all cards were collected by the instructor and tabulated into an Excel sheet, and the data were projected onto a white board for a group analysis and discussion. The Excel file was opened with the software JMP (SAS) and the analysis was performed in front of the students. Finally, the Excel file was uploaded onto the online learning management system to be accessible to all the students. The instructor encouraged the students to replicate the analysis by using the Excel file online and by using the statistical software downloadable from the University's website. Students could also be asked to write a laboratory report about their findings.

Faculty instructions

This exercise required two hours of preparatory activities the day before the first week, and one hour for each subsequent week. The most time-consuming part was the preparation of the XLD plates. Details for media preparation, time requested, storage, and safety tips are described in Appendix 1.

During the activities, students proceeded at their own pace, with the instructor passing through the benches observing student progress, assuring adherence to safety procedures, and addressing questions and concerns. Approximately two hours were required to accomplish experiments in the first week, including infection and plating the initial inoculum. Approximately two hours were required to accomplish experiments in the second week. During the second week, students harvested vegetables, stomached them and plated a small aliquot on the XLD medium. Two different stomacher stations were set up to have students rotating in an organized manner, making it an easier process. During the third week, two hours were required to accomplish the experiment, collect the results, and carry out collective statistical analysis of the data. The third week mainly involved counting colonies, preparing the Excel sheet and conducting the statistical analysis (Fig. 2, panel C).

Learning objective 3 is the statistical analysis, which we do not discuss here in detail. The instructor is left to use any software or methodology that he/she expects to be more favorable. However, we would strongly recommend the instructor run both the ANOVA and Tukey separations to determine whether the results reveal any statistically significant differences in cell counts among the vegetables. In our experience, students could then appreciate that cherry tomatoes (small sizes) are less conductive to *Salmonella* proliferation than the larger-size tomato (Fig. 2C). Both yellow and red bell peppers were the most conducive, and onion exhibited significantly lower *Salmonella* proliferation. The reasons for these differences in proliferation may range from the presence of biocidal substances in onion, to a different plant defense response, to production of secondary metabolites, to availability of easily metabolizable nutrients.

To better focus the hypothesis and current knowledge, the references for three recent papers about the interaction of human pathogens and plants were provided at the end of the student handout (Appendix 2). For example, the paper by Marvasi and colleagues (11) stresses the role of ethylene and the contribution of this plant hormone to the changes in plant physiology that affect proliferation of *Salmonella*. In addition, the work by Hann and Micallef suggests that the immunity conferred by tomato genes could be responding to *Salmonella* outbreaks (5).

Finally, students could be asked to write a laboratory report about their findings. The laboratory report may be formative or summative, graded, and included in the students' final examination mark. The student handout (Appendix 2) can be included with the laboratory report. The report can include an Introduction, Materials and Methods, Results, and Discussion, in addition to a Bibliography section.

Suggestions for determining student learning

To assess student learning outcomes in this laboratory, we designed a pre- and post- content test that consisted of five questions in the following formats: multiple choice, true or false, and fill in the blanks (Fig. 3 and Appendix 4). We also designed a checklist to determine the students' proficiency while conducting different laboratory practices (Fig. 4). At the end of the laboratory experience, a rubric was provided to the students for the evaluation of the activities conducted (Appendix 4, Table 1). The use of a student handout is strongly encouraged (Appendix 2), especially if provided one week in advance, for students to familiarize themselves with the materials and the workflow of the laboratory. In addition, a laboratory report can be used as an assessment method to measure student learning.

Sample data

Figure 2 provides illustrations showing an example of the wound and an example of how *Salmonella* looks on XLD. A typical result, including the Tukey means separation for each vegetable, is shown in Figure 2C. In Appendix 3, we have included an example of a completed student's report card.

Safety issues

This laboratory activity should take place at the end of the semester (or year), in order for the students to have demonstrated competency working with BSL1 before working on this BSL2 activity. The laboratory procedures and/or practices outlined in the submission and in the laboratory must adhere to the ASM Guidelines for Biosafety in Teaching Laboratories (4). Students should wear standard laboratory protections (i.e., lab coat, closed-toed shoes, goggles, and gloves) at all times. For the manipulation of *Salmonella*, students should be requested to always work under a biosafety cabinet (BSL level 2), wearing goggle and
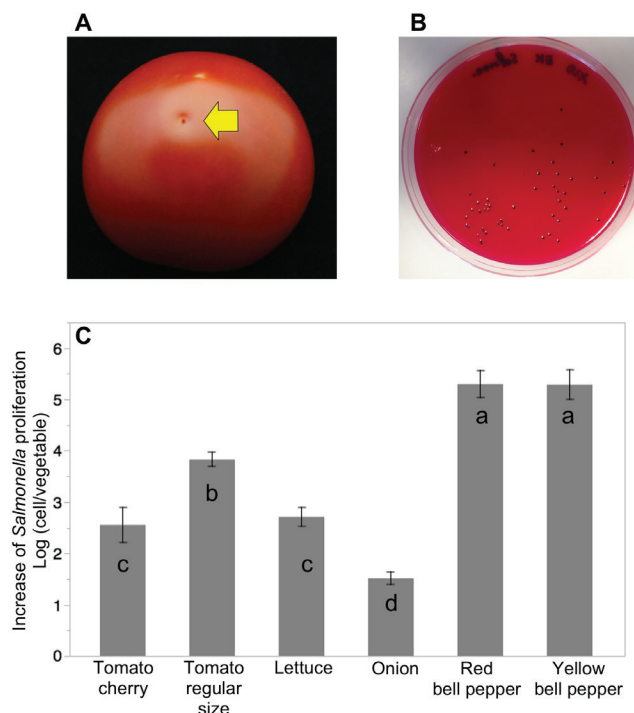
FIGURE 2. Main findings reported after the three-week laboratory experience. Panel A, the yellow arrow shows an example of wound where a 3-µL drop containing *Salmonella* was inoculated under the pericarp. Panel B, an example of *Salmonella* colonies on XLD. Panel C, final results. Different letters represent significantly different means. Error bars are the standard error. Cherry tomatoes (small sizes) supported *Salmonella* less than the larger-size tomatoes. Peppers are the most conducive, and onions are the vegetable with a significantly lower *Salmonella* proliferation. XLD = xylose lysine deoxycholate agar.

gloves. Biohazard transparent boxes containing the inoculated fruits should be stored during incubation and properly labeled with a "biohazard label." The stomacher should be placed in the biosafety cabinet and contained within a spill tray. The contaminated waste should be autoclaved and disposed of according to the institution's policy and regulations. The instructor or the technician must be in charge of autoclaving the biohazard waste.

In order to limit the biohazard potential, *Salmonella enterica* sv Typhimurium LT2 was used. *Salmonella* LT2 is an avirulent strain, due to the mutation of the gene *rpoS* (14). At the beginning and at the end of the experiments, students were instructed on disposal procedures of the biohazard material.

DISCUSSION

Field-testing and evidence of student learning

Data were collected from 10 undergraduates enrolled in the Module "Dissertation" (BIO3330) and 20
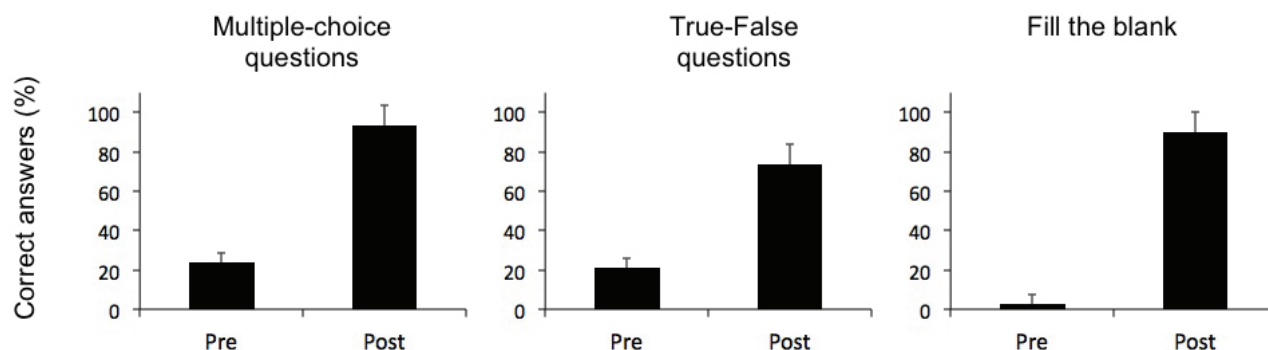
Pre - Post assesment quiz resuts

FIGURE 3. Percent correct answers in the pre- and posttests to assess student learning after the three-week laboratory experience. All the comparisons between the pre- and posttests are significantly different (Student's *t*-test, $p = 0.05$). Error bars represent the standard error. Please refer to Appendix 4 for the typology of questions.

undergraduates enrolled in "Environmental and Health Stressors – Microbiology section" (BIO1605) laboratories at the Natural Science Department of Middlesex University, London, UK. The microbiology laboratory sections were organized in these courses during the spring semester in 2015. To evaluate learning gains, students were given a pre-/posttest (Appendix 4). Results from the test showed a significant increase in student learning, with an average increase from 15.8% to 85.5% correct answers (Fig. 3). Specifically, on multiple-choice questions, the number of correct answers increased from 23.7% to 93.3%; the increase was also significant on True/False questions from 21.2% to 73.3%. A more open-ended "fill in the blank" test also demonstrated a significant increase, from 2.5% to 90.0% correct answers. All differences between the pre- and posttests were statistically significant (Student's *t*-test, $p < 0.0001$).

In addition, laboratory skills were evaluated during the laboratory exercises. For this evaluation, the instructor used a checklist of five practices (Fig. 4) to evaluate the students while they conducted their tasks. Prior to the laboratory, the instructor prepared one checklist per student and evaluated their performance based on a rubric (see below) while the students were working. The scale of the rubric used was: "very good" (5 points): the student was proficient at the task every time; "good" (3 points): the student was able to conduct the task most of the time (failing only on 1 or 2 occasions); "poor" (1 point): the student failed on more than three occasions (14). No significant differences among the averages were reported (significant probabilities at 0.05), revealing that no major difficulties were detected while conducting the experiments. However, minor difficulties were reported in pipetting properly (Fig. 4E). Students needed to be supervised closely by the instructor, as their pipetting skills were not sufficient to aliquot the exact volume.
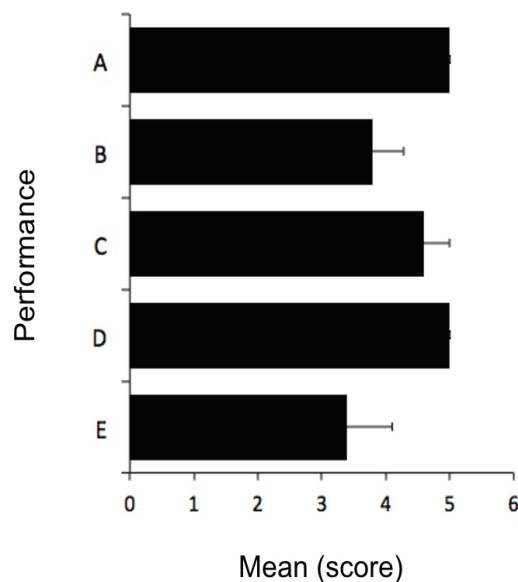
Performance indicators

FIGURE 4. Assessment for student performance. The scale ranges from very good (5), to good (3) to poor (1). Tasks assigned: A) The student is able to properly infect the fruit. B) The student can discriminate *Salmonella* colonies on XLD. C) The student can properly plate the XLD plate. D) The student can properly use the stomacher. E) The student is able to pipette properly. XLD = xylose lysine deoxycholate agar.

Students' evaluation of the laboratory activities. At the end of the three-week lesson, students were asked to evaluate the laboratory experience on a scale of one to five, where 1 indicated complete disagreement and 5 indicated complete agreement (Table 1). Surprisingly, 100% of the students completely agreed that the

TABLE 1.
Students' evaluation of the laboratory activities.

Question	Disagree		Neutral		Agree
	1	2	3	4	5*
1. I enjoyed the exercises	•	•	•	•	100%
2. The experiments and the materials were well explained	•	•	•	•	100%
3. The experiments and the materials were well organized	•	•	•	•	100%
4. The experiments were easy to perform	•	•	•	•	100%
5. The experiments were well-designed to improve my knowledge in proliferation of *Enterobacteriaceae* in plant	•	•	•	•	100%
6. I learned how to recognize *Salmonella* colonies on XLD	•	•	•	•	100%

*These percentages are averaged from the cards collected from 10 students.

exercises were well explained, organized, and easy to perform. Such a response is a reasonable indicator that this laboratory exercise could be implemented in any undergraduate microbiology course to effectively teach the role of vegetables in the proliferation of *Salmonella*.

Possible modification

The laboratory exercise can be easily modified for other settings. Many other vegetables and fruits can be tested and compared, including dry fruits and nuts. The laboratory exercise can also be performed in two weeks. In such case, a video visualizing the procedure carried out during week one will have to be shown. The students will then harvest the vegetables and plate the samples onto XLD in the first week. During the second week, students count colonies and conduct the statistical analysis.

To reduce the biohazard potential, *Salmonella* can be replaced with *E. coli* (BSL1). In this case colonies appear yellow on XLD.

SUPPLEMENTAL MATERIALS

Appendix 1: Materials for the instructor: Procedures for media preparation, inoculation of the strain, consumables, and equipment required
Appendix 2: Student laboratory handout and answer key
Appendix 3: Report card and example of results
Appendix 4: Pre- and post-activity assessment and answer key

ACKNOWLEDGMENTS

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