# Journal of Emerging Investigators

## Information Packet

Hello Students and Teachers,

We hope to provide you with some information to help you navigate the world of scientific publishing.

We have compiled a few items to assist you in the process of submitting a manuscript. First we have a manuscript help sheet and a sample manuscript as it would look before editors have prepared it for publication. The sample paper should be used as a rubric to setting up a manuscript, and should not be used for its scientific content. This should help you understand the format of a paper: abstract, introduction, results, discussion and methods/materials. You should also take note of things like paragraph breakups and the use of subheadings. Specific instructions for formatting a manuscript are on our website.

The next items attached include an overview of the scientific review process and the reviewers' comments of the sample paper. It is not the intention of the reviewers to nitpick grammar or appear antagonistic. Instead, the purpose of the reviews is to provide students with critical feedback that will help students better understand scientific concepts as well as the process of science writing. These reviews will provide you with an idea of the kind of things that reviewers will critique in a paper. While the majority of comments can easily be addressed in the text, there will be circumstances where additional experiments will be required. Once reviews are received, you, the author, are expected to address the comments made by the reviewers and revise the manuscript accordingly. Upon resubmission you should include a cover-letter addressing the changes you made or provide reasons as to why the suggested changes cannot or should not be made (it will then be at the discretion of the editor to accept the paper without the suggested changes).

We thank you for your interest in JEI, if you have any questions at all feel free to visit our website at **www.emerginginvestigators.org** or email us at **questions@emerginginvestigators.org.**

Sincerely,

The JEI Editorial Board

# JEI Manuscript Help Sheet

At JEI we are excited to give students an opportunity to be stimulated and engaged by basic science and allow students an opportunity to present their original work. This help sheet gives information on what is required of each manuscript submitted to JEI. More details can be found on our website.

We **STRONGLY** encourage hypothesis-driven research:

- The manuscript should be focused around a central question that can be tested.
- How do the experiments involved test/examine that central question?
- How do the results of the manuscript alter the original question?

**Manuscript Basics** - There are 7 components every manuscript must have:

1. Title page- This page includes the title of the manuscripts along with the author names listed in order of contribution with the teacher/mentor listed last. The authors' and teacher/mentor's schools should be listed.
2. Abstract – A summary of the entire manuscript highlighting the hypothesis, experiments performed and overall conclusions. (250 words Max)

3. Introduction – A summary of current field of research on the topic. The introduction should provide the context for the studies in the manuscript and cite primarily current scientific literature to support hypothesis (some online references are acceptable).

4. Results – A description of the results from the experiments in the study. A complete results section will reference all figures present in the manuscript. Interpretation and explanation of experimental results should be saved for the discussion not the results.

5. Discussion – Interpretation of all experiments and an explanation on how they agree with/disagree with the overall hypothesis. We encourage developing "a working model" that allows the data from the manuscript to be placed in the context of the overall hypothesis.

6. Methods – Description of techniques or protocols used to generate the results, with enough detail to allow replication of experiments by readers. Materials should also be described within the context of the techniques/protocol description.

7. References – All work that is cited throughout the manuscript (including the methods section) should be listed here in the order they are referenced in the paper. We require references to be in the MLA format.

**Scientific Integrity**

We at JEI take scientific integrity very seriously – This includes but is not limited to the following:

<u>Original research</u>

- The students must perform all the experiments described in the manuscript.
- The students must be the primary author, and therefore write the manuscript.
- The study should address novel experiments and ideas.

<u>Honest research</u>

- The data shown must be an honest reflection of actual results and not altered to fit a hypothesis.
- The experiments described must actually be performed and the real data shown in the manuscript.
- All other manuscripts, ideas, hypotheses that are not original but referenced must be cited.

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# Overview of the Scientific Review Process

**Authors Submit the Manuscript**

The teacher/senior author submits the completed manuscript on the JEI website.

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**Editor Receives the Manuscript**

A JEI editor verifies that the manuscript is in accordance with our submission guidelines and distributes it to three scientific reviewers (PhD candidates with expertise in the relevant field).

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**Reviewers Assess the Manuscript**

The JEI reviewers analyze the manuscript, including the interest of the scientific question, the quality of the scientific writing, the believability of the results, and the soundness of the conclusions.

↓

**Authors Receive Reviewer Comments and Manuscript Decision**

An editor will provide the authors with the reviewer comments, which consist of suggestions to improve the manuscript (such as a topic that should be included in the introduction, an alternative interpretation of a result, or an additional experiment that could provide insight into the scientific question). These comments are designed not only to improve the manuscript but also to help the students better understand both this project and the general process of scientific inquiry. The editor will also inform the authors if the manuscript has been accepted for publication, and if so, the changes that must be made before the manuscript can be published.

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**JEI Publishes the Manuscript**

Once the required revisions have been performed, the JEI copy-editors will prepare the manuscript for publication and the manuscript will be published on the JEI website.

# In Vitro Modeling of the Efficacy of Probiotic Inhibition of E. coli in the Intestinal Tract

**Peter Giunta**
**Eoin Moriarty**
**Lawrence Johnson**

**Abstract:**

Our project examined the efficacy of commercially available probiotics of inhibiting E. coli in the intestines. Probiotic supplements contain live microorganisms that are purported to maintain a balanced, healthy gut flora. We hypothesize that due to the hostile acidic environment found in the stomach, probiotic organisms Lactobacillus acidophilus and Lactobacillus casei do not survive the digestion process to colonize the intestinal tract. Without colonization, they cannot, therefore, confer the implied health benefits to the host. With a series of in vitro assays, we tested the effect of three commercially available probiotic treatments as possible direct inhibitors of E. coli (Escherichia coli K-12). In addition we directly applied L. acidophilus and L. casei to established lawns of E. coli.

No zones of inhibition were visible on any plates in either trial. After recording the data from trial one, each plate was re-inoculated according to protocols to ensure that incubation produced visible growth consistent with Lactobacillus acidophilus morphology. No zones of inhibition in E. coli were detected after demonstrable Lactobacillus acidophilus growth.

After repeated challenge and extended incubation it was shown that Lactobacillus acidophilus (in pill and capsule form) can survive stomach passage to form colonies in our in vitro system. However no direct inhibitory effect of the E. coli was evident. Based on our model, any probiotic effects do not appear to be a result of direct inhibition. Other proposed mechanisms of competitive exclusion or binding interference may be the source of probiotic effects. Examination of these mechanisms were beyond the scope of our experimental design.

**Introduction:**

The term Probiotic is used to describe live micro-organisms that can yield a health benefit on a host (1). The discovery that bacteria can actually be helpful to the immune system dates to the early 20th century. Elie Metchnikoff theorized about the health benefits of lactic acid bacteria. He believed that aging is caused by cumulative assaults of toxic bacteria that, over time, colonize in the digestive tract. He further theorized that these negative effects could be mitigated and perhaps regulated using lactic acid. He noted that Bulgarian peasants consumed fermented milk, and had consistently long and healthy lives (12). Based on this theory, he drank sour milk multiple times per day, hoping to prolong his life. These observations helped to prompt an extensive field of research in the effects of these bacteria that promote health-Probiotics.

When a child is born, it leaves the completely sterile environment that is the womb and enters a world teeming with bacteria. Upon exiting the womb an infant collect a myriad of bacteria that will grow on and within its body. Many of the strains of bacteria will remain with him for the duration of his life (5). These bacteria are extremely numerous, with a magnitude of 10 times that of the amount of cells in our body. There is an equally impressive level of diversity in these commensal bacteria; ranging from about 400 to 1000 different strains (5). As these commensal organisms become residents colonizing their preferred human micro niches, they can exclude or out compete pathogenic strains of bacteria. E. coli, a resident of the colon, synthesizes vitamin K, a key component in normal blood clotting. Life in a relatively germ free industrialized world has shielded humans from exposure to microorganisms during the time of immunity passed on by mother's milk. This lack of early immunological challenge lead to epidemics of polio and has further promoted the theory that allowing or even fostering the colonization of Probiotic bacteria is beneficial for a number of reasons (5).

Probiotics is a very loose term used to describe many different bacteria. Probiotics must be living organisms, and they must also be healthy to the person or host that receives the bacteria. The vast majority of reputed Probiotics come from the genera Bifidobacterium and Lactobacillus(13).

Within Bifidobacterium and Lactobacillus, there is much variability in the reported Probiotic effects. For example, Bifidobacterium Infantis 35624 has been scientifically studied to ease symptoms of IBS (Irritable-Bowel Syndrome), which is disease of the lower intestinal tract that can cause abdominal pain (4). However, a different strand of that same genus can have a completely different function. Bifidobacterium Lactis Bb-12 has been shown to help the immune system and promote digestive tranquility (4). A study was done in Israel among children ages 4-10 months in Childcare Centers over a

12 week period. The children were administered this strain of Probiotic, and they showed improvement in episodes of Diarrhea over that time(4). The same variation can be found in the Aspergillus, Lactobacillus, Saccharomyces, Enterococcus, and any other genera of Probiotics.

These Probiotics have been studied in numerous trials. Some studies have yielded very promising, though modest, results as far as maintaining a balance of commensal bacteria in our bodies (1). It is clear that the Probiotic effects of the studied bacteria are not as dramatic or emphatic as the negative effects of ingesting pathogenic strains of E. coli. Nevertheless, people want a way that they can include these Probiotics into their diets and obtain any health benefits from these bacteria (1). That is why many companies have released hundreds of food and pill products in a bewildering array, which contain Probiotics. When choosing a Probiotic product, it is often difficult to discern whether a product has been shown to work or is simply labeled Probiotic for monetary gain (13).

The purported effect of commercially available Probiotics is to reinstate healthy bacteria, and maintain a balance of healthy gut flora. We have some skepticism about the effectiveness of probiotics. In order to colonize the intestinal tract, a probiotic supplement would have to survive the stomach's acidic environment and remain viable as it passes into the intestines. We hypothesized that probiotics do not survive the digestion process and therefore cannot yield any benefits on the host. Through a series of in vitro assays, we tested the effect of three commercial probiotics as direct inhibitors of Escherichia coli in the intestines. In addition, we directly applied strains of Lactobacillus acidophilus and casei to bacterial overlays of E. coli.

**Results:**

Experimentation was carried out in two parts. Part one examines the probiotics' unhindered efficacy of E. coli inhibition. Part two examines the influence of digestion on E. coli inhibition. In part one, crude preparations of probiotic strains Lactobacillus Acidophilus and Lactobacillus casei were propagated using a Tomato-Juice Yeast-Extract Milk Medium for luxurious bacterial growth (It should be noted that the isolated strains were chosen due to their widespread commercial use. Additionally, we were able to match the selected strains with monoculture probiotic supplements used in testing). Each strain was then inoculated into 4 wells cut from bacterial overlays of the E. coli. A well was cut in the center of each plate to serve as the control (the control for part one was deionized water). This same procedure was duplicated for diluted preparations of Lactobacillus Acidophilus in pill and capsule form, and Lactobacillus casei in a yogurt preparation. Inoculated plates were incubated overnight and examined for inhibition zones.

In part two of the experiment commercial preparations were subjected to "stomach-acid" solution to simulate the digestion process to our most accurate degree. Preparations remained in the solution for 4 hours (approximately half the normal transit time in the human stomach) at $37^0$C and 360 RPMs (to simulate peristalsis). Following transit the digested preparations were neutralized and inoculated onto E. coli lawns (note that the control for these preparations was stomach acid rather than deionized water). Plates were then incubated and subsequently examined for zones of inhibition. The procedure was then repeated for a second trial, with minor modifications in the incubation period and isolated strain growth. Analysis of the inoculated quadrants showed visible Lactobacillus acidophilus growth consistent with Lactobacillus acidophilus morphology in both digested and crude preparations. This means that based on our experimental parameters, Lactobacillus acidophilus in pill and capsule preparations can survive the digestion process and colonize in the intestines. The efficiency of the pill and capsule could be attributed to the stearate coating that protects the colonies during passage. This is consistent with lack of colonies present in plates containing yogurt and strain preparations, which lack a protective covering. Despite the bacterial growth no zones of inhibition were present on any plates in either trial. The probable justification for this result is that isolated strains Lactobacillus acidophilus and Lactobacillus casei do not directly inhibit E. coli. However, direct inhibition is only one inhibitory property that probiotics are purported to exhibit. Our experimental design was only focused on the detection of direct

inhibition. Other possible causes of the inconclusiveness of the results are the E. coli strain used, or the agar used in the bacterial overlays of E. coli.

**Trial 1**

| | Diameter of Zone of Inhibition (mm) | | | | |
|---|---|---|---|---|---|
| **Inoculum** | Quadrant 1 | Quadrant 2 | Quadrant 3 | Quadrant 4 | Control (Distilled water) |
| **Lactobacillus Acidophilus (Pill)** | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm |
| **Lactobacillus Acidophilus (Capsule)** | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm |
| **Lactobacillus Casei (Yoghurt)** | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm |
| **Lactobacillus Acidophilus (Strain)** | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm |
| **Lactobacillus Casei (Strain)** | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm |

**Trial 1**

| | | Diameter of Zone of Inhibition (mm) | | | |
|---|---|---|---|---|---|
| **Inoculum** | Quadrant 1 | Quadrant 2 | Quadrant 3 | Quadrant 4 | Control (Stomach Acid) |
| **Lactobacillus Acidophilus (Pill)** | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm |
| **Lactobacillus Acidophilus (Capsule)** | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm |
| **Lactobacillus Casei (Yoghurt)** | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm |

**Trial 2**

| Inoculum | Diameter of Zone of Inhibition (mm) | | | | |
|---|---|---|---|---|---|
| | Quadrant 1 | Quadrant 2 | Quadrant 3 | Quadrant 4 | Control (Distilled water) |
| **Lactobacillus Acidophilus (Pill)** | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm |
| **Lactobacillus Acidophilus (Capsule)** | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm |
| **Lactobacillus Casei (Yoghurt)** | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm |
| **Lactobacillus Acidophilus (Strain)** | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm |
| **Lactobacillus Casei (Strain)** | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm |

**Trial 2**

| Inoculum | Diameter of Zone of Inhibition (mm) | | | | |
|---|---|---|---|---|---|
| | Quadrant 1 | Quadrant 2 | Quadrant 3 | Quadrant 4 | Control (Stomach Acid) |
| **Lactobacillus Acidophilus (Pill)** | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm |
| **Lactobacillus Acidophilus (Capsule)** | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm |
| **Lactobacillus Casei (Yoghurt)** | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm | 0.7 mm |

**Discussion:**

As stated, no zones of inhibition were present on any plates in either trial, despite bacterial growth from commercial preparations of Lactobacillus acidophilus. This development supports the notion that the examined strains are not direct inhibitors of E. coli. Direct inhibition represents only one possible inhibitory mechanism however, and it is the only mechanism that our parameters allowed us to measure. Probiotics are purported to inhibit pathogens in numerous ways, including direct inhibition (emitting an organic acid to eliminate pathogenic bacteria), nutrient competition, pH regulation (changes the pH of its environment to displace pathogens), and binding interference (binds to the intestinal villi to block pathogens). If the examined strains displace pathogens in one of these other ways, our experimental design would be insufficient. Furthermore, the examination of binding interference was particularly difficult due to our school's biosafety level. The E. coli strain used (Escherichia coli K-12) is a model of the pathogenic strain (Escherichia coli 0157:H7). Our strain was specifically chosen because it does not

bind to receptor sites in the intestines, making it safe for laboratory use. Without this property it is impossible to view binding interference as method of inhibition.

Another possible reason why there were no visible zones of inhibition may be due to the type of agar used in the experiment. Our assumption was that nutrient agar (being a general medium for growing bacteria) would grow the strains of bacteria we used. However, certain bacteria require more than the macronutrients (carbohydrates, lipids, etc.) provided by the nutrient agar. It is possible that the probiotics require micronutrients (vitamins, minerals, etc.) in order to grow. The tomato juice used in the Tomato Juice Yeast-Extract Milk Medium may provide these micronutrients that the nutrient agar lacks. Without these essential nutrients, it is possible that the probiotic strains were unable to compete with the E. coli for nutrients.

Our experimental data shows no sign of direct inhibition. No solid conclusions can be drawn from this result. Our parameters were limited to a specific aspect of probiotics' inhibitory properties. To gain a more comprehensive understanding of probiotics' role as commensal bacteria, we need to look at a other mechanisms of its purported effects. Future research could include gaining access to a pathogenic strain of E. coli and modeling binding interference on a host epithelium. Then we could consider our research to be more comprehensive as well as more conclusive.

**Materials:**

- 18 plastic Petri dishes
- Erlenmeyer flasks
- 1 bottle of dehydrated nutrient agar (5.0g pancreatic digest of gelatin, 3.0g beef extract, 15.0g agar, pH after autoclaving = 6.8±.2 at 25deg. C)
- 1 P.1000 micropipette, 1 P.200 micropipette
- Plastic pipettes
- 10 micro centrifuge tubes
- 1 hot water bath (Edvotek 1.8L, catalog no. 539)
- 1 incubator (Quincy lab model 20-140E)
- 1 bottle of pepsin (enzyme)
- 1 bottle of 1M HCL (Hydrochloric Acid)
- 1 gallon of low-fat skim milk
- 1 bottle of marmite yeast extract
- 2 cans of Nature Made canned whole tomatoes
- 2 gallons distilled water
- 1 triple beam balance (scale no. 1967)
- 1 bottle of solid KOH (Potassium Hydroxide)
- 2 50 mL beakers
- 1 pH meter (Vernier Labquest program)
- 1 pH electrode (Vernier pH sensor)
- 1 pH 7.0 buffer solution (Potassium phosphate monobasic sodium Hydroxide buffer 0.5 molar 5b107k-500)
- 3 rolls of pH paper (1 roll pHydrion 0-13pH, 1 roll pHydrion 0-4 pH, and 1 roll pHydrion 6-8 pH)
- 1 box of filter paper (Central Scientific Company, 9cm qualitative smooth filter paper, no. 13250, Cambridge Mass)
- 1 box of cheesecloth wipes (Fischer Scientific CAT NO. 06-665-29 100%reagent cotton)
- 3 magnetic stirrers (1 Corning PC-620D, 2 Corning PC-420D)
- 3 magnetic bars
- 4 autoclavable bottles (Pyrex)
- 2 Bio-Rad plastic inoculating loops
- 2 metal inoculating loops

- 2 mortar and pestles
- 1 100mL graduated cylinder
- 1 Bunsen burner
- 1 tube of Escherichia coli k-12 (ordered from Carolina Biological)
- 1 tube of Lactobacillus Acidophilus (ordered from Carolina Biological)
- 1 tube of Lactobacillus Casei (ordered from Carolina Biological)
- 1 bottle of Acidophilus capsules
- 1 bottle of Acidophilus Pills
- 1 package of Yakult brand yogurt (fortified with Lactobacillus Casei)

**Methods:**

Part 1: Making the crude preparations of our experiment (ideal conditions for probiotics to grow).

Making the bacterial lawn of E. coli-

3.75 grams of nutrient agar (CAT no. WLDFI-02 Dehydrated Nutrient Agar, 5.0g pancreatic digest of gelatin, 3.0g beef extract, 15.0g agar per liter) were diluted in 500mL distilled water for a .75% concentration. The solution was heated to dissolve the agar, and autoclaved at 121deg. C for 15 minutes. 1.5mL of the agar solution were placed in 8 micro centrifuge tubes using a micropipette (P.1000). The tubes of agar were then inoculated with .1mL of E. coli (using P200 micropipette). The inoculated medium was then suspended over a water bath (Edvotek, 1.8L, catalog no.539) at 45deg. C to equilibrate. Once equilibrated, the centrifuge tubes were capped and shaken, and poured over 8 plastic Petri dishes of pre-poured nutrient agar. The plates were shaken gently to spread the medium over its surface area, and incubated at 37deg. C for 24 hours. When removed the following day, the dished exhibited an overlay of E. coli bacteria for optimum visibility of zones of inhibition.

Synthesizing Tomato Juice Yeast-Extract-Milk Medium and propagating probiotic strain growth-

Tomato juice was filtered using an aspirator connected to an Erlenmeyer flask, and poured over a Cheesecloth wipe (Fischer Scientific Cheesecloth Wipes, CAT NO. 06-665-29, 100%pure reagent cotton). The filtered tomato juice was refrigerated and stored for later use.

One Normal KOH (potassium hydroxide) was prepared by measuring 14.024g of solid KOH and diluted in 250mL of de-ionized water. This KOH solution was used in a titration to bring the tomato juice to a neutral pH (so the milk in the medium does not curdle). After calibrating the pH electrode (Vernier pH sensor), the Tomato juice (pH approximately 4.5) was placed on a magnetic stirrer (Corning PC-620D), and stirred at 1150rpm with a magnetic bar. The 1 normal KOH solution was placed into the 500mL beaker of tomato juice using a plastic pipette, checking every .5 increase in pH with pH paper (pHydrion 0-13pH). Once neutralized to pH 7.0, the tomato juice could be mixed with the rest of the ingredients required for our probiotic culture medium. Quantities used in the medium were scaled down to .176 of the recommended amounts (the tomato juice being the limiting reagent). 19.245g of low-fat skim milk was weighed using a balance (scale no. 1967). .88g of yeast extract was measured using the standard "weigh-out" technique. The 158.4mL of distilled water was measured using a 100mL volumetric. Once the ingredients were measured, they were transferred into an autoclavable Pyrex bottle, and shaken vigorously until homogeneous. Contents were autoclaved at 113deg. C for 20 minutes.

After cooling the bottle to room temperature, the medium was poured into 2 autoclavable bottles (rinsed with bleach solution). The bottles were then inoculated with our strains of Lactobacillus Acidophilus and Casei (source: Carolina Biological) using Bio-Rad plastic inoculating loops. Each bottle was labeled with its respective strain, and incubated at 37deg. C for 24 hours. When removed, the bottles of culture medium displayed cloudiness that indicated bacterial growth.

Inoculating the plates-

1 pill and 1 capsule of Acidophilus were crushed with a mortar and pestle. The crushed pills and capsules were diluted in distilled water (the capsule was not soluble in water). A plastic pipette was then used to pipette 1 drop of acidophilus pill solution onto four wells in the bacterial lawn of E. coli. The wells were cut using a flamed inoculating loop. A fifth well was cut in the center of the Petri dish and distilled water

was placed onto it. The distilled water acted as a control for each Petri dish. This method was repeated for the acidophilus capsule, Casei yogurt, acidophilus strain and casei strain. Each plate was labeled with their inoculum and control. The plates were then left out of the incubator for approximately 4 hours to react with the agar and were then placed into the incubator at 37deg. C for 24 hours. The next day the plates were examined for zones of inhibition.

Part 2: Making a more accurate representation of the conditions for bacterial growth in the intestinal tract

Making the model of the stomach-

100mL of 1 molar Hydrochloric Acid was added to a 1000mL Erlenmeyer flask using a graduated cylinder. 900mL of distilled water was added to the Erlenmeyer flask using another graduated cylinder. Then 0.5 g of Pepsin was measured using a triple beam balance and placed into the Erlenmeyer flask. The solution was mixed gently to ensure homogeneity.

Passage through the stomach-

Three magnetic stirrers (1 PC-620 D and 2 PC 420-D) were set up in order to simulate peristalsis in the stomach. Three Erlenmeyer flasks were filled with 125mL of the stomach acid solution and a magnetic bar was placed in each flask. Dropped into each flask was one serving of each of our forms of probiotics (excluding the strains). The temperatures of the stirrers were also set to 37deg. C to accurately simulate the heat of a human stomach. The flasks were left on the stirrers for a transit time of 2.5 hours (normal transit time for a human stomach is 4 hours). After the digestion process was complete, each of the model stomachs were neutralized to pH 7.0 (stomach acid is neutralized before entering the intestine) using 1N KOH. Once neutralized, plates of E. coli were inoculated using the method of inoculation shown in part 1. The plates were then given time to react with the agar and placed in the incubator at 37deg. C for 24 hours. The following day plates were examined for zones of inhibition. After examination, plates from this trial were re-inoculated with each inoculum to maximize the possibility of bacterial growth. The plates were then re-incubated at 37deg. C for 24 hours.

The above process was repeated for a second trial, with slight modifications. For the second trial, the E. coli was inoculated after just 6 hours of incubation, so the probiotics could inhibit growth while the E. coli was in its log-phase. Also, when inoculating the E. coli with our crude preparations, we used the strains directly from the test tubes of probiotics purchased from Carolina Biological. This was to ensure that the plates were inoculated with live bacteria.

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**Figure Legends:**

Figure 1. (A) Crude preparations of Lactobacillus acidophilus (in diluted pill and capsule form, as well as strain form) and Lactobacillus casei (in yogurt and strain form) were applied directly to bacterial overlays of E. coli and incubated for 24 hours. Plates were then examined for zones of inhibition. The control for this part of the experiment was distilled water. Note that the .7mm reading on the chart indicates the width of the well cut to inoculate the preparations and does not indicate inhibitory effects. Also note that each plate was re-inoculated and re-incubated after examination in trial one. (B) Digested preparations of Lactobacillus acidophilus (in pill and capsule form) and Lactobacillus casei (in yogurt form) were applied directly to bacterial overlays of E. coli and incubated for 24 hours. Plates were then examined for zones of inhibition. The control for part two was stomach acid. Note that the isolated strains were not subjected to stomach acid treatment, due to the impracticality of ingesting an isolated strain of probiotic. Note that the .7mm reading on the chart indicates the width of the well cut to inoculate the preparations and does not indicate inhibitory effects. Also note that each plate was re-inoculated and re-incubated after examination in trial one.

Figure 2. (A) Crude preparations of Lactobacillus acidophilus (in diluted pill and capsule form, as well as strain form) and Lactobacillus casei (in yogurt and strain form) were applied directly to bacterial overlays of E. coli and incubated for 6 hours. Plates were then examined for zones of inhibition. The control for this part of the experiment was distilled water. Note that the .7mm reading on the chart indicates the width of the well cut to inoculate the preparations and does not indicate inhibitory effects. Also note that each plate was re-inoculated and re-incubated after examination in trial one.(B) Digested preparations of Lactobacillus acidophilus (in pill and capsule form) and Lactobacillus casei (in yogurt form) were applied directly to bacterial overlays of E. coli and incubated for 6 hours. Plates were then examined for zones of inhibition. The control for part two was stomach acid. Note that the isolated strains were not subjected to stomach acid treatment, due to the impracticality of ingesting an isolated strain of probiotic. Note that the .7mm reading on the chart indicates the width of the well cut to inoculate the preparations and does not indicate inhibitory effects. Also note that each plate was re-inoculated and re-incubated after examination in trial one.

# SAMPLE EDITOR'S LETTER AND REVIEWS

**Greetings from the Journal of Emerging Investigators,**

Thank you for submitting your manuscript entitled "In VitroModeling of the Efficacy of Probioitic Inhibition of E. coli in the Intestinal Tract" to the Journal of Emerging Investigators (JEI). You addressed an interesting/relevant scientific question with appropriate scientific techniques. Furthermore, it was clever to test whether your probiotic bacteria can survive an invitro model of stomach conditions. However, the manuscript requires several changes to both (1) increase the accuracy/clarity of the written manuscript and (2) improve the believability of the experimental results. Thus, your manuscript has been accepted with major revisions.

Below, we have listed both the major and minor revisions that should be performed prior to re-submission. Following these required revisions are the comments made by the scientific reviewers that read your manuscript. Please address these revisions to the best of your ability. When addressing these revisions, we highly recommend that the student(s) consult with their research mentor(s). Once you complete the revisions, please resubmit the revised manuscript, the figures, and a cover letter (which states what revisions were performed and provides a rational for the revisions that were not performed) on the JEI website. As major revisions are required, the revised manuscript will then be subjected to a second round of review to determine if the manuscript is acceptable for publication.

Please do not hesitate to contact us if you have any questions. And thank you once again for submitting your manuscript to the Journal of Emerging Investigators. We applaud your excellent scientific work and look forward to your re-submission.

Sincerely,
Christopher Wells
JEI Senior Editor

**Major Revisions**

1.Throughout. A scientific manuscript must be clear so that a reader can both understand and analyze what was done. Although your manuscript successfully describes several aspects of your project, there are many cases where it is unclear how and/or why certain things were done (these will be discussed in more detail below). You should always try to clearly explain the thought processes behind your decisions, be it about reagents, experimental type/design/technique/protocol/conditions, etc., to the reader. This will help the reader follow your logic progression and provide a deeper understanding of both (1) the data and conclusions from individual experiments and (2) the more general concepts and how they fit into the bigger picture.
2.Throughout. Please clarify your definition of "inhibition" and use it appropriately throughout the text. Do you mean killing? Or prevention of growth? In general, in scientific writing is very important to use the correct terminology so that the reader understands exactly what you have done.
3.Results. In your inhibition experiments, you include a negative control (distilled water) to show that some aspect of your experimental setup is not producing unexpected growth inhibition. This is very

good! However, you also need a positive control to demonstrate that your experimental setup works. This will show that your assay can actually detect growth inhibition ofE. coli. This can be done using a known antibiotic, acid, alcohol, or, most preferably, another bacterial strain known to inhibit E. coli growth.

4.Results. To determine whether or not the probiotic bacteria can inhibit E. coli growth, it is important to show that that the probiotic bacteria themselves actually grow in the inhibition experiments! Although you attempted to address this issue, your manuscript was not clear in several instances. For example, you did indicate that the commercial cultures could grow in the TJYEMM medium (as indicated by cloudiness). But do they grow effectively on the nutrient plate? Furthermore, do you have evidence that the probiotic bacteria from the pill, capsule, and yogurt can grow after crushing and resuspension? To address this, you do note that the inoculated quadrants showed visible L. acidophilus growth consistent with its morphology. How exactly were the colonies identified based on mophology? Do you have images or data for this? Also, a reference for this verification technique needs to be included. And what about L. casei– is there any evidence of growth on the plates? To simplify this confusion, it would be best to demonstrate growth of all of these different probiotic strains by applying the same amount of sample that was added to the E. coli lawns to a fresh nutrient plate without E. coli. This will show that the preparations you are adding to the E. coli lawns are in fact viable. Furthermore, a picture of the growth inhibition plates would also be helpful to demonstrate the growth of the probiotic strains in these conditions.

5.Results. The above positive controls may demonstrate that your assay can detect E. coli growth inhibition. However, we do not believe that it is typical to detect inhibition by placing the potential inhibitory strain is a cut-out well (where, depending on the details of this technique, the probiotic strain may not be in close-enough proximity with the E. coli). The strength of this manuscript thus may be greatly improved by performing an alternative method to examine the inhibition of E. coli growth by different probiotic sample preparations. Some straight forward examples are as follows:

Filter disc assay (preferred). Drop a normalized amount of each probiotic sample onto a small circle of autoclaved (sterile) filter paper. These filter discs can then be placed directly onto the top of an exponential and/or stationary phase E. coli lawn, as the filter disc will contain the spread or diffusion of the probiotic species, abolishing the need to cut wells. After a period of incubation, measure the size of the circle of inhibition around the filter discs. A dilution series (see minor revisions, 4f) of the probiotic samples can be added to a number of filter discs, which can then be arrayed similarly to the spotting assay described below.

Serial dilution spotting assay. Grow up the different probiotic samples and generate a normalized serial dilution series (see minor revision, 4f) for each, i.e. a series from 100-10-5, where each specific dilution for each probiotic has the same number of cells, i.e. the 100for each probiotic has the same number of cells, etc. Then spot the same volume of each of these dilutions in a line across an exponential and/or stationary phase E. coli lawn. On the typical agar plate, a 4x4 or 5x5 spot grid can be made using 0.005 mL spots without the spots spreading and running into each other. A Google image search with “Serial dilution spotting assay” will return nice examples. The spots are then given time to dry, similar to the reaction time you allowed after inoculation the lawns with the probiotics, before being placed in the incubator.

Liquid culture assay. Grow the E. coli in liquid media both in the presence and absence of the different probiotic samples. Then plate out serial dilutions (see minor revisions, 4f) of these cultures and count the number of E. coli cells in each condition. If there is growth inhibition, there should be less E. coli cells in the co-cultured samples as opposed to the ones only containing E. coli. This is a little more involved as it requires you to normalize both the starting amount of E. coli cells as well as each probiotic species. It also requires you to be able to distinguish between E. coli and probiotic colonies on the plates relatively easily.

**Reviewer's Comments to Authors**

**Reviewer 1**

Overall, this is fantastic work. However, the authors must show that their assay works before this can be published. To do so, I recommend inclusion of one final experiment to demonstrate observable inhibition of E. coli growth in their assay. I would suggest known antibiotics, strong acid, or another bacterial strain known to inhibit E. coli growth. Aside from this critique, the manuscript was well-written and timely. The questions asked were clearly-framed and important.

1 – Formatting. Please include author affiliations and contact information. I assume this will be taken care of in the final copy.
2 – Abstract. One sentence suggestion: why do you suspect probiotics should inhibit E. coli? Would an observed inhibition imply a health benefit? Please also (very briefly – one word) define E. coli inhibition (growth? motility? etc.).
3 – Introduction. Elie Metchnikoff story is awesome!
4 – "Life in a relatively germ free industrialized world has shielded humans from exposure to microorganisms during the time of immunity passed on by mother's milk." I'm not sure I totally believe this. Can you find a reference to support this idea?
5 – Introduction: "strand" --> "strain"
6 – Results. Please divide this section into two parts, divided by sub-headings. The parts are mentioned in the text. First go through part one (with no mention of part two), stating the findings for this section. Then go through part two, saying something like, "We were then interested to determine whether probiotic strains could survive a simulated passage through the human stomach, and how this treatment would affect our observations from part one."
7 – Results. Do you have pictures of your plates? It would help the reader understand the experimental setup.
8 – Results. 360rpm on what instrument?
9 – Results. Speculations regarding lack of E. coli inhibition should be reserved for the discussion.
10 – Results. How did you "match the selected strains with monoculture probiotic supplemements used in testing?" This seems important. Should it really be in
parentheses?
12 – Discussion. "No solid conclusions can be drawn from this result." BUT YOU DID NICE WORK! Solid conclusions can rarely be drawn from results. But that doesn't make good experiments (like yours) worthless. Please replace this sentence with another one (or just remove it). Suggestion: "In our simple assay, we were unable to observe inhibition of E. coli growth by commercial probiotic preparations."

**Review 2**

This manuscript examines the ability of commercial Lactobacillus strains to inhibit the growth of Escherichia coli. The authors use standard and novel in vitro techniques in an attempt to investigate both a direct inhibitory effect of Lactobacilli on E. coli growth, as well as an inhibitory effect after passage through simulated acidic stomach conditions. The hypotheses behind these experiments are sound and the topic is of considerable interest, however the writing needs polishing, as there are numerous grammatical and terminology errors. The discussion is very good and addresses numerous issues with the interpretation of the data. The results are believable, but would be significantly strengthened by doing other experiments to allow for clearer interpretation. I would recommend this manuscript for publication with considerable revisions. Listed below are additional comments and experiments the authors should consider.

Throughout. The first time scientific names of bacteria are used, write out the full genus and species name (e.g.Lactobacillus casei). After this, refer to it in shortened format (e.g.L. casei), where the genus initial is capitalized and the species name is in lower case. Also, when discussing multiple species of the same genus, pluralize the genus name (e.g.Bifidobacteria and Lactobacilli).

Abstract. In the third paragraph, clarify that L. acidophilus survives simulated stomach passage, as all experiments are in vitro.

Introduction. The introduction details a number of important concepts, however additional background is needed on certain points. A good history of probiotics is given, but there is no background on E.coli, which is the target bacterium of the study. In the second paragraph, diversity among commensal bacteria is mentioned, but there is no explanation about what a commensal is.
Introduction. In the first paragraph, Elie Metchnikoff's studies may have been some of the first observations in the early days of studying probiotics. It cannot be stated, however, that his notations on Bulgarian peasants and his own subsequent consumption of sour milk led to a new field of study – probiotics.
Introduction. The last few sentences of paragraph 2 do not make sense. "Life in a relatively germ free industrialized world has shielded humans from exposure to microorganisms during the time of immunity passed on by mother's milk." The meaning of this sentence is unclear. "This lack of early immunological challenge lead to epidemics of polio and has further promoted the theory that allowing or even fostering the colonization of Probiotic bacteria is beneficial for a number of reasons." Lack of immunological challenge did not: 1) lead to polio epidemics, or 2) promote a theory.
Introduction. Change one word each in the second and third sentences of paragraph 4. "However, a different strand of that same genus can have a completely different function. Bifidobacterium lactisBb-12 has been shown to help the immune system and promote digestive tranquility." Change strand to strain and tranquility to balance.
Introduction. Clarify the last sentence in paragraph 4. "The same variation can be found in the Aspergillus, Lactobacillus, Saccharomyces, Enterococcus, and any other genera of Probiotics." Do you mean there is the same variation in the effect of bacterial strains in these genera?
Results. In the third sentence of paragraph 1, why explain why tomato-juice yeast-extract milk medium was chosen. If it is standard growth medium for these bacteria, state that. If not, explain why it was chosen. Also, remove "luxurious" from the sentence.
Results. Did you test whether or not the probiotics in the pill and capsule (after being crushed and resuspended) are alive? This is a necessary control, as you're assuming that they're still alive and well because the company's label says so. Also, can they survive the experiment you do? If, after resuspending them and inoculating them into the well, the Lactobacilli die shortly thereafter,
this could confound your results.
Results. How did you control for the concentrations of bacteria added to the wells? Do you know the same amount (e.g. 1 million bacteria) were added to each well, and was it the same amount between capsule, pill and strain alone?
Results. In the third paragraph, you discuss your stomach-acid solution. How did you come up with this formula? Also, you mention the normal transit time through the human stomach – do you have a reference for this?
Results. Always give your reasoning for conducting an experiment a certain way. Your reader should be able to follow along with your thought process. In the last sentence of the second paragraph ("The procedure was then repeated for a second trial, with minor modifications in the incubation period and isolated strain growth."), there is no explanation as to why you repeated the experiment
with different conditions.
Results. Refer to the figures when you discuss those results. The reader should be able to read the text and know which figure to look at.

Results. The final several sentences of the last paragraph belong in the discussion, not the results section, as you're postulating why things may or may not have worked. Here are some additional points: In the second to last sentence, you state that your design was focused on detecting direct inhibition, but your assay actually looks at indirect inhibition. Because you aren't attempting to grow the Lactobacilli on your plate with wells, a more likely way the Lactobacilli could inhibit E. coli is through secreted products – indirect inhibition. Additionally, in the final sentence you mention that the inconclusiveness may be due to the E. coli strain used. Specifically what about the strain used may have led to the ambiguity? Maybe this strain is less susceptible to Lactobacilli, so in the future testing other strains may give more insight. (Again, all of this belongs in the discussion section.)

Results. Clarify (in the text, not just figure legends) that the 7mm listed in your figures as zones of inhibition are actually the well size – and not a 'zone of inhibition.

Discussion. In the fourth sentence of the first paragraph, you list the ways probiotics inhibit pathogens. Please give a reference.

Discussion. In the final sentence of the first paragraph ("Without this property it is impossible to view binding interference…"), change the word view to investigate.

Discussion. When discussing future research, maybe it would make sense to consider (either doing yourself or suggesting for future work) co-cultures or competitions between strains to test interactions or ability to inhibit growth in a different manner than on plates. Co-culture E. coli and a Lactobacillus strain and plate out dilutions after set periods of growth to see if the E. coli counts are diminished as compared to E. coli growth from a pure culture. Control for concentration by using optical density, for example. Along these lines, why was the overlay technique chosen? Is it based on the assumption that E. coli is already colonized in the intestine and you're therefore simulating that by introducing Lactobacilli to existing E. coli lawns?

Methods. Several points need to be clarified in "Part 2: passage through the stomach." First, you state that one serving of each probiotic was dropped into the flask. Define one serving. Second, explain why you chose 2.5 hours for transit time when you state that the normal transit is 4 hours. Third, state the amount of time plates reacted ("The plates were then given tie to react with the agar and placed in the incubator…"). Fourth, why re-inoculate the same plates? Wouldn't the E. coli on these plates be in or almost in stationary phase, and thus any inhibitory effect would be muted because the bacteria are no longer growing much?

Methods. In "Part 2: passage through the stomach," explain why you chose 2.5 hours for transit time when you state that normal transit is 4 hours. Figures. There is no difference between the legends of Figures 1 and 2. The point is to show different things, so the legends should be (at least slightly) different. Also, the figures need to be labeled so the reader knows which is which.

# Incorporating JEI in the classroom

There are several ways that JEI can be used in your classroom to provide a rich learning experience for your students:

1. JEI can primarily be used to encourage hypothesis-driven research by your students. Students can gain a great deal from the process of engaging in hypothesis-driven research, and JEI provides a forum to encourage this way of learning and allows students to publish their original work, much in the same way that professional scientists strive to do every day. Students' own research or written work can be directly submitted to JEI in the form of:

Research Articles: original research based on Individual/small group science fair-based projects or other classroom-based projects.

Letters to the Editor: short opinion pieces written by the student(s) about current science news or other exciting topics of interest

Review Articles: summaries written by the student(s) on a topic of interest that directly references current scientific articles or other reliable sources

- bacteria vs. viruses: what's the difference?
- stem cells: what are they and what can they be used for?
- yogurt and your digestive system: can bacteria be beneficial?

2. If you do not have space in your curriculum to allow students to write and submit their own work to JEI, JEI can also be used as a tool for your students to learn from other students' previously published JEI articles:

Students can be encouraged to pick a published article from JEI that looks interesting and write a short summary of the chosen article, including some future directions or questions that should still be addressed, such as:

Where could the authors have used better controls for their experiments?
What experiments should be done next?
If you had been the scientist running this project, what would you have done differently? How can this research be used in the future to improve ___(human health, the environment, etc.)__?
Why is this research important?!

**With your help, we sincerely hope that this journal encourages a deep fascination and life-long interest in science in your students!**