Methods Article

Methods for testing effects of prebiotic on *Lactobacillus* species and *Escherichia coli* co-cultures*

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This paper provides the methods for testing prebiotics on Lactobacillus growth, both individually and in co-culture with Escherichia coli K12. While prior research examines the effects of different prebiotics on the growth of various Lactobacillus species, their effects on Lactobacillus - E. coli concomitantly remain unexplored. Lactobacillus, a gram-positive bacterium, plays a key role in lactose breakdown. Certain strains of Lactobacillus are known for their sugar fermenting properties which can convert lactose to lactic acid instead of gas, thus diminishing some lactose intolerance symptoms. Our paper aims to shed light on the effects of prebiotics on Lactobacillus growth, potentially providing a pathway to discover new strategies for alleviating lactose intolerance symptoms. Additionally, the methods outlined could potentially be used to investigate the ability of Lactobacillus to inhibit E. coli growth under certain conditions. The methodology involves preparing De Man, Rogosa and Sharpe (MRS) broths with different prebiotic concentrations, inoculating them with Lactobacillus and E. coli or either alone, and incubating them before plating on MRS Petri dishes though our design does not address the underlying mechanisms of Lactobacillus-E. coli interactions. Some strains of E. coli are beneficial and necessary for a healthy gut. However, the human microbiota is a careful balance between "friendly" or helpful bacteria and harmful bacteria. Future research could investigate these interactions and explore the effects with potentially pathogenic strains of E. coli.

Keywords: Prebiotics, *Escherichia coli*, *Lactobacillus*, MRS media

This paper outlines the procedures for creating *Lactobacillus* - *E. coli* cocultures in order to test the effects of prebiotics on *Lactobacillus* growth and its subsequent effects on *E. coli*. This includes the preparation of De Man, Rogosa and Sharpe (MRS) media with modifications to decrease the cost burden. This will allow us in the future to fully investigate the effects of paramylon, a Euglena byproduct, on *Lactobacillus* - *E. coli* cocultures.

Prior research examines the effects of different prebiotics on the growth of various *Lactobacillus* species, however, their effects on *Lactobacillus* - *E. coli* concomitantly remain unexplored (Dai et al., 2022). *Lactobacillus* is a gram-positive bacteria that aids in the breakdown of lactose to lactic acid instead of gas, one of the main symptoms of lactose intolerance. While there have been no human trials, research suggests that the strain *L. acidophilus* mitigates the symptoms caused by lactose intolerance (Pakadaman et al, 2016). Additionally, research indicates

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that certain *Lactobacillus* strains exhibit inhibitory properties towards *E. coli.* though the mechanisms for this are not understood.

Preparing a modified De Man, Rogosa and Sharpe media

Lactobacillus grows best on De Man, Rogosa, and Sharpe (MRS) media as many strains are auxotrophic. This media can be expensive to purchase premade, as such this section outlines the methods needed to prepare MRS media and can be easily scaled up to decrease associated costs. Other modifications were made to decrease costs following the work of Zhang et. al. (2020). Ammonium citrate, a chelating agent that provides micronutrients to the media was omitted and beef extract was substituted with additional yeast extract, this will provide the media with proteins, minerals, and vitamins but will exclude any creatine and creatinine. Additionally, Tween 80 was substituted for Tween 20 as they are very similar in function, with few differences being solubility, hydrophilicity, and lipophilicity. Finally, sodium acetate was excluded not to decrease cost but due to its function as a suppressant of non-lactic acid-producing bacteria.

Requirements

Time: 45 min
Equipment:
- Weigh boat
- Scoopula
- 250 mL beaker
- 500 mL Erlenmeyer flask
- 250 mL, 100 mL, 25 mL graduated cylinders
- Scale
- Autoclave

Materials (Note 1):
- 10 g peptone
- 10 g yeast extract
- 20 g glucose
- 2 g Potassium phosphate
- 0.2 g Magnesium sulfate heptahydrate
- 0.8 g Tween 20
- 1 L distilled water
- Probiotic of choice (ex. 12.5 g Euglena powder)

Procedures

1. Weigh out amounts of all materials and place them in a 250 mL graduated cylinder.
2. Fill up to 250 mL with distilled water.
3. Pour into an Erlenmeyer flask with 750 mL of distilled water and mix well.
4. Autoclave at 121ºC for 15 min (Note 2).

Preparing Petri dishes and broth

This is necessary to culture the bacteria. The broths will be used to test the effects of the prebiotics while the Petri dishes will allow one to observe the results.

Requirements

Time: 20 min
Equipment:
- 500 mL Erlenmeyer flasks
- three 100 mL Erlenmeyer flasks
- 18 mL Petri dishes (Note 3)
- six 50 mL media bottles (Note 3)
- Scale
- Scoopula
- Thermometer
- Hot plate

Materials:
- 1 L prepared MRS media with Prebiotic of choice (Note 4)
- 14 g agar powder

Procedures

Petri dishes
1. Remove MRS media from the autoclave.
2. Add 14 g of agar powder to 550 mL of MRS media.
3. Heat until boiling for 1 min and let cool until 55oC.
4. Pour approximately 30 mL into each Petri dish.
5. Leave to solidify.

Broth
1. Pour 100 mL of MRS media into three flasks.
2. Add the desired amount of prebiotic to each flask at varying

concentrations.
3. Pour 50 mL each into three properly labeled media bottles for a total of six cultures

Inoculating broths

Requirements

Time: 30 min
Equipment:
Bunsen burner
1 mL metal inoculation loop
Gloves
70% Isopropyl alcohol
Materials:
E. coli K12 culture
Lactobacillus culture (Note 5)
Prepared 50 mL MRS broths

Procedures

1. Wipe down the work area with isopropyl alcohol.
2. Hold the inoculating loop over the Bunsen burner to sterilize.
3. Dip loop into *Lactobacillus* culture and then into one of the six MRS broths.
4. Resterilize the loop and repeat for the other five broths sterilizing the loop between each.
5. Sterilize the loop and dip into *E. coli* culture then dip into one of the broths.
6. Resterilize the loop and repeat for the two other broths with different prebiotic concentrations.
7. This should leave you with three broths that are *E. coli* - *Lactobacillus* co-cultures at different prebiotic concentrations and three broths that are solely Lactobacillus cultures at different prebiotic concentrations.

Plating samples

Plating the samples on Petri dishes allows one to quantify the concentration of bacteria by counting the number of CFUs. This will allow us to determine how the bacteria interacted with each other and the prebiotics.

Requirements

Time: 20 min
Equipment:
Serial pipette
24 test tubes
Materials:
Prepared Petri dishes
Prepared broths
200 mL distilled water

Procedures

1. Fill each test tube with 9 mL of distilled water.
2. For each broth pipette 1 mL into a test tube (label 1:10).
3. Then to continue the serial dilution pipette 1 mL of the 1:10 test tube into a new test tube (label 1:100) and repeat twice more for a dilution of 1:10,000.
4. Pipette 1 mL of the 1:100, 1:1,000, and 1:10,000 dilution from each broth onto properly labeled MRS Petri dishes.
5. Leave upside down for 48 hours before counting the number of CFUs in each (Note 6).

Safe Disposal

When working with bacteria, it is of utmost importance to properly dispose of all cultures. All bacteria strains cultured were classified as Biosafety Level 1 and adherence to the proper safety measures according to that designation were followed. Biohazardous materials are decontaminated prior to disposal through the use of the autoclave before being placed in a red biohazard-marked bag then disposed of in the solid waste container.

Requirements

Time: 10 min
Equipment:
Large container
Materials:
Bleach
Water
Prepared Petri dishes
Prepared media bottles
Prepared test tubes

Procedures

1. Fill a large container with 10% bleach solution.
2. Soak Petri dishes, media bottles, and test tubes in solution for 20 min. minimum.
3. Rinse and dispose of materials or wash and reuse.

Notes

1. Tween 20 can be substituted for Tween 80.
2. Media can be left in the autoclave until it is used in subsequent steps to ensure it remains sterile.
3. The number of Petri dishes and media bottles listed is dependent on the number of trials and number of concentrations desired. This protocol assumes three trials at three different concentrations.
4. Amounts vary based on the chosen prebiotic for the development of this procedure; we used paramylon at concentrations of 1, 5, and 10% (w/v).
5. This culture we created ourselves by isolating *Lactobacillus* strains in Kefir yogurt.

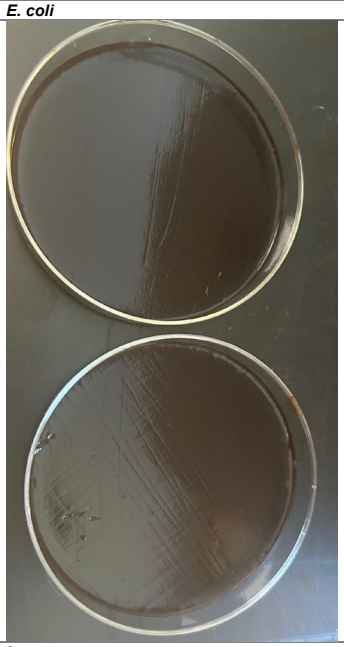
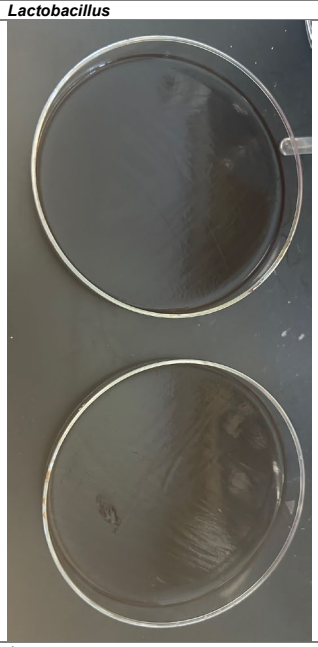
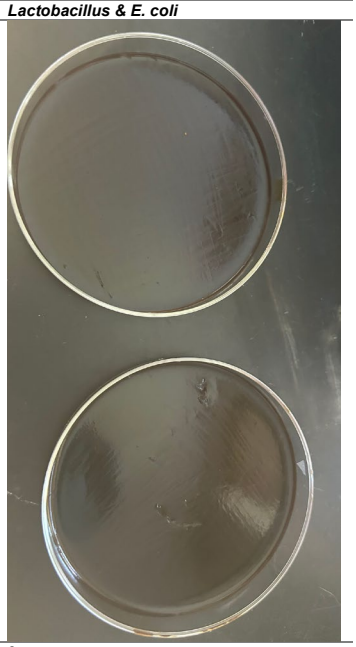
Discussion

Our methodology is overall very straightforward. The main drawback comes for the cost of the MRS agar. However, that was minimized through our protocol modifications. It allows one to culture *E. coli* and *Lactobacillus* at varying prebiotic concentrations relatively easily. The results of our methodology with paramylon have not yet been fully observed, however the methodology was successful so far.

Next Steps

The next step of our research would be performing the full-scale experiment. However, beyond that investigation of the interactions between *Lactobacillus* and *E. coli* would be advisable, particularly addressing the question of how lactic acid produced by *Lactobacillus* may contribute to *E. coli* inhibition when euglena prebiotics are present. Additionally, the effects of different prebiotics could be investigated, including isolating specific nutrients such as paramylon, linoleic acid, and carotenoids (Dai et al., 2022). As there are few human test trials, our next research lab can observe the

Table 1.

Petri dish samples	***E. coli***	***Lactobacillus***	***Lactobacillus & E. coli***
Pictures			
# of Colonies	9	1	6

effects of our increased *Lactobacillus* cultures by adding these probiotics into common food and testing if they have an effect on those who are lactose intolerant.

Author contributions

T.H. and M.D. developed and executed methods. T.H. and M.D. wrote the abstract, procedures, notes, and discussions. T.H. wrote the background, next steps, and acknowledgments. M.D. created the video and provided a table with the results.

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