Enumeration and Identification of *Listeria monocytogenes* on Ready-to-Eat (RTE) Foods

Food Microbiology Lab 8

Overview

In this lab, students will perform the enumeration and identification of *Listeria monocytogenes* on commercial frankfurters.**"hot dogs"** or **"sausages"**)(The experiment includes inoculation, serial dilution, and spread plating on the selective medium Modified Oxford Agar (MOX). Presumptive colonies will be identified using Gram staining, the catalase test, and the 12L® Listeria test kit. A portion of this work will require students to complete the tasks independently.

Objective

To determine the population of *Listeria monocytogenes* on inoculated chicken through spread plating and to identify the organism using Gram staining, the catalase test, and the 12L® biochemistry test.

Key Materials

- *Listeria monocytogenes* (Biosafety Level-2 organism)
- Commercial frankfurters (hot dogs)
- Buffered peptone water (100% and 0.1%)
- Whirl® food sampling bags
- Stomacher
- Modified Oxford Agar (MOX), Tryptic Soy Agar (TSA), and Blood Agar
- Hydrogen peroxide (H_2O_2) reagent for catalase testing
- Gram staining reagents
- 12L® Listeria test kit

Introduction

Listeria monocytogenes is a Gram-positive, non-spore-forming, facultative bacterium capable of surviving and growing in diverse food products such as dairy, meat, poultry, vegetables, and seafood. Its psychrotrophic nature and ability to withstand adverse environmental conditions make it a persistent pathogen in food-processing environments.

Between 1998 and 2002, *L. monocytogenes* was implicated in several multistate outbreaks in the United States linked to RTE meat products, including commercially cured ham. As a result, the USDA-FSIS implemented strict regulations requiring RTE meat processors to adopt one of three control measures for *L. monocytogenes*:

1. A post-lethality treatment (e.g., steam, pressure, or antimicrobial agents) combined with an antimicrobial process.
2. Either a post-lethality treatment or an antimicrobial process.
3. Enhanced sanitation practices with increased microbial verification testing.

Lab Procedure

1. Inoculation of Frankfurters

- Each group will receive two frankfurter links.
- Inoculate the surface of each frankfurter (5–6 cm^2) with 0.2 mL of *L. monocytogenes* using a sterile bent plastic rod.
- Allow the inoculated hotdogs to rest at room temperature for 10 minutes to facilitate bacterial attachment.

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2. Enumeration of *L. monocytogenes*

1. Place the two inoculated frankfurters into a Whirl® food sampling bag.
2. Add 100 mL of 0.1% buffered peptone water (BPW) to the bag and seal it.
3. Shake the bag vigorously for 30 seconds.
4. Perform a serial dilution using 9.0 or 9.9 mL of 0.1% BPW solution to achieve final dilutions of 10^{-2} and 10^{-4}.
5. Spread-plate 0.1 mL of each dilution onto MOX and TSA plates.
6. Incubate the plates at 35°C for 48 hours.
7. Count the colonies on the plates and record the results in your notebook. Use the following formula for calculations: $\text{Log10 CFU/g} = \text{Log}_{10}\left[\frac{\text{Final CFU on plates}}{\text{Dilution factor}} \times \frac{100}{112}\right]$ Log10 CFU/g=Log10 [Dilution factorFinal CFU on plates ×112100]

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3. Identification of *L. monocytogenes*

A. Gram Staining

- *L. monocytogenes* should appear as Gram-positive, single, large rod-shaped bacteria.
- Steps:

    1. Add crystal violet to the slide for 60 seconds, then rinse.
    2. Add Gram iodine for 60 seconds, then rinse.
    3. Decolorize with 95% alcohol for 10–15 seconds, then rinse.

4. Counterstain with safranin, then rinse and observe under the microscope.

B. Catalase Test

- *L. monocytogenes* is catalase-positive (bubbles form).
- Steps:

1. Place a drop of H_2O_2 on a clean glass slide.
2. Pick a presumptive colony from the MOX plate using a sterilized loop.
3. Immerse the colony into the H_2O_2 and observe for bubbles.

C. Hemolysis Test

- Streak the presumptive colony onto a blood agar plate.
- Observe hemolytic activity:
  - *L. monocytogenes* typically exhibits beta-hemolysis (clear, transparent zone).

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4. Contents and Description of the 12L® Listeria Test Kit:
The 12L® Listeria test kit includes a set of wells, each containing a specific substrate to test for enzymatic or biochemical activity in *Listeria* spp. The reactions in these wells provide a unique profile to identify and differentiate *Listeria monocytogenes* from other *Listeria* species.

1.Wells with Substrates: Preloaded with specific substrates that react with Listeria spp., causing color changes for metabolic differentiation. 2.Listeria Suspension Medium: Used to prepare bacterial suspensions for inoculating the wells.3.Holding Tray with Lid: Holds the test strip securely during incubation.4.Hemolysis Reagent: Detects hemolytic activity in well 12, indicating β-hemolysis.

Biochemical Tests in Each Well:

Well No.	Substrate	Negative Reaction	Positive Reaction
1	Esculin (ESC)	Pink or brown	Black
2	Mannitol (MAN)	Purple	Yellow, brown, or straw
3	Xylose (XYL)	Purple	Yellow, brown, or straw
4	Arabitol (ARL)	Purple	Yellow, brown, or straw
5	Ribose (RIB)	Purple	Yellow, brown, or straw
6	Rhamnose (RHA)	Purple	Yellow, brown, or straw
7	Trehalose (TRE)	Purple	Yellow, brown, or straw
8	Tagatose (TAG)	Purple	Yellow, brown, or straw
9	Glucose-1-phosphate (G1P)	Purple	Yellow, brown, or straw
10	Methyl-D-glucose (MDG)	Purple	Yellow, brown, or straw
11	Methyl-D-mannose (MDM)	Purple	Yellow, brown, or straw
12	Hemolysin (HEM)	Red cell deposit	Partial or complete lysis

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Procedure for Using the 12L® Test Kit:

1. Pick 4–5 suspected colonies from Modified Oxford Agar (MOX).
2. Suspend the colonies in the Listeria suspension medium.
3. Dispense **4 drops (100 µL)** of the suspension into each test well.
4. Add **1 drop of hemolysis reagent** to well 12.
5. Place the strip in the holding tray and replace the lid.
6. Incubate at **35°C ± 2°C for 24 hours**.
7. Record the results by comparing the color changes in each well to the provided interpretation chart.

Key Identifying Reactions for *Listeria* spp.:

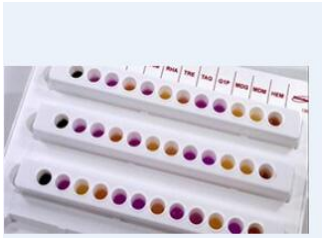
- Positive reactions in **well 1 (Esculin)**, **well 4 (Arabitol)**, and **well 7 (Trehalose)** are characteristic of *Listeria monocytogenes*.
- β-hemolysis in **well 12** or on blood agar confirms the identification.

Expected Results

- *Listeria monocytogenes* will produce typical black colonies on MOX agar due to esculin hydrolysis.
- Gram staining will confirm its morphology as Gram-positive rods.
- Positive catalase and beta-hemolysis tests further confirm the identification.
- The 12L® test kit will validate biochemical properties consistent with *L. monocytogenes*.

Notes for Students

Ensure strict adherence to biosafety protocols when handling *Listeria monocytogenes*.Document all observations thoroughly in your laboratory notebook.Be prepared to analyze and interpret your results during the discussion section of the lab



Oxoid™ Microbact™ Listeria 12L Kit