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# Inhibition of L. Monocytogenes Growth in Dairy Productions with Lactose Monolaurate

Yao Chen

# INHIBITION OF *L. MONOCYTOGENES* GROWTH IN DAIRY PRODUCTS WITH LACTOSE MONOLAURATE

by

Yao Chen

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

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Approved:

| | |
|---|---|
| Marie K. Walsh<br>Major Professor | Brian A. Nummer<br>Committee Member |
| Robert E. Ward<br>Committee Member | Mark R. McLellan<br>Vice President for Research and<br>Dean of the School of Graduate Studies |

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# ABSTRACT

Inhibition of *L. monocytogenes* Growth in Dairy Products

with Lactose Monolaurate

by

Yao Chen, Master of Science

Utah State University, 2013

Major Professor: Dr. Marie K. Walsh
Department: Nutrition and Food Sciences

*Listeria monocytogenes* leads to severe health problems and is the third leading cause of death among the major 5 pathogens. A synthesized novel sugar ester, lactose monolaurate (LML), has antimicrobial properties against *Listeria monocytogenes.* The minimum bactericidal concentration (MBC) of LML is less than 5 mg/mL (9.5 mM) in growth media. To determine which moiety of LML dominates in its bacteriostatic activities, the antibacterial effect of lactose, lauric acid and Tween 20 were tested. Lactose has no inhibition effect on *Listeria*. Lauric acid and Tween 20 had some antimicrobial effect (3.48 and 1.59 log reduction respectively), but did not have a bactericidal effect as LML did. To determine the antibacterial effect of LML on *L. monocytogenes* a 5-strain cocktail of *L. monocytogenes* with an initial concentration of approximately 5 log CFU/mL was incubated in milk, yogurt and cottage cheese. The effects were determined via plate counts after 24-hour incubation at 37°C. LML had at least a 4 log reduction and killed all the bacteria at 5 mg/mL in fat-free milk, fat-free

drinkable yogurt, 1% fat drinkable yogurt, and fat-free cottage cheese. LML also showed bacteriostatic effect in low-fat milk, whole milk, 1.5% fat drinkable yogurt, and 2% fat cottage cheese with a log reduction varying from 3.54 to 4.35. These tests showed that the antibacterial effect of LML was related to the fat content of the dairy products as well as temperature. LML only inhibited *Listeria* at room temperature (37°C) and showed no inhibitive effects at refrigeration temperature (4°C). LML can inhibit the viable but nonculturable state of *Listeria monocytogenes* for up to 6 weeks at room temperature.

(66 pages)

PUBLIC ABSTRACT

**Inhibition of *L. monocytogenes* Growth in Dairy Products with Lactose Monolaurate**

Foodborne illness is a very severe problem, and it is now a serious issue throughout the world. Within the 5 pathogens that cause more than 90 percent of foodborne illnesses, *Listeria monocytogenes* ranks the third. *L. monocytogenes causes* the highest death rate among all foodborne pathogens, and it represents high risks to pregnant women, fetuses and people with weak immune systems. Therefore, inhibition of *Listeria* in food becomes a relevant topic in food industry and scientific field nowadays.

Adding antibacterial agents into food products is considered as an effective method to inhibit *Listeria* growth. My research was focused on characterization of a novel sugar ester, lactose monolaurate (LML), with respect to antimicrobial activity. Previous research showed that LML was antimicrobial to *L. monocytogenes* with minimum bactericidal concentrations (MBC) less than 5 mg/mL in growth media. The objectives of this research were to determine if LML showed the same antibacterial activity in various dairy products.

My results found that LML showed a bactericidal effect on *Listeria* in fat-free milk, fat-free drinkable yogurt, 1% drinkable yogurt, fat-free cottage cheese and 2% fat cottage cheese. With skim milk, whole milk and 1.5% fat yogurt, LML had bacteriostatic effect. Moreover, LML did not show significant inhibition on *Listeria* in cream. Therefore, the antibacterial activity of LML was dependent on the fat content of the food product.

# ACKNOWLEDGMENTS

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I also want to thank Ashwini Wagh for training me to operate the synthesizing and preliminary experiments, and I am thankful for Guneev Sandhu for her assistance in some other experiments.

Finally, my research wouldn't have been accomplished without the support of my parents, Zhongliang Chen and Meirong Gu, and especially my husband, Hao Liu, for giving me confidence and encouragement during these two years.

With warm regards,

Yao Chen

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| BHI | Brain heart infusion |
| HPLC | High performance liquid chromatography |
| LML | Lactose monolaurate |
| MBC | Minimum bactericidal concentration |
| MIC | Minimum inhibitory concentration |

# LITERATURE REVIEW

## Synthesis of Lactose Monolaurate

Sugar esters are available commercially (Sisterna, The Netherlands and Mitsubishi-Kagaku Foods Corporation, Japan) and are used in a variety of applications in the food, pharmaceutical, and personal care industries. They are classified as non-ionic surfactants and examples include sorbitan monooleate (polysorbate 80), sucrose palmitate, and sucrose oleate. They are available in a variety of hydrophilic-lipophilic balances for use in oil-in-water and water-in-oil emulsions (Walsh et al., 2009). Lactose monolaurate (LML) is a novel sugar ester recently synthesized, and it is not commercially available. LML was synthesized with immobilized lipases in an organic solution from vinyl laurate and lactose as the substrates. LML has been shown to be bactericidal against some gram-positive microorganisms in growth media, namely *L. monocytogenes* and *Mycobacteria* at concentrations ranging from 1 to 5 mg/mL (Wagh et al., 2012). This thesis will investigate the bactericidal activity of LML in food products.

## Foodborne Illnesses

Foodborne illness is a very serious and worldwide problem. It has been estimated that 14 foodborne pathogens cause 14.1 billion in cost of illness per year. More than 90 percent of this health burden is caused by 5 pathogens; *L. monocytogenes* ranks third after *Salmonella spp* and *Toxoplasma gondii* (Batz et al., 2011). Certain pathogen-food combinations have been identified as the main cause of most foodborne illness. According to Scallan's estimates (Scallan et al., 2011), *L. monocytogenes* ranks 24$^{th}$ in

pathogens cause annual domestic foodborne illnesses cases, and it leads 255 cases of death annually.

Disease outcome trees characterize the symptoms, severities, and likelihood of major health states, such as hospitalization and death, associated with each of the 14 pathogens (Batz et al., 2011), is shown below in Figure 1. A "cost of illness" is associated with each health state in each tree. *L. monocytogenes* costs 2,655 million, which ranks 3rd in cost of illness among 14 food pathogens cause 14.1 billion dollars.

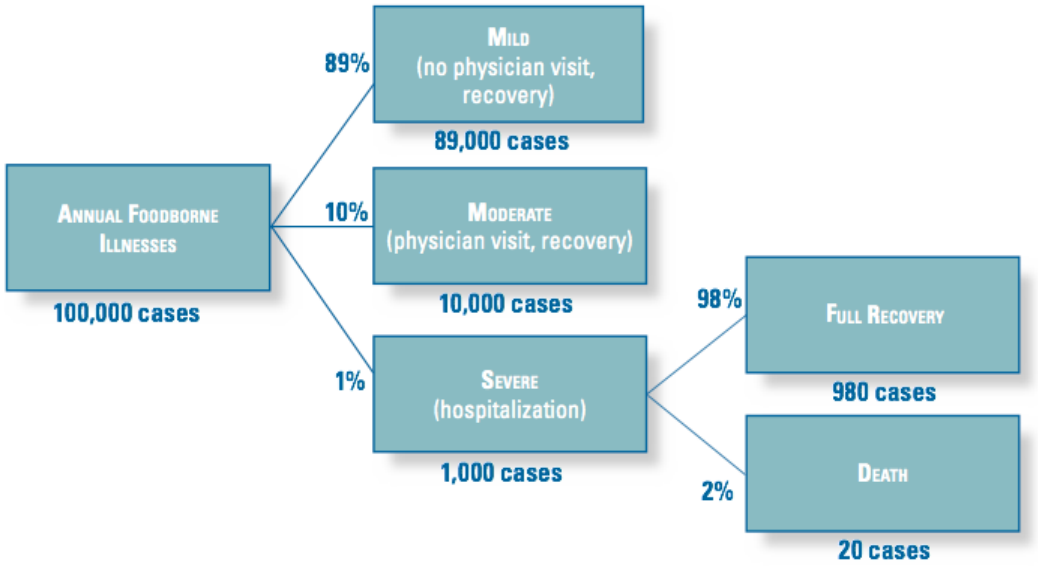


**Figure 1.** Example disease outcome tree for a hypothetical pathogen (from Batz et al., 2011).

*L. monocytogenes* also represents risks to pregnant women and developing fetuses. Congenital listeriosis can lead to miscarriage, stillbirth, and neonatal death, as well as lifelong complications ranging from mild learning disabilities to severe mental impairment, permanently blurry vision, neurological disorders, and paralysis (CDC) (Mead et al., 1999).

There are a limited number of pathogen-food combinations associated with most foodborne illnesses (Batz et al., 2011). *L. monocytogenes* in deli meat ranks third (USDA 2010), and *L. monocytogenes* in dairy products ranks fifth in pathogen-food combinations in terms of annual disease burden (Voetsch et al., 2007). While there have been significant gains over the last decade in reducing contamination rates of pre-sliced, packaged meats (USDA, 2010), numerous studies have found that retail-sliced deli meats have significantly higher prevalence and levels of *L. monocytogenes* than pre-sliced and packaged meats (Gombas et al., 2003; Endrikat et al., 2010).

*L. monocytogenes* is a high-risk pathogen and can cause serious health problems, even death. It also leads to a high financial loss every year. Therefore, inhibiting the growth of *L. monocytogenes* in dairy and meat products will be investigated in this thesis.

**Evaluation of Potentially Hazardous Foods**

A microbiological challenge test is a method to determine if spoilage microorganisms, including pathogens, could grow in food and be able to cause illness ((U.S. Food and Drug Administration, 2013). When conducting a microbiological challenge study, a number of factors must be considered. These include the selection of appropriate pathogens or surrogates, the level of challenge inoculum, the inoculum preparation and method of inoculation, the duration of the study, formulation factors, storage conditions, and sample analyses (Vestergaard, 2001).

According to Vestergaard's study (Vestergaard, 2001), *L. monocytogenes* may be used in challenge studies for modified atmosphere packaged products and dairy products. Multiple specific strains of the target pathogens are used in the challenge test.

The inoculum level used in the microbiological challenge study depends on validating a step in the process designed to reduce microbial numbers. If the inoculum level is too low, the product would appear to be stable or safe after the test, but actually it is not. On the other hand, if the inoculum level is too high, the product would not be stable or safe. When validating a process lethality step, such as heat processing or high-pressure processing, it is usually necessary to use a high inoculum level to demonstrate the reduction of the target pathogens. In microbiological challenge testing, inoculum preparation is an important part. The challenge organisms should be grown in specific media and under certain conditions for optimum growth.

The method of inoculation is another important factor in the microbiological challenge testing. A specific inoculation method based on the food type must be done which represents the correct water activity, pH, salt level, packing and storage method of the product. If the product has a high water activity (>0.96), the challenge inoculum may be directly inoculated into the product with mixing, using a minimal amount of sterile water or buffer as a carrier. One main principle is not changing the critical parameters of the product formulation undergoing challenge. It is recommended to use no less than three replicates for each sampling time throughout the challenge study (U.S. Food and Drug Administration, 2013). The minimum of replicates may be variable for particular cases. Other factors should be taken into consideration in the challenge study such as duration of the study, formulation factors and storage conditions.

**Antimicrobial Properties of Sugar Esters**

Sugar fatty-acid esters are used as emulsifiers in foods. They also have antimicrobial effects (Kato a d Arima, 1971; Hathcox and Beuchat, 1996). Commercial

sucrose esters are used mostly in Japan in canned beverages for inhibiting the growth of spore forming bacteria (Wang 2004). They can be synthesized via immobilized lipases in an organic solvent. The structure of LML is shown in Figure 2.

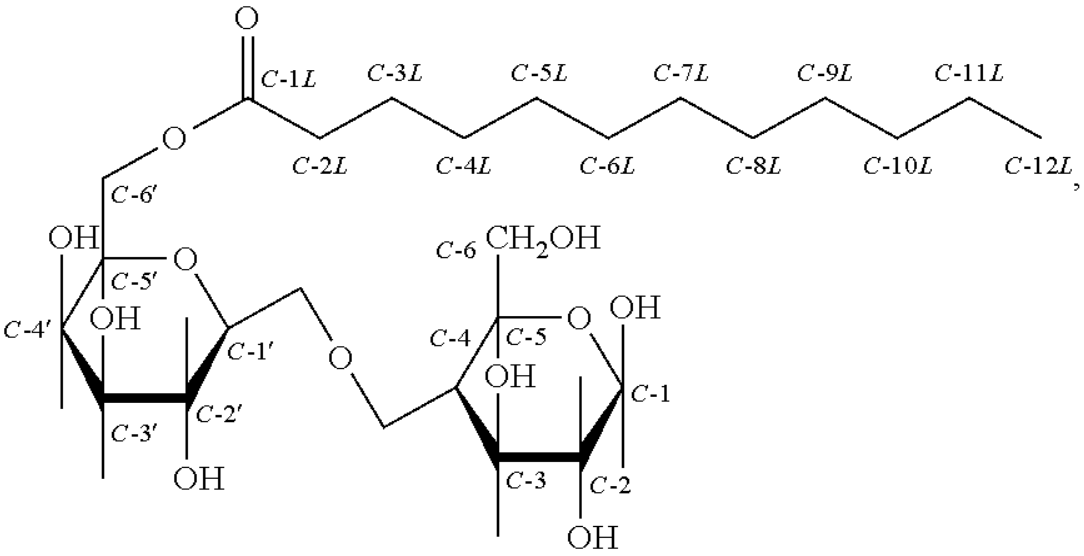


**Figure 2.** Atom numbering scheme for LML.

The antimicrobial activity of sugar fatty-acid esters is related to their structure. The antimicrobial activity of sugar esters depends on the sugar, number and type of esterified fatty acids, and the degree of esterification (Smith et al., 2008). Fatty acids with more than 8 carbons have no inhibitory effect to Gram-negative bacteria (Devulapalle et al., 2004; Watanabe et al., 2000). Yeasts are inhibited by fatty acids with 10 to 12 carbons (Piao et al., 2006). Gram-positive bacteria are less resistant to the slightly longer chain fatty acid ester (Ferrier et al., 2005).

Table 1 lists some conflicting antimicrobial data on the effectiveness of sugar esters. Some reports showed inhibition of Gram-negative bacteria (*E. coli* and *Vibro parahaemolyticus*) while others reported no inhibition. There are three publications on the use of esters in food systems with inhibitory effects against some spoilage organisms

and foodborne pathogens (*Listeria*). The Sisterna products L70-C (sucrose laurate) and SP70-C (sucrose stearate) have selective growth-inhibiting properties and were used in some of the above listed studies, as were the Ryoto sugar esters P-1670 (sucrose laurate) and P-1670 (sucrose palmitate) from Mitsubishi.

Recent research (Wagh et al., 2012) showed that LML is bactericidal against 4 clinical isolates of *L. monocytogenes* and three different *Mycobacteria*. The minimum bactericidal concentration (MBC) for *L. monocytogenes* was lower than 5 mg/mL and the MBC for *Mycobacteria* was only 1 mg/mL in bacterial growth media.

**Table 1.** Publications reporting the antimicrobial effects of sugar esters

| Ref | Esters | Organism | Effect | Medium |
|---|---|---|---|---|
| Wagh et al. 2012 | Synthesized lactose monolaurate | Various Gram positive and Gram negative | Bactericidal against *Listeria monocytogenes* and *Mycobacteria* | Growth media |
| Xiao et al. 2011 | Commercial sucrose monolaurate | *E. coli* 0157:H7 | Strong inhibition at 10 mg/mL with sodium hypochlorite | Spinach |
| Habulin et al. 2008 | commercial and synthesized sucrose and fructose palmitate and laurate | *Bacillus cereus*<br><br>*E. coli* K12 | Strong inhibition (75-96%) against *B. cereus* with sucrose laurate at 1% concentration at 3 days<br><br>Limited (10%) inhibitation against *E. coli* with all esters | Growth media |
| Piao et al. 2006 | Various synthesized erythritol and xylitol esters | Various Gram positive and negative | Strong inhibitory effect with xylitol monolaurate against *B. cereus*. All esters were ineffective against *E. coli* | Growth media and plates |
| Ferrier et al. 2005 | Various synthesized sugar esters | Various Gram positive and negative | Sucrose and maltose laurate inhibited *Bacillus* at 0.5%. Limited inhibitation (26%) against *E. coli* at 0.4%. | Growth media |
| Devulapalle et al. 2004 | Maltose laurate, maltotriose laurate, sucrose laurate | *Streptococcus mutans* | All esters suppressed the growth at 0.05- 2% concentration of esters | Growth media and plates |
| Yang et al. 2003 | Sucrose and glucose esters | Spoilage organisms *Z. bailii* and *L fructivorans* | 1% sucrose esters of laurate, myristate or palmitate inhibited the growth of the organisms in salad dressing and were more effective than 0.1% sodium benzoate | Salad dressing |
| Watanabe et al. 2000 | 23 different synthesized sugar esters | *Streptococcus mutans* | Galactose and sucrose laurates inhibited growth at <0.05% | Microbial media |
| Shearer et al. 2000 | Sucrose laurate, palmitate and stearate | *Bacillus* and *Clostridium spores* | A combined treatment of sucrose laurate (1%), 392 MPa pressure provided a 3-5.5 log10 DFU/mL reduction of Bacillus in milk and beef. | Various foods |

The synthesis efficiency of LML depends on the activity of the immobilized lipase and the type of organic solvent (e.g. ethanol, isopropanol, tert-amyl alcohol, acetone). For each new commercial lipase used, the efficiency of LML synthesis must be determined; therefore, this thesis also investigated the synthesis of LML as dependent on the lipase and solvent.

**Regulations of Sugar Esters**

Sugar fatty-acid esters can be added into food as emulsifiers as stated in the Code of Federal Regulation title 21 section 170.3 (o) (8), and they are allowed in dairy products (U.S. Code of Federal Regulation title 21 section 172.859 (c) (1)). The usage of sugar esters in dairy products ranges between 0.1% (0.1 mg/mL) to 0.5% (0.5 mg/mL), and the maximum usage of sugar fatty acid is 5% (5 mg/mL) (U.S. Code of Federal Regulation title 21 section 172.859 (b) (2)).

# HYPOTHESES AND OBJECTIVES

The hypotheses of this study were:

1. Synthesis of LML is influenced by various lipases.
2. LML is bactericidal to *L. monocytogenes* in foods.

The objectives of this study were:

1. Find the most efficient lipase to synthesize LML.
2. Compare the bactericidal effects of LML, lauric acid, and Tween 20 in BHI media at 0.01 to 5 mg/mL with one strain of *L. monocytogenes*.
3. Investigate the bactericidal effects of LML and lauric acid in different kinds of dairy products (milk, yogurt, and cheese) at concentrations from 1 to 5 mg/mL to find the MBC with a 5-strain cocktail of *L. monocytogenes*.
4. Compare the bactericidal effects of LML in dairy products under room temperature and refrigerator temperature.
5. Confirm that LML inhibited the viable but non-culturable state of a 5-strain cocktail of *L. monocytogenes* in BHI media.

# MATERIALS AND METHODS

## Materials

Materials and equipment included a HPLC (Beckman), microtitre well plate reader (HTS Ole 7000), spectrophotometer (Beckman), stomacher (AESAP 1068), shaker (Lab-Line), disperser (IKA T25), incubator (Utah State University), 48 well microtiter well plates (Becton Dickinson), brain heart infusion (BD), granulated agar (BD), and acetonitrile (HPLC grade, Thermo Fisher). Lactose (Proliant), molecular sieves (3A), anhydrous isopropanol, vinyl laurate, ethanol, Tween 20, and Tween 80 were from Sigma Aldrich. Immobilized lipases *R. miehei* (RM1, RM2 and RM3), *Thermomyces launuginose* (TM1, TM2 and TM3), *Pseudomonas cepacia* (PC1, PC2 and PC3), *Candida antarctica* (CA1), *Candida rugosa* (CR), *Rhiyzopus oryzae* (RO), and *Candida antarctica B* (CAB1 and CAB2) were from Sigma Aldrich. Phosphate buffer saline (PBS) (pH 7.4) was from Thermo Fisher. Palcam broth, agar base and supplements with polymixin B, acriflavine and ceftazidime were from Neogen. *L. monocytogenes* isolates (FSL C1-056, FSL J1-177, FSL N1-227, FSL N3-013, FSL R2-499) were obtained from Dr. Martin Wiedmann, director of the International Life Sciences Institute North American Database at Cornell University. Sterile milks (fat free, 1% fat and 1.5% fat) and cream (8% fat) were purchased from Gossner Foods, Inc (Logan UT). Drinkable yogurts (fat free, 1% fat and 1.5% fat) and cottage cheese (fat free and 2% fat made from cultured milk) were purchased locally.

**LML Synthesis**

To set up a 3 mL LML synthesis reaction, 44 mg of lactose was added to 3 mL of anhydrous isopropanol with 10% dried molecular sieves, followed by the addition of 0.124 mL of vinyl laurate and 90 mg of one of the 14 immobilized lipases. The reactions were assembled in 4 mL glass vials (Utah State University) and incubated on a shaker at 55°C for 3 days. The reactions were analyzed at room temperature by the HPLC (Beckman System Gold 125 Solvent Module) equipped with a Luna 5 micron C18 (2) 100 Å column (250 mm × 4.6 mm, Phenomenex, Torrance, CA, USA). The mobile phase consists of a gradient from 10% acetonitrile: water (40:60) to 100% acetonitrile: water (95:5), with a flow rate of 1.0 mL/min over 24 minutes. Products and standards were detected with an evaporative light scattering detector at 60 °C with a nitrogen gas pressure of 3.65 bar.

**Inoculum Preparation**

Five strains of *L. monocytogenes* (FSL C1-056, FSL J1-177, FSL N1-227, FSL N3-013, FSL R2-499) were used in antimicrobial testing in dairy products. Freezer stocks were kept at -80°C. An aliquot of each freezer stock, 20 µL, was transferred into 15 mL of fresh BHI media and was grown with shaking at 37°C for 24 hours. The optical density (OD) at 600 nm for each strain was adjusted to 0.2 with BHI media.

The 5-strain cocktail was prepared by mixing 2 mL aliquots of each strain into a sterile, 50 mL centrifuge tube. Phosphate-buffered saline solution (100 mL) was added and the sample was centrifuged (3000 RPM for 10 minutes). The cell precipitate was re-suspended in 10 mL of PBS and centrifuged, then re-suspended and centrifuged again. The 5-strain cocktail stock was kept at -20°C.

Freezer stocks of all microorganisms were kept at -20°C prior to growth in BHI media at 37°C. After 1 hour of thawing, 100 µL of culture was inoculated into 15 mL of BHI media. Cells were grown with shaking at 37°C for 24 hours. The overnight culture, 315 µL, was subcultured into 12 mL of BHI media, and incubated in a shaker at 37°C for 4 hours. The OD of the culture was measured by using the spectrophotometer at 600 nm. When the OD approached 0.2 nm, an aliquot of 30 µL was subcultured again into 30 mL of BHI media and this was used in the experiments.

**Antibacterial Test on *L. monocytogenes* N3-013 in BHI Media**

Treatments were created with LML, lauric acid, lactose, and Tween 20 using 20 mg/mL stocks in 50% of ethanol-water, with ethanol-water as a control with *L. monocytogenes* N3-013. *L. monocytogenes* was grown as described above for the 5-strain cocktail. The total volume of each replicate was 0.5 mL. The concentrations of treatments were 0.01, 0.05, 0.1, 1, 3 and 5 mg/mL. The final ethanol content of 6 concentrations in both treatment and controls were 0.025%, 0.125%, 0.25%, 2.5%, 7.5% and 12.5% respectively. For each concentration of LML, lauric acid, and Tween 20, there were 6 replicates for both control and treatment. Microtiter well plates were incubated in a shaker at 37°C for 24 hours. The number of cells in each treatment was determined by enumeration via plate counts. A t-test of the controls and the treatments was done to determine significance.

**Antibacterial Test in Dairy Products**

The 5-strain cocktail stock, 100 µL, was inoculated into 15 mL of BHI media and grown with shaking at 37°C for 24 hours. An aliquot, 315 µL, was subcultured into 12 mL of BHI media, then incubated in a shaker at 37°C for 4 hours. When the OD

approached 0.2, an aliquot, 30 µL, was subcultured again into 30 mL each of sterile milk, yogurt, and cottage cheese. LML (in 50% ethanol:water) was added to samples at concentrations of 1, 3, or 5 mg/mL for milk and 3 and 5 mg/mL for yogurt and cottage cheese. The total volume of each sample was 0.5 mL. For each concentration of LML, there were 6 replicates for both control (50% ethanol: water solution) and treatment. The final ethanol concentration in 1, 3, and 5 mg/mL of both treatment and controls were 2.5%, 7.5%, and 12.5% respectively. Microtiter well plates were incubated in a shaker at 37°C or in a fridge at 5°C for 24 hours. Cells were diluted with PBS solution and plated onto a PALCAM agar plate, and incubated at 37°C. Cells were enumerated via plate counts after 24 hours. Prior to antimicrobial testing, the cottage cheese was homogenized via a stomacher. A t-test of the controls and the treatments was done to determine significance.

**Homogenization**

Five milliliter of whole milk was kept at 50 °C in a 15 mL sterile tube. Add 25 or 40 mg of dry LML products into the aliquot. LML was homogenized into milk sample by using a disperser (IKA T 25 digital ULTRA-TURRAX) for 5 minutes (25000 RPM). The homogenization procedure was done in a biosafety cabinet. All equipment used in homogenization were required to be sterilized.

**Probiotics Test of Dairy Products**

Fat-free yogurt and cottage cheese were treated with or without 5 mg/mL of LML. The total volume of each sample was 0.5 mL. Six replicates for both control and treatment were added into microtiter well plates and incubated in a shaker at 37°C for 24

hours. Cells were diluted using PBS solution, then 0.1 mL of the diluted aliquot was placed onto a LB agar plate and incubated at 37°C for 24 hours. Total cells (probiotic) were enumerated via plate counts after 24 hours without LML treatment.

**Confirmation of VBNC State of *L. monocytogenes***

The *L. monocytogenes* 5-strain cocktail was cultured in BHI media with 5 mg/mL of LML for 24 hours. The treated cultures were diluted 0, 10, 50, 100 and 1000 fold, and allowed to grow at both 37°C and 5°C. The number of cells via plate counts was determined once a week for 6 weeks.

# RESULTS AND DISCUSSION

## Synthesis of LML

Immobilized lipases were used to synthesize LML. The immobilized enzyme technique can allow enzymes to be held in place throughout the reaction, following which they are easily separated from the products and can be used again, thus helping cut the synthesis cost.

This synthesizing reaction is a reversible, esterification reaction, so the reaction should proceed in the absence of water. With lipases, the esterification reaction takes place under non-aqueous conditions, while the hydrolysis reaction takes place under aqueous conditions.

Different kinds of lipases were used to synthesis LML. The yield of LML was measured by HPLC after 3 days. Enzymes RM1, TM3, and PC2 were the top three highest yield of final product (Table 2). In terms of purity, TM3 and PC2 were more satisfactory, since these two lipases had less impurity peaks. Therefore, TM3 and PC2 were chosen to use in the further synthesizing experiments.

**Table 2.** Yield of synthesized LML of each enzyme after 3-day reaction

| S/No | Enzyme | LML Peak Area | LML Peak Area % | Total Number of Peaks | Numbers of LML Peaks | Amount of LML mg/mL |
|---|---|---|---|---|---|---|
| 1 | RM1 | 5.539 | 47.567 | 4 | 1 | 5.54 |
| 2 | RM2 | 2.52 | 25.71 | 7 | 1 | 1.09 |
| 3 | RM3 | 0 | 0 | 2 | 0 | 0 |
| 4 | TM1 | 1.78 | 34.2 | 4 | 1 | 0.72 |
| 5 | TM2 | 0.7289 | 46.68 | 3 | 1 | 0.26 |
| 6 | TM3 | 7.89 | 88.08 | 3 | 1 | 3.59 |
| 7 | PC1 | 0.40 | 23.22 | 3 | 0 | 0.1 |
| 8 | PC2 | 6.31 | 100 | 1 | 1 | 2.85 |
| 9 | PC3 | 0.83 | 41.04 | 3 | 1 | 0.3 |
| 10 | RO | 0 | 0 | 1 | 0 | 0 |
| 11 | CR | 0 | 0 | 1 | 0 | 0 |
| 12 | CA1 | 2.70 | 77.55 | 2 | 0 | 1.18 |
| 13 | CAB1 | 0 | 0 | 0 | 0 | 0 |
| 14 | CAB2 | 0 | 0 | 0 | 0 | 0 |

**Antibacterial Test on *L. monocytogenes* N3-013 in BHI Media**

Previous research from Wagh et al. (2012) showed that LML inhibited the growth of 5 isolates of *L. monocytogenes.* This paper prompted the decision to use up to 5 mg/mL of LML as treatment in dairy products.

Lactose, lauric acid (Figure 3) and Tween 20 (Figure 4) were used to determine which moiety of LML was responsible for its bactericidal activities. Tween 20 is a sugar ester (sorbitan monolaurate) similar to LML, and it can also be used as an emulsifier. So, testing Tween 20 and comparing it with LML showed the influence of the sugar on the antibacterial effect.


**Figure 3.** Structure of lauric acid.

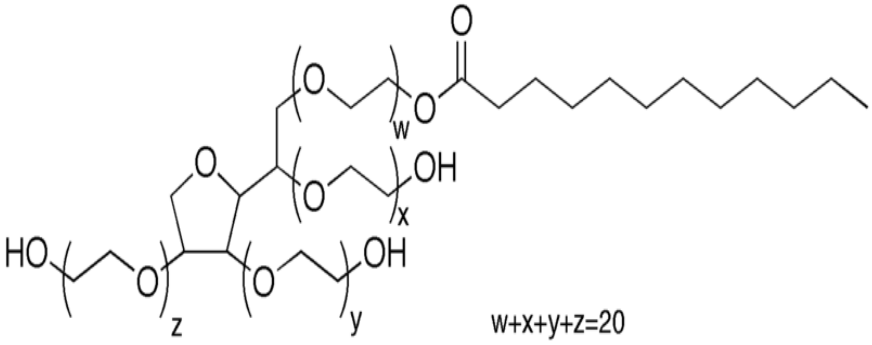


**Figure 4.** Structure of Tween 20.

LML started to show an inhibitory effect at 0.01 mg/mL (0.02 mM) and had a bactericidal effect at 5 mg/mL (Figure 5). The MBC was between 3 (5.7 mM) and 5 mg/mL (9.5 mM). The MIC of LML was lower than the result from Wagh et al., (2012), but the MBC conformed with their results. With 5 mg/mL of LML, a 4.3 ± 0.04 log reduction of *Listeria* was achieved (raw data is given in appendix A).

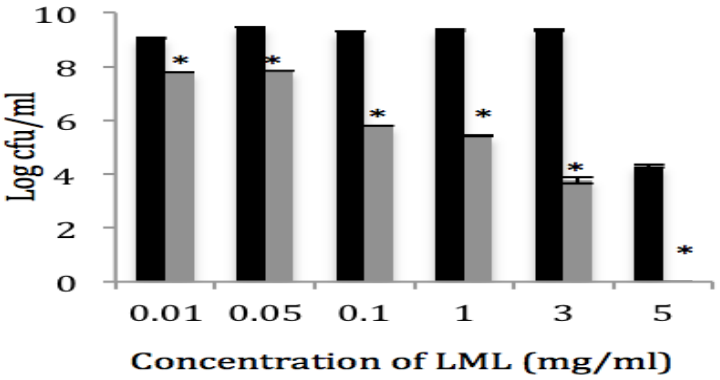


**Figure 5.** Average counts (log CFU/mL) of *L. monocytogenes* N3-013 treated with 0.01, 0.05, 0.1, 1, 3 or 5 mg/mL of LML in BHI media after 24 hours. The black bars are controls and the grey bars are treatments. The error bars represent the standard deviation and the asterisks indicate significant difference between control and treatment.

Lactose had no effect on inhibiting *Listeria.* There were no significant difference between control and treatment after 24 hours (data not shown). Lauric acid showed a similar result as LML, but the antibacterial effect was not as great as LML (Figure 6). The MIC of lauric acid was 0.1 mg/mL (0.5 mM) and the MBC was greater than 5 mg/mL. Some bacteria survived after being treated with 5 mg/mL of lauric acid. The log reduction of *Listeria* was 3.5 ± 0.2.

Monolaurin (glyceryl monolaurate), as an emulsifier, can be added into food. Wang et al. (1992) found that the MBC of monolaurin against *L. monocytogenes* in BHI was 10 to 20 mg/mL. In Nobmann et al. (2009), lauric ester of methyl alpha-D-

glucopyranoside and lauric ester of methyl alpha-D-mannopyranoside showed the highest growth-inhibitory effect with MIC values of 0.04 mM against *L. monocytogenes*, which was similar to the MIC reported in this paper (0.02 mM). The MIC for lauric acid in their studies with *L. monocytogenes* ranged from 0.63 to 1.25 mM, which was higher than the lauric esters, and was close to the MIC reported here (0.5 mM).

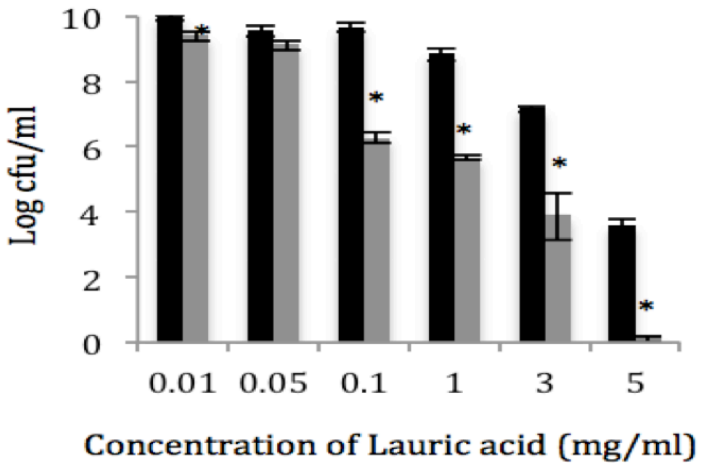


**Figure 6.** Average counts (log CFU/mL) of *L. monocytogenes* N3-013 treated with 0.01, 0.05, 0.1, 1, 3 or 5 mg/mL of lauric acid in BHI media after 24 hours. The black bars are controls and the grey bars are treatments. The error bars represent the standard deviation and the asterisks indicate significant difference between control and treatment.

Tween 20 showed a bacteriostatic effect when the concentration of treatment was 5 mg/mL and the log reduction of *Listeria* was only 1.6 ± 0.08 (Figure 7). In Figures 5, 6 and 7, 5 mg/mL of control (50% of ethanol) resulted in a significant decrease compared with 3 mg/mL of control, which was because of the difference between the ethanol concentrations. The final ethanol contents of 3 and 5 mg/mL controls were 7.5% and 12.5%. In 5 mg/mL of control, the final ethanol concentration was almost twice that in 3 mg/mL of control, so the larger amount of ethanol killed much more bacteria than 3

mg/mL of control. However, 5 mg/mL of LML could still achieve a 4.3 log reduction, which still showed significant bactericidal properties.

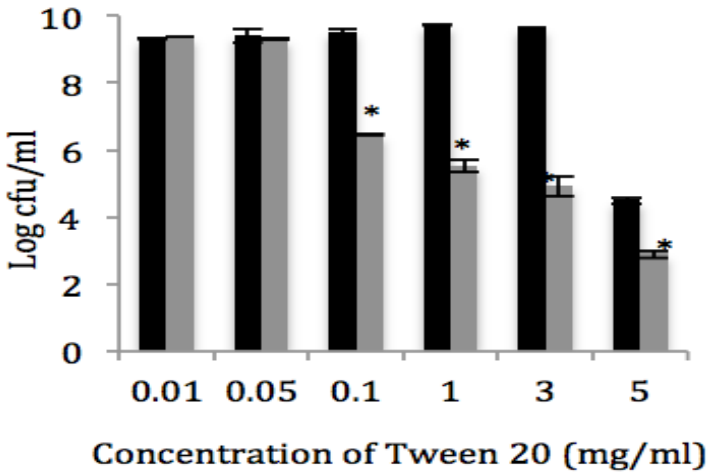


**Figure 7.** Average counts (log CFU/mL) of *L. monocytogenes* N3-013 treated with 0.01, 0.05, 0.1, 1, 3 or 5 mg/mL of Tween 20 in BHI media after 24 hours. The black bars are controls and the grey bars are treatments. The error bars represent the standard deviation and the asterisks indicate significant difference between control and treatment.

As with the Smith et al. (2009) study, they also found that the carbohydrate moiety is involved in the antimicrobial activity of the sugar esters and that the nature of the bond (ether or ester between the sugar and fatty acid) also has a significant effect on efficacy. The MBC and MIC of LML is lower than that for a similar sugar ester, Tween-20, and similar to lauric acid, therefore it is believed that the lauric acid is the critical moiety for bactericidal activity and the lactose influences this activity. Therefore, lauric acid moiety conveyed the antibacterial properties of LML. The type of sugar attached to the lauric acid and the degree of esterification results in different antibacterial properties.

**Antibacterial Test in Milk and Cream**

When testing the in dairy products, a *L. monocytogenes* 5-strain cocktail was inoculated into the products. Testing a 5-strain cocktail instead of a single strain made it more practical since it is very possible to have more than one isolate growing in products. To test the antibacterial effect of LML, it was necessary to simulate a potentially real situation of *Listeria* contamination in a food system. The initial inoculating concentration of *Listeria* was 5 log CFU/mL, which is a very severe contamination level that could almost never happen in real life. So, a 4 log reduction of *Listeria* can be considered very effective.

In the milk experiment, 1, 3 and 5 mg/mL of LML were used. This was based on the results from the growth media experiment, which showed that 0.01, 0.1 and 0.5 mg/mL of LML did not have significant inhibitory effects. In fat free milk, 5 mg/mL of LML showed a bactericidal effect on the growth of *Listeria* (raw data is given in appendix B). A 4.45 ± 0 log reduction was achieved (Figure 8).

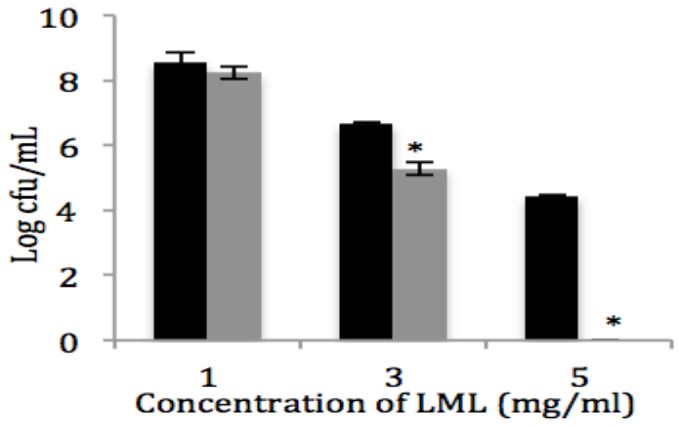


**Figure 8.** Average counts (log CFU/mL) of *L. monocytogenes* 5-strain cocktail treated with 1, 3 or 5 mg/mL of LML in fat-free milk after 24 hours. The black bars are controls and the grey bars are treatments. The error bars represent the standard deviation and the asterisks indicate significant difference between control and treatment

With low-fat and whole milk, 5 mg/mL (9.5 mM) of LML inhibited the growth of *Listeria* significantly, but LML was not bactericidal (Figures 9, 10). The log reductions at 5 mg/mL of *Listeria* in these two milks were 4.01 ± 0.13 and 4.25 ± 0.10. LML had no inhibitory effect on *Listeria* in cream (8% fat) at concentrations up to 5 mg/mL of LML (data not shown). These results were similar to previous results of antibacterial tests of LML in BHI media on single strain of *L. monocytogenes*. With each concentration, there is a corresponding control that contains the same amount of ethanol, which in itself inhibited the growth of *L. monocytogenes* in certain level. The antimicrobial effect in the 3 and 5 mg/mL samples may have been due to the compounding stress of both the ethanol and the LML.

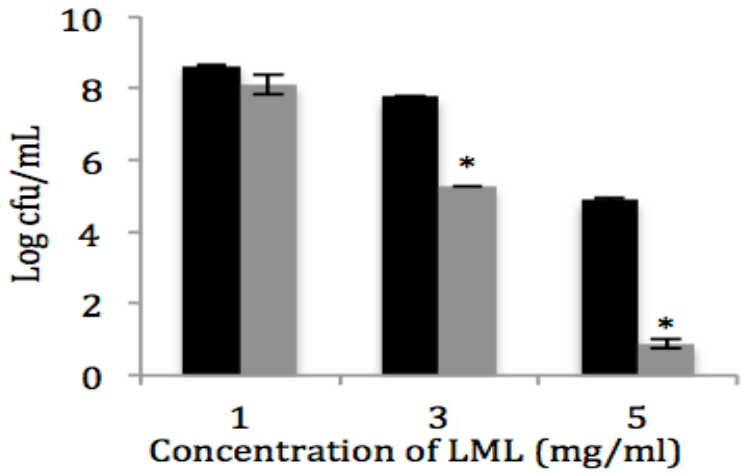


**Figure 9.** Average counts (log CFU/mL) of *L. monocytogenes* 5-strain cocktail treated with 1, 3 or 5 mg/mL of LML in low-fat milk after 24 hours. The black bars are controls and the grey bars are treatments. The error bars represent the standard deviation and the asterisks indicate significant difference between control and treatment.

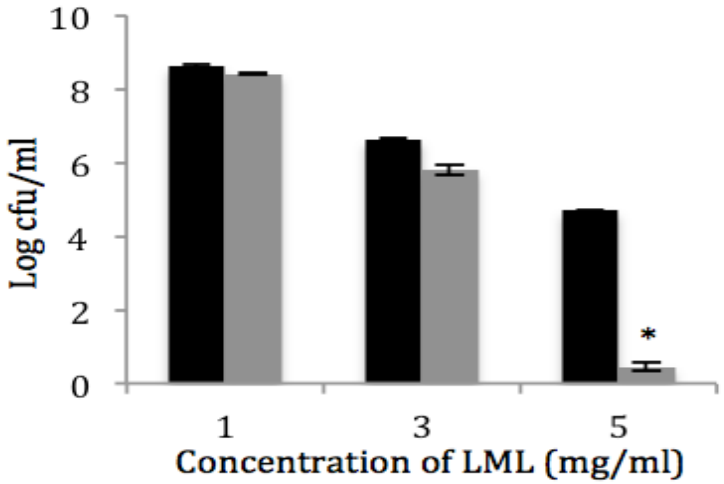


**Figure 10.** Average counts (log CFU/mL) of *L. monocytogenes* 5-strain cocktail treated with 1, 3 or 5 mg/mL of LML in whole milk after 24 hours. The black bars are controls and the grey bars are treatments. The error bars represent the standard deviation and the asterisks indicate significant difference between control and treatment.

The antibacterial effect of LML was related to the fat content of the dairy products. Because of the amphipathic nature of LML, it may have partially dissolved into the fat phase of low-fat milk, whole milk, and cream. As a result, there were less LML in the system can be used to kill bacteria. So, the higher the fat content in the products, the weaker the bacteriostatic effect of LML will become.

Directly homogenizing LML into dairy reduced its antibacterial effect. Our results showed that 5 mg/mL of LML did not have significant inhibitory effect on *Listeria* in whole milk (data not shown). Based on this result, the concentration of LML was raised up to 8 mg/mL. However, 8 mg/mL of LML, which was higher than the maximum usage level that USDA regulation (5 mg/mL), did not have bactericidal effect on *L. monocytogenes*. Another possible reason leaded this unsatisfactory result was because of the contamination of the sample. Therefore, homogenize LML directly into milk was considered infeasible.

**Antibacterial Test in Drinkable Yogurt**

Based on the results from the milk experiments, 1 mg/mL of LML did not have a significant bacteriostatic effect. So, in the drinkable yogurt and cottage cheese experiments, only 3 and 5 mg/mL of both control and treatment were used.

In fat free and 1% fat drinkable yogurt, 5 mg/mL (9.5 mM) of LML killed all the viable *Listeria* corresponding to a 4.08 ± 0 and 4.35 ± 0.77 log reduction respectively (Figures 11, 12) (raw data is given in appendix C). Because of the increasing of fat content in 1.5% fat yogurt, 5 mg/mL of LML showed a bacteriostatic effect and resulted a 3.54 ± 0.31 log reduction in *Listeria* (Figure 13). The antibacterial effects of LML in yogurt were similar to the effects in milk. However, the control cells of yogurt were lower than milk, which may because of the different growth environment for *Listeria*. *Listeria* grows at pH values between 4.3 and 9.4 with an optimum at pH 7 (Faber and Peterkin, 1991). Since the pH value of milk was around 7, more *Listeria* control cells grew in milk than in yogurt (pH valued around 4.5).

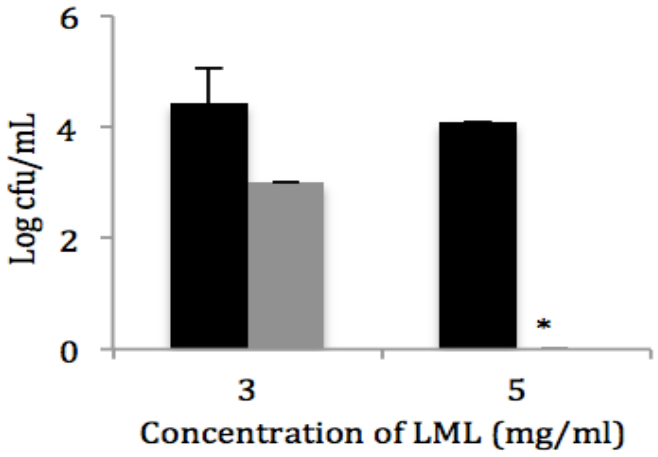


**Figure 11.** Average counts (log CFU/mL) of *L. monocytogenes* 5-strain cocktail treated with 3 or 5 mg/mL of LML in fat-free drinkable yogurt after 24 hours. The black bars are controls and the grey bars are treatments. The error bars represent the standard deviation and the asterisks indicate significant difference between control and treatment.

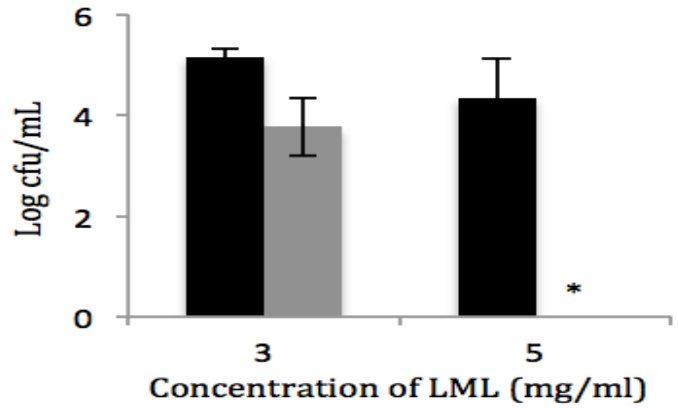


**Figure 12.** Average counts (log CFU/mL) of *L. monocytogenes* 5-strain cocktail treated with 3 or 5 mg/mL of LML in 1% fat drinkable yogurt after 24 hours. The black bars are controls and the grey bars are treatments. The error bars represent the standard deviation and the asterisks indicate significant difference between control and treatment.

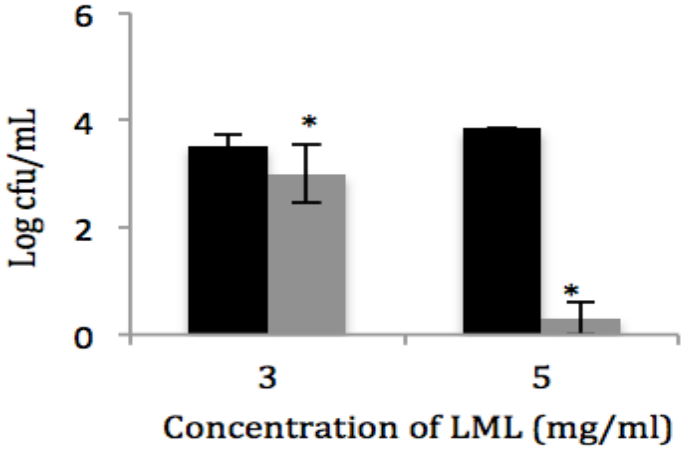


**Figure 13.** Average counts (log CFU/mL) of *L. monocytogenes* 5-strain cocktail treated with 3 or 5 mg/mL of LML in 1.5% fat drinkable yogurt after 24 hours. The black bars are controls and the grey bars are treatments. The error bars represent the standard deviation and the asterisks indicate significant difference between control and treatment.

Since the yogurt used in this research contained probiotics (*Lactobacillus reuteri* and *Biodobacterium lactis*), it was necessary to measure the CFU/mL of the probiotics in each yogurt to determine whether LML had any bacteriostatic effect on the probiotics. Average count of probiotics in fat free yogurt was 7.33 log CFU/mL. After treated with 5 mg/mL of LML for 24 hours, there were no viable probiotics. Therefore, LML killed all the viable *Listeria* and the probiotics in fat-free and 1% fat drinkable yogurt, and is bacteriostatic in 1.5% fat drinkable yogurt.

**Antibacterial Test in Cottage Cheese**

In fat-free and 2% fat cottage cheese, there was no viable *Listeria* culture after being treated with 5 mg/mL (9.5 mM) of LML (Figures 14, 15). The log reduction of *Listeria* in both cottage cheese after being treated for 24 hours were 4.0 ± 0.17 and 4.05 ± 0.13 respectively (raw data is given in appendix D).

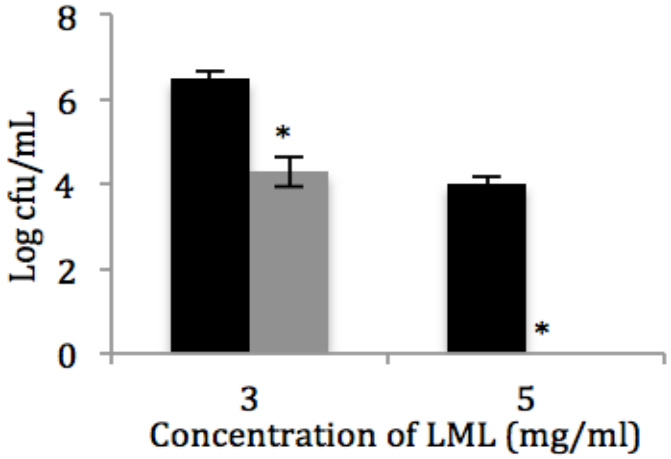


**Figure 14.** Average counts (log CFU/mL) of *L. monocytogenes* 5-strain cocktail treated with 3 or 5 mg/mL of LML in fat-free cottage cheese after 24 hours. The black bars are controls and the grey bars are treatments. The error bars represent the standard deviation and the asterisks indicate significant difference between control and treatment

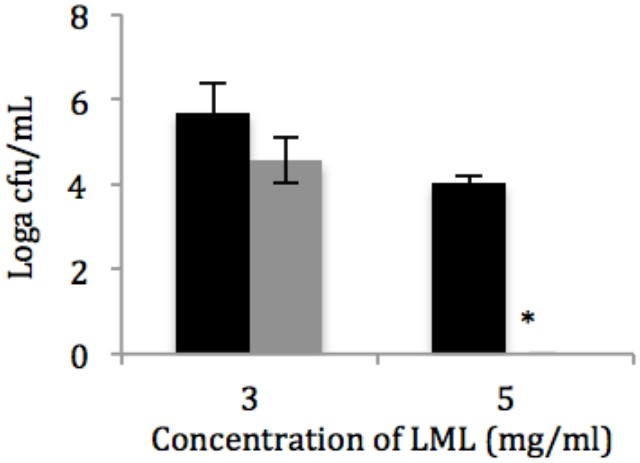


**Figure 15.** Average counts (log CFU/mL) of *L. monocytogenes* 5-strain cocktail treated with 3 or 5 mg/mL of LML in 2% fat cottage cheese after 24 hours. The black bars are controls and the grey bars are treatments. The error bars represent the standard deviation and the asterisks indicate significant difference between control and treatment.

Similar to yogurt, growth of the control cells in cottage cheese was lower than in milk, which may have been due to the low pH value of cheese (around 5.1). The cottage cheese also contained probiotics, and the average CFU/mL of the probiotics was 7 log CFU/mL. After being treated with 5 mg/mL of LML for 24 hours, there were no probiotics. Therefore, LML killed all the viable *Listeria* and the probiotics in fat free and 2% fat cottage cheese, and is bacteriostatic in 1.5% fat drinkable yogurt. In summary, 5 mg/mL of LML killed all the *Listeria* in several dairy products and could achieve a 4 log reduction.

Sugar ester as an emulsifier should be mixed or homogenized into dairy products to decrease droplet size or air bubble size and to get the emulsifier distributed on the surface of the interface (Tual et al., 2006). Another concern is the ethanol content in dairy products. With 5 mg/mL of LML, the final ethanol concentration is 12.5%. Whether this

high content of ethanol in diary products would affect the flavor or shelf life is unknown. For further study, reducing the ethanol content by improving synthesizing technology needs to be done. Sensory testing is also necessary in further study.

**Antimicrobial Test at 5°C**

Dairy products are usually kept in a refrigerator. Therefore, antibacterial tests should also be done at refrigeration temperature. Additionally, LML is a fatty acid ester, and the solubility of ester is temperature related. Previous research has noted that LML only dissolves in warm water or in a binary solvent with equal volumes of ethanol and water only at 22°C or higher. In order to keep LML completely dissolved, this experiment needs to be conducted under room temperature. Otherwise, while using partially dissolved LML, the antibacterial effect will be reduced significantly.

Five mg/mL of LML were treated in each kind of dairy product and were cultivated at refrigeration temperature (5°C). However, the result was not satisfactory (data not shown). There was no significant inhibition on *Listeria* growth. To use LML in dairy products, the treated products need to be kept at room temperature. However, keeping dairy products at room temperature will shorten their shelf life.

**Confirmation of VBNC State of *L. monocytogenes***

*L. monocytogenes* has been reported to enter a VBNC state when exposed to stress conditions such as resource depletion, extreme temperature, high osmolarity and pressure (Oliver, 2005; Dreux et al., 2007). The morphology of *L. monocytogenes* changed from the normal rod shape to cocci in chains when exposed to LML at 1 mg/mL, which is consistent with a stressed state (Wen et al., 2009; Wagh et al., 2012). Therefore,

the hypothesis that *L. monocytogenes* was not in a VBNC state when treated with LML was tested. Five isolates of *L. monocytogenes* cocktail were treated with 5 mg/mL LML and was diluted to reduce the effect of surfactant. Dilutions up to 1000 fold resulted in 10 to 100 CFU/mL and 5 mg/mL LML. The culture was allowed to keep growing for 6 weeks at both 37°C and 5°C, and cells were enumerated once a week. No viable *Listeria* culture was detected for up to 6 weeks (data not shown). Five mg/mL of LML effectively inhibited the VNBC state of *L. monocytogenes*.

# CONCLUSIONS

The immobilized lipases TM3 and PC2 were the two most effective immobilized lipases to synthesis LML. Lauric acid moiety is responsible for the bacteriostatic effect of LML. The type of sugar used to synthesize sugar ester and the degree of esterification leads to different antibacterial properties.

Five mg/mL of LML can effectively inhibit the growth of *L. monocytogenes* in growth media and can achieve approximately a 4 log reduction in dairy products. The MBC of LML on *Listeria* was between 3 and 5 mg/mL.

With fat-free milk, fat-free drinkable yogurt, 1% fat drinkable yogurt, fat-free cottage cheese, and 2% fat cottage cheese, 5 mg/mL of LML killed all the viable *Listeria* culture corresponding to a 4 to 4.45 log reduction. With low-fat milk, whole milk, and 1.5% fat drinkable yogurt, LML had a bacteriostatic effect and achieved a 3.54 to 4.01 log reduction of *Listeria*. LML did not show significant inhibition on *Listeria* in cream. The antibacterial effect of LML was related to the fat content of the dairy products. With a higher content of fat, the antibacterial effect was weaker due to the amphipathic nature of LML.

Since LML is a sugar ester, its solubility is temperature related. LML only dissolved in warm conditions. Therefore, LML did not show a bacteriostatic effect at 5 °C.

Five mg/mL of LML can inhibit the VBNC state of *Listeria* for up to 6 weeks.

# FUTURE RESEARCH

Although sugar ester has been reported tasteless and odorless, the effect of adding LML into dairy products on the taste is still known. A sensory to investigate on the taste, texture and odor of the dairy products with LML is necessary in future research. Studies of the antibacterial effect of LML in other kind of food such as meat can also be done in future.

With 5 mg/mL of LML, the ethanol content was 12.5%, which was had microbial inhibitory effects. Recent research has shown that the ethanol content of the solvent o to solubilize LML can be reduced by adding dimethyl sulfoxide (DMSO).

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# APPENDICES

**Appendix A. Antibacterial Test on *L. monocytogenes* N3-013 in BHI Media**

Antibacterial effect of LML (Control) on *L. monocytogenes* N3-013 in BHI Media

| Concentration of Control (mg/mL) | Average Counts (log CFU/mL) | Error |
|---|---|---|
| 0.01 | 9.05 | 0.02 |
| 0.05 | 9.47 | 0 |
| 0.1 | 9.30 | 0 |
| 1 | 9.35 | 0.06 |
| 3 | 9.36 | 0.03 |
| 5 | 4.30 | 0.05 |

Error represents the standard deviation divided by square root of sample size.

Antibacterial effect of LML (Treatment) on *L. monocytogenes* N3-013 in BHI Media

| Concentration of LML (mg/mL) | Average Counts (log CFU/mL) | Error |
|---|---|---|
| 0.01 | 7.78 | 0 |
| 0.05 | 7.85 | 0 |
| 0.1 | 5.80 | 0.012 |
| 1 | 5.42 | 0.024 |
| 3 | 3.78 | 0.12 |
| 5 | 0 | 0 |

Error represents the standard deviation divided by square root of sample size.

Antibacterial effect of LML on *L. monocytogenes* N3-013 in BHI Media

| Concentration (mg/mL) | 0.01 | 0.05 | 0.1 | 1 | 3 | 5 |
|---|---|---|---|---|---|---|
| P-value | 0.001 | <0.0001 | <0.0001 | 0.0005 | 0.001 | 0.0002 |

Antibacterial effect of Lauric Acid (Control) on *L. monocytogenes* N3-013 in BHI Media

| Concentration of Control (mg/mL) | Average Counts (log CFU/mL) | Error |
|---|---|---|
| 0.01 | 9.95 | 0.07 |
| 0.05 | 9.70 | 0.17 |
| 0.1 | 9.64 | 0.14 |
| 1 | 8.82 | 0.19 |
| 3 | 7.16 | 0.09 |
| 5 | 3.58 | 0.20 |

Error represents the standard deviation divided by square root of sample size.

Antibacterial effect of Lauric Acid (Treatment) on *L. monocytogenes* N3-013 in BHI Media

| Concentration of Lauric Acid (mg/mL) | Average Counts (log CFU/mL) | Error |
|---|---|---|
| 0.01 | 9.37 | 0.14 |
| 0.05 | 9.09 | 0.15 |
| 0.1 | 6.28 | 0.16 |
| 1 | 5.66 | 0.08 |
| 3 | 3.87 | 0.72 |
| 5 | 0.10 | 0.09 |

Error represents the standard deviation divided by square root of sample size.

Antibacterial effect of Lauric Acid on *L. monocytogenes* N3-013 in BHI Media

| Concentration (mg/mL) | 0.01 | 0.05 | 0.1 | 1 | 3 | 5 |
|---|---|---|---|---|---|---|
| P-value | 0.01 | 0.11 | 0.001 | <0.0001 | <0.0001 | 0.0008 |

Antibacterial effect of Tween 20 (Control) on *L. monocytogenes* N3-013 in BHI Media

| Concentration of Control (mg/mL) | Average Counts (log CFU/mL) | Error |
|---|---|---|
| 0.01 | 9.32 | 0.01 |
| 0.05 | 9.41 | 0.21 |
| 0.1 | 9.52 | 0.06 |
| 1 | 9.73 | 0.01 |
| 3 | 9.63 | 0.018 |
| 5 | 4.48 | 0.08 |

Error represents the standard deviation divided by square root of sample size.

Antibacterial effect of Tween 20 (Treatment) on *L. monocytogenes* N3-013 in BHI Media

| Concentration of Tween 20 (mg/mL) | Average Counts (log CFU/mL) | Error |
|---|---|---|
| 0.01 | 9.36 | 0 |
| 0.05 | 9.3 | 0.01 |
| 0.1 | 6.455 | 0.06 |
| 1 | 5.51 | 0.18 |
| 3 | 4.91 | 0.31 |
| 5 | 2.89 | 2.89 |

Error represents the standard deviation divided by square root of sample size.

Antibacterial effect of Tween 20 on *L. monocytogenes* N3-013 in BHI Media

| Concentration (mg/mL) | 0.01 | 0.05 | 0.1 | 1 | 3 | 5 |
|---|---|---|---|---|---|---|
| P-value | 0.095 | 0.76 | 0.0009 | 0.004 | 0.008 | 0.014 |

**Appendix B. Antibacterial Test on *L. monocytogenes* 5 Clinical Isolates Cocktail in Milk Products**

Antibacterial effect of LML (Control) on *L. monocytogenes* 5 Clinical Isolates Cocktail in Fat-Free Milk

| Concentration of Control (mg/mL) | Average Counts (log CFU/mL) | Error |
|---|---|---|
| 1 | 9.35 | 0.057 |
| 3 | 9.355 | 0.03 |
| 5 | 4.3 | 0.047 |

Error represents the standard deviation divided by square root of sample size.

Antibacterial effect of LML (Treatment) on *L. monocytogenes* 5 Clinical Isolates Cocktail in Fat-Free Milk

| Concentration of LML (mg/mL) | Average Counts (log CFU/mL) | Error |
|---|---|---|
| 1 | 8.23 | 0.38 |
| 3 | 5.29 | 0.4 |
| 5 | 0 | 0 |

Error represents the standard deviation divided by square root of sample size.

Antibacterial effect of LML on *L. monocytogenes* 5 Clinical Isolates Cocktail in Fat-Free Milk

| Concentration (mg/mL) | 1 | 3 | 5 |
|---|---|---|---|
| P-value | 0.60 | 0.04 | <0.0001 |

Antibacterial effect of LML (Control) on *L. monocytogenes* 5 Clinical Isolates Cocktail in Low-Fat Milk

| Concentration of Control (mg/mL) | Average Counts (log CFU/mL) | Error |
|---|---|---|
| 1 | 8.62 | 0.088 |
| 3 | 7.77 | 0.037 |
| 5 | 4.90 | 0.074 |

Error represents the standard deviation divided by square root of sample size.

Antibacterial effect of LML (Treatment) on *L. monocytogenes* 5 Clinical Isolates Cocktail in Fat-Free Milk

| Concentration of LML (mg/mL) | Average Counts (log CFU/mL) | Error |
|---|---|---|
| 1 | 8.11 | 0.54 |
| 3 | 5.28 | 0.032 |
| 5 | 0.89 | 0.27 |

Error represents the standard deviation divided by square root of sample size.

Antibacterial effect of LML on *L. monocytogenes* 5 Clinical Isolates Cocktail in Low-Fat Milk

| Concentration (mg/mL) | 1 | 3 | 5 |
|---|---|---|---|
| P-value | 0.27 | 0.0002 | 0.002 |

Antibacterial effect of LML (Control) on *L. monocytogenes* 5 Clinical Isolates Cocktail in Whole Milk

| Concentration of Control (mg/mL) | Average Counts (log CFU/mL) | Error |
|---|---|---|
| 1 | 8.625 | 0.09 |
| 3 | 6.64 | 0.057 |
| 5 | 4.7 | 0 |

Error represents the standard deviation divided by square root of sample size.

Antibacterial effect of LML (Treatment) on *L. monocytogenes* 5 Clinical Isolates Cocktail in Whole Milk

| Concentration of LML (mg/mL) | Average Counts (log CFU/mL) | Error |
|---|---|---|
| 1 | 8.42 | 0.057 |
| 3 | 5.8 | 0.28 |
| 5 | 0.45 | 0.212 |

Error represents the standard deviation divided by square root of sample size.

Antibacterial effect of LML on *L. monocytogenes* 5 Clinical Isolates Cocktail in Whole Milk

| Concentration (mg/mL) | 1 | 3 | 5 |
|---|---|---|---|
| P-value | 0.115 | 0.05 | 0.039 |

**Appendix C. Antibacterial Test on *L. monocytogenes* 5 Clinical Isolates Cocktail in Drinkable Yogurt**

Antibacterial effect of LML (Control) on *L. monocytogenes* 5 Clinical Isolates Cocktail in fat-free drinkable yogurt

| Concentration of Control (mg/mL) | Average Counts (log CFU/mL) | Error |
|---|---|---|
| 3 | 4.43 | 0.72 |
| 5 | 4.08 | 0 |

Error represents the standard deviation divided by square root of sample size.

Antibacterial effect of LML (Treatment) on *L. monocytogenes* 5 Clinical Isolates Cocktail in fat-free drinkable yogurt

| Concentration of LML (mg/mL) | Average Counts (log CFU/mL) | Error |
|---|---|---|
| 3 | 3 | 0.62 |
| 5 | 0 | 0 |

Error represents the standard deviation divided by square root of sample size.

Antibacterial effect of LML on *L. monocytogenes* 5 Clinical Isolates Cocktail in fat-free drinkable yogurt

| Concentration (mg/mL) | 3 | 5 |
|---|---|---|
| P-value | 0.19 | 0.006 |

Antibacterial effect of LML (Control) on *L. monocytogenes* 5 Clinical Isolates Cocktail in 1% fat drinkable yogurt

| Concentration of Control (mg/mL) | Average Counts (log CFU/mL) | Error |
|---|---|---|
| 3 | 5.16 | 0.16 |
| 5 | 4.35 | 0.77 |

Error represents the standard deviation divided by square root of sample size.

Antibacterial effect of LML (Treatment) on *L. monocytogenes* 5 Clinical Isolates Cocktail in 1% fat drinkable yogurt

| Concentration of LML (mg/mL) | Average Counts (log CFU/mL) | Error |
|---|---|---|
| 3 | 3.767 | 0.57 |
| 5 | 0 | 0 |

Error represents the standard deviation divided by square root of sample size.

Antibacterial effect of LML on *L. monocytogenes* 5 Clinical Isolates Cocktail in 1% fat drinkable yogurt

| Concentration (mg/mL) | 3 | 5 |
|---|---|---|
| P-value | 0.15 | 0.0003 |

Antibacterial effect of LML (Control) on *L. monocytogenes* 5 Clinical Isolates Cocktail in 1.5% fat drinkable yogurt

| Concentration of Control (mg/mL) | Average Counts (log CFU/mL) | Error |
|---|---|---|
| 3 | 3.53 | 0.19 |
| 5 | 3.845 | 0 |

Error represents the standard deviation divided by square root of sample size.

Antibacterial effect of LML (Treatment) on *L. monocytogenes* 5 Clinical Isolates Cocktail in 1.5% fat drinkable yogurt

| Concentration of LML (mg/mL) | Average Counts (log CFU/mL) | Error |
|---|---|---|
| 3 | 3 | 0.55 |
| 5 | 0.31 | 0.31 |

Error represents the standard deviation divided by square root of sample size.

Antibacterial effect of LML on *L. monocytogenes* 5 Clinical Isolates Cocktail in 1.5% fat drinkable yogurt

| Concentration (mg/mL) | 3 | 5 |
|---|---|---|
| P-value | 0.034 | 0.001 |

**Appendix D. Antibacterial Test on *L. monocytogenes* 5 Clinical Isolates Cocktail in Cottage Cheese**

Antibacterial effect of LML (Control) on *L. monocytogenes* 5 Clinical Isolates Cocktail in fat-free cottage cheese

| Concentration of Control (mg/mL) | Average Counts (log CFU/mL) | Error |
|---|---|---|
| 3 | 6.477 | 0.30 |
| 5 | 4 | 0.17 |

Error represents the standard deviation divided by square root of sample size.

Antibacterial effect of LML (Treatment) on *L. monocytogenes* 5 Clinical Isolates Cocktail in fat-free cottage cheese

| Concentration of LML (mg/mL) | Average Counts (log CFU/mL) | Error |
|---|---|---|
| 3 | 4.30 | 0.36 |
| 5 | 0 | 0 |

Error represents the standard deviation divided by square root of sample size.

Antibacterial effect of LML on *L. monocytogenes* 5 Clinical Isolates Cocktail in fat-free cottage cheese

| Concentration (mg/mL) | 3 | 5 |
|---|---|---|
| P-value | 0.024 | 0.0004 |

Antibacterial effect of LML (Control) on *L. monocytogenes* 5 Clinical Isolates Cocktail in 2% fat cottage cheese

| Concentration of Control (mg/mL) | Average Counts (log CFU/mL) | Error |
|---|---|---|
| 3 | 4.61 | 0.70 |
| 5 | 4.04 | 0.13 |

Error represents the standard deviation divided by square root of sample size.

Antibacterial effect of LML (Treatment) on *L. monocytogenes* 5 Clinical Isolates Cocktail in 2% fat cottage cheese

| Concentration of LML (mg/mL) | Average Counts (log CFU/mL) | Error |
|---|---|---|
| 3 | 4.55 | 0.54 |
| 5 | 0 | 0 |

Error represents the standard deviation divided by square root of sample size.

Antibacterial effect of LML on *L. monocytogenes* 5 Clinical Isolates Cocktail in 2% fat cottage cheese

| Concentration (mg/mL) | 3 | 5 |
|---|---|---|
| P-value | 0.63 | 0.0002 |