University of Alberta

Effect of Bacterial Stress Response on Pathogen Enumeration and its Implications for Food Safety

by

Huaiyu Wang

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science
in
Food Science and Technology

Agricultural, Food and Nutritional Science

© Huaiyu Wang
Spring 2011
Edmonton, Alberta

Permission is hereby granted to the University of Alberta Libraries to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only. Where the thesis is converted to, or otherwise made available in digital form, the University of Alberta will advise potential users of the thesis of these terms.

The author reserves all other publication and other rights in association with the copyright in the thesis and, except as herein before provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatsoever without the author's prior written permission.

Examining Committee

David Bressler, Agricultural, Food and Nutritional Science

Lynn McMullen, Agricultural, Food and Nutritional Science

Michael Gänzle, Agricultural, Food and Nutritional Science

Tracy Raivio, Biological Sciences

This thesis is dedicated to my parents and my husband,
for their love and support.

ABSTRACT

To determine the impact of stress response on enumeration, cell association status and the viability of *Escherichia coli* DH5α, *Staphylococcus aureus* ATCC 13565 and *Listeria monocytogenes* CDC 7762 were evaluated using fluorescence microscopy and were compared with the outcomes of traditional plate count and optical density measurements. Fluorescence microscopy revealed that organic acid stress (acetic and lactic, pH 2.7-3.3) induced cell clumping with little loss of viability in *Escherichia coli* DH5α. Significantly lower values for cell enumeration were found for plate counts and OD_{600} measurement, likely due to cell clumping in response to organic acid stress. Gram-negative bacteria *Escherichia coli* DH5α showed higher levels of clumping and subsequent resistance against organic acid stress. Increased cell surface hydrophobicity was found in cells that exhibited more evident clumping. However, inorganic acid stress (hydrochloric and sulfuric, pH 3.0-3.3) induced only very low level of clumping in stationary-phase *Escherichia coli* DH5α and almost no clumping in other cultures. Osmotic stress, heat and cold shock were not found to induce cell clumping. It has been determined that traditional enumeration methods have significantly underestimated the number of viable bacterial cells when organic acid stress is involved. Plate counts and OD_{600} measurement therefore need to be reassessed as tools for accurate evaluation of pathogens in food industry.

ACKNOWLEDGMENTS

I would like to thank my supervisor Dr. David Bressler for his guidance, patience and encouragement throughout my graduate study.

I would like to thank my supervisory committee member Dr. Lynn McMullen, and my examining committee members, Dr. Michael Gänzle and Dr. Tracy Raivio for all their support and contributions to this thesis. Special thanks to Dr. Michael Gänzle for use of fluorescence microscope in his lab.

I would like to thank Dr. Daniel Barreda for his valuable advice on fluorescence microscopy. I would also like to thank Loredana Dorobantu from Dr. Murray Gray's group in the Department of Chemical and Materials Engineering for technical support on contact angle measurement. Many thanks to Dr. Xuejun Sun from Cell Imaging Facility in the Department of Oncology for his dedicated assistance on my final fluorescence microscopy results.

I would like to thank former and present lab members for their support and friendship. My special gratitude is contributable to Dr. Natisha Stashko for her valuable suggestions and dedicated help in preparing my thesis.

Lastly but not least, I would like to express my deepest appreciations to my parents, my parents-in-law, my dearest husband, all of my family members and friends, for their love and support.

TABLE OF CONTENTS

Title Page
Examining Committee Page
Dedication
Abstract
Acknowledgements
Table of Contents
List of Tables
List of Figures
List of Symbols and Abbreviations

CHAPTER 1 INTRODUCTION..... 1

1.1 BACKGROUND INFORMATION..... 1
1.1.1 Project Background..... 1
1.1.2 Objectives 2
1.2 BACTERIAL STRESS RESPONSE..... 3
1.2.1 Stress and Stress Response 3
1.2.2 Bacterial Stress Response and Food Safety 6
1.2.3 Mechanisms of Bacterial Stress Response..... 8
1.2.3.1 General Stress Response 8
1.2.3.2 Heat-Shock Response 14
1.2.3.3 Cold-Shock Response 15
1.2.3.4 Acid Stress Response..... 17
1.2.3.4.1 Low-pH Stress Response in Gram-negative Bacteria..... 18
1.2.3.4.2 Low-pH Stress Response in Gram-positive Bacteria..... 21
1.2.3.4.3 Versatile Roles of Weak Organic Acids 24

1.2.3.5 Osmotic Stress Response 25
1.3 THE PATHOGENICITY OF FOODBORNE PATHOGENS 28
1.3.1 *Escherichia coli* 28
1.3.1.1 The Organism and its Characteristics 28
1.3.1.2 Association with Food 29
1.3.1.3 Virulence Factors and Pathogenicity 30
1.3.2 *Staphylococcus aureus* 32
1.3.2.1 The Organism and its Characteristics 32
1.3.2.2 Association with Food 33
1.3.2.3 Virulence Factors and Pathogenicity 34
1.3.3 *Listeria monocytogenes* 35
1.3.3.1 The Organism and its Characteristics 35
1.3.3.2 Association with Food 36
1.3.3.3 Virulence Factors and Pathogenicity 37
1.3.4 Stress Response and Bacterial Virulence 38
1.4 TRADITIONAL AND EMERGING ENUMERATION METHODOLOGIES 39
1.4.1 Traditional Enumeration Methods 39
1.4.1.1 Standard Plate Count 39
1.4.1.2 Turbidity (Optical Density) Measurement 41
1.4.1.3 Other Enumeration Methods 44
1.4.2 Emerging Methodologies 46
1.4.2.1 Viability Staining using Fluorescence Microscopy 46
1.4.2.1.1 Contact Angle Measurement 49
1.5 CONCLUSIONS 51

CHAPTER 2 EXPERIMENTAL PROCEDURES 53

2.1 BACTERIAL STRAINS AND GROWTH CONDITIONS 53

2.1.1 Bacterial Strains and Culture Media 53

2.1.2 Growth Conditions 53

2.2 STRESS TREATMENT PROCEDURE 54

2.2.1 Acid Stress Assay 54

2.2.2 Heat Shock Assay 56

2.2.3 Cold Shock Assay 56

2.2.4 Osmotic Stress Assay 56

2.3 MICROSCOPY 57

2.3.1 Preliminary Optical Microscopy 57

2.3.2 Fluorescence Microscopy Using Viability Staining 57

2.3.2.1 LIVE/DEAD® *Bac*Light™ Bacterial Viability Staining 57

2.3.2.2 Fluorescence Microscopy 58

2.4 STANDARD PLATE COUNT 59

2.5 TURBIDITY (OD_{600}) MEASUREMENT 59

2.6 CONTACT ANGLE MEASUREMENT 60

2.7 STATISTICAL ANALYSIS 61

CHAPTER 3 RESULTS 62

3.1 EFFECT OF ORGANIC ACID STRESS 63

3.1.1 Stationary-Phase *E. coli* DH5α 64

3.1.1.1 Fluorescence Microscopy 64

3.1.1.2 Standard Plate Count 67

3.1.1.3 Turbidity (OD_{600}) Measurement 68
3.1.2 Mid-log Phase *E. coli* DH5α 70
3.1.2.1 Fluorescence Microscopy 70
3.1.2.2 Standard Plate Count 71
3.1.2.3 Turbidity (OD_{600}) Measurement 75
3.1.3 Mid-log Phase *S. aureus* ATCC 13565 77
3.1.3.1 Fluorescence Microscopy 77
3.1.3.2 Standard Plate Count 77
3.1.3.3 Turbidity (OD_{600}) Measurement 81
3.1.4 Mid-log Phase *L. monocytogenes* CDC 7762 83
3.1.4.1 Fluorescence Microscopy 83
3.1.4.2 Standard Plate Count 83
3.1.4.3 Turbidity (OD_{600}) Measurement 87
3.2 Effect of Inorganic Acid Stress 89
3.2.1 Stationary-Phase *E. coli* DH5α 89
3.2.1.1 Fluorescence Microscopy 89
3.2.1.2 Standard Plate Count 92
3.2.1.3 Turbidity (OD_{600}) Measurement 93
3.2.2 Mid-log Phase *E. coli* DH5α 95
3.2.2.1 Fluorescence Microscopy 95
3.2.2.2 Standard Plate Count 95
3.2.2.3 Turbidity (OD_{600}) Measurement 98
3.2.3 Mid-log Phase *S. aureus* ATCC 13565 99
3.2.3.1 Fluorescence Microscopy 99

3.2.3.2 Standard Plate Count 99
3.2.3.3 Turbidity (OD_{600}) Measurement 102
3.2.4 Mid-log Phase *L. monocytogenes* CDC 7762 104
3.2.4.1 Fluorescence Microscopy 104
3.2.4.2 Standard Plate Count 104
3.2.4.3 Turbidity (OD_{600}) Measurement 107
3.3 EFFECT OF OTHER STRESSES 108
3.3.1 Osmotic Stress 108
3.3.2 Heat-Shock 108
3.3.3 Cold-Shock 108
3.4 CONTACT ANGLE MEASUREMENT 109
3.4.1 Acetic Acid Stress 111
3.4.1.1 Stationary-phase *E. coli* DH5α 111
3.4.1.2 Mid-log Phase *S. aureus* ATCC 13565 111
3.4.2 Lactic Acid Stress 112
3.4.2.1 Stationary-phase *E. coli* DH5α 112
3.4.2.2 Mid-log Phase *S. aureus* ATCC 13565 112
CHAPTER 4 DISCUSSION 113
4.1 EFFECT OF STRESS RESPONSE ON BACTERIAL ENUMERATION 113
4.1.1 Effect of Organic Acid Stress 114
4.1.2 Effect of Inorganic Acid Stress 117
4.1.3 Effect of Other Stresses 119
4.2 POTENTIAL CHANGES IN CELL SURFACE PROPERTIES 119

4.3 LIMITATIONS OF TRADITIONAL ENUMERATION METHODS IN FOOD INDUSTRY 123

CHAPTER 5 CONCLUSIONS AND FUTURE WORK 126

BIBLIOGRAPHY 129

APPENDIX A ADDITIONAL FLUORESCENCE MICROSCOPY IMAGES 147

APPENDIX B STATISTICAL DATA ON ACID STRESS 187

APPENDIX C ADDITIONAL OPTICAL MICROSCOPY IMAGES 196

LIST OF TABLES

Table 1.1 Main listeriosis outbreaks in North America and Europe..... 37

Table 2.1 Culture media used in this study..... 53

Table 2.2 Acid solutions used in this study. 55

Table 3.1 Cell surface hydrophobicity determined by contact angle measurements for cells of stationary-phase *E. coli* DH5α and mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of acetic acid and lactic acid for 1 and 5 h. 110

Table B.1 Standard plate count (A) and optical density (B) results for cells of stationary-phase *E. coli* DH5α after exposure to a range of concentrations of acetic acid and lactic acid for 1, 5 and 24 h. 188

Table B.2 Standard plate count (A) and optical density (B) results for cells of mid-log phase *E. coli* DH5α after exposure to a range of concentrations of acetic acid and lactic acid for 1, 5 and 24 h. 189

Table B.3 Standard plate count (A) and optical density (B) results for cells of mid-log phase *S. aureus* ATCC 13565 after exposure to a range of concentrations of acetic acid and lactic acid for 1, 5 and 24 h. 190

Table B.4 Standard plate count (A) and optical density (B) results for cells of mid-log phase *L. monocytogenes* CDC 7762 after exposure to a range of concentrations of acetic acid and lactic acid for 1, 5 and 24 h. 191

Table B.5 Standard plate count (A) and optical density (B) results for cells of stationary-phase *E. coli* DH5α after exposure to a range of concentrations of hydrochloric acid and sulfuric acid for 1, 5 and 24 h. 192

Table B.6 Standard plate count (A) and optical density (B) results for cells of mid-log phase *E. coli* DH5α after exposure to a range of concentrations of hydrochloric acid and sulfuric acid for 1, 5 and 24 h. 193

Table B.7 Standard plate count (A) and optical density (B) results for cells of mid-log phase *S. aureus* ATCC 13565 after exposure to a range of concentrations of hydrochloric acid and sulfuric acid for 1, 5 and 24 h. 194

Table B.8 Standard plate count (A) and optical density (B) results for cells of mid-log phase *L. monocytogenes* CDC 7762 after exposure to a range of concentrations of hydrochloric acid and sulfuric acid for 1, 5 and 24 h..... 195

LIST OF FIGURES

Figure 1.1 The interrelation among physiological states of microorganisms under different stress levels..... 5

Figure 1.2 The RssB-ClpXP two-component system in the regulation of σ^S proteolysis in *E. coli*..... 11

Figure 1.3 The regulation of σ^B activity via a partner-switching mechanism in *B. subtilis*, *S. aureus* and *L. monocytogenes*..... 12

Figure 1.4 Mechanisms of acid stress response in Gram-negative bacteria 20

Figure 1.5 Mechanisms of acid stress response in Gram-positive bacteria 22

Figure 1.6 Schematic diagram of a spectrophotometer. 43

Figure 1.7 Illustration of the hypothesis that stress responses may affect the cell association and further the cell concentration in suspensions..... 44

Figure 1.8 Schematic diagram of the approach used for fluorescence microscopy. 47

Figure 1.9 Contact angle (θ) of a drop of liquid on a solid surface. 50

Figure 1.10 Young's equation for Contact Angles..... 50

Figure 2.1 Illustration of the contact angle measurement. The contact angle (θ) is measured between the baseline of the water droplet on bacterial lawn and the tangent at water/hexadecane interface..... 60

Figure 3.1 An example of one set fluorescence microscopy images acquired for *E. coli* DH5α depicting live bacterial cells (A), dead bacterial cells (B), total bacterial cells (C) and a merged image of live and dead bacterial cells (D)..... 62

Figure 3.2 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of acetic acid for 1, 5 and 24 h..... 65

Figure 3.3 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of lactic acid for 1, 5 and 24 h..... 66

Figure 3.4 Cell counts determined by standard plate count methods for cells of stationary-phase *E. coli* DH5α after exposure to a range of concentrations of acetic acid (A) and lactic acid (B) for 1, 5 and 24 h. 68

Figure 3.5 Cell concentration determined by optical density measurements for cells of stationary-phase *E. coli* DH5α after exposure to a range of concentrations of acetic acid (A) and lactic acid (B) for 1, 5 and 24 h 69

Figure 3.6 Fluorescence microscopy images of mid-log phase *E. coli* DH5α after exposure to different concentrations of acetic acid for 1, 5 and 24 h……...72

Figure 3.7 Fluorescence microscopy images of mid-log phase *E. coli* DH5a after exposure to different concentrations of lactic acid for 1, 5 and 24 h……...73

Figure 3.8 Cell counts determined by standard plate count methods for cells of mid-log phase *E. coli* DH5α after exposure to a range of concentrations of acetic acid (A) and lactic acid (B) for 1, 5 and 24 h. 74

Figure 3.9 Cell concentration determined by optical density measurements for cells of mid-log phase *E. coli* DH5α after exposure to a range of concentrations of acetic acid (A) and lactic acid (B) for 1, 5 and 24 h. 76

Figure 3.10 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of acetic acid for 1, 5 and 24 h. 78

Figure 3.11 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of lactic acid for 1, 5 and 24 h. 79

Figure 3.12 Cell counts determined by standard plate count methods for cells of mid-log phase *S. aureus* ATCC 13565 after exposure to a range of concentrations of acetic acid (A) and lactic acid (B) for 1, 5 and 24 h 80

Figure 3.13 Cell concentration determined by optical density measurements for cells of mid-log phase *S. aureus* ATCC 13565 after exposure to a range of concentrations of acetic acid (A) and lactic acid (B) for 1, 5 and 24 h. 82

Figure 3.14 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of acetic acid for 1, 5 and 24 h. 84

Figure 3.15 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of lactic acid for 1, 5 and 24 h. 85

Figure 3.16 Cell counts determined by standard plate count methods for cells of mid-log phase *L. monocytogenes* CDC 7762 after exposure to a range of concentrations of acetic acid (A) and lactic acid (B) for 1, 5 and 24 h..... 86

Figure 3.17 Cell concentration determined by optical density measurements for cells of mid-log phase *L. monocytogenes* CDC 7762 after exposure to a range of concentrations of acetic acid (A) and lactic acid (B) for 1, 5 and 24 h..... 88

Figure 3.18 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of hydrochloric acid for 1, 5 and 24 h. Cell clumping was observed under each acid treatment condition, whereas a considerable proportion of cells still existed as single cells..... 90

Figure 3.19 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of sulfuric acid for 1, 5 and 24 h. Cell clumping was observed under each acid treatment condition, whereas a considerable proportion of cells still existed as single cells. 91

Figure 3.20 Cell counts determined by standard plate count methods for cells of stationary-phase *E. coli* DH5α after exposure to a range of concentrations of hydrochloric acid (A) and sulfuric acid (B) for 1, 5 and 24 h..... 92

Figure 3.21 Cell concentration determined by optical density measurements for cells of stationary-phase *E. coli* DH5α after exposure to a range of concentrations of hydrochloric acid (A) and sulfuric acid (B) for 1, 5 and 24 h..... 94

Figure 3.22 Fluorescence microscopy images of mid-log phase *E. coli* DH5α after exposure to different concentrations of hydrochloric acid and sulfuric acid for 1, 5 and 24 h..... 96

Figure 3.23 Cell counts determined by standard plate count methods for cells of mid-log phase *E. coli* DH5α after exposure to a range of concentrations of hydrochloric acid (A) and sulfuric acid (B) for 1, 5 and 24 h..... 97

Figure 3.24 Cell concentration determined by optical density measurements for cells of mid-log phase *E. coli* DH5α after exposure to a range of concentrations of hydrochloric acid (A) and sulfuric acid (B) for 1, 5 and 24 h..... 98

Figure 3.25 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of hydrochloric acid and sulfuric acid for 1, 5 and 24 h..... 100

Figure 3.26 Cell counts determined by standard plate count methods for cells of mid-log phase *S. aureus* ATCC 13565 after exposure to a range of concentrations of hydrochloric acid (A) and sulfuric acid (B) for 1, 5 and 24 h..... 101

Figure 3.27 Cell concentration determined by optical density measurements for cells of mid-log phase *S. aureus* ATCC 13565 after exposure to a range of concentrations of hydrochloric acid (A) and sulfuric acid (B) for 1, 5 and 24 h 103

Figure 3.28 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of hydrochloric acid and sulfuric acid for 1, 5 and 24 h..... 105

Figure 3.29 Cell counts determined by standard plate count methods for cells of mid-log phase *L. monocytogenes* CDC 7762 after exposure to a range of concentrations of hydrochloric acid (A) and sulfuric acid (B) for 1, 5 and 24 h..... 106

Figure 3.30 Cell concentration determined by optical density measurements for cells of mid-log phase *L. monocytogenes* CDC 7762 after exposure to a range of concentrations of hydrochloric acid (A) and sulfuric acid (B) for 1, 5 and 24 h..... 107

Figure A.1 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of acetic acid for 1 h..... 148

Figure A.2 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of acetic acid for 5 h..... 149

Figure A.3 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of acetic acid for 24 h.. 150

Figure A.4 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of lactic acid for 1 h.. 151

Figure A.5 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of lactic acid for 5 h. 152

Figure A.6 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of lactic acid for 24 h.. .. 153

Figure A.7 Fluorescence microscopy images of mid-log phase *E. coli* DH5α after exposure to different concentrations of acetic acid for 1 h.. 154

Figure A.8 Fluorescence microscopy images of mid-log phase *E. coli* DH5α after exposure to different concentrations of acetic acid for 5 h. 155

Figure A.9 Fluorescence microscopy images of mid-log phase *E. coli* DH5α after exposure to different concentrations of acetic acid for 24 h. 156

Figure A.10 Fluorescence microscopy images of mid-log phase *E. coli* DH5α after exposure to different concentrations of lactic acid for 1 h. 157

Figure A.11 Fluorescence microscopy images of mid-log phase *E. coli* DH5α after exposure to different concentrations of lactic acid for 5 h. 158

Figure A.12 Fluorescence microscopy images of mid-log phase *E. coli* DH5α after exposure to different concentrations of lactic acid for 24 h. ... 159

Figure A.13 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of acetic acid for 1 h. 160

Figure A.14 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of acetic acid for 5 h.. 161

Figure A.15 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of acetic acid for 24 h..... 162

Figure A.16 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of lactic acid for 1 h.. 163

Figure A.17 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of lactic acid for 5 h. 164

Figure A.18 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of lactic acid for 24 h..... 165

Figure A.19 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of acetic acid for 1 h..... 166

Figure A.20 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of acetic acid for 5 h..... 167

Figure A.21 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of acetic acid for 24 h..... 168

Figure A.22 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of lactic acid for 1 h. 169

Figure A.23 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of lactic acid for 5 h. 170

Figure A.24 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of lactic acid for 24 h. 171

Figure A.25 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of hydrochloric acid for 1 h. 172

Figure A.26 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of hydrochloric acid for 5 h. 173

Figure A.27 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of hydrochloric acid for 24 h. 174

Figure A.28 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of sulfuric acid for 1 h.. 175

Figure A.29 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of sulfuric acid for 5 h.. 176

Figure A.30 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of sulfuric acid for 24 h.. 177

Figure A.31 Fluorescence microscopy images of mid-log phase *E. coli* DH5α after exposure to different concentrations of hydrochloric acid and sulfuric acid for 1 h. 178

Figure A.32 Fluorescence microscopy images of mid-log phase *E. coli* DH5α after exposure to different concentrations of hydrochloric acid and sulfuric acid for 5 h. 179

Figure A.33 Fluorescence microscopy images of mid-log phase *E. coli* DH5α after exposure to different concentrations of hydrochloric acid and sulfuric acid for 24 h. 180

Figure A.34 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of hydrochloric acid 181

Figure A.35 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of hydrochloric acid and sulfuric acid for 5 h. 182

Figure A.36 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of hydrochloric acid and sulfuric acid for 24 h. 183

Figure A.37 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of hydrochloric acid and sulfuric acid for 1 h..... 184

Figure A.38 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of hydrochloric acid and sulfuric acid for 5 h..... 185

Figure A.39 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of hydrochloric acid and sulfuric acid for 24 h..... 186

Figure C.1 Optical microscopy images (10 ×) of stationary-phase *E. coli* DH5α after exposure to low temperatures, high temperatures and high concentrations of sodium chloride for 1, 5 and 24 h..... 197

Figure C.2 Optical microscopy images (10 ×) of mid-log phase *E. coli* DH5α after exposure to low temperatures, high temperatures and high concentrations of sodium chloride for 1, 5 and 24 h..... 198

Figure C.3 Optical microscopy images (10 ×) of mid-log phase *S. aureus* ATCC 13565 after exposure to low temperatures, high temperatures and high concentrations of sodium chloride for 1, 5 and 24 h..... 199

LIST OF SYMBOLS AND ABBREVIATIONS

θ	contact angle
σ	sigma factor
ANOVA	analysis of variance
APT	All Purpose Tween medium
AR	acid resistance
ATCC	American Type Culture Collection
ATP	adenosine 5'-triphosphate
ATR	acid tolerance response
a_w	water activity
BHI	Brain Heart Infusion medium
cAMP	cyclic adenosine monophosphate
CDC	Centers for Disease Control and Prevention (USA)
CFU	colony forming unit
cGMP	cyclic guanosine monophosphate
CIRCE	controlling inverted repeat of chaperone expression
CRP	cAMP receptor protein
Cy3	cyanine dye 3
Da	dalton
DAEC	diffusely adherent *E. coli*
DIC	differential interference contrast
DNA	deoxyribonucleic acid
EAEC	enteroaggregative *E. coli*
E. coli	*Escherichia coli*
EHEC	enterohemorrhagic *E. coli*
EIEC	enteroinvasive *E. coli*
EPEC	enteropathogenic *E. coli*
ETEC	enterotoxigenic *E. coli*
FDA	Food and Drug Administration (USA)
FITC	fluorescein isothiocyanate

GABA	γ-aminobutyric acid
GAD	glutamate decarboxylase
GC-content	guanine-cytosine content
GLM	generalized linear model
HCl	hydrochloric acid
H_2O_2	hydrogen peroxide
HrcA	heat-shock regulation at CIRCE elements
LB	Luria-Bertani medium
LEE	locus of enterocyte effacement
L. monocytogenes	*Listeria monocytogenes*
LPS	lipopolysaccharide
M	molarity, mol/L
MNEC	meningitis-associated *E. coli*
MPN	most probable number
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
OD_{600}	optical density at 600 nm
ppGpp	guanosine-3',5'-bispyrophosphate
PP2C	protein phosphatase 2C
P	probability
pH	potentiometric hydrogen ion concentration
pH_i	intracellular pH
pH_o	external pH
PI	propidium iodide
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
rpm	revolutions per minute
RTE	ready-to-eat
SAS	Statistical Analysis Software
S. aureus	*Staphylococcus aureus*
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

UDP-glucose	uridine diphosphate glucose
UPEC	uropathogenic *E. coli*
USDA	United States Department of Agriculture
UV	ultraviolet
VBNC	viable but non-culturable
v/v	volume/volume percentage
w/v	weight/volume percentage
w/w	weight/weight percentage
× *g*	times the force of gravity, the unit of relative centrifugal force

Chapter 1 Introduction

1.1 Background Information

1.1.1 Project Background

Bacteria do not always live in optimal growth conditions, and are therefore subjected to various environmental stresses during their life cycle. To eliminate harmful factors or to minimize damages from such stress, bacteria exhibit stress-induced responses (Hazen & Stahl, 2006; Hengge-Aronis, 2002b; Storz & Hengge-Aronis, 2000). In addition to previous theoretical and mechanistic stress response studies, research efforts have focused on the impact of bacterial stress response on food processing and preservation techniques in food industry (Abee & Wouters, 1999; Archer, 1996; Yousef & Courtney, 2003). Food processing and food preservation conditions exert different types of stress on bacteria, including: acid stress induced by organic acids present in the food (Brul & Coote, 1999; Brul *et al.*, 2002; Ricke, 2003); osmotic stress caused by high salt content (Abee & Wouters, 1999; Gutierrez *et al.*, 1995; Koutsoumanis *et al.*, 2003); heat shock stress induced by mild heat treatments (Dowds & Browne, 2001; Wang & Doyle, 1998); and cold shock stress induced by low temperature storage (Russell, 2002). These stresses may alter bacterial growth and gene expression, and potentially impact the evaluation of bacterial viability.

Although a wealth of molecular, immunological, physical and chemical detection techniques have been developed in the last several decades to evaluate the viability of bacteria, the standard plate count technique is still by far the most

widely used method for quantifying the number of viable cells in the food industry. Other traditional enumeration tools used in microbiology and related fields include direct microscopic count, most probable number and turbidity measurement (Adams & Moss, 2008; Jay *et al.*, 2005). However, these methods are typically performed without taking into consideration the pre-treatment conditions that may impose stresses on bacteria. The literature lacks systematic study of how stress incurred by the pre-treatment of bacteria impacts the outcome of the enumeration.

Preliminary research in our lab revealed that 0.2 M acetic acid stress (pH 2.70) induced an obvious association, or clumping, of *Staphylococcus aureus*, and that clumped bacterial cultures had significantly decreased plate counts. This finding suggested that the existing enumeration methods might have dramatically underestimated the total number of viable bacteria because of the change of cell association or survival mechanisms under stress conditions.

If bacterial stress responses dramatically affect the cell association and thereby dramatically affect the pathogen enumeration, the enumeration methods currently employed in the food industry, as well as the evaluation of food processing and preservation would need to be re-assessed with regard to altered bacterial association and behavior under stress conditions.

1.1.2 Objectives

The goal of this thesis was to study the impact of bacterial stress response on the outcome of current enumeration methods. The first objective was to investigate the influence of various stresses (including acids, temperature and

high salts) on cell association and enumeration of different foodborne pathogens, including a Gram-negative model microorganism *Escherichia coli*, a Gram-positive model microorganism *Staphylococcus aureus*, and an important foodborne pathogen *Listeria monocytogenes*. The second objective was to evaluate and compare the outcomes of traditional enumeration methods, including plate counts and OD_{600}, to microscopic observations of cell association and viability under those stress conditions. The last objective was to provide novel and comprehensive information on the effect of stress responses in the area of food safety.

In this chapter, the microbial mechanisms of several important food industry-related stress responses (heat shock, cold shock, low pH and weak acid stress, and osmotic stress) and some well-characterized response systems will be briefly reviewed. The pathogenicity of individual foodborne pathogens *E. coli*, *S. aureus* and *L. monocytogenes* will also be described. Lastly, some traditional enumeration methods and their limitations in terms of stress responses will be discussed. This will provide an understanding of the current research on bacterial stress response, with emphasis on its promising effect in the fields of food safety.

1.2 Bacterial Stress Response

1.2.1 Stress and Stress Response

Bacteria experience a variety of environmental stresses throughout their life cycle. Since the stresses imposed by the environment vary in intensity and are dependent on the bacterial species, there is no universal definition for stress thus far. In the study of bacterial stress responses, stress can be defined as any change

in the optimal growth conditions that reduces growth rate; or any environmental factor that damages cellular components in the absence of a cellular response; or any situation that induces the expression of genes that are known to respond to specific environmental conditions (Storz & Hengge-Aronis, 2000).

Stresses encountered by bacteria can be categorized into three types (Yousef & Courtney, 2003):

1. Physical stress, including heat, cold, freezing, osmotic, high pressure, dehydration, irradiation, pulsed electric field, *etc*.
2. Chemical stress, including acids, salts and oxidants.
3. Nutritional stress, including nutrient starvation.

During the cell life cycle, bacteria are constantly adapting to the changing environment which can either affect their optimal growth rate or result in severe damage to the cells. The process of bacteria adapting to non-optimal growth environments is termed "bacterial stress response" (Abee *et al.*, 2004). This stress response is crucial to the growth and survival of bacteria. Different bacteria have developed different stress responses to survive in their natural environments (Hengge-Aronis, 2002b; Price, 2000).

The interrelation among physiological states of microorganisms under different stress levels is shown in Figure 1.1 (Yousef & Courtney, 2003). After sensing a mild or sub-lethal stress, microbial cells become stressed, and a spectrum of adaptive responses may be induced. This adaptation enhances the resistance of bacteria so that stress-adapted cells can quickly recover. When cells are subjected to moderate stress, some of them typically cannot resist and may

become injured. The injured cells can either recover by a repair progress or die after suffering a more severe stress.

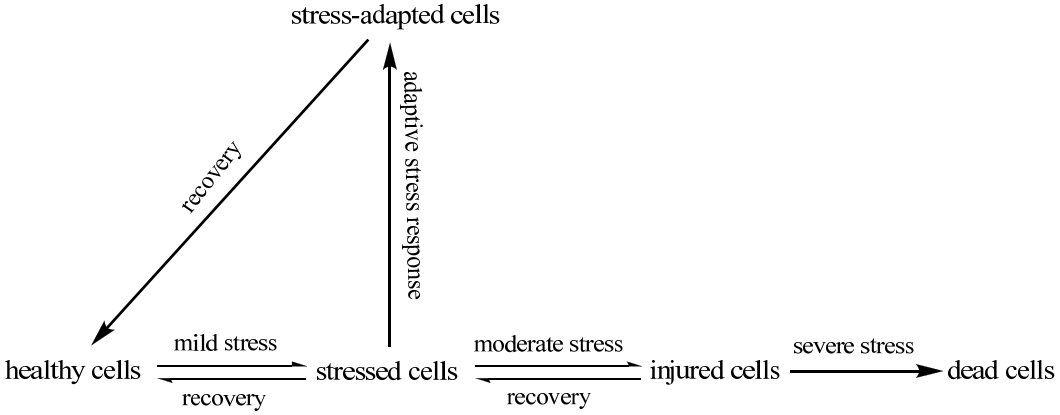


Figure 1.1 The interrelation among physiological states of microorganisms under different stress levels (adapted from Yousef and Courtney, 2003).

Once exposed to mild stress, bacteria can develop resistance to counteract subsequent higher levels of homologous stresses. It has been shown that *E. coli* grown at a sub-lethal pH (5.0) showed greater acid resistance to normally lethal pH (3.0 or 3.5), whereas the same strains grown at pH 7.0 failed to survive after exposure to pH 3.0 or 3.5 for a short period of time (Goodson & Rowbury, 1989b). In addition, bacteria can also gain resistance to heterologous stresses after exposure to a mild stress. This multiple stress adaptation is referred to as cross protection (Rodriguez-Romo & Yousef, 2005). For instance, a related study on *L. monocytogenes* indicates that adaptation to sub-lethal levels of acid (HCl, pH 4.5 to 5.0), ethanol (5%, v/v), NaCl (7%, w/v) or heat (45^{o}C for 1 h) significantly increased the resistance to lethal levels of H_2O_2 (0.1%, w/v); adaptation to sub-lethal levels of acid (HCl, pH 5.0) or heat (45^{o}C for 1 h) significantly increased the resistance to lethal levels of ethanol (17.5%, v/v); and adaptation to sub-lethal

levels of ethanol (5%, v/v) or heat (45^{o}C for 1 h) significantly increased the resistance to lethal levels of NaCl (25%, w/v) (Lou & Yousef, 1997). Therefore, the adaptive responses enhance the ability of bacteria to resist various stresses, either homologous or heterologous.

1.2.2 Bacterial Stress Response and Food Safety

The food production chain consists of many steps, from the pre-harvest phase to post-harvest processing, storage and distribution. Throughout the entire food production chain, bacteria are subjected to various stresses (Yousef & Courtney, 2003). For example, in the pre-harvest stage of the food industry, heat and cold shocks can be caused by temperature changes. Acid stresses can be induced by adjustment of irrigation water pH, acid rain or silage fermentation. Osmotic stresses can be associated to soil salinity or extra salts deposited by irrigation water. Finally, nutritional stresses can result in a nutrient-lacking environment (Yousef & Courtney, 2003). During food processing and storage procedures, heat shock may be caused by mild heat treatments or temperature control failures (Dowds & Browne, 2001; Wang & Doyle, 1998). Cold shock can be induced by refrigeration conditions (Russell, 2002). Acid stresses can be imposed by food preservatives (food antimicrobials) or acidulants (Brul & Coote, 1999; Brul *et al.*, 2002; Kwon & Ricke, 1998; Ricke, 2003). And osmotic stresses can occur in the desiccation process or by addition of high concentrations of osmotically active compounds (such as salts or sugars) (Abee & Wouters, 1999; Gutierrez *et al.*, 1995; Koutsoumanis *et al.*, 2003).

In addition, bacterial stress response has been drawing much attention with the emergence of innovative minimal processing techniques (Abee & Wouters, 1999; Yousef & Courtney, 2003). These emerging techniques include high hydrostatic pressure processing, irradiation (e.g. γ-ray, X-ray, UV, microwave), pulsed electric field processing, and so on. Since the novel techniques, which are aimed to meet consumers' demands for better nutrition and higher sensory quality, use less heavily processed or preserved procedures, a series of adaptive stress responses may be more likely to be induced under the milder treatment conditions.

Moreover, research indicates that stress response may influence the expression of virulence factors. It has been demonstrated that acid-tolerant mutants of *L. monocytogenes* (O'Driscoll *et al.*, 1996) and *Salmonella enterica* serovar Typhimurium (Riesenberg-Wilmes *et al.*, 1996) exhibited increased virulence. It was also shown that *Salmonella enterica* Enteritidis PT4 with enhanced heat and acid tolerance has been found more virulent in mice (Humphrey *et al.*, 1996). Corresponding cellular stress response machineries were also found to account for the increased virulence. It was suggested that a two-component system LisR-LisK, which plays an important role in stress response, is essential for the virulence of *L. monocytogenes* (Cotter *et al.*, 1999). A two-component system PhoP-PhoQ, which regulates the acid stress response in *Salmonella enterica* serovar Typhimurium, has been shown to affect the expression of virulence genes (Bearson *et al.*, 1998; Gahan & Hill, 1999). In conclusion, the adaptive stress responses developed by foodborne pathogens not

only provide them with resistance against more severe hostile environments, but they also increase their virulence. Therefore, stress response by pathogens during food production and subsequent *in vivo* digestion presents great potential threat to the health of consumers.

1.2.3 Mechanisms of Bacterial Stress Response

1.2.3.1 General Stress Response

A general stress response can be evoked by a variety of stress conditions. This provides pathogens potential resistance against a broad spectrum of stresses. The general stress response is usually, but not always, characterized by reduced growth rates or the entry into the stationary phase of growth (Hengge-Aronis, 2002b). The regulation of general stress response is controlled by sigma factors, including σ^S in Gram-negative bacteria and σ^B in Gram-positive bacteria. σ^S (encoded by *rpoS* gene) and σ^B (encoded by *sigB* gene) are sigma subunits of RNA polymerases and have homologous functions. Gram-negative bacteria *Escherichia coli* and Gram-positive bacteria *Bacillus subtilis* are two microorganisms that are well-characterized in stress response studies (Hengge-Aronis, 1999; Hengge-Aronis, 2002b; Price, 2000). This section will use these two model bacterial systems to explain the mechanism by which the σ factors operate.

In Gram-negative bacteria, such as *E. coli*, σ^S is not expressed at any detectable levels during rapid growth under ideal conditions (Hengge-Aronis, 2002b). Nevertheless, the σ^S factor accumulates when the bacterial cell is exposed

to stresses, and this leads to the expression of more than 70 genes that are involved in stress adaptation (Gruber & Gross, 2003; Hengge-Aronis, 2002b). The regulation of σ^S occurs through a network of different levels of cellular biological activities, from transcription to translation and post-translational protein modification (σ^S proteolysis) (Hengge-Aronis, 2000).

At the transcription level, *rpoS*, the gene encoding for factor σ^S, is negatively controlled by cAMP-CRP complex and positively controlled by histidine sensor kinase BarA, polyphosphate, small molecules such as guanosine-3',5'-bispyrophosphate (ppGpp) and homoserine lactone (Gentry *et al.*, 1993; Huisman & Kolter, 1994; Lange & Hengge-Aronis, 1994; Lange *et al.*, 1995; Mukhopadhyay *et al.*, 2000; Shiba *et al.*, 1997).

It was proposed that the *rpoS* translation is triggered when stress conditions alter the *rpoS* mRNA secondary structure to improve its accessibility to ribosomes and accordingly enhance gene translation (Hengge-Aronis, 2000). The RNA-binding protein Hfq (or HF-I), histone-like protein HU and some small regulatory RNAs (e.g. DsrA, RprA) have been identified to play a role in stimulating translation (Balandina *et al.*, 2001; Lease & Belfort, 2000; Majdalani *et al.*, 1998; Majdalani *et al.*, 2001; Muffler *et al.*, 1996b; Muffler *et al.*, 1997b), whereas the histone-like protein H-NS, small RNA OxyS and small molecule UDP-glucose are considered as translation inhibitors (Barth *et al.*, 1995; Hengge-Aronis, 2002a; Wassarman, 2002; Yamashino *et al.*, 1995; Zhang *et al.*, 1998).

Stresses, such as carbon starvation, high osmolarity, high temperature and low pH, are able to stabilize σ^S and prevent its degradation at the cellular level

(Bearson *et al.*, 1996; Lange & Hengge-Aronis, 1994; Muffler *et al.*, 1996a; Muffler *et al.*, 1997a). ATP-dependent protease ClpXP and response regulator RssB are two essential components in the regulation of σ^S proteolysis (Hengge-Aronis, 2000). RssB acts as a specific direct σ^S recognition factor, whose affinity for σ^S is modulated by the phosphorylation of its receiver domain (Becker *et al.*, 1999; Bouché *et al.*, 1998; Klauck *et al.*, 2001). Upon binding to the phosphorylated RssB, σ^S is then transferred to the ClpXP protease to form a σ^S•RssB-P•ClpXP complex (Figure 1.2). σ^S is subsequently unfolded and completely degraded in a process that involves the releasing of RssB by an ATP hydrolysis-dependent mechanism (Hengge-Aronis, 2002b; Klauck *et al.*, 2001; Zhou *et al.*, 2001). Figure 1.2 also shows that several mechanisms have been proposed to play roles in the regulation of the proteolysis of σ^S under stress conditions (Hengge-Aronis, 2002a). Stress may affect the phosphorylation of RssB and therefore interfere with the formation of σ^S•RssB-P•ClpXP complex. Stress may also directly downregulate the cellular level of RssB. Furthermore, stress may affect level of free core RNA polymerase which, instead of RssB, could also bind to σ^S. The association of σ^S and core RNA polymerase readily competes with that of σ^S and RssB. This could potentially inhibit the degradation of σ^S. (Hengge-Aronis, 2002a).

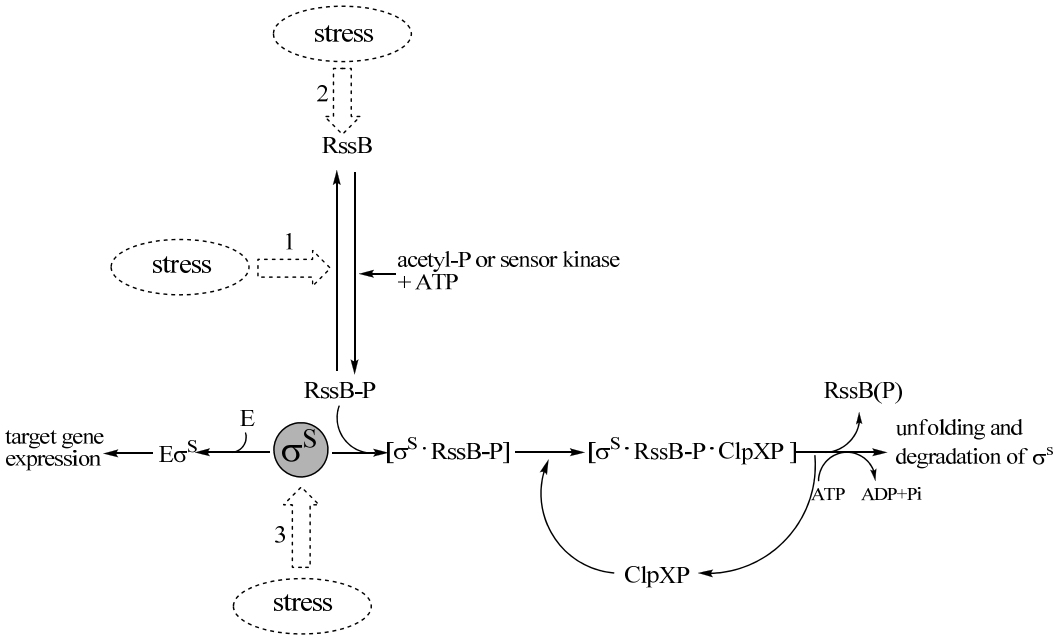


Figure 1.2 The RssB-ClpXP two-component system in the regulation of σ^S proteolysis in *E. coli* (adapted from Hengge-Aronis, 2002a). Under nonstress conditions, phosphorylated RssB directly interacts with σ^S and delivers it to the ClpXP protease. Under stress conditions, stress signals may result in (1) dephosphorylation of RssB; (2) downregulation of the cellular RssB content; (3) association of σ^S with core RNA polymerase, which readily competes with RssB and may inhibit the degradation of σ^S.

σ^B is a central regulator of the general stress response in Gram-positive genera with low GC-content, which include *Bacillus*, *Staphylococcus* and *Listeria* spp. (Ferreira *et al.*, 2004; Price, 2000; van Schaik & Abee, 2005). The activity of σ^B is modulated by a partner-switching mechanism involved with two crucial regulators, the anti-sigma factor RsbW and the anti-anti-sigma factor RsbV as shown in Figure 1.3 (Benson & Haldenwang, 1993a; Benson & Haldenwang, 1993b; Delumeau *et al.*, 2004; Dufour & Haldenwang, 1994; Yang *et al.*, 1996). RsbW can either bind to σ^B as an inhibitor; or bind to RsbV and release σ^B. The relative affinity of RsbV and σ^B for RsbW is determined by the phosphorylation

state of RsbV. In unstressed cells, RsbV cannot bind to RsbW due to its phosphorylation by the serine kinase activity of RsbW. RsbW binds to σ^B to form an RsbW-σ^B complex, and the activity of σ^B is inhibited (Dufour & Haldenwang, 1994; Yang *et al.*, 1996). However, the affinity of RsbV towards RsbW greatly increases when RsbV is dephosphorylated by PP2C-type phosphatases when exposing to several stresses (Delumeau *et al.*, 2004; Vijay *et al.*, 2000). In this case, RsbV binds to RsbW and forms an alternative complex RsbW-RsbV. Therefore, σ^B is released from the RsbW-σ^B complex to interact with RNA polymerase and promote transcription (Delumeau *et al.*, 2004; Price, 2000).

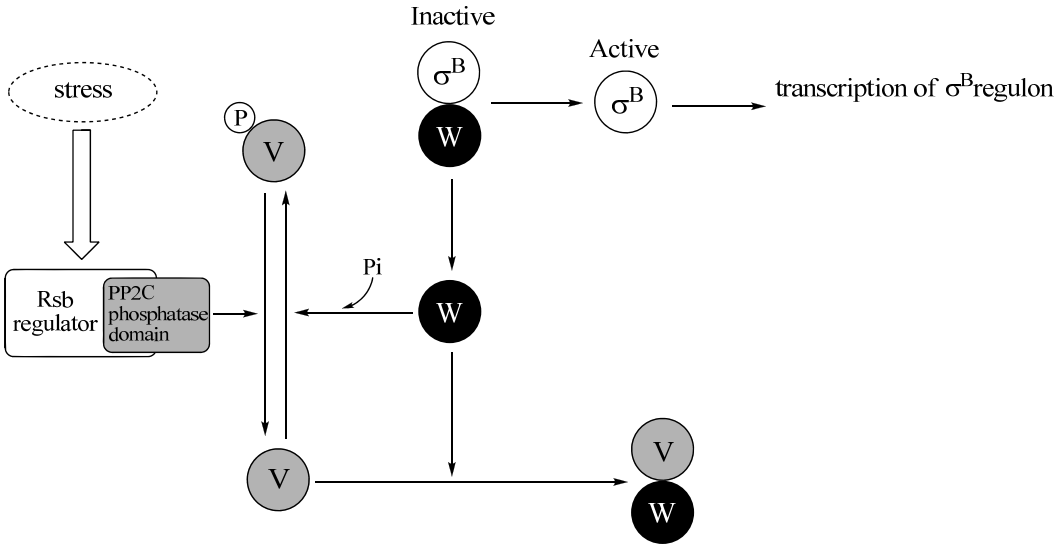


Figure 1.3 The regulation of σ^B activity via a partner-switching mechanism in *B. subtilis*, *S. aureus* and *L. monocytogenes* (adapted from van Schaik and Abee, 2005). The anti-sigma factor RsbW and the anti-anti-sigma factor RsbV are two major regulators. Under nonstress conditions, σ^B is in an inactive state by binding to anti-sigma factor RsbW. The kinase activity of RsbW can phosphorylate anti-anti-sigma factor RsbV to inhibit its affinity. Under stress conditions, PP2C-type phosphatases can dephosphorylate RsbV to promote its affinity with RsbW. σ^B is therefore released and associated with core RNA polymerase to initiate transcription of stress-related genes.

Recent studies described below have shown that all the phosphatases involved in the regulation of σ^B, as well as other PP2C-type phosphatases, share high homology in the C-terminal catalytic domains. However, these phosphatases have different N-terminal domains which function in stress signal sensing (Delumeau *et al.*, 2004; van Schaik & Abee, 2005). It has been shown that there are considerable differences in the upstream regulation of Rsb (regulator of σ^B) in different bacteria, probably as a result of the structural differences of the N-terminal domain of different PP2C-type phosphatases (Delumeau *et al.*, 2004). RsbU and RsbP, for example, are two *B. subtilis* phosphatases that are responsible for different stress responses. RsbU is required for response to environmental stresses that include conditions such as heat, acid, salt or ethanol stress. RsbP is required for response to energy stresses that are usually referred to starvation for glucose, phosphate or oxygen. RsbU contains an N-terminal domain that can bind to another regulator RsbT to promote the activity of the C-terminal catalytic domain. RsbP contains an N-terminal PAS domain that can bind to another regulator RsbQ, but the detailed activation is still not clear (Delumeau *et al.*, 2004; Vijay *et al.*, 2000). In addition to the difference in the N-terminal domain of different phosphatases that are responsible for different stress responses, similar phosphatases from different bacteria may also have different N-terminal structures and would therefore operate differently. For instance, RsbU from *L. monocytogenes* contains an N-terminal RsbT-binding domain that is similar to that of *B. subtilis* RsbU (Chaturongakul & Boor, 2004; Delumeau *et al.*, 2004). It can be stimulated by both environmental and energy stress. However, *S. aureus*

RsbU does not have the N-terminal RsbT-binding domain and its activation mechanism is still under investigation (Delumeau *et al.*, 2004; Palma & Cheung, 2001).

In addition to the central regulatory functions in a general stress response, σ^B also plays an important role in post-transcriptional regulations, such as small RNA binding protein Hfq in *L. monocytogenes*, staphylococcal accessory regulator A /SarA in *S. aureus* (Bischoff *et al.*, 2004; Bronner *et al.*, 2004; Christiansen *et al.*, 2004), as well as the expression of virulence factors (such as internalin and positive regulatory factor A /PrfA in *L. monocytogenes*) (Kazmierczak *et al.*, 2003; Kazmierczak *et al.*, 2005).

In conclusion, the sigma factors σ^S and σ^B function as the master regulators of general stress response in Gram-negative and Gram-positive bacteria, respectively. They contribute to bacterial resistance to a variety of stresses.

1.2.3.2 Heat-Shock Response

Conventional food preservation treatments at elevated temperatures for short periods of time, or innovative minimal processing techniques under mild heating conditions, induce heat-shock stress in foodborne bacteria (Abee & Wouters, 1999; Earnshaw *et al.*, 1995). Heat causes damage to macromolecular cell components including ribosomes and proteins (Abee & Wouters, 1999).

Bacteria mainly respond to heat shock by transiently over-expressing heat shock proteins to repair or destroy damaged cellular components (Arsène *et al.*, 2000; Yura & Nakahigashi, 1999). Heat shock proteins are highly conserved proteins in a variety of microorganisms (Lindquist, 1986). Some heat shock

proteins act as molecular chaperones that assist the folding or assembly of heat-damaged proteins, such as DnaK (and its co-chaperones DnaJ, GrpE), GroEL (and it co-chaperone GroES). These heat shock proteins are found to be responsible for the modulation of protein folding pathways by preventing misfolding/aggregation, and facilitating refolding/assembly of proteins (Georgopoulos & Welch, 1993). In addition, heat shock proteins also include ATP-dependent proteases that degrade heat damaged proteins, such as ClpAP and Lon in *E. coli* (Yura & Nakahigashi, 1999), ClpC and ClpP in *B.subtilis* (Kruger *et al.*, 2001).

In Gram-negative bacteria, the transcription of heat-inducible genes is regulated by a master regulon σ^{32}, as well as two other minor regulons σ^{E} and σ^{54} (Kuczynska-Wisnik *et al.*, 2001; Yura & Nakahigashi, 1999). On the other hand, three classes of heat-inducible genes are controlled by relevant regulatory factors in Gram-positive bacteria. Class I chaperon genes are encoded by CIRCE-HrcA system; class II genes, the majority of general stress genes, are regulated by σ^{B}; and class III genes, which include the rest of heat-inducible genes, are controlled by either class III stress gene repressor or other unrevealed mechanisms (Yura & Nakahigashi, 1999).

Therefore, understanding the role of heat shock proteins and the regulation of heat-inducible genes would help us elucidate the mechanism of the bacterial response to heat-stress conditions.

1.2.3.3 Cold-Shock Response

Since refrigeration and freezing are the most common methods to preserve foods, it is very important to understand how bacteria, especially some foodborne

psychrotrophic pathogens such as *L. monocytogenes* and *Yersinia enterocolitica*, respond to low temperature conditions (Abee & Wouters, 1999). Bacterial stress response induced by temperature downshift involves the over-expression of two types of proteins: the cold shock proteins and the cold acclimation proteins. It has been suggested that both of the two proteins play roles in the bacterial adaptation to cold stress (Graumann & Marahiel, 1996; Phadtare *et al.*, 1999). Cold shock proteins are rapidly and transiently over-expressed in response to a sudden decrease in temperature. In contrast, cold acclimation proteins are synthesized during growth at sustained low temperatures via rapid induction but slow over-expression.

Bacteria respond to cold shock by synthesizing DNA- and RNA-binding proteins to counteract the cold-induced stabilization of nucleic acid secondary structures that may reduce the efficiency of transcription, translation and DNA replication (Phadtare, 2004). It has been demonstrated that many cold shock proteins function by binding to DNA and RNA. In *E. coli*, for instance, CspA, CspB, CspG and CspI function as RNA/DNA chaperones, CsdA is a ribosome associated protein with RNA unwinding activity, RbfA is a ribosome binding factor, NusA is involved in termination and anti-termination of transcription, and PNP acts as a ribonuclease (Phadtare *et al.*, 1999; Phadtare, 2004).

Secondly, bacteria can also minimize harm resulted from abnormally low temperature by modifying their cell membrane to maintain optimum fluidity (Russell *et al.*, 1995; Russell, 2002). Appropriate membrane fluidity is required for the movement of molecules across the cell membrane and the survival of

bacteria cells. However, membrane fluidity is usually decreased as a result of temperature downshifts. It has been proposed that some bacteria can adapt to low temperature and keep optimal membrane fluidity by increasing the degree of unsaturation and/or shortening chain length of fatty acids. In *L. monocytogenes*, for example, an increase of unsaturated fatty acid (C 18:1), as well as an increase of shorter-chain fatty acid (e.g. increasing C 15:0 at the expense of C 17:0) contribute to membrane fluidity in response to low temperature (Russell *et al.*, 1995) .

Furthermore, bacteria can synthesize or transport compatible solutes in response to temperature downshift (Abee & Wouters, 1999). It has been reported that trehalose plays an important role in the low temperature-resistance in *E. coli* and its synthesis is induced by cold shock (Kandror *et al.*, 2002). Another example is that in *L. monocytogenes*, glycine betaine and carnitine are transported in response to cold shock (Angelidis *et al.*, 2002; Angelidis & Smith, 2003; Ko *et al.*, 1994).

1.2.3.4 Acid Stress Response

Bacteria may experience drastic pH changes in natural habitats, food processing, as well as in the gastrointestinal tract of the host (Abee & Wouters, 1999; Bearson *et al.*, 1997). Acid stress is defined as the combined biological effect of low pH (inorganic acids) and weak (organic) acids present in the microbial environment (Abee & Wouters, 1999; Bearson *et al.*, 1997). It has been discovered that Gram-negative and Gram-positive microorganisms use a series of

different but sometimes overlapping strategies in response to low-pH stress, as described below (Bearson *et al.*, 1997; Cotter & Hill, 2003; Foster, 2000).

1.2.3.4.1 Low-pH Stress Response in Gram-negative Bacteria

In Gram-negative bacteria, acid tolerance response (ATR) and acid resistance (AR) are two well-characterized response systems to relieve low-pH stress (Figure 1.4). The ATR system is referred to as an acid survival system in log or stationary-phase cells that can function in minimal glucose medium and protect cells at pH as low as 3.0. The AR system is an acid survival system in stationary-phase cells and complex medium Luria-Bertani (LB) broth. It is able to protect cells at pH 2.5 and below (Lin *et al.*, 1995). The ATR and AR systems have been well studied in *S. enterica* serovar Typhimurium and *E. coli*, respectively (Audia *et al.*, 2001; Bearson *et al.*, 1997; Lin *et al.*, 1995). Bacteria may have one or both of the response systems. It has been shown that *E. coli* has both ATR and AR systems, whereas *S. enterica* serovar Typhimurium only contains ATR systems and *Shigella flexneri* only occupies glutamate- and arginine-dependent AR systems (Lin *et al.*, 1995). In this section, *S. enterica* serovar Typhimurium and *E. coli* will be used as models to demonstrate the mechanism of ATR and AR systems, respectively.

Bacteria have different types of **ATR systems** in different growth stages. The log-phase ATR in *S. enterica* serovar Typhimurium includes three systems with different functions (Figure 1.4). The first type of ATR system is able to trigger an emergency pH homeostasis that allows the cell to maintain a relatively constant intracellular pH (pH_i) over a broad range of external pH values (pH_o)

(Audia *et al.*, 2001; Bearson *et al.*, 1997). It was shown that low pH-inducible amino acid decarboxylases (e.g. lysine, ornithine, and arginine) contribute to the maintenance of pH_i homeostasis (Bearson *et al.*, 1997; Park *et al.*, 1996). A second system is involved in the synthesis of acid shock proteins (Bearson *et al.*, 1997). The main function of acid shock proteins has been proposed to be protecting and/or repairing acid-induced damage to macromolecules. The synthesis of acid shock proteins is regulated by regulation factor σ^S (previously mentioned in Section 1.2.3.1), transcriptional regulator PhoP and iron regulator Fur. The third type of ATR system repairs damaged DNA in response to low-pH stress (Audia *et al.*, 2001). Transcriptional regulator Ada and DNA polymerase I *polA* have been shown to regulate DNA repair under acidic conditions.

The stationary-phase ATR in *S. enterica* serovar Typhimurium consists of two types of systems: σ^S-dependent system that is triggered by entry into stationary phase, and σ^S-independent system that is involved in the synthesis of acid shock proteins under the control of OmpR (Audia *et al.*, 2001; Bang *et al.*, 2000) (Figure 1.4).

The **AR system** in *E. coli* is composed of three independent systems (Figure 1.4). The first type of AR system is referred to as oxidative or glucose-repressed acid resistance (Audia *et al.*, 2001). This resistance system, as implied by its name, is repressed if glucose is present in the growth media (Bearson *et al.*, 1997). It is induced by acid stress and is found to be highly dependent on the σ^S regulator. In addition, it has been proposed that this system could be regulated by cAMP and cAMP receptor protein (CRP) (Castanie-Cornet *et al.*, 1999).

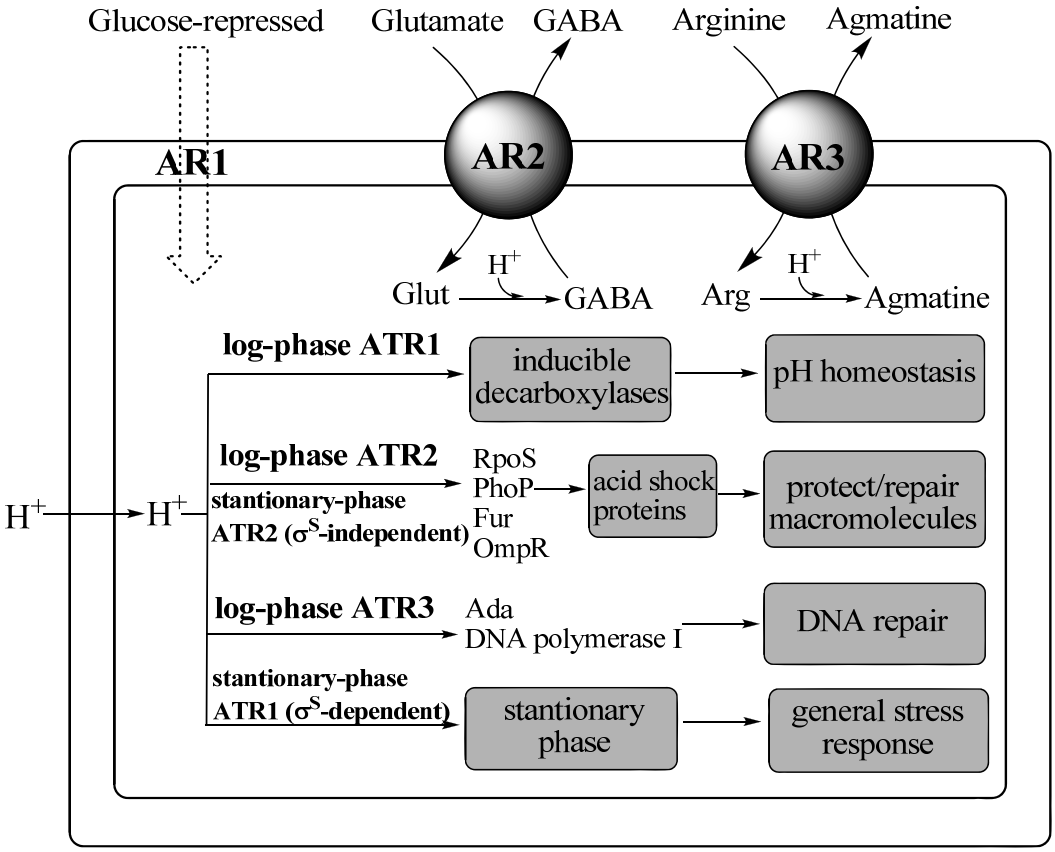


Figure 1.4 Mechanisms of acid stress response in Gram-negative bacteria (adapted from Bearson *et al.*, 1997 and Audia *et al.*, 2001). ATR and AR are two types of systems used to represent low-pH response under different conditions. Log-phase ATR1: synthesis of emergency pH homeostasis which is induced by amino acid decarboxylases. Log-phase ATR2: synthesis of acid shock proteins to protect and/or repair acid-induced damage to macromolecules, acid shock proteins are regulated by σ^S, PhoP and Fur. Log-phase ATR3: DNA repair which is regulated by Ada and DNA polymerase I. Stationary-phase ATR1: σ^S-dependent mechanism which is triggered by entry into stationary phase. Stationary-phase ATR2: σ^S-independent mechanism which is involved in the synthesis of acid shock proteins under the control of OmpR. AR1: oxidative or glucose-repressed acid resistance. AR2: glutamate-dependent acid resistance. AR3: arginine-dependent acid resistance.

The second AR system is glutamate-dependent acid resistance system (Audia *et al.*, 2001). The operation of this system requires the presence of glutamate under acidic conditions (Lin *et al.*, 1995; Lin *et al.*, 1996). It was

shown that this type of acid resistance is regulated by the enzymatic activity of glutamate decarboxylase (GadA or GadB) and the putative glutamate/γ-aminobutyric acid (GABA) antiporter GadC (Castanie-Cornet *et al.*, 1999; Hersh *et al.*, 1996).

The third AR system is called arginine-dependent acid resistance (Audia *et al.*, 2001). It was shown that the action of this system is triggered by the addition of arginine during pH 2.5 acid challenge (Lin *et al.*, 1995; Lin *et al.*, 1996). This type of acid resistance is controlled by arginine decarboxylase (AdiA) and the regulatory protein CysB (Castanie-Cornet *et al.*, 1999; Shi & Bennett, 1994).

The regulatory systems of ATR and AR in Gram-negative bacteria are extremely complex. Good understanding of these systems would provide valuable insights into the strategy employed by bacteria to survive low pH conditions.

1.2.3.4.2 Low-pH Stress Response in Gram-positive Bacteria

In contrast to the well-studied ATR and AR systems in Gram-negative bacteria, the mechanisms of low-pH stress response in Gram-positive bacteria are still under development. Based on a review by Cotter and Hill (2003), there are six types of systems involved in the resistance against low-pH stress in Gram-positive bacteria. These systems are shown in Figure 1.5 and will be described in detail.

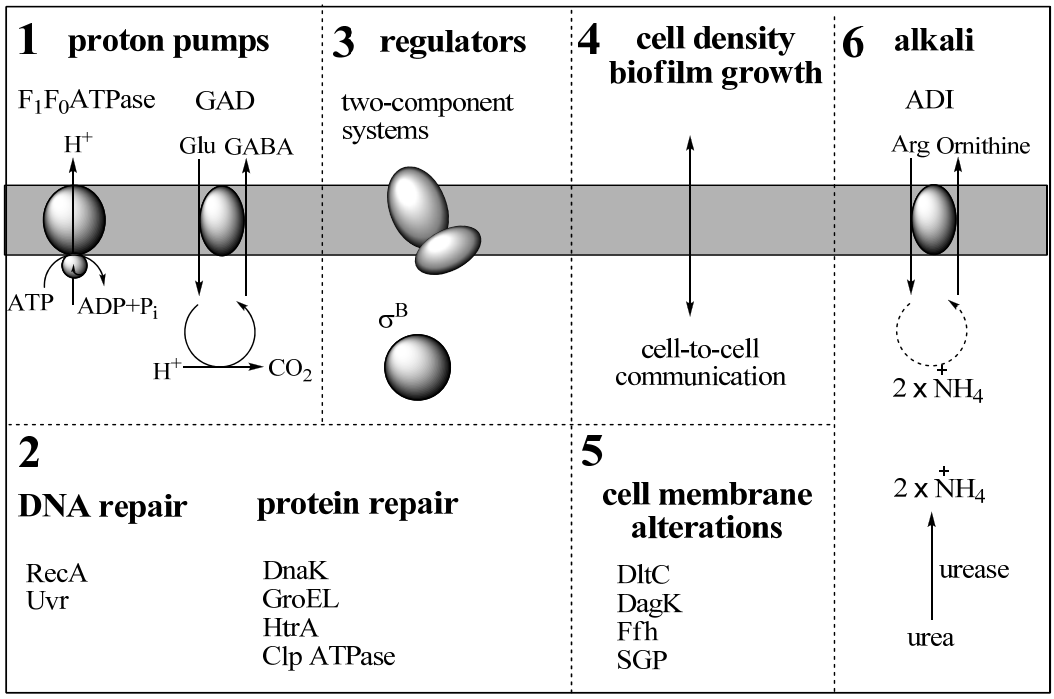


Figure 1.5 Mechanisms of acid stress response in Gram-positive bacteria (adapted from Cotter and Hill, 2003).

Proton pumps are utilized by Gram-positive bacteria to transport protons out of the cell so as to increase the intracellular pH (pH_i) when encountered acidic environment (Figure 1.5 section 1). These proton pumps include F_1F_0-ATPase complex and glutamate decarboxylase (GAD) system in *L. monocytogenes* (Cotter *et al.*, 2000; Cotter *et al.*, 2001).

Some proteins can be synthesized to protect and/or repair acid-induced damage to macromolecules (Figure 1.5 section 2). These protective/repairing proteins include RecA and Uvr involved in DNA damage repair, as well as chaperones (e.g. Dnak, GroEL) and proteases (e.g. Clp ATPase, HtrA) involved in protein damage repair. Each of these proteins has been investigated in the studies on the acid stress response of oral streptococci *Streptococcus mutans* (Diaz-Torres & Russell, 2001; Jayaraman *et al.*, 1997; Lemos *et al.*, 2001;

Wilkins *et al.*, 2002). The chaperon GroEL has also been shown to be expressed in *L. monocytogenes* during acid stress (Phan-Thanh *et al.*, 2000).

Some regulators, such as σ^B and two-component signal transduction systems, are involved in response to low pH (Figure 1.5 section 3). The functions of σ^B have been described previously in the general stress response section. A two-component signal transduction system is typically comprised of a membrane-associated histidine kinase sensor and a cytoplasmic response regulator. The two-component system LisR-LisK plays an important role in stress response (e.g. acid, cold, osmotic stress) and virulence in *L. monocytogenes* (Cotter *et al.*, 1999; Gandhi & Chikindas, 2007).

Changes in cell density and biofilm growth are able to play roles in the modulation of acid adaptation by affecting cell-to-cell communication (Figure 1.5 section 4. This phenomenon was mainly observed in *Streptococcus mutans* (Li *et al.*, 2001).

The properties of cell membranes, such as membrane architecture, composition, stability and activity, can be altered to protect cells from acidic environment (Figure 1.5 section 5). Proteins involved in this mechanism include DltC, DagK, Ffh and SGP. These proteins have been identified in *Streptococcus mutans* (Baev *et al.*, 1999; Boyd *et al.*, 2000; Gutierrez *et al.*, 1999; Yamashita *et al.*, 1993).

The production of alkali can neutralize acids using the enzymatic activity of urease or arginine deiminase (Figure 1.5 section 6). This mechanism has been

found in *Streptococcus mutans* and other oral streptococci (Clancy *et al.*, 2000; Curran *et al.*, 1995).

1.2.3.4.3 Versatile Roles of Weak Organic Acids

In contrary to the relatively well-studied low-pH stress response, the mechanism of weak organic acid stress response seems to lack information in the scientific literature (Beales, 2004). Weak organic acids (e.g. acetic, lactic, propionic, benzoic, sorbic, citric acid, *etc*.) have a long history of use in food as antimicrobials or acidulants (Brul & Coote, 1999; Brul *et al.*, 2002; Ricke, 2003). In addition, weak organic acids (e.g. acetic acid and lactic acid) have been applied as spray sanitizers in the decontamination procedures on meat carcasses (Berry & Cutter, 2000; Eggenberger-Solorzano *et al.*, 2002; Hirshfield *et al.*, 2003). Furthermore, some short-chain fatty acids (e.g. acetate, propionate, and butyrate) are present in the gastrointestinal tracts of humans and animal hosts at high concentrations (Kwon & Ricke, 1998). Therefore, it is important to understand the potential role of weak organic acids in the stress response system.

Studies have been conducted on the impact of weak organic acids on acid stress response in Gram-negative bacteria *E. coli* and *S. enterica serovar* Typhimurium. It has been demonstrated that pre-adaptation to weak organic acids and their salts can protect bacteria from subsequent lethal concentrations of inorganic acids (Guilfoyle & Hirshfield, 1996; Kwon & Ricke, 1998). For example, a 1 h exposure to 0.1% propionic or butyric acids (pH 6.5) was able to enhance the survival of *E. coli* in inorganic acid with a pH value of 3.5 (Guilfoyle & Hirshfield, 1996), and a 1 h-exposure to acetate or propionate (pH 7.0) granted

S. enterica serovar Typhimurium the resistance against pH 3.0 acidic condition (Kwon & Ricke, 1998). Interestingly, it was shown that pre-adaptation to organic acids cannot protect bacteria from subsequent higher concentrations of the same organic acids (Baik *et al.*, 1996).

Pre-adaption to inorganic acids also has an effect on resistance to weak organic acids. It was suggested that the ATR system in *S. enterica* serovar Typhimurium can be activated and provide protection against organic acids (Baik *et al*., 1996). Research indicates that a 1 h-pretreatment with HCl (pH 4.4) can protect the log-phase *S. enterica* serovar Typhimurium against acetate, propionate and benzoate whereas a 1 h-pretreatment with HCl (pH 5.5) can protect the stationary-phase bacteria against acetate and propionate but not against benzoate.

However, each of the previously described experiments involved the use of inorganic acids (HCl), either in pre-treatments or pH adjustment of organic acid solutions. The stress response to organic acid has never been evaluated in a system devoid of inorganic acid.

1.2.3.5 Osmotic Stress Response

Increasing the osmotic pressure (i.e. lowering water activity, a_w) is one of the most widely used methods to preserve foods. Bacteria may encounter osmotic stress in the presence of high concentrations of osmotically active substances (e.g. salts and sugars) or under dry conditions (Abee & Wouters, 1999).

Bacteria can accumulate compatible solutes in the cytoplasm in response to hyperosmotic stress. Compatible solutes are small organic molecules that remain water-soluble at high concentrations without affecting intracellular

structures and metabolic activities (Abee & Wouters, 1999; Gutierrez *et al.*, 1995). The common compatible solutes in bacteria include betaine, proline, carnitine, trehalose, glycerol, sucrose, mannitol, glucitol, ectoine, glycine betaine, proline, betaine and small peptides (Abee & Wouters, 1999). The compatible solutes can be accumulated either by de novo synthesis in the cytoplasm of the cell (such as glutamate, proline, ectoine, trehalose and sucrose), and/or by transport from the environment (such as glycine and betaine) (Csonka & Hanson, 1991).

The internal osmotic pressure in bacterial cells is usually greater than that of the external environment (e.g. media). This results in osmotic pressure exerted towards the outside of the cell wall. This outward force is termed turgor pressure, which is considered to provide an essential mechanical force for cell elongation (Csonka, 1989). Bacteria cells have to maintain turgor in order to survive the osmotic pressure of the surrounding medium. When the osmotic pressure of the environment is increased, a temporary loss of turgor pressure creates osmotic stress. In response, bacteria then increase the internal osmotic pressure by raising their internal levels of compatible solute and accordingly, restore the turgor pressure. This homeostatic process is termed osmoregulation (Csonka, 1989; Gutierrez *et al.*, 1995). In this process, the regulation of the *in situ* synthesis and/or import of compatible solutes play an important role.

Betaine, for example, is the most efficient compatible solute in response to osmotic stress in a variety of bacteria. The transporter systems for betaine can be categorized into two groups. The first group is the binding protein-dependent, ATP-driven transporters, such as ProU in *E. coli* and *S. enterica* serovar

Typhimurium, as well as OpuA, OpuB and OpuC in *B. subtilis*. The second group is the ion motive force-driven transporters, such as ProP in *E. coli* and *S. enterica* serovar Typhimurium, OpuD and OpuE in *B. subtilis*, BetL in *L. monocytogenes*, as well as betaine and proline transporters in *S. aureus* (Abee & Wouters, 1999).

General stress response sigma factors (σ^S and σ^B) play a significant role in regulating the gene expression involved in compatible solute synthesis or transporter systems (Hengge-Aronis, 1996; Volker *et al.*, 1999). For example, betaine transporter gene *proP* and trehalose synthesis gene *otsAB* are regulated by σ^S in *E. coli* (Culham *et al.*, 2001; Xu & Johnson, 1997). The proline transporter gene *opuE* in *B. subtilis* and the *ctc* gene involved in osmotolerance in *L. monocytogenes* are both mediated by σ^B (Blohn *et al.*, 1997; Gardan *et al.*, 2003).

In addition to accumulating compatible solute, bacteria are also able to alter the membrane composition in response to osmotic stress. They may increase the proportion of anionic phospholipids and/or glycolipids when external a_w is lowered. For example, the addition of 2% (w/v) NaCl to the growth medium triggered an increase in the ratio of diphosphatidylglycerol: phosphatidylglycerol in *L. monocytogenes* (Russell *et al.*, 1995).

In brief, two types of mechanisms could be triggered in response to hyperosmotic stress conditions. These include the accumulation of compatible solutes and modification of cell membrane lipid composition.

1.3 The Pathogenicity of Foodborne Pathogens

1.3.1 *Escherichia coli*

1.3.1.1 The Organism and its Characteristics

E. coli is a Gram-negative, short rod-shaped, non-spore-forming organism. It belongs to the family of Enterobacteriaceae and is the predominant facultative anaerobe in the human colonic flora (Bhunia, 2008). Although most of *E. coli* strains are harmless symbionts in the intestinal lumen, there are a number of pathogenic forms that can cause a wide variety of diseases. Three general clinical syndromes caused by pathogenic *E. coli* strains are urinary tract infections, sepsis/meningitis, and enteric/diarrheal disease (Nataro & Kaper, 1998). Moreover, typically nonpathogenic strains are also able to cause infections in debilitated/ immunosuppressed hosts, or in hosts of which gastrointestinal barriers are violated.

E. coli can be classified by the serogroup that is solely defined by the type of their O antigens (or lipopolysaccharide/LPS antigens), or by the serotype based on a combination of their O, H (flagellar) and K (capsular) antigens (Kaper *et al.*, 2004; Nataro & Kaper, 1998) . In addition, pathogenicity is also used to classify *E. coli* strains. Intestinal pathogenic *E. coli* can be subcategorized into six virotypes (or pathotypes), including enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC), based on their virulence properties (Bhunia, 2008; Kaper *et al.*, 2004). In addition, extraintestinal infection-related *E. coli* strains are often subcategorized based on

the target tissue that they are infecting, such as uropathogenic *E. coli* (UPEC) which causes urinary tract infections and meningitis-associated *E. coli* (MNEC) which is responsible for meningitis and sepsis (Kaper *et al.*, 2004). Among the *E. coli* strains mentioned above, pathotypes EHEC, EPEC, ETEC and EIEC are considered as foodborne pathogens (FDA/CFSAN, 2009).

1.3.1.2 Association with Food

EHEC cause diseases from mild non-bloody diarrhea to severe bloody diarrhea (hemorrhagic colitis), and even life-threatening complication such as hemolytic uremic syndrome (FDA/CFSAN, 2009; Kaper, 2005). *E. coli* O157:H7 is the most important EHEC serotype that poses threat to public health in North America (Kaper *et al.*, 2004). Based upon a review of *E. coli* O157:H7 outbreaks in the United States from 1982 to 2002, food is the predominant mode of transmission (61% of 8,598 outbreak-related cases) and ground beef is a common food vehicle (33% of 5,269 foodborne-related cases) (Rangel *et al.*, 2005). Contaminated spinach and lettuce have been associated with three major multistate *E. coli* O157:H7 outbreaks in fall 2006 (CDC, 2008). Other foods that have been associated with *E. coli* O157:H7 outbreaks worldwide include raw/undercooked meat products (e.g. hamburger, roasted beef), raw milk, unpasteurized apple juices, dry-cured salami, and alfalfa sprouts (Buchanan & Doyle, 1997; FDA/CFSAN, 2009; Pigott, 2008; Rangel *et al.*, 2005).

EPEC primarily cause infant diarrhea in developing countries (Kaper, 2005). However, foods such as raw beef and chicken have been implicated in EPEC outbreaks (FDA/CFSAN, 2009).

ETEC are the leading bacterial cause of diarrhea in underdeveloped countries with insufficient medical facilities, as well as the most common cause of traveler's diarrhea (Kaper, 2005). Water contamination by human sewage and infected food handlers are considered to be causes of ETEC-related food contamination (FDA/CFSAN, 2009).

EIEC infections can lead to profuse diarrhea in humans. Any food contamination associated with human feces, either from ill individuals or contaminated water, can contribute to EIEC outbreaks. Raw beef and unpasteurized milk have also been reported to be sources of EIEC infections (FDA/CFSAN, 2009).

1.3.1.3 Virulence Factors and Pathogenicity

Adhesins and toxins are two major virulence factors involved in the pathogenicity of intestinal pathogenic *E. coli* (Kaper *et al.*, 2004). Pathogenic *E. coli* possesses specific adhesins that assist them to colonize and adhere to the intestinal epithelial cells. These adhesins include fimbriae, intimin, colonization factor antigens, Type IV fimbriae named bundle-forming pili, and short surface-associated filaments. In addition, some pathogenic *E. coli* strains are able to synthesize toxins, such as Shiga toxins in EHEC, heat-labile enterotoxin and heat-stable enterotoxin in ETEC.

Furthermore, it has been reported that the locus of enterocyte effacement (LEE) plays an important role in the pathogenicity of EPEC and numerous EHEC (Jores *et al.*, 2004). The LEE is a pathogenicity island required for the formation of attaching and effacing lesion on epithelial cells of humans and animals during

the infection of EPEC, EHEC, and other related bacteria. The LEE encodes a type III secretion system, an outer membrane adhesin (intimin), translocated intimin receptor and a number of secreted proteins involved in the signal transduction (Jores *et al.*, 2004; Nataro & Kaper, 1998).

EHEC adhere to the colon and induce an attaching and effacing lesion. EHEC first adhere to intestinal epithelial cells using fimbriae. A signal is then transmitted to the host cell via a type III secretion system. EHEC are finally in intimate contact with the host cell, and this step is mediated by intimin (Bhunia, 2008). Besides attaching and effacing lesion, a unique feature of EHEC is their ability to produce Shiga toxins in the colon. The functions of Shiga toxins include damaging renal endothelial cells, inducing apoptosis in intestinal epithelial cells and mediating local damage in the colon (Bhunia, 2008; Kaper *et al.*, 2004).

EPEC adhere to the small bowel enterocytes and induce the characteristic attaching and effacing lesion. EPEC firstly bind to intestinal epithelial cells and express adhesins such as bundle-forming pili, intimin, and a short surface-associated filament named EspA. EPEC then adhere to intestinal epithelial cells using these adhesins. A signal is subsequently transmitted to the host cell via a type III secretion system. EPEC are finally in intimate contact with the host cell; this step leads to accumulation of cytoskeletal components and thereby the formation of a "pedestal-like" structure underneath the attached bacteria (Bhunia, 2008; Kaper *et al.*, 2004; Nataro & Kaper, 1998).

ETEC adhere to the small bowel enterocytes using adhesins (e.g. CFAs, TibA) and then produce heat-labile and/or heat-stable enterotoxins (Bhunia, 2008;

Kaper *et al.*, 2004). The heat-labile enterotoxins can increase cAMP level and the heat-stable enterotoxins can increase cGMP level. The elevation in cAMP and cGMP levels stimulates secretion of water and electrolytes into the small intestine, thereby causing watery diarrhea (Kaper *et al.*, 2004).

EIEC invade the colonic epithelial cell, lyse the phagosome and move through the cell by nucleating actin microfilaments. EIEC could either move laterally through the epithelium by direct cell-to-cell spread, or exit and re-enter the baso-lateral plasma membrane (Kaper *et al.*, 2004). Although EIEC are biochemically, genetically and pathogenically closely related to *Shigella* spp., they do not produce Shiga toxins (Kaper *et al.*, 2004; Nataro & Kaper, 1998). Instead, a 63 kDa-toxin encoded by the plasmid-borne *sen* gene, has been reported to cause watery diarrhea (Nataro & Kaper, 1998).

1.3.2 *Staphylococcus aureus*

1.3.2.1 The Organism and its Characteristics

S. aureus is a Gram-positive, facultative anaerobic, non-sporeforming coccus, appearing as short chains or grape-like clusters. *S. aureus* has received much attention as an important foodborne pathogen mainly due to its salt-tolerance (10-15%) and relative resistance to dry and heat conditions (Bhunia, 2008; Le Loir *et al.*, 2003). Some strains are able to grow in up to 20% NaCl, and the minimum water activity ($a_w$min) for growth is 0.83 in aerobic conditions.

Staphylococcal food poisoning is a gastrointestinal illness caused by foods contaminated with enterotoxins produced by *S. aureus*. It is one of the most common foodborne diseases reported worldwide (Pigott, 2008). It has been

estimated that 185,060 cases of staphylococcal food poisoning occur annually in the United States (Mead & Slutsker, 1999). The most common symptoms of staphylococcal food poisoning are nausea, vomiting, abdominal cramps, diarrhea, and prostration. Headache, muscle cramping, and transient changes in blood pressure and pulse rate may also be found in more severe cases. Death is very rarely caused by staphylococcal food poisoning, but could occur in cases where particularly susceptible populations, such as elderly, infants, and severely debilitated persons, are involved (FDA/CFSAN, 2009; Le Loir *et al.*, 2003).

1.3.2.2 Association with Food

Foods associated with staphylococcal food poisoning include meat and meat products, poultry and egg products, seafood, salads, creamy food prepared with milk, sandwich fillings, and dairy products (FDA/CFSAN, 2009). Food handlers and equipment surfaces (e.g. meat grinder's knives) are considered the main sources of contamination in staphylococcal food poisoning. Multiple handling procedures would increase the possibility of *S. aureus* infection (FDA/CFSAN, 2009; Le Loir *et al.*, 2003; Pigott, 2008). Moreover, as enterotoxins are produced in *S. aureus* at 10-46^{o}C, temperature below 60^{o}C would allow the growth of *S. aureus* and the production of enterotoxins (Bhunia, 2008). Therefore, foods that require considerable handling during preparation and that undergo temperature abuse after preparation are frequently involved in staphylococcal food poisoning.

1.3.2.3 Virulence Factors and Pathogenicity

S. aureus produces a large variety of exotoxins, including staphylococcal enterotoxins, toxic shock syndrome toxin, and staphylococcal enterotoxin-like toxins (Dinges *et al.*, 2000; Thomas *et al.*, 2009). These toxins are responsible for specific acute clinical syndromes such as toxic shock syndrome, foodborne intoxication, and staphylococcal scarlet fever (a mild form of toxic shock syndrome) (Thomas *et al.*, 2006). In particular, staphylococcal enterotoxins are the major virulence factors of staphylococcal food poisoning. There are 19 serologically distinct staphylococcal enterotoxins designated from SEA through SEV (not including SEF, SES and SET) (Bhunia, 2008; Thomas *et al.*, 2006). Staphylococcal enterotoxins function as potent gastrointestinal toxins and superantigens that stimulate non-specific T-cell proliferation (Balaban & Rasooly, 2000). After consuming *S. aureus*-contaminated food, staphylococcal enterotoxins are absorbed and cause typical gastroenteritis. They stimulate vagus nerve endings in stomach linings and the medullary vomiting center, and induce a violent emetic symptom. Staphylococcal enterotoxins also cause damage to the intestinal epithelial cells, resulting in the destruction of intestinal villi (Bhunia, 2008).

Resistance of staphylococcal enterotoxins to heat and gastrointestinal proteases are the two major factors that make *S. aureus* a major threat in terms of food safety (Balaban & Rasooly, 2000). First, staphylococcal enterotoxins exhibit a high level of heat stability, and it was shown that SEA is stable at 121 °C for 28 min in canned mushroom products (Anderson *et al.*, 1996). This heat stability is

dependent on environmental factors (such as pH and salt concentration) that are related to the toxin denaturation (Bhunia, 2008). Second, staphylococcal enterotoxins are tolerant of proteolytic cleavage by gastrointestinal proteases such as pepsin, trypsin, chymotrypsin, papain, and rennin (Bhunia, 2008; Le Loir *et al.*, 2003). This digestive resistance allows staphylococcal enterotoxins to stay intact in the gastrointestinal tract where they induce the symptoms of staphylococcal food poisoning by interacting with staphylococcal enterotoxin receptors.

1.3.3 *Listeria monocytogenes*

1.3.3.1 The Organism and its Characteristics

L. monocytogenes is a Gram-positive, rod-shaped, facultative anaerobic, non-sporeforming organism. It has been recognized as a significant foodborne pathogen since the early 1980s, when numerous outbreaks of foodborne listeriosis were reported in North America (Bhunia, 2008; McLauchlin, 1997). *L. monocytogenes* can survive and grow in a wide range of environmental conditions such as refrigeration temperatures (2 to 4°C), broad pH ranges (4.1 to 9.6) and high salt concentration (10%) (Bhunia, 2008; Gandhi & Chikindas, 2007).

L. monocytogenes can cause listeriosis, a rare but severe disease with high hospitalization and case-fatality rates (Gandhi & Chikindas, 2007; Lianou & Sofos, 2007; McLauchlin, 1997). Listeriosis primarily affects elderly persons, pregnant women, infants (both unborn and newly delivered), and immunocompromised individuals (e.g. patients with AIDS or immunosuppressive medication). The early symptoms of listeriosis are fever, muscle aches, and gastrointestinal symptoms (e.g. nausea or diarrhea). More severe symptoms

include sepsis, meningitis, and endocarditis. Infected pregnant women may experience only a mild flu-like illness, but infection can lead to miscarriage, stillbirth, or premature delivery (Bhunia, 2008; Gandhi & Chikindas, 2007; McLauchlin, 1997).

In addition, it has been reported recently that *L. monocytogenes* was established as a cause of acute, self-limited, febrile gastroenteritis in healthy individuals (Ooi & Lorber, 2005). At least seven outbreaks of foodborne gastroenteritis due to *L. monocytogenes* have been well documented in the United States and Europe from 1993 to 2001 (Aureli *et al.*, 2000; Dalton *et al.*, 1997; Frye *et al.*, 2002; Ooi & Lorber, 2005).

1.3.3.2 Association with Food

L. monocytogenes can be found in a wide variety of raw and processing foods, including meat, vegetables, fish, and dairy products (FDA/CFSAN, 2009; Gandhi & Chikindas, 2007; Khelef *et al.*, 2006). Post-processing contamination of products with *L. monocytogenes* has become a major concern since those products are eaten without further cooking (Bhunia, 2008). Ready-to-eat (RTE) or minimally processed foods such as hotdogs, deli meats, and soft cheeses have been implicated in several outbreaks of listeriosis since the 1980s (Khelef *et al.*, 2006; Lianou & Sofos, 2007) (Table 1.1). FDA currently has a zero-tolerance policy in place for *L. monocytogenes* in RTE foods (Gandhi & Chikindas, 2007).

Table 1.1 Main listeriosis outbreaks in North America and Europe (adapted from Khelef *et al.*, 2006[a], Public Health Agency of Canada, 2008[b], and Public Health Agency of Canada, 2009[c]).

Year	Location	Number of cases	Perinatal cases (%)	Mortality rate (%)	Source of contamination
1980–1981[a]	Canada	41	83	34	Coleslaw
1983[a]	New England, USA	49	14	29	Pasteurized milk
1983–1984[a]	Switzerland	57	9	32	Soft cheese
1985[a]	California, USA	142	65	34	Mexican cheese
1986–1987[a]	Pennsylvania, USA	36	11	44	Unknown
1989[a]	Connecticut, USA	10	20	10	Shrimps
1992[a]	France	38	82	32	Deli meats (rillettes)
1993[a]	Italy	39	Unknown	Unknown	Rice salad
1994[a]	Illinois, USA	45	Unknown	Unknown	Chocolate milk
1997[a]	Italy	1566	Unknown	Unknown	Corn salad
1998–1999[a]	United States	1001	Unknown	Unknown	Hot dogs
1999[a]	France	32	12	21	Pork deli meats
2002[a]	Illinois, USA	43	28	31	Turkey deli meats
2008[b, c]	Canada	57	Unknown	39	Deli meats, wieners (Maple Leaf Foods)

1.3.3.3 Virulence Factors and Pathogenicity

The pathogenic mechanism of *L. monocytogenes* is a complex process. It can be subdivided into intestinal and systemic phases. In the intestinal phase of infection, bacteria first colonize in the intestine and subsequently translocate through the mucosal barrier to either the circulatory or lymphatic system for systemic dissemination. This phase consists of three steps: adhesion and invasion; lysis of vacuole to release bacteria; and intracellular growth (Bhunia, 2008). During systemic cell-to-cell spread, *L. monocytogenes* is transported by dendritic cells or macrophages to the liver, spleen, lymph nodes, brain, and to the placenta (in pregnant women) (Bhunia, 2008; Portnoy *et al.*, 2002).

Many surface associated and secreted proteins are major virulence factors in *L. monocytogenes*. Internalin, *Listeria* adhesion protein, autolysin amidase, cell wall hydrolase (p60), and virulence protein are responsible for adhesion and invasion to intestinal epithelial cells. Listeriolysin O and phospholipase C contribute to the lysis of vacuole. Actin polymerization protein (ActA) initiates actin polymerization which facilitates bacterial movement inside the cytoplasm (Bhunia, 2008; Posfay-Barbe & Wald, 2009). Furthermore, positive regulatory factor A (PrfA) plays a central role in the transcription of virulence genes in *L. monocytogenes* (Chaturongakul *et al.*, 2008; Posfay-Barbe & Wald, 2009). It has been demonstrated that most virulence genes are regulated by *prfA* (the gene encoding PrfA) (Bhunia, 2008; Posfay-Barbe & Wald, 2009). Strains lacking functional PrfA are considered non-virulent (Posfay-Barbe & Wald, 2009).

1.3.4 Stress Response and Bacterial Virulence

As described in Section 1.2, the sigma factors σ^S and σ^B play a pivotal role in the regulation of general stress response. In addition to their contribution to bacterial resistance to environmental stress, sigma factors also regulate virulence gene expression in foodborne pathogens (Kazmierczak *et al.*, 2005). It has been indicated that σ^B regulates the transcription of *prfA*, the gene encoding the global virulence regulator PrfA in *L. monocytogenes* (Chaturongakul *et al.*, 2008; Kazmierczak *et al.*, 2005). In addition, the two-component regulatory system which regulates specific stress response is involved in the expression of virulence genes (Gahan & Hill, 1999; Gandhi & Chikindas, 2007). These two-component systems include LisR-LisK in *L. monocytogenes* (Cotter *et al.*, 1999; Gandhi &

Chikindas, 2007), as well as PhoP-PhoQ in *S. enterica* serovar Typhimurium (Bearson *et al.*, 1998; Gahan & Hill, 1999). All these findings reveal that there is a significant relationship between stress response and bacterial virulence.

1.4 Traditional and Emerging Enumeration Methodologies

1.4.1 Traditional Enumeration Methods

1.4.1.1 Standard Plate Count

Standard plate count, also called aerobic plate count or viable plate count, is one of the most widely used methods of evaluate the number of viable bacterial cells in food products (Jay *et al.*, 2005). This technology is based on the ability of bacteria to form colonies on a nutrient agar surface. Each colony on the plate represents a colony forming unit (CFU) which, in theory, is derived from a single viable bacterial cell. Each approved methodology has a defined acceptable counting range, such as 25-250 or 30-300 CFU per plate (Adams & Moss, 2008; FDA/CFSAN, 1998; Health Canada, 1989).

Standard plate count is a conventional bacterial analytical method approved by Food and Drug Administration (FDA), United States Department of Agriculture (USDA/FSIS) and Health Canada (FDA/CFSAN, 1998; Health Canada, 1989; USDA/FSIS, 1998). In addition, CFU/g of indicator organisms has been used as an important standard in ICMSF food sampling plans (International Commission on Microbiological Specifications for Foods, 1986) and in other food microbiological criteria in Canada (Health Canada, 1999; Health Canada, 2004).

However, there are several limitations of this method. First, using CFU to determine the number of viable cells is based on the assumption that each colony originates from a single cell. However, for bacterial strains that grow in clusters or chains and cannot be completely separated into single cells during sample preparation, the resultant CFU could be much lower than the actual number of viable bacterial cells (Montville & Matthews, 2008; Morton, 2001). Second, agar plates may not be the most suitable culture conditions for some bacterial species, such as the WS6 phylogenetic division of bacteria (Dojka *et al.*, 2000). It has been reported that this uncultured phylogenetic division of bacteria is mainly distributed in marine and soil environments (Dojka *et al.*, 2000; Stackebrandt & Embley, 2000). Therefore, the growth of these bacteria under plate counting conditions could be compromised and may not accurately reflect the viability of corresponding bacterial cells. Third, bacteria could exist in a viable but non-culturable (VBNC) state, where they cannot form colonies on agar media. The amount of bacteria in the VBNC state would therefore be severely underestimated when using the standard plate count method (Colwell, 2000). This phenomenon has been found with bacteria such as *E. coli*, *Campylobacter jejuni*, *Vibrio cholera* and several other strains from natural environments (Byrd & Colwell, 1990; Colwell, 2000; Rollins & Colwell, 1986; Xu *et al.*, 1982). Last, the plate count procedure is tedious and time-consuming. Repeated plate preparation and serial dilutions are required, and results are acquired after 24 to 48 h of incubation.

With the above limitations of the standard plate count method, we proposed that the standard plate count may dramatically underestimate the total

number of bacteria, as a result of the change of cell association or survival mechanisms during the stress responses.

1.4.1.2 Turbidity (Optical Density) Measurement

Turbidity measurement using spectrophotometer is a rapid method to estimate cell concentrations in a suspension (Koch, 1970; Madigan *et al.*, 2003). The turbidity, or optical density (OD), of a bacterial suspension can be directly read from a spectrophotometer at a particular wavelength such as 540 nm, 600 nm or 660 nm (Madigan *et al.*, 2003). In addition to its use as a traditional way to establish bacterial growth curves, this method also plays an important role in monitoring cell growth during laboratory experiments and fermentation production. It is demonstrated that a linear relationship exists between turbidity (or OD) and the amount of cells (or dry weight) at low turbidities (Madigan *et al.*, 2003).

Because bacterial suspensions are usually turbid and milky (or at least light-colored), the underlying principle for concentration measurement of bacterial suspensions is somewhat different from that for concentration measurement on clear and colored solution samples (Koch, 1994). In the case of clear and colored solutions (e.g. solutions of proteins or DNA), the reading on the UV-Visible spectrophotometer reflects the ability of the dissolved sample to absorb light at certain wavelengths (Figure 1.6A). The relationship of absorbance versus wavelength can be plotted as an absorption spectrum, which can be used to identify certain functional groups (or chromophores) by comparing the spectrum to the characteristic maximum absorbance of known chromophores. Moreover,

the absorbance increases as a linear function of the concentration within certain concentration ranges, and is usually used to determine the amount of chromophore-bearing compound in its solution. This relationship is described by the Beer–Lambert law (Wood & Paterek, 1994):

$$A = \varepsilon \ell C = \log (I_0 / I) = \log (1 / T)$$

where A is the absorbance of liquid samples; ε is the molar extinction coefficient of the compound that absorbs at a certain wavelengths; ℓ is the length of the light path in centimeters; C is the molar concentration of the absorbing species; I_0 is the intensity of incident light; I is the intensity of transmitted light; T, termed transmittance, is the ratio of I to I_0. Since ε and ℓ are constant in the equation, C is directly proportional to A and inversely proportional to logarithm of T.

On the other hand, the principle underlying the concentration measurement of turbid suspensions is based on the light scattering rather than light absorption (Koch, 1994). As light passes through the suspension, part of the incident light is scattered by the surface of the cells, and only unscattered light can reach the photoelectric cell and be turned into a digital reading by the terminal output (Figure 1.6B). The linear relationship of the reading and the cell density can also be correlated by the Beer–Lambert law described above, except that optical density (OD) is used in the place of absorbance (A) as a more precise term. Since ε and ℓ are constant in the equation, OD is directly proportional to C (cell concentrations) and inversely proportional to logarithm of T within in certain concentration range.

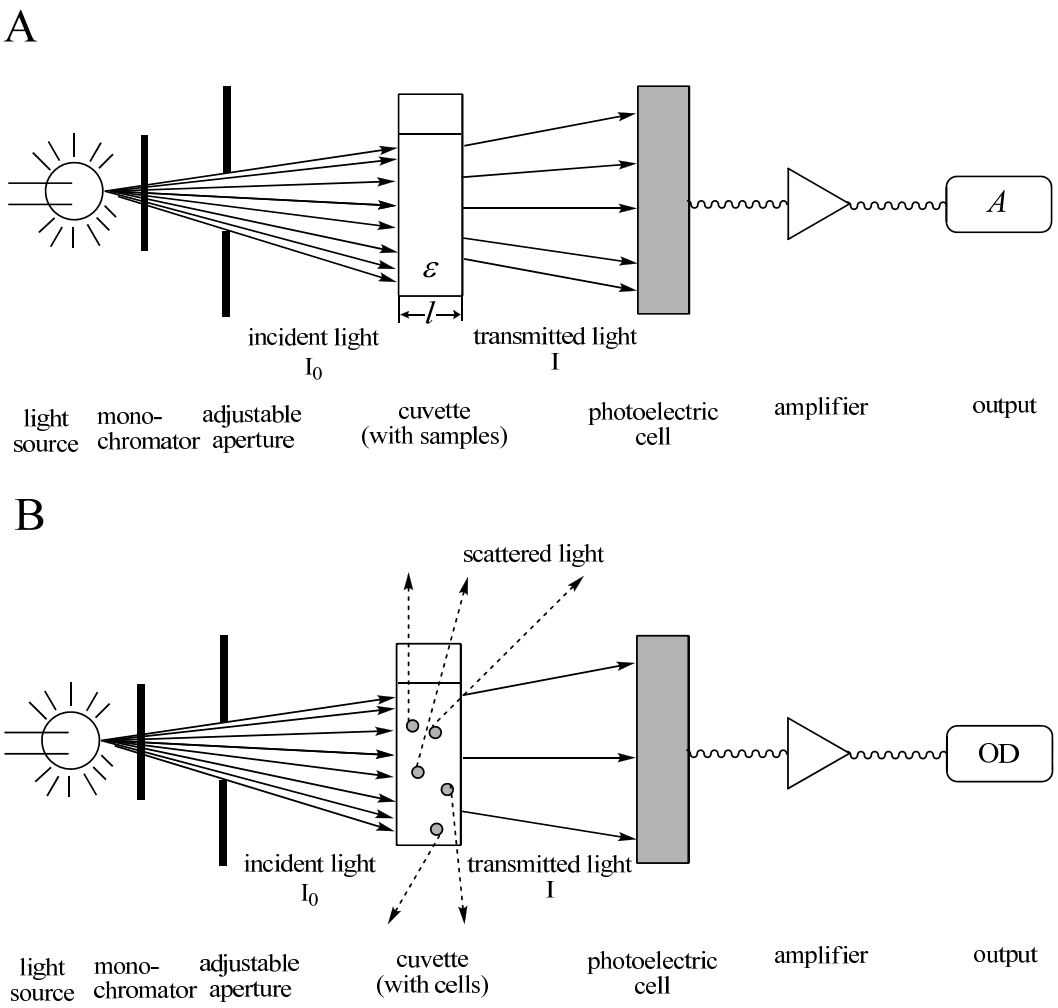


Figure 1.6 Schematic diagram of a spectrophotometer. (A) Absorbance measurement of clear and colored solutions. (B) Turbidity (optical density) measurement of bacterial suspensions.

As previously mentioned in Section 1.4.1.1, the cell association change induced by stress responses might affect the results obtained by plate count methods and lead to underestimation of viable cells. Similarly, as the associations might change the distribution of cells in suspensions, stress responses might also affect the evaluation of cell concentration by OD measurement. For instance, if cells aggregate together as a response induced by specific stress treatments, a smaller portion of the incident light would be scattered as it travels through the

suspension. As a result, the intensity of transmitted lights would be increased and a smaller OD value would be recorded by the spectrophotometer (Figure 1.7). This will lead to the underestimation of cell concentration.

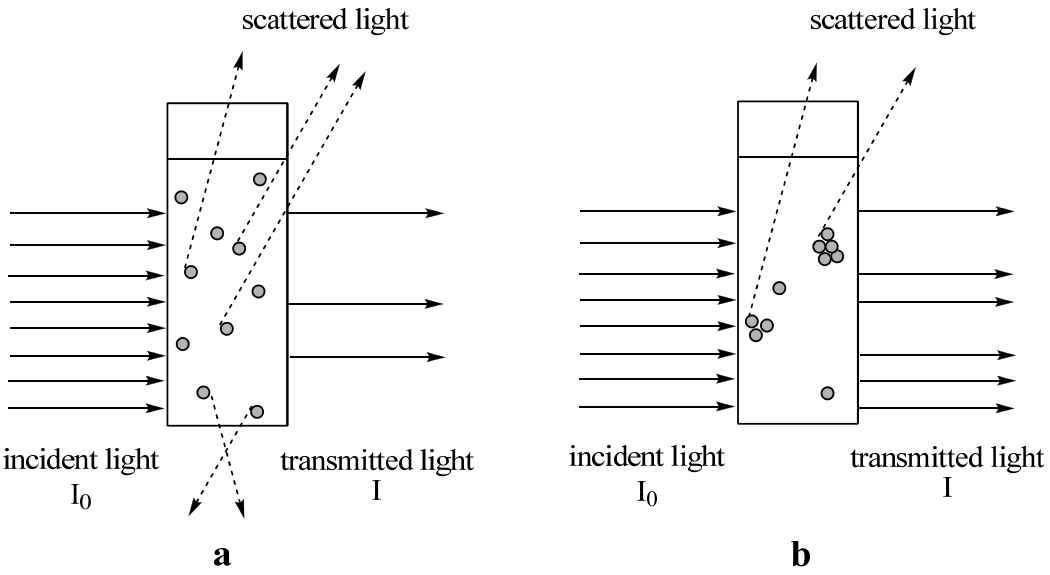


Figure 1.7 Illustration of the hypothesis that stress responses may affect the cell association and further the cell concentration in suspensions. (a) Cell suspensions under nonstress conditions. (b) Cell suspensions under stress conditions. Specific stress treatments may cause the cells to clump together and give a smaller OD value. The cell concentration would be consequently underestimated.

1.4.1.3 Other Enumeration Methods

In addition to the standard plate count and turbidity (optical density) measurement, other alternative enumeration methods used in microbiology include most probable number (MPN) count and direct microscopic count.

MPN count is a statistics-based method for estimating the number of viable microorganism in a sample (Montville & Matthews, 2008). It is particularly used to estimate low concentrations of microorganisms (<100/g) in samples such as milk, food, water and soil (Montville & Matthews, 2008;

USDA/FSIS, 1998). This method involves a minimum of three serial dilutions and several inoculated tubes (usually 3, 5 or 10) per dilution. After incubation, the pattern of positive-growth and negative-growth tubes is used to derive the "most probable number" of bacteria from a statistical chart (Montville & Matthews, 2008). However, as implied by its name, MPN count only indicates the most likely number of bacteria in a sample (Swanson *et al.*, 2001). The result is not as precise as the plate count for samples containing high concentrations of bacteria (USDA/FSIS, 1998). Another limitation of this method is that it is time-consuming. A large number of test tubes with media and serial dilutions are required, and results are acquired after 24 to 48 h incubation.

Direct microscopic count using a counting chamber (e.g. Petroff-Hausser counting chamber) is a rapid and simple method to enumerate the total number of bacteria. Cell association status and size can be assessed in the same step (Jay *et al.*, 2005). The counting chamber contains a precisely machined grid, with each square having a known area. The number of cells present in each square can be counted under the microscope. The total number is then calculated from the average of cell number per square and the corresponding culture volumes (Madigan *et al.*, 2003). There are several limitations of this method. First, dead cells are not distinguishable from live cells. Second, food particles are not always distinguishable from microorganisms. Third, small cells are difficult to observe and therefore can be missed during counting. Last, it is not suitable for cell suspensions of low density since few bacteria can be observed under the microscope (Jay *et al.*, 2005; Madigan *et al.*, 2003).

1.4.2 Emerging Methodologies

As previously described, both standard plate counts and turbidity (optical density) measurements, the two most widely used traditional enumeration methods, could underestimate the total number of bacterial cells when stress responses are induced by stresses from the cells' environment. These stresses are inseparably associated with food processing and preservation techniques in today's food industry. If survival mechanisms induced by specific stress conditions trigger changes in cell association (such as clumping) and subsequently cause the underestimation of cell numbers when using traditional enumeration methods, it would pose a serious threat to food safety and public health. Therefore, there is a need to study the impact of stress response on cell enumeration by traditional techniques and to introduce a new technique that more accurately determines the amount and viability of cells, as well as a contact angle measurement to further investigate the changes in cell surface.

1.4.2.1 Viability Staining using Fluorescence Microscopy

A fluorescence microscope is an optical microscope in which a beam of light with a defined wavelength is used to excite the fluorochrome in the specimen that consequently emits absorbed energy in the form of fluorescence. Detection with a fluorescence microscope is based upon the high-energy excitation of a fluorochrome in the specimen, followed by a lower-energy emission (Taylor & Salmon, 1989; The Nobel Foundation, 2008; Wood & Paterek, 1994). The main components of a fluorescence microscope include a light source,

an excitation filter, an emission filter, a fluorochrome in the specimen, a dichroic mirror (or beamsplitter), an objective, and an ocular (Figure 1.8).

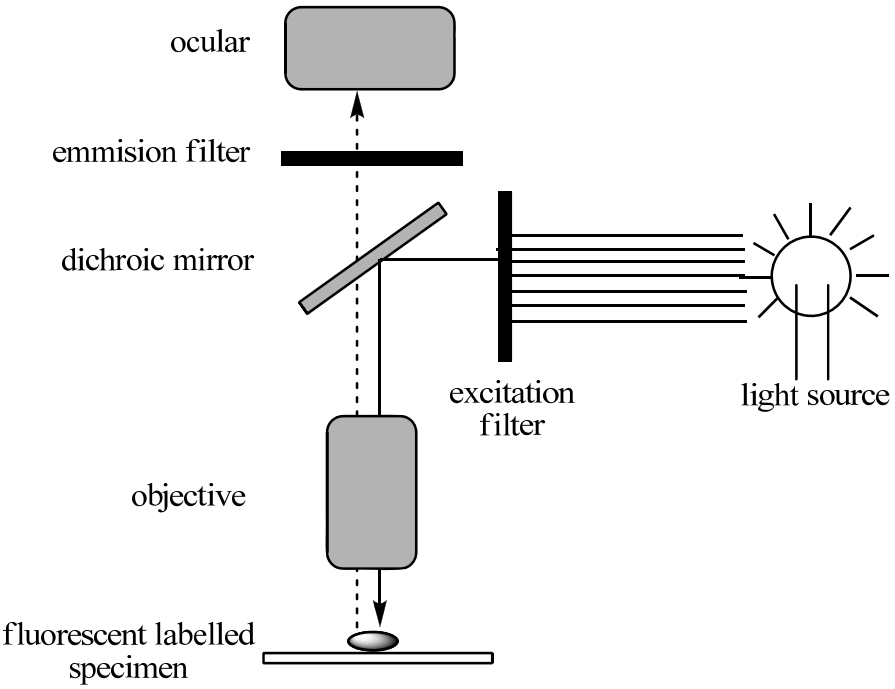


Figure 1.8 Schematic diagram of the approach used for fluorescence microscopy. The multispectral light produced by light source first passes through the excitation filter. A specific wavelength of light (usually in the shorter wavelength, such as ultraviolet, blue or green regions of the visible spectrum) is selected to excite the fluorochrome in the specimen. The excitation light is reflected by fluorochrome and the dichroic mirror, a longer wavelength (or lower intensity) of fluorescent light is then emitted.

Fluorescence microscopy has become an essential tool in microbiology and biology. In particular, some systems, such as the LIVE/DEAD® *Bac*Light™ bacterial viability staining, have been developed to test bacterial viability based on fluorescence microscopy (Boulos *et al.*, 1999; Braux *et al.*, 1997). LIVE/DEAD® *Bac*Light™ bacterial viability stain is a novel nucleic acid binding stain that can differentiate live and dead cells based on the cell membrane

integrity. Using LIVE/DEAD® *Bac*Light™ bacterial viability stain and fluorescence microscopy, it is possible to determine both the viable and total number of bacteria in a single step (Molecular Probes, 2004).

The LIVE/DEAD® *Bac*Light™ stain kit is composed of two fluorescent stains: SYTO 9 and propodium iodide (PI). These two stains penetrate bacterial cell membranes in different fashions, bind to the DNA with different affinity and give off fluorescence of different colors (Molecular Probes, 2004). The excitation/emission maximum for SYTO 9 is 480/500 nm, the excitation/emission maximum for PI is 490/635 nm (Molecular Probes, 2004). The wavelength 450-495 nm corresponds to blue light, wavelength 495-570 nm corresponds to green light and wavelength 620-750 nm corresponds to red light (Bruno & Svorono, 2006). Therefore, when choosing appropriate excitation/emission filters, blue light can excite SYTO 9 to emit green fluorescent light, and green light can excite PI to emit red fluorescent light.

The green stain SYTO 9 is a relatively small molecule that penetrates both intact membranes of live cells and damaged membranes of dead cells. In contrast, the red stain PI is a larger molecule that can only penetrate dead bacteria cells through their damaged membranes (Braux *et al.*, 1997). Since PI has a stronger affinity towards nucleic acid compared to SYTO 9, dead bacterial cells with damaged membranes would give off red fluorescence when the two stains are used simultaneously. In brief, when with a mixture of both stains in an appropriate ratio, live bacteria with intact cell membranes stain fluorescence

green, while dead bacteria with damaged membranes stain fluorescence red (Molecular Probes, 2004).

This stain system has been described as a reliable tool to evaluate cell viability in a variety of bacterial strains including *E. coli* and *S. aureus* (Boulos *et al.*, 1999; Braux *et al.*, 1997; Molecular Probes, 2004). Some studies have also shown its good staining capability in *L. monocytogenes* (Flekna *et al.*, 2007; Nexmann Jacobsen *et al.*, 1997; Swarts *et al.*, 1998).

1.4.2.1.1 Contact Angle Measurement

Contact angle measurement is an economical and user friendly standard method to determine the hydrophobic properties of the cell surface (Krekeler *et al.*, 1989; van der Mei *et al.*, 1998). In this study, we are going to apply the contact angle measurement to further investigate the changes in cell surface if the assumption that stress response does affect the cell association is confirmed.

Contact angle (θ) is a quantitative measure of the wetting of a solid surface by a certain liquid (Good, 1992). It is the angle formed by a liquid droplet at the solid-liquid-vapor or solid-liquid-liquid three-phase boundary (Figure 1.9). A large angle formed by a water droplet indicates that the substrate surface is relatively hydrophobic and a smaller contact angle suggests more hydrophilic characteristics. Generally a surface is considered to be hydrophobic if the water-solid surface contact angle is greater than 90 degrees, and is considered hydrophilic if the angle is less than 90 degrees.

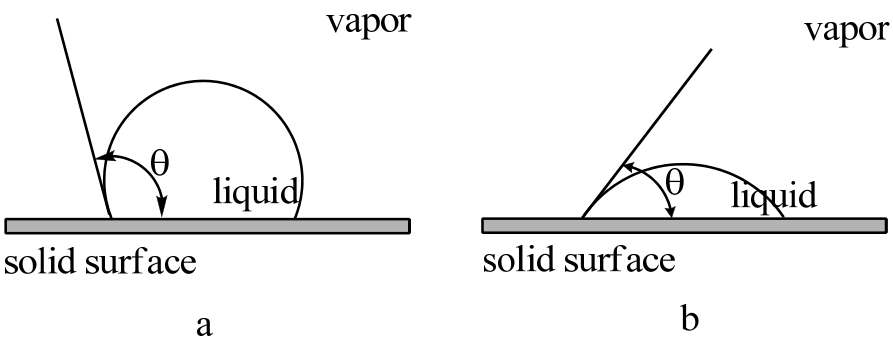


Figure 1.9 Contact angle (θ) of a drop of liquid on a solid surface (adapted from Good, 1992). (a) A liquid droplet placed on a hydrophobic surface (large contact angle). (b) A liquid droplet placed on a hydrophilic surface (small contact angle).

The theory of contact angle measurement is based on Thomas Young's hypothesis that the competition between the cohesive forces of a liquid to itself and the adhesive forces between a liquid and a solid surface result in an equilibrium contact angle which is constant and specific to a certain system. The equation that is used to describe this situation is (Neufeld *et al.*, 1980):

$$\cos \theta = (\gamma_{SV} - \gamma_{SL}) / \gamma_{LV}$$

where θ is the equilibrium contact angle, γ_{SV} is the solid surface free energy, γ_{SL} is the interfacial tension between solid and liquid, and γ_{LV} is the liquid surface tension (Figure 1.10).

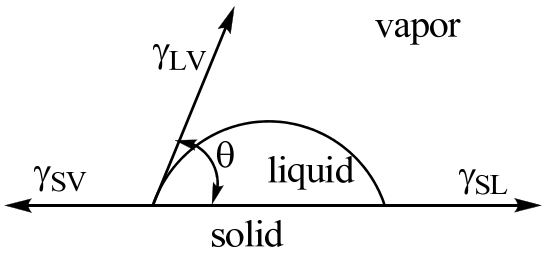


Figure 1.10 Young's equation for Contact Angles (adapted from Hahn-Hägerdal *et al.*, 1986).

In addition to the commonly used solid-liquid-vapor three-phase, contact angle can also measured in a solid-water-oil system to determine the hydrophobicity of cell surfaces. The advantage of using a solid-water-oil three-phase system is that more accurate measurements can be obtained because of the extremely low interfacial tension (Hahn-Hägerdal *et al.*, 1986; Krekeler *et al.*, 1989). It also brings minimal perturbation to the physiological characteristics of the cell surface because cells do not have to be dried during the preparation process (Neufeld *et al.*, 1980). This solid-water-oil system was employed in this study to measure the cell surface contact angle.

1.5 Conclusions

Bacteria experience a variety of environmental stresses throughout their life cycle. To eliminate harmful factors or to minimize damages from such stress, bacteria exhibit a network of stress-induced responses. Recent studies of bacterial stress responses have aroused more concerns in the food industry since various stresses are frequently involved in food processing and preservation techniques (such as heat shock, cold shock, acid stress and osmotic stress). Research indicates that the adaptive stress responses developed by foodborne pathogens not only contribute their resistance to more severe hostile environments, but also influence the expression of virulence factors.

Traditional enumeration methods (such as standard plate count, turbidity measurement) are performed without taking into consideration the pre-treatment conditions that may impose stresses on bacteria. However, there has been no systematic research on how stress brought about by pre-treatment of bacteria

relates to the outcome of the enumeration. We proposed that the existing enumeration methods might have dramatically underestimated the total number of bacteria because of the change of cell association or survival mechanisms under stress conditions. Therefore, if the stress responses do dramatically affect the pathogen enumeration, the potential hazards of present enumeration methods used in the food industry, as well as the evaluation of food processing and preservation will be disclosed.

Chapter 2 Experimental Procedures

2.1 Bacterial Strains and Growth Conditions

2.1.1 Bacterial Strains and Culture Media

Escherichia coli DH5α, *Staphylococcus aureus* ATCC 13565 and *Listeria monocytogenes* CDC 7762 were used in this study. The liquid and plate culture media of each strain are listed in Table 2.1. All culture media were purchased from Fisher Scientific (Ottawa, ON, Canada) and manufactured by Becton, Dickinson & Co. (Sparks, MD, USA).

Table 2.1 Culture media used in this study.

Strains	Source	Liquid Culture	Plate Culture
E. coli DH5α	Gibco BRL[a]	Luria-Bertani (LB) broth	LB agar: LB broth with 1.5% (w/v) agar
S. aureus ATCC 13565	ATCC[b]	Brain Heart Infusion (BHI) broth	Baird-Parker agar base with Egg Yolk Tellurite Enrichment
L. monocytogenes CDC 7762	CDC[c]	All Purpose Tween (APT) broth	APT agar: APT broth with 1.5% (w/v) agar

[a]Gaithersburg, MD, USA
[b]ATCC: American Type Culture Collection
[c]CDC: Centers for Disease Control and Prevention (USA)

2.1.2 Growth Conditions

The stock culture of each strain was prepared as per the following procedure. Liquid media were inoculated with a single bacterial colony from a

fresh streak plate, and incubated overnight at 37°C, 200 rpm in a digital platform shaker (Innova 44R, New Brunswick Scientific, Edison, NJ, USA). The culture was separated into sterile 1.5 mL microcentrifuge tubes with sterile glycerol (16% v/v) and stored at -80°C for further use.

For each experiment, bacteria were grown by transferring the stock culture to 100-fold volume of liquid medium (e.g. 1 mL stock culture to 100 mL liquid medium) and incubating at 37°C, 200 rpm until either mid-log phase or stationary phase, as required. Generally, mid-log phase bacteria were grown until the OD_{600} reached 1.0 (Ultrospec 4300 *pro* UV/Visible Spectrophotometer, GE Healthcare, Piscataway, NJ, USA) and stationary phase bacteria were grown overnight (12 h).

2.2 Stress Treatment Procedure

Cell culture (30 mL) was added to 50 mL sterile polypropylene centrifuge tubes (Corning Incorporated, Corning, NY, USA) and cells were harvested by centrifugation (accuSpin 400, Fisher Scientific, Pittsburgh, PA, USA) at 7500 × *g* for 15 min. After the supernatant was removed, the cell pellets were resuspended in 10 mL of NaCl (0.85% w/v) and centrifuged at 7500 × *g* for 15 min. This wash step was repeated three times. The control for stress studies was prepared by diluting the washed cell pellet with 0.85% NaCl solution until an OD_{600} of 1.0 was reached.

2.2.1 Acid Stress Assay

The organic and inorganic acid solutions used in this study are listed in (Table 2.2). They were prepared aseptically by filtration through 0.22 μm pore

size syringe filters (Fisher Scientific, Ottawa, ON, Canada). An aliquot of acid solution was removed for pH measurement using a digital pH meter (Accumet BASIC AB15, Fisher Scientific, Pittsburgh, PA, USA).

Table 2.2 Acid solutions used in this study.

Name	Manufacturer	Concentration	pH
acetic acid (glacial)	MP Biomedicals, LLC Solon, Ohio, USA	0.5 M	2.46
		0.2 M	2.70
		0.1 M	2.90
		0.05 M	3.12
		0.01 M	3.64
		0.001 M	5.10
lactic acid (≥85%, natural)	Sigma-Aldrich, Co. Milwaukee, WI, USA	0.05 M	2.50
		0.02 M	2.78
		0.01 M	3.01
		0.005 M	3.29
		0.001 M	4.87
hydrochloric acid	Fisher Scientific Nepean, ON, Canada	0.001 M	2.99
		0.0005 M	3.37
sulfuric acid	Fisher Scientific Nepean, ON, Canada	0.0005 M	3.00
		0.00025 M	3.29

To study the effect of acid stress on bacterial enumerations, cell pellets were treated by being gently suspended in different concentrations of acetic acid, lactic acid, hydrochloric acid, and sulfuric acid. The volumes of the acid were consistent to that of the 0.85% NaCl solution used in the preparation of the control. All resultant cultures were incubated at 37°C and sampled at different

time intervals for optical (or fluorescence) microscopy, plate count, optical density and contact angle analyses.

2.2.2 Heat Shock Assay

For heat shock studies, cell pellets were resuspended in 0.85% NaCl solution and the volumes were equal to those used in the preparation of the control. Cultures were incubated in a water bath at 55^{o}C and 60^{o}C, respectively. All resultant cultures were sampled at different time intervals for optical (or fluorescence) microscopy, plate count, and optical density analyses.

2.2.3 Cold Shock Assay

For cold shock studies, cell pellets were resuspended in 0.85% NaCl solution and the volumes were equal to those used in the preparation of the control. Cultures were incubated at -20°C, 4^{o}C, and 10^{o}C, respectively. The -20°C treatment was carried out in a freezer. The resultant cultures were subsequently thawed at room temperature for 15 min and sampled at different time intervals for optical (or fluorescence) microscopy, plate count, and optical density analyses. The 4^{o}C and 10^{o}C treatments were carried out in a refrigerator and a water bath, respectively. The resultant cultures were directly sampled at different time intervals and subjected to the analyses described above.

2.2.4 Osmotic Stress Assay

The osmotic stress environment was created using sodium chloride solutions with various concentrations. Because the solubility of NaCl in water at 25^{o}C is 35.9 g/100 mL (w/v), the highest concentration that a NaCl solution can

reach is 26.4% (w/w) (35.9 g/135.9 = 26.4%) (Patnaik, 2003). Therefore, NaCl solutions with concentrations of 5%, 15% and 25% (w/w) were used in this study.

To determine the effect of osmotic stress on bacterial enumerations, cell pellets were gently suspended in different concentrations of NaCl solutions. The volumes of the NaCl solutions were consistent to that of the 0.85% NaCl solution used in the preparation of the control. All resultant cultures were incubated at 37°C and sampled at different time intervals for optical (or fluorescence) microscopy, plate count, and optical density analyses.

2.3 Microscopy

2.3.1 Preliminary Optical Microscopy

One droplet of culture was directly observed under an optical microscope (Hund Wetzlar H 600 Wilozyt K, Helmut Hund GmbH, Wetzlar, Germany) equipped with a digital camera (EOS 350D, Canon Canada Inc., Mississauga, ON, Canada) every hour after acid, salt, heat and cold treatments, as described in Section 2.2. If the desired association change (i.e. cell clumping) was observed, a live/dead fluorescent staining method was employed to assess the bacterial viability.

2.3.2 Fluorescence Microscopy Using Viability Staining

2.3.2.1 LIVE/DEAD® *Bac*Light™ Bacterial Viability Staining

The LIVE/DEAD® *Bac*Light™ stain kit (L7012, Molecular Probes Inc., Eugene, OR, USA) was used to access bacterial viability in this study. The kit is composed of two nucleic acid stains: green-fluorescent SYTO 9 and red-

fluorescent propidium iodide (PI). When using a mixture of both stains, live bacteria stain fluorescence green, while dead bacteria stain fluorescence red.

The stain mixture was prepared by mixing equal volumes of SYTO 9 and PI in a 1.5 mL microcentrifuge tube. Freshly prepared stain mixture (0.6 μL) was added to 200 μL of the sample cultures and incubated at room temperature in the dark for 15 min. Stained culture (7 μL) was placed between a glass slide and a coverslip and observed under a fluorescence microscope.

2.3.2.2 Fluorescence Microscopy

Sample cultures were examined at 1 h, 5 h and 24 h using a Zeiss Axioplan2 upright microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY, USA) equipped with a Zeiss plan-Apochromat 100× 1.40 oil DIC objective. The fluorescein isothiocyanate (FITC) and Cyanine3 (Cy3) filters were selected according to the excitation/emission spectra of stains: SYTO 9 (480/500 nm) and PI (490/635 nm). In addition, a differential interference contras (DIC) filter was selected to represent all bacteria. Images were captured with a Photometrics CoolSNAP HQ monochrome camera (Roper Scientific Inc., Tucson, AZ, USA) mounted on the microscope and were analyzed by MetaMorph software (Molecular Devices, Downingtown, PA, USA).

For each sample, three images were acquired using the FITC, Cy3 and DIC filters, respectively. The first image represents live bacteria with fluorescent green, the second image represents dead bacteria with fluorescent red, and the third image represents all bacteria. A fourth merged image was automatically

generated by overlapping the first two images (live and dead) using MetaMorph software to show the viable status of each sample.

Proper exposure time of each strain was determined using control samples before the actual imaging. The live control, which was suspended in 0.85% NaCl as described in Section 2.2, was used to determine the exposure time for green (live) strains. The dead control, an autoclaved bacterial culture suspended in 0.85% NaCl, was used to optimize settings for imaging red (dead) stains.

A minimum of 10 randomly chosen fields of each sample were subjected to fluorescence microscopic analysis. All experiments were repeated on a minimum of three different days.

2.4 Standard Plate Count

Sample cultures were serially diluted in peptone saline water (0.1% peptone and 0.85% NaCl) at 1 h, 5 h and 24 h after each treatment. Appropriate dilutions (0.1 mL) were spread on agar plates, as described in Section 2.1.1. All plates were incubated in a 37°C incubator for 48 h. Plate counts were performed in triplicate, and the plates with 30-300 colonies were counted. Data were converted to log CFU/mL prior to statistical analysis.

2.5 Turbidity (OD_{600}) Measurement

The optical density (OD_{600}) of sample cultures was determined using a spectrophotometer at 1 h, 5 h and 24 h after treatment. Well-mixed sample cultures (1.5 mL) were placed in a cuvette (semimicro polystyrene, Fisher Scientific, Ottawa, ON, Canada) and measured at each time interval. A 0.85%

NaCl solution was used as blank to determine the background absorbance. Each measurement was performed in triplicate and the mean value was calculated.

2.6 Contact Angle Measurement

The contact angle measurement was used to further investigate the changes in cell surface hydrophobicity in this study. The contact angle was measured with a FTA200 goniometer (First Ten Angstroms, Portsmouth, VA, USA) in a solid-water-oil system (Figure 2.1).

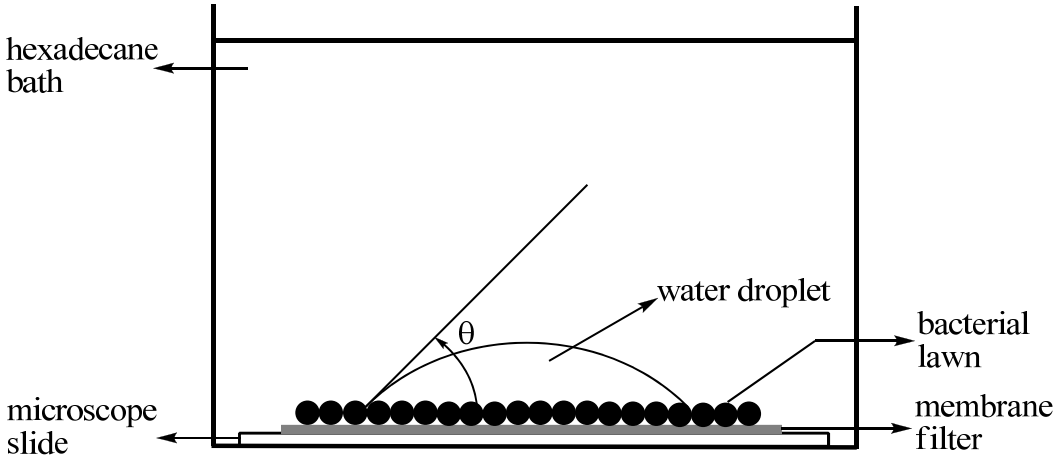


Figure 2.1 Illustration of the contact angle measurement. The contact angle (θ) is measured between the baseline of the water droplet on bacterial lawn and the tangent at water/hexadecane interface.

After the treatment steps described in Section 2.2, 50 mL of each sample culture was filtered through a 0.22 μm polyvinylidene fluoride membrane filter (Durapore® PVDF, diameter 25mm, Millipore Co., Billerica, MA, USA) to form a lawn of bacterial cells on the filter. The bacteria-loaded filter was mounted on a microscope slide using double-sided adhesive tape (PELCO Tabs™, Carbon

Conductive Tabs, Ted Pella Inc., Redding, CA, USA), and immersed in an n-hexadecane solution (99% purity, Fisher Scientific Co. Nepean, ON, Canada). Sterile distilled water (10 μL) was added to the surface of the bacterial lawn and the contact angle between the baseline of the water droplet and the tangent at water/hexadecane interface was measured using a FTA200 goniometer. Each contact angle value was measured at 1 min intervals within 10 min after the addition of the water droplet. The values measured at the 3 min time point in each treatment were used for statistical analysis. The goniometer was calibrated with a sapphire standard ball (First Ten Angstroms, Portsmouth, VA, USA) before each experiment. The contact angle value was the mean of four independent measurements.

2.7 Statistical Analysis

Standard plate count, turbidity (OD_{600}) and contact angle measurement data were analyzed by using a two-way ANOVA (GLM procedure of SAS®, SAS Institute Inc., Cary, NC, USA) separately. Different treatment concentrations and different treatment periods were fixed effects. Least squares means were estimated and separated using the pdiff option when fixed effects were significant ($P < 0.05$).

Chapter 3 Results

The objective of this study was to provide an in-depth evaluation of how traditional enumeration methods for bacteria are affected by identified stresses in the food industry. In this study, three bacterial strains were enumerated by means of standard plate count and optical density measurements post-stress. The cultures were then further evaluated using fluorescence microscopy to identify changes in cell association status and viability. For each microscopic evaluation described in this section, a set of four fluorescence microscopy images were acquired. Figure 3.1 shows a general example, using *E. coli* DH5α, of the microscopy data that was obtained for each strain and treatment.

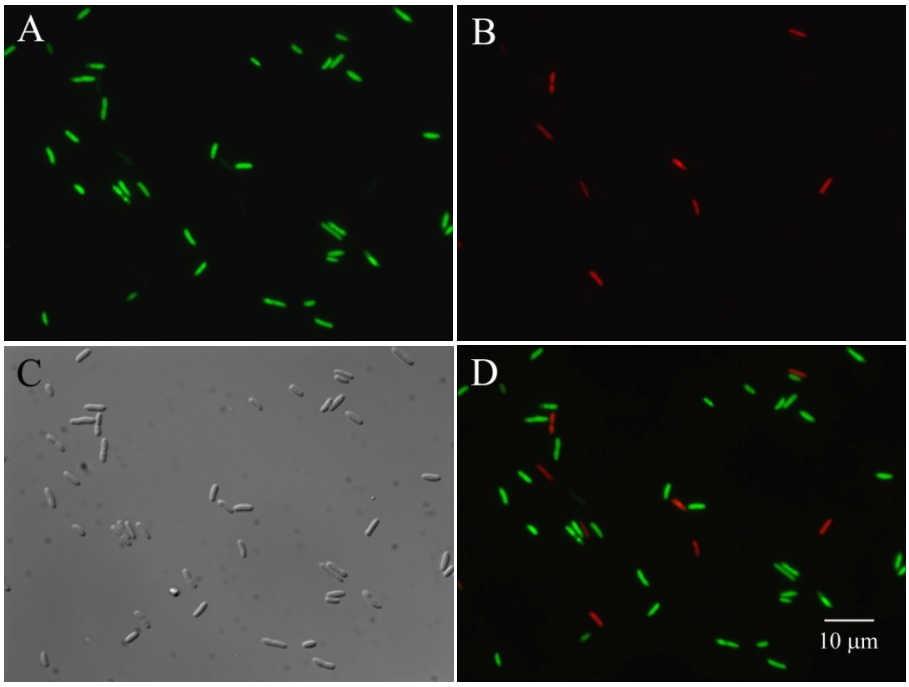


Figure 3.1 An example of one set fluorescence microscopy images acquired for *E. coli* DH5α depicting live bacterial cells (A), dead bacterial cells (B), total bacterial cells (C) and a merged image of live and dead bacterial cells (D).

In Figure 3.1A and 3.1B, the green and red fluorescent cells are live bacterial cells stained with SYTO 9 and dead bacterial cells stained with PI, respectively. Figure 3.1C shows all visible bacterial cells (live and dead) using differential interference contrast light microscopy. Figure 3.1D is automatically generated within the detection system by overlap of Figure 3.1A and Figure 3.1B. This results section will only report the overlapping images, which simultaneously display live and dead cells. Complete sets of images are provided in Appendix A. Each fluorescence microscopic image was obtained from the same volume of bacterial culture and randomly chosen from a minimum of 30 independent microscopic fields. Some images may not reflect the same range of cell numbers. However, the overall number and pattern of bacterial cells did not significantly change across the 30 fields if not further mentioned. The following sections describe the effect of various stresses on *E. coli* DH5α, *S. aureus* ATCC 13565, and *L. monocytogenes* CDC 7762 in relationship to cell association status, viability and enumeration.

3.1 Effect of Organic Acid Stress

To examine the impact of organic acid stress on the survival and cell association of *E. coli* DH5α, *S. aureus* ATCC 13565, and *L. monocytogenes* CDC 7762, bacterial cells were treated for 1 h, 5 h and 24 h with acetic acid and lactic acid of different concentrations/pH as shown in Table 2.2. The cells were stained with LIVE/DEAD® *Bac*Light™ viability stains and observed by fluorescence microscopy. Standard plate count and optical density measurements were

performed to compare the outcomes of these two traditional enumeration methods with the fluorescence microscopy analyses.

3.1.1 Stationary-Phase *E. coli* DH5α

3.1.1.1 Fluorescence Microscopy

The fluorescence microscopy analyses of stationary-phase *E. coli* DH5α cells treated with acetic acid and lactic acid solutions for periods of 1 h, 5 h and 24 h are shown in Figures 3.2 and 3.3, respectively. It was found that bacterial cells tended to aggregate when treated with either acetic or lactic acid of higher concentrations (or lower pH). After 1 h treatment, initial indications of clumping were observed at acetic acid concentrations of 0.05 M and 0.1 M. More consistent clumping was observed after 1 h at 0.2 M and 0.5 M acetic acid, as well as 0.005 M - 0.05 M lactic acid. For both acid treatments of 5 and 24 h, significant clumping was observed at concentrations beyond 0.05 M acetic acid and 0.005 M lactic acid with no visible difference between the two time treatments. Some individual cells were seen when the samples were treated with 0.2 M and 0.5 M acetic acids, as well as 0.02 M and 0.05 M lactic acids, however, the majority of cells were positioned in a clumping pattern.

These results also demonstrate that the majority of cells were still alive after either acetic or lactic acid treatment. Upon treatment with 0.2 M and 0.5 M acetic acid, the apparent number of dead cells slightly increased with an extended treatment time of 5 or 24 h. Additionally, a considerable proportion of cells died after a 1 h exposure to 0.05 M lactic acid.

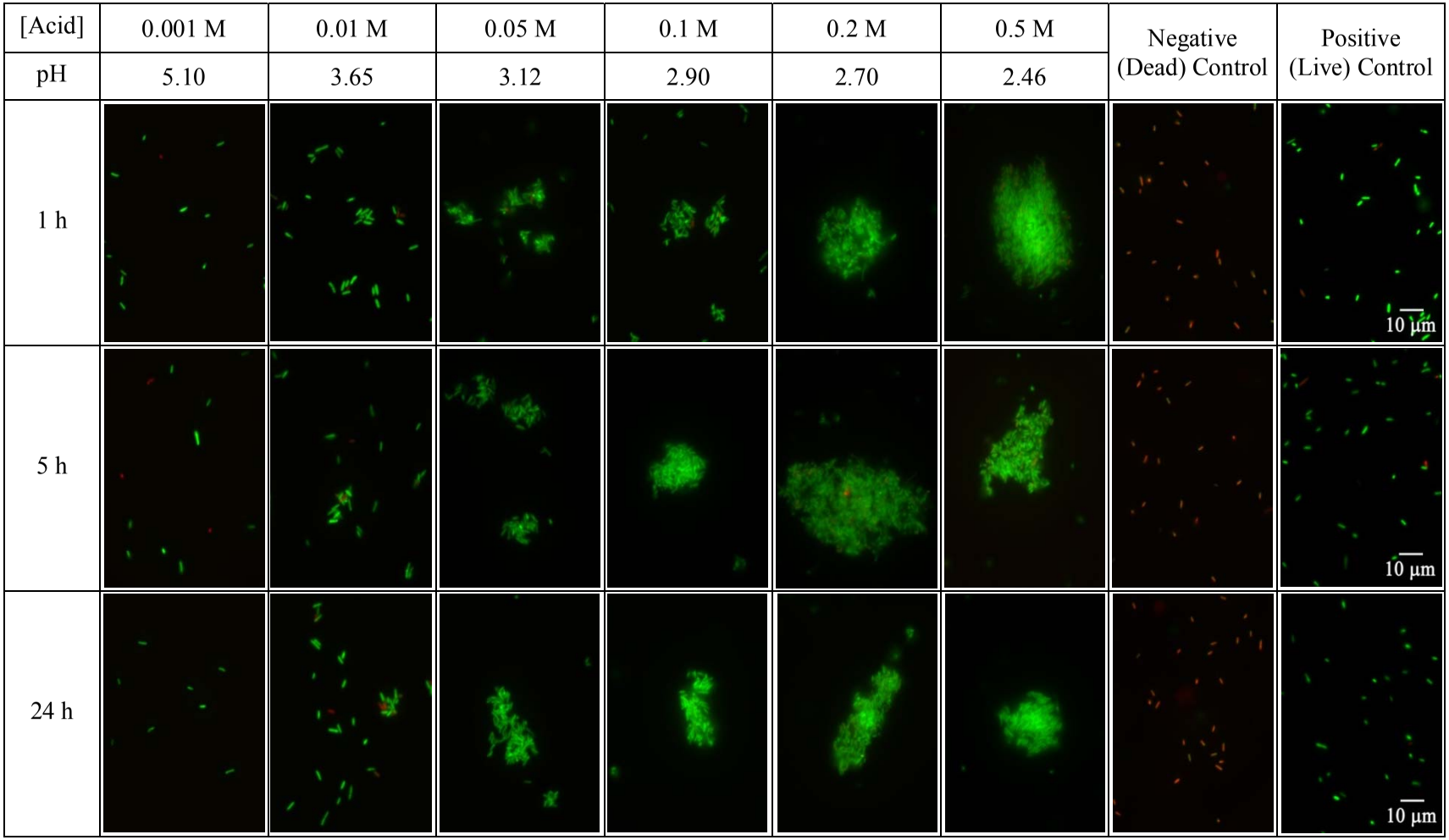


Figure 3.2 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of acetic acid for 1, 5 and 24 h.

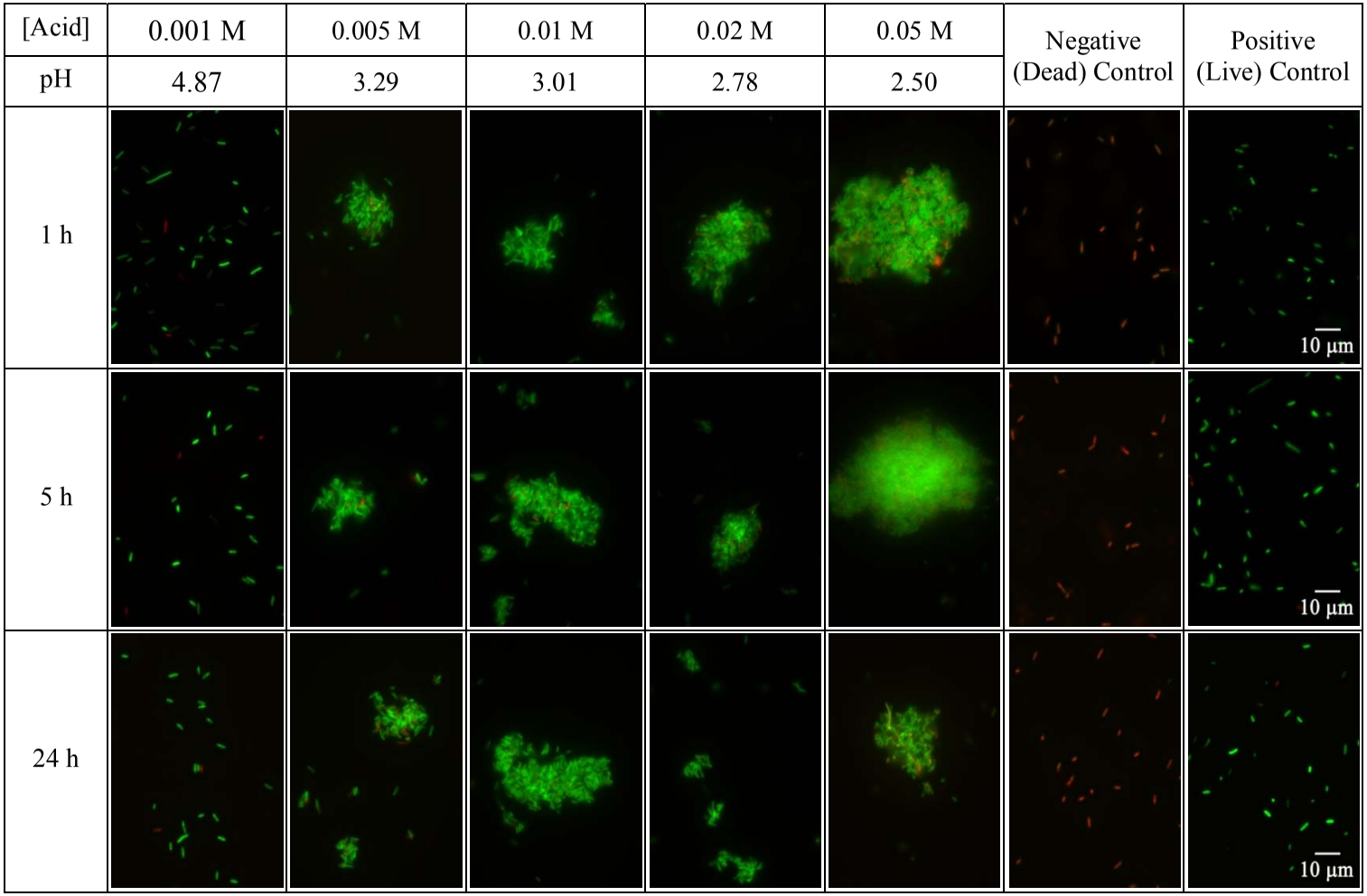


Figure 3.3 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of lactic acid for 1, 5 and 24 h.

3.1.1.2 Standard Plate Count

In the corresponding plate count study, we have demonstrated that the viable counts of stationary-phase *E. coli* DH5α were dramatically reduced when the cells were treated with either acetic or lactic acid of higher concentrations (lower pH) or for longer times (Figure 3.4).

The plate count results of each acid treated sample were compared to those of the control experiment after same periods of treatment. As shown in Figure 3.4A, the counts of 0.01 M and 0.05 M acetic acid treatments significantly decreased by 1.45 and 3.82 log units ($P < 0.05$) respectively after 5 h, and no colonies formed if the acid treatment was prolonged to 24 h. The counts of 0.1 M and 0.2 M treatments significantly decreased by 0.73 and 1.67 log units ($P < 0.05$) respectively after 1 h, and no colonies formed when the treatment time was extended to 5 or 24 h. When the sample was treated with 0.5 M acetic acid, no colonies formed within the 24 h treatment. See Appendix B Table B.1 for detailed statistical data.

As represented in Figure 3.4B, a similar reduction was observed with lactic acid treatments. The counts of cells treated with 0.005 M lactic acid significantly decreased by 2.21 log units ($P < 0.05$) after 5 h, and no colonies formed after 24 h. The counts of 0.01 M and 0.02 M treatments significantly decreased by 0.69 and 1.66 log units ($P < 0.05$) respectively after 1 h, and no colonies formed after 5 and 24 h. When the samples were treated with 0.05 M lactic acid, no colonies were detected within the 24 h treatment. See Appendix B Table B.1 for detailed statistical data.

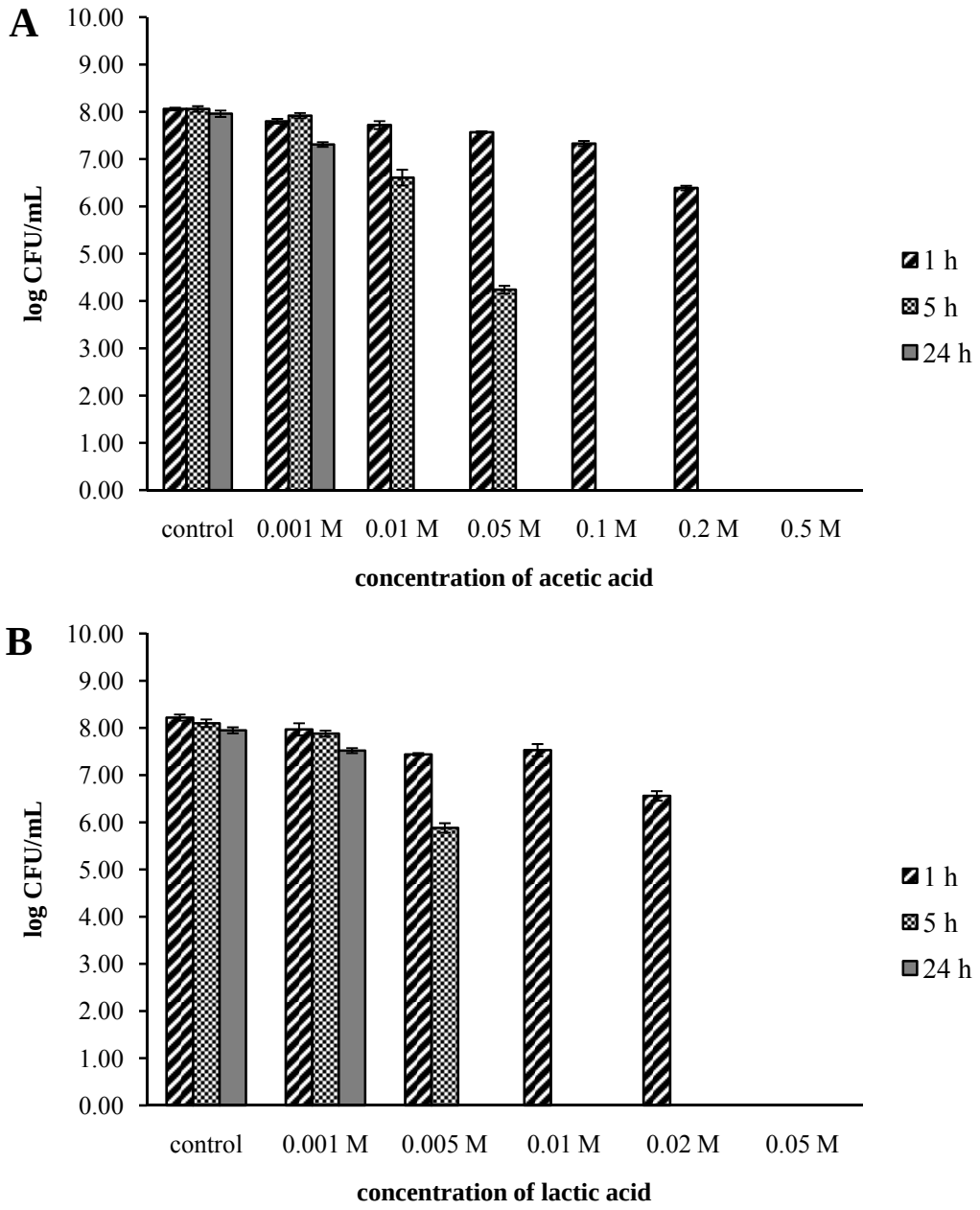


Figure 3.4 Cell counts determined by standard plate count methods for cells of stationary-phase *E. coli* DH5α after exposure to a range of concentrations of acetic acid (A) and lactic acid (B) for 1, 5 and 24 h (mean ± SEM; n=9).

3.1.1.3 Turbidity (OD_{600}) Measurement

As shown in Figure 3.5, although there were no significant differences ($P > 0.05$) in OD_{600} values over time for each acetic acid and lactic acid concentration,

significant decreases ($P < 0.05$) were observed at each treatment concentration as compared to the control.

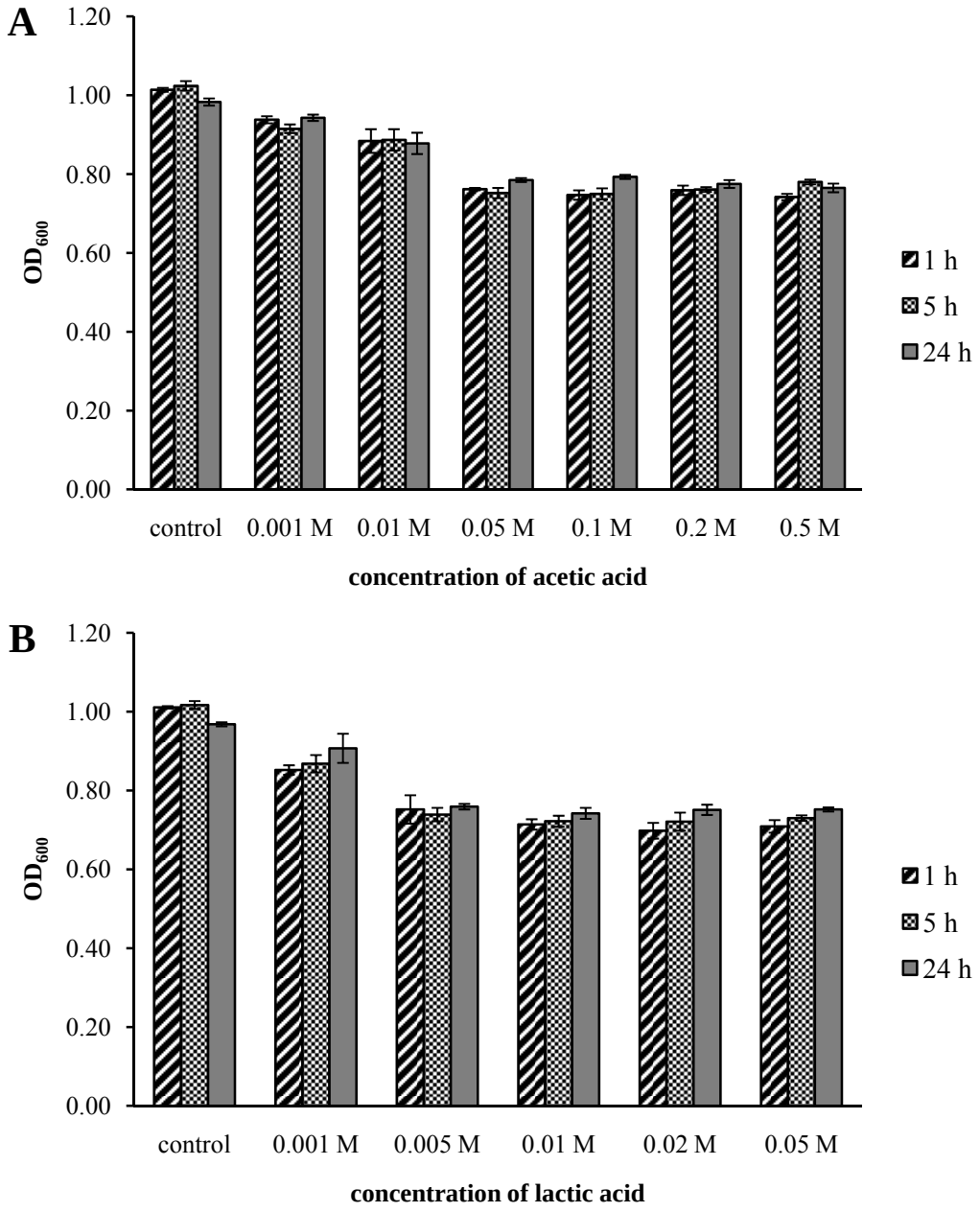


Figure 3.5 Cell concentration determined by optical density measurements for cells of stationary-phase *E. coli* DH5α after exposure to a range of concentrations of acetic acid (A) and lactic acid (B) for 1, 5 and 24 h (mean ± SEM; n=9).

For acetic acid treatments, there was a 7%, 12% and 24% observed decrease ($P < 0.05$) in OD_{600} values at 0.001 M, 0.01 M, and 0.05 to 0.5 M

concentrations as compared to the control, respectively. See Appendix B Table B.1 for detailed statistical data.

For lactic acid treatments, there was approximately a 12% and 27% decrease ($P < 0.05$) in OD_{600} values at 0.001 M, as well as 0.005 to 0.05 M concentrations as compared to the control, respectively. See Appendix B Table B.1 for detailed statistical data.

3.1.2 Mid-log Phase *E. coli* DH5α

3.1.2.1 Fluorescence Microscopy

Figures 3.6 and 3.7 provide the fluorescence microscopy analyses of mid-log phase *E. coli* DH5α cells treated with acetic acid and lactic acid for periods of 1 h, 5 h and 24 h, respectively. These figures demonstrate that mid-log phase cells could clump together when treated with acetic and lactic acid of higher concentrations (or lower pH) or for longer periods.

After 1 h treatment, initial indications of clumping were detected at acetic acid concentrations of 0.05 M and 0.1 M, as well as lactic acid concentrations of 0.005 M and 0.01 M. The clumping became more evident when the treatment time was extended to 5 or 24 h. When the concentrations were increased to 0.2 M acetic acid and 0.02 M lactic acid, obvious clumping was observed after 1 h treatment, with minor visible changes in cell aggregation after 5 or 24 h. Additionally, the clumping resulted by both organic acid stresses on mid-log phase *E. coli* were not as dramatic as observed on stationary-phase cells.

In terms of cell viability, it is demonstrated that most bacteria were still alive after 0.05 M, 0.1 M and 0.2 M acetic acid treatments for 5 h. The apparent

number of dead cells increased when the treatment time was extended to 24 h. In addition, increased dead cells were observed after 0.5 M acetic acid treatment for 1 h. Most bacteria were still alive after 1 h of lactic acid treatment, and the apparent number of dead cells increased when treated with 0.005 M, 0.01 M, 0.02 M and 0.05 M lactic acids for 5 or 24 h.

3.1.2.2 Standard Plate Count

The plate count study indicates that the viable counts of mid-log phase *E. coli* DH5α were dramatically reduced when the cells were treated with either acetic or lactic acid of higher concentrations (lower pH) or for longer times (Figure 3.8).

The counts of cells treated with 0.01 M acetic acid significantly decreased by 1.50 log units ($P < 0.05$) after 1 h, with a further decrease of 5.72 log units ($P < 0.05$) after 5 h, and no colony formation after 24 h (Figure 3.8A). The counts of 0.05 M and 0.1 M treatments significantly decreased by 2.46 and 5.46 log units ($P < 0.05$) respectively after 1 h, and no colonies formed when the treatment time was extended to 5 or 24 h. When the sample was treated with 0.2 M and 0.5 M acetic acids, no colonies formed within the 24 h. See Appendix B Table B.2 for detailed statistical data.

The counts of cells treated with 0.005 M, 0.01 M and 0.02 M lactic acids significantly decreased by 1.91, 4.57 and 5.28 log units ($P < 0.05$) respectively after 1 h, and no colonies formed when the treatment time was extended to 5 or 24 h ((Figure 3.8B). Exposure of cells to 0.05 M lactic acid did not produce any colonies within the 24 h. See Appendix B Table B.2 for detailed statistical data.

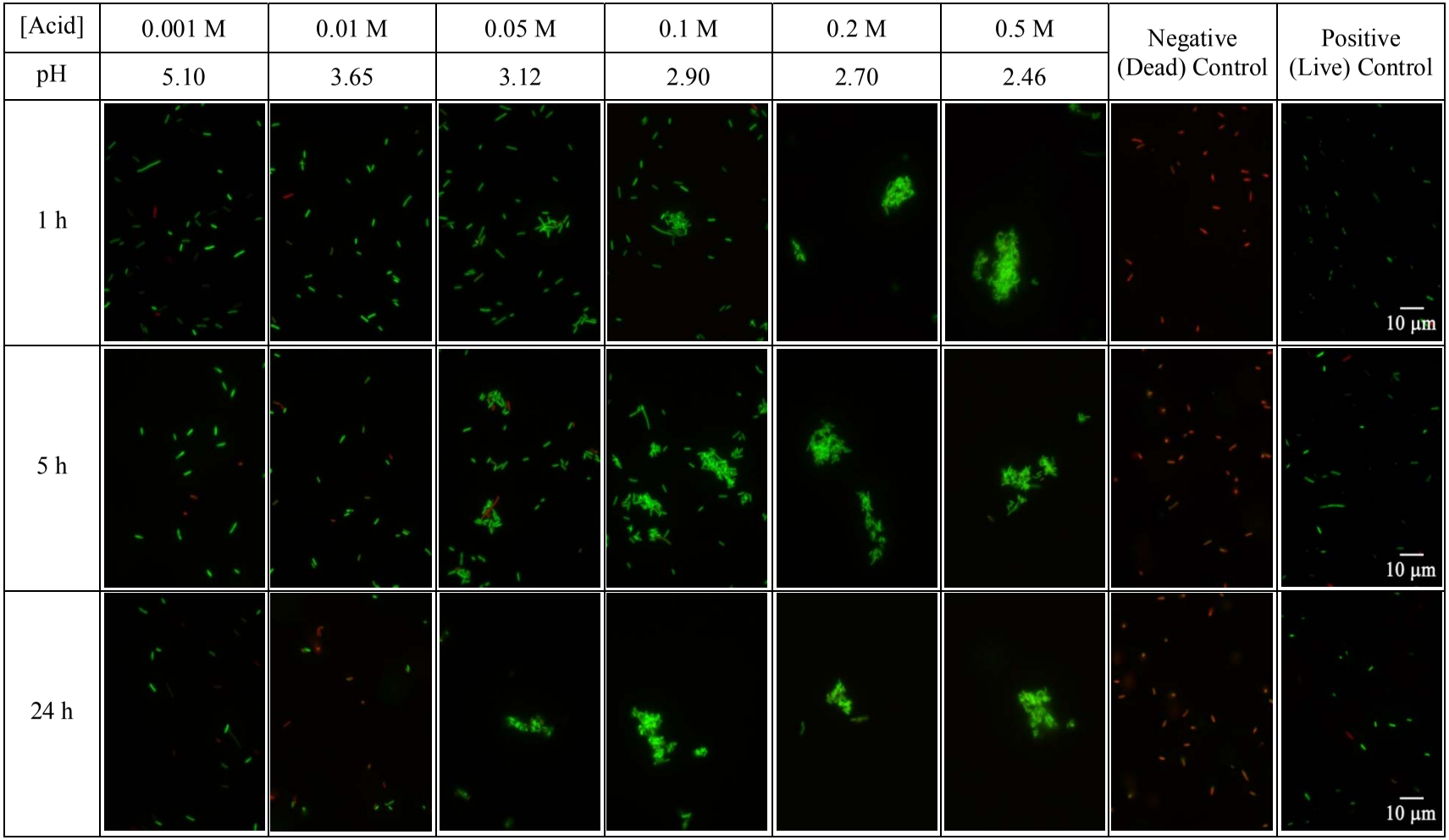


Figure 3.6 Fluorescence microscopy images of mid-log phase *E. coli* DH5α after exposure to different concentrations of acetic acid for 1, 5 and 24 h.

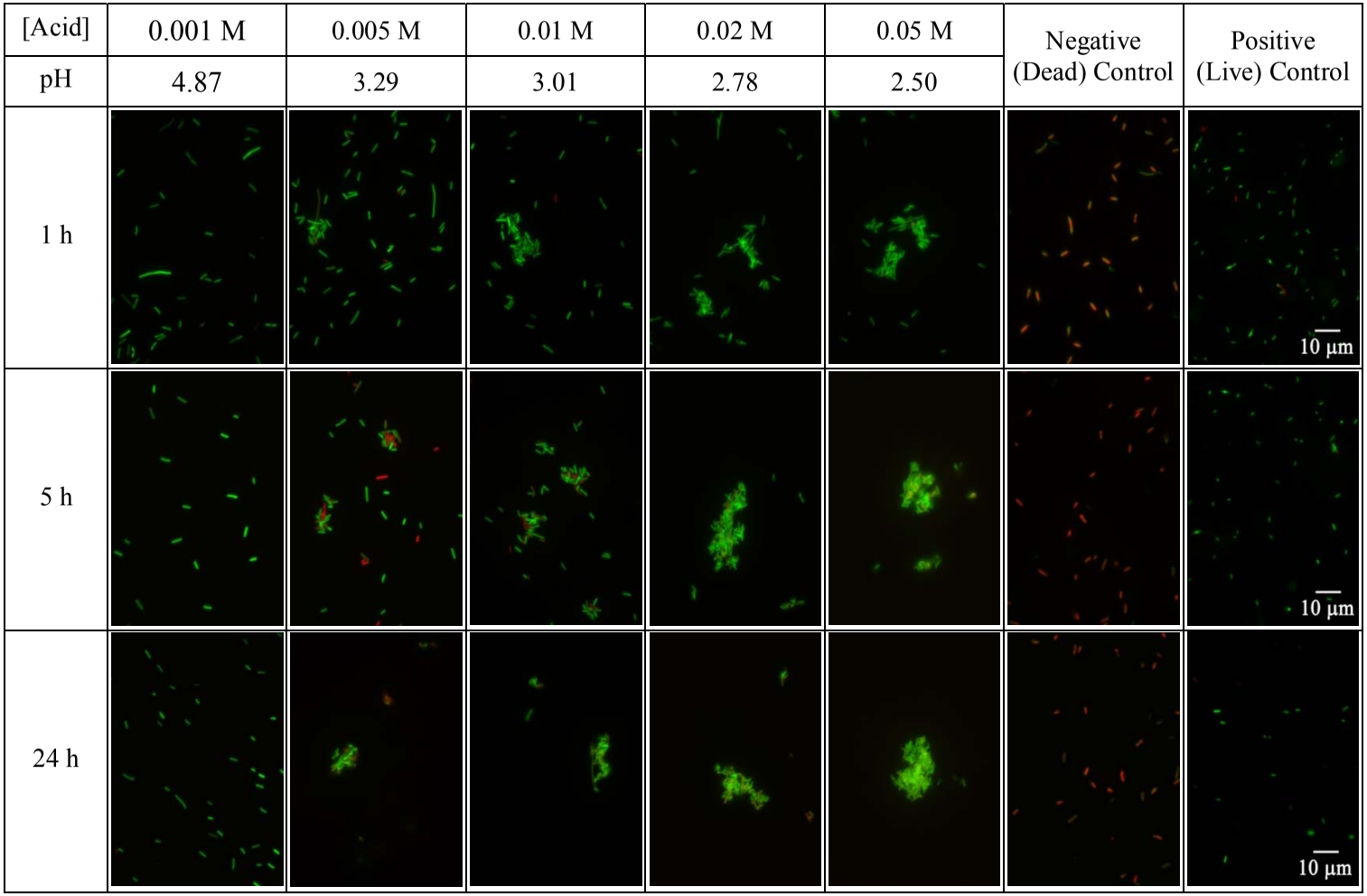


Figure 3.7 Fluorescence microscopy images of mid-log phase *E. coli* DH5a after exposure to different concentrations of lactic acid for 1, 5 and 24 h.

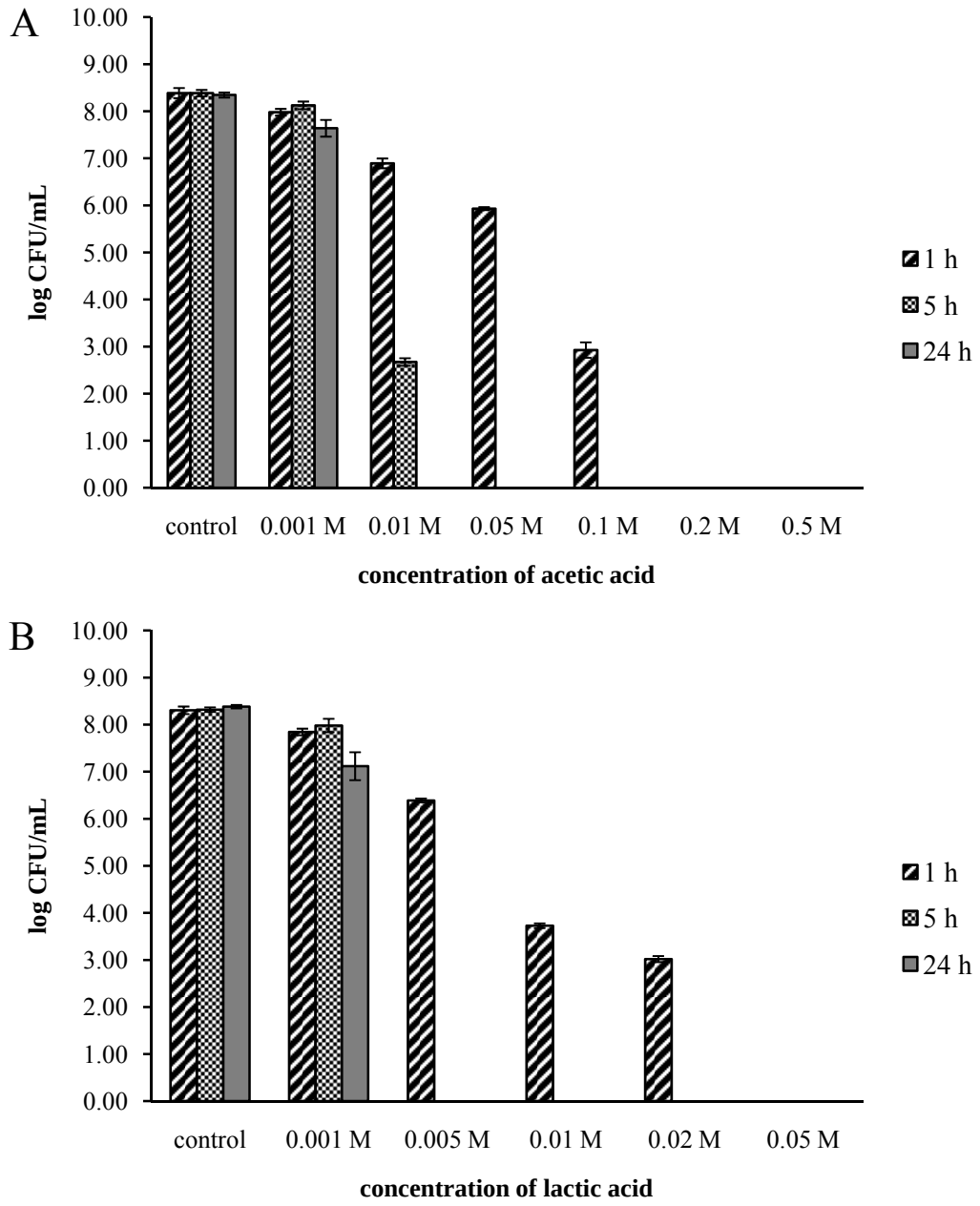


Figure 3.8 Cell counts determined by standard plate count methods for cells of mid-log phase *E. coli* DH5α after exposure to a range of concentrations of acetic acid (A) and lactic acid (B) for 1, 5 and 24 h (mean ± SEM; n=9).

3.1.2.3 Turbidity (OD_{600}) Measurement

As represented in Figure 3.9, the OD_{600} value of control experiments showed a significant decrease ($P < 0.05$) over time, whereas the OD_{600} values of all acetic acid and lactic acid treated samples showed a significant increase ($P < 0.05$) over time. Significant decreases ($P < 0.05$) in OD_{600} values were observed in all acid treated samples as compared to the control at 1 h. In contrast, significant increases ($P < 0.05$) in OD_{600} values were observed in all acid treated samples as compared to the control at 24 h, with exception of the 0.5 M acetic acid treatment. After 5 h treatment, a significant difference ($P < 0.05$) in OD_{600} values was detected among all acetic acid treatments, as well as the 0.001 M and 0.05 M lactic acid treatments as compared to the control, respectively.

The overall OD_{600} values were generally reduced with increasing concentrations (lower pH) of either acetic or lactic acid at each specific time point. There was no significant difference ($P > 0.05$) in OD_{600} values among cells exposed to 0.05 M, 0.1 M and 0.2 M acetic acid, as well as among cells exposed to 0.005 M, 0.01 M and 0.02 M lactic acid at any time point, respectively. See Appendix B Table B.2 for detailed data.

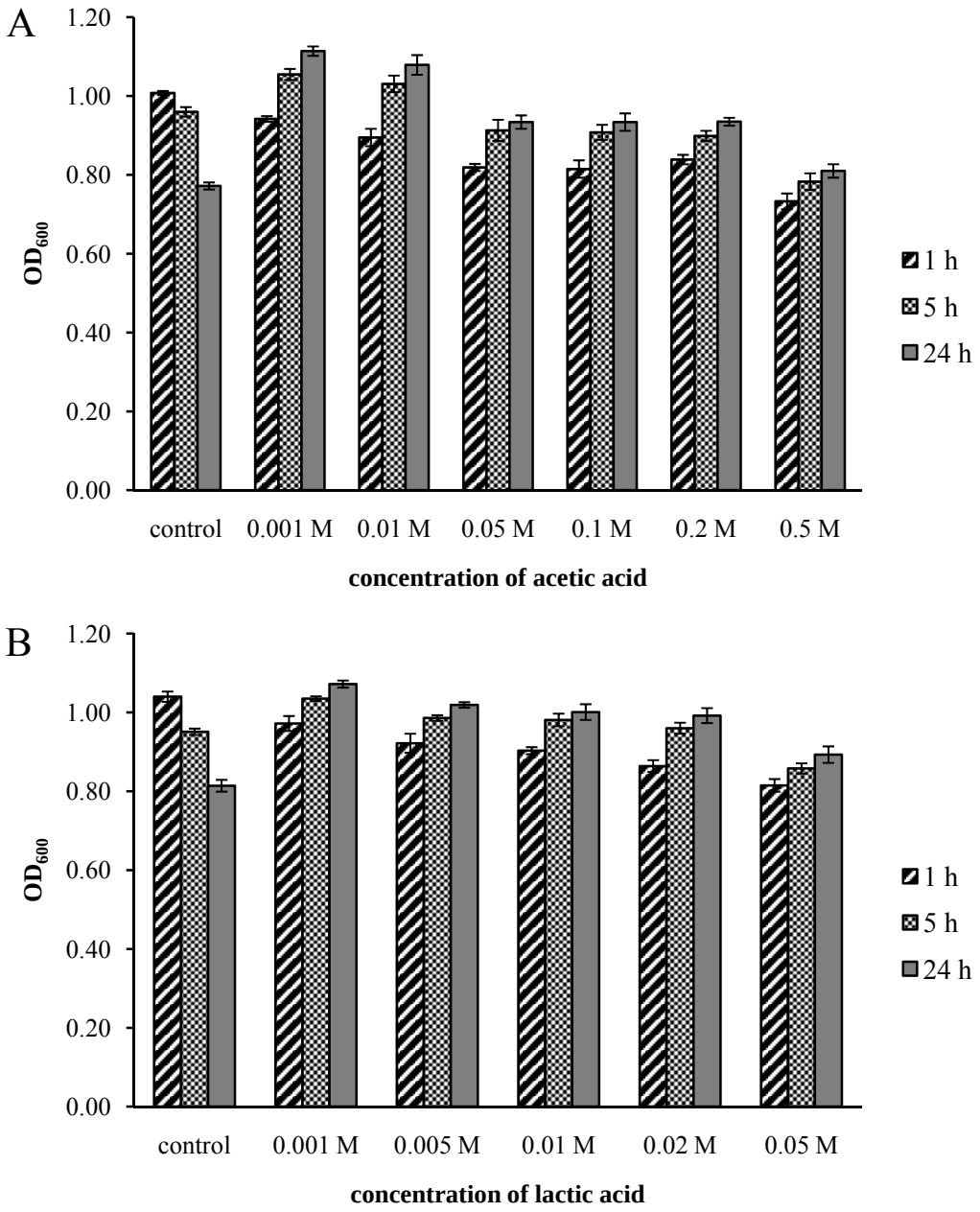


Figure 3.9 Cell concentration determined by optical density measurements for cells of mid-log phase *E. coli* DH5α after exposure to a range of concentrations of acetic acid (A) and lactic acid (B) for 1, 5 and 24 h (mean ± SEM; n=9).

3.1.3 Mid-log Phase *S. aureus* ATCC 13565

3.1.3.1 Fluorescence Microscopy

The fluorescence microscopy analyses of mid-log phase *S. aureus* ATCC 13565 cells treated with acetic acid and lactic acid for periods of 1 h, 5 h and 24 h are shown in Figures 3.10 and 3.11, respectively. It was observed that 0.05 M, 0.1 M and 0.2 M acetic acid, as well as 0.01 M and 0.02 M lactic acid could induce several small cell clumps after 1 h treatment with most cells in a viable state. If the treatment time was extended to 5 h or longer, cell clumping seemed to decrease over time, and no apparent increase in the numbers of dead cells were found.

3.1.3.2 Standard Plate Count

The plate count results in Figure 3.12 indicate that the viable counts of mid-log phase *S. aureus* ATCC 13565 were reduced when the cells were treated with either acetic or lactic acid of higher concentrations (lower pH) or for longer times.

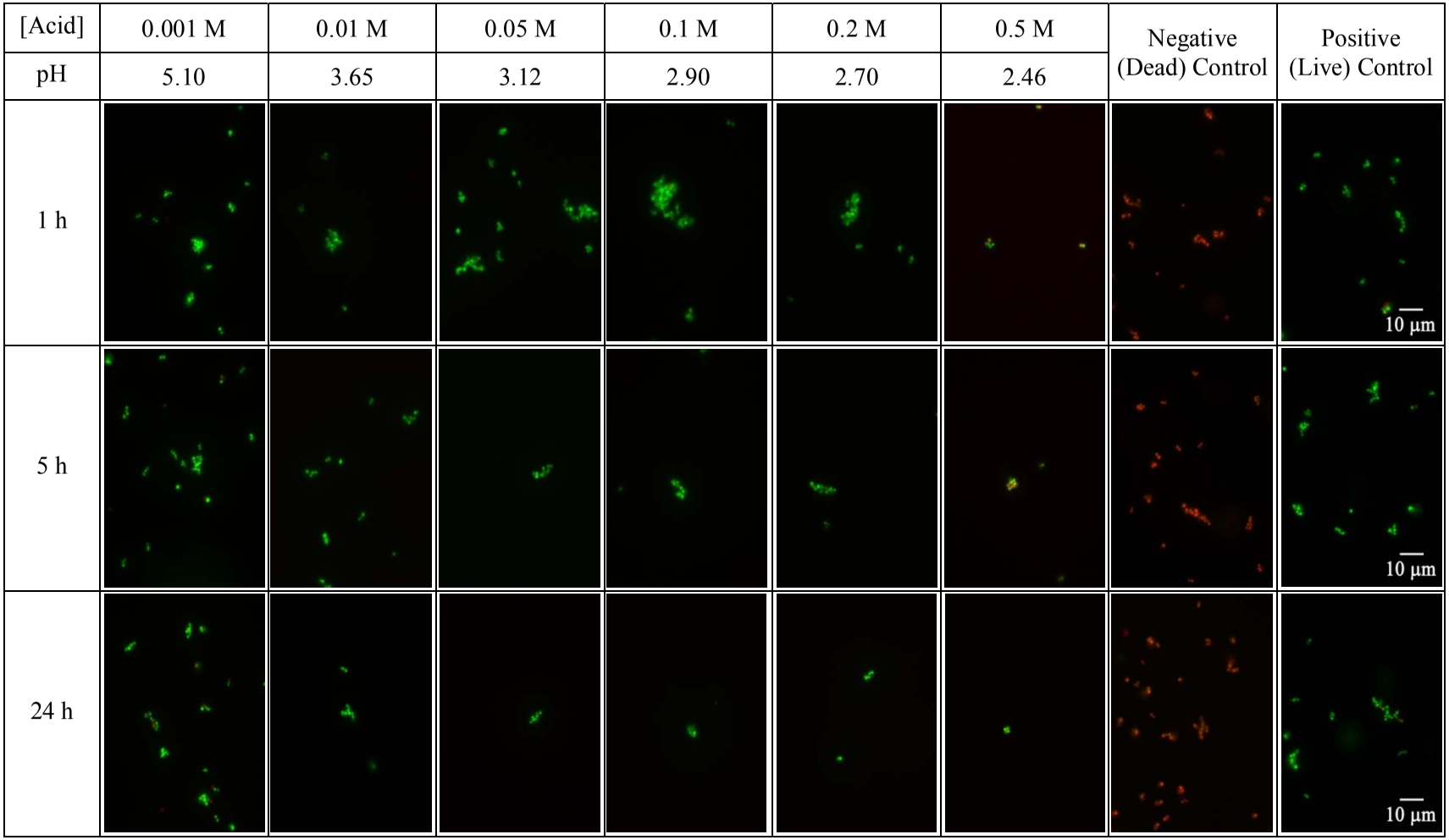


Figure 3.10 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of acetic acid for 1, 5 and 24 h.

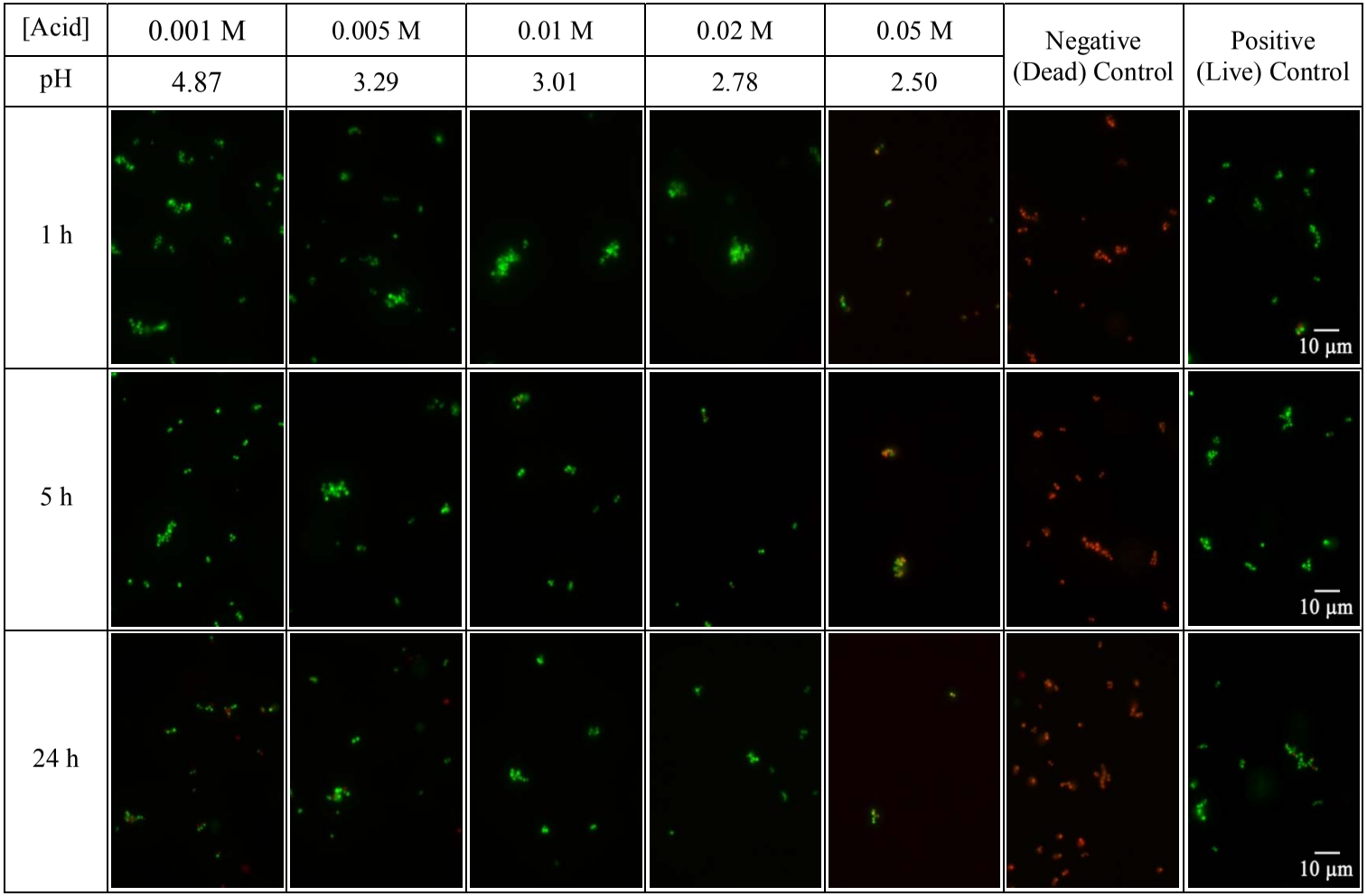


Figure 3.11 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of lactic acid for 1, 5 and 24 h.

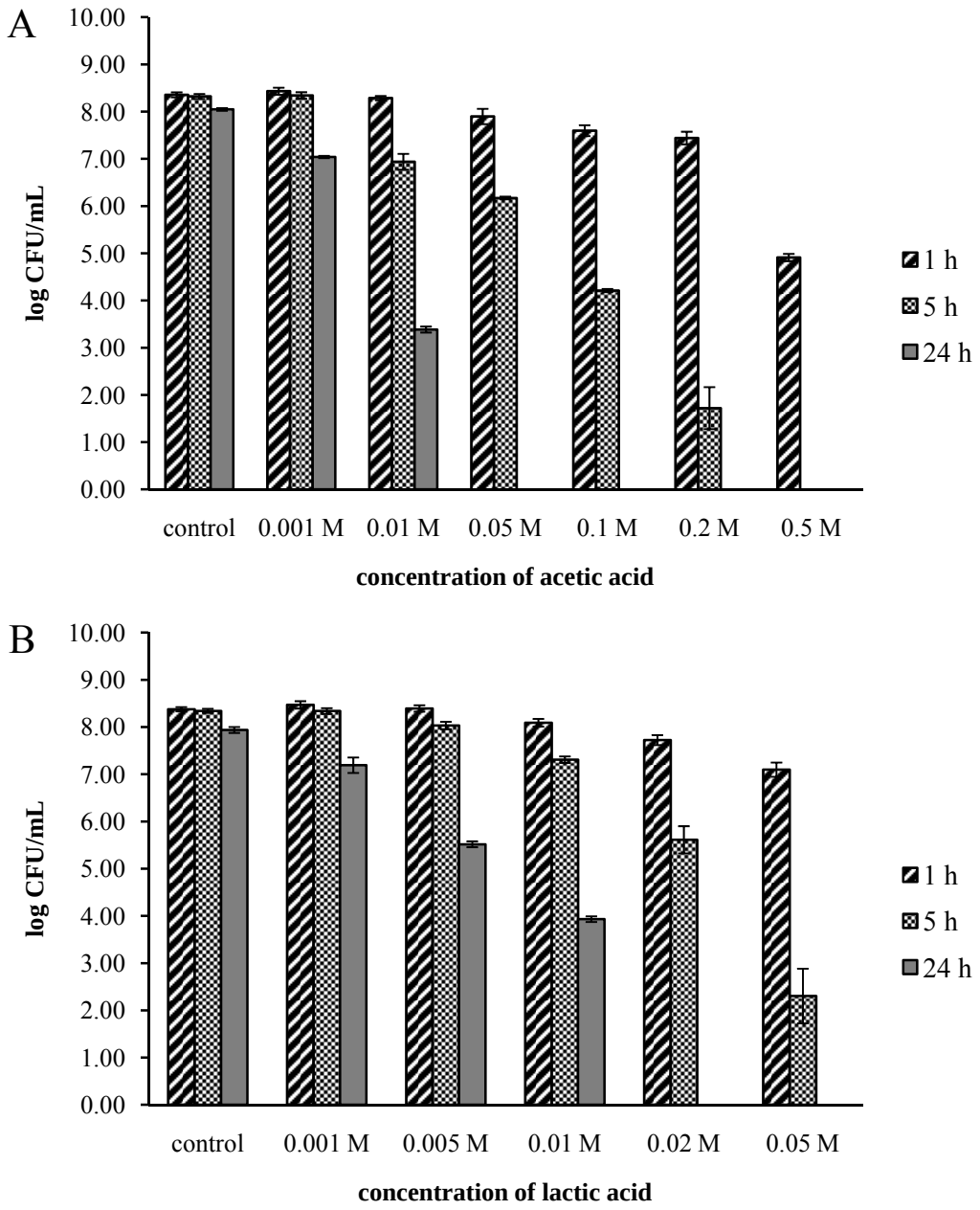


Figure 3.12 Cell counts determined by standard plate count methods for cells of mid-log phase *S. aureus* ATCC 13565 after exposure to a range of concentrations of acetic acid (A) and lactic acid (B) for 1, 5 and 24 h (mean ± SEM; n=9).

The counts of cells treated with 0.01 M acetic acid significantly decreased by 1.38 log units ($P < 0.05$) as compared to that of the control after 5 h treatment, and decreased by 4.66 log units ($P < 0.05$) after 24 h (Figure 3.12A). The counts of cells exposed to 0.05 M, 0.1 M and 0.2 M acetic acid significantly decreased

by 2.15, 4.11 and 6.60 log units ($P < 0.05$), respectively, as compared to that of the control after 5 h treatment, and no colonies formed after 24 h. When the samples were treated with 0.5 M acetic acid, the counts significantly decreased by 3.45 log units ($P < 0.05$) after 1 h, and no colonies formed when the treatment time was extended to 5 or 24 h. See Appendix B Table B.3 for detailed statistical data.

The counts of cells exposed to 0.005 M lactic acid significantly decreased by 2.42 log units ($P < 0.05$) as compared to that of the control after 24 h treatment (Figure 3.12B). The counts of 0.01 M treatment significantly decreased by 1.03 log units ($P < 0.05$) as compared to that of the control after 5 h treatment, and decreased by 4.01 log units ($P < 0.05$) after 24 h. The counts of 0.02 M and 0.05 M treatments significantly decreased by 2.73 and 6.04 log units respectively ($P < 0.05$) after 5 h, and no colonies formed after 24 h. See Appendix B Table B.3 for detailed statistical data.

3.1.3.3 Turbidity (OD_{600}) Measurement

Results for optical density measurements of mid-log phase *S. aureus* ATCC 13565 shown in Figure 3.13 demonstrated that control, 0.05 to 0.5 M acetic acid treatments, and 0.005 to 0.05 M lactic acid treatments showed significant decreases ($P < 0.05$) in OD_{600} values over time, whereas 0.001 M and 0.01 M acetic acid treatments, as well as 0.001 M lactic acid treatment did not change significantly ($P > 0.05$) over time. The OD_{600} values were generally reduced at each time point when the cells were treated with either acetic or lactic acid of higher concentrations (lower pH).

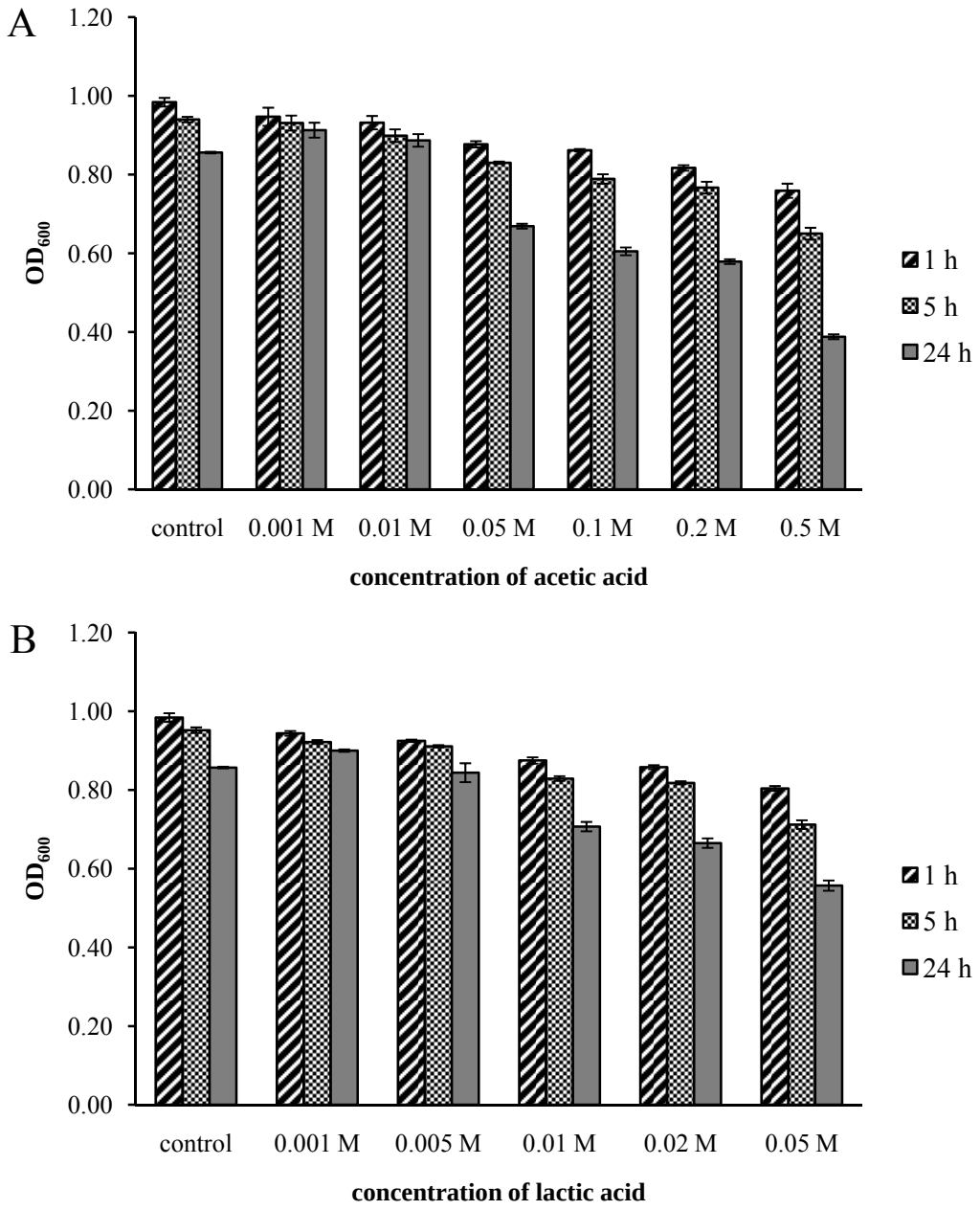


Figure 3.13 Cell concentration determined by optical density measurements for cells of mid-log phase *S. aureus* ATCC 13565 after exposure to a range of concentrations of acetic acid (A) and lactic acid (B) for 1, 5 and 24 h (mean ± SEM; n=9).

Treatments of *S. aureus* cells with 0.001 M and 0.01 M acetic acid, as well as 0.001 M and 0.005 M lactic acid always showed comparable or less than 6% difference in OD_{600} values as compared to the control. A significant decrease ($P < 0.05$) in OD_{600} values was observed for cells exposed to 0.05 to 0.5 M acetic acid,

as well as 0.01 to 0.05 M lactic acid as compared to the control at 1, 5 and 24 h. The cells treated with 0.5 M acetic acid and 0.05 M lactic acid always had the lowest OD_{600} values at any given time. See Appendix B Table B.3 for detailed statistical data.

3.1.4 Mid-log Phase *L. monocytogenes* CDC 7762

3.1.4.1 Fluorescence Microscopy

The fluorescence microscopy analyses of mid-log phase *L. monocytogenes* CDC 7762 cells treated with acetic acid and lactic acid solutions for periods of 1 h, 5 h and 24 h are shown in Figures 3.14 and 3.15, respectively. Slight cell clumping was detected in 0.2 M acetic acid and 0.02 M lactic acid treatments after 24 h, as well as in 0.5 M acetic acid and 0.05 M lactic acid treatments after 1 h, respectively. More evident clumping was observed in 0.5 M acetic acid and 0.05 M lactic acid treatments after 5 and 24h.

In terms of the cell viability, it was found that most of the cells were dead after 1 h at concentrations higher than 0.05 M acetic acid and 0.005 M lactic acid. In addition, most bacteria were dead when treated with 0.01 M acetic acid for 5 h.

3.1.4.2 Standard Plate Count

The plate count results in Figure 3.16 indicate that the viable counts of mid-log phase *L. monocytogenes* CDC 7762 were dramatically reduced when the cells were treated with either acetic acid or lactic acid of higher concentrations (lower pH) or for longer times.

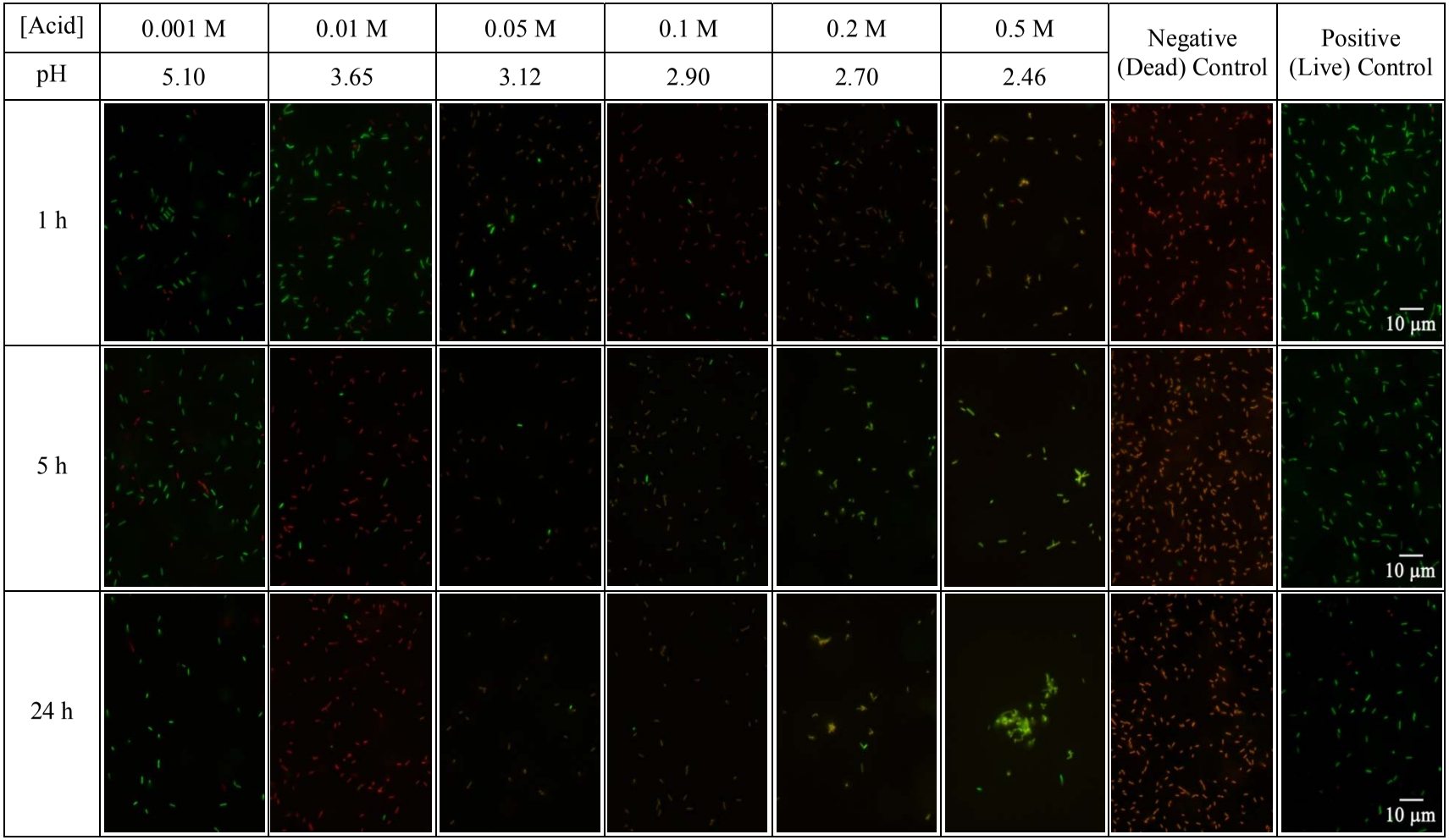


Figure 3.14 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of acetic acid for 1, 5 and 24 h.

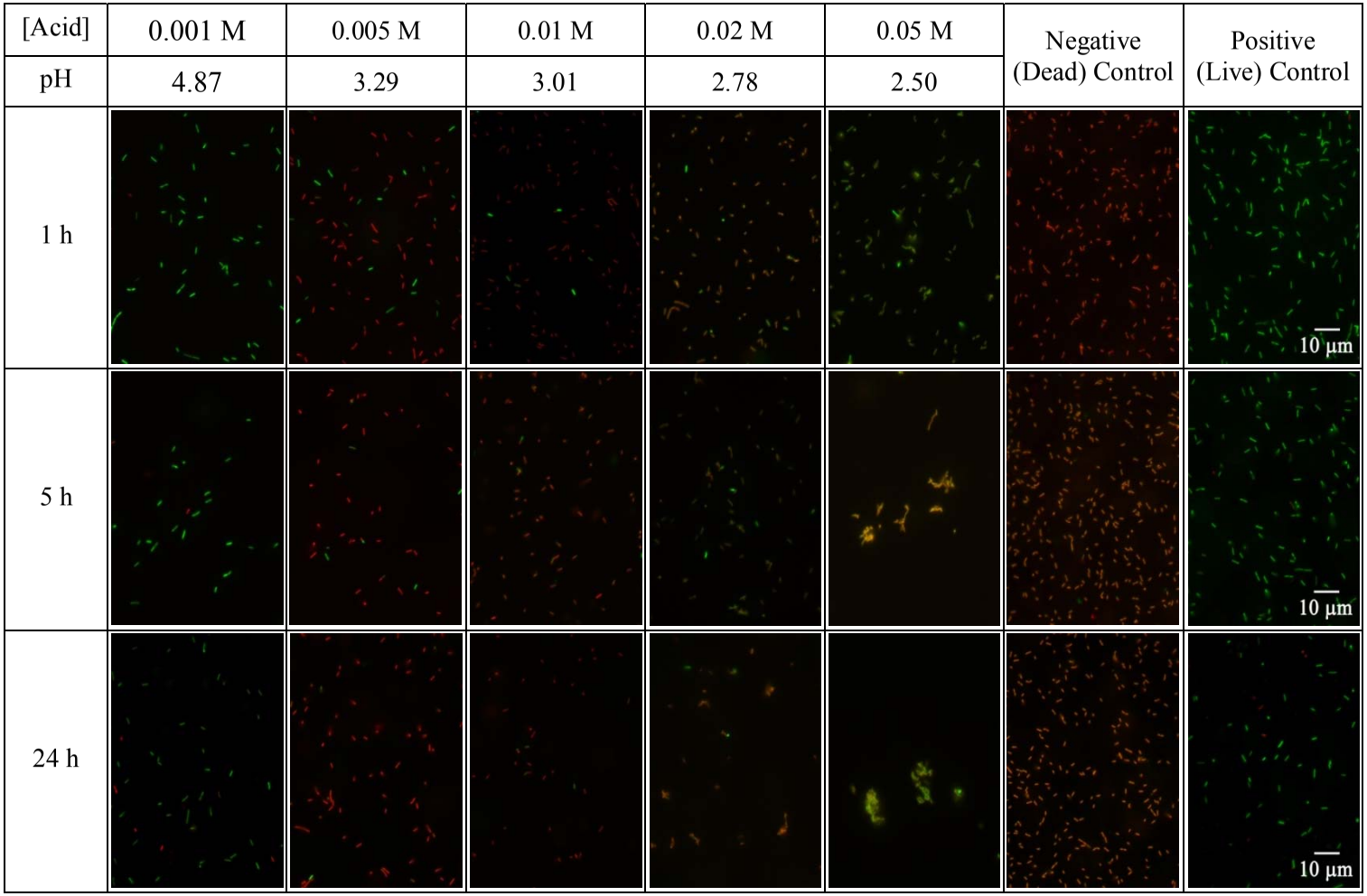


Figure 3.15 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of lactic acid for 1, 5 and 24 h.

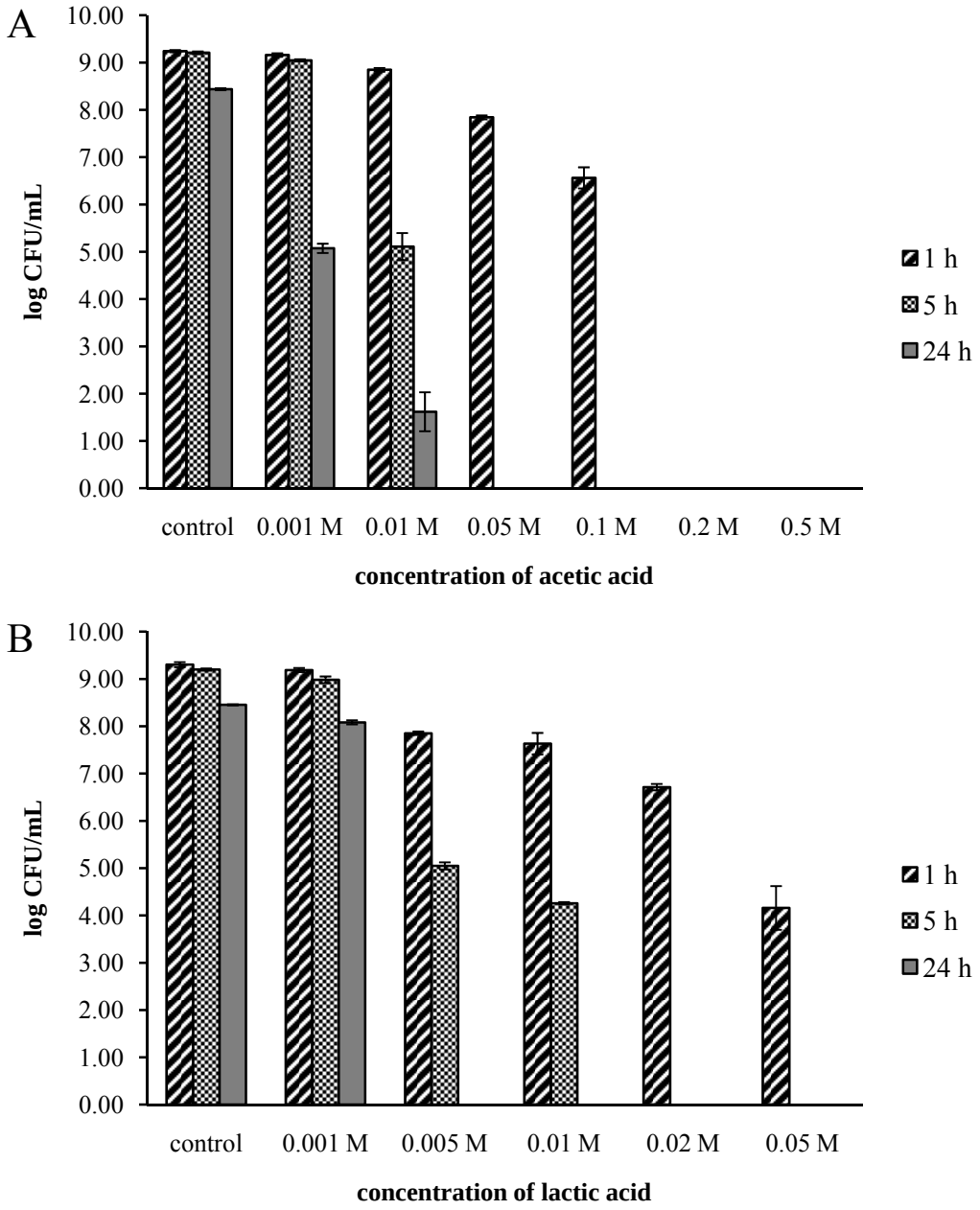


Figure 3.16 Cell counts determined by standard plate count methods for cells of mid-log phase *L. monocytogenes* CDC 7762 after exposure to a range of concentrations of acetic acid (A) and lactic acid (B) for 1, 5 and 24 h (mean ± SEM; n=9).

As shown in Figure 3.16A, the counts of cells exposed to 0.001 M acetic acid significantly decreased by 3.37 log units ($P < 0.05$) as compared to that of the control after 24 h treatment. The counts of cells exposed to 0.01 M acetic acid significantly decreased by 4.10 log units ($P < 0.05$) as compared to that of the

control after 5 h treatment, and decreased by 6.82 log units ($P < 0.05$) after 24 h. The counts of cells exposed to 0.05 M and 0.1 M acetic acid significantly decreased by 1.40 and 2.68 log units respectively ($P < 0.05$) after 1 h, and no colonies formed when the treatment time was extended to 5 or 24 h. When the samples were treated with 0.2 M and 0.5 M acetic acids, no colonies were observed within the 24 h treatment. See Appendix B Table B.4 for detailed statistical data.

As shown in Figure 3.16B, the counts of cells exposed to 0.005 M and 0.01 M lactic acid significantly decreased by 1.45 and 1.67 log units ($P < 0.05$), respectively, as compared to that of the control after 1 h treatment, decreased by 4.15 and 4.94 log units respectively ($P < 0.05$) after 5 h, and no colonies formed after 24 h. The counts of 0.02 M and 0.05 M lactic acid treatments significantly decreased by 2.59 and 5.14 log units respectively ($P < 0.05$) after 1 h, and no colonies formed after 5 or 24 h. See Appendix B Table B.4 for detailed statistical data.

3.1.4.3 Turbidity (OD_{600}) Measurement

The optical density results in Figure 3.17 indicate that the OD_{600} values of control and all acid treated samples showed significant decreases ($P < 0.05$) over time. The OD_{600} values were reduced when the cells were treated with either acetic or lactic acid of higher concentrations (lower pH). Significant increases ($P < 0.05$) in OD_{600} values were observed in all acid treated samples as compared to the control at 1 and 24 h, as well as in all acid treated samples at 5 h with

exception of the 0.5 M acetic acid and 0.05 M lactic acid treatments. See Appendix B Table B.4 for detailed statistical data.

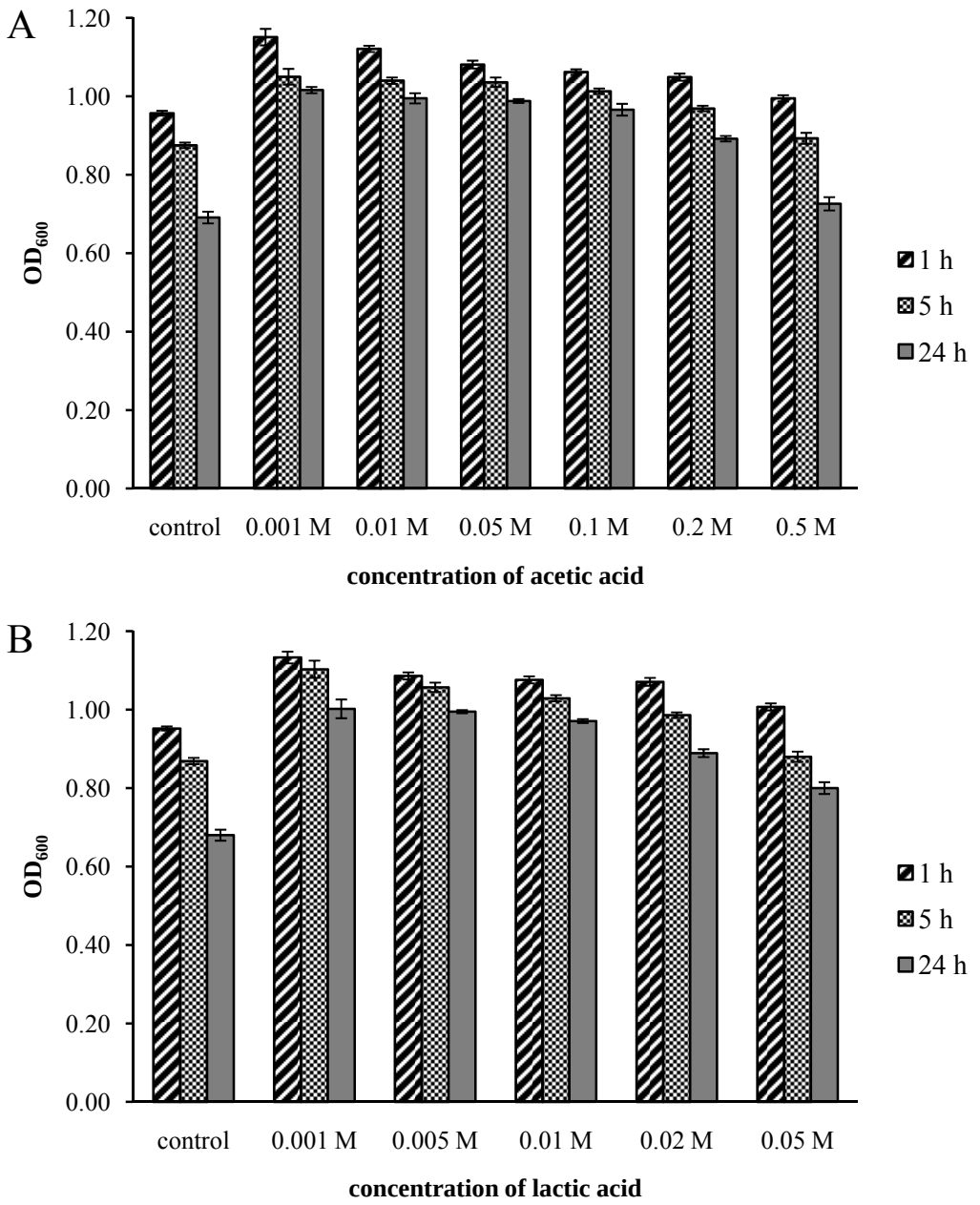


Figure 3.17 Cell concentration determined by optical density measurements for cells of mid-log phase *L. monocytogenes* CDC 7762 after exposure to a range of concentrations of acetic acid (A) and lactic acid (B) for 1, 5 and 24 h (mean ± SEM; n=9).

3.2 Effect of Inorganic Acid Stress

As described in Section 3.1, the acetic acid or lactic acid treatments with pH 3.0 to 3.3 could induce dramatic cell clumping in stationary-phase and mid-log phase *E. coli* DH5α within 24 h, as well as some visible clumping in mid-log phase *S. aureus* ATCC 13565 within 1 h, and most bacteria were still alive according to the fluorescence microscopy analyses. In order to gain insight into a broader scope of acid stresses, hydrochloric acid and sulfuric acid in the same pH range was used to investigate if there are any similar changes in cell aggregation under inorganic acid stress treatments.

3.2.1 Stationary-Phase *E. coli* DH5α

3.2.1.1 Fluorescence Microscopy

The fluorescence microscopy analyses of stationary-phase *E. coli* DH5α cells treated with hydrochloric acid and sulfuric acid solutions for periods of 1 h, 5 h and 24 h are shown in Figures 3.18 and 3.19, respectively. When treated with either hydrochloric acid or sulfuric acid for 1 h, some bacteria tended to aggregate together, while others were shown to exist as single cells. In contrast to what was observed with organic acid treatments, although obvious clumping was observed under all inorganic acid treatment conditions, a considerable proportion of cells were still in single-cell status. Images for each treatment condition are included in Figures 3.18 and 3.19 to illustrate this variability across the slide. In terms of cell viability, some *E. coli* cells died after a 1 h treatment. The apparent number of dead cells increased dramatically after 5 h and 24 h treatments.

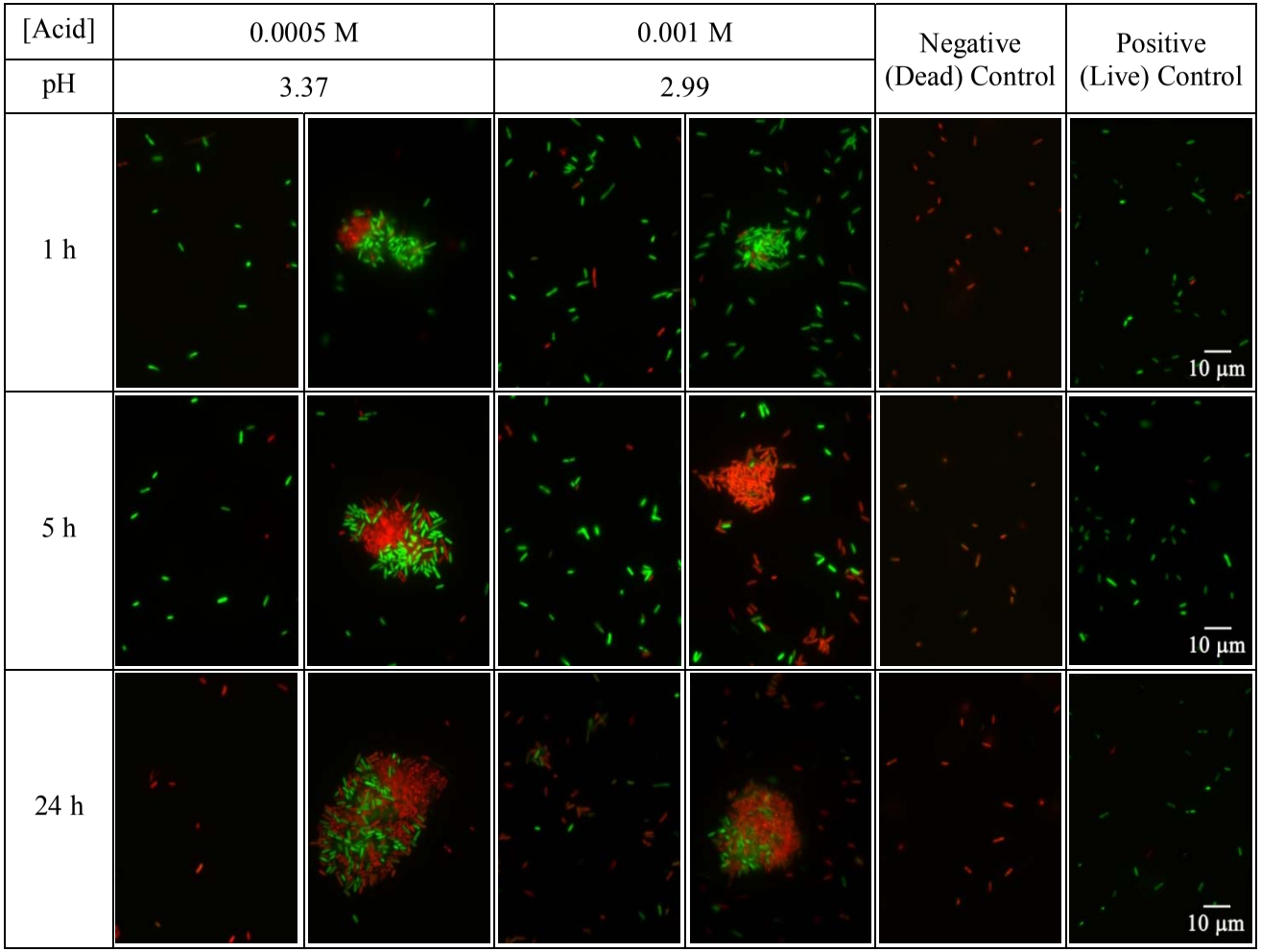


Figure 3.18 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of hydrochloric acid for 1, 5 and 24 h. Cell clumping was observed under each acid treatment condition, whereas a considerable proportion of cells still existed as single cells.

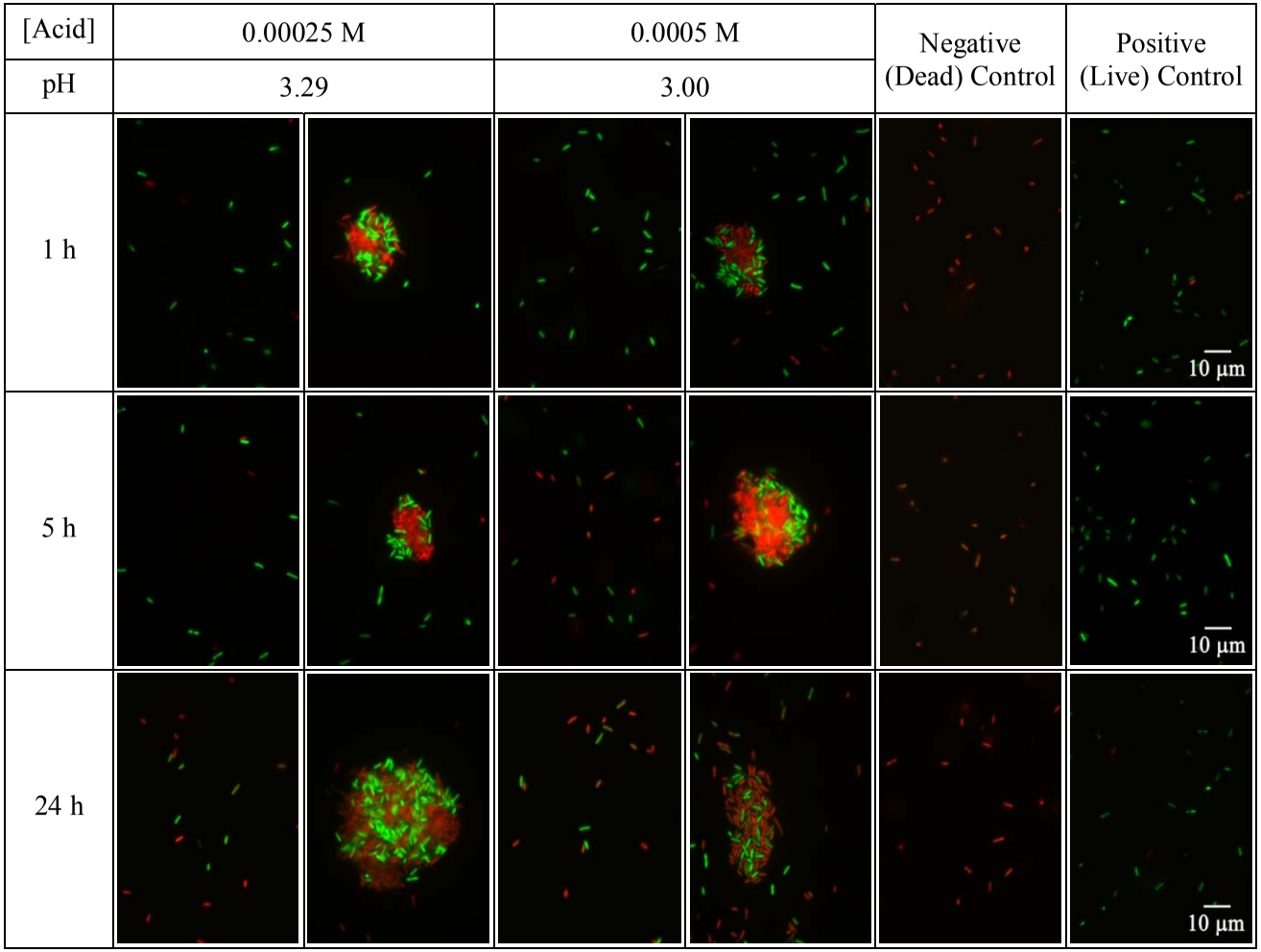


Figure 3.19 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of sulfuric acid for 1, 5 and 24 h. Cell clumping was observed under each acid treatment condition, whereas a considerable proportion of cells still existed as single cells.

3.2.1.2 Standard Plate Count

As shown in Figure 3.20, the viable counts of stationary-phase *E. coli* DH5α were reduced when the cells were treated with either hydrochloric acid or sulfuric acid of higher concentrations (lower pH) or for longer times.

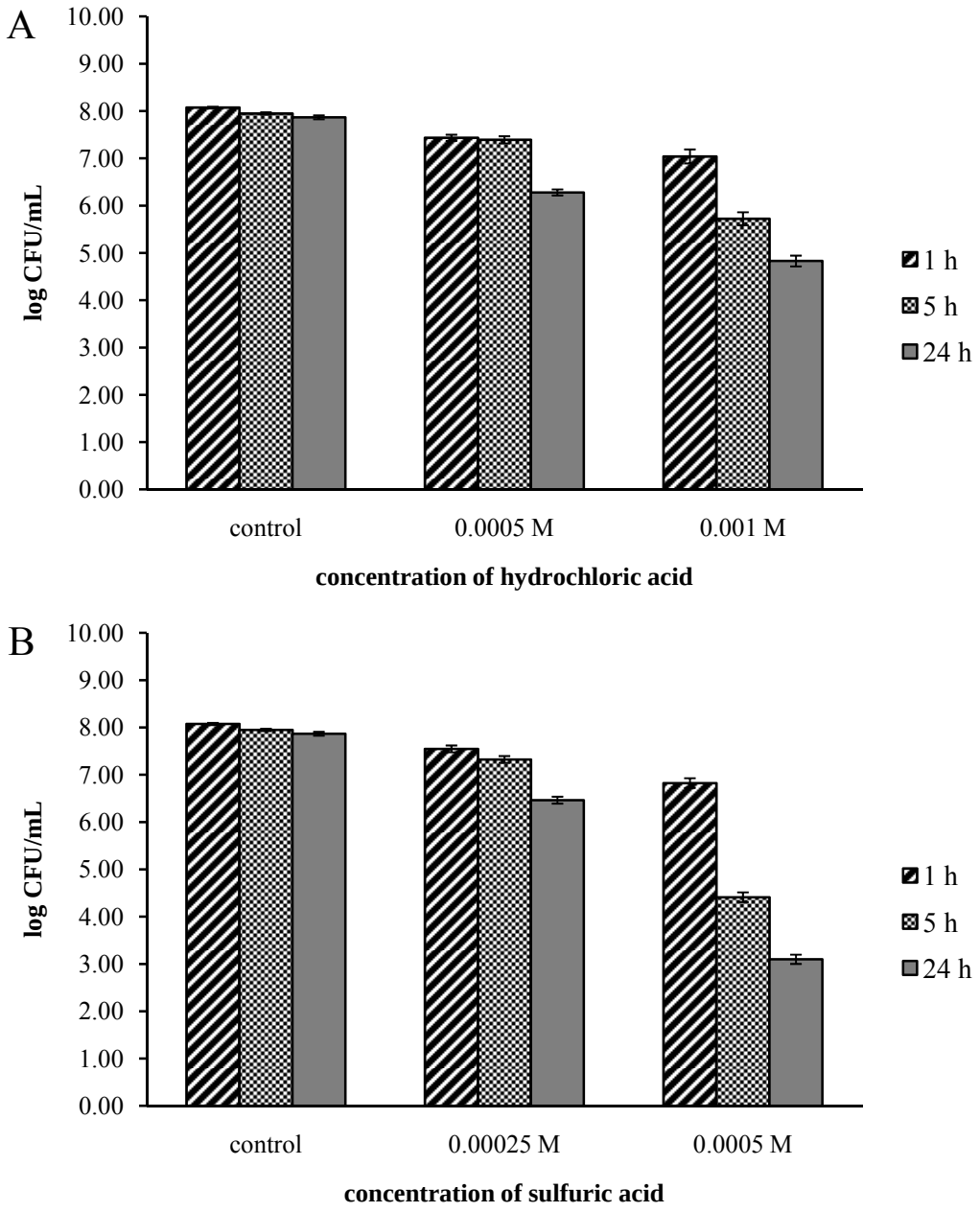


Figure 3.20 Cell counts determined by standard plate count methods for cells of stationary-phase *E. coli* DH5α after exposure to a range of concentrations of hydrochloric acid (A) and sulfuric acid (B) for 1, 5 and 24 h (mean ± SEM; n=9).

The counts of cells exposed to 0.0005 M hydrochloric acid significantly decreased by 1.59 log units ($P < 0.05$) as compared to that of the control after 24 h (Figure 3.20A). The counts of cells exposed to 0.001 M hydrochloric acid significantly decreased by 1.03, 2.23 and 3.04 log units ($P < 0.05$) after 1, 5 and 24 h, respectively, as compared to that of the control. See Appendix B Table B.5 for detailed statistical data.

The counts of cells exposed to 0.00025 M sulfuric acid significantly decreased by 1.41 log units ($P < 0.05$) as compared to that of the control after 24 h (Figure 3.20B). The counts of cells exposed to 0.0005 M sulfuric acid significantly decreased by 1.25, 3.54 and 4.77 log units ($P < 0.05$) after 1, 5 and 24 h, respectively, as compared to that of the control. See Appendix B Table B.5 for detailed statistical data.

3.2.1.3 Turbidity (OD_{600}) Measurement

As shown in Figure 3.21, significant decreases ($P < 0.05$) in OD_{600} values were observed for each inorganic acid treatment as compared to the control, whereas no significant differences ($P > 0.05$) were observed over time within each treatment.

For cells treated with hydrochloric acid, there was approximately a 21% and 30% difference ($P < 0.05$) in OD_{600} values at 0.0005 M and 0.001 M, respectively, as compared to the control. For cells treated with sulfuric acid, there was approximately an 18% and 28% difference ($P < 0.05$) in OD_{600} values at 0.00025 M and 0.0005 M, respectively, as compared to the control. See Appendix B Table B.5 for detailed statistical data.

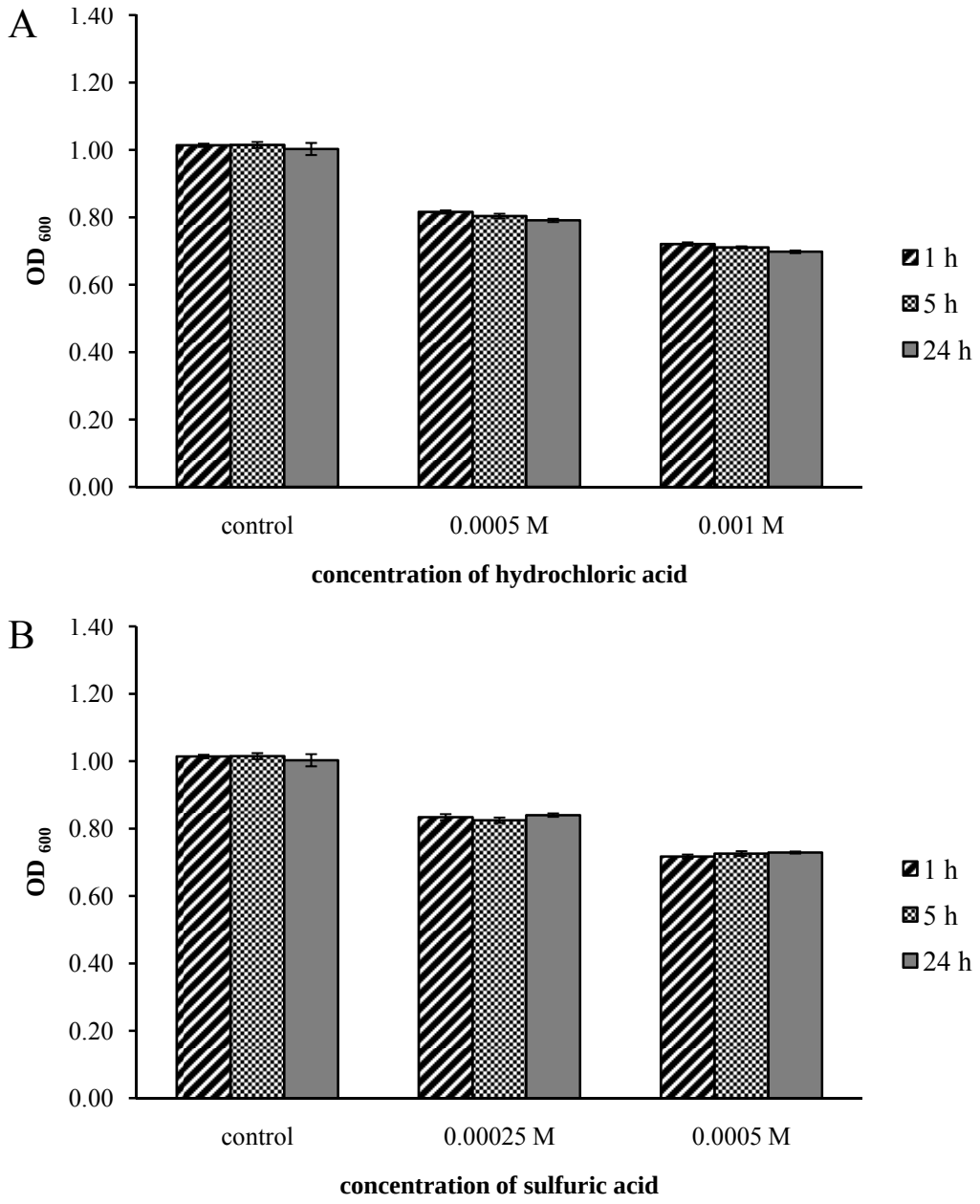


Figure 3.21 Cell concentration determined by optical density measurements for cells of stationary-phase *E. coli* DH5α after exposure to a range of concentrations of hydrochloric acid (A) and sulfuric acid (B) for 1, 5 and 24 h (mean ± SEM; n=9).

3.2.2 Mid-log Phase *E. coli* DH5α

3.2.2.1 Fluorescence Microscopy

Figure 3.22 provides the fluorescence microscopy results of mid-log phase *E. coli* DH5α cells treated with hydrochloric acid and sulfuric acid for periods of 1 h, 5 h and 24 h respectively. It was found that only hydrochloric acid could induce several slight cell clumps after 24 h treatment. For all the samples treated with inorganic acid, the apparent number of dead cells highly increased over time..

3.2.2.2 Standard Plate Count

The plate count results shown in Figure 3.23 indicate that the viable counts of mid-log phase *E. coli* DH5α were reduced when the cells were treated with either hydrochloric acid or sulfuric acid of higher concentrations (lower pH) or for longer times.

As shown in Figure 3.23A, the counts of cells treated with 0.0005 M hydrochloric acid significantly decreased by 2.22 and 4.76 log units ($P < 0.05$) after 5 and 24 h, respectively, as compared to that of the control. The counts of cells treated with 0.001 M hydrochloric acid significantly decreased by 1.18, 3.56 and 5.13 log units ($P < 0.05$) after 1, 5 and 24 h, respectively, as compared to that of the control. See Appendix B Table B.6 for detailed statistical data.

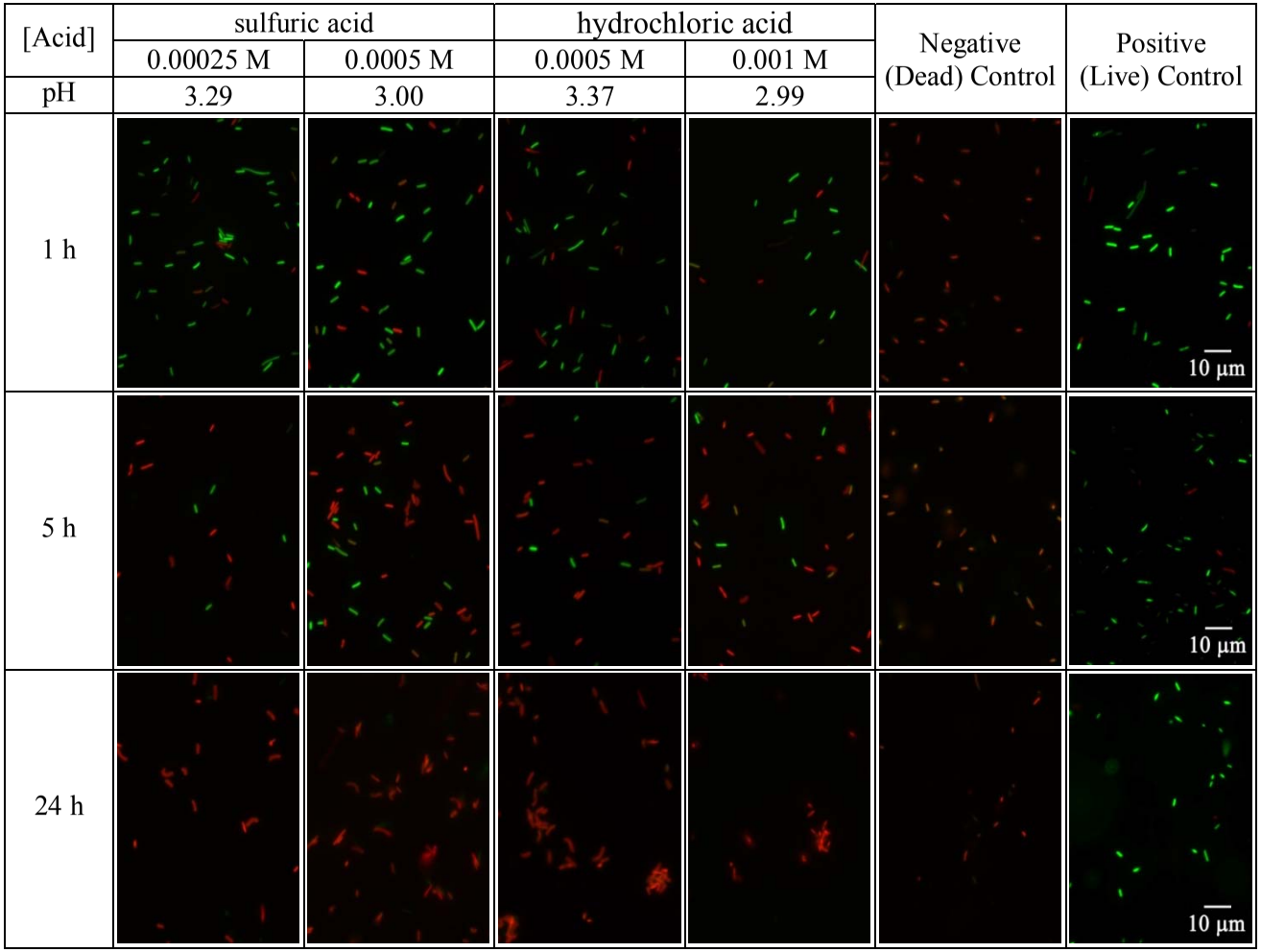


Figure 3.22 Fluorescence microscopy images of mid-log phase *E. coli* DH5α after exposure to different concentrations of hydrochloric acid and sulfuric acid for 1, 5 and 24 h.

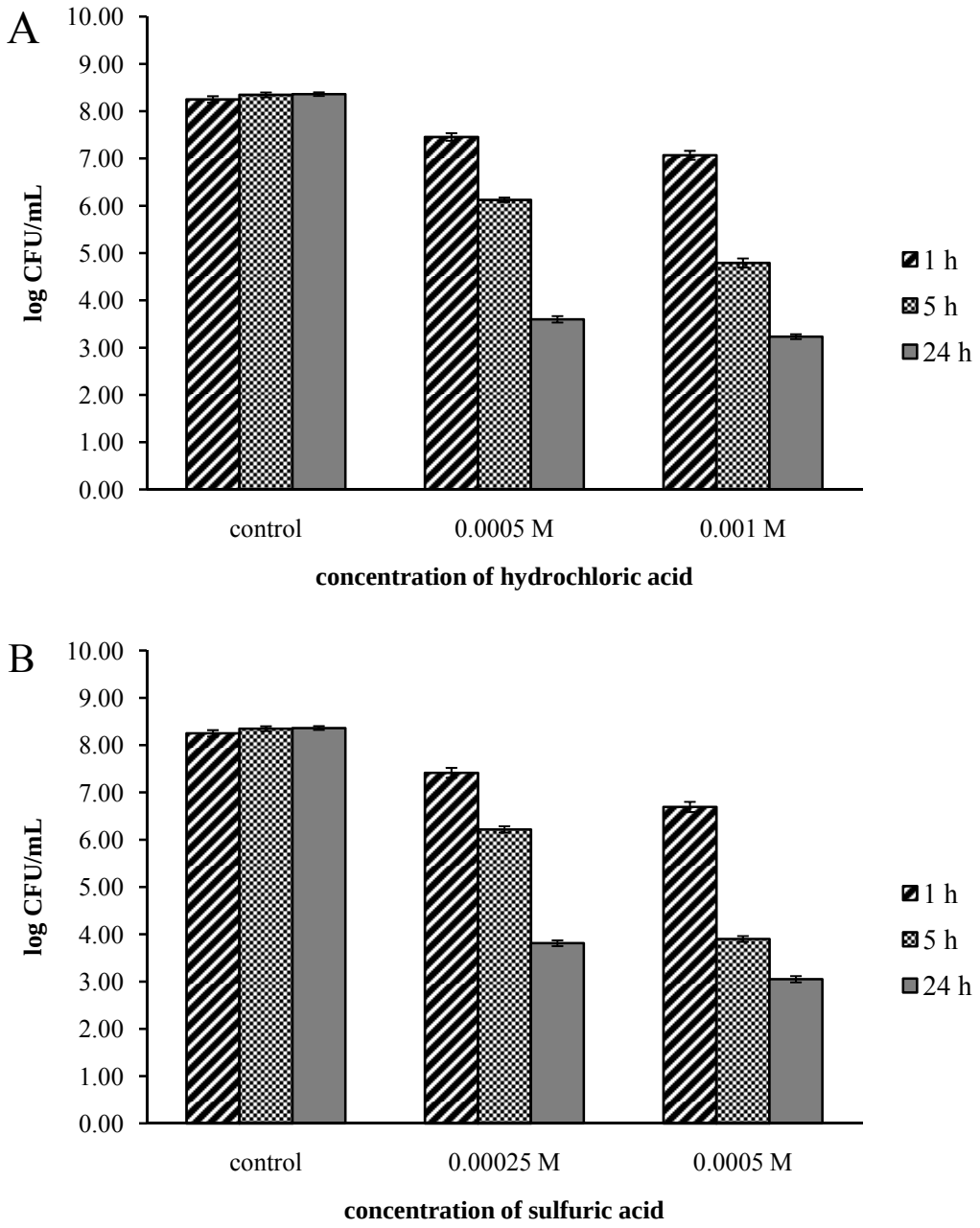


Figure 3.23 Cell counts determined by standard plate count methods for cells of mid-log phase *E. coli* DH5α after exposure to a range of concentrations of hydrochloric acid (A) and sulfuric acid (B) for 1, 5 and 24 h (mean ± SEM; n=9).

As depicted in Figure 3.23B, the counts of cells treated with 0.00025 M sulfuric acid significantly decreased by 2.13 and 4.55 log units ($P < 0.05$) after 5 and 24 h, respectively, as compared to that of the control. The counts of cells

treated with 0.0005 M sulfuric acid significantly decreased by 1.56, 4.45 and 5.31 log units ($P < 0.05$) after 1, 5 and 24 h, respectively, as compared to that of the control. See Appendix B Table B.6 for detailed statistical data.

3.2.2.3 Turbidity (OD_{600}) Measurement

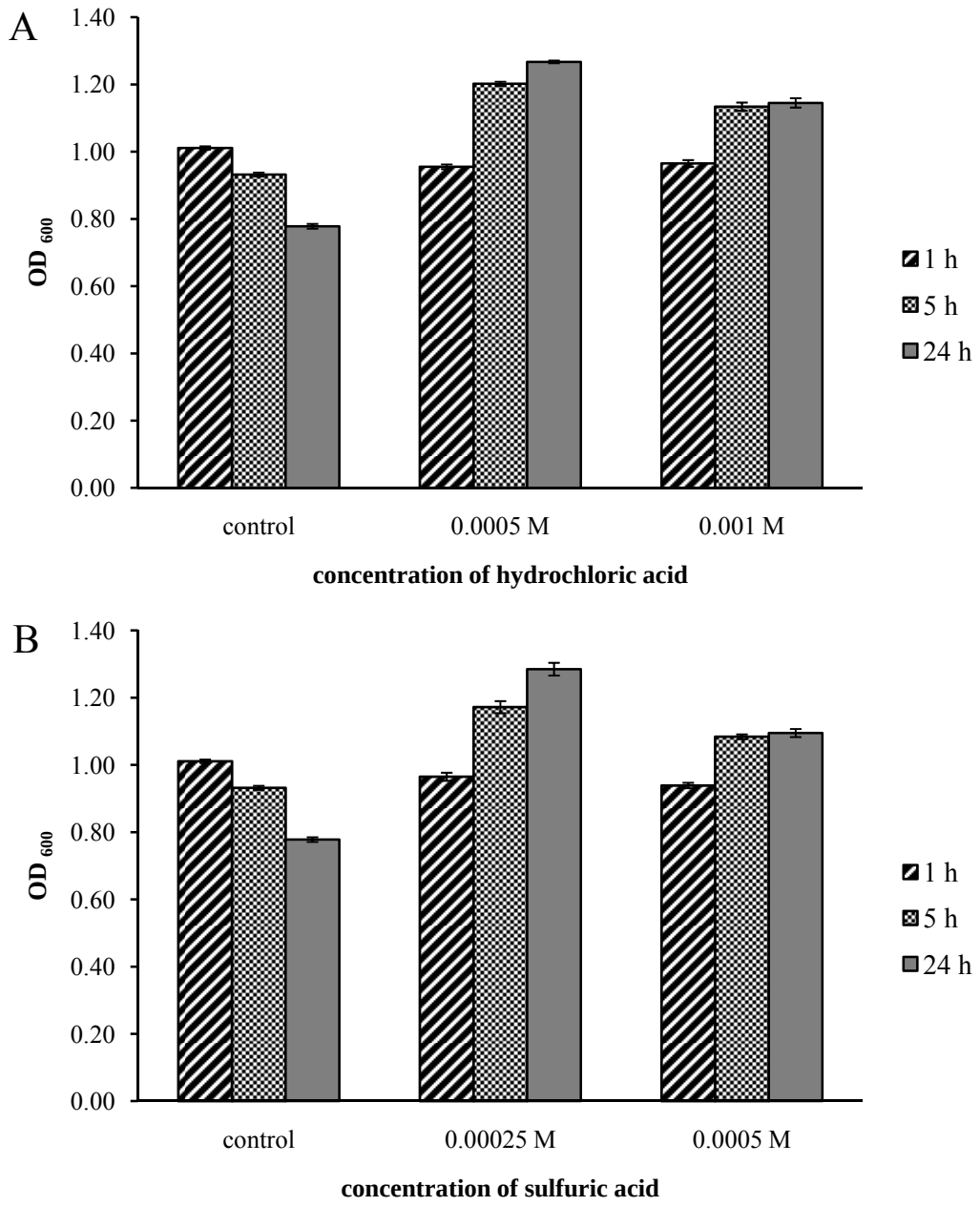


Figure 3.24 Cell concentration determined by optical density measurements for cells of mid-log phase *E. coli* DH5α after exposure to a range of concentrations of hydrochloric acid (A) and sulfuric acid (B) for 1, 5 and 24 h (mean ± SEM; n=9).

As shown in Figure 3.24, the OD_{600} value of control samples showed significant decreases ($P < 0.05$) over time, whereas the OD_{600} values of all inorganic acid treated samples showed significant increases ($P < 0.05$) over time. Significant decreases ($P < 0.05$) in OD_{600} values were observed in all acid treated samples as compared to the control at 1 h. In contrast, significant increases ($P < 0.05$) were observed in all acid treated samples as compared to the control at 5 and 24 h. In addition, The OD_{600} values were generally reduced when the cells were treated with either hydrochloric or sulfuric acid of higher concentrations (lower pH) at 5 and 24 h. See Appendix B Table B.6 for detailed statistical data.

3.2.3 Mid-log Phase *S. aureus* ATCC 13565

3.2.3.1 Fluorescence Microscopy

Figure 3.25 presents the fluorescence microscopy results of mid-log phase *S. aureus* ATCC 13565 cells treated with hydrochloric acid and sulfuric acid for periods of 1 h, 5 h and 24 h, respectively. It was demonstrated that none of the two inorganic acids induced cell clumping within the 24 h exposure, and most bacteria were alive in each treatment.

3.2.3.2 Standard Plate Count

As shown in Figure 3.26, the viable counts of mid-log phase *S. aureus* ATCC 13565 were reduced when the cells were treated with either hydrochloric acid or sulfuric acid of higher concentrations (lower pH) or for longer periods.

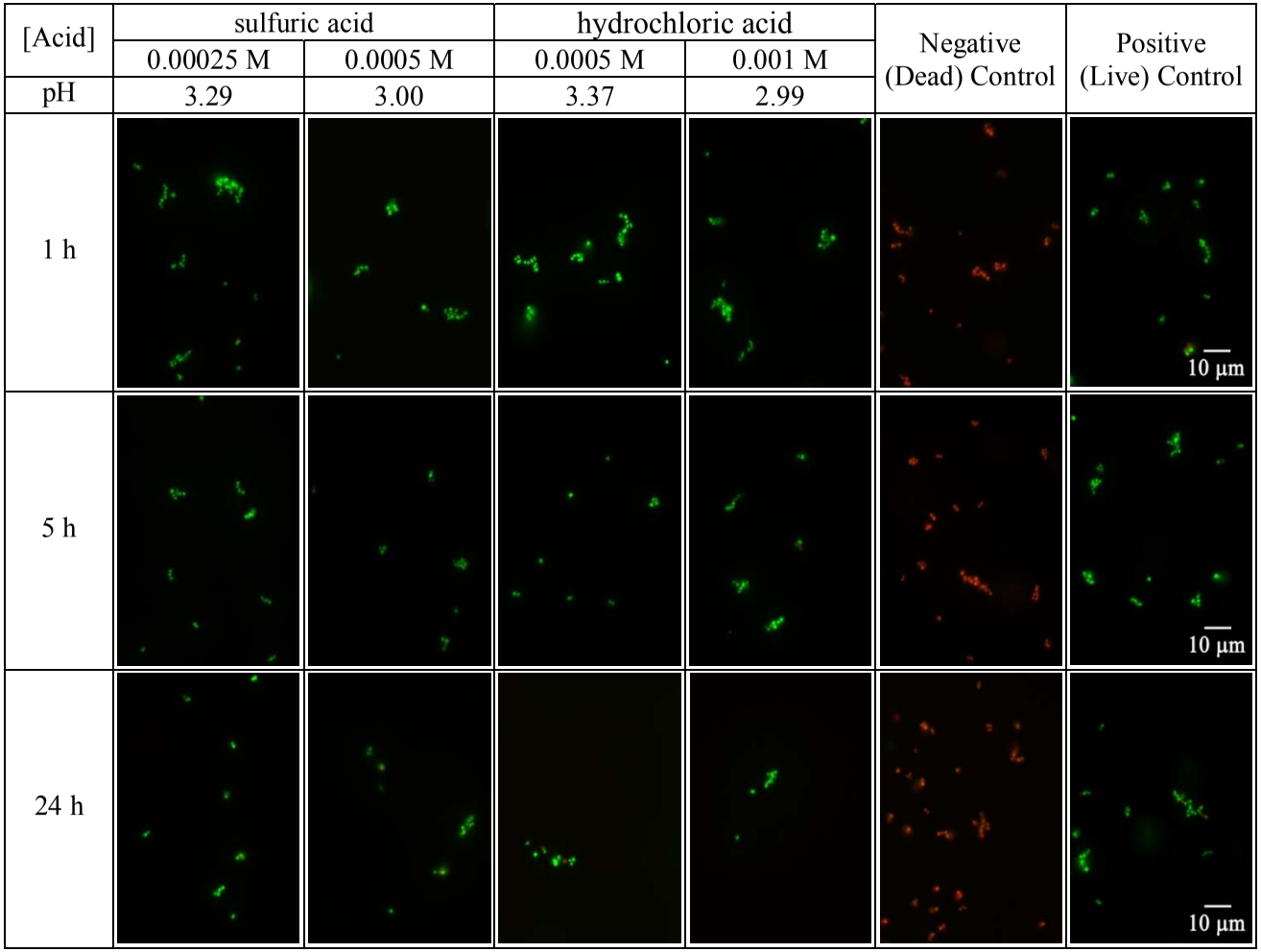


Figure 3.25 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of hydrochloric acid and sulfuric acid for 1, 5 and 24 h.

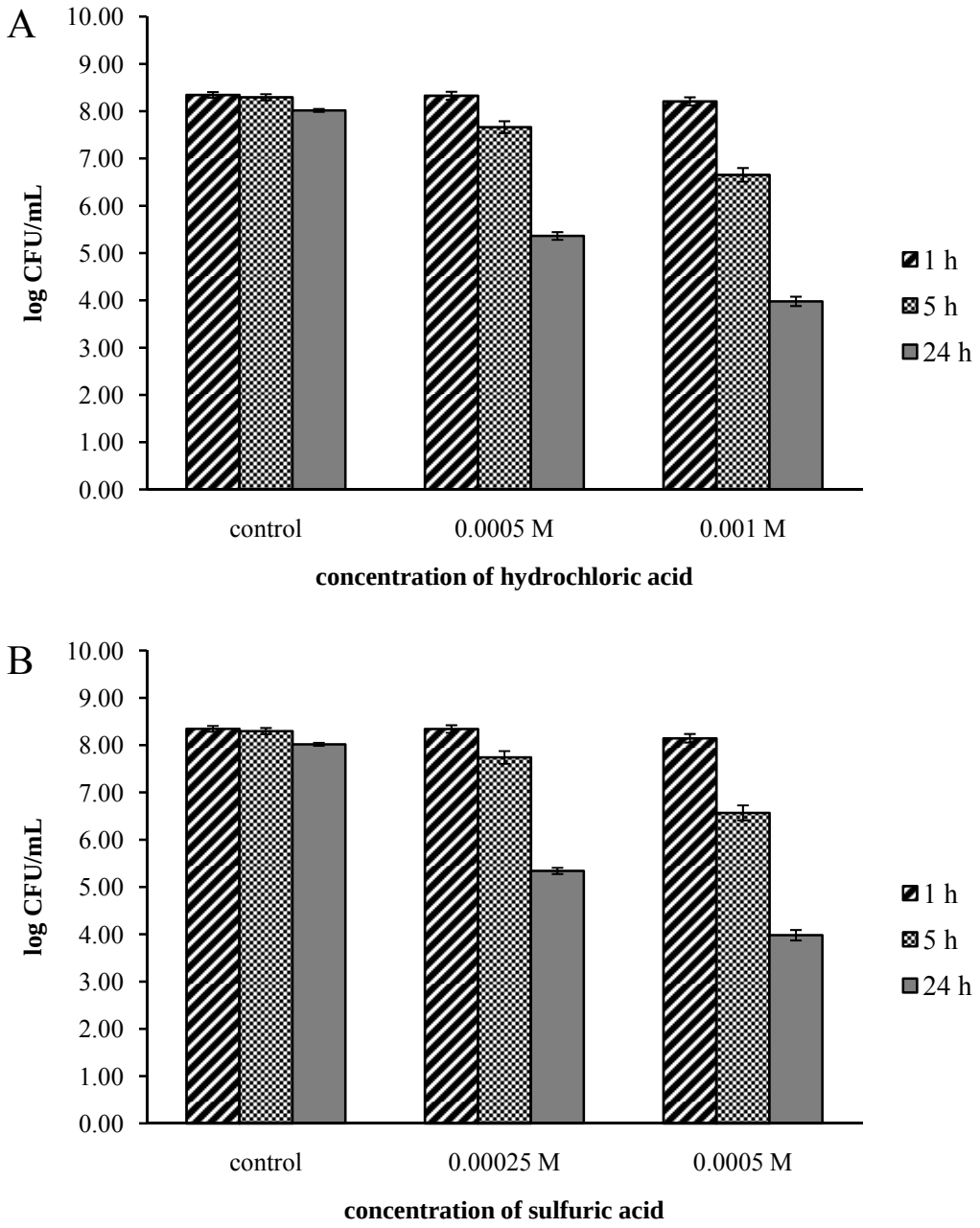


Figure 3.26 Cell counts determined by standard plate count methods for cells of mid-log phase *S. aureus* ATCC 13565 after exposure to a range of concentrations of hydrochloric acid (A) and sulfuric acid (B) for 1, 5 and 24 h (mean ± SEM; n=9).

For hydrochloric acid treatments, the cell counts for the 0.0005 M treatment significantly decreased by 2.66 log units ($P < 0.05$) as compared to that of the control after 24 h. The cell counts for the 0.001 M treatment significantly decreased by 1.65 and 4.04 log units ($P < 0.05$) after 5 and 24 h, respectively, as compared to that of the control. For sulfuric acid treatment, the cell counts for the 0.00025 M treatment significantly decreased by 2.68 log units ($P < 0.05$) as compared to that of the control after 24 h. The cell counts for the 0.0005 M treatment significantly decreased by 1.73 and 4.04 log units ($P < 0.05$) after 5 and 24 h, respectively, as compared to that of the control. See Appendix B Table B.7 for detailed statistical data.

3.2.3.3 Turbidity (OD_{600}) Measurement

According to the optical density results shown in Figure 3.27, all control, hydrochloric acid and sulfuric acid treatments showed significant decreases ($P < 0.05$) in OD_{600} values over time. Furthermore, significant decreases ($P < 0.05$) in OD_{600} values were observed in all acid treated samples as compared to the control at 1, 5 and 24 h. In addition, there were no significant differences ($P > 0.05$) in OD_{600} values between 0.0005 M and 0.001 M hydrochloric acid treatments, as well as between 0.00025 M and 0.0005 M sulfuric acid treatments at 1 and 5 h, respectively. See Appendix B Table B.7 for detailed statistical data.

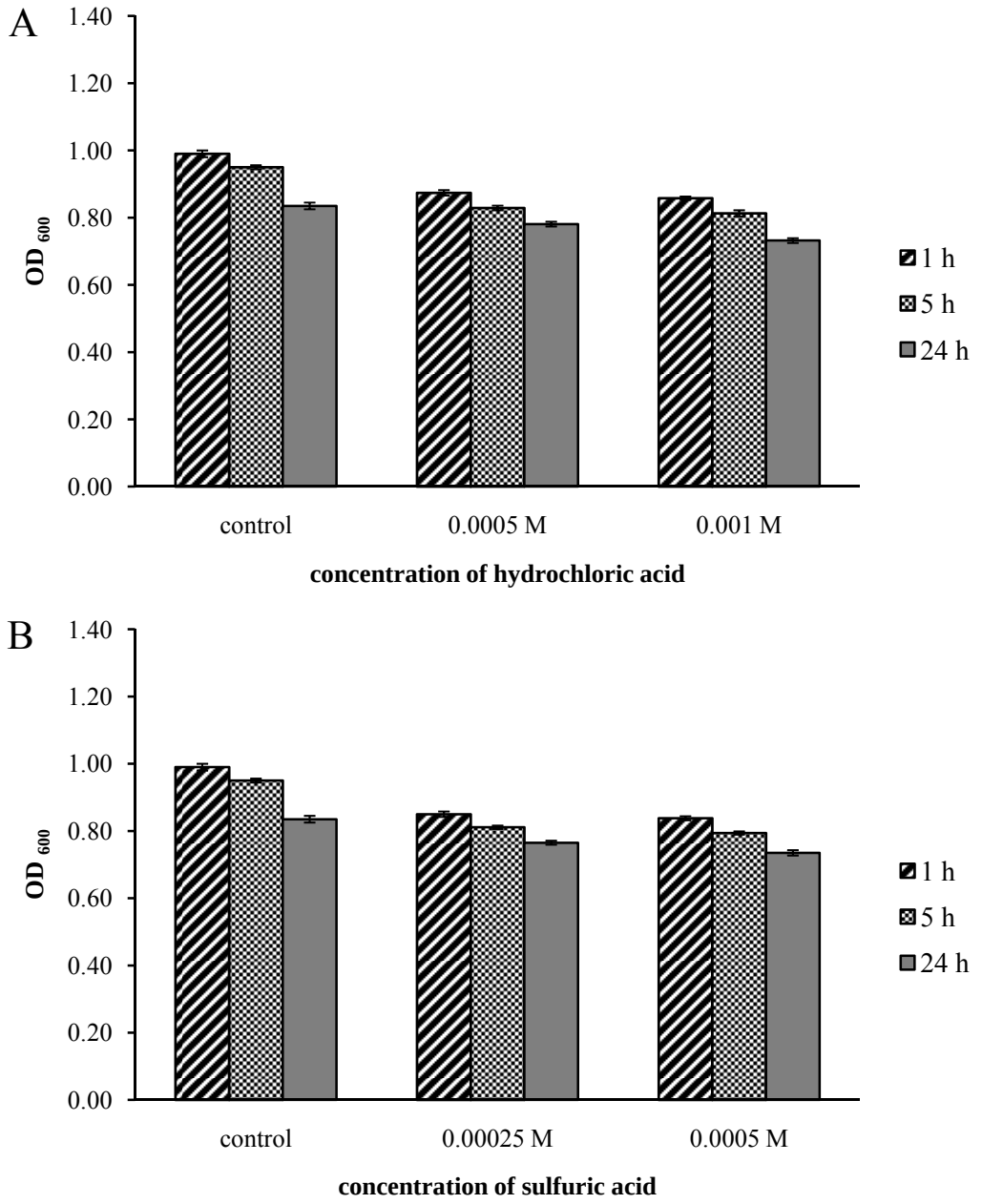


Figure 3.27 Cell concentration determined by optical density measurements for cells of mid-log phase *S. aureus* ATCC 13565 after exposure to a range of concentrations of hydrochloric acid (A) and sulfuric acid (B) for 1, 5 and 24 h (mean ± SEM; n=9).

3.2.4 Mid-log Phase *L. monocytogenes* CDC 7762

3.2.4.1 Fluorescence Microscopy

The fluorescence microscopy analyses of mid-log phase *L. monocytogenes* CDC 7762 cells treated with hydrochloric acid and sulfuric acid for periods of 1 h, 5 h and 24 h are shown in Figure 3.28. It was found that neither inorganic acids could induce cell clumping within the 24 h treatment. The apparent number of dead cells greatly increased over time.

3.2.4.2 Standard Plate Count

As shown in Figure 3.29, the viable counts of mid-log phase *L. monocytogenes* CDC 7762 were reduced when the cells were treated with either hydrochloric acid or sulfuric acid of higher concentrations (lower pH) or for longer times. For hydrochloric acid treatment, the cell counts for the 0.0005 M treatment significantly decreased by 1.71 and 2.88 log units ($P < 0.05$) after 5 and 24 h, respectively, as compared to that of the control. The cell counts for the 0.001 M treatment significantly decreased by 1.88, 3.19 and 7.18 log units ($P < 0.05$) after 1, 5 and 24 h, respectively, as compared to that of the control. For sulfuric acid treatment, the cell counts for the 0.00025 M treatment significantly decreased by 1.39 and 4.55 log units ($P < 0.05$) after 5 and 24 h, respectively, as compared to that of the control. The cells counts for the 0.0005 M treatment significantly decreased by 2.02 and 4.49 log units ($P < 0.05$) after 1 and 5 h, respectively, as compared to that of the control, and no colonies formed after 24 h. See Appendix B Table B.8 for detailed statistical data.

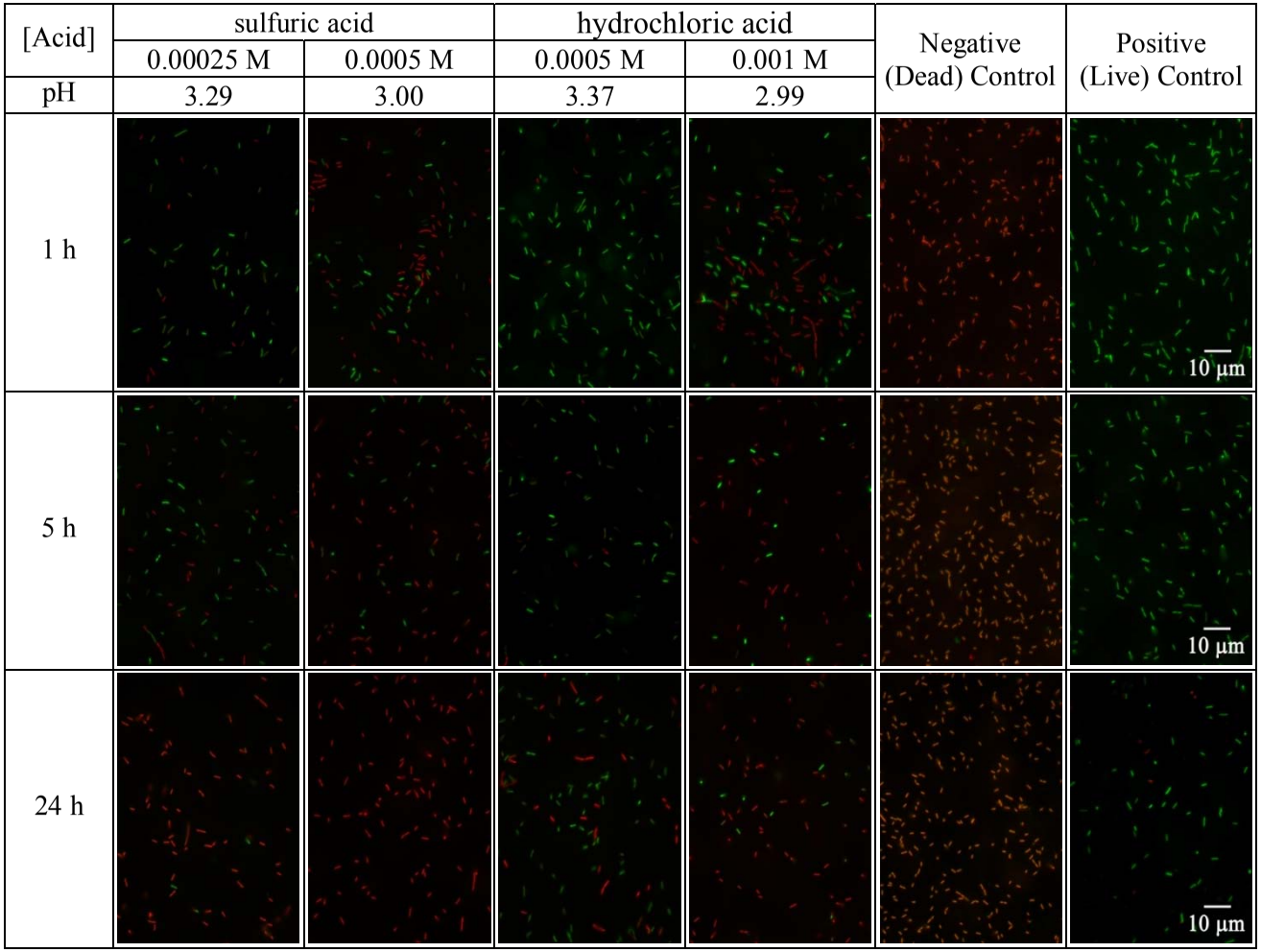


Figure 3.28 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of hydrochloric acid and sulfuric acid for 1, 5 and 24 h.

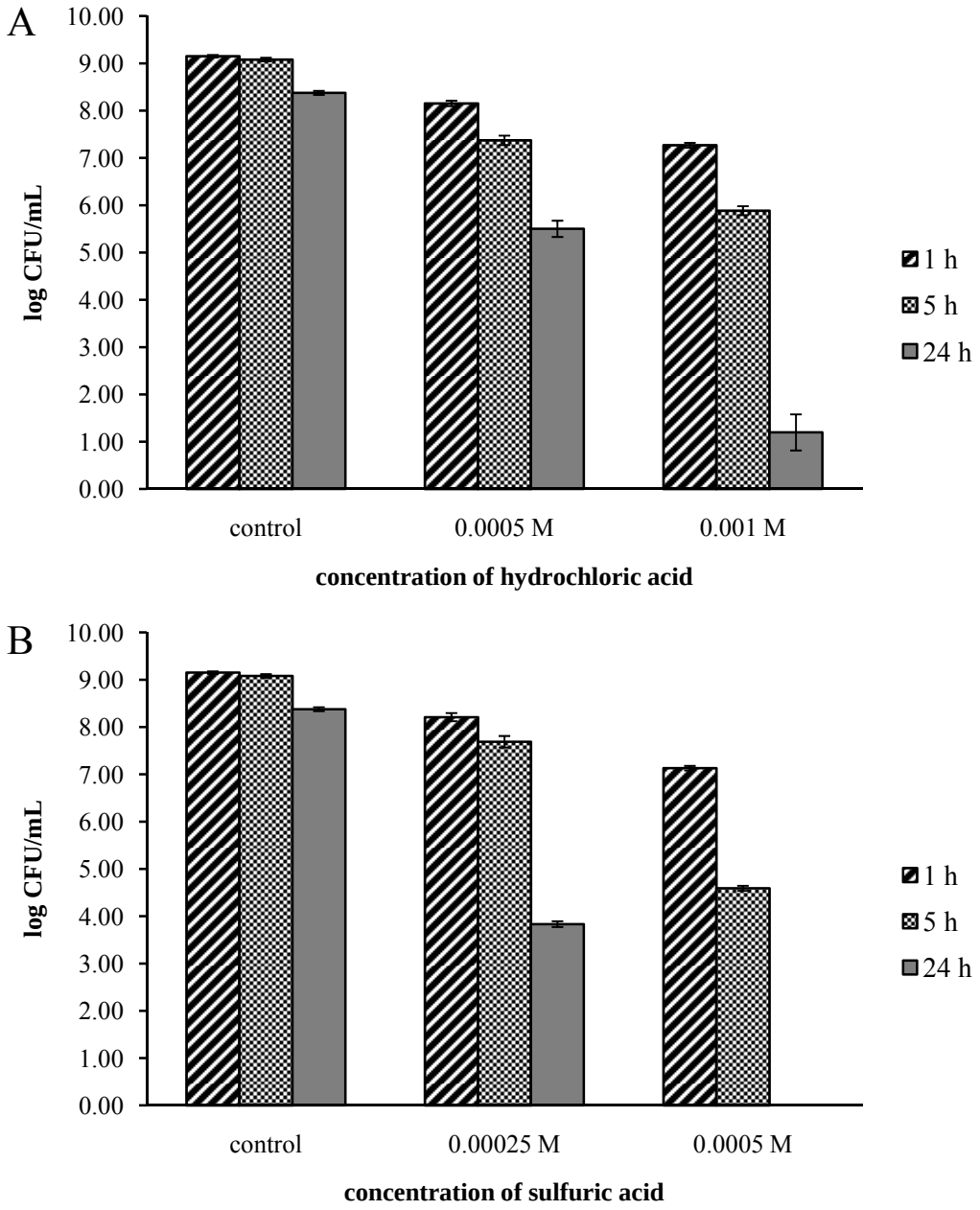


Figure 3.29 Cell counts determined by standard plate count methods for cells of mid-log phase *L. monocytogenes* CDC 7762 after exposure to a range of concentrations of hydrochloric acid (A) and sulfuric acid (B) for 1, 5 and 24 h (mean ± SEM; n=9).

3.2.4.3 Turbidity (OD_{600}) Measurement

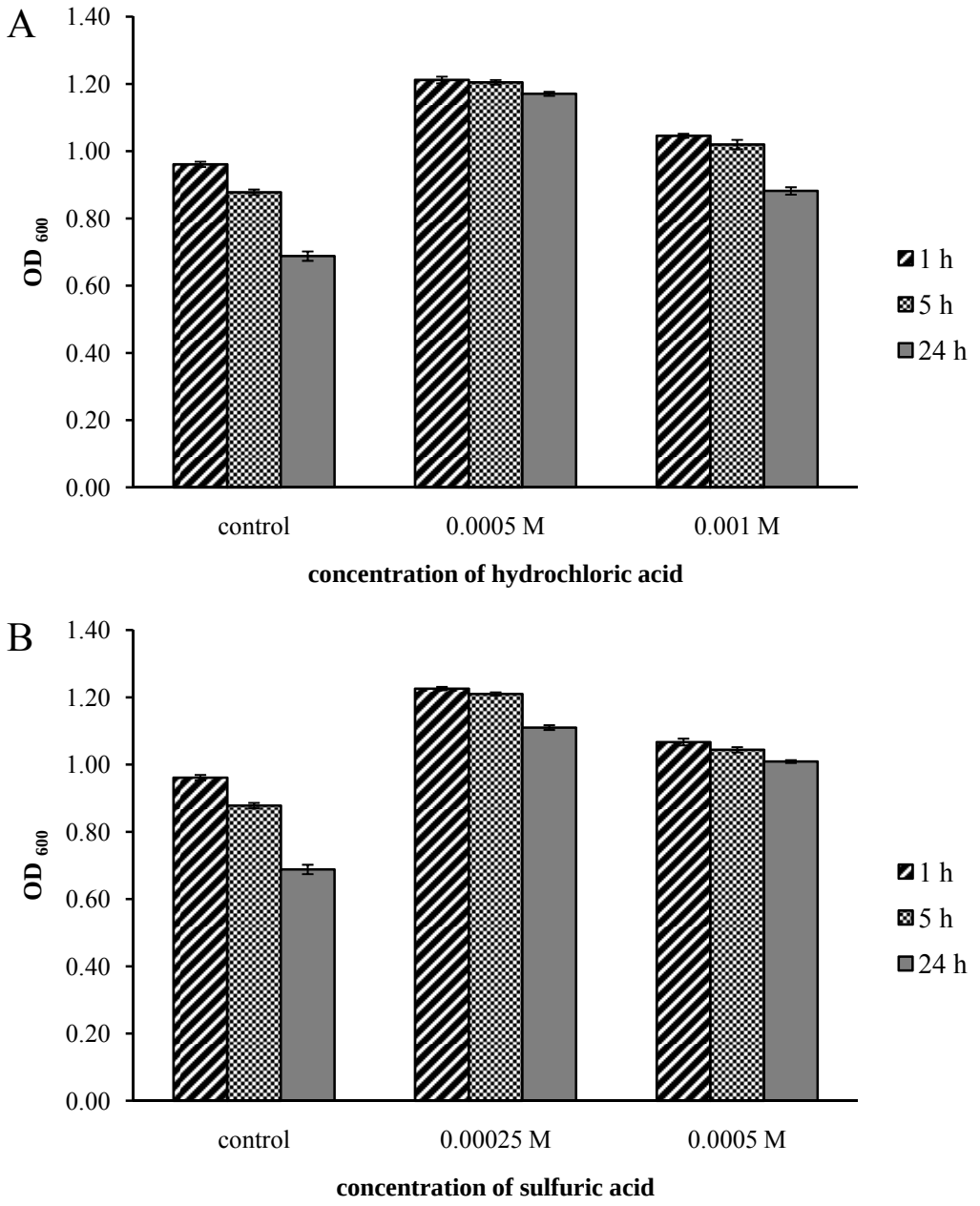


Figure 3.30 Cell concentration determined by optical density measurements for cells of mid-log phase *L. monocytogenes* CDC 7762 after exposure to a range of concentrations of hydrochloric acid (A) and sulfuric acid (B) for 1, 5 and 24 h (mean ± SEM; n=9).

According to the optical density results shown in Figure 3.30, the OD_{600} values of control samples significantly decreased ($P < 0.05$) over time, whereas all inorganic acid treated samples had a significant decrease ($P < 0.05$) at 24 h.

Significant increases ($P < 0.05$) in OD_{600} values were observed in all acid treated samples as compared to the control at 1, 5 and 24 h. In general, the OD_{600} values were reduced when the cells were treated with either hydrochloric or sulfuric acid of higher concentrations (lower pH). See Appendix B Table B.8 for detailed statistical data.

3.3 Effect of Other Stresses

3.3.1 Osmotic Stress

In the osmotic stress study, results have demonstrated that high concentrations of sodium chloride (5%, 15% and 25%) did not cause evident cell clumping of stationary-phase and mid-log phase *E. coli* DH5α, as well as mid-log phase *S. aureus* ATCC 13565 within the 24 h treatment. See Appendix C for optical microscopy results.

3.3.2 Heat-Shock

In the heat shock study, results have demonstrated that high temperatures (55°C and 60°C) did not induce evident cell clumping of stationary-phase and mid-log phase *E. coli* DH5α, as well as mid-log phase *S. aureus* ATCC 13565 within the 24 h treatment. See Appendix C for optical microscopy results.

3.3.3 Cold-Shock

In the cold shock study, low temperatures (-20°C, 4°C, and 10°C) did not induce evident cell clumping of stationary-phase and mid-log phase *E. coli* DH5α, as well as mid-log phase *S. aureus* ATCC 13565 within the 24 h treatment. See Appendix C for optical microscopy results.

3.4 Contact Angle Measurement

As previously shown in Section 3.1, higher concentrations (lower pH) of organic acid induced cell clumping of stationary-phase and mid-log phase *E. coli* DH5α cells at 1, 5 and 24 h, whereas lower concentrations (higher pH) of organic acid did not. The same was true for mid-log phase *S. aureus* ATCC 13565, however, it only occurred at 1 h.

To investigate potential changes in cell surface hydrophobicity in response to organic acid stress, contact angle measurements of stationary-phase *E. coli* DH5α and mid-log phase *S. aureus* ATCC 13565 cells treated with 0.1 M and 0.01 M acetic acids, as well as 0.01 M and 0.001 M lactic acids were obtained. As described in Section 2.6, each contact angle value was measured at 1 min intervals within 10 min after the addition of the water droplet on the surface of the bacterial lawn. The values measured at the 3 min time point in each treatment were used for statistical analysis. See Table 3.1 for detailed statistical data.

Table 3.1 Cell surface hydrophobicity determined by contact angle measurements for cells of stationary-phase *E. coli* DH5α and mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of acetic acid and lactic acid for 1 and 5 h.

A	strain	[acid]	pH	contact angles (degree)* at different treatment times	
				1 h	5 h
acetic acid	*E. coli* DH5α	control	6.57	5.16 ± 0.24^{Aa}	5.57 ± 0.36^{Aa}
		0.01 M	3.65	5.08 ± 1.82^{Aa}	7.73 ± 1.72^{Aa}
		0.1 M	2.90	28.01 ± 3.30^{Ba}	38.70 ± 2.18^{Bb}
	S. aureus ATCC 13565	control	6.57	7.81 ± 0.30^{Aa}	6.27 ± 1.30^{Aa}
		0.01 M	3.65	12.16 ± 0.97^{Ba}	5.81 ± 0.33^{Ab}
		0.1 M	2.90	12.45 ± 0.71^{Ba}	7.76 ± 1.30^{Ab}
B					
lactic acid	*E. coli* DH5α	control	6.57	6.05 ± 0.51^{Aa}	6.66 ± 0.34^{Aa}
		0.001 M	4.87	5.80 ± 0.55^{Aa}	5.71 ± 0.85^{Aa}
		0.01 M	3.01	15.55 ± 1.48^{Ba}	23.65 ± 1.16^{Bb}
	S. aureus ATCC 13565	control	6.57	7.99 ± 2.55^{Aa}	6.70 ± 0.38^{Aa}
		0.001 M	4.87	13.77 ± 1.34^{Ba}	17.76 ± 2.02^{Ba}
		0.01 M	3.01	15.03 ± 1.42^{Ba}	14.83 ± 1.84^{Ba}

* Values represent mean ± standard error of the mean (n=4). Values with same uppercase letters (A, B, C) within columns are not significantly different ($P > 0.05$) among acid treatments. Values with same lowercase letters (a, b, c) within rows are not significantly different ($P > 0.05$) among treatment times.

3.4.1 Acetic Acid Stress

3.4.1.1 Stationary-phase *E. coli* DH5α

As shown in Table 3.1A, *E. coli* DH5α cells treated with 0.1 M acetic acid had significantly larger ($P < 0.05$) contact angle values as compared to the control and 0.01 M acetic acid treatment. The contact angle values of cells exposed to 0.1 M acetic acid significantly increased by 22.85 and 33.13 degrees ($P < 0.05$) after 1 and 5 h, respectively, as compared to that of the control. In addition, the contact angle values of cells exposed to 0.1 M acetic acid at 1 h significantly increased by 10.69 degrees ($P < 0.05$) when the treatment time was extended to 5 h. Although no significant differences ($P > 0.05$) were observed between control and 0.01 M acetic acid treatment, these results suggest that treatment with higher concentrations (or lower pH) of acetic acid or with longer treatment times increased the contact angle values and cell surface hydrophobicity.

3.4.1.2 Mid-log Phase *S. aureus* ATCC 13565

As shown in Table 3.1A, the angle values of 0.01 M and 0.1 M acetic acid treatments on *S. aureus* ATCC 13565 cells significantly increased by 4.35 and 4.64 degrees ($P < 0.05$) respectively as compared to the control at 1 h, and subsequently decreased to comparable values to that of the control ($P > 0.05$) at 5 h. No significant differences ($P > 0.05$) were observed between the concentration of acetic acid. These results indicate that acetic acid only increased the cell surface hydrophobicity of *S. aureus* after a 1 h treatment. The hydrophobicity of *S. aureus* cell surfaces decreased when treated for longer times, and higher concentrations (or lower pH) of acetic acid did not significantly affect the results.

3.4.2 Lactic Acid Stress

3.4.2.1 Stationary-phase *E. coli* DH5α

Similar to the acetic acid stress, the contact angle values of 0.01 M lactic acid treatment were significantly higher ($P < 0.05$) than those of the control and 0.001 M treatment (Table 3.1B). The contact angle values of cells exposed to 0.01 M lactic acid significantly increased by 9.50 and 16.99 degrees ($P < 0.05$) after 1 and 5 h, respectively, as compared to that of the control. Furthermore, the contact angle values of cells exposed to 0.01 M lactic acid at 1 h significantly increased by 8.10 degrees ($P < 0.05$) when the treatment time was extended to 5 h. As for the 0.001 M lactic acid treatment, there was no significant difference ($P > 0.05$) in contact angle values as compared to the control. These results indicate that when treated with 0.01 M lactic acid, both higher concentrations (lower pH) and longer treatment time (5 h) significantly increased the contact angle values and cell surface hydrophobicity.

3.4.2.2 Mid-log Phase *S. aureus* ATCC 13565

Shown in Table 3.1B, the contact angle values of cells exposed to 0.001 M and 0.01 M lactic acid significantly increased by 5.78 and 7.04 degrees ($P < 0.05$) respectively as compared to that of the control after 1 h, and by 11.06 and 8.13 degrees respectively after 5 h. No significant differences ($P > 0.05$) in contact angle values were observed between the concentration of lactic acid. These results suggest that the *S. aureus* cell surface becomes more hydrophobic when treated with lactic acid, but no significant difference was observed with increased acid concentrations or treatment times.

Chapter 4 Discussion

4.1 Effect of Stress Response on Bacterial Enumeration

This project aimed to study the impact of stress response on bacterial viability and changes in cell association using microscopy and traditional enumeration methods. Three different organisms were selected: *E. coli* DH5α, a Gram-negative bacteria; *S. aureus* ATCC 13565, a Gram-positive organism; and *L. monocytogenes* CDC 7762, a Gram-positive foodborne pathogen that has received heightened awareness in recent years after it was implicated in outbreaks of listeriosis resulting in a large recall of ready-to-eat meats (Lianou & Sofos, 2007; Public Health Agency of Canada, 2008; Public Health Agency of Canada, 2009). In order to survive various environmental conditions that they may encounter in natural habitats, during food processing and storage, as well as *in vivo* digestion, bacteria have developed adaptive strategies in response to the stresses (Abee & Wouters, 1999; Bearson *et al.*, 1997; Yousef & Courtney, 2003).

In food systems, acid stress has been described as the combined biological effect of low pH (typically inorganic) and weak organic acids present in the natural food environment or as a result of further processing, such as the fermentation or addition of preservatives (Abee & Wouters, 1999; Bearson *et al.*, 1997). Although the low pH stress response of bacteria has been studied extensively, few studies have elucidated the mechanism of weak organic acid stress response (Barua *et al.*, 2002; Beales, 2004; Hirshfield *et al.*, 2003). Moreover, previous studies on stress induced by organic acid have involved the use of inorganic acid (i.e. HCl), either in pre-treatment experiments or for the pH

adjustment of organic acid solutions (Baik *et al.*, 1996; Berry & Cutter, 2000; Goodson & Rowbury, 1989a; Guilfoyle & Hirshfield, 1996; Kwon & Ricke, 1998; Lin *et al.*, 1996; Rice *et al.*, 2005). As such, there lacks knowledge related to bacterial stress response to organic acid in a system devoid of inorganic acid. Therefore, this study independently investigated the effect of organic and inorganic acid stress in an effort to better understand their individual effects. Additionally, the effect of other food industry-related stresses, heat shock, cold shock, as well as osmotic stress has also been included for comparison.

4.1.1 Effect of Organic Acid Stress

Standard plate count results indicated that treatments with either acetic or lactic acid dramatically affect the enumeration of stationary-phase *E. coli* DH5α, mid-log phase *E. coli* DH5α, *S. aureus* ATCC 13565 and *L. monocytogenes* CDC 7762 grown on agar plates. This was reflected by decreased to undetectable plate counts when lowering pH (pH 2.5-3.7) or increasing exposure time of acid treatment (Figures 3.4, 3.8, 3.12 and 3.16 respectively). These observations agree with earlier acid stress studies showing that pH values at or below 3.0 were normally lethal to bacterial cells if they have not experienced pre-adaptation to sub-lethal pHs (Cebrián *et al.*; Chan *et al.*, 1998; Koutsoumanis *et al.*, 2003; Lou & Yousef, 1997; Paul & Hirshfield, 2003).

However, the fluorescence microscopy and optical density results demonstrated that the observed reduction in viable counts using the standard plate count method is not solely because of cell death, but largely the result of changes in cell association. With Gram-negative bacteria, direct exposure of stationary-

phase and mid-log phase *E. coli* DH5α to higher concentrations of acetic acid and lactic acid (pH 2.7-3.3) induced evident cell clumping with little loss of viability (Figures 3.2, 3.3, 3.6 and 3.7). The extent of cell aggregation and resistance to organic acid with increasing exposure was more pronounced with the stationary phase cells when compared to mid-log phase *E. coli* DH5α. By clumping together as the response to organic acid stress, mid-log phase *E. coli* DH5α were able to survive pH 2.7 to 3.3 for at least 5 h; stationary-phase *E. coli* DH5α were able to survive pH 2.7 to 3.3 for 24 h, and even developed resistance to acetic acid with pH as low as 2.5, for 24 h. These results indicate that viability of *E. coli* DH5α cells is not significantly compromised by treatment with organic acid. In the case of Gram-positive bacteria, similar cell clumping was observed in *S. aureus* ATCC 13565 within pH range of 2.7 to 3.1 at 1 h and in *L. monocytogenes* CDC 7762 within pH range of 2.5 to 2.8 at 5 or 24 h (Figures 3.10, 3.11, 3.14 and 3.15 respectively). Generally, the clumping was not as dramatic as seen in Gram-negative *E. coli* DH5α. Cell clumping level was decreased in *S. aureus* ATCC 13565 when treatment time is extended to 5 h or longer. And immediate cell death was detected in *L. monocytogenes* CDC 7762 at pH below 3.3. This finding indicates that *L. monocytogenes* CDC 7762 were less resistant to organic acid when compared to *E. coli* DH5α and *S. aureus* ATCC 13565 cells. In addition, Gram-negative *E. coli* DH5α cells showed greater degree of cell clumping when compared to Gram-positive *S. aureus* ATCC 13565 and *L. monocytogenes* CDC 7762. As for the optical density results, it is demonstrated that the OD_{600} values of clumped bacteria samples were always lower than those of non-clumped samples

(Figures 3.5, 3.9, 3.13 and 3.17 respectively). Significantly decreased OD_{600} values were observed in clumped stationary-phase *E. coli* DH5α cells all the time, as well as in clumped mid-log phase *E. coli* DH5α and *S. aureus* ATCC 13565 cells at 1 h, as compared to the control.

The fluorescence microscopy results provided direct evidence of cell death as well as changes in cell association in response to the stress. Based on these results, it was observed that organic acid stress induced cell clumping with little loss of viability in Gram-negative *E. coli* DH5α. With Gram positive *S. aureus* and *L. monocytogenes*, the same form of aggregation is not as evident as seen in Gram-negative *E. coli*, as further discussed in Section 4.2.

The standard plate count, one of the most widely used traditional enumeration methods, seemed to be affected by the change in cell association. It has been indicated that treatment with organic acid dramatically decreased plate counts, and the decrease in the plate counts was associated with the changes in the cell aggregation status. These findings support the hypothesis mentioned in the Section 1.4.1.1 that the changes in cell association status in response to organic acid stress may lead to the underestimation of the plate count result and the total viable number of bacteria. Additionally, since immediate cell death was observed in *L. monocytogenes* CDC 7762, their compromised plate counts could just be a reflection of the live/dead status.

Decreased optical density values were observed in clumped stationary-phase *E. coli* DH5α cells, as well as in clumped mid-log phase *E. coli* DH5α and *S. aureus* ATCC 13565 cells at 1 h, after the organic acid treatment. These

findings support the hypothesis mentioned in the Section 1.4.1.2 that the change in cell association in response to organic acid stress affects the distribution of cells in suspension, and therefore causes the underestimation of cell concentration. In addition, it is felt that the optical density method may not serve as an accurate representation of cell population. The OD_{600} values observed in each bacterial strain did not show the same trend. For example, the OD_{600} values of control samples in mid-log phase *E. coli* DH5α decreased with prolonged incubation, whereas all other treatments increased (Figure 3.9). The OD_{600} values of control experiments in mid-log phase *L. monocytogenes* CDC 7762 were always lower than those of the acid treated samples (Figure 3.17). Therefore, it does not seem to be feasible to use OD_{600} values to evaluate the cell population, especially when compared to the control samples.

4.1.2 Effect of Inorganic Acid Stress

The standard plate count results in this study indicate that either hydrochloric acid or sulfuric acid at pH 3.0 and 3.3 decreased cell counts (Figures 3.20, 3.23, 3.26 and 3.29 respectively), which is consistent with earlier findings that pH values at or below 3.0 were normally lethal to bacterial cells if they have not experienced pre-adaptation to sub-lethal pHs (Cebrián *et al.*; Chan *et al.*, 1998; Koutsoumanis *et al.*, 2003; Lou & Yousef, 1997; Paul & Hirshfield, 2003). In the fluorescence microscopy studies, cell clumping was only observed in some of stationary-phase *E. coli* DH5α, a considerable proportion of clumped bacteria were dead, and the apparent number of dead single cells increased over time (Figures 3.18 and 3.19). Additionally, the apparent number of dead mid-log *E.*

coli DH5α, *S. aureus* ATCC 13565 and *L. monocytogenes* CDC 7762 cells increased over time (Figures 3.22, 3.25 and 3.28 respectively). These findings indicate that the viability of tested bacteria was compromised by treatment with inorganic acid with pH range of 3.0-3.3. Moreover, since cell clumping was either not observed or found in very low intensity, the standard plate results seemed consistent with the live/dead status upon treatment with inorganic acid. However, any clumping would indicate some degree of inaccuracy during enumeration with conventional methods. Similar to organic acid stress study, the optical density result seemed to not be a reliable representation of cell population (Figures 3.21, 3.24, 3.27, and 3.30 respectively).

Our fluorescence microscopy results indicate that organic acids could induce clumping reaction of bacterial cells, whereas such response was either not observed or found in very low level with inorganic acid treatment in the same pH range. It has been generally proposed that weak organic acids not only lower the intracellular pH (pH_i) as would be expected in the case of inorganic acids, but also result in the intracellular accumulation of the dissociated organic acid anions. The latter effect would likely contribute to the change in the turgor pressure and may trigger a series of stress response that are different from those caused by inorganic acids (Foster, 1999; Hirshfield *et al.*, 2003). This could be one reason why different fluorescence microscopy results were observed in organic acid and inorganic acid treatments.

4.1.3 Effect of Other Stresses

In the osmotic stress, heat-shock and cold-shock studies, all tested treatments did not induce evident cell clumping of stationary-phase and mid-log phase *E. coli* DH5α, as well as mid-log phase *S. aureus* ATCC 13565 within 24 h. These findings suggest that high concentrations of sodium chloride, high temperatures and low temperatures do not have effects on the association (clumping) of bacteria. There is hence no evidence showing that plate count and optical density measurements could be underestimated as results of cell aggregation under such conditions.

4.2 Potential Changes in Cell Surface Properties

This study demonstrated that although traditional enumeration methods, such as standard plate count, may indicate a loss of cell viability when bacteria are exposed to organic and inorganic acids, further fluorescence microscopy analysis had proven it to be a consequence of cell clumping. This suggests that standard plate count results do not serve as good standard of lethality under certain stress conditions. Moreover, Gram-negative and Gram-positive bacteria seem to act differently in response to organic acid stress, with Gram-negative bacteria showing more pronounced clumping as well as more live cells. This could result from different changes in cell surface structures or properties of Gram-negative and Gram-positive bacteria in response to the environmental stress.

Gram-negative and Gram-positive bacteria have complicated cytoplasmic membrane structures. The cytoplasmic membrane primarily consists of phospholipid bilayers and proteins. Each phosoholipid molecule includes a

hydrophilic polar head (phosphate and glycerol) and a hydrophobic nonpolar tail (fatty acid). Phospholipid bilayers are arranged with hydrophilic heads pointing outwards and hydrophobic tails packed inside (Madigan *et al.*, 2003).

Gram-positive bacteria and Gram-negative bacteria are mainly different in the structures of their cell walls. The cell wall of Gram-positive bacteria contains a thick layer of peptidoglycan and some embedded components (teichoic acids, polysaccharide and proteins). In contrast, the cell wall of Gram-negative bacteria contains a much thinner layer of peptidoglycan, as well as an additional characteristic layer named the outer membrane. The outer membrane is mainly composed of lipopolysaccharide (LPS, including O-antigen, core polysaccharide and lipid A), phospholipid, porin protein and lipoprotein. Lipid A, the hydrophobic region of LPS, associates with phospholipid to form a "second lipid bilayer", which is functionally similar to the cytoplasmic phospholipid bilayer (Madigan *et al.*, 2003; Nikaido & Vaara, 1985; Russell, 2003). Bacterial membranes, especially the outer membrane, serve as a selective permeability barrier to the environment. It has been suggested that the permeation resistance to hydrophobic (nonpolar) molecules is provided by the hydrophilic barrier near the membrane surface (such as the outer part of LPS, or polar head of phosoholipid), while permeation resistance to hydrophilic (polar) molecules is mainly provide by the hydrophobic barrier in the center of the membrane (such as nonpolar fatty acid tail of lipid A or phosoholipid). In addition, large hydrophilic molecules (> 600 Da) can be impeded by porin protein channels (Nikaido & Vaara, 1985; Nikaido, 2003; Subczynski *et al.*, 1994).

Some studies suggest that bacteria may modify their cell surfaces in response to various stress conditions. It has been demonstrated that *L. monocytogenes* can maintain optimum membrane fluidity by increasing the degree of unsaturation and/or by shortening chain length of fatty acids in response to cold shock (Russell *et al.*, 1995; Russell, 2002). An increased ratio of diphosphatidylglycerol/phosphatidylglycerol was observed in *L. monocytogenes* in a study on osmotic stress (Russell *et al.*, 1995). More recently, it has been shown that *E. coli* produce higher levels of saturated or cyclopropane fatty acid at low pH, suggesting that bacteria may increase their membrane rigidity to reduce proton permeability (Brown *et al.*, 1997; Dlamini & Buys, 2009; Shabala & Ross, 2008). In addition, it has been reported that *Helicobacter pylori* may alter the structure of lipopolysaccharide (LPS) in response to acid stress (McGowan *et al.*, 1998). The full expression of LPS has been suggested to be indispensable for resistance against acetic acid and other short chain fatty acids in Shiga toxin-producing *E. coli* and *Salmonella enterica* serovar Typhimurium (Barua *et al.*, 2002). However, there are few studies that clearly correlate the changes in association and cell surface hydrophobicity with bacterial response to environmental stresses.

The contact angle results indicate that treatment with organic acid at pH 3.0 (0.1 M acetic acid and 0.01 M lactic acid) or with longer treatment times (5 h) brings about significant increases in the contact angle values and surface hydrophobicity in stationary-phase *E. coli* DH5α (Table 3.1). The pH 3.0 organic acid treatment and longer treatment time (5 h) were related to more evident cell

clumping. The relation between cell clumping and increased hydrophobicity was consistent with previous observation that the deletion of *ycfR*, a putative gene encoding outer membrane multiple stress resistance protein, caused significant cell aggregation and increased cell surface hydrophobicity for *E.coli* K-12 (Zhang *et al.*, 2007).

In contrast, the increase in cell surface hydrophobicity of mid-log phase *S. aureus* ATCC 13565 was not as significant as was observed in stationary-phase *E. coli* DH5α (Table 3.1). A relatively small increase in contact angle value (less than 10 degrees) was detected in each of the organic acid treatments except for the acetic acid treatments at 5 h. It appears that, with the exception of the result of the 5 h treatment, neither higher concentrations (or lower pH) of organic acid nor longer treatment times have significant effects on cell surface hydrophobicity. Therefore, it would be hard to conclude if there exists a relationship between the cell aggregation and the change in hydrophobicity in *S. aureus* cells.

It seems reasonable to suggest that the differences in the relationships between cell clumping and cell surface hydrophobicity in *E. coli* DH5α and *S. aureus* ATCC 13565, in response to organic acid stress, are due to different cell surface structures of Gram-negative and Gram-positive bacteria.

In this study, higher concentrations (or lower pH) of organic acid induced more dramatic clumping and more cell surface hydrophobicity in Gram-negative *E. coli* DH5α, suggesting that the cell surface may be altered to be more hydrophobic to reduce the penetration of hydrophilic acetic or lactic acid solvent.. One of the possible modifications in response to the organic acid stress could be

the expression of some specific hydrophobic proteins. It has been demonstrated that low pH stress (pH 5.8) leads to increased cell surface hydrophobicity and synthesis of specific outer membrane proteins in *Salmonella enterica* serovar serovar Typhimurium (Leyer & Johnson, 1993), suggesting that the up-regulation of the expression of hydrophobic proteins may play a role in acid stress response. In addition, the fatty acyl chain of lipid A may be rearranged to provide better protection against organic acid. Lipid A serves as the hydrophobic region of LPS and associates with phospholipid to form a "second lipid bilayer" in the outer membrane of Gram-negative bacteria. It has been indicated that modification of lipid A plays an important role in response to environmental stimuli (Trent, 2004). However, more studies need to be done to reveal the detailed mechanism of structural change under the organic acid stress conditions. As for the Gram-positive *S. aureus* ATCC13565, the thicker and more rigid layer of peptidoglycan, as well as teichoic acids, would likely contribute to their lower level of changes in cell aggregation and cell surface hydrophobicity.

4.3 Limitations of Traditional Enumeration Methods in Food Industry

Standard plate count result is an important parameter to evaluate the amount of viable bacterial cells. It also reflects the growth capability of bacterial cells in certain environments. This method has been widely used in food industry (FDA/CFSAN, 1998; Health Canada, 1999; Health Canada, 2004; International Commission on Microbiological Specifications for Foods, 1986; USDA/FSIS, 1998), as well as in stress response studies (Culham *et al.*, 2001; Dlamini & Buys,

2009; Kenny *et al.*, 2009; Lin *et al.*, 1995; Lin *et al.*, 1996; Rice *et al.*, 2005; Wang & Doyle, 1998). The optical density (OD_{600}) result is a conventional parameter to evaluate the cell concentrations in a suspension. It is usually used to estimate bacterial growth during laboratory experiments and fermentation production (Madigan *et al.*, 2003).

In this study, cell association changes in response to organic acid stress result in significantly compromised plate count results, strongly suggesting that standard plate count is not a reliable enumeration method when taking into consideration the impact of certain stress responses. Decreased optical density values, resulting from cell aggregation, were observed in stationary-phase *E. coli* DH5α at all time points, as well as in mid-log phase *E. coli* DH5α and *S. aureus* ATCC 13565 at 1 h. These prove that turbidity (optical density) measurement is also not a dependable enumeration method when stress response is involved.

Numerous studies have suggested that the usually optimal growth conditions in the laboratory may not represent the actual physiology environment in foods and bacteria my exhibit different growth patterns (Abee & Wouters, 1999; Yousef & Courtney, 2003). Additionally, it has been demonstrated that the adaptive stress responses developed by foodborne pathogens not only contribute to the resistance against more severe hostile environments (either homologous or heterologous), but also enhance their virulence (Humphrey *et al.*, 1996; O'Driscoll *et al.*, 1996; Riesenberg-Wilmes *et al.*, 1996; Rodriguez-Romo & Yousef, 2005). Therefore, bacteria that experience stress conditions may underestimate the microbial risk in food system. Hence, the traditional enumeration methods used in

food industry, such as standard plate count, need to be reassessed, with the impact of stress response taken into consideration.

Short-chain fatty acids with concentration of 0.1 to 0.3 M have been widely used as preservatives in food products (Dorsa, 1997; Hardin *et al.*, 1995; Jay *et al.*, 2005; Ricke, 2003). It also has been reported that a 1 to 3% concentration (v/v) of acetic acid or lactic acid can be used to wash and sanitize animal carcasses without affecting the desirable sensory properties of meat (Smulders & Greer, 1998). The 1 % and 2% concentration of acetic acid are in the range of 0.1 M to 0.2 M and 0.2 M to 0.5 M in this study, respectively. Taking into account the change of cell aggregation or survival mechanisms during the stress responses that may be associated with food processing and preservation techniques, the currently used enumeration methods, such as standard plate count, may significantly underestimate the total number of pathogens. It would cause a potential threat to food safety and public health. Evaluation of the impact of stress response on current enumeration methods, as well as potential solutions to this problem, would play significant roles in food safety assessment.

Chapter 5 Conclusions and Future Work

This study attempted to evaluate the influence of bacterial stress response on cell association status and its consequent impact on the traditional enumeration methods. Three different microorganisms, Gram-negative *E. coli* DH5α, Gram-positive *S. aureus* ATCC 13565 and Gram-positive foodborne pathogen *L. monocytogenes* CDC 7762, were selected. The effect of four types of food industry-related stresses, acid stress, osmotic stress, heat shock and cold shock, has been investigated. In addition, the effect of organic and inorganic acid stress has been studied independently.

According to the results obtained in this study, the following conclusions can be stated.

1. Organic acid (acetic and lactic acid) stress had effect on cell association (clumping) with little loss of viability in *E. coli* DH5α and *S. aureus* ATCC 13565 cells.

2. The plate counts of clumped bacterial cultures significantly decreased when organic acid stress is involved, even to undetectable values. In addition, the optical density values significantly decreased in clumped stationary-phase *E. coli* DH5α cells at all time points, as well as in clumped mid-log phase *E. coli* DH5α and *S. aureus* ATCC 13565 cells at 1 h, when organic acid stress is involved. It could therefore be concluded that the standard plate count method and optical density measurement dramatically underestimated the number of bacteria because of cell aggregation in response to organic acid stress.

3. A relationship between the cell clumping and the increased cell surface hydrophobicity (larger contact angle values) was observed in stationary-phase *E. coli* DH5α, but not in mid-log phase *S. aureus* ATCC 13565. This suggested that the cell surface of Gram-negative bacteria may be altered to be more hydrophobic in order to reduce the penetration of hydrophilic acetic acid or lactic acid. The difference in the behaviour of Gram-negative and Gram-positive bacteria in response to organic acid stress is likely due to their different cell membrane structures.

4. There was no or very little cell association change (clumping) when the acid stress was induced by inorganic acid (hydrochloric and sulfuric acid). The standard plate count results were consistent with the live/dead status upon treatment with inorganic acid.

5. Osmotic stress, heat shock and cold shock did not have effect on cell association (clumping). There is thereby no evidence showing that standard plate count and optical density measurement could be compromised as results of cell aggregation in response to such stress conditions.

6. Taking into consideration the impact of organic acid stress response, the traditional enumeration methods used in food industry, such as standard plate count, need to be reassessed. This is important since organic acids are widely used as antimicrobials or carcass spray sanitizers.

Future work will mainly focus on two aspects: the detailed mechanism of cell surface structure changes in response to organic acid stress and further

evaluation of the effect of organic acid stress on traditional enumeration methods in food industry.

It was observed that *E. coli* DH5α modify their cell surface hydrophobicity to adapt to organic acid stress. *S. aureus* ATCC 13565 was also shown to be capable of similar modifications, but to a lesser extent. The detailed changes in cell surface may involve modification or expression of specific membrane protein, as well as modification of lipid A fatty acyl chains. Experiments such as SDS-PAGE, gas chromatography separation, as well as mass spectral analysis should be conducted to further analyse these cell surface components.

It has been indicated that the adaptive stress responses developed by foodborne pathogens not only contribute their resistance to more severe hostile environments (either homologous or heterologous), but also enhance the virulence. Therefore, the effect of organic acid-related cross protection (or cross-resistance response) on traditional enumeration methods will need to be investigated. Additionally, a broad spectrum of foodborne pathogens (especially Gram-negative pathogens) can be subjected to various stresses (especially organic acid stress) to provide more detailed information of the impact of stress response on enumeration methods. Lastly, and importantly, the analysis of the effect of organic acid stress in food samples will need to be carried out.

Bibliography

Abee, T. & Wouters, J. A. (1999). Microbial stress response in minimal processing. *Int J Food Microbiol* **50**, 65-91.

Abee, T., van Schaik, W. & Siezen, R. J. (2004). Impact of genomics on microbial food safety. *Trends Biotechnol* **22**, 653-660.

Adams, M. R. & Moss, M. O. (2008). *Food Microbiology* 3rd edn. Cambridge, UK RSC Publishing.

Anderson, J. E., Beelman, R. R. & Doores, S. (1996). Persistence of serological and biological activities of staphylococcal enterotoxin A in canned mushrooms. *J Food Prot* **59**, 1292-1299.

Angelidis, A. S., Smith, L. T., Hoffman, L. M. & Smith, G. M. (2002). Identification of OpuC as a chill-activated and osmotically activated carnitine transporter in *Listeria monocytogenes*. *Appl Environ Microbiol* **68**, 2644-2650.

Angelidis, A. S. & Smith, G. M. (2003). Role of the glycine betaine and carnitine transporters in adaptation of *Listeria monocytogenes* to chill stress in defined medium. *Appl Environ Microbiol* **69**, 7492-7498.

Archer, D. L. (1996). Preservation microbiology and safety: evidence that stress enhances virulence and triggers adaptive mutations. *Trends Food Sci Technol* **7**, 91-95.

Arsène, F., Tomoyasu, T. & Bukau, B. (2000). The heat shock response of *Escherichia coli*. *Int J Food Microbiol* **55**, 3-9.

Audia, J. P., Webb, C. C. & Foster, J. W. (2001). Breaking through the acid barrier: an orchestrated response to proton stress by enteric bacteria. *Int J Med Microbiol* **291**, 97-106.

Aureli, P., Fiorucci, G. C., Caroli, D., Marchiaro, G., Novara, O., Leone, L. & Salmaso, S. (2000). An outbreak of febrile gastroenteritis associated with corn contaminated by *Listeria monocytogenes*. *N Engl J Med* **342**, 1236-1241.

Baev, D., England, R. & Kuramitsu, H. K. (1999). Stress-induced membrane association of the *Streptococcus mutans* GTP-binding protein, an essential G protein, and investigation of its physiological role by utilizing an antisense RNA strategy. *Infect Immun* **67**, 4510-4516.

Baik, H. S., Bearson, S., Dunbar, S. & Foster, J. W. (1996). The acid tolerance response of *Salmonella typhimurium* provides protection against organic acids. *Microbiology* **142**, 3195-3200.

Balaban, N. & Rasooly, A. (2000). Staphylococcal enterotoxins. *Int J Food Microbiol* **61**, 1-10.

Balandina, A., Claret, L., Hengge-Aronis, R. & Rouviere-Yaniv, J. (2001). The *Escherichia coli* histone-like protein HU regulates *rpoS* translation. *Mol Microbiol* **39**, 1069.

Bang, I. S., Kim, B. H., Foster, J. W. & Park, Y. K. (2000). OmpR regulates the stationary-phase acid tolerance response of *Salmonella enterica* serovar Typhimurium. *J Bacteriol* **182**, 2245-2252.

Barth, M., Marschall, C., Muffler, A., Fischer, D. & Hengge-Aronis, R. (1995). Role for the histone-like protein H-NS in growth phase-dependent and osmotic regulation of σ^S and many σ^S -dependent genes in *Escherichia coli*. *J Bacteriol* **177**, 3455-3464.

Barua, S., Yamashino, T., Hasegawa, T., Yokoyama, K., Torii, K. & Ohta, M. (2002). Involvement of surface polysaccharides in the organic acid resistance of Shiga Toxin-producing *Escherichia coli* O157:H7. *Mol Microbiol* **43**, 629-640.

Beales, N. (2004). Adaptation of microorganisms to cold temperatures, weak acid preservatives, low pH, and osmotic stress: a review. *Compr Rev Food Sci F* **3**, 1-20.

Bearson, B. L., Wilson, L. & Foster, J. W. (1998). A low pH-inducible, PhoPQ-dependent acid tolerance response protects *Salmonella typhimurium* against inorganic acid stress. *J Bacteriol* **180**, 2409-2417.

Bearson, S., Benjamin, W., Jr, Swords, W. & Foster, J. (1996). Acid shock induction of RpoS is mediated by the mouse virulence gene mviA of *Salmonella typhimurium*. *J Bacteriol* **178**, 2572-2579.

Bearson, S., Bearson, B. & Foster, J. W. (1997). Acid stress responses in enterobacteria. *FEMS Microbiol Lett* **147**, 173-180.

Becker, G., Klauck, E. & Hengge-Aronis, R. (1999). Regulation of RpoS proteolysis in *Escherichia coli*: the response regulator RssB is a recognition factor that interacts with the turnover element in RpoS. *Proc Natl Acad Sci U S A* **96**, 6439-6444.

Benson, A. K. & Haldenwang, W. G. (1993a). Regulation of σ^B levels and activity in *Bacillus subtilis*. *J Bacteriol* **175**, 2347-2356.

Benson, A. K. & Haldenwang, W. G. (1993b). *Bacillus subtilis* σ^B is regulated by a binding protein (RsbW) that blocks its association with core RNA polymerase. *Proc Natl Acad Sci U S A* **90**, 2330-2334.

Berry, E. D. & Cutter, C. N. (2000). Effects of acid adaptation of *Escherichia coli* O157:H7 on efficacy of acetic acid spray washes to decontaminate beef carcass tissue. *Appl Environ Microbiol* **66**, 1493-1498.

Bhunia, A. K. (2008). *Foodborne Microbial Pathogens: Mechanisms and Pathogenesis* New York, USA: Springer Science+ Bussiness Media, LLC.

Bischoff, M., Dunman, P., Kormanec, J., Macapagal, D., Murphy, E., Mounts, W., Berger-Bachi, B. & Projan, S. (2004). Microarray-based analysis of the *Staphylococcus aureus* σ^B regulon. *J Bacteriol* **186**, 4085-4099.

Blohn, C. v., Kempf, B., Kappes, R. M. & Bremer, E. (1997). Osmostress response in *Bacillus subtilis*: characterization of a proline uptake system (OpuE) regulated by high osmolarity and the alternative transcription factor sigma B. *Mol Microbiol* **25**, 175-187.

Bouché, S., Klauck, E., Fischer, D., Lucassen, M., Jung, K. & Hengge-Aronis, R. (1998). Regulation of RssB-dependent proteolysis in *Escherichia coli*: a role for acetyl phosphate in a response regulator-controlled process. *Mol Microbiol* **27**, 787.

Boulos, L., Michèle, P., Barbeau, B., Coallier, J. & Desjardins, R. (1999). LIVE/DEAD® *Bac*Light™: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *J Microbiol Methods* **37**, 77-86.

Boyd, D. A., Cvitkovitch, D. G., Bleiweis, A. S., Kiriukhin, M. Y., Debabov, D. V., Neuhaus, F. C. & Hamilton, I. R. (2000). Defects in D-alanyl-lipoteichoic acid synthesis in *Streptococcus mutans* results in acid sensitivity. *J Bacteriol* **182**, 6055-6065.

Braux, A.-S., Minet, J., Tamanai-Shacoori, Z., Riou, G. & Cormier, M. (1997). Direct enumeration of injured *Escherichia coli* cells harvested onto membrane filters. *J Microbiol Methods* **31**, 1-8.

Bronner, S., Monteil, H. & Prévost, G. (2004). Regulation of virulence determinants in *Staphylococcus aureus*: complexity and applications. *FEMS Microbiol Rev* **28**, 183-200.

Brown, J. L., Ross, T., McMeekin, T. A. & Nichols, P. D. (1997). Acid habituation of *Escherichia coli* and the potential role of cyclopropane fatty acids in low pH tolerance. *Int J Food Microbiol* **37**, 163-173.

Brul, S. & Coote, P. (1999). Preservative agents in foods: mode of action and microbial resistance mechanisms. *Int J Food Microbiol* **50**, 1-17.

Brul, S., Coote, P., Oomes, S., Mensonides, F., Hellingwerf, K. & Klis, F. (2002). Physiological actions of preservative agents: prospective of use of modern microbiological techniques in assessing microbial behaviour in food preservation. *Int J Food Microbiol* **79**, 55-64.

Bruno, T. J. & Svorono, P. D. N. (2006). *CRC Handbook of Fundamental Spectroscopic Correlation Charts*, 1st edn. Boca Raton, FL CRC Press.

Buchanan, R. L. & Doyle, M. P. (1997). Foodborne disease significance of *Escherichia coli* O157: H7 and other Enterohemorrhagic *E. coli*. *Food Technol-Chicago* **51**, 69-76.

Byrd, J. J. & Colwell, R. R. (1990). Maintenance of plasmids pBR322 and pUC8 in nonculturable *Escherichia coli* in the marine environment. *Appl Environ Microbiol* **56**, 2104-2107.

Castanie-Cornet, M.-P., Penfound, T. A., Smith, D., Elliott, J. F. & Foster, J. W. (1999). Control of acid resistance in *Escherichia coli*. *J Bacteriol* **181**, 3525-3535.

CDC (2008). Summary of notifiable diseases - United States, 2006. *Morb Mortal Wkly Rep* **55**, 1-94.

Cebrián, G., Sagarzazu, N., Pagán, R., Condón, S. & Mañas, P. Development of stress resistance in *Staphylococcus aureus* after exposure to sublethal environmental conditions. *Int J Food Microbiol* **In Press, Corrected Proof**.

Chan, P. F., Foster, S. J., Ingham, E. & Clements, M. O. (1998). The *Staphylococcus aureus* alternative sigma factor σ^B controls the environmental stress response but not starvation survival or pathogenicity in a mouse abscess model. *J Bacteriol* **180**, 6082-6089.

Chaturongakul, S. & Boor, K. J. (2004). RsbT and RsbV contribute to σ^B-dependent survival under environmental, energy, and intracellular stress conditions in *Listeria monocytogenes*. *Appl Environ Microbiol* **70**, 5349-5356.

Chaturongakul, S., Raengpradub, S., Wiedmann, M. & Boor, K. J. (2008). Modulation of stress and virulence in *Listeria monocytogenes*. *Trends Microbiol* **16**, 388-396.

Christiansen, J. K., Larsen, M. H., Ingmer, H., Sogaard-Andersen, L. & Kallipolitis, B. H. (2004). The RNA-binding protein Hfq of *Listeria monocytogenes*: role in stress tolerance and virulence. *J Bacteriol* **186**, 3355-3362.

Clancy, K. A., Pearson, S., Bowen, W. H. & Burne, R. A. (2000). Characterization of recombinant, ureolytic *Streptococcus mutans* demonstrates an inverse relationship between dental plaque ureolytic capacity and cariogenicity. *Infect Immun* **68**, 2621-2629.

Colwell, R. R. (2000). Viable but nonculturable bacteria: a survival strategy. *J Infect Chemother* **6**, 121-125.

Cotter, P. D., Emerson, N., Gahan, C. G. M. & Hill, C. (1999). Identification and disruption of *lisRK*, a genetic locus encoding a two-component signal transduction system involved in stress tolerance and virulence in *Listeria monocytogenes*. *J Bacteriol* **181**, 6840-6843.

Cotter, P. D., Gahan, C. G. M. & Hill, C. (2000). Analysis of the role of the *Listeria monocytogenes* F_0F_1-ATPase operon in the acid tolerance response. *Int J Food Microbiol* **60**, 137-146.

Cotter, P. D., Gahan, C. G. M. & Hill, C. (2001). A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. *Mol Microbiol* **40**, 465.

Cotter, P. D. & Hill, C. (2003). Surviving the acid test: responses of Gram-positive bacteria to low pH. *Microbiol Mol Biol Rev* **67**, 429-453.

Csonka, L. N. (1989). Physiological and genetic responses of bacteria to osmotic stress. *Microbiol Mol Biol Rev* **53**, 121-147.

Csonka, L. N. & Hanson, A. D. (1991). Prokaryotic osmoregulation: genetics and physiology. *Annu Rev Microbiol* **45**, 569-606.

Culham, D. E., Lu, A., Jishage, M., Krogfelt, K. A., Ishihama, A. & Wood, J. M. (2001). The osmotic stress response and virulence in pyelonephritis isolates of *Escherichia coli*: contributions of RpoS, ProP, ProU and other systems. *Microbiology* **147**, 1657-1670.

Curran, T., Lieou, J. & Marquis, R. (1995). Arginine deiminase system and acid adaptation of oral streptococci. *Appl Environ Microbiol* **61**, 4494-4496.

Dalton, C. B., Austin, C. C., Sobel, J., Hayes, P. S., Bibb, W. F., Graves, L. M., Swaminathan, B., Proctor, M. E. & Griffin, P. M. (1997). An outbreak of gastroenteritis and fever due to *Listeria monocytogenes* in milk. *N Engl J Med* **336**, 100-106.

Delumeau, O., Dutta, S., Brigulla, M., Kuhnke, G., Hardwick, S. W., Völker, U., Yudkin, M. D. & Lewis, R. J. (2004). Functional and structural characterization of RsbU, a stress signaling protein phosphatase 2C. *J Biol Chem* **279**, 40927-40937.

Diaz-Torres, M. L. & Russell, R. R. B. (2001). HtrA protease and processing of extracellular proteins of *Streptococcus mutans*. *FEMS Microbiol Lett* **204**, 23-28.

Dinges, M. M., Orwin, P. M. & Schlievert, P. M. (2000). Exotoxins of *Staphylococcus aureus*. *Clin Microbiol Rev* **13**, 16-34.

Dlamini, B. C. & Buys, E. M. (2009). Survival and growth of acid adapted *Escherichia coli* strains in broth at different pH levels. *J Food Saf* **29**, 484-497.

Dojka, M. A., Harris, J. K. & Pace, N. R. (2000). Expanding the Known Diversity and Environmental Distribution of an Uncultured Phylogenetic Division of Bacteria. *Appl Environ Microbiol* **66**, 1617-1621.

Dorsa, W. J. (1997). New and Established Carcass Decontamination Procedures Commonly Used in the Beef-Processing Industry. *J Food Prot* **60**, 1146-1151.

Dowds, B. C. A. & Browne, N. (2001). Heat and salt stress in the food pathogen *Bacillus cereus*. *J Appl Microbiol* **91**, 1085-1094.

Dufour, A. & Haldenwang, W. G. (1994). Interactions between a *Bacillus subtilis* anti-sigma factor (RsbW) and its antagonist (RsbV). *J Bacteriol* **176**, 1813-1820.

Earnshaw, R. G., Appleyard, J. & Hurst, R. M. (1995). Understanding physical inactivation processes: combined preservation opportunities using heat, ultrasound and pressure. *Int J Food Microbiol* **28**, 197-219.

Eggenberger-Solorzano, L., Niebuhr, S. E., Acuff, G. R. & Dickson, J. S. (2002). Hot water and organic acid interventions to control microbiological contamination on hog carcasses during processing. *J Food Prot* **65**, 1248-1252.

FDA/CFSAN (**1998**, 05/14/2009). *Bacteriological Analytical Manual*. 8th Edition, Revision A. Retrieved 05/14/2009, from http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm063346.htm.

FDA/CFSAN (**2009**, 06/18/2009). *The bad bug book: foodborne pathogenic microorganisms and natural toxins handbook*. Retrieved 06/18/2009, from www.cfsan.fda.gov/~mow/intro.html.

Ferreira, A., Gray, M., Wiedmann, M. & Boor, K. J. (2004). Comparative genomic analysis of the sigB operon in *Listeria monocytogenes* and in other Gram-positive bacteria. *Curr Microbiol* **48**, 39-46.

Flekna, G., Stefanic, P., Wagner, M., Smulders, F. J. M., Mozina, S. S. & Hein, I. (2007). Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide (EMA) compromises EMA/real-time PCR. *Res Microbiol* **158**, 405-412.

Foster, J. W. (1999). When protons attack: microbial strategies of acid adaptation. *Curr Opin Microbiol* **2**, 170-174.

Foster, J. W. (2000). Microbial responses to acid stress. In *Bacterial Stress Response*, pp. 99-115. Edited by G. Storz & R. Hengge-Aronis. Washington, D.C.: American Society for Microbiology.

Frye, D., Zweig, R., Sturgeon, J., Tormey, M., LeCavalier, M., Lee, I., Lawani, L. & Mascola, L. (2002). An outbreak of febrile gastroenteritis associated with delicatessen meat contaminated with *Listeria monocytogenes*. *Clin Infect Dis* **35**, 943-949.

Gahan, C. G. M. & Hill, C. (1999). The relationship between acid stress responses and virulence in *Salmonella typhimurium* and *Listeria monocytogenes*. *Int J Food Microbiol* **50**, 93-100.

Gandhi, M. & Chikindas, M. L. (2007). *Listeria*: a foodborne pathogen that knows how to survive. *Int J Food Microbiol* **113**, 1-15.

Gardan, R., Duche, O., Leroy-Setrin, S., the European Listeria Genome Consortium & Labadie, J. (2003). Role of ctc from *Listeria monocytogenes* in osmotolerance. *Appl Environ Microbiol* **69**, 154-161.

Gentry, D. R., Hernandez, V. J., Nguyen, L. H., Jensen, D. B. & Cashel, M. (1993). Synthesis of the stationary-phase sigma factor σ^S is positively regulated by ppGpp. *J Bacteriol* **175**, 7982-7989.

Georgopoulos, C. & Welch, W. J. (1993). Role of the major heat shock proteins as molecular chaperones. *Annu Rev Cell Biol* **9**, 601-634.

Good, R. J. (1992). Contact angle, wetting, and adhesion: a critical review *J Adhes Sci Technol* **6**, 1269-1302.

Goodson, M. & Rowbury, R. J. (1989a). Resistance of acid-habituated *Escherichia coli* to organic acids and its medical and applied significance. *Lett Appl Microbiol* **8**, 211-214.

Goodson, M. & Rowbury, R. J. (1989b). Habituation to normally lethal acidity by prior growth of *Escherichia coli* at a sub-lethal acid pH value. *Lett Appl Microbiol* **8**, 77-79.

Graumann, P. & Marahiel, M. A. (1996). Some like it cold: response of microorganisms to cold shock. *Arch Microbiol* **166**, 293-300.

Gruber, T. M. & Gross, C. A. (2003). Multiple sigma subunits and the partitioning of bacterial transcription space. *Annu Rev Microbiol* **57**, 441-467.

Guilfoyle, D. E. & Hirshfield, I. N. (1996). The survival benefit of short-chain organic acids and the inducible arginine and lysine decarboxylase genes for *Escherichia coli*. *Lett Appl Microbiol* **22**, 393-396.

Gutierrez, C., Abee, T. & Booth, I. R. (1995). Physiology of the osmotic stress response in microorganisms. *Int J Food Microbiol* **28**, 233-244.

Gutierrez, J. A., Crowley, P. J., Cvitkovitch, D. G., Brady, L. J., Hamilton, I. R., Hillman, J. D. & Bleiweis, A. S. (1999). *Streptococcus mutans ffh*, a gene encoding a homologue of the 54 kDa subunit of the signal recognition particle, is involved in resistance to acid stress. *Microbiology* **145**, 357-366.

Hahn-Hägerdal, B., Hosono, K., Zaehrisson, A. & Bornman, C. H. (1986). Polyethylene glycol and electric field treatment of plant protoplasts: characterization of some membrane properties. *Physiol Plant* **67**, 359-364.

Hardin, M. D., Acuff, G. R., Lucia, L. M., Oman, J. S. & Savell, J. W. (1995). Comparison of Methods for Decontamination from Beef Carcass Surlaces. *J Food Prot* **58**, 368-374.

Hazen, T. C. & Stahl, D. A. (2006). Using the stress response to monitor process control: pathways to more effective bioremediation. *Curr Opin Biotechnol* **17**, 285-290.

Health Canada (**1989**, 11/16/2009). *Compendium of analytical methods*. Retrieved 11/16/2009, from www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/index-eng.php.

Health Canada (**1999**, 02/01/2000). *Guidelines for raw ground beef products found positive for Escherichia coli O157:H7*. Retrieved 02/01/2000, from www.hc-sc.gc.ca/fn-an/legislation/guide-ld/guidelines_raw_ground_beef-directives_boeuf_hache_cru-eng.php.

Health Canada (**2004**, 02/21/2001). *Policy on Listeria monocytogenes in Ready-to-Eat Foods*. Retrieved 02/21/2001, from www.hc-sc.gc.ca/fn-an/legislation/pol/policy_listeria_monocytogenes_politique_toc-eng.php.

Hengge-Aronis, R. (1996). Back to log phase: sigma S as a global regulator in the osmotic control of gene expression in *Escherichia coli*. *Mol Microbiol* **21**, 887-893

Hengge-Aronis, R. (1999). Interplay of global regulators and cell physiology in the general stress response of *Escherichia coli*. *Curr Opin Microbiol* **2**, 148-152.

Hengge-Aronis, R. (2000). The general stress response in *Escherichia coli*. In *Bacterial Stress Response*, pp. 161-178. Edited by G. Storz & R. Hengge-Aronis. Washington, D.C.: American Society for Microbiology.

Hengge-Aronis, R. (2002a). Signal transduction and regulatory mechanisms involved in control of the σ^S (RpoS) subunit of RNA polymerase. *Microbiol Mol Biol Rev* **66**, 373-395.

Hengge-Aronis, R. (2002b). Recent insights into the general stress response regulatory network in *Escherichia coli*. *J Mol Microbiol Biotechnol* **4**, 341-346.

Hersh, B., Farooq, F., Barstad, D., Blankenhorn, D. & Slonczewski, J. (1996). A glutamate-dependent acid resistance gene in *Escherichia coli*. *J Bacteriol* **178**, 3978-3981.

Hirshfield, I. N., Terzulli, S. & O'Byrne, C. (2003). Weak organic acids: a panoply of effects on bacteria. *Sci Prog* **86**, 245(225).

Huisman, G. W. & Kolter, R. (1994). Sensing starvation: a horseserine lactone-dependent signaling pathway in *Escherichia coli*. *Science* **265**, 537.

Humphrey, T. J., Williams, A., McAlpine, K., Lever, M. S., Guard-Petter, J. & Cox, J. M. (1996). Isolates of *Salmonella enterica* Enteritidis PT4 with enhanced heat and acid tolerance are more virulent in mice and more invasive in chickens. *Epidemiol Infect* **117**, 79-88.

International Commission on Microbiological Specifications for Foods (1986). *Microorganisms in foods 2 : sampling for microbiological analysis: principles and specific applications* 2nd edition edn. Toronto: University of Toronto Press.

Jay, J. M., Loessner, M. J. & Golden, D. A. (2005). *Modern Food Microbiology*, 7th edn. New York, NY: Springer Science + Business Media, Inc.

Jayaraman, G. C., Penders, J. E. & Burne, R. A. (1997). Transcriptional analysis of the *Streptococcus mutans hrcA*,*grpE* and *dnaK* genes and regulation of expression in response to heat shock and environmental acidification. *Mol Microbiol* **25**, 329-341.

Jores, J., Rumer, L. & Wieler, L. H. (2004). Impact of the locus of enterocyte effacement pathogenicity island on the evolution of pathogenic *Escherichia coli*. *Int J Med Microbiol* **294**, 103-113.

Kandror, O., DeLeon, A. & Goldberg, A. L. (2002). Trehalose synthesis is induced upon exposure of *Escherichia coli* to cold and is essential for viability at low temperatures. *Proc Natl Acad Sci U S A* **99**, 9727-9732.

Kaper, J. B., Nataro, J. P. & Mobley, H. L. T. (2004). Pathogenic *Escherichia coli*. *Nat Rev Microbiol* **2**, 123(118).

Kaper, J. B. (2005). Pathogenic *Escherichia coli*. *Int J Med Microbiol* **295**, 355-356.

Kazmierczak, M. J., Mithoe, S. C., Boor, K. J. & Wiedmann, M. (2003). *Listeria monocytogenes* σ^B regulates stress response and virulence functions. *J Bacteriol* **185**, 5722-5734.

Kazmierczak, M. J., Wiedmann, M. & Boor, K. J. (2005). Alternative sigma factors and their roles in bacterial virulence. *Microbiol Mol Biol Rev* **69**, 527-543.

Kenny, J. G., Ward, D., Josefsson, E., Jonsson, I.-M., Hinds, J., Rees, H. H., Lindsay, J. A., Tarkowski, A. & Horsburgh, M. J. (2009). The *Staphylococcus aureus* Response to Unsaturated Long Chain Free Fatty Acids: Survival Mechanisms and Virulence Implications. *PLoS One* **4**, e4344.

Khelef, N., Lecuit, M., Buchrieser, C., Cabanes, D., Dussurget, O. & Cossart, P. (2006). *Listeria monocytogenes* and the genus *Listeria*. In *The Prokaryotes*, pp. 404-476.

Klauck, E., Lingnau, M. & Hengge-Aronis, R. (2001). Role of the response regulator RssB in σ^S recognition and initiation of σ^S proteolysis in *Escherichia coli*. *Mol Microbiol* **40**, 1381-1390.

Ko, R., Smith, L. T. & Smith, G. M. (1994). Glycine betaine confers enhanced osmotolerance and cryotolerance on *Listeria monocytogenes*. *J Bacteriol* **176**, 426-431.

Koch, A. L. (1970). Turbidity measurements of bacterial cultures in some available commercial instruments. *Anal Biochem* **38**, 252-259.

Koch, A. L. (1994). Growth Measurement. In *Methods for general and molecular bacteriology* pp. 248-276. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, D.C.: American Society for Microbiology.

Koutsoumanis, K. P., Kendall, P. A. & Sofos, J. N. (2003). Effect of food processing-related stresses on acid tolerance of *Listeria monocytogenes*. *Appl Environ Microbiol* **69**, 7514-7516.

Krekeler, C., Ziehr, H. & Klein, J. (1989). Physical methods for characterization of microbial cell surfaces. *Cell Mol Life Sci* **45**, 1047-1055.

Kruger, E., Zuhlke, D., Witt, E., Ludwig, H. & Hecker, M. (2001). Clp-mediated proteolysis in Gram-positive bacteria is autoregulated by the stability of a repressor. *EMBO J* **20**, 852-863.

Kuczynska-Wisnik, D., Laskowska, E. & Taylor, A. (2001). Transcription of the ibpB heat-shock gene is under control of σ^{32}- and σ^{54}-promoters, a third regulon of heat-shock response. *Biochem Biophys Res Commun* **284**, 57-64.

Kwon, Y. M. & Ricke, S. C. (1998). Induction of acid resistance of *Salmonella typhimurium* by exposure to short-chain fatty acids. *Appl Environ Microbiol* **64**, 3458-3463.

Lange, R. & Hengge-Aronis, R. (1994). The cellular concentration of the σ^{S} subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation, and protein stability. *Genes Dev* **8**, 1600-1612.

Lange, R., Fischer, D. & Hengge-Aronis, R. (1995). Identification of transcriptional start sites and the role of ppGpp in the expression of *rpoS*, the structural gene for the σ^{S} subunit of RNA polymerase in *Escherichia coli*. *J Bacteriol* **177**, 4676-4680.

Le Loir, Y., Baron, F. & Gautier, M. (2003). *Staphylococcus aureus* and food poisoning. *Genet Mol Res* **2**, 63-76.

Lease, R. A. & Belfort, M. (2000). A trans-acting RNA as a control switch in *Escherichia coli*: DsrA modulates function by forming alternative structures. *Proc Natl Acad Sci U S A* **97**, 9919-9924.

Lemos, J. A. C., Chen, Y.-Y. M. & Burne, R. A. (2001). Genetic and physiologic analysis of the *groE* operon and role of the HrcA repressor in stress gene regulation and acid tolerance in *Streptococcus mutans*. *J Bacteriol* **183**, 6074-6084.

Leyer, G. J. & Johnson, E. A. (1993). Acid adaptation induces cross-protection against environmental stresses in *Salmonella typhimurium*. *Appl Environ Microbiol* **59**, 1842-1847.

Li, Y.-H., Hanna, M. N., Svensater, G., Ellen, R. P. & Cvitkovitch, D. G. (2001). Cell density modulates acid adaptation in *Streptococcus mutans*: implications for survival in biofilms. *J Bacteriol* **183**, 6875-6884.

Lianou, A. & Sofos, J. N. (2007). A review of the incidence and transmission of *Listeria monocytogenes* in ready-to-eat products in retail and food service environments. *J Food Prot* **70**, 2172-2198.

Lin, J., Lee, I., Frey, J., Slonczewski, J. & Foster, J. (1995). Comparative analysis of extreme acid survival in *Salmonella typhimurium*, *Shigella flexneri*, and *Escherichia coli*. *J Bacteriol* **177**, 4097-4104.

Lin, J., Smith, M., Chapin, K., Baik, H., Bennett, G. & Foster, J. (1996). Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*. *Appl Environ Microbiol* **62**, 3094-3100.

Lindquist, S. (1986). The heat-shock response. *Annu Rev Biochem* **55**, 1151-1191.

Lou, Y. & Yousef, A. (1997). Adaptation to sublethal environmental stresses protects *Listeria monocytogenes* against lethal preservation factors. *Appl Environ Microbiol* **63**, 1252-1255.

Madigan, M. T., Martinko, J. M. & Parker., J. (2003). *Brock biology of microorganisms* 10th edn. Upper Saddle River, NJ: Prentice Hall/Pearson Education, Inc.

Majdalani, N., Cunning, C., Sledjeski, D., Elliott, T. & Gottesman, S. (1998). DsrA RNA regulates translation of RpoS message by an anti-antisense mechanism, independent of its action as an antisilencer of transcription. *Proc Natl Acad Sci U S A* **95**, 12462-12467.

Majdalani, N., Chen, S., Murrow, J., St John, K. & Gottesman, S. (2001). Regulation of RpoS by a novel small RNA: the characterization of RprA. *Mol Microbiol* **39**, 1382-1394.

McGowan, C. C., Necheva, A., Thompson, S. A., Cover, T. L. & Blaser, M. J. (1998). Acid-induced expression of an LPS-associated gene in *Helicobacter pylori*. *Mol Microbiol* **30**, 19-31.

McLauchlin, J. (1997). The pathogenicity of *Listeria monocytogenes*: a public health perspective. *Rev Med Microbiol* **8**, 1-14.

Mead, P. S. & Slutsker, L. (1999). Food-related illness and death in the United States. *Emerg Infect Dis* **5**, 607-625.

Molecular Probes (**2004**, 07/15/2004). *LIVE/DEAD® BacLight™ Bacterial Viability Kits*. Retrieved 07/15/2004, from www.probes.invitrogen.com/media/pis/mp07007.pdf.

Montville, T. J. & Matthews, K. R. (2008). *Food microbiology : an introduction* 2nd edn. Washington, DC ASM Press.

Morton, R. D. (2001). Aerobic Plate Count. In *Compendium of methods for the microbiological examination of foods*. Edited by F. P. Downes & K. Itō. Washington, DC American Public Health Association.

Muffler, A, Traulsen, D., Lange, R. & Hengge-Aronis, R. (1996a). Posttranscriptional osmotic regulation of the σ^S subunit of RNA polymerase in *Escherichia coli*. *J Bacteriol* **178**, 1607-1613.

Muffler, A., Fischer, D. & Hengge-Aronis, R. (1996b). The RNA-binding protein HF-I, known as a host factor for phage Qβ RNA replication, is essential for *rpoS* translation in *Escherichia coli*. *Genes Dev* **10**, 1143-1151.

Muffler, A., Barth, M., Marschall, C. & Hengge-Aronis, R. (1997a). Heat shock regulation of σ^S turnover: a role for DnaK and relationship between stress responses mediated by σ^S and σ^{32} in *Escherichia coli*. *J Bacteriol* **179**, 445-452.

Muffler, A., Traulsen, D., Fischer, D., Lange, R. & Hengge-Aronis, R. (1997b). The RNA-binding protein HF-I plays a global regulatory role which is largely, but not exclusively, due to its role in expression of the σ^S subunit of RNA polymerase in *Escherichia coli*. *J Bacteriol* **179**, 297-300.

Mukhopadhyay, S., Audia, J. P., Roy, R. N. & Schellhorn, H. E. (2000). Transcriptional induction of the conserved alternative sigma factor RpoS in *Escherichia coli* is dependent on BarA, a probable two-component regulator. *Mol Microbiol* **37**, 371-381.

Nataro, J. P. & Kaper, J. B. (1998). Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev* **11**, 142-201.

Neufeld, R. J., Zajic, J. E. & Gerson, D. F. (1980). Cell Surface Measurements in Hydrocarbon and Carbohydrate Fermentations. *Appl Environ Microbiol* **39**, 511-517.

Nexmann Jacobsen, C., Rasmussen, J. & Jakobsen, M. (1997). Viability staining and flow cytometric detection of *Listeria monocytogenes*. *J Microbiol Methods* **28**, 35-43.

Nikaido, H. & Vaara, M. (1985). Molecular basis of bacterial outer membrane permeability. *Microbiol Mol Biol Rev* **49**, 1-32.

Nikaido, H. (2003). Molecular Basis of Bacterial Outer Membrane Permeability Revisited. *Microbiol Mol Biol Rev* **67**, 593-656.

O'Driscoll, B., Gahan, C. & Hill, C. (1996). Adaptive acid tolerance response in *Listeria monocytogenes*: isolation of an acid-tolerant mutant which demonstrates increased virulence. *Appl Environ Microbiol* **62**, 1693-1698.

Ooi, S. T. & Lorber, B. (2005). Gastroenteritis due to *Listeria monocytogenes*. *Clin Infect Dis* **40**, 1327-1332.

Palma, M. & Cheung, A. L. (2001). σ^B Activity in *Staphylococcus aureus* is controlled by RsbU and an additional factor(s) during bacterial growth. *Infect Immun* **69**, 7858-7865.

Park, Y.-K., Bearson, B., Bang, S. H., Bang, I. S. & Foster, J. W. (1996). Internal pH crisis, lysine decarboxylase and the acid tolerance response of *Salmonella typhimurium*. *Mol Microbiol* **20**, 605-611.

Patnaik, P. (2003). *Handbook of Inorganic Chemicals* New York: McGraw-Hill.

Paul, B. & Hirshfield, I. (2003). The effect of acid treatment on survival and protein expression of a laboratory K-12 strain *Escherichia coli*. *Res Microbiol* **154**, 115-121.

Phadtare, S., Alsina, J. & Inouye, M. (1999). Cold-shock response and cold-shock proteins. *Curr Opin Microbiol* **2**, 175-180.

Phadtare, S. (2004). Recent developments in bacterial cold-shock response. *Curr Issues Mol Biol* **6**, 125-136.

Phan-Thanh, L., Mahouin, F. & Alig, S. (2000). Acid responses of *Listeria monocytogenes*. *Int J Food Microbiol* **55**, 121-126.

Pigott, D. C. (2008). Foodborne illness. *Emerg Med Clin North Am* **26**, 475-497.

Portnoy, D. A., Auerbuch, V. & Glomski, I. J. (2002). The cell biology of *Listeria monocytogenes* infection: the intersection of bacterial pathogenesis and cell-mediated immunity. *J Cell Biol* **158**, 409-414.

Posfay-Barbe, K. M. & Wald, E. R. (2009). Listeriosis. *Semin Fetal Neonatal Med* **14**, 228-233.

Price, C. W. (2000). Protective function and regulation of the general stress response in *Bacillus subtilis* and related Gram-positive bacteria. In *Bacterial*

Stress Response, pp. 179-197. Edited by G. Storz & R. Hengge-Aronis. Washington, D.C.: American Society for Microbiology.

Public Health Agency of Canada (**2008**, 08/23/08). *Link between listeriosis outbreak strain and maple leaf foods products confirmed*. Retrieved 08/23/08, from www.phac-aspc.gc.ca/media/nr-rp/2008/2008_13-eng.php.

Public Health Agency of Canada (**2009**, 04/17/09). *Listeria monocytogenes outbreak (Final Update)*. Retrieved 04/17/09, from www.phac-aspc.gc.ca/alert-alerte/listeria/listeria_2009-eng.php.

Rangel, J. M., Sparling, P. H., Crowe, C., Griffin, P. M. & Swerdlow, D. L. (2005). Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982-2002. *Emerg Infect Dis* **11**, 603-609.

Rice, K. C., Nelson, J. B., Patton, T. G., Yang, S.-J. & Bayles, K. W. (2005). Acetic Acid Induces Expression of the *Staphylococcus aureus* cidABC and lrgAB Murein Hydrolase Regulator Operons. *J Bacteriol* **187**, 813-821.

Ricke, S. (2003). Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. *Poult Sci* **82**, 632-639.

Riesenberg-Wilmes, M., Bearson, B., Foster, J. & Curtis, R., 3d (1996). Role of the acid tolerance response in virulence of *Salmonella typhimurium*. *Infect Immun* **64**, 1085-1092.

Rodriguez-Romo, L. & Yousef, A. (2005). Cross-protective effects of bacterial stress. In *Understanding Pathogen Behaviour: Virulence, Stress Response and Resistance*, pp. 128-151. Edited by M. Griffiths. Boca Raton: CRC Press LLC.

Rollins, D. M. & Colwell, R. R. (1986). Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. *Appl Environ Microbiol* **52**, 531-538.

Russell, A. D. (2003). Bacterial outer membrane and cell wall penetration and cell destruction by polluting chemical agents and physical conditions. *Sci Prog* **86**, 283(229).

Russell, N. J., Evans, R. I., ter Steeg, P. F., Hellemons, J., Verheul, A. & Abee, T. (1995). Membranes as a target for stress adaptation. *Int J Food Microbiol* **28**, 255-261.

Russell, N. J. (2002). Bacterial membranes: the effects of chill storage and food processing. An overview. *Int J Food Microbiol* **79**, 27-34.

Shabala, L. & Ross, T. (2008). Cyclopropane fatty acids improve *Escherichia coli* survival in acidified minimal media by reducing membrane permeability to H^+ and enhanced ability to extrude H^+. *Res Microbiol* **159**, 458-461.

Shi, X. & Bennett, G. N. (1994). Effects of rpoA and cysB mutations on acid induction of biodegradative arginine decarboxylase in *Escherichia coli*. *J Bacteriol* **176**, 7017-7023.

Shiba, T., Tsutsumi, K., Yano, H. & other authors (1997). Inorganic polyphosphate and the induction of *rpoS* expression. *Proc Natl Acad Sci U S A* **94**, 11210-11215.

Smulders, F. J. M. & Greer, G. G. (1998). Integrating microbial decontamination with organic acids in HACCP programmes for muscle foods: prospects and controversies. *Int J Food Microbiol* **44**, 149-169.

Stackebrandt, E. & Embley, T. M. (2000). Diversity of uncultured microorganisms in the environment. In *Nonculturable Microorganisms in the Environment*, pp. 57-76. Edited by R. R. Colwell & D. J. Grimes. Washing D.C.: ASM Press.

Storz, G. & Hengge-Aronis, R. (2000). Preface. In *Bacterial Stress Response*, pp. xiii. Edited by G. Storz & R. Hengge-Aronis. Washington, D.C.: American Society for Microbiology.

Subczynski, W. K., Wisniewska, A., Yin, J.-J., Hyde, J. S. & Kusumi, A. (1994). Hydrophobic Barriers of Lipid Bilayer Membranes Formed by Reduction of Water Penetration by Alkyl Chain Unsaturation and Cholesterol. *Biochemistry* **33**, 7670-7681.

Swanson, K. M. J., Petran, R. L. & Hanlin, J. H. (2001). Culture Methods for Enumeration of Microorganisms. In *Compendium of methods for the microbiological examination of foods*. Edited by F. P. Downes & K. Itō. Washington, DC American Public Health Association.

Swarts, A. J., Hastings, J. W., Roberts, R. F. & von Holy, A. (1998). Flow Cytometry Demonstrates Bacteriocin-Induced Injury to *Listeria monocytogenes*. *Curr Microbiol* **36**, 266-270.

Taylor, D. L. & Salmon, E. D. (1989). Basic Fluorescence Microscopy In *Fluorescence microscopy of living cells in culture*, pp. 207-236. Edited by Y.-L. Wang & D. L. Taylor. San Diego, Calif: Academic Press.

The Nobel Foundation (**2008**). *The Fluorescence Microscope*. from www.nobelprize.org/educational_games/physics/microscopes/fluorescence/.

Thomas, D., Dauwalder, O., Brun, V., Badiou, C., Ferry, T., Etienne, J., Vandenesch, F. & Lina, G. (2009). *Staphylococcus aureus* superantigens elicit redundant and extensive human Vβ patterns. *Infect Immun* **77**, 2043-2050.

Thomas, D. Y., Jarraud, S., Lemercier, B., Cozon, G., Echasserieau, K., Etienne, J., Gougeon, M.-L., Lina, G. & Vandenesch, F. (2006). Staphylococcal enterotoxin-like toxins U2 and V, two new staphylococcal superantigens arising from recombination within the enterotoxin gene cluster. *Infect Immun* **74**, 4724-4734.

Trent, M. S. (2004). Biosynthesis, transport, and modification of lipid A. *Biochem Cell Biol* **82**, 71-86.

USDA/FSIS (**1998**, 09/15/2009). *Microbiology Laboratory Guidebook*. 3rd. Retrieved 09/15/2009, from www.fsis.usda.gov/Science/Microbiological_Lab_Guidebook/index.asp.

van der Mei, H. C., Bos, R. & Busscher, H. J. (1998). A reference guide to microbial cell surface hydrophobicity based on contact angles. *Colloids Surf, B* **11**, 213-221.

van Schaik, W. & Abee, T. (2005). The role of σ^B in the stress response of Gram-positive bacteria - targets for food preservation and safety. *Curr Opin Biotechnol* **16**, 218-224.

Vijay, K., Brody, M. S., Fredlund, E. & Price, C. W. (2000). A PP2C phosphatase containing a PAS domain is required to convey signals of energy stress to the σ^B transcription factor of *Bacillus subtilis*. *Mol Microbiol* **35**, 180-188.

Volker, U., Maul, B. & Hecker, M. (1999). Expression of the σ^B-dependent general stress regulon confers multiple stress resistance in *Bacillus subtilis*. *J Bacteriol* **181**, 3942-3948.

Wang & Doyle (1998). Heat shock response enhances acid tolerance of *Escherichia coli* O157 : H7. *Lett Appl Microbiol* **26**, 31-34.

Wassarman, K. M. (2002). Small RNAs in bacteria: diverse regulators of gene expression in response to environmental changes. *Cell* **109**, 141-144.

Wilkins, J. C., Homer, K. A. & Beighton, D. (2002). Analysis of *Streptococcus mutans* proteins modulated by culture under acidic conditions. *Appl Environ Microbiol* **68**, 2382-2390.

Wood, W. A. & Paterek, J. R. (1994). Physical Analysis. In *Methods for general and molecular bacteriology* pp. 467-511. Edited by P. Gerhardt, R. G. E. Murray,

W. A. Wood & N. R. Krieg. Washington, D.C.: American Society for Microbiology.

Xu, H. S., Roberts, N., Singleton, F. L., Attwell, R. W., Grimes, D. J. & Colwell, R. R. (1982). Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. *Microb Ecol* **8**, 313-323.

Xu, J. & Johnson, R. C. (1997). Activation of RpoS-dependent *proP* P2 transcription by the Fis protein in vitro. *J Mol Biol* **270**, 346-359.

Yamashino, T., Ueguchi, C. & Mizuno, T. (1995). Quantitative control of the stationary phase-specific sigma factor, σ^S, in *Escherichia coli*: involvement of the nucleoid protein H-NS. *EMBO J* **14**, 594-602.

Yamashita, Y., Takehara, T. & Kuramitsu, H. K. (1993). Molecular characterization of a *Streptococcus mutans* mutant altered in environmental stress responses. *J Bacteriol* **175**, 6220-6228.

Yang, X., Kang, C. M., Brody, M. S. & Price, C. W. (1996). Opposing pairs of serine protein kinases and phosphatases transmit signals of environmental stress to activate a bacterial transcription factor. *Genes Dev* **10**, 2265-2275.

Yousef, A. E. & Courtney, P. D. (2003). Basics of stress adaptation and implications in new-generation foods. In *Microbial Stress Adaptation and Food Safety*, pp. 1-30. Edited by A. E. Yousef & V. K. Juneja. Boca Raton: CRC Press LLC.

Yura, T. & Nakahigashi, K. (1999). Regulation of the heat-shock response. *Curr Opin Microbiol* **2**, 153-158.

Zhang, A., Altuvia, S., Tiwari, A., Argaman, L., Hengge-Aronis, R. & Storz, G. (1998). The OxyS regulatory RNA represses *rpoS* translation and binds the Hfq (HF-I) protein. *EMBO J* **17**, 6061-6068.

Zhang, X.-S., Garcia-Contreras, R. & Wood, T. K. (2007). YcfR (BhsA) Influences *Escherichia coli* Biofilm Formation through Stress Response and Surface Hydrophobicity. *J Bacteriol* **189**, 3051-3062.

Zhou, Y., Gottesman, S., Hoskins, J. R., Maurizi, M. R. & Wickner, S. (2001). The RssB response regulator directly targets σ^S for degradation by ClpXP. *Genes Dev* **15**, 627-637.

Appendix A Additional Fluorescence Microscopy Images

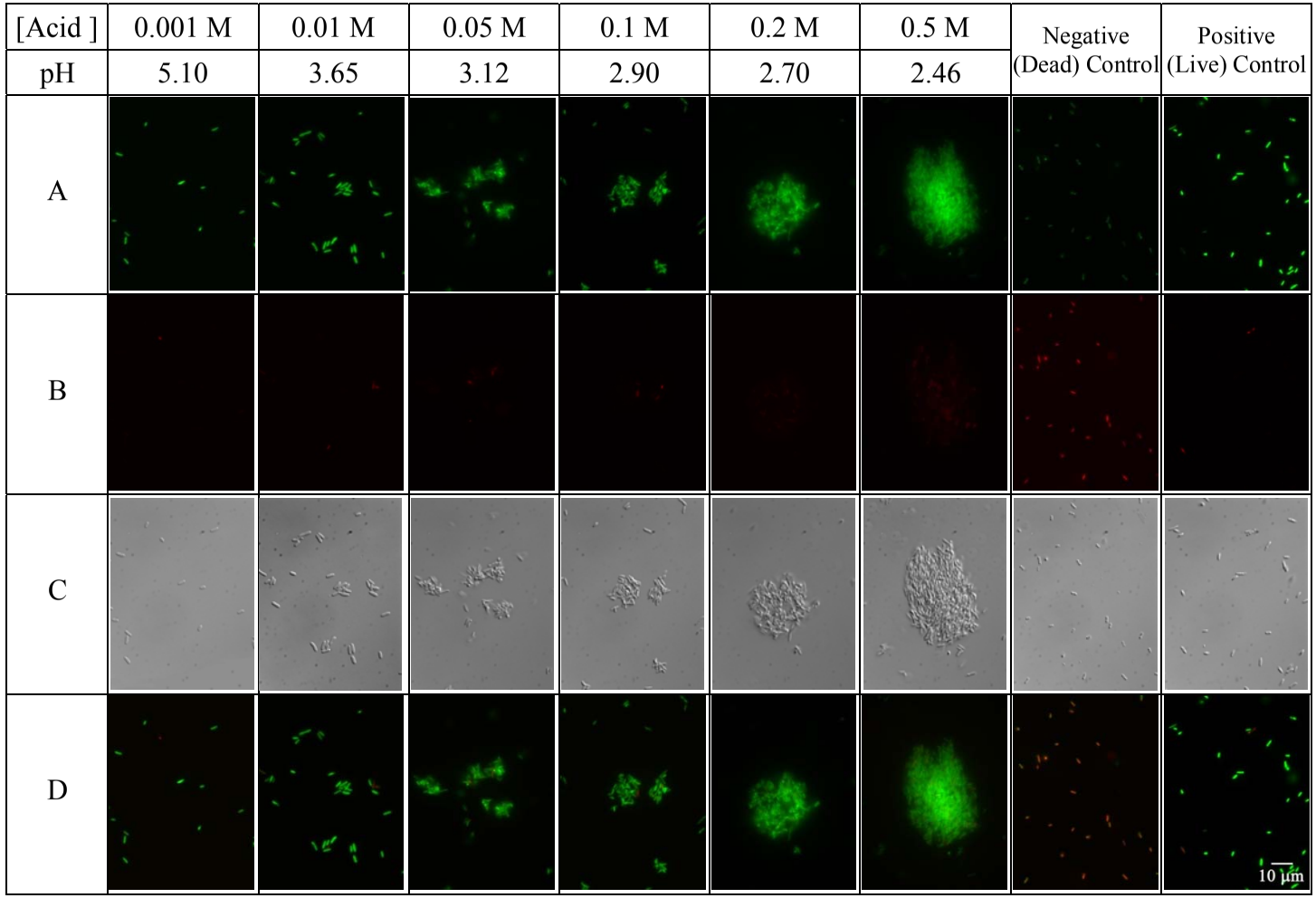


Figure A.1 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of acetic acid for 1 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

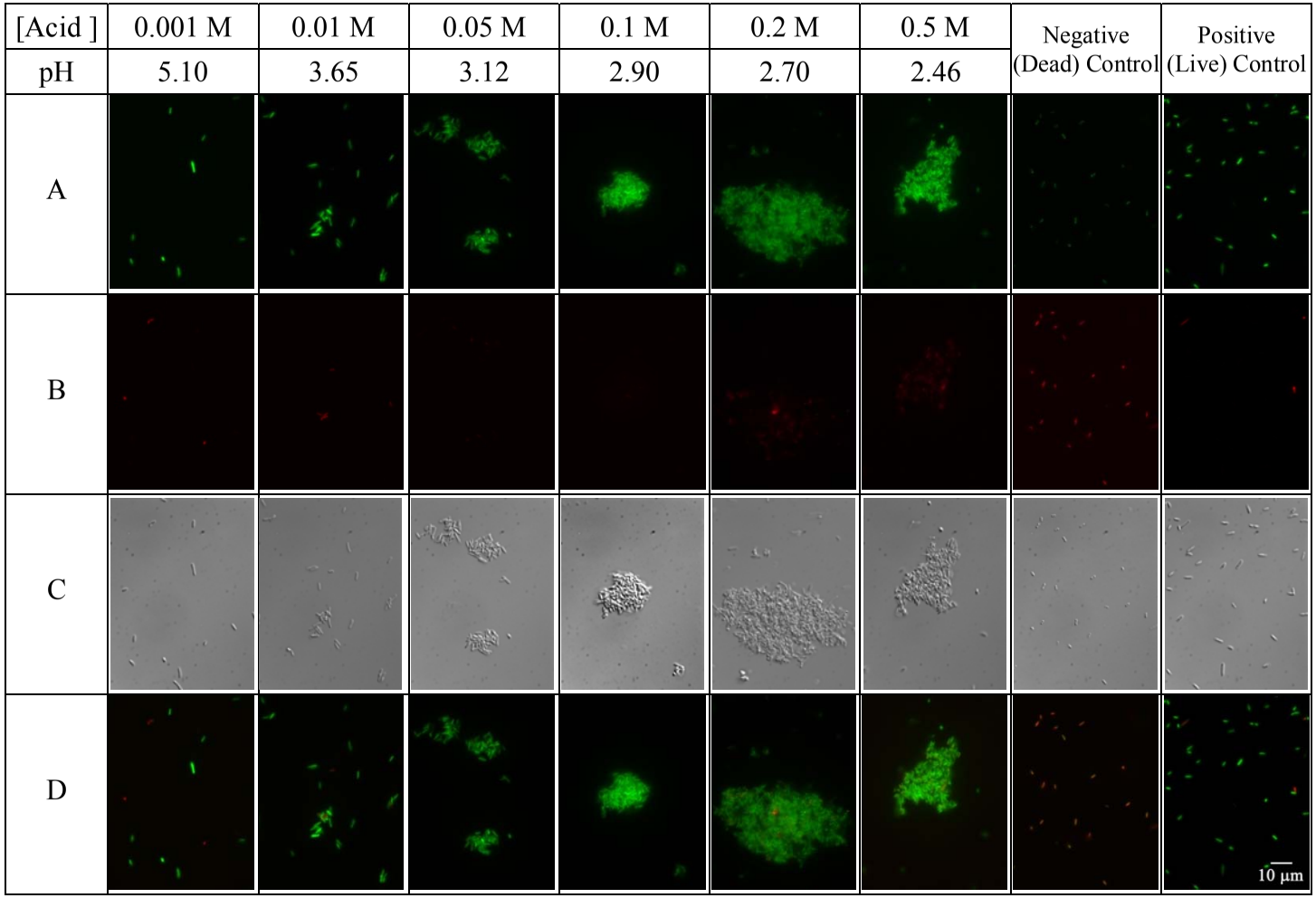


Figure A.2 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of acetic acid for 5 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

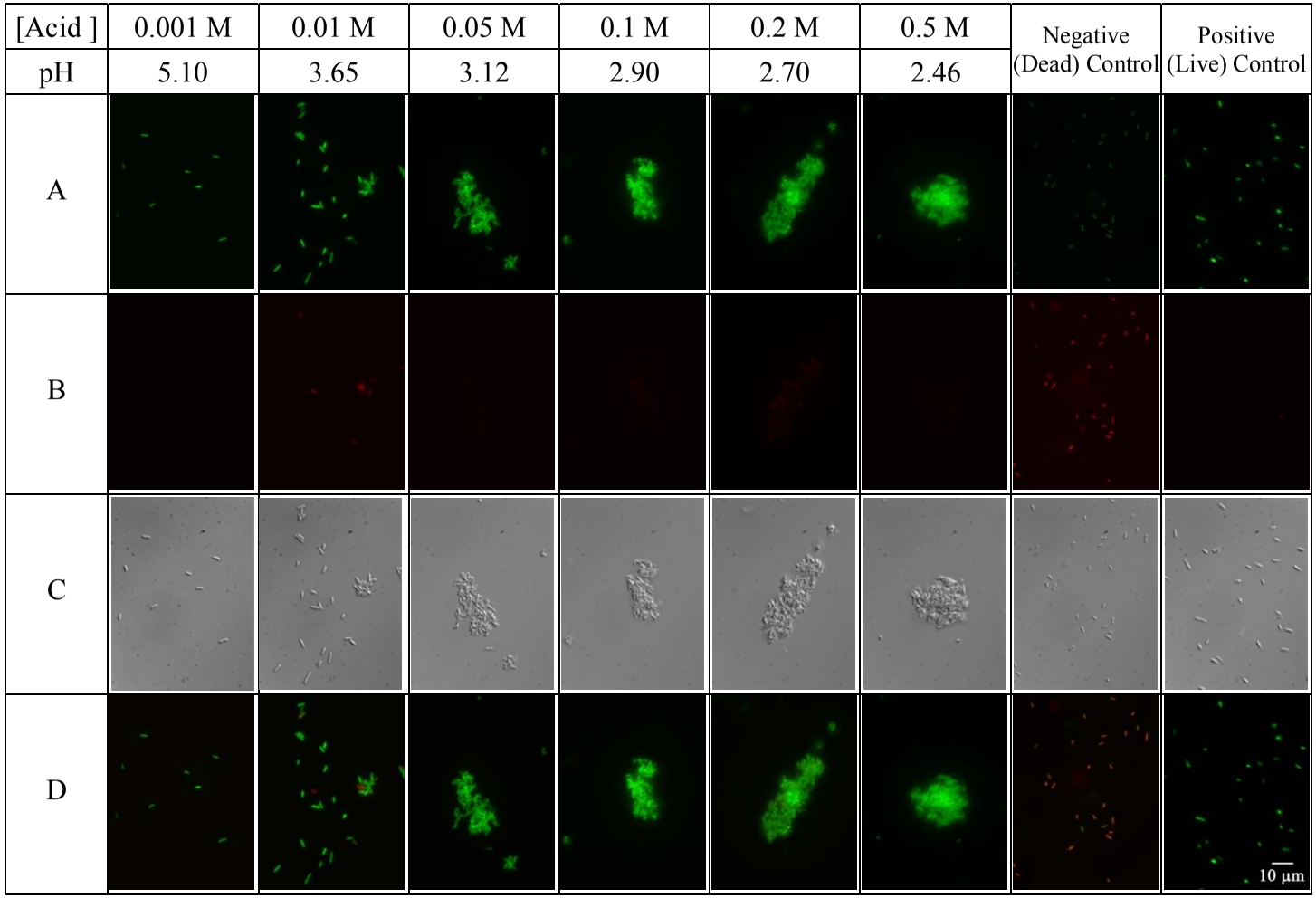


Figure A.3 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of acetic acid for 24 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

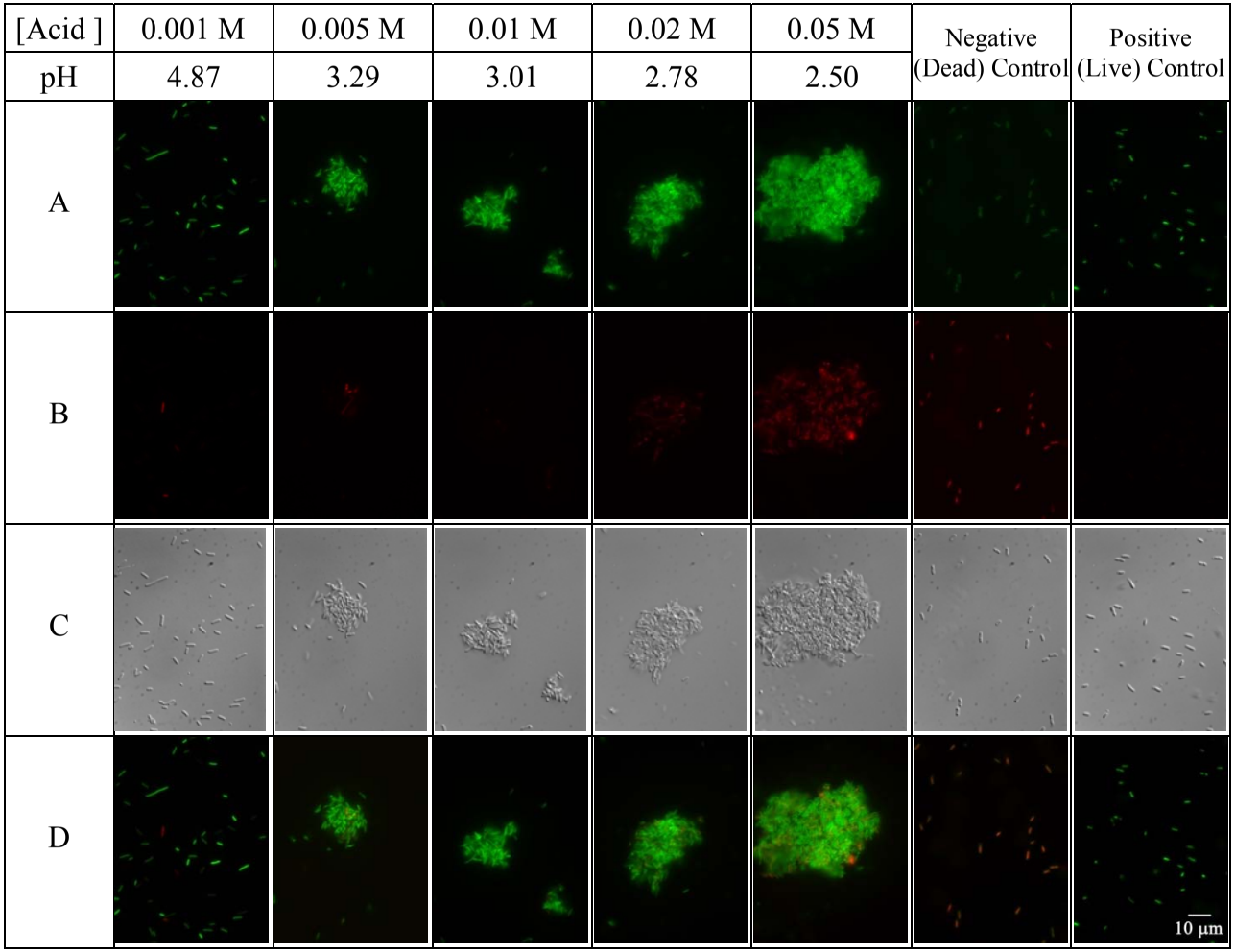


Figure A.4 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of lactic acid for 1 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

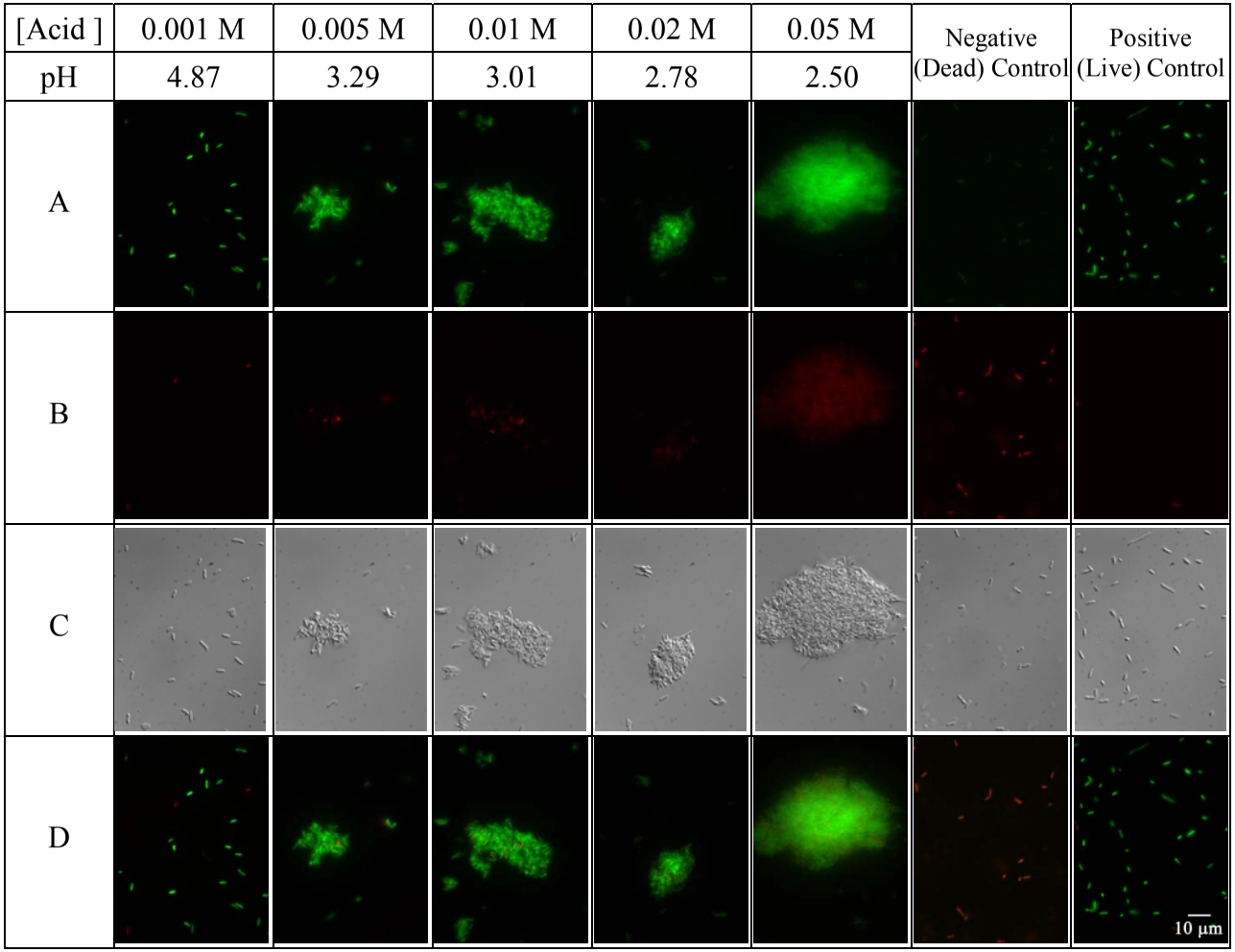


Figure A.5 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of lactic acid for 5 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

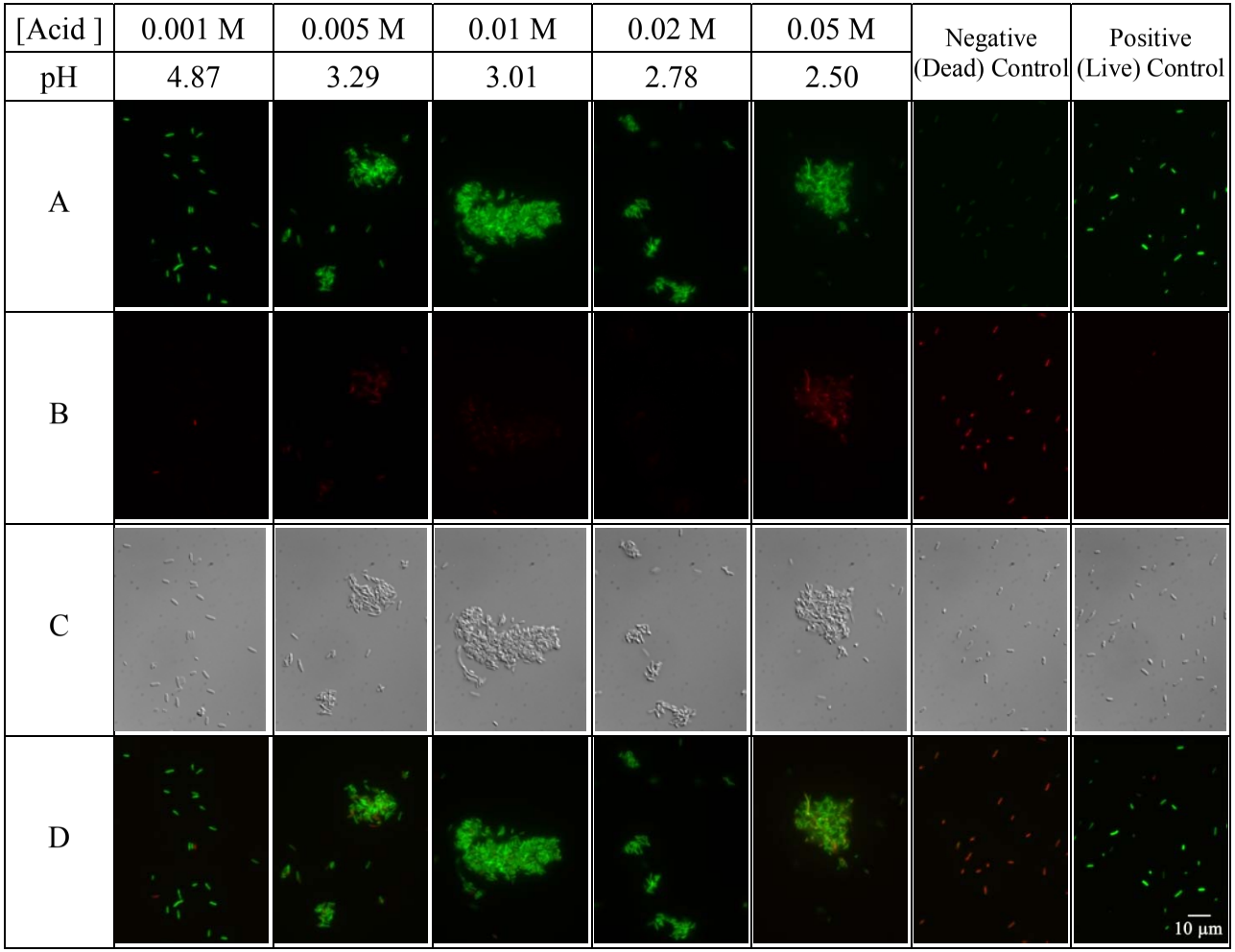


Figure A.6 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of lactic acid for 24 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

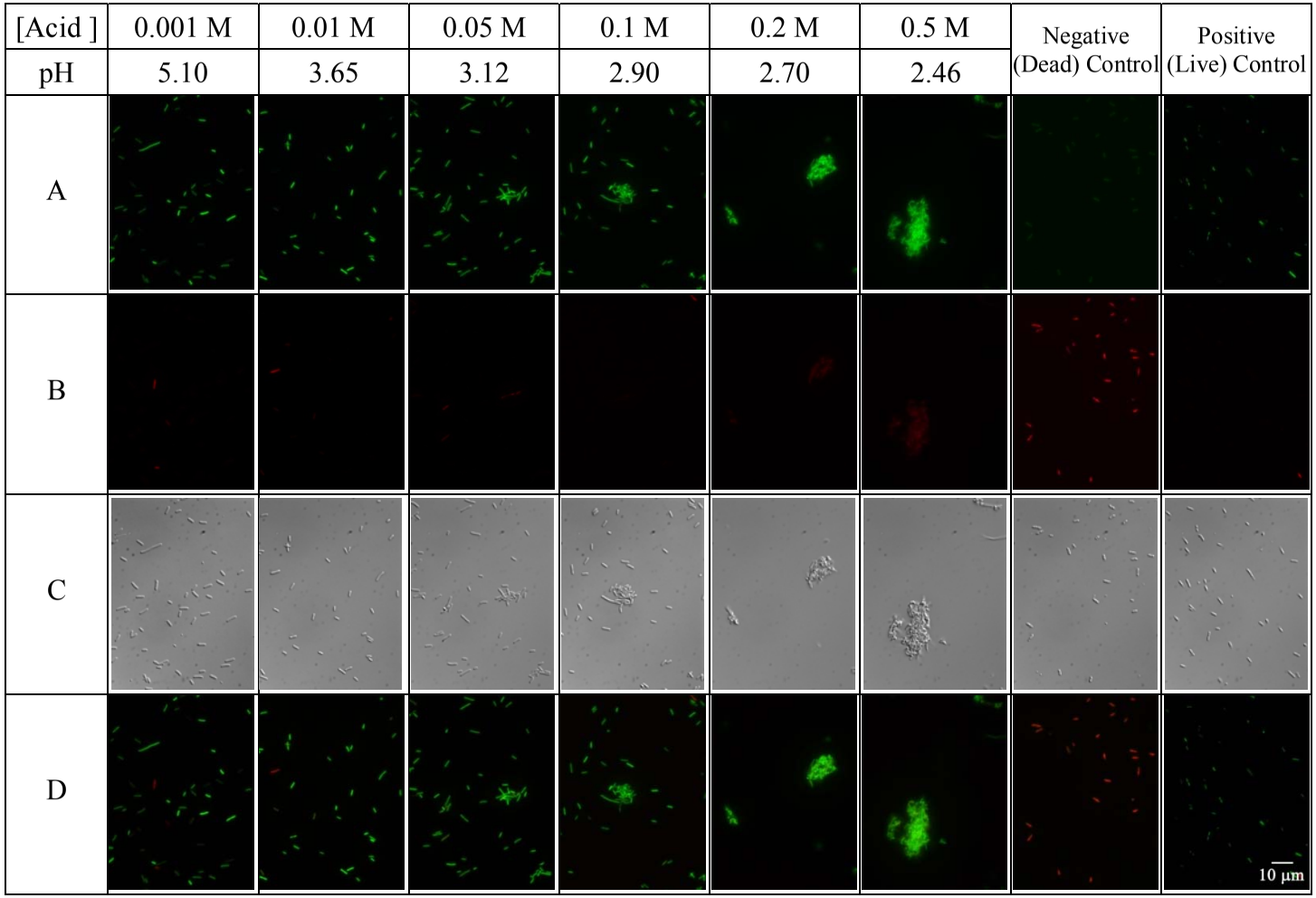


Figure A.7 Fluorescence microscopy images of mid-log phase *E. coli* DH5α after exposure to different concentrations of acetic acid for 1 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

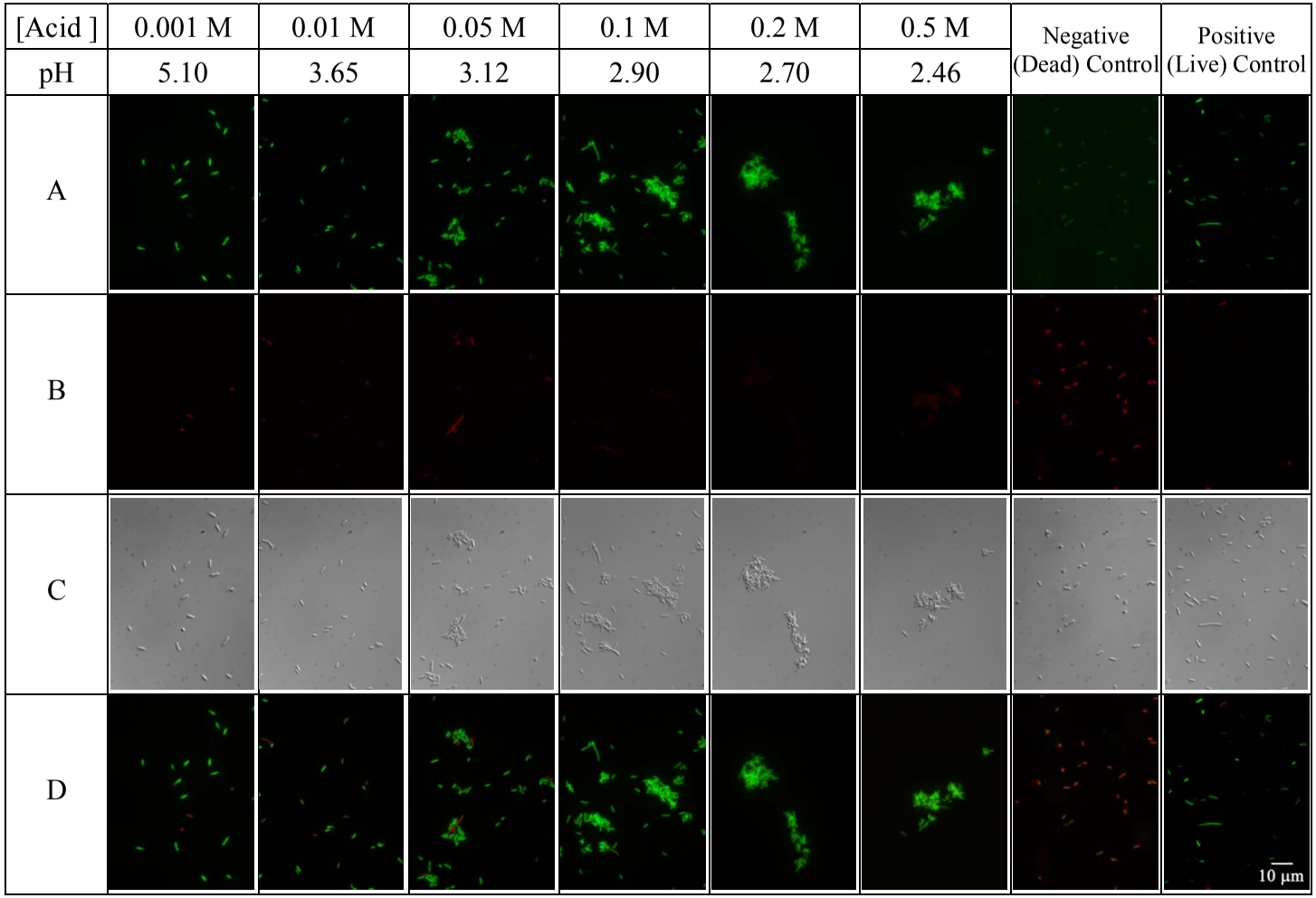


Figure A.8 Fluorescence microscopy images of mid-log phase *E. coli* DH5α after exposure to different concentrations of acetic acid for 5 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

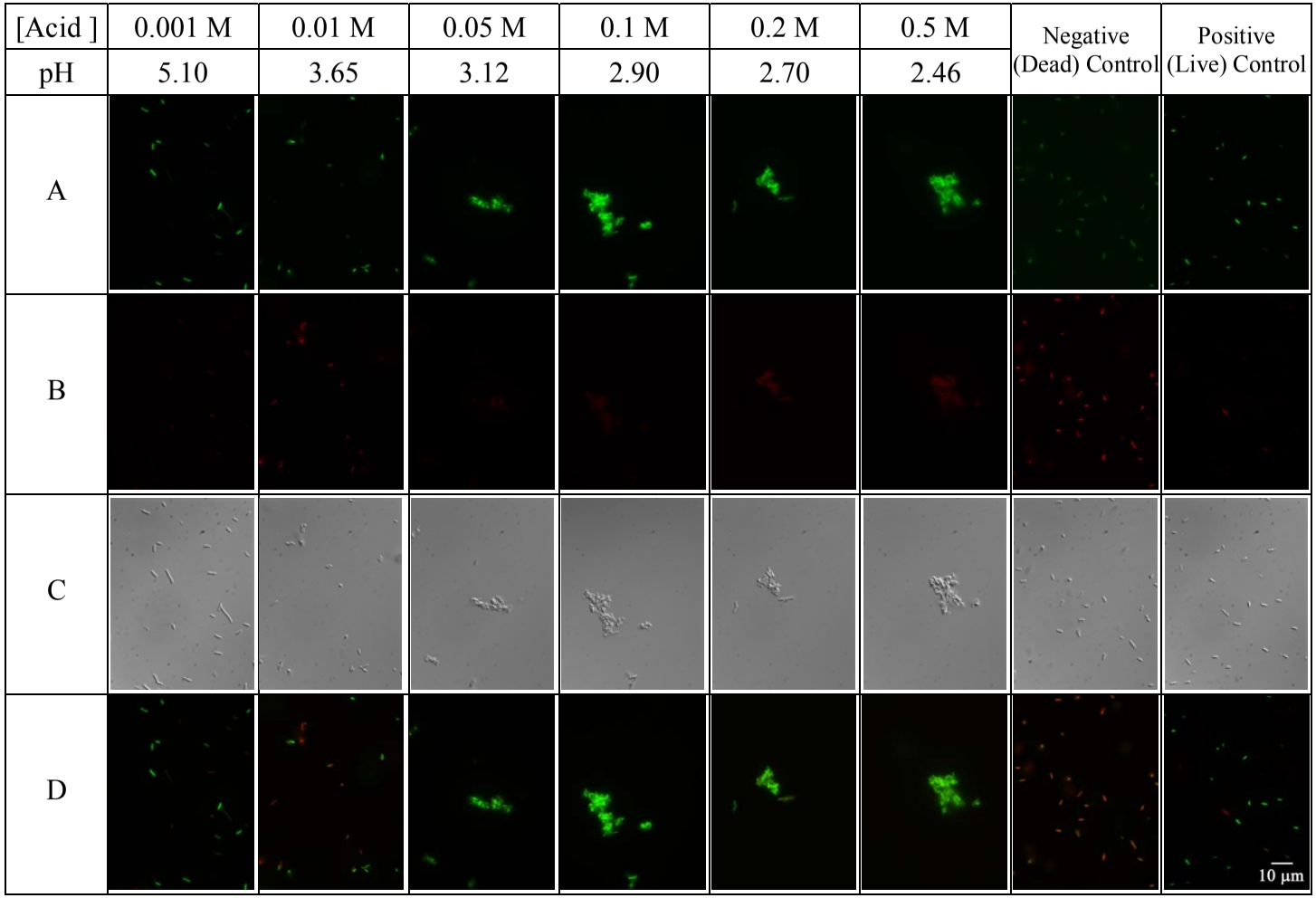


Figure A.9 Fluorescence microscopy images of mid-log phase *E. coli* DH5α after exposure to different concentrations of acetic acid for 24 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

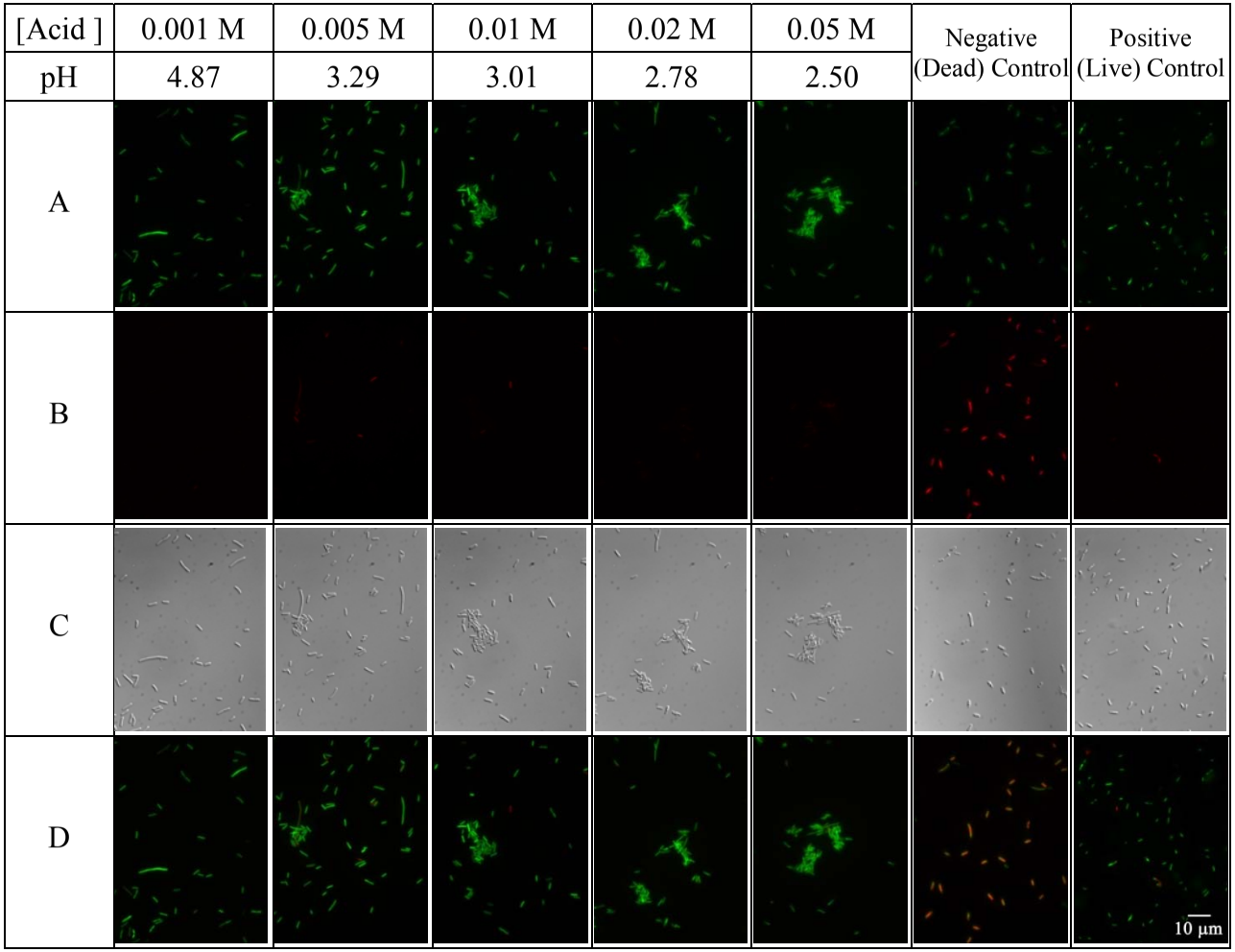


Figure A.10 Fluorescence microscopy images of mid-log phase *E. coli* DH5α after exposure to different concentrations of lactic acid for 1 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

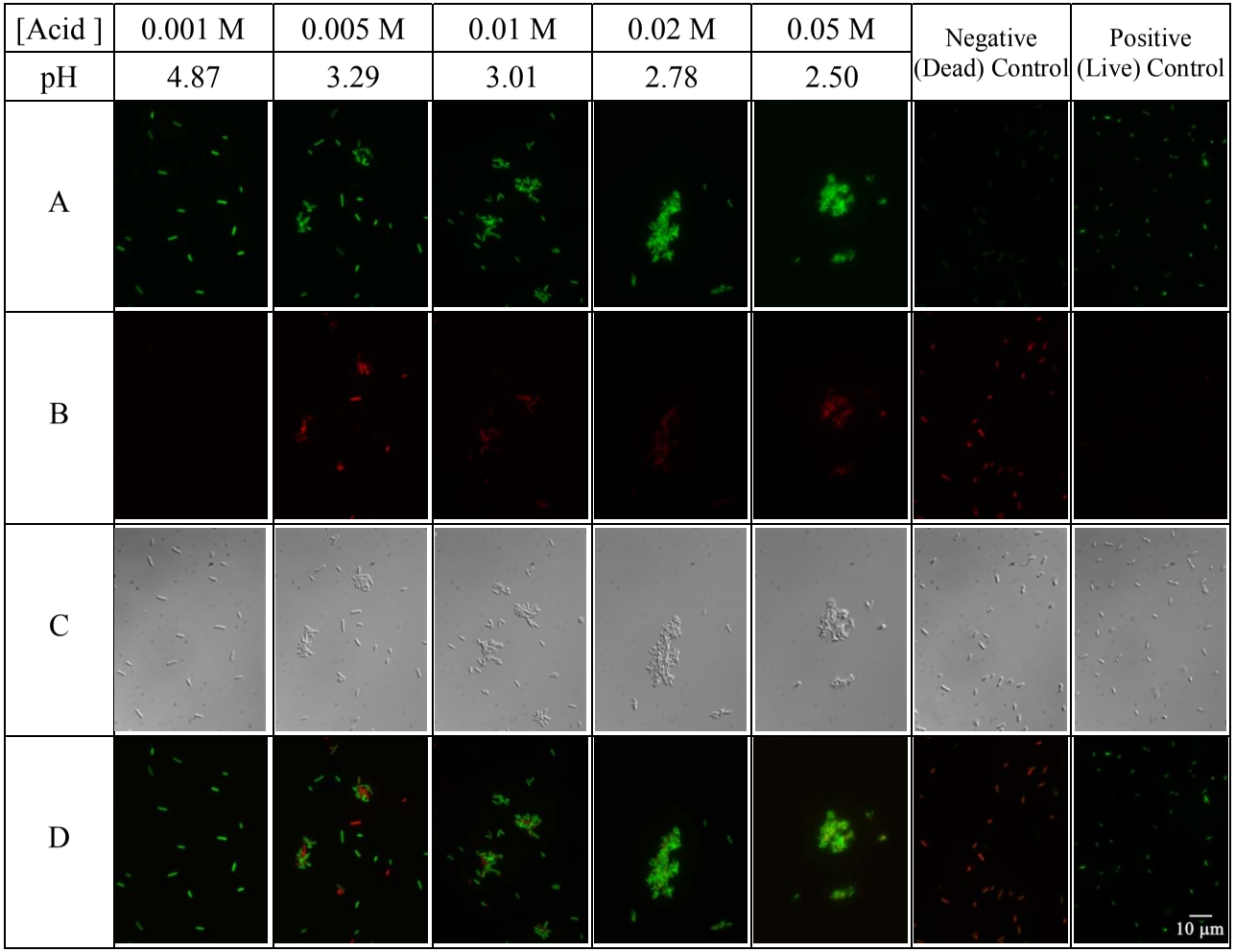


Figure A.11 Fluorescence microscopy images of mid-log phase *E. coli* DH5α after exposure to different concentrations of lactic acid for 5 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

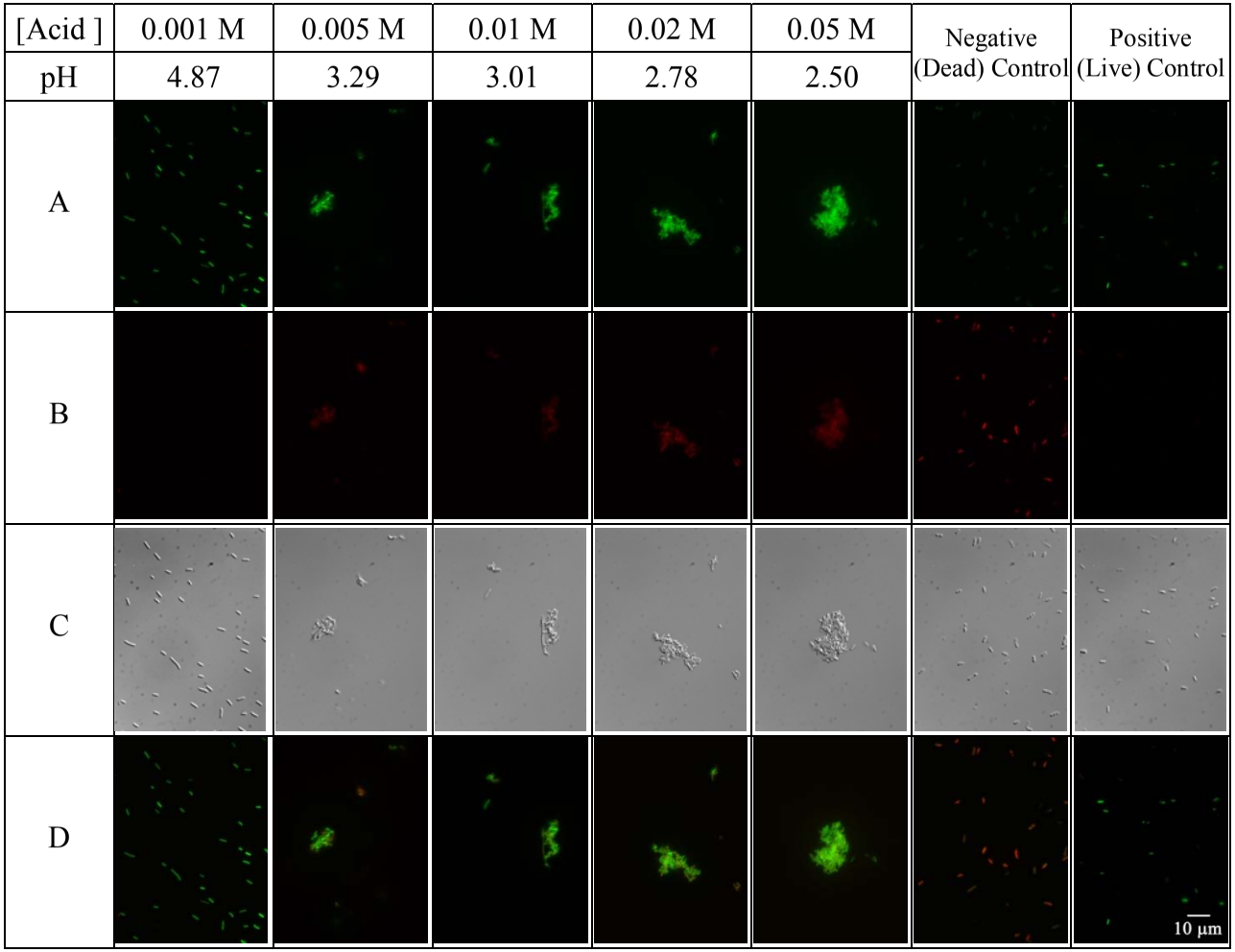


Figure A.12 Fluorescence microscopy images of mid-log phase *E. coli* DH5α after exposure to different concentrations of lactic acid for 24 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

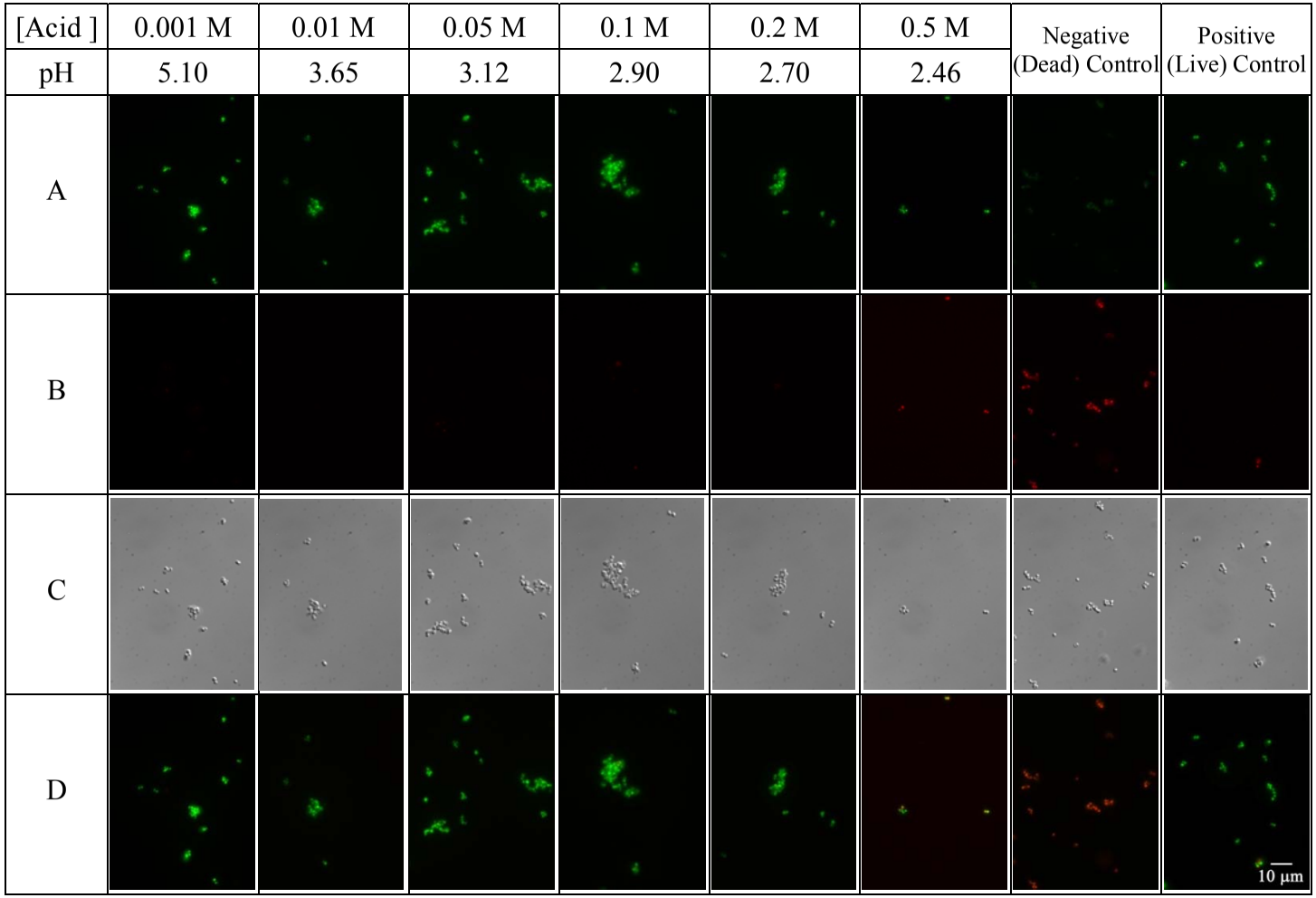


Figure A.13 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of acetic acid for 1 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

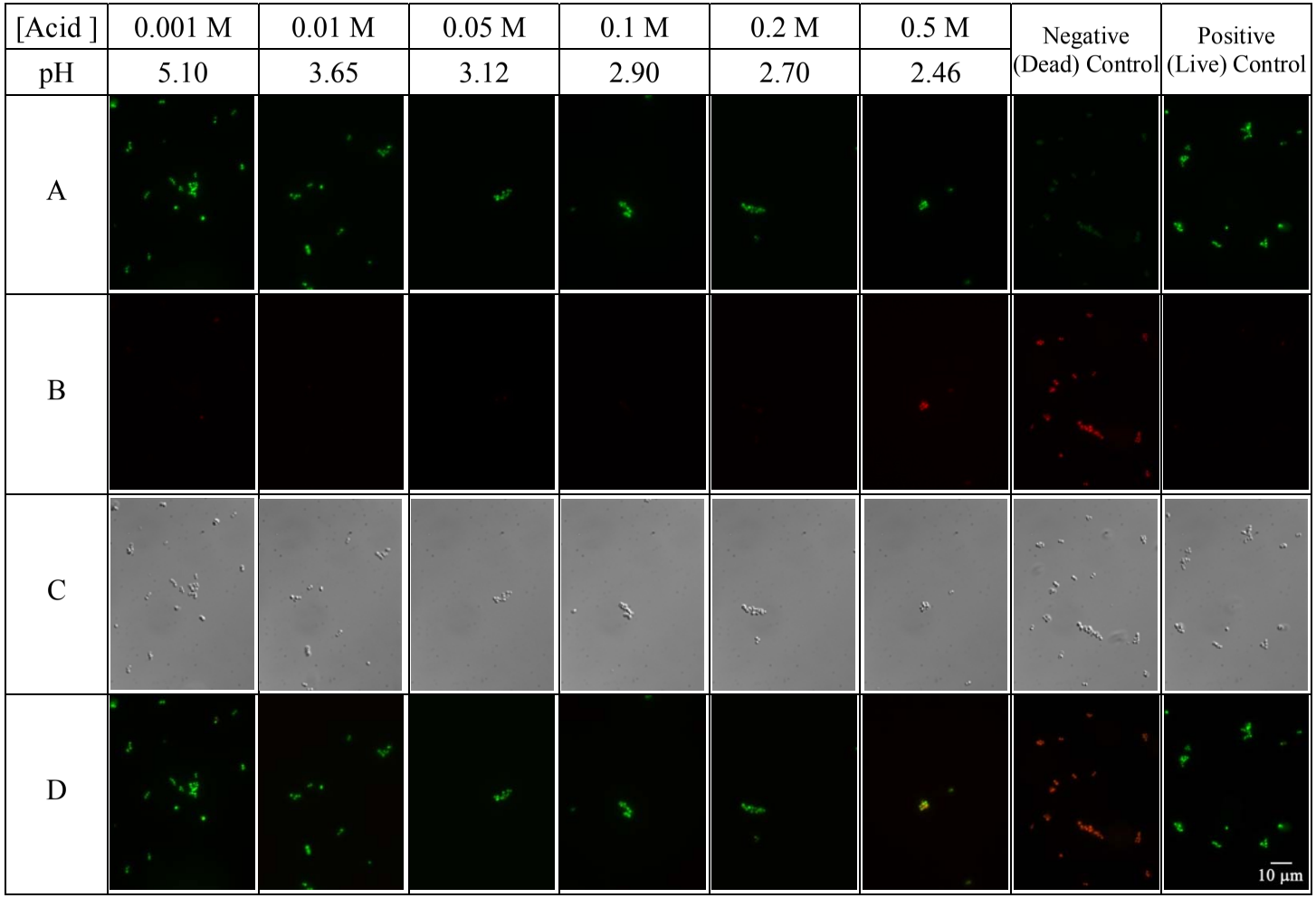


Figure A.14 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of acetic acid for 5 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

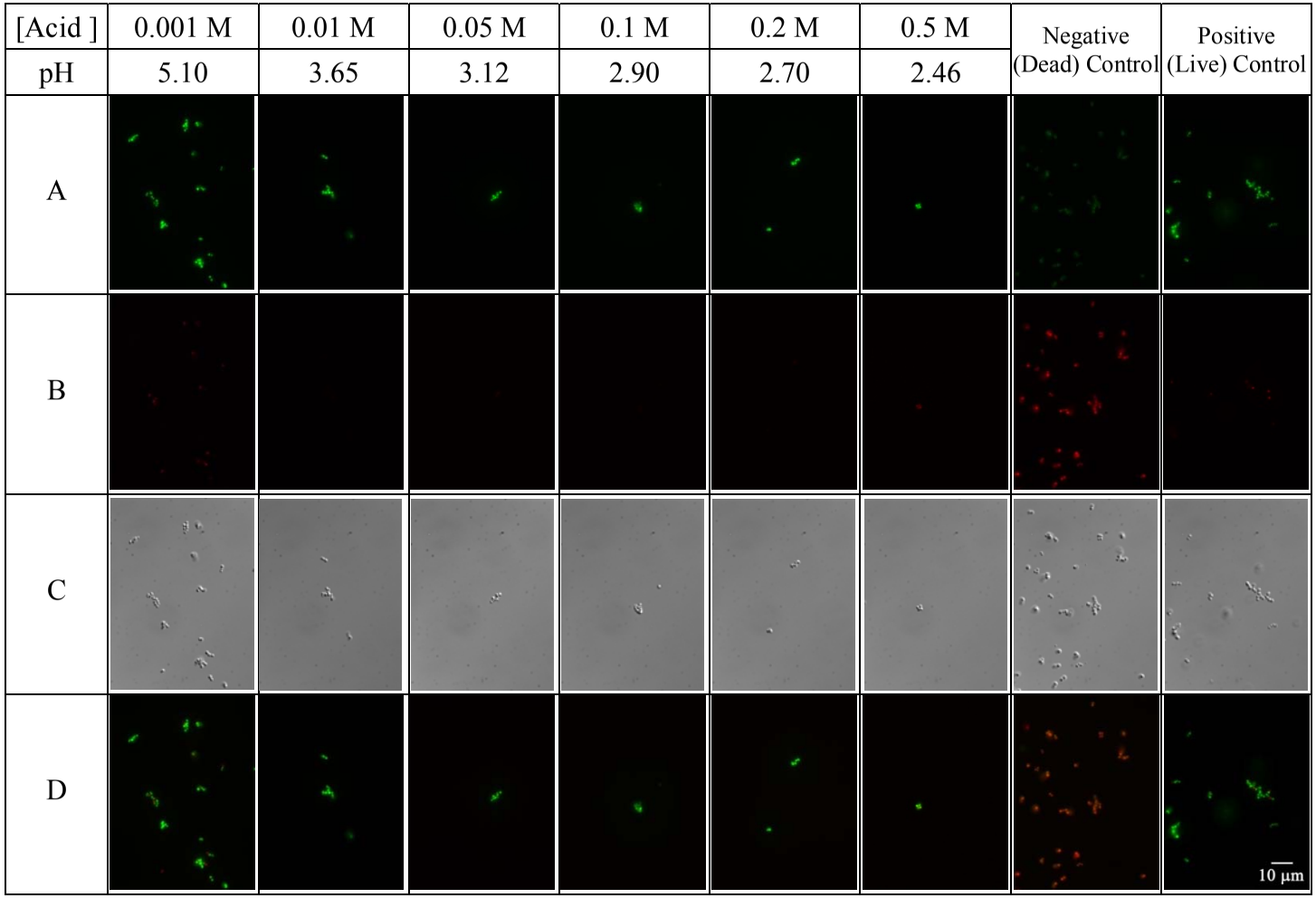


Figure A.15 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of acetic acid for 24 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

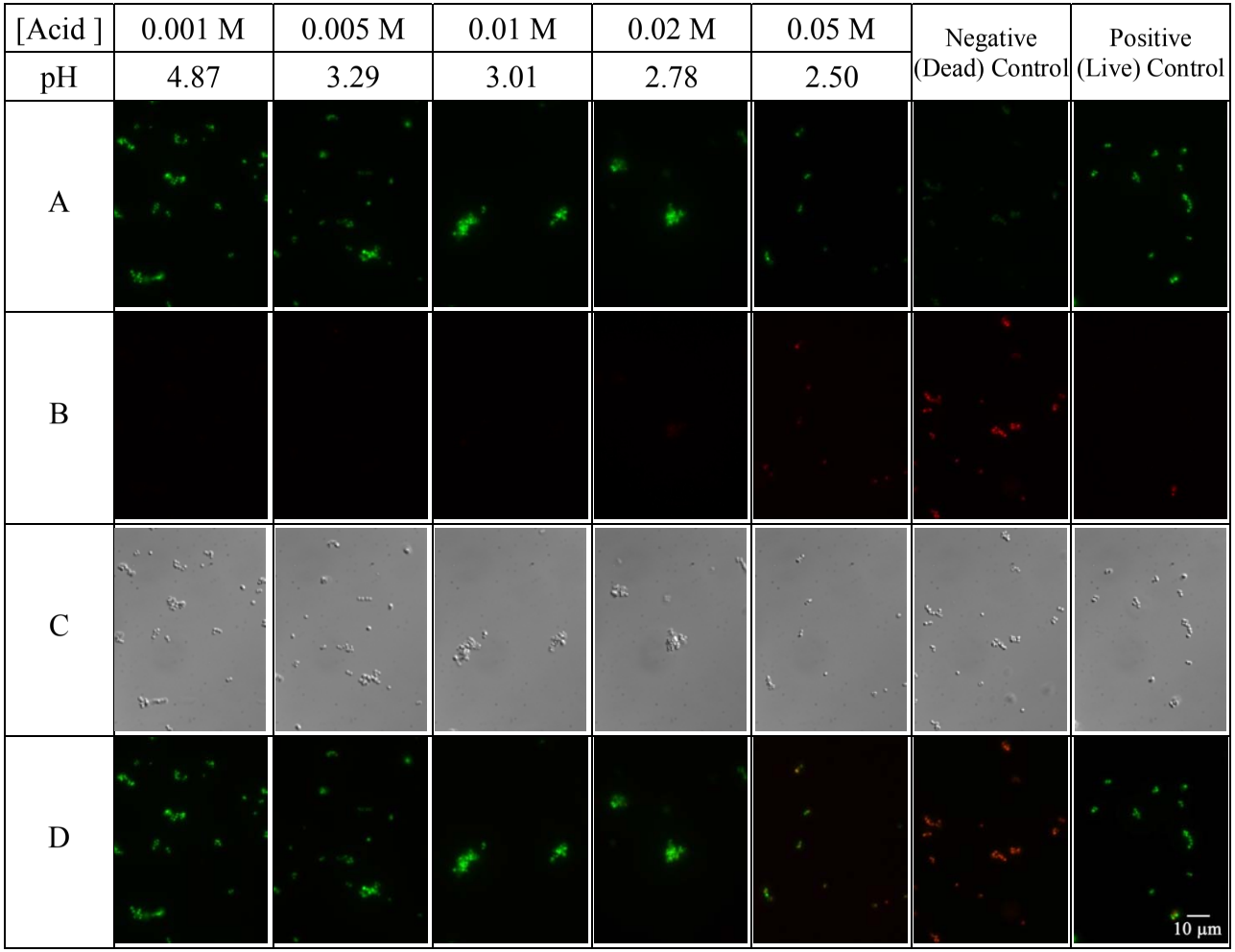


Figure A.16 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of lactic acid for 1 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

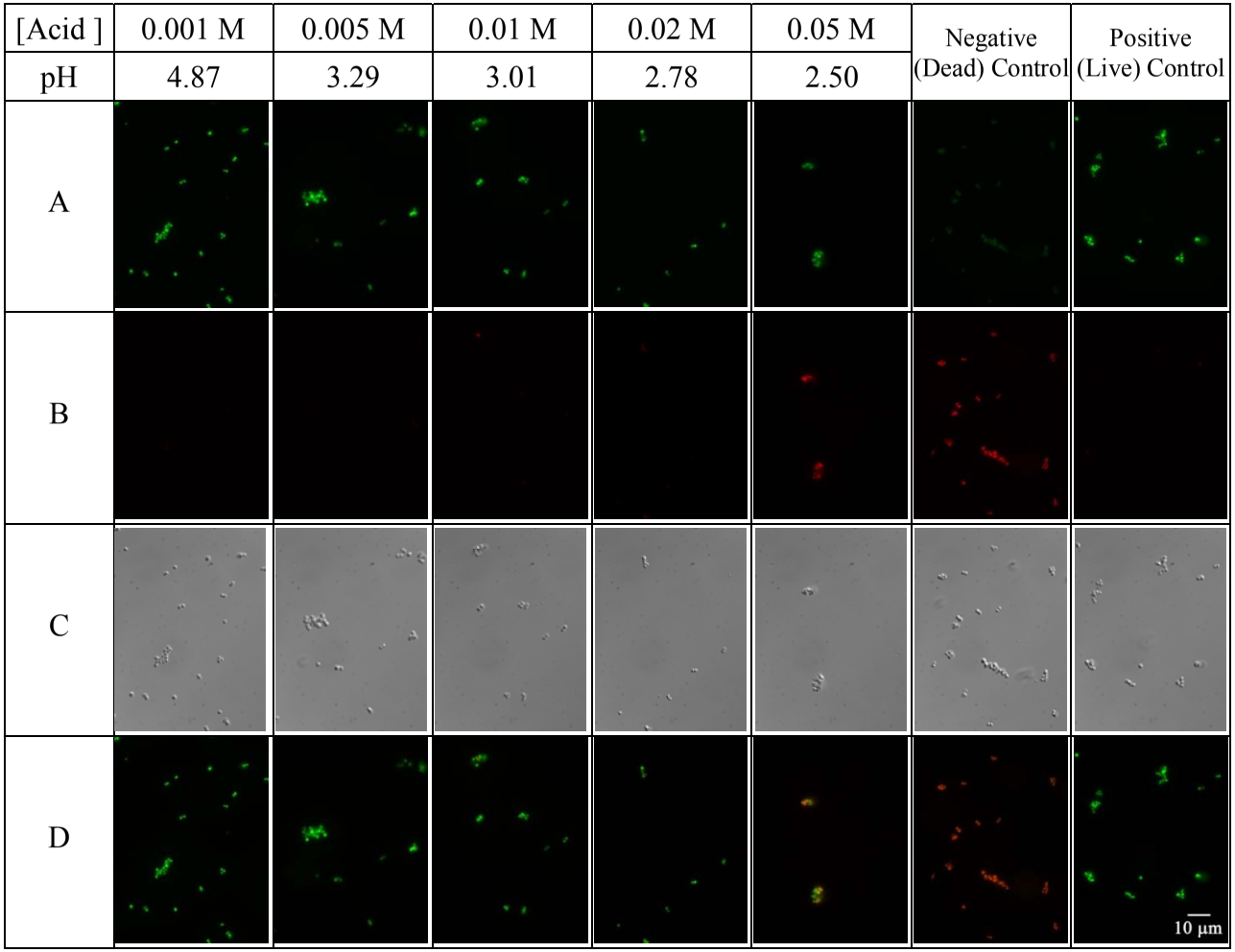


Figure A.17 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of lactic acid for 5 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

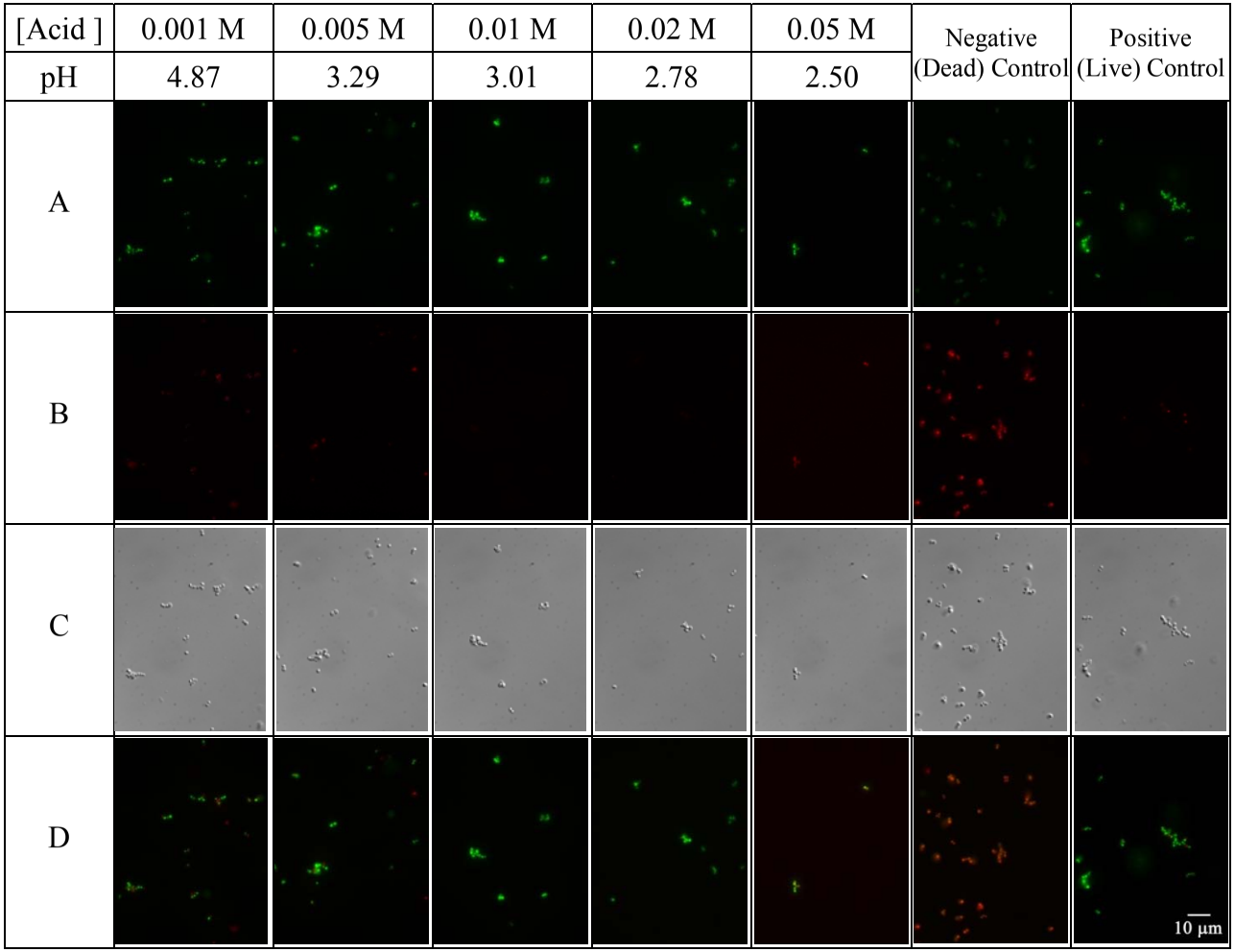


Figure A.18 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of lactic acid for 24 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

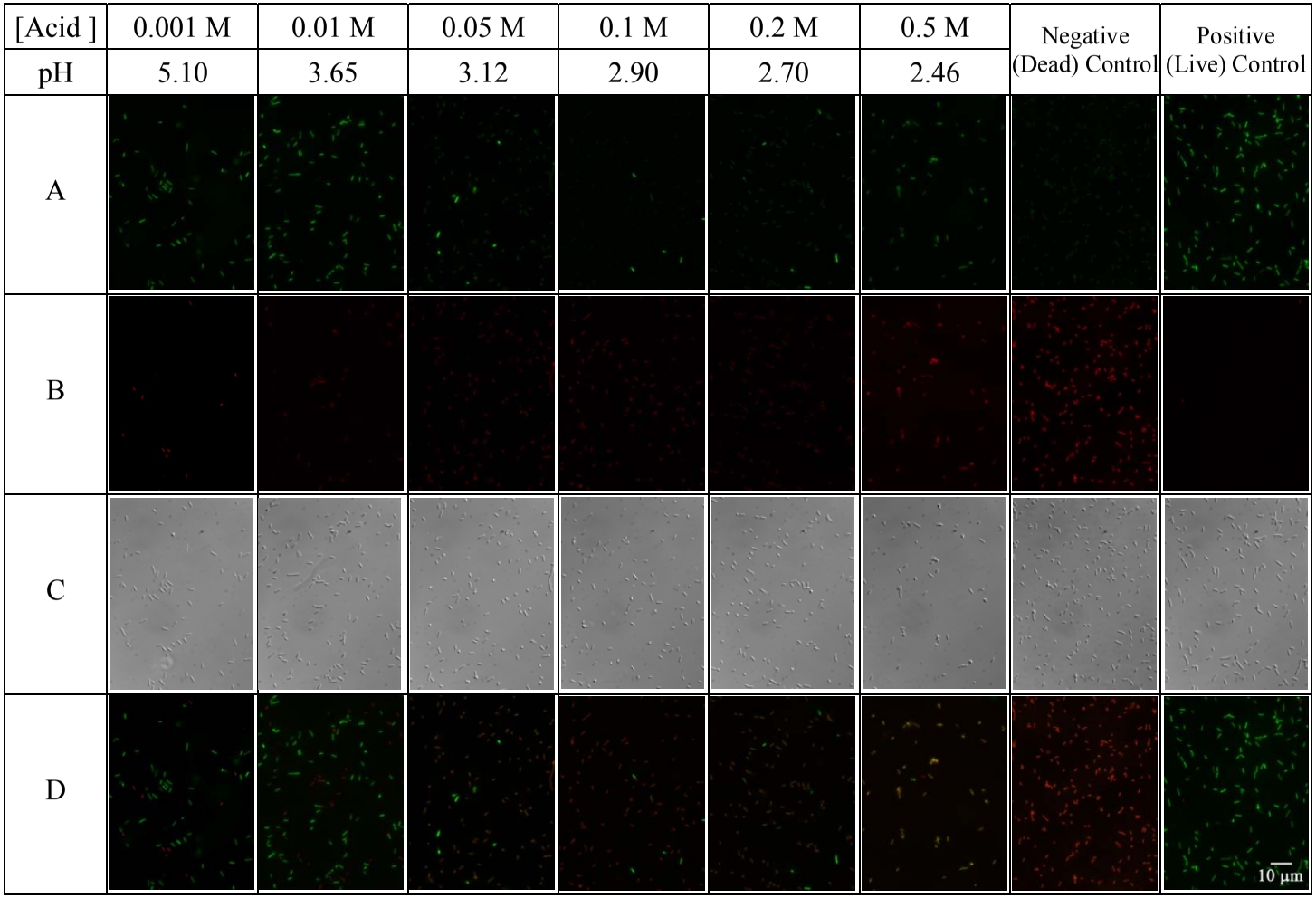


Figure A.19 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of acetic acid for 1 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

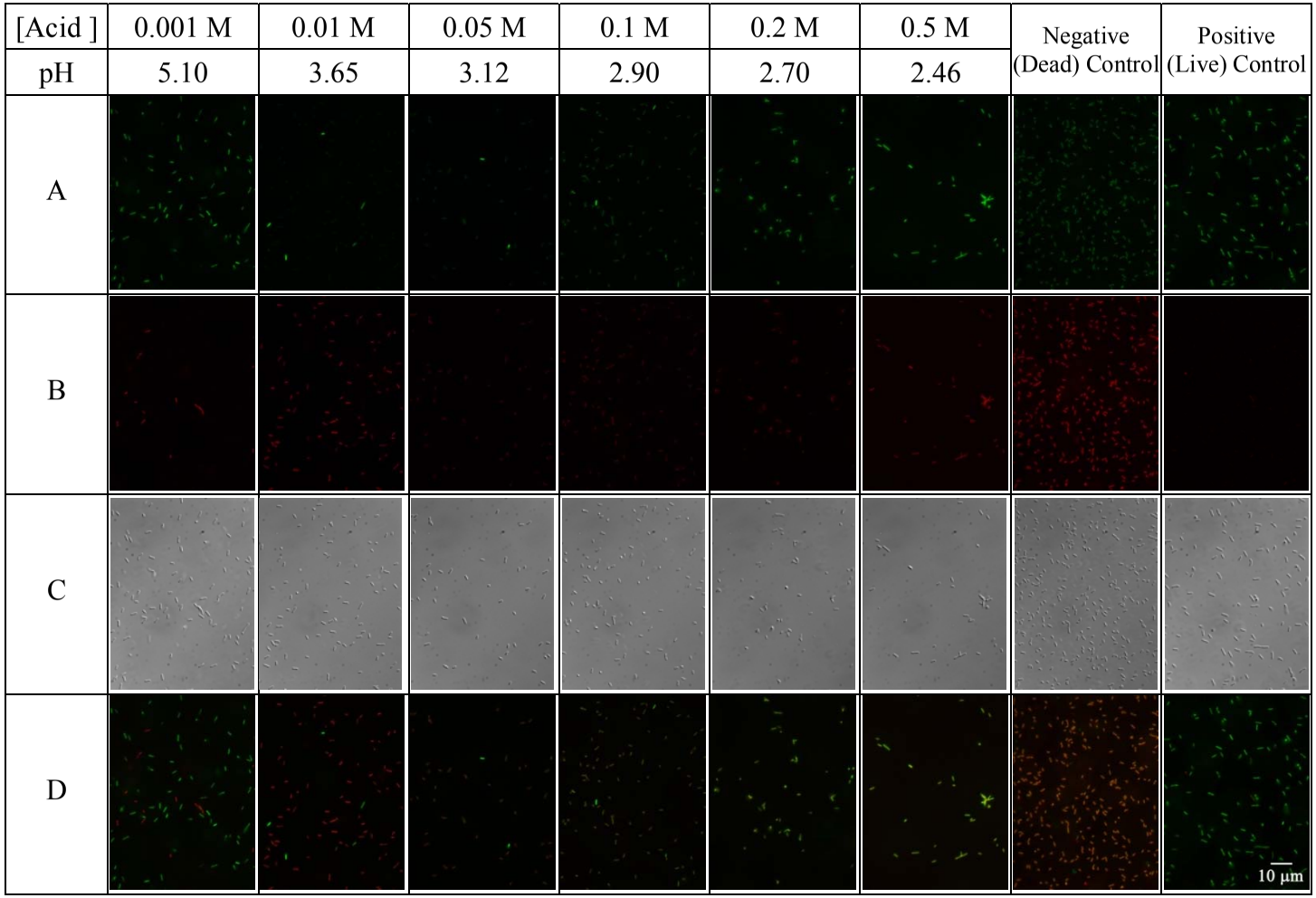


Figure A.20 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of acetic acid for 5 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

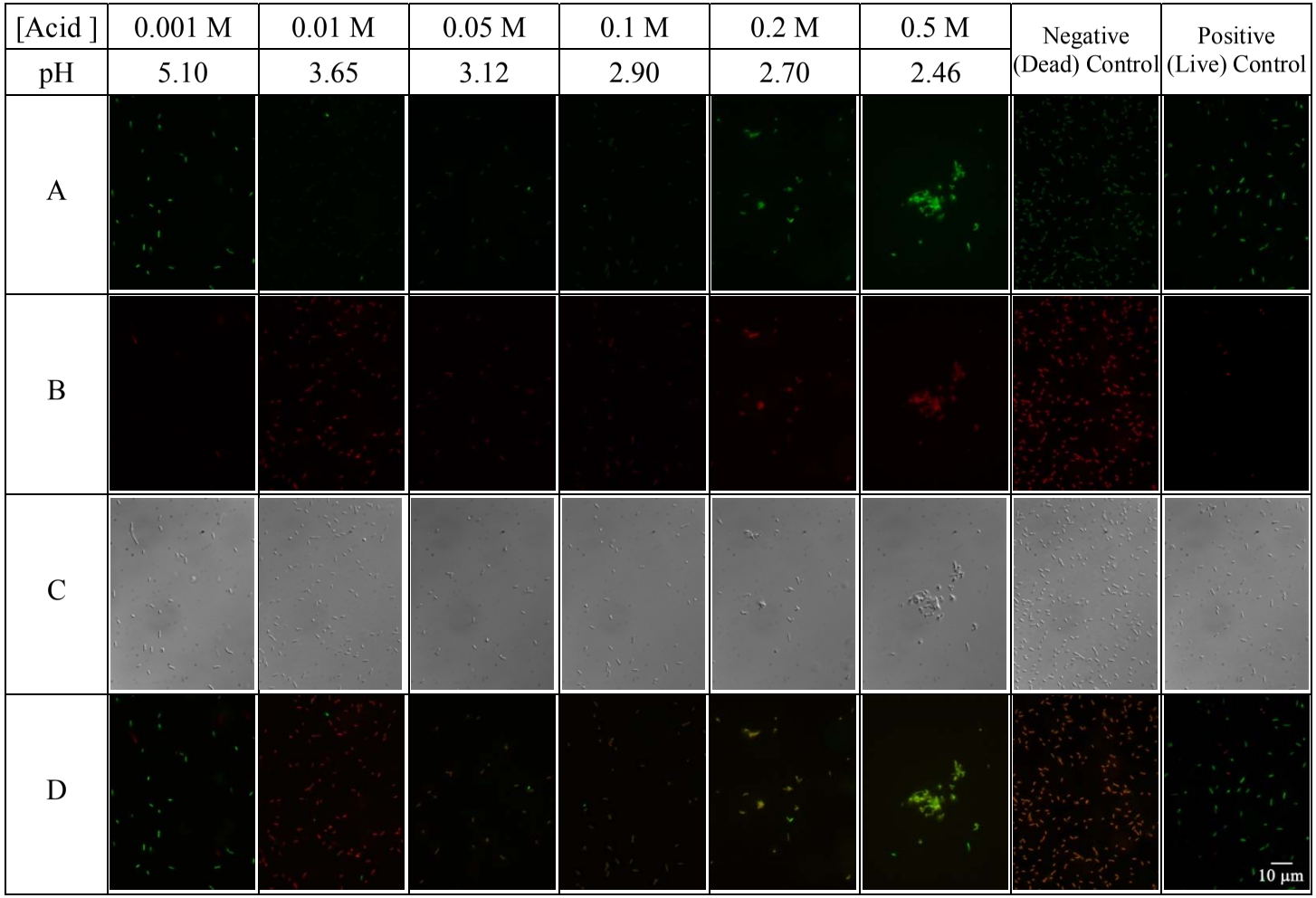


Figure A.21 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of acetic acid for 24 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

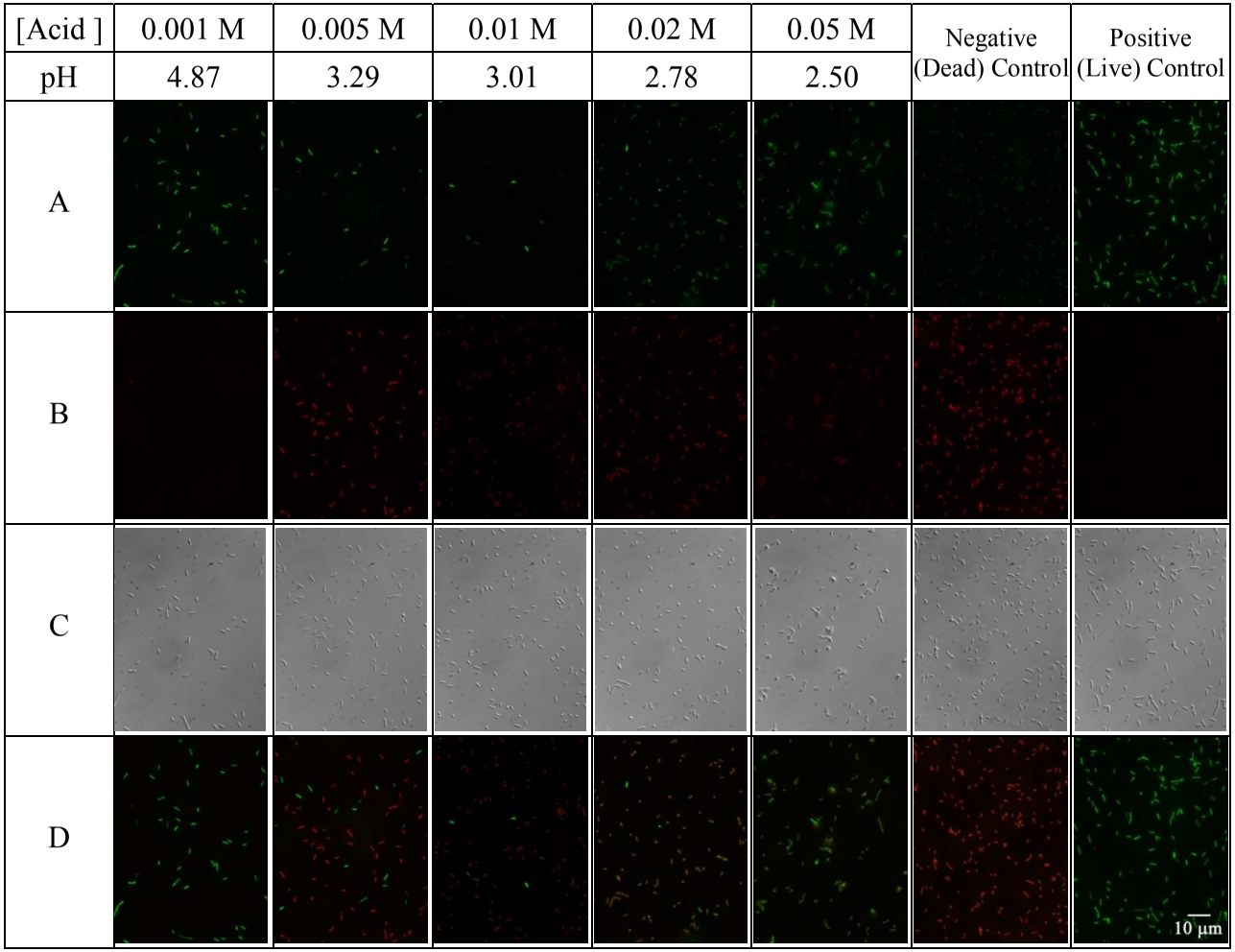


Figure A.22 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of lactic acid for 1 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

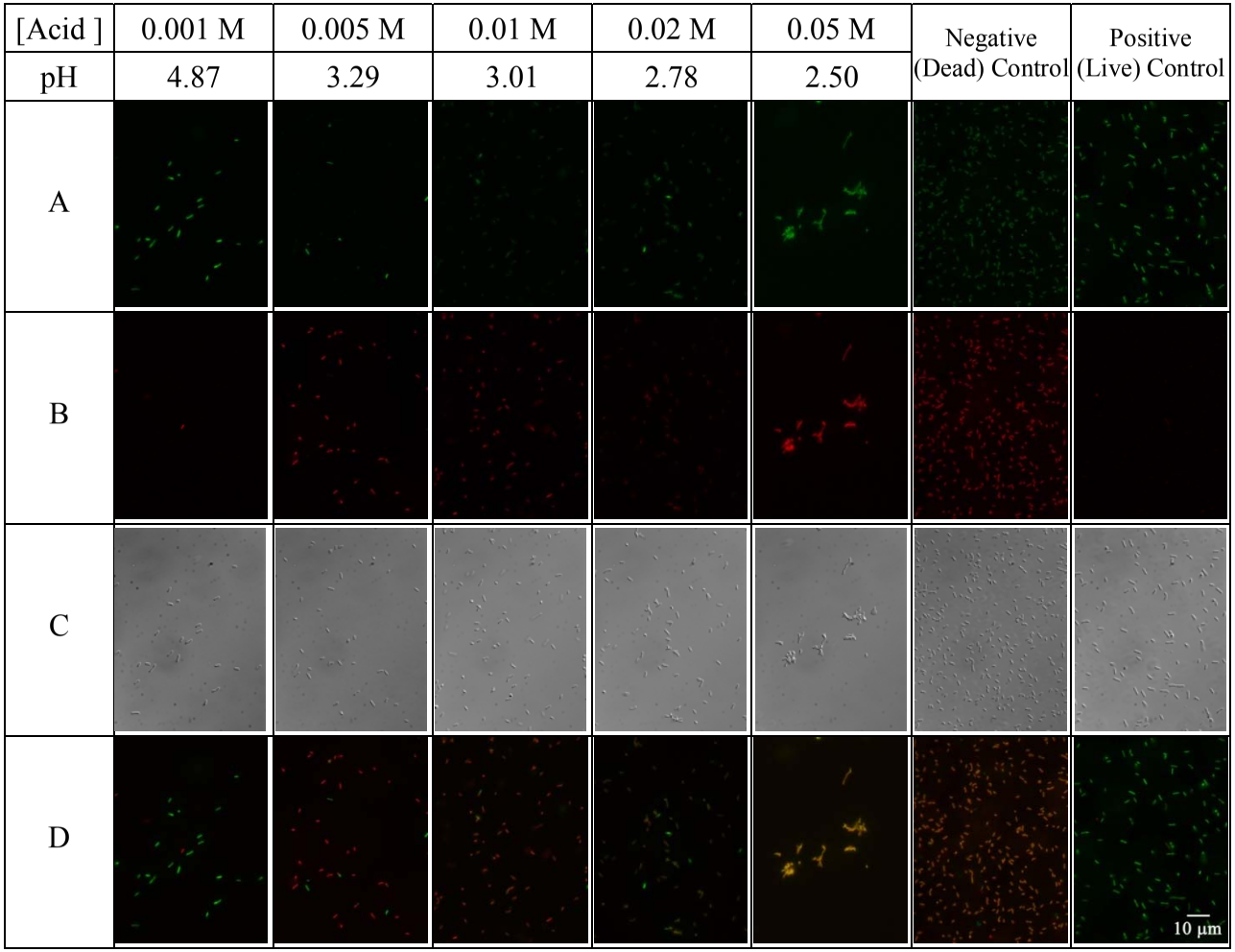


Figure A.23 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of lactic acid for 5 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

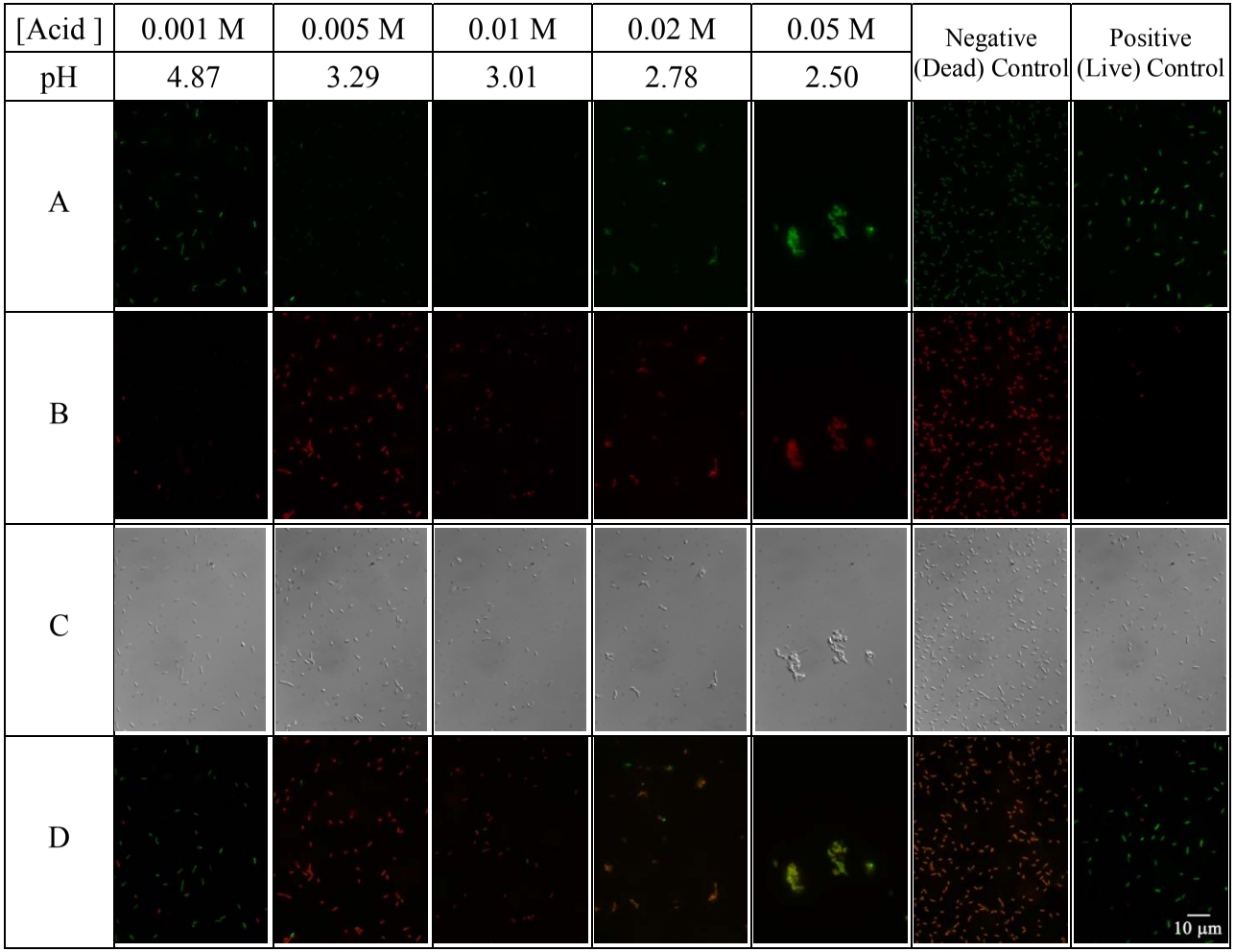


Figure A.24 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of lactic acid for 24 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

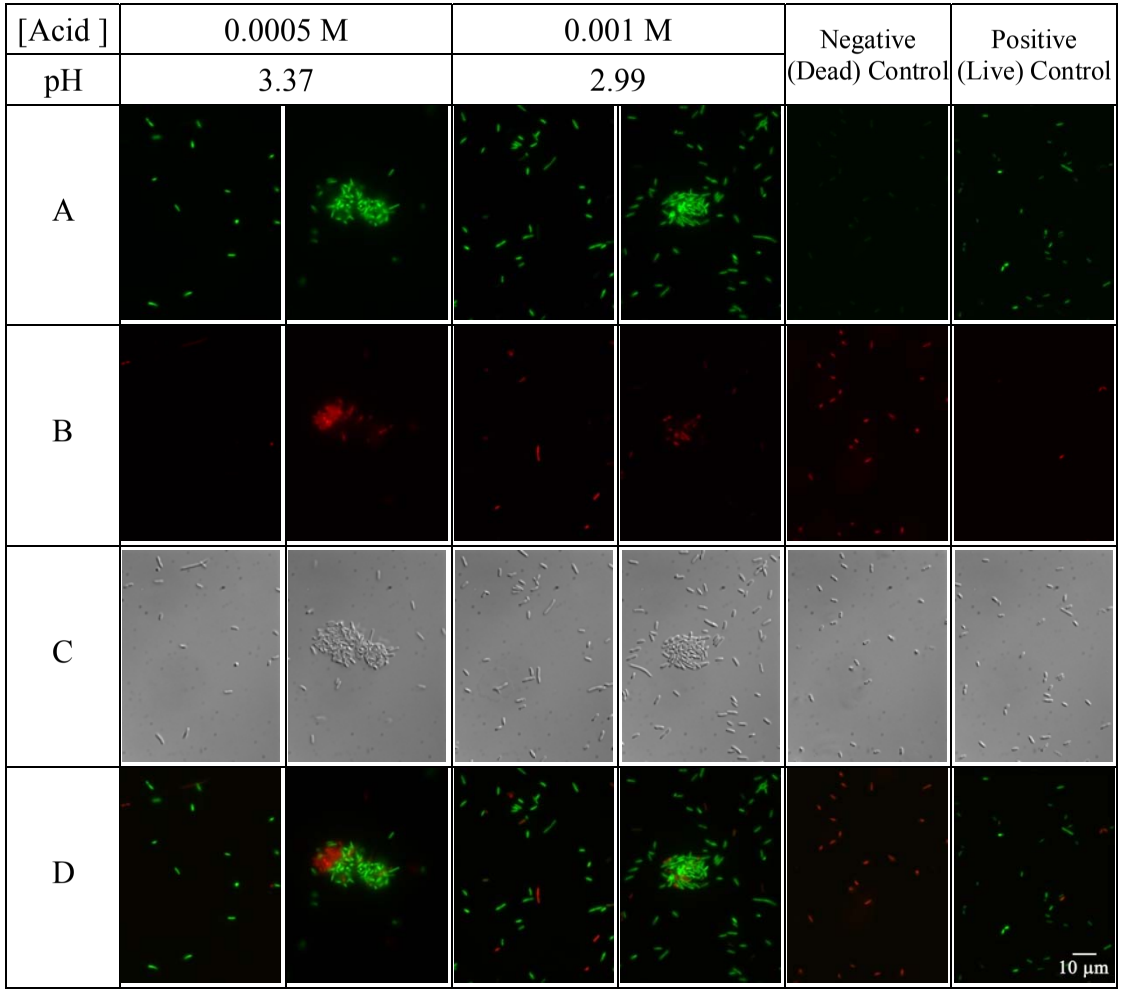


Figure A.25 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of hydrochloric acid for 1 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

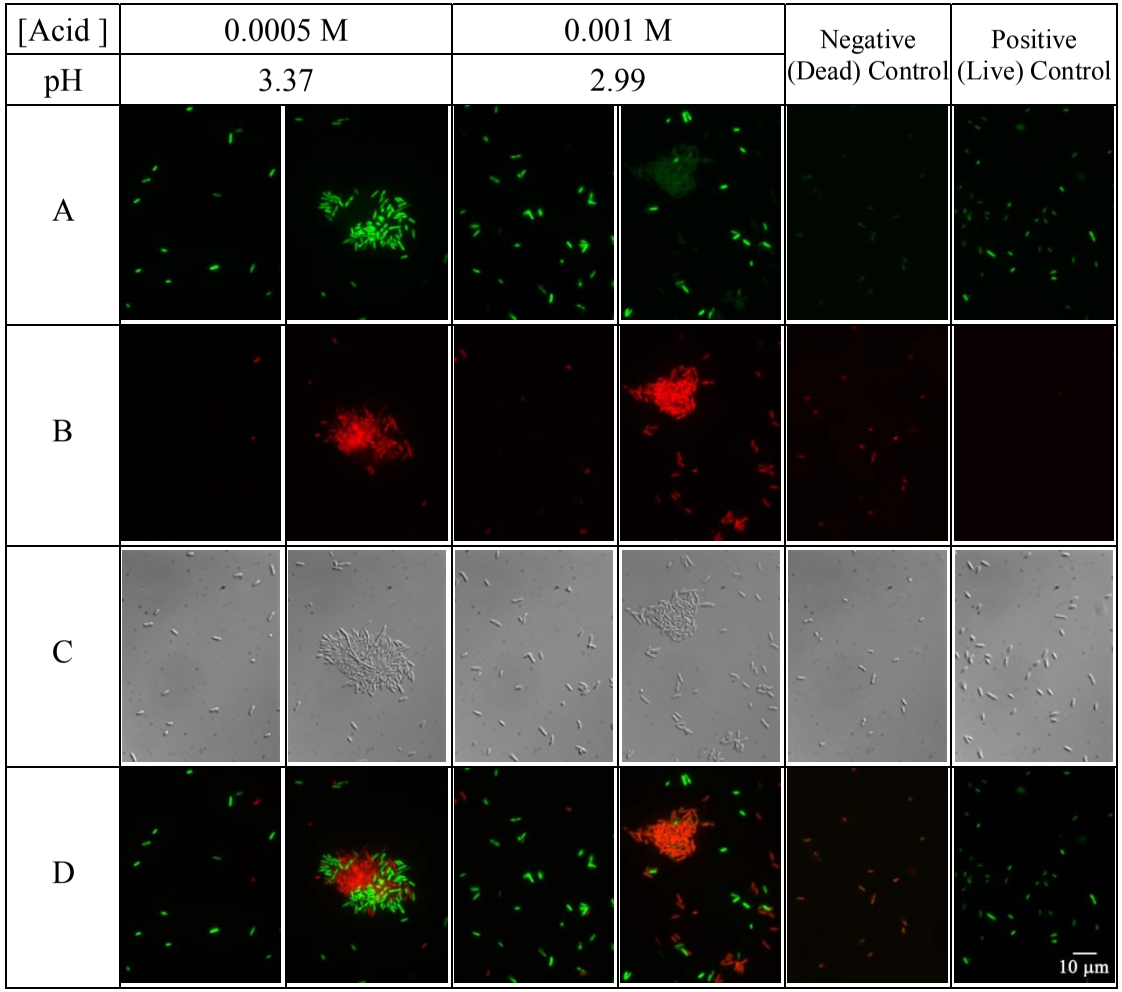


Figure A.26 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of hydrochloric acid for 5 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

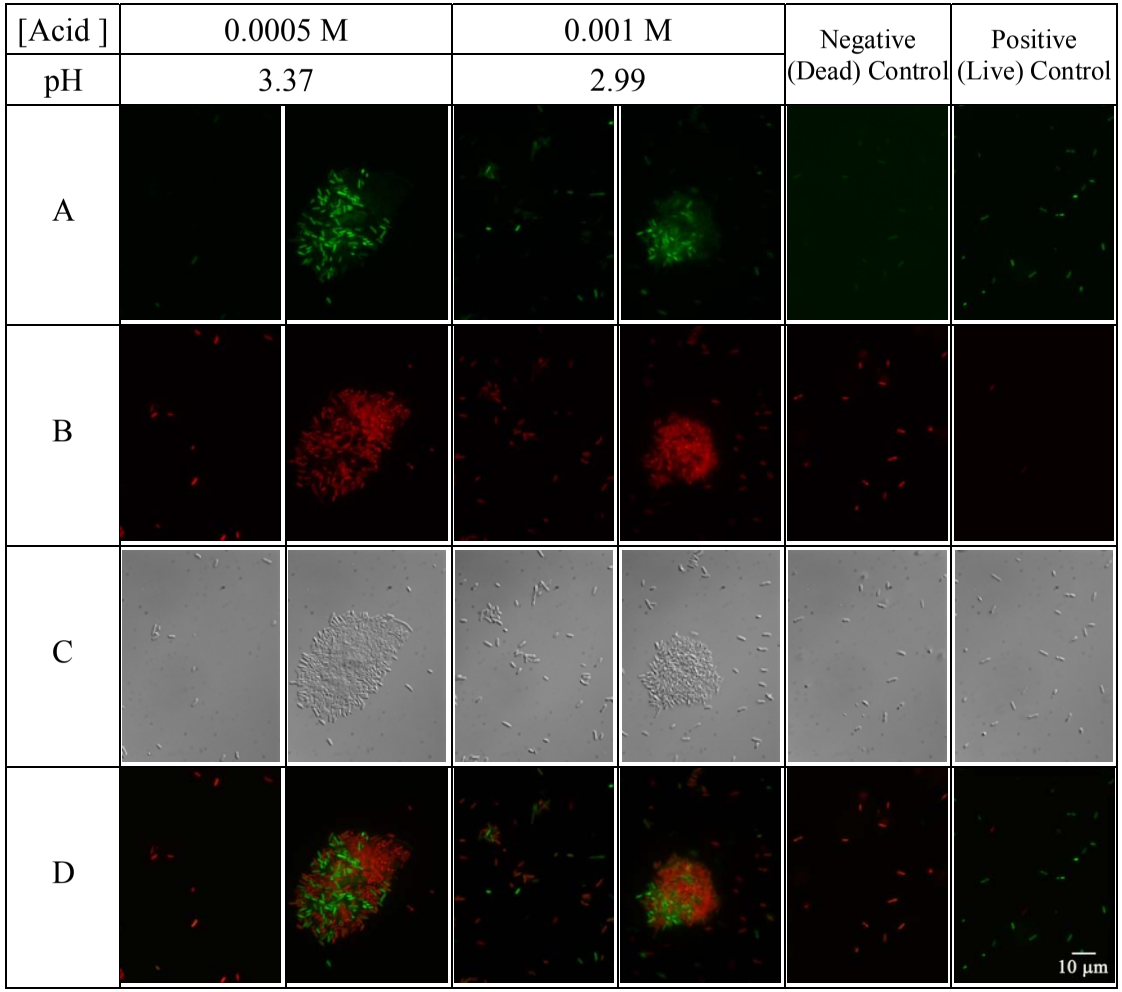


Figure A.27 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of hydrochloric acid for 24 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

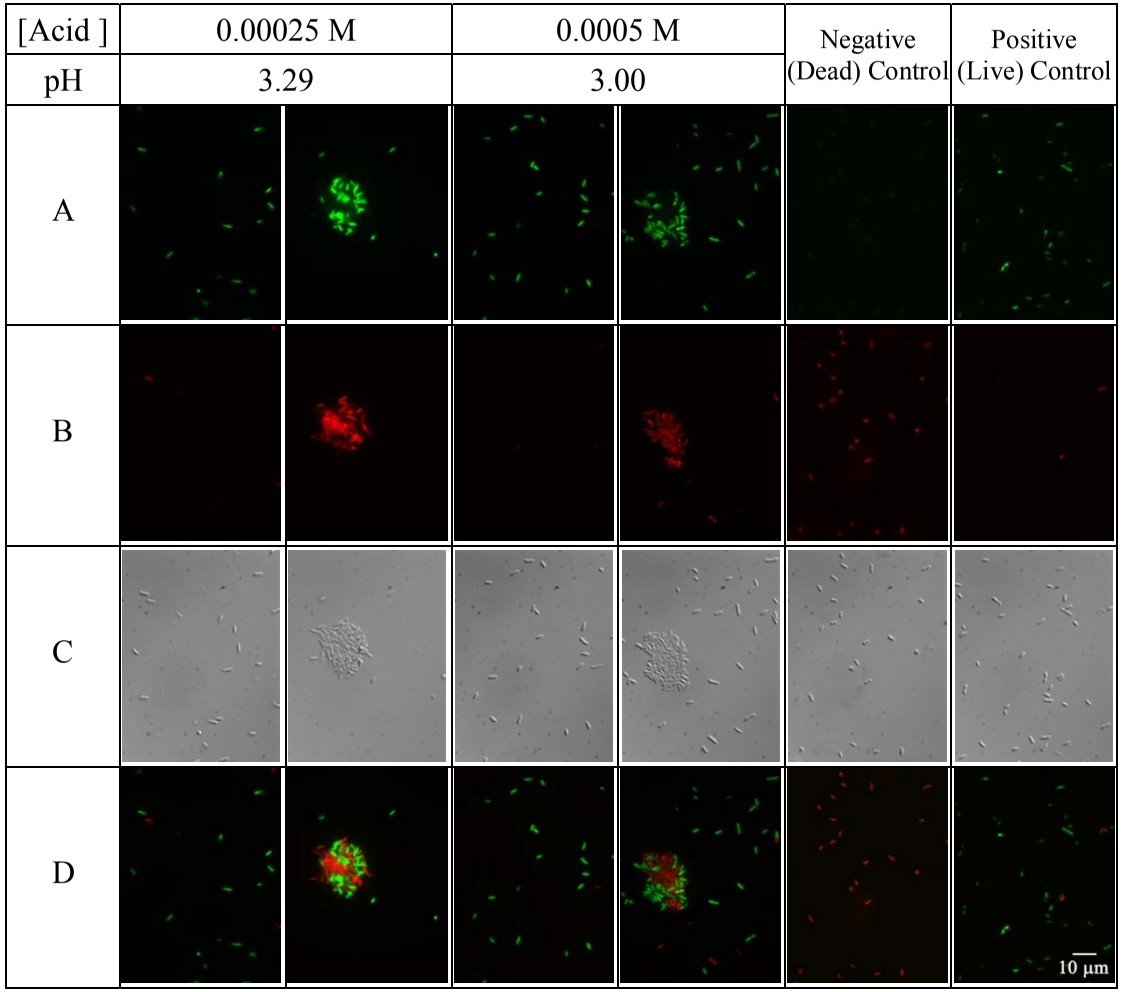


Figure A.28 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of sulfuric acid for 1 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

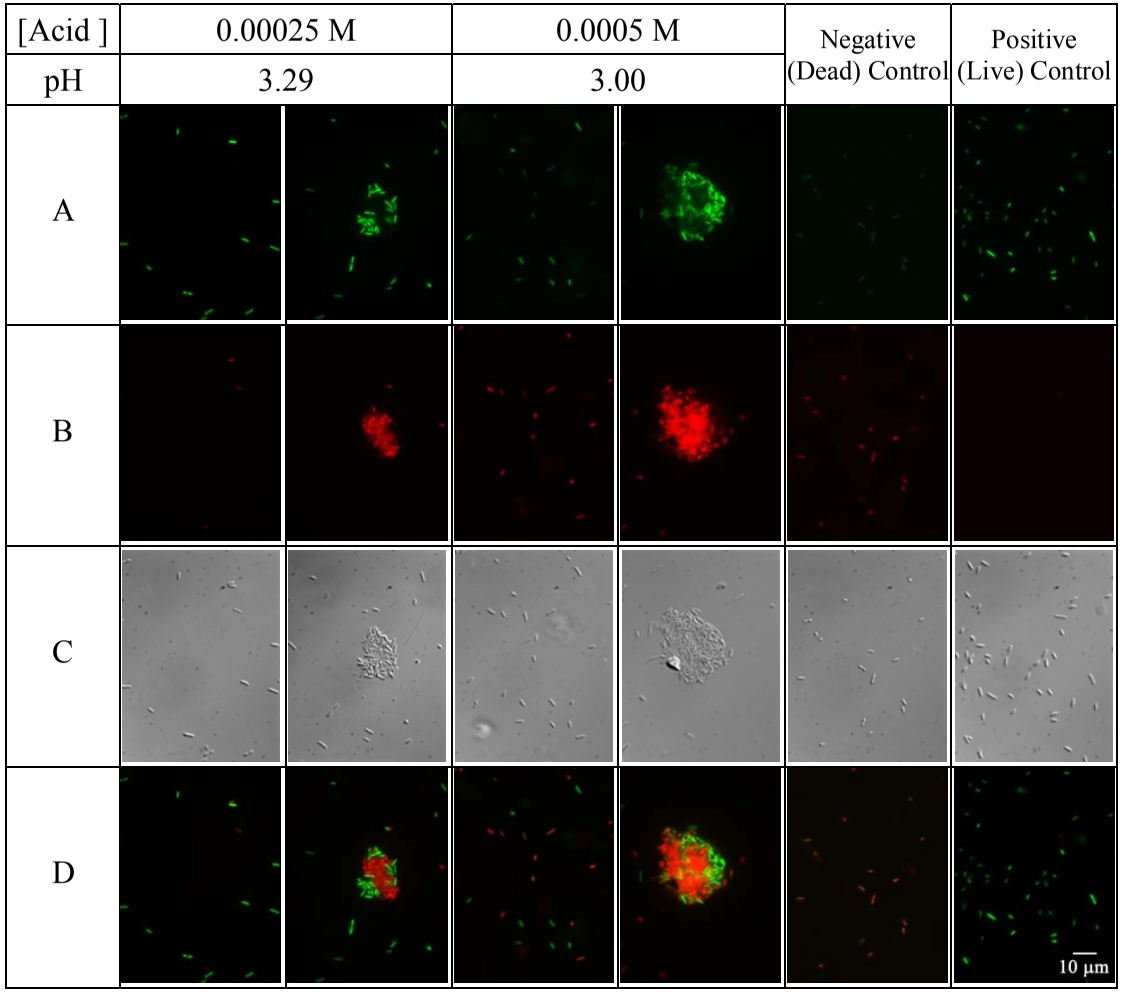


Figure A.29 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of sulfuric acid for 5 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

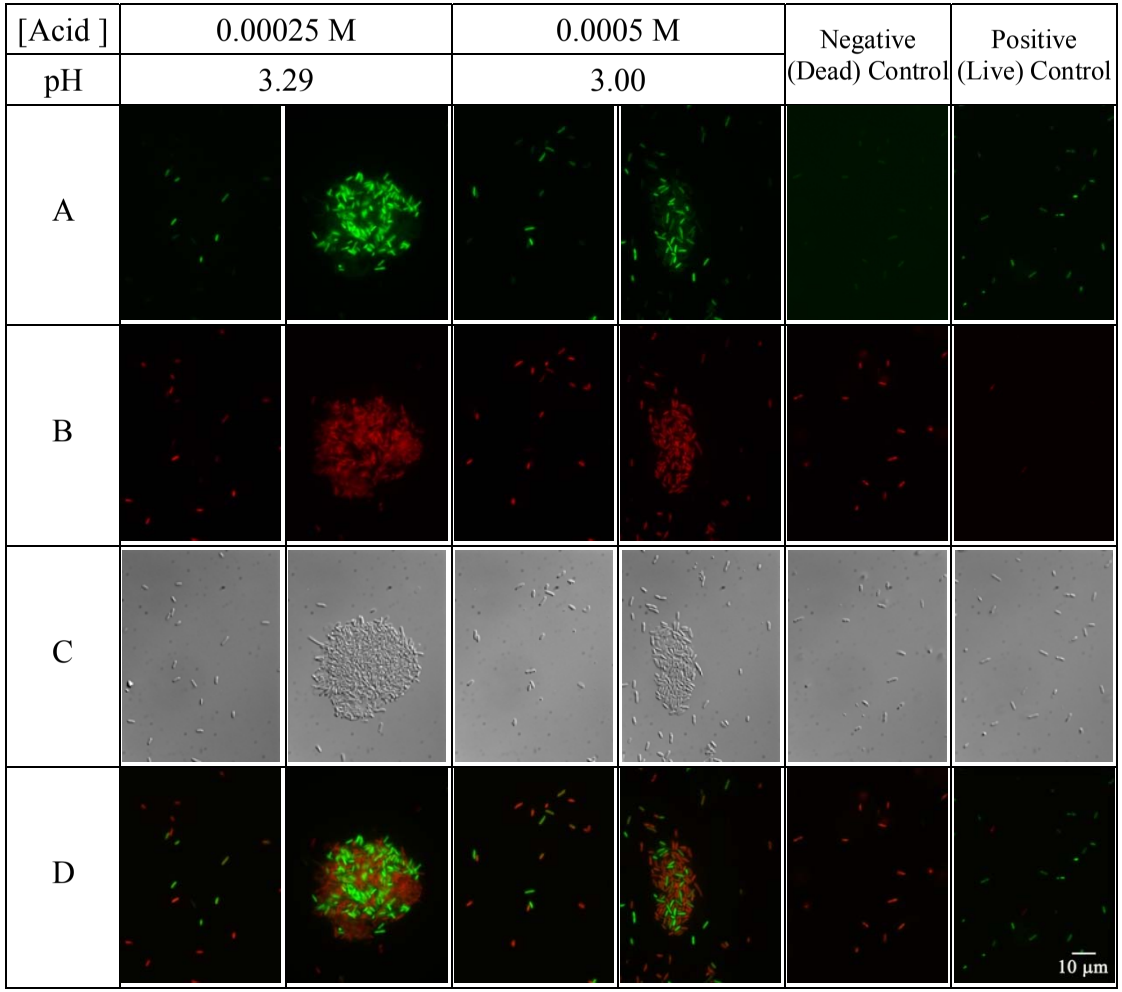


Figure A.30 Fluorescence microscopy images of stationary-phase *E. coli* DH5α after exposure to different concentrations of sulfuric acid for 24 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

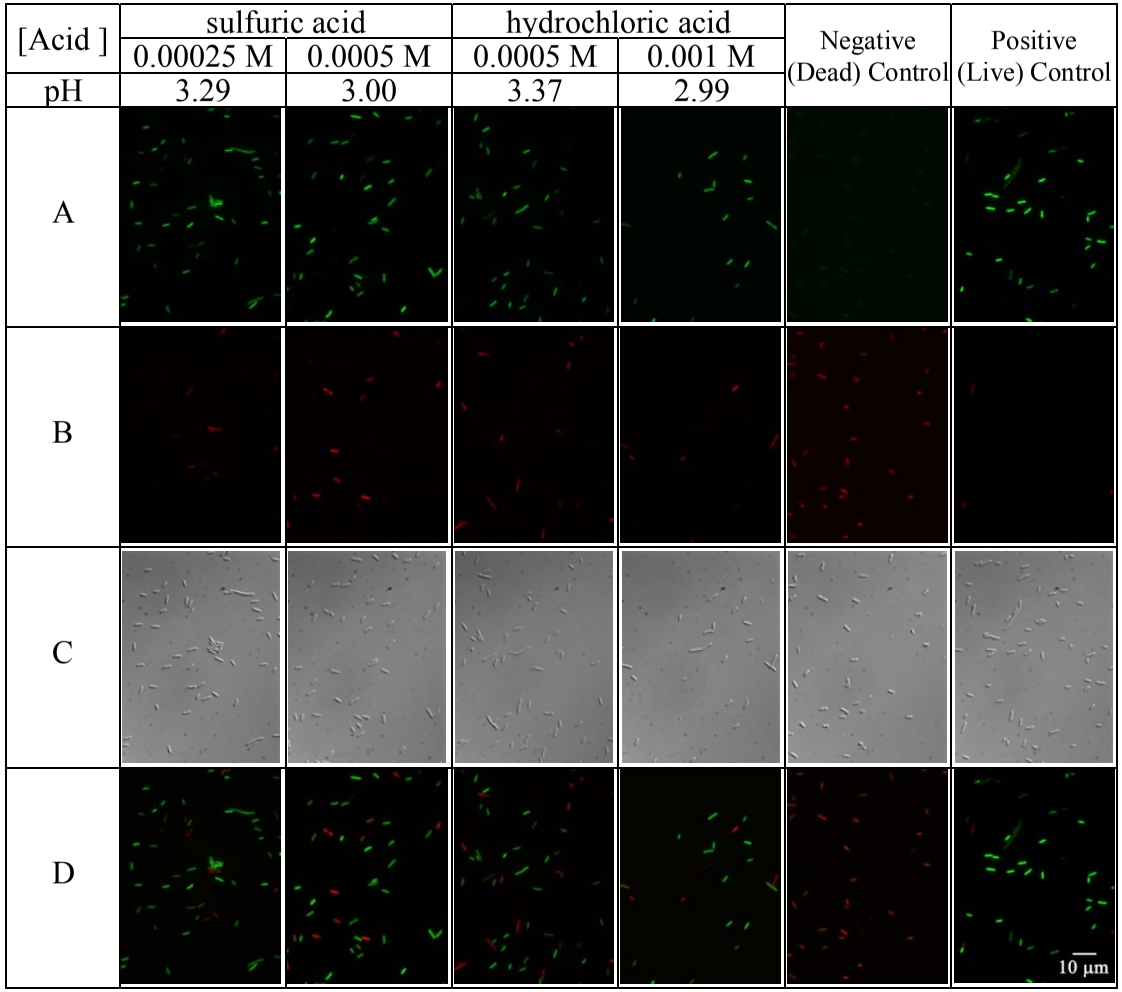


Figure A.31 Fluorescence microscopy images of mid-log phase *E. coli* DH5α after exposure to different concentrations of hydrochloric acid and sulfuric acid for 1 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

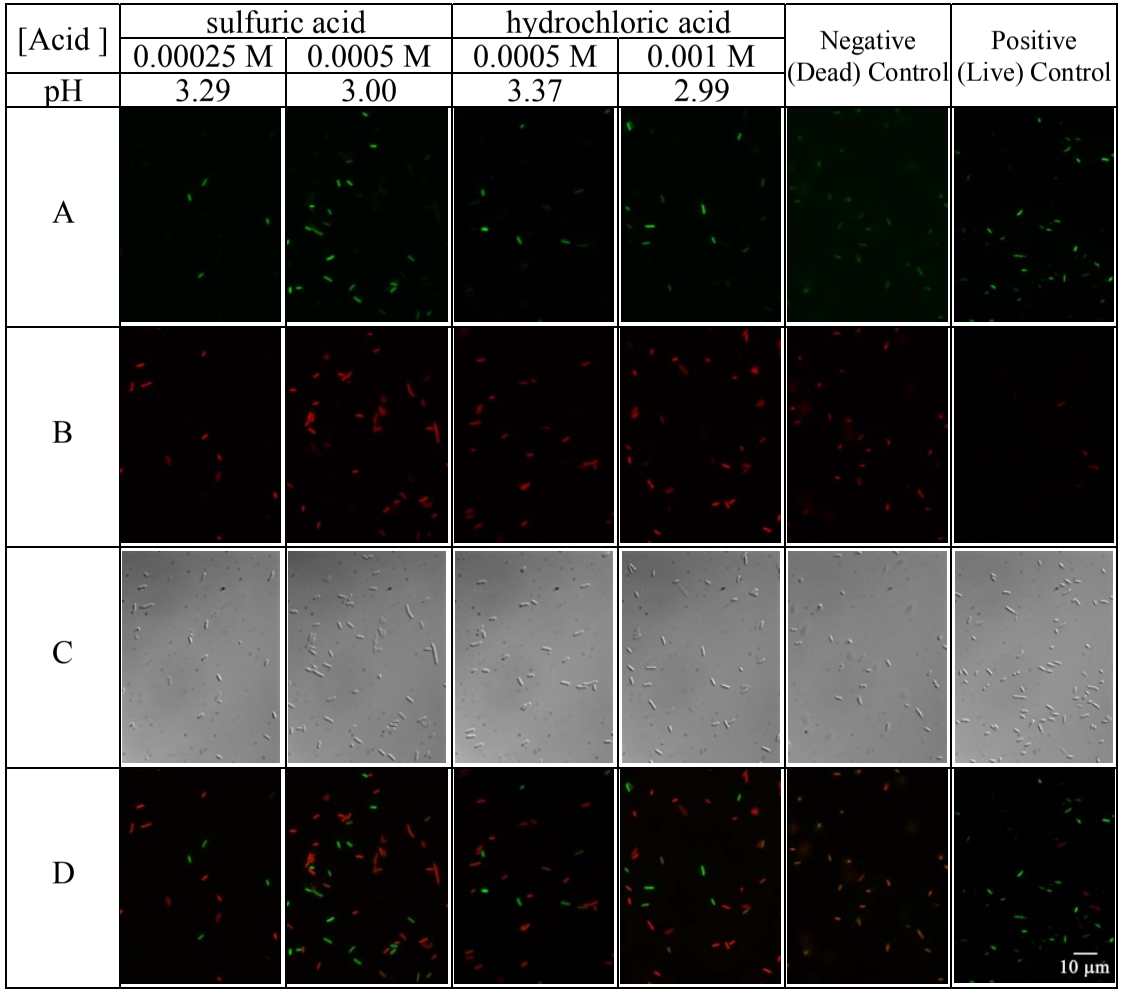


Figure A.32 Fluorescence microscopy images of mid-log phase *E. coli* DH5α after exposure to different concentrations of hydrochloric acid and sulfuric acid for 5 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

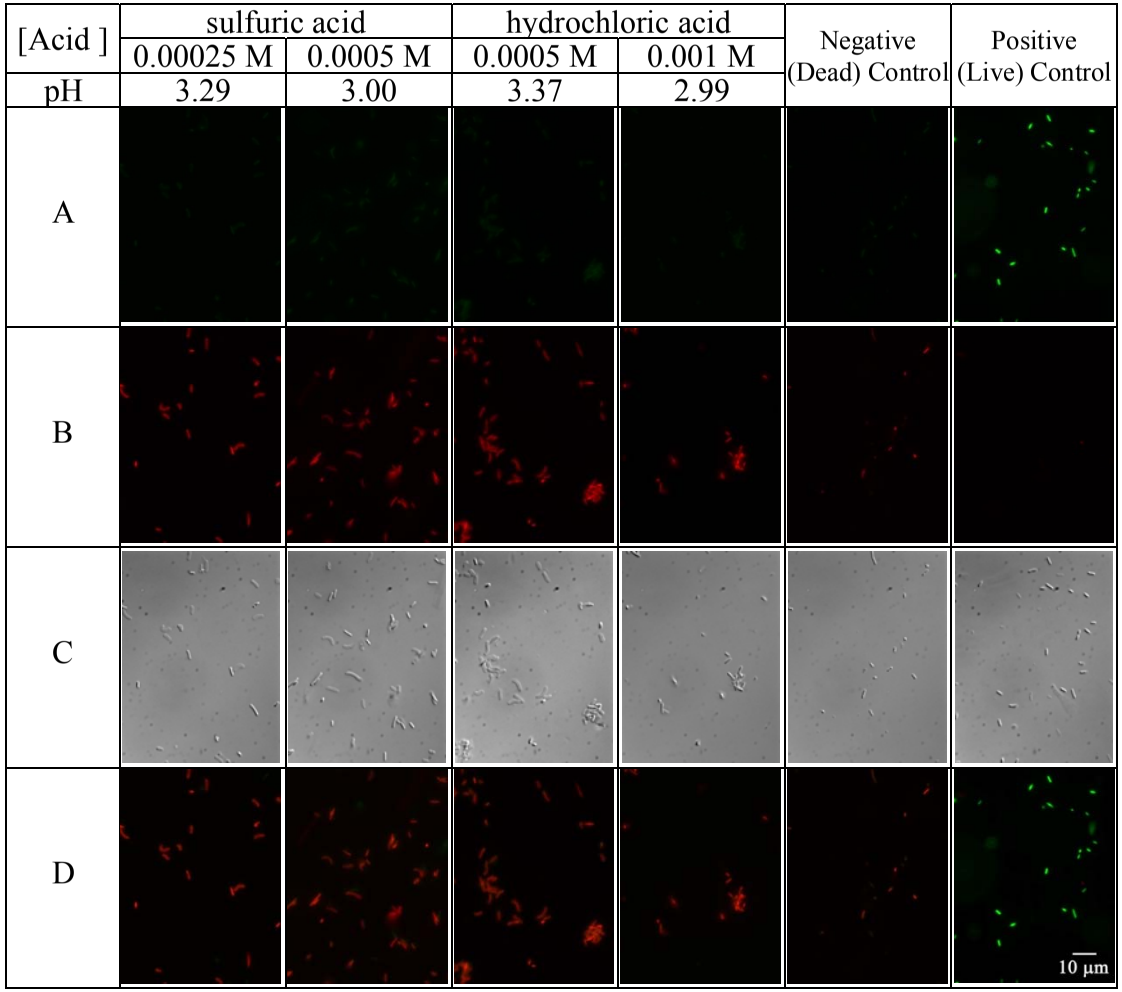


Figure A.33 Fluorescence microscopy images of mid-log phase *E. coli* DH5α after exposure to different concentrations of hydrochloric acid and sulfuric acid for 24 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

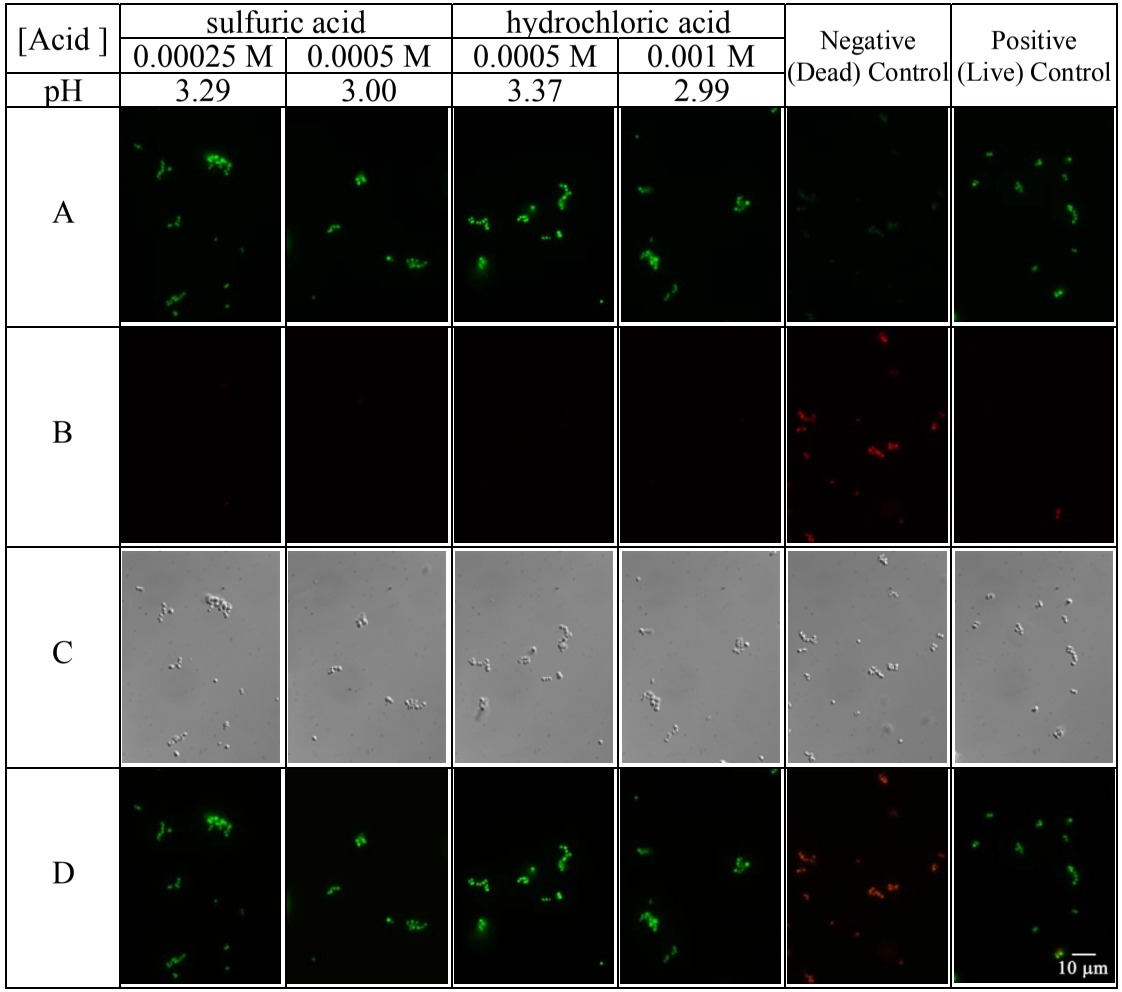


Figure A.34 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of hydrochloric acid and sulfuric acid for 1 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

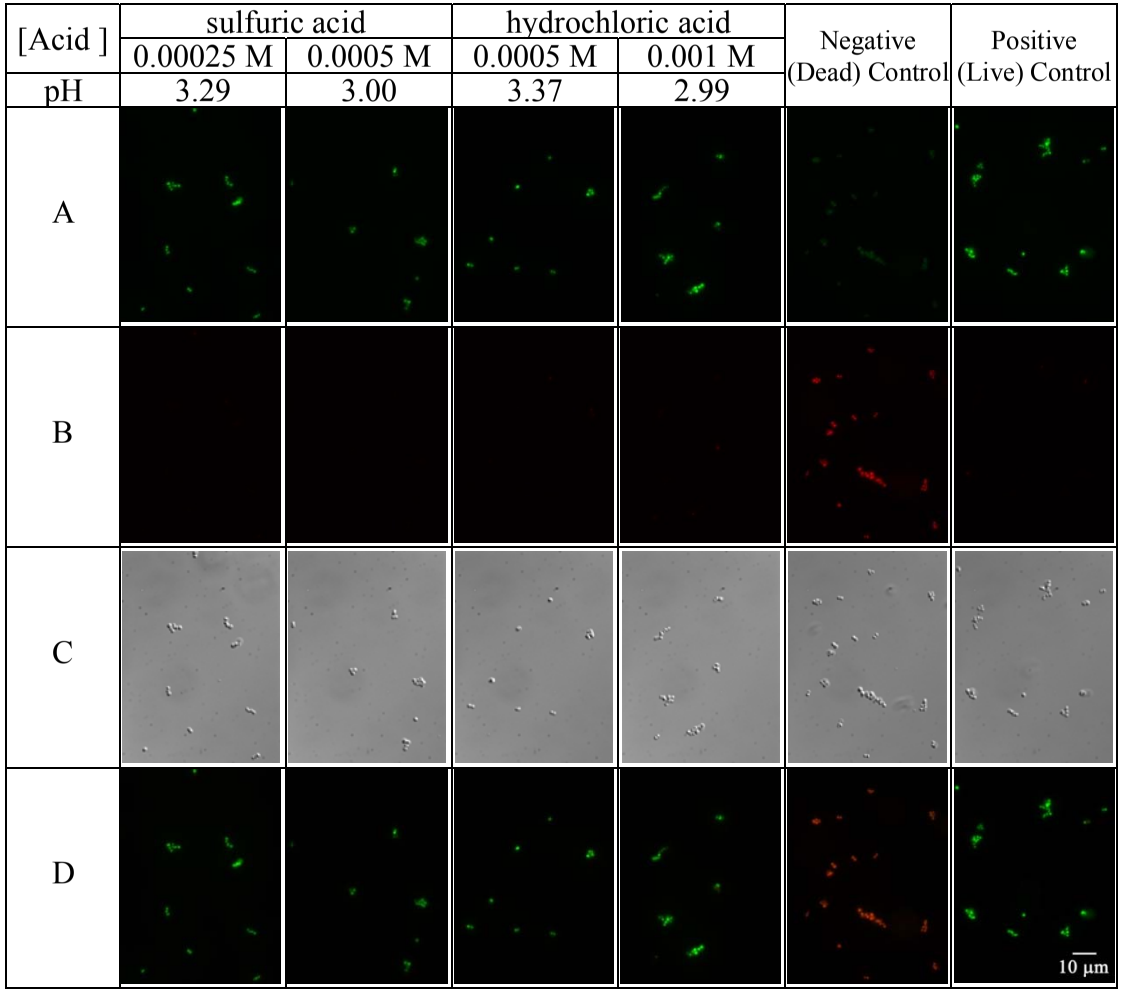


Figure A.35 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of hydrochloric acid and sulfuric acid for 5 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

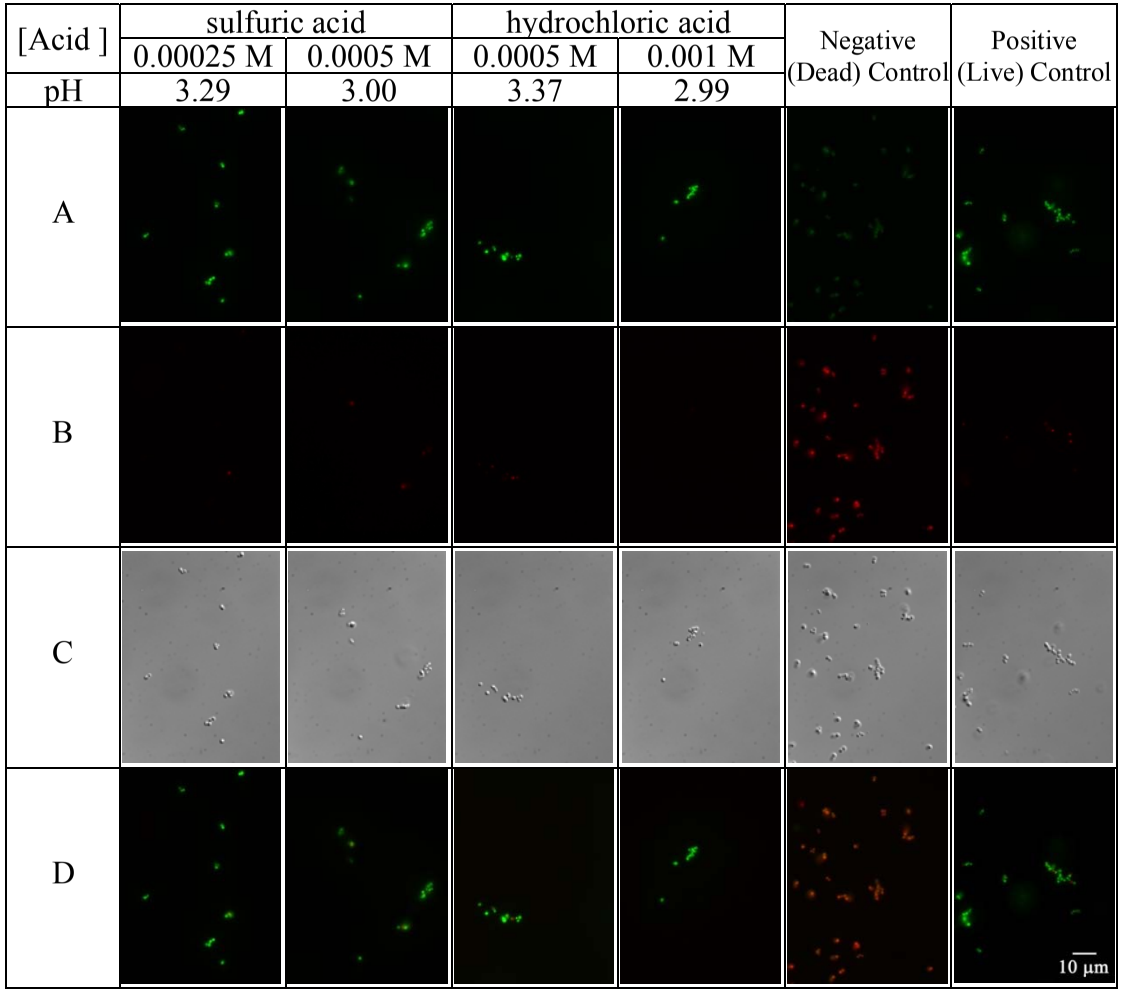


Figure A.36 Fluorescence microscopy images of mid-log phase *S. aureus* ATCC 13565 after exposure to different concentrations of hydrochloric acid and sulfuric acid for 24 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

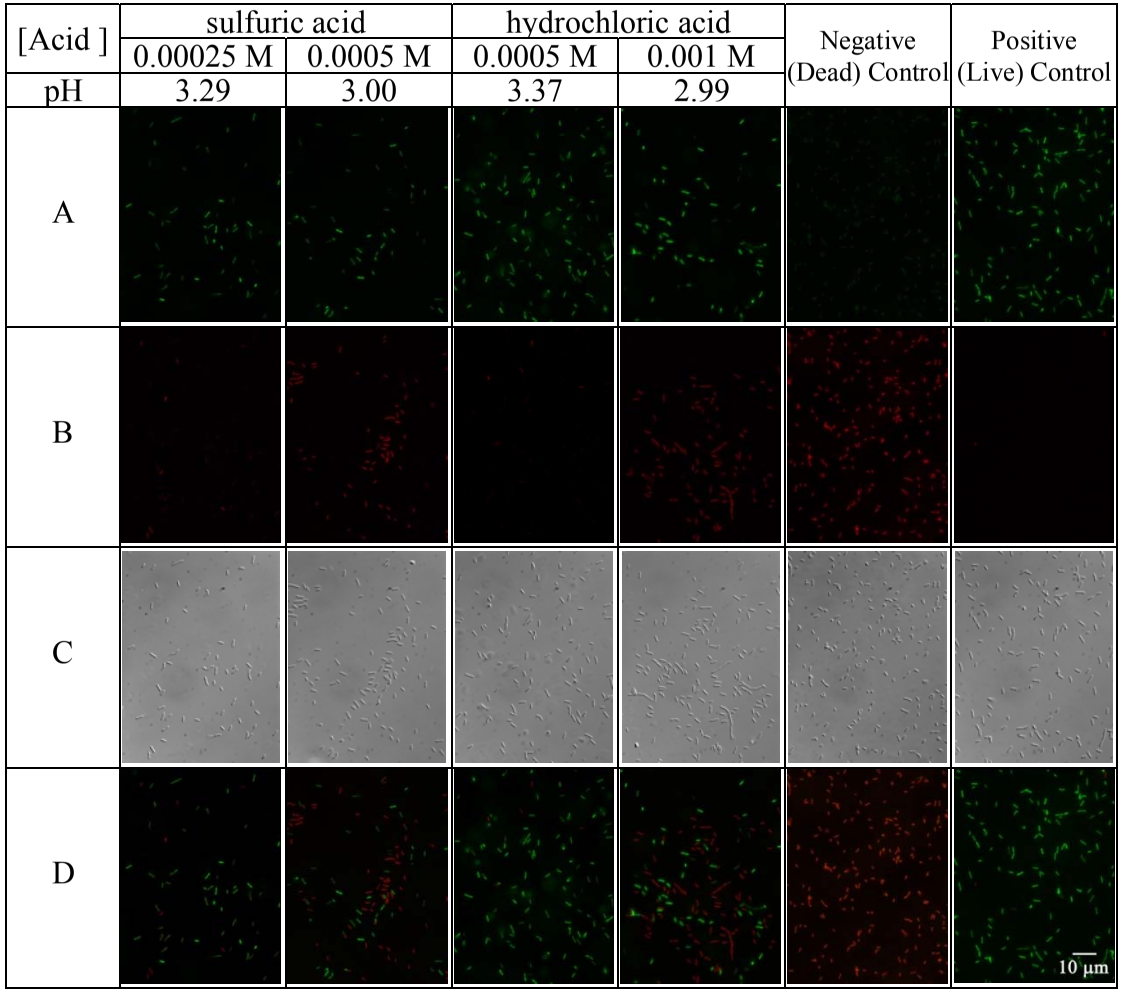


Figure A.37 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of hydrochloric acid and sulfuric acid for 1 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

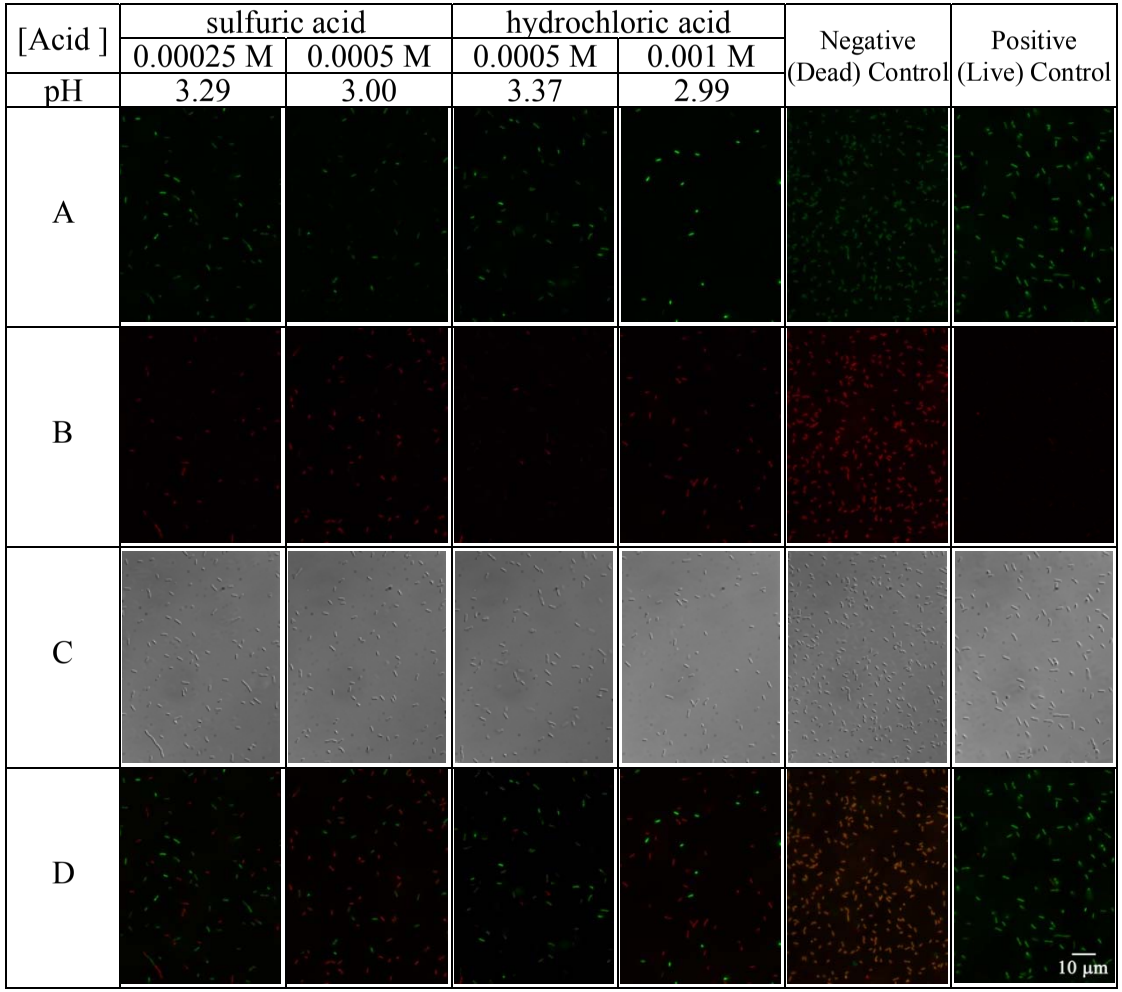


Figure A.38 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of hydrochloric acid and sulfuric acid for 5 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

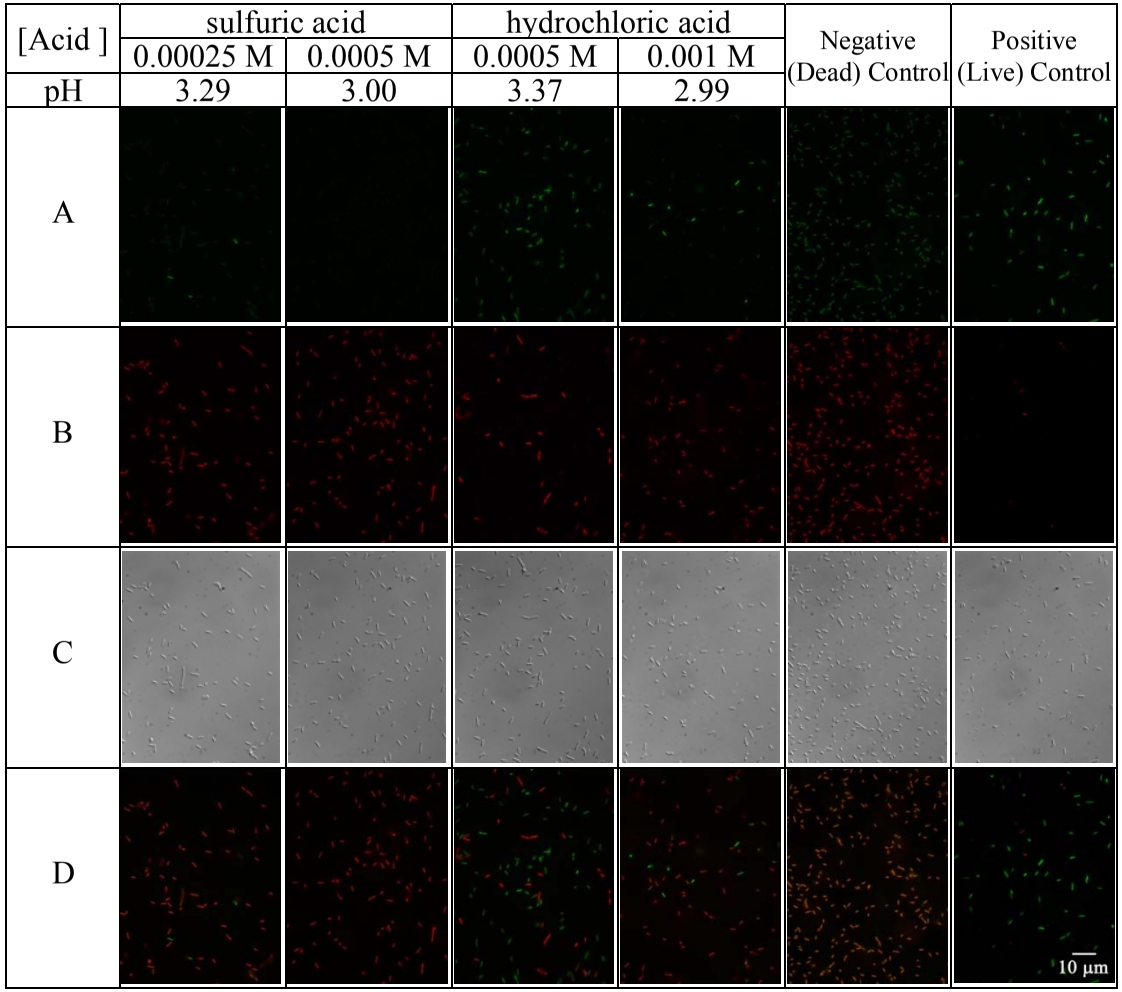


Figure A.39 Fluorescence microscopy images of mid-log phase *L. monocytogenes* CDC 7762 after exposure to different concentrations of hydrochloric acid and sulfuric acid for 24 h. (A) live bacterial cells, (B) dead bacterial cells, (C) total bacterial cells, (D) a merged image of live and dead bacterial cells.

Appendix B Statistical Data on Acid Stress

Table B.1 Standard plate count (A) and optical density (B) results for cells of stationary-phase *E. coli* DH5α after exposure to a range of concentrations of acetic acid and lactic acid for 1, 5 and 24 h.

A	[acid]	pH	Viable count (log CFU/mL)*		
			1 h	5 h	24 h
acetic acid	control	6.57	8.06 ± 0.03^{Aa}	8.06 ± 0.06^{Aa}	7.96 ± 0.07^{Aa}
	0.001 M	5.10	7.80 ± 0.05^{Ba}	7.92 ± 0.06^{Aa}	7.31 ± 0.05^{Bb}
	0.01 M	3.65	7.72 ± 0.08^{Ba}	6.61 ± 0.17^{Bb}	0.00 ± 0.00^{Cc}
	0.05 M	3.12	7.57 ± 0.02^{Ca}	4.24 ± 0.08^{Cb}	0.00 ± 0.00^{Cc}
	0.1 M	2.90	7.33 ± 0.05^{Da}	0.00 ± 0.00^{Db}	0.00 ± 0.00^{Cb}
	0.2 M	2.70	6.39 ± 0.05^{Ea}	0.00 ± 0.00^{Db}	0.00 ± 0.00^{Cb}
	0.5 M	2.46	0.00 ± 0.00^{Fa}	0.00 ± 0.00^{Da}	0.00 ± 0.00^{Ca}
lactic acid	control	6.57	8.22 ± 0.07^{Aa}	8.10 ± 0.08^{Aa}	7.95 ± 0.06^{Aa}
	0.001 M	4.87	7.97 ± 0.13^{Ba}	7.88 ± 0.06^{Ba}	7.52 ± 0.05^{Bb}
	0.005 M	3.29	7.44 ± 0.03^{Ca}	5.89 ± 0.10^{Cb}	0.00 ± 0.00^{Cc}
	0.01 M	3.01	7.53 ± 0.13^{Ca}	0.00 ± 0.00^{Db}	0.00 ± 0.00^{Cb}
	0.02 M	2.78	6.56 ± 0.10^{Da}	0.00 ± 0.00^{Db}	0.00 ± 0.00^{Cb}
	0.05 M	2.50	0.00 ± 0.00^{Ea}	0.00 ± 0.00^{Da}	0.00 ± 0.00^{Ca}

B	[acid]	pH	OD_{600}*		
			1 h	5 h	24 h
acetic acid	control	6.57	1.01 ± 0.01^{Aa}	1.02 ± 0.01^{Aa}	0.98 ± 0.01^{Aa}
	0.001 M	5.10	0.94 ± 0.01^{Ba}	0.92 ± 0.01^{Ba}	0.94 ± 0.01^{Ba}
	0.01 M	3.65	0.88 ± 0.03^{Ca}	0.89 ± 0.03^{Ca}	0.88 ± 0.03^{Ca}
	0.05 M	3.12	0.76 ± 0.01^{Da}	0.75 ± 0.01^{Da}	0.79 ± 0.01^{Da}
	0.1 M	2.90	0.75 ± 0.01^{Da}	0.75 ± 0.01^{Da}	0.79 ± 0.01^{Da}
	0.2 M	2.70	0.76 ± 0.01^{Da}	0.76 ± 0.01^{Da}	0.78 ± 0.01^{Da}
	0.5 M	2.46	0.74 ± 0.01^{Da}	0.78 ± 0.01^{Da}	0.77 ± 0.01^{Da}
lactic acid	control	6.57	1.01 ± 0.01^{Aa}	1.02 ± 0.01^{Aa}	0.97 ± 0.01^{Aa}
	0.001 M	4.87	0.85 ± 0.01^{Ba}	0.87 ± 0.02^{Ba}	0.91 ± 0.04^{Ba}
	0.005 M	3.29	0.75 ± 0.02^{Ca}	0.74 ± 0.02^{Ca}	0.76 ± 0.01^{Ca}
	0.01 M	3.01	0.71 ± 0.01^{Ca}	0.72 ± 0.01^{Ca}	0.74 ± 0.01^{Ca}
	0.02 M	2.78	0.70 ± 0.02^{Ca}	0.72 ± 0.02^{Ca}	0.75 ± 0.01^{Ca}
	0.05 M	2.50	0.71 ± 0.02^{Ca}	0.73 ± 0.01^{Ca}	0.75 ± 0.01^{Ca}

*Values represent mean ± standard error of the mean (n=9). Values with same uppercase letters (A, B, C, D, E, F) within columns are not significantly different ($P > 0.05$) among acid treatments. Values with same lowercase letters (a, b, c) within rows are not significantly different ($P > 0.05$) among treatment times.

Table B.2 Standard plate count (A) and optical density (B) results for cells of mid-log phase *E. coli* DH5α after exposure to a range of concentrations of acetic acid and lactic acid for 1, 5 and 24 h.

A	[acid]	pH	Viable count (log CFU/mL)*		
			1 h	5 h	24 h
acetic acid	control	6.57	8.39 ± 0.11^{Aa}	8.39 ± 0.07^{Aa}	8.35 ± 0.05^{Aa}
	0.001 M	5.10	7.98 ± 0.07^{Ba}	8.13 ± 0.08^{Ba}	7.64 ± 0.18^{Bb}
	0.01 M	3.65	6.89 ± 0.10^{Ca}	2.67 ± 0.08^{Cb}	0.00 ± 0.00^{Cc}
	0.05 M	3.12	5.93 ± 0.03^{Da}	0.00 ± 0.00^{Db}	0.00 ± 0.00^{Cb}
	0.1 M	2.90	2.93 ± 0.16^{Ea}	0.00 ± 0.00^{Db}	0.00 ± 0.00^{Cb}
	0.2 M	2.70	0.00 ± 0.00^{Fa}	0.00 ± 0.00^{Da}	0.00 ± 0.00^{Ca}
	0.5 M	2.46	0.00 ± 0.00^{Fa}	0.00 ± 0.00^{Da}	0.00 ± 0.00^{Ca}
lactic acid	control	6.57	8.30 ± 0.08^{Aa}	8.32 ± 0.05^{Aa}	8.38 ± 0.04^{Aa}
	0.001 M	4.87	7.84 ± 0.07^{Ba}	7.98 ± 0.14^{Ba}	7.12 ± 0.30^{Bb}
	0.005 M	3.29	6.39 ± 0.04^{Ca}	0.00 ± 0.00^{Cb}	0.00 ± 0.00^{Cb}
	0.01 M	3.01	3.73 ± 0.05^{Da}	0.00 ± 0.00^{Cb}	0.00 ± 0.00^{Cb}
	0.02 M	2.78	3.02 ± 0.06^{Ea}	0.00 ± 0.00^{Cb}	0.00 ± 0.00^{Cb}
	0.05 M	2.50	0.00 ± 0.00^{Fa}	0.00 ± 0.00^{Ca}	0.00 ± 0.00^{Ca}

B	[acid]	pH	OD_{600}*		
			1 h	5 h	24 h
acetic acid	control	6.57	1.01 ± 0.01^{Aa}	0.96 ± 0.01^{Ab}	0.77 ± 0.01^{Ac}
	0.001 M	5.10	0.94 ± 0.01^{Ba}	1.06 ± 0.01^{Bb}	1.11 ± 0.01^{Bc}
	0.01 M	3.65	0.90 ± 0.02^{Ca}	1.03 ± 0.02^{Bb}	1.08 ± 0.03^{Bc}
	0.05 M	3.12	0.82 ± 0.01^{Da}	0.91 ± 0.03^{Cb}	0.93 ± 0.02^{Cb}
	0.1 M	2.90	0.82 ± 0.02^{Da}	0.91 ± 0.02^{Cb}	0.93 ± 0.02^{Cb}
	0.2 M	2.70	0.84 ± 0.01^{Da}	0.90 ± 0.01^{Cb}	0.94 ± 0.01^{Cb}
	0.5 M	2.46	0.73 ± 0.02^{Ea}	0.78 ± 0.02^{Db}	0.81 ± 0.02^{Ab}
lactic acid	control	6.57	1.04 ± 0.01^{Aa}	0.95 ± 0.01^{Ab}	0.81 ± 0.02^{Ac}
	0.001 M	4.87	0.97 ± 0.02^{Ba}	1.04 ± 0.01^{Bb}	1.07 ± 0.01^{Bb}
	0.005 M	3.29	0.92 ± 0.02^{Ca}	0.99 ± 0.01^{Ab}	1.02 ± 0.01^{Cb}
	0.01 M	3.01	0.90 ± 0.01^{Ca}	0.98 ± 0.02^{Ab}	1.00 ± 0.02^{Cb}
	0.02 M	2.78	0.86 ± 0.02^{Ca}	0.96 ± 0.01^{Ab}	0.99 ± 0.02^{Cb}
	0.05 M	2.50	0.82 ± 0.02^{Da}	0.86 ± 0.01^{Cb}	0.89 ± 0.02^{Db}

*Values represent mean ± standard error of the mean (n=9). Values with same uppercase letters (A, B, C, D, E, F) within columns are not significantly different ($P > 0.05$) among acid treatments. Values with same lowercase letters (a, b, c) within rows are not significantly different ($P > 0.05$) among treatment times.

Table B.3 Standard plate count (A) and optical density (B) results for cells of mid-log phase *S. aureus* ATCC 13565 after exposure to a range of concentrations of acetic acid and lactic acid for 1, 5 and 24 h.

A	[acid]	pH	Viable count (log CFU/mL)*		
			1 h	5 h	24 h
acetic acid	control	6.57	8.36 ± 0.05^{Aa}	8.32 ± 0.05^{Aa}	8.05 ± 0.03^{Aa}
	0.001 M	5.10	8.43 ± 0.07^{Aa}	8.34 ± 0.07^{Aa}	7.04 ± 0.02^{Bb}
	0.01 M	3.65	8.29 ± 0.04^{Aa}	6.94 ± 0.17^{Bb}	3.39 ± 0.06^{Cc}
	0.05 M	3.12	7.90 ± 0.16^{Ba}	6.17 ± 0.03^{Cb}	0.00 ± 0.00^{Dc}
	0.1 M	2.90	7.60 ± 0.12^{Ba}	4.21 ± 0.03^{Db}	0.00 ± 0.00^{Dc}
	0.2 M	2.70	7.44 ± 0.13^{Ba}	1.72 ± 0.45^{Eb}	0.00 ± 0.00^{Dc}
	0.5 M	2.46	4.91 ± 0.08^{Ca}	0.00 ± 0.00^{Fb}	0.00 ± 0.00^{Db}
lactic acid	control	6.57	8.38 ± 0.05^{Aa}	8.34 ± 0.04^{Aa}	7.94 ± 0.06^{Aa}
	0.001 M	4.87	8.47 ± 0.08^{Aa}	8.34 ± 0.06^{Aa}	7.19 ± 0.16^{Bb}
	0.005 M	3.29	8.39 ± 0.07^{Aa}	8.03 ± 0.08^{Aa}	5.52 ± 0.06^{Cb}
	0.01 M	3.01	8.09 ± 0.08^{Aa}	7.31 ± 0.07^{Bb}	3.93 ± 0.06^{Dc}
	0.02 M	2.78	7.72 ± 0.11^{Ba}	5.61 ± 0.29^{Cb}	0.00 ± 0.00^{Ec}
	0.05 M	2.50	7.10 ± 0.15^{Ca}	2.30 ± 0.58^{Db}	0.00 ± 0.00^{Ec}

B	[acid]	pH	OD_{600}*		
			1 h	5 h	24 h
acetic acid	control	6.57	0.98 ± 0.01^{Aa}	0.94 ± 0.01^{Ab}	0.86 ± 0.00^{Ac}
	0.001 M	5.10	0.95 ± 0.02^{Ba}	0.93 ± 0.02^{Aa}	0.91 ± 0.02^{Ba}
	0.01 M	3.65	0.93 ± 0.02^{Ba}	0.90 ± 0.02^{Ba}	0.89 ± 0.02^{Aa}
	0.05 M	3.12	0.88 ± 0.01^{Ca}	0.83 ± 0.00^{Cb}	0.67 ± 0.01^{Cc}
	0.1 M	2.90	0.86 ± 0.00^{Ca}	0.79 ± 0.01^{Db}	0.61 ± 0.01^{Dc}
	0.2 M	2.70	0.82 ± 0.01^{Da}	0.77 ± 0.02^{Db}	0.58 ± 0.01^{Dc}
	0.5 M	2.46	0.76 ± 0.02^{Ea}	0.65 ± 0.02^{Eb}	0.39 ± 0.01^{Ec}
lactic acid	control	6.57	0.98 ± 0.01^{Aa}	0.95 ± 0.01^{Ab}	0.86 ± 0.00^{Ac}
	0.001 M	4.87	0.94 ± 0.01^{Ba}	0.92 ± 0.01^{Ba}	0.90 ± 0.00^{Ba}
	0.005 M	3.29	0.93 ± 0.00^{Ba}	0.91 ± 0.00^{Ba}	0.84 ± 0.02^{Ab}
	0.01 M	3.01	0.88 ± 0.01^{Ca}	0.83 ± 0.01^{Cb}	0.71 ± 0.01^{Cc}
	0.02 M	2.78	0.86 ± 0.01^{Ca}	0.82 ± 0.00^{Cb}	0.67 ± 0.01^{Dc}
	0.05 M	2.50	0.80 ± 0.01^{Da}	0.71 ± 0.01^{Db}	0.56 ± 0.01^{Ec}

*Values represent mean ± standard error of the mean (n=9). Values with same uppercase letters (A, B, C, D, E, F) within columns are not significantly different ($P > 0.05$) among acid treatments. Values with same lowercase letters (a, b, c) within rows are not significantly different ($P > 0.05$) among treatment times.

Table B.4 Standard plate count (A) and optical density (B) results for cells of mid-log phase *L. monocytogenes* CDC 7762 after exposure to a range of concentrations of acetic acid and lactic acid for 1, 5 and 24 h.

A	[acid]	pH	Viable count (log CFU/mL)*		
			1 h	5 h	24 h
acetic acid	control	6.57	9.24 ± 0.02^{Aa}	9.21 ± 0.03^{Aa}	8.44 ± 0.02^{Ab}
	0.001 M	5.10	9.16 ± 0.04^{Aa}	9.05 ± 0.02^{Aa}	5.07 ± 0.10^{Bb}
	0.01 M	3.65	8.85 ± 0.04^{Aa}	5.11 ± 0.29^{Bb}	1.62 ± 0.41^{Cc}
	0.05 M	3.12	7.84 ± 0.04^{Ba}	0.00 ± 0.00^{Cb}	0.00 ± 0.00^{Db}
	0.1 M	2.90	6.56 ± 0.22^{Ca}	0.00 ± 0.00^{Cb}	0.00 ± 0.00^{Db}
	0.2 M	2.70	0.00 ± 0.05^{Da}	0.00 ± 0.00^{Ca}	0.00 ± 0.00^{Da}
	0.5 M	2.46	0.00 ± 0.00^{Da}	0.00 ± 0.00^{Ca}	0.00 ± 0.00^{Da}
lactic acid	control	6.57	9.30 ± 0.05^{Aa}	9.20 ± 0.02^{Aa}	8.45 ± 0.01^{Ab}
	0.001 M	4.87	9.19 ± 0.04^{Aa}	8.98 ± 0.07^{Aa}	8.08 ± 0.04^{Bb}
	0.005 M	3.29	7.85 ± 0.04^{Ba}	5.05 ± 0.08^{Bb}	0.00 ± 0.00^{Cc}
	0.01 M	3.01	7.63 ± 0.23^{Ba}	4.26 ± 0.03^{Cb}	0.00 ± 0.00^{Cc}
	0.02 M	2.78	6.71 ± 0.07^{Ca}	0.00 ± 0.00^{Db}	0.00 ± 0.00^{Cb}
	0.05 M	2.50	4.16 ± 0.46^{Da}	0.00 ± 0.00^{Db}	0.00 ± 0.00^{Cb}

B	[acid]	pH	OD_{600}*		
			1 h	5 h	24 h
acetic acid	control	6.57	0.96 ± 0.01^{Aa}	0.88 ± 0.01^{Ab}	0.69 ± 0.02^{Ac}
	0.001 M	5.10	1.15 ± 0.02^{Ba}	1.05 ± 0.02^{Bb}	1.02 ± 0.01^{Bc}
	0.01 M	3.65	1.12 ± 0.01^{Ba}	1.04 ± 0.01^{Bb}	1.00 ± 0.01^{Bc}
	0.05 M	3.12	1.08 ± 0.01^{Ca}	1.04 ± 0.01^{Bb}	0.99 ± 0.01^{Bc}
	0.1 M	2.90	1.06 ± 0.01^{Ca}	1.01 ± 0.01^{Bb}	0.97 ± 0.02^{Bc}
	0.2 M	2.70	1.05 ± 0.01^{Ca}	0.97 ± 0.01^{Cb}	0.89 ± 0.01^{Cc}
	0.5 M	2.46	1.00 ± 0.01^{Da}	0.89 ± 0.01^{Ab}	0.73 ± 0.02^{Dc}
lactic acid	control	6.57	0.95 ± 0.01^{Aa}	0.87 ± 0.01^{Ab}	0.68 ± 0.01^{Ac}
	0.001 M	4.87	1.13 ± 0.02^{Ba}	1.10 ± 0.02^{Ba}	1.00 ± 0.02^{Bb}
	0.005 M	3.29	1.09 ± 0.01^{Ca}	1.06 ± 0.01^{Ca}	1.00 ± 0.00^{Bb}
	0.01 M	3.01	1.08 ± 0.01^{Ca}	1.03 ± 0.01^{Cb}	0.97 ± 0.01^{Bc}
	0.02 M	2.78	1.07 ± 0.01^{Ca}	0.99 ± 0.01^{Db}	0.89 ± 0.01^{Cc}
	0.05 M	2.50	1.01 ± 0.01^{Da}	0.88 ± 0.01^{Ab}	0.80 ± 0.02^{Dc}

*Values represent mean ± standard error of the mean (n=9). Values with same uppercase letters (A, B, C, D, E, F) within columns are not significantly different ($P > 0.05$) among acid treatments. Values with same lowercase letters (a, b, c) within rows are not significantly different ($P > 0.05$) among treatment times.

Table B.5 Standard plate count (A) and optical density (B) results for cells of stationary-phase *E. coli* DH5α after exposure to a range of concentrations of hydrochloric acid and sulfuric acid for 1, 5 and 24 h.

A	[acid]	pH	Viable count (log CFU/mL)*		
			1 h	5 h	24 h
HCl	control	6.57	8.07 ± 0.02^{Aa}	7.95 ± 0.03^{Aa}	7.87 ± 0.04^{Aa}
	0.0005 M	3.37	7.43 ± 0.07^{Ba}	7.39 ± 0.08^{Ba}	6.28 ± 0.06^{Bb}
	0.001 M	2.99	7.04 ± 0.15^{Ca}	5.72 ± 0.14^{Cb}	4.83 ± 0.11^{Cc}
H_2SO_4	control	6.57	8.07 ± 0.02^{Aa}	7.95 ± 0.03^{Aa}	7.87 ± 0.04^{Aa}
	0.00025 M	3.29	7.55 ± 0.13^{Ba}	7.32 ± 0.07^{Ba}	6.46 ± 0.07^{Bb}
	0.0005 M	3.00	6.82 ± 0.22^{Ca}	4.41 ± 0.10^{Cb}	3.10 ± 0.10^{Cc}

B	[acid]	pH	OD_{600}*		
			1 h	5 h	24 h
HCl	control	6.57	1.01 ± 0.01^{Aa}	1.02 ± 0.01^{Aa}	1.00 ± 0.02^{Aa}
	0.0005 M	3.37	0.82 ± 0.01^{Ba}	0.80 ± 0.01^{Ba}	0.79 ± 0.01^{Ba}
	0.001 M	2.99	0.72 ± 0.01^{Ca}	0.71 ± 0.00^{Ca}	0.70 ± 0.00^{Ca}
H_2SO_4	control	6.57	1.01 ± 0.01^{Aa}	1.02 ± 0.01^{Aa}	1.00 ± 0.02^{Aa}
	0.00025 M	3.29	0.83 ± 0.01^{Ba}	0.83 ± 0.01^{Ba}	0.84 ± 0.01^{Ba}
	0.0005 M	3.00	0.72 ± 0.01^{Ca}	0.73 ± 0.01^{Ca}	0.73 ± 0.00^{Ca}

*Values represent mean ± standard error of the mean (n=9). Values with same uppercase letters (A, B, C) within columns are not significantly different ($P > 0.05$) among acid treatments. Values with same lowercase letters (a, b, c) within rows are not significantly different ($P > 0.05$) among treatment times.

Table B.6 Standard plate count (A) and optical density (B) results for cells of mid-log phase *E. coli* DH5α after exposure to a range of concentrations of hydrochloric acid and sulfuric acid for 1, 5 and 24 h.

A	[acid]	pH	Viable count (log CFU/mL)*		
			1 h	5 h	24 h
HCl	control	6.57	8.25 ± 0.07^{Aa}	8.35 ± 0.05^{Aa}	8.36 ± 0.04^{Aa}
	0.0005 M	3.37	7.46 ± 0.08^{Ba}	6.13 ± 0.05^{Bb}	3.60 ± 0.07^{Bc}
	0.001 M	2.99	7.07 ± 0.09^{Ca}	4.79 ± 0.10^{Cb}	3.23 ± 0.05^{Cc}
H_2SO_4	control	6.57	8.25 ± 0.07^{Aa}	8.35 ± 0.05^{Aa}	8.36 ± 0.04^{Aa}
	0.00025 M	3.29	7.41 ± 0.11^{Ba}	6.22 ± 0.07^{Bb}	3.81 ± 0.06^{Bc}
	0.0005 M	3.00	6.69 ± 0.11^{Ca}	3.90 ± 0.06^{Cb}	3.05 ± 0.07^{Cc}

B	[acid]	pH	OD_{600}*		
			1 h	5 h	24 h
HCl	control	6.57	1.01 ± 0.01^{Aa}	0.93 ± 0.01^{Ab}	0.78 ± 0.01^{Ac}
	0.0005 M	3.37	0.96 ± 0.01^{Ba}	1.20 ± 0.01^{Bb}	1.27 ± 0.00^{Bc}
	0.001 M	2.99	0.97 ± 0.01^{Ba}	1.13 ± 0.01^{Cb}	1.15 ± 0.01^{Cb}
H_2SO_4	control	6.57	1.01 ± 0.01^{Aa}	0.93 ± 0.01^{Ab}	0.78 ± 0.01^{Ac}
	0.00025 M	3.29	0.97 ± 0.01^{Ba}	1.17 ± 0.02^{Bb}	1.29 ± 0.02^{Bc}
	0.0005 M	3.00	0.94 ± 0.01^{Ba}	1.08 ± 0.01^{Cb}	1.10 ± 0.01^{Cb}

*Values represent mean ± standard error of the mean (n=9). Values with same uppercase letters (A, B, C) within columns are not significantly different ($P > 0.05$) among acid treatments. Values with same lowercase letters (a, b, c) within rows are not significantly different ($P > 0.05$) among treatment times.

Table B.7 Standard plate count (A) and optical density (B) results for cells of mid-log phase *S. aureus* ATCC 13565 after exposure to a range of concentrations of hydrochloric acid and sulfuric acid for 1, 5 and 24 h.

A	[acid]	pH	Viable count (log CFU/mL)*		
			1 h	5 h	24 h
HCl	control	6.57	8.34 ± 0.06^{Aa}	8.30 ± 0.07^{Aa}	8.02 ± 0.03^{Ab}
	0.0005 M	3.37	8.33 ± 0.08^{Aa}	7.66 ± 0.13^{Bb}	5.36 ± 0.08^{Bc}
	0.001 M	2.99	8.21 ± 0.09^{Aa}	6.65 ± 0.15^{Cb}	3.98 ± 0.10^{Cc}
H_2SO_4	control	6.57	8.34 ± 0.06^{Aa}	8.30 ± 0.07^{Aa}	8.02 ± 0.03^{Ab}
	0.00025 M	3.29	8.34 ± 0.08^{Aa}	7.74 ± 0.14^{Bb}	5.34 ± 0.07^{Bc}
	0.0005 M	3.00	8.14 ± 0.09^{Aa}	6.57 ± 0.16^{Cb}	3.98 ± 0.11^{Cc}

B	[acid]	pH	OD_{600}*		
			1 h	5 h	24 h
HCl	control	6.57	0.99 ± 0.01^{Aa}	0.95 ± 0.01^{Ab}	0.84 ± 0.01^{Ac}
	0.0005 M	3.37	0.87 ± 0.01^{Ba}	0.83 ± 0.01^{Bb}	0.78 ± 0.01^{Bc}
	0.001 M	2.99	0.86 ± 0.01^{Ba}	0.81 ± 0.01^{Bb}	0.73 ± 0.01^{Cc}
H_2SO_4	control	6.57	0.99 ± 0.01^{Aa}	0.95 ± 0.01^{Ab}	0.84 ± 0.01^{Ac}
	0.00025 M	3.29	0.85 ± 0.01^{Ba}	0.81 ± 0.01^{Bb}	0.77 ± 0.01^{Bc}
	0.0005 M	3.00	0.84 ± 0.01^{Ba}	0.79 ± 0.01^{Bb}	0.74 ± 0.01^{Cc}

*Values represent mean ± standard error of the mean (n=9). Values with same uppercase letters (A, B, C) within columns are not significantly different ($P > 0.05$) among acid treatments. Values with same lowercase letters (a, b, c) within rows are not significantly different ($P > 0.05$) among treatment times.

Table B.8 Standard plate count (A) and optical density (B) results for cells of mid-log phase *L. monocytogenes* CDC 7762 after exposure to a range of concentrations of hydrochloric acid and sulfuric acid for 1, 5 and 24 h.

A	[acid]	pH	Viable count (log CFU/mL)*		
			1 h	5 h	24 h
HCl	control	6.57	9.15 ± 0.03^{Aa}	9.08 ± 0.04^{Aa}	8.38 ± 0.04^{Ab}
	0.0005 M	3.37	8.15 ± 0.06^{Ba}	7.37 ± 0.10^{Bb}	5.50 ± 0.17^{Bc}
	0.001 M	2.99	7.27 ± 0.05^{Ca}	5.89 ± 0.10^{Cb}	1.20 ± 0.38^{Cc}
H_2SO_4	control	6.57	9.15 ± 0.03^{Aa}	9.08 ± 0.04^{Aa}	8.38 ± 0.04^{Ab}
	0.00025 M	3.29	8.21 ± 0.09^{Ba}	7.69 ± 0.12^{Bb}	3.83 ± 0.06^{Bc}
	0.0005 M	3.00	7.13 ± 0.05^{Ca}	4.59 ± 0.05^{Cb}	0.00 ± 0.00^{Cc}

B	[acid]	pH	OD_{600}*		
			1 h	5 h	24 h
HCl	control	6.57	0.96 ± 0.01^{Aa}	0.88 ± 0.01^{Ab}	0.69 ± 0.01^{Ac}
	0.0005 M	3.37	1.21 ± 0.01^{Ba}	1.21 ± 0.01^{Ba}	1.17 ± 0.01^{Bb}
	0.001 M	2.99	1.05 ± 0.01^{Ca}	1.02 ± 0.01^{Ca}	0.88 ± 0.01^{Cb}
H_2SO_4	control	6.57	0.96 ± 0.01^{Aa}	0.88 ± 0.01^{Ab}	0.69 ± 0.01^{Ac}
	0.00025 M	3.29	1.23 ± 0.01^{Ba}	1.21 ± 0.01^{Ba}	1.11 ± 0.01^{Bb}
	0.0005 M	3.00	1.07 ± 0.01^{Ca}	1.04 ± 0.01^{Ca}	1.01 ± 0.00^{Cb}

*Values represent mean ± standard error of the mean (n=9). Values with same uppercase letters (A, B, C) within columns are not significantly different ($P > 0.05$) among acid treatments. Values with same lowercase letters (a, b, c) within rows are not significantly different ($P > 0.05$) among treatment times.

Appendix C Additional Optical Microscopy Images

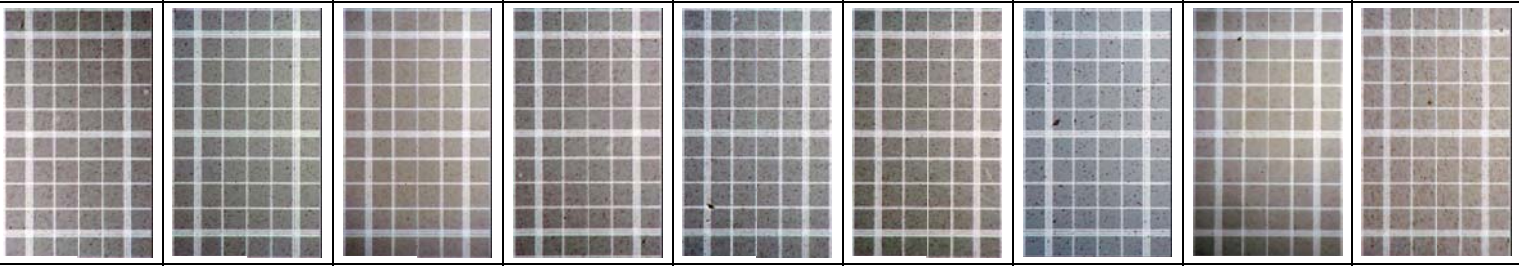
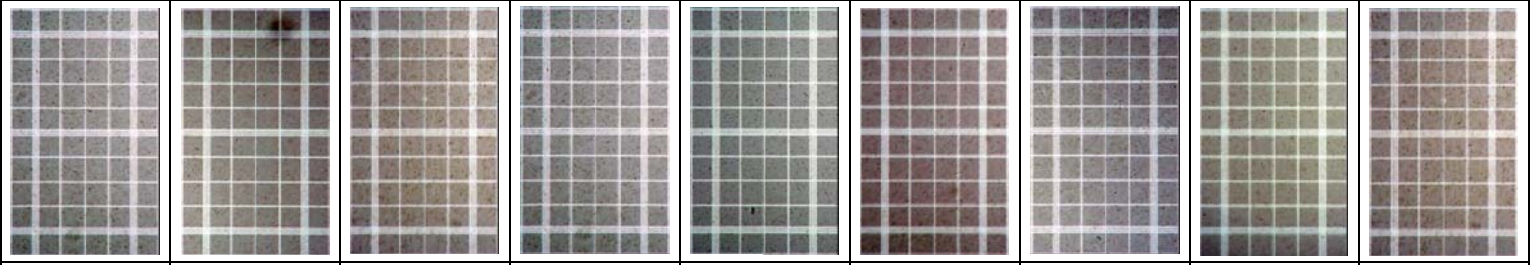
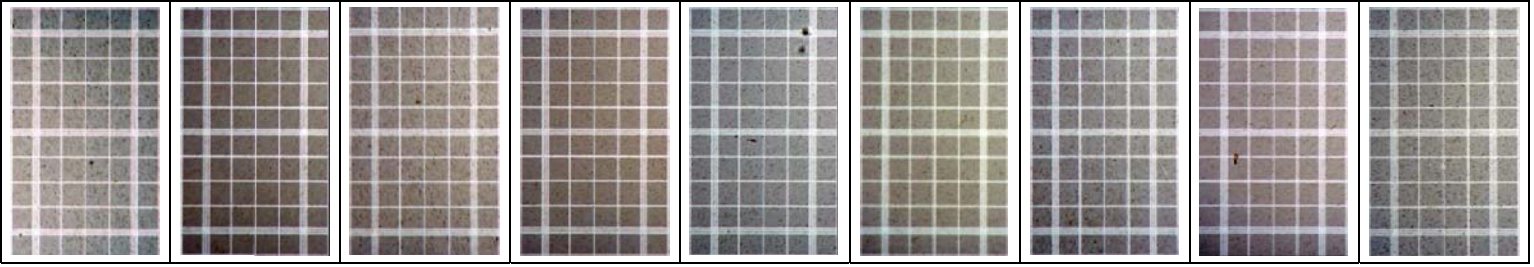
treatment	low temperature			high temperature		sodium chloride			control
	-20°C	4°C	10°C	55°C	60°C	5%	15%	25%	
1 h									
5 h									
24 h									

Figure C.1 Optical microscopy images (10 ×) of stationary-phase *E. coli* DH5α after exposure to low temperatures, high temperatures and high concentrations of sodium chloride for 1, 5 and 24 h.

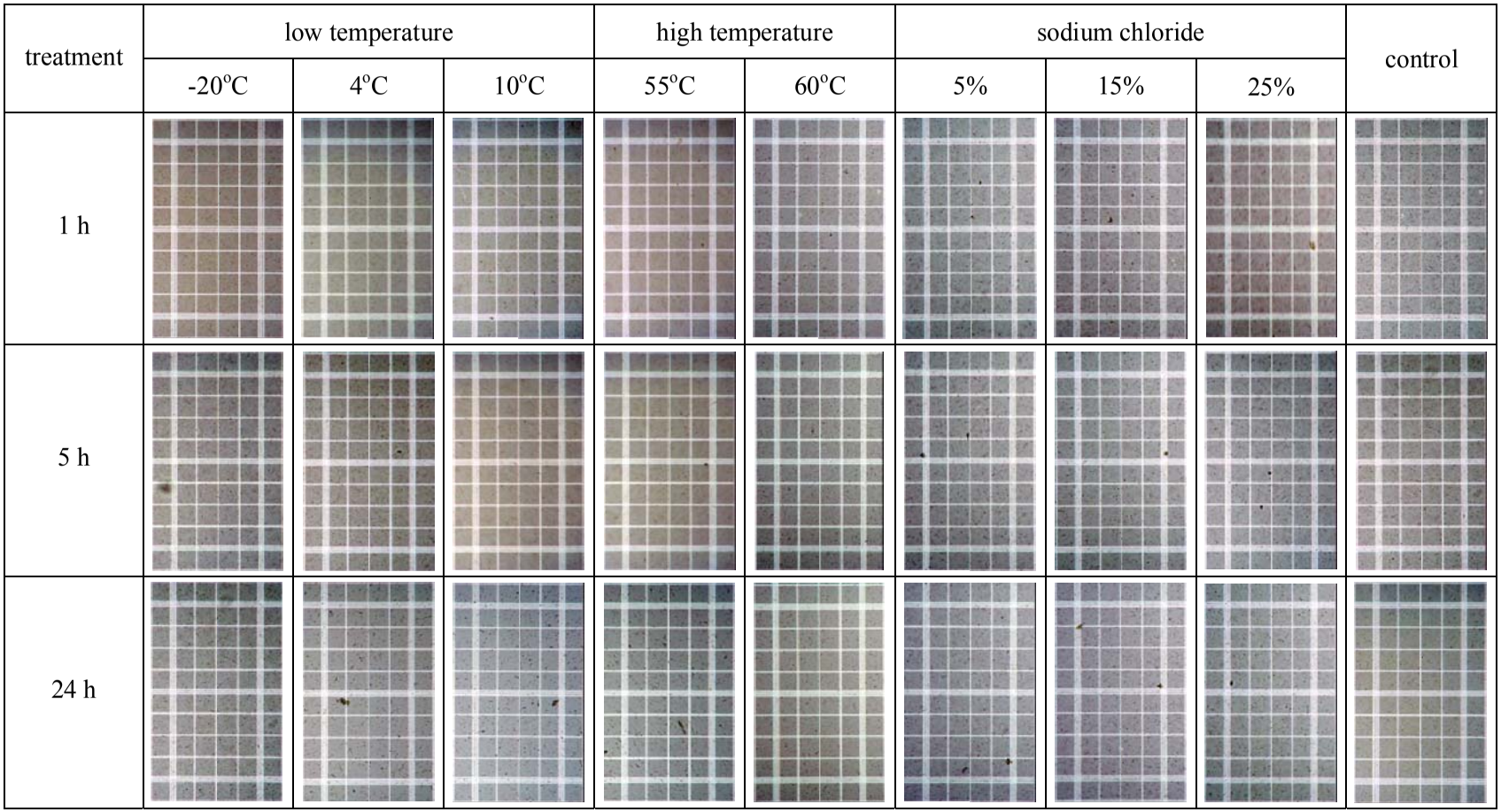


Figure C.2 Optical microscopy images (10 ×) of mid-log phase *E. coli* DH5α after exposure to low temperatures, high temperatures and high concentrations of sodium chloride for 1, 5 and 24 h.

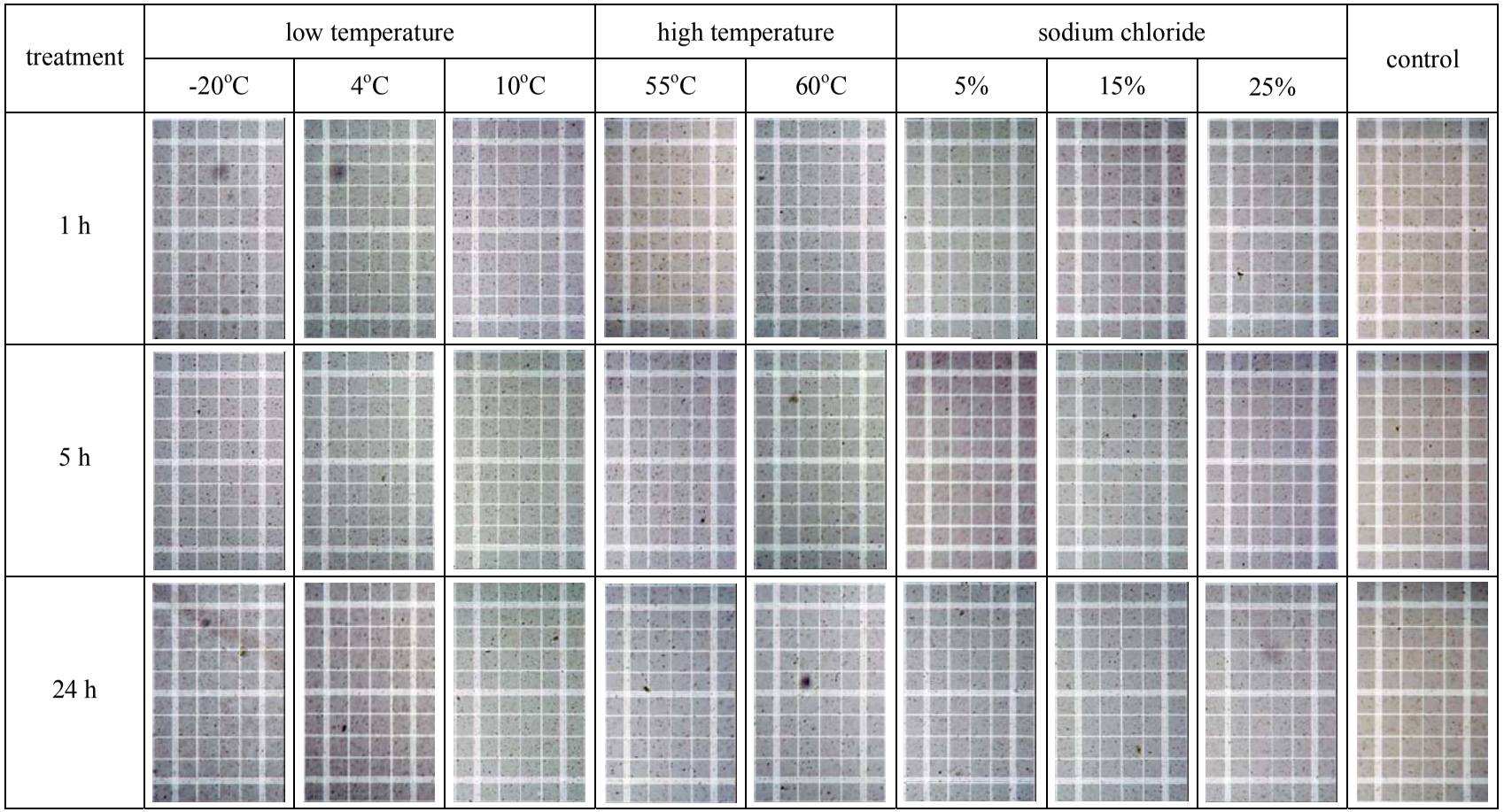


Figure C.3 Optical microscopy images (10 ×) of mid-log phase *S. aureus* ATCC 13565 after exposure to low temperatures, high temperatures and high concentrations of sodium chloride for 1, 5 and 24 h.