Acoustic Emission of *Lactococcus lactis* ssp. *lactis* C2 Infected with Three Bacteriophages c2, sk1 and ml3

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Acoustic emission of *Lactococcus lactis* ssp. *lactis* C2 infected with three bacteriophages c2, sk1 and ml3.

---

**THESIS**

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Agriculture, Food and Environment at the University of Kentucky

By

Luxi Meng
Lexington, Kentucky

Director: Dr. Hicks, Professor of Animal and Food Science
Lexington, Kentucky

2016

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Acoustic emission of *Lactococcus lactis* ssp. *lactis* C2 infected with three bacteriophages c2, sk1 and ml3.

The objective of this research was to monitor the Acoustic Emission (AE) produced by *Lactococcus lactis* ssp. *lactis* C2 infected with three bacteriophages (c2, sk1 and ml3 at 90 min) using an acoustic emission-monitoring device which was designed at University of Kentucky in Lexington KY. The acoustic emission data was collected and then analyzed. These Acoustic Emission (AE) data suggested that bacteriophage ml3, sk1 and c2 can easily be distinguished by the differences in Absolute Energy (ABE), Centroid Frequency (CF) and Peak Frequency (PF) signals. The AE data suggested that bacteriophage sk1 and c2 caused greater stress on the *lactis* C2 than bacteriophage ml3. When the bacteriophage were added to the *lactis* C2 at 90 min, the host bacteria shifted from cell division processes to the development of bacteriophage replications. When the bacteriophage replications were initiated, the AE information being emitted increased significantly. The distinguishable difference produced by AE could provide a novel method to identify different bacteriophage and track certain metabolic and cell growth parameters of the host bacteria. Further research is still needed to identify where the AE information is being generated and whether all bacteriophage are as different as those used in this study.

KEYWORDS: Acoustic Emissions, *Lactococcus lactis* ssp. *lactis* C2, bacteriophage, M17 medium, absolute energy, centroid frequency, peak frequency

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January 26, 2016
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This thesis is dedicated to my lovely professor and my dear parents:

Thanks to my professor unreserved help and guidance and thanks to my parents continuous support in my life and spirit

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# TABLE OF CONTENTS

ACKNOWLEDGEMENTS .................................................................................................................. iii

TABLE OF CONTENTS .................................................................................................................. iv

LIST OF TABLES .......................................................................................................................... vii

LIST OF FIGURES ....................................................................................................................... viii

CHAPTER 1 ................................................................................................................................... 1

INTRODUCTION .................................................................................................................................. 1

CHAPTER 2 ................................................................................................................................... 3

LITERATURE REVIEW ................................................................................................................... 3

2.1 Sound propagation .................................................................................................................... 3
    2.1.1 Categories of waves ............................................................................................................ 3
    2.1.2 Properties of Transverse Sound waves .............................................................................. 5
    2.1.3 Sound wave propagation ................................................................................................... 6
    2.1.4 Sound wave travel speeds ................................................................................................. 6

2.2 Acoustic emission background ............................................................................................... 8
    2.2.1 Acoustics definition .......................................................................................................... 8
    2.2.2 Acoustics Emission ............................................................................................................ 8
    2.2.3 Key terminology in Acoustic Emission study ................................................................. 10

2.3 Bacteria growth and communication .................................................................................... 10
    2.3.1 *Lactococcus lactis* ssp. *lactis* C2 bacteria growth ...................................................... 11
    2.3.2 Bacteria communication through chemical signals ......................................................... 11
    2.3.3 Bacteria communications through physical signals ........................................................ 13

2.4 Bacteriophage development and the mechanism of infection ............................................... 14
    2.4.1 The mechanism of bacteria cell lyse by bacteriophage .................................................. 15
    2.4.2 Infection of *Lactococcus lactis* ssp. *lactis* C2 with c2, sk1 and ml3 bacteriophage .... 16
    2.4.3 The effect of CaCl₂ solution on bacteriophage infection ................................................ 18

2.5 Generations of acoustic emission study by Dr. Hick’s team .................................................. 20
    2.5.1 The first generation of acoustic emission-monitoring device ....................................... 20
    2.5.2 The second generation of acoustic emission-monitoring device .................................... 20
    2.5.3 The third generation of acoustic emission-monitoring device ..................................... 21
    2.5.4 The fourth generation of acoustic emission-monitoring device .................................. 21
CHAPTER 3 ................................................................................................................................. 24

MATERIALS AND METHODS .................................................................................................. 24

3.1 Biological and chemical supplies ......................................................................................... 24
3.2 Stock of Bacterial strain and bacteriophages ....................................................................... 25
3.3 Preparation of medium for bacteriological and phage assays .............................................. 25
  3.3.1 Preparation of M17 medium ............................................................................................ 26
  3.3.2 Preparation of skim milk .................................................................................................. 26
  3.3.3 Preparation of bottom agar and top agar ....................................................................... 26
  3.3.4 Preparation of CaCl2 solution ......................................................................................... 27
3.4 Selection of C2 culture to make new stock culture ................................................................. 27
3.5 Bacteria and bacteriophages propagation to make new stocks ............................................ 27
  3.5.1 Bacteria propagation ....................................................................................................... 28
  3.5.2 Bacteriophages propagation ............................................................................................ 28
3.6 Enumeration of stock bacteriophages ................................................................................... 28
3.7 Working bacteria culture preparation for acoustic emission monitoring ................................ 29
3.8 Latest generation of acoustic emission-monitoring device ...................................................... 30
  3.8.1 Setup of apparatus before data collection ....................................................................... 31
  3.8.2 Acoustic emission sensor ............................................................................................... 33
  3.8.3 Modified acoustic emission cylinder tube with voltage measurement ............................. 34
  3.8.4 Acoustic emission data collection .................................................................................. 38
  3.8.5 Optical density and voltage measurements during acoustic emission data collection ...... 39
3.9 Estimate growth of Lactococcus lactis ssp. lactis C2 concentration during the acoustic emission data collection ................................................................. 40
3.10 Statistical analysis of acoustic emission data ...................................................................... 40

CHAPTER 4 ................................................................................................................................. 43

RESULTS AND DISCUSSION ................................................................................................... 43

4.1 Growth of Lactococcus lactis ssp. lactis C2 .......................................................................... 43
4.2 Monitoring bacteriophage activity during host growth and lysis ........................................... 46
  4.2.1 Absorbance measurements ............................................................................................. 46
  4.2.2 Comparison of Voltage and Absorbance measurements .............................................. 47
4.3 Acoustic Emission of Lactococcus lactis ssp. lactis C2 from 0 to 90 min ......................... 51
  4.3.2 Absolute Energy emissions during lag phase ................................................................. 52
  4.3.3 Changes in Centroid and Peak frequencies in the lag phase ......................................... 52
4.4 Statistic results of acoustic emission data from 0-240 minutes .......................................... 56
4.5 Acoustic emission data from 90-240 minute ...................................................................... 58
  4.5.1 Log phase growth ......................................................................................................... 58
4.6 Effect of bacteriophage on *L. lactis* ssp *lactis* C2 from 90-120 min............... 61
4.7 Determination of bacteriophage cycles......................................................... 63
   4.7.1 Infection cycle of ml3. ................................................................. 63
   4.7.2 Infection cycle of sk1. ................................................................. 64
   4.7.3 Infection cycle of c2................................................................. 64
4.8 Effect of bacteriophage infection on host, *lactis* C2, stress................... 65

CHAPTER 5 ................................................................................................................................. 70
CONCLUSION AND FUTURE WORK ..................................................................................... 70
BIBLIOGRAPHY ......................................................................................................................... 72
VITA ............................................................................................................................................. 76
LIST OF TABLES
Table 1. Speed of sound in different medium at specific temperature conditions (gases, liquids and solids) ................................................................................................................................................. 7
Table 2. Biological and chemical supplies ................................................................................................................. 24
Table 3. Composition of DifcoTM M17 broth (37.25g) made to1000ml with purified water ..... 25
Table 4. The composition of top agar mixture to overlay on the bottom agar plate................. 29
Table 5. Enumeration of bacteriophage in working culture ................................................................. 46
LIST OF FIGURES

Figure 1 Sound waves are longitudinal waves................................................................. 4
Figure 2 Schematic of transvers wave. ............................................................................. 4
Figure 3 A basic sine wave .............................................................................................. 5
Figure 4. Lindsay's Wheel of Acoustics illustrates nearly all the fields of acoustics field (Lindsey, 1964). .......................................................... 9
Figure 5. Examples of autoinducers used by Gram-negative and Gram-positive bacteria....... 13
Figure 6. The lytic bacteriophage infection cycle of lytic bacteriophage. ....................... 16
Figure 7. The first generation of acoustic emission-monitoring device (figures and photos obtained from Hicks photo library).................................................... 22
Figure 8. The second generation of acoustic emission-monitoring device (figures and photos obtained from Hicks phot library)................................................................. 23
Figure 9. The third generation of acoustic emission-monitoring device (figures and photos obtained from Hicks photo library)................................................................. 23
Figure 10. The complete set up of acoustic emission-monitoring device (included the dual acoustic emission compartment, computer system and voltage meter)................. 32
Figure 11. Inside view of one of the environmental chambers. Arrow shows tube and tube holder. ..................................................................................................................... 32
Figure 12. Clear PVC tube containing an o-ring and R-6α sensor ...................................... 32
Figure 13. Acoustic sensor (α-serials Model R6α-SNAD 52) .......................................... 33
Figure 14. Modified acoustic emission PVC tube. ......................................................... 35
Figure 15. Schematic modified acoustic emission PVC tube............................................. 36
Figure 16. The controller circuit board .......................................................................... 37
Figure 17. Volt Ohm meter (VOM) used to read voltage measurement ......................... 38
Figure 18. Lactis C2 culture growth curve as measured by CFU and OD600nm ............... 45
Figure 19. Absorbance and voltage measurement without bacteriophage infection .......... 45
Figure 20. Photograph of raw data being received from the Acoustic Energy sensor showing Hits vs Time as recorded on channel 4. Time is in seconds on x axis and number of Hits is on y axis. ................................................................................................................................. 46
Figure 21. Average absorbance of Lactococcus lactis ssp. lactis C2 with and without being infected by bacteriophage ml3, sk1 and c2 ................................................................. 48
Figure 22. Average absorbance and voltage measurement when Lactococcus lactis ssp. lactis C2 was infected with bacteriophage ml3................................................................. 49
Figure 23. Average absorbance and voltage measurement when Lactococcus lactis ssp. lactis C2 was infected with bacteriophage sk1........................................................... 49
Figure 24. Average absorbance and voltage measurement when Lactococcus lactis ssp. lactis C2 was infected with bacteriophage c2 ............................................................. 50
Figure 25. Comparison of Absolute Energy (ABE) LSMEANs for each treatment from 0-90 min. ......................................................................................................................... 53
Figure 26. Comparison of Centroid frequency (CF) LSMEANS for each treatment from 0-90 min ..................................................................................................................... 54
Figure 27. Comparison of Peak frequency (PF) LSMEANS for each treatment from 0-90 min. 54
Figure 28. Absolute Energy (ABE) LSMEANS for 5 minutes interval from 0-90 min ............. 54
Figure 29. Centroid frequency (CF) LSMEANS for 5 minutes interval from 0-90 min ........ 55
Figure 30. Peak Frequency (PF) LSMEANs for 5 minutes interval from 0-90 min ............... 55
Figure 31. Absolute energy (ABE) LSMEANS for each bacteriophage between 0-240min .... 57
Figure 32. Centroid Frequency (CF) LSMEANS for each bacteriophage between 0-240 min... 57
Figure 33. Peak Frequency (PF) LSMEANS for each bacteriophage between 0-240 min ....... 58
Figure 34. Absolute energy (ABE) emitted for each bacteriophage between 90-240 min .... 59
Figure 35. Centroid Frequency (CF) LSMEANS for each bacteriophage between 90-240 min. 60
Figure 36. Peak Frequency (PF) LSMEANS for each bacteriophage in 90-240 min .............. 60
Figure 37. Absolute Energy (ABE) LSMEANS from 90-120 min ........................................ 62
Figure 38. Centroid Frequency (CF) LSMEANS from 90-120 min ........................................ 62
Figure 39. Peak Frequency (PF) LSMEANS from 90-120 min ........................................... 63
Figure 40. ABE LSMEANS for 5 min interval from 90-120 min ........................................... 66
Figure 41. Centroid Frequency LSMEANS for 5 min interval from 90-120 min ................... 67
Figure 42. PF LSMEANS for 5 min interval from 90-120 min ............................................. 68
Figure 43. Centroid Frequency LSMEANs for effect treatment and time from 90-120 min ... 68
CHAPTER 1

INTRODUCTION

1. Introduction

Acoustic emission (AE) is the phenomena of acoustic waves being generated in materials, objects, humans or even small bacteria. An AE is caused by a rapid release of stress energy, which then propagates elastic waves.

This research monitored AE produced by bacteria in a growth medium. Bacteria can produce chemical signaling as well as physical signaling when they are in their growth cycle (Matsuhashi et al., 1998; Stephan and Bassler, 2001; Ng WL, 2009; Rutherford and Bassler, 2012). Many microbiologists have focused on the chemical signaling produced by bacteria (Rutherford and Bassler, 2012) known as quorum sensing in the last decade while a few others (Matsuhashi et al., 1998; Pelling et al., 2004) have reported on physical signaling in bacteria.

Bacteria have been observed to respond to sound signals such as ultrasound, but also to generate sound signals as well. Matsuhashi at al. (1998) reported continuous single sine waves produced by a speaker at frequencies of 6-10, 18-22 and 28-38 kHz promoted colony formation in Bacillus carboniphilus. They also, observed the detection of sound waves emitted from Bacillus subtilis cells at frequencies between 8 to 43 kHz with board peaks at 8.5, 19, 29 and 37 kHz by a sensitive microphone system. They suggested that the detected sound waves were a function of growth-regulatory-signals, which they called “biosonics” between cells.

C. L. Hicks and associates (Hicks et al., 2004; Wardani et al., 2010; Ghosh et al., 2013; ChapotChartier and Kulakauskas, 2014) at University of Kentucky, Department of Animal and Food Science started to investigate this novel approach in 2007. The monitoring device for the acoustic emissions was developed in partnership with TriboFlow Separations in Lexington, KY. Several generations of apparatus were designed to enhance the sensitivity of the equipment. Initial studies on AE were conducted on Escherichia coli and Lactococcus lactis ssp. lactis C2 during their growth cycle in lag, log and stationary phases and where bacteriophage were used to infect Lactococcus lactis ssp. lactis C2 (Wardani et al., 2010; Ghosh et al., 2013; Cox, 2014). T.
Cox (Cox, 2014) reported that three strains of *Escherichia coli* (5024-parent, 8279-mutant and 8279-unrelated strain) generate different AE’s thus producing a unique fingerprint of sound which could contribute to bacteria identification. Agustin et al. (2010) conducted a study to distinguish the sound of infection by two lactococcal bacteriophage (sk1 and ml3) using an acoustic emission monitoring technique and found a difference between infection cycles for bacteriophage sk1 and ml3.

The objective of this research was to monitor the acoustic emissions produced by *Lactococcus lactis* ssp. lactis C2 infected with three bacteriophages (c2, sk1 and ml3 at 90 min) using a new detector setup that was 8 times more sensitive than that used by Agustin et al. (2010) and to monitor the bacteria growth for up to 4 hours. The data were then analyze in a more complex manner to differentiate the three different bacteriophages, to partially characterize the bacteria metabolism with or without phage presence, and some of host metabolic sounds prior to infection.
CHAPTER 2

LITERATURE REVIEW

2.1 Sound propagation

Vibration in different objects generates sound. Sound can propagates as audible mechanical waves in media, e.g. gases, liquids and solids. Sound cannot travel without a medium. In a vacuum condition, the sound stops its propagation. The mechanical waves are not capable of transmitting energy in a vacuum.

2.1.1 Categories of waves

Waves can be divided into two categories: mechanical waves and electromagnetic waves. Electromagnetic wave is a kind of wave, which does not require a medium to propagate, whereas, mechanical wave requires a medium to transfer. Electromagnetic waves have a wide range of waves such as light, microwaves, X-rays, infrared, ultraviolet rays and radio waves. Mechanical waves can be divided into longitudinal waves and transvers waves. Sound waves are typical longitudinal waves, which requires a medium for transfer. The direction of wave travel is parallel to the displacement of medium. Figure 1 shows the features of longitudinal waves. These waves consist of a compression region, a rarefaction region and a wavelength. The compression region represents the area of high pressure, while the rarefaction region indicates the area of low pressure. The wavelength, \( \lambda \) is the distance between two close compression regions. A transvers wave is showed in Figure 2. By definition, the direction of wave travel is perpendicular to the displacement of medium particles. The wavelength of a transvers wave is the distance between two crests.
Figure 1 Sound waves are longitudinal waves.

The direction of wave travel is parallel to the displacement of medium.

Figure 2 Schematic of transvers wave.

The direction of wave travel is perpendicular to the displacement of medium particles.
2.1.2 Properties of Transverse Sound waves

A basic sine wave is illustrated in Figure 3. Sound waves can be characterized similarly by the properties of: frequency, wavelength, amplitude, speed of sound and wave number. The wavelength of a sine sound can be measured between two successive peaks or any two corresponding points on the cycle (Pohlmann, 2001). The distance travelled by the sound wave during one cycle of vibration is called the wavelength (Drury, 2004). Amplitude is the unit to reflect the strength or power of a wave signal. Amplitude corresponds with the intensity of wave as it travels. The loudness of the sound depends on the amplitude or intensity of wave itself.

Frequency is the number of times the wavelength occurs in one second. The frequency (f) specifies the number of cycles per second, measured in hertz (Pohlmann, 2001). Sound waves that humans hear, range between 20 to 20000 Hz (Ogunsote, 2007). Frequency and wavelength are related in the following equation:

\[ \text{Wavelength (ft)} = \frac{\text{speed of sound (ft/sec)}}{\text{Frequency (Hz)}} \]

which can be also written as: Frequency = speed of sound /wavelength (Pohlmann, 2001).
2.1.3 Sound wave propagation

Propagation of sound is the transmission of acoustic energy through medium via a sound wave (Ogunsote, 2007). When the sound wave gets further away from the source, the amplitude of the sound wave decreases. The reduction of sound level is referred to as a change in attenuation. During the sound wave propagation, the wave can be refracted, reflected and attenuated by different medium to varying extents. Sound propagation can be affected by three properties of the media as it travels through a different medium. The first factor is the relationship between density and pressure of the medium. Temperature affects this relationship by decreasing density as temperature increases. Thus, a change in the refraction of a sound wave occurs when the temperature changes. The second factor is the viscosity of the medium. The viscosity of medium can determine the rate at which the sound is attenuated. The third factor is the motion of medium. The sound wave propagation can be dependent on the motion of the medium itself.

2.1.4 Sound wave travel speeds

Sound travels at different speeds through different materials (Drury, 2004). The speed of sound is a term used to describe the speed of sound waves passing through an elastic medium (Ogunsote, 2007). Table 1 presents several speeds of sound in different medium at specific temperatures. The speed of sound tends to be greater in a solid medium. In general, the speed of sound in solids is greater than in liquids and the speed of sound in liquids is greater than in gases.

The speed of sound \( c \) is given by following general equation:
\[
c = \sqrt{\frac{c}{\rho}}
\]
where \( c \) is the coefficient of stiffness and \( \rho \) is the density of the medium (Ogunsote, 2007). Therefore, the speed of sound decreases as the density of medium decreases and increases with the coefficient of stiffness. The equation for calculating the speed of sound varies with the medium. In solids, the speed of sound is given by:
\[
c_{\text{solids}} = \sqrt{\frac{E}{\rho}}, \text{ where } E \text{ is Young’s modulus and } \rho \text{ is the density of the medium.}
\]
In fluids, the speed of sound is given by:
\[
c_{\text{fluids}} = \sqrt{\frac{K}{\rho}}, \text{ where } K \text{ is the adiabatic bulk modulus and } \rho \text{ is still the density of the medium.}
\]
For the calculation of the speed of sound in gases, \( \gamma p \) replaces the \( K \) in the equation above, where \( \gamma \) is the adiabatic index also known as isentropic expansion factor and \( p \) is the pressure of gas condition (Ogunsote, 2007).
### Table 1. Speed of sound in various substances (CRC Handbook)

<table>
<thead>
<tr>
<th>Gases (°C)</th>
<th>Substance</th>
<th>Speed of Sound (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carbon Dioxide</td>
<td>259</td>
</tr>
<tr>
<td></td>
<td>Hydrogen</td>
<td>1284</td>
</tr>
<tr>
<td></td>
<td>Helium</td>
<td>965</td>
</tr>
<tr>
<td></td>
<td>Nitrogen</td>
<td>334</td>
</tr>
<tr>
<td></td>
<td>Oxygen</td>
<td>316</td>
</tr>
<tr>
<td></td>
<td>Air (21% Oxygen, 78% Nitrogen)</td>
<td>331</td>
</tr>
<tr>
<td></td>
<td>Air (20°C)</td>
<td>344</td>
</tr>
<tr>
<td>Liquids (25°C)</td>
<td>Glycerol</td>
<td>1904</td>
</tr>
<tr>
<td></td>
<td>Sea Water (3.5% salinity)</td>
<td>1535</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>1493</td>
</tr>
<tr>
<td></td>
<td>Mercury</td>
<td>1450</td>
</tr>
<tr>
<td></td>
<td>Kerosene</td>
<td>1324</td>
</tr>
<tr>
<td></td>
<td>Methyl Alcohol</td>
<td>1103</td>
</tr>
<tr>
<td></td>
<td>Carbon Tetrachloride</td>
<td>926</td>
</tr>
<tr>
<td>Solids</td>
<td>Diamond</td>
<td>12000</td>
</tr>
<tr>
<td></td>
<td>Pyrex Glass</td>
<td>5640</td>
</tr>
<tr>
<td></td>
<td>Iron</td>
<td>5960</td>
</tr>
<tr>
<td></td>
<td>Granite</td>
<td>6000</td>
</tr>
<tr>
<td></td>
<td>Aluminum</td>
<td>5100</td>
</tr>
<tr>
<td></td>
<td>Brass</td>
<td>4700</td>
</tr>
<tr>
<td></td>
<td>Copper (annealed)</td>
<td>4760</td>
</tr>
<tr>
<td></td>
<td>Gold</td>
<td>3240</td>
</tr>
<tr>
<td></td>
<td>Lead (annealed)</td>
<td>2160</td>
</tr>
<tr>
<td></td>
<td>Rubber (gum)</td>
<td>1550</td>
</tr>
</tbody>
</table>

Table 1. Speed of sound in different medium at specific temperature conditions (gases, liquids and solids)

2.2 Acoustic emission background

2.2.1 Acoustics definition

The American National Standards Institute (ANSI) has defined Acoustic as “the science of sound, including its production, transmission and its effects, including biological and psychological effects.” The study area of acoustics includes the generation of acoustic, the propagation of acoustics, the reception of acoustics, the effect of acoustics, and the control of it. Acoustics is the interdisciplinary science that deals with the study of all mechanical waves in gases, liquids and solids including vibration, sound, ultrasound, and infrasound (Richard and Stork, 1982). The wheel in Figure 4 was created by Lindsey to illustrate the four places where acoustics play an important role, that being earth science, engineering, life science and the arts. The outer circle of the wheel lists the various disciplines in acoustics and the inner circle includes the fields where acoustics play an important role.

2.2.2 Acoustics Emission

Measurement of acoustic emissions from bacteria is the beginning of the new field in bacteriology. Normally acoustic emissions are used to detect or examine the behavior of materials. The general concept of acoustic emission is defined as the class of phenomena in which transient waves are generated by a rapid release of energy from a localized source or sources within a material (ASTM). In the review of acoustics emission and acoustic-ultrasonic techniques for wood and wood based composites, acoustic emission (AE) is a technique used for nondestructive testing (NDT) (Kawamoto, 2002). Also Kawamoto included that NDT is defined as the technical method to examine materials or components in ways that do not impair future usefulness and serviceability of that material (Kawamoto, 2002).
Figure 4. Lindsay's Wheel of Acoustics illustrates nearly all the fields of acoustics field (Lindsey, 1964).
2.2.3 Key terminology in Acoustic Emission study

A typical acoustic emission system involves sensors, preamplifiers, AE detection systems and an AE collection device. The AE signal was first detected using a sensor, then pre-amplified, and filtered both in the preamplifiers and on the PCI-2 board. Acoustic Emission was defined as elastic waves generated by the rapid release of energy from sources within a material. The waveform was detected when the filter signal was passed through signal processing. The waveform is processed for its total energy, frequency and intensity. The key terminology of acoustic emission is used to describe the process from sensor to data signal detection. These terminology were included in the PCI-2 Based AE System User’s Manual (Corporation, 2003).

2.3 Bacteria growth and communication

Microorganism survive and grow when environmental conditions are favorable, such as oxygen and nutrients in carbon, nitrogen, sulfur and phosphorus. Carbon can make up of 50% of the cell dry weight. Nitrogen is important to form amino acid, RNA and DNA in cells and also makes up around 14% of the dry cell weight. Based on the ability of oxygen utilization and tolerance, microbes can be divided into five groups, which are: obligate aerobes, facultative anaerobes, obligate anaerobes, aerotolerant anaerobes and microaerophiles (Brannan, 1997).

In addition, microorganisms also have a variety of physical requirements for their growth, including appropriate temperature, osmotic pressure and pH. Dependence on their pH tolerance, microbes can be classified as three groups: acidophiles, neutrophils and alkaliphiles. Based on the preferred temperature ranges, microbes are classified as psychrophiles, psychrotrophs, mesophiles, thermophiles and hyperthermophiles (Anthony et al., 2015).

By definition, communication is a process of signal exchange between sender and receiver through a medium (Reguera, 2011). Microbial communication through chemical signals has been researched by many investigators (Stephan and Bassler, 2001; Visick and Fuqua, 2005; Ng and Bassler, 2009; Ng WL, 2009), while the research on microbial communication through physical signals has been very limited and restricted. Reguera (2011) suggests that various microorganisms may generate and respond to three physical signals: sound waves, electromagnetic radiation and electric currents and that the modes of microbial communication via physical signals could be widely existing in nature.
2.3.1 *Lactococcus lactis* ssp. *lactis* C2 bacteria growth

*Lactococcus lactis* subsp. *lactis* C2 is a mesophilic bacteria that has an optimal temperature for growth of around 30°C (Chen et al., 2015). Temperature and pH influence both the growth rate and the amount of lactic acid produced from lactose. The optimal pH for *Lactococcus lactis* growth and lactic acid formation is about pH 6 (Ed and Barbel, 1999).

*Lactococcus lactis* ssp. *lactis* C2 bacteria lacks the various biosynthetic pathways to produce the nutrients they need for growth, so they require nutrients such as amino acids, vitamins, minerals and carbon sources to support their growth at a suitable rate (Jensen and Hammer, 1993). M17 medium has been a commonly used growth medium for *Lactococcus lactis* ssp. *lactis* C2 since 1975 (Terzaghi and Sandine, 1975). M17 medium is commercially available without a carbon source. Thus, various carbon sources can be added to this medium. Since lactococcal species are limited in various biosynthetic pathways, the main metabolic pathway for *Lactococcus lactis* ssp. *lactis* C2 is anaerobic fermentation pathway. In this fermentation, *Lactococcus lactis* ssp. *lactis* C2 produces lactic acid from the appropriate available carbon sources within the medium. The carbon source used by *Lactococcus lactis* ssp. *lactis* C2, includes glucose, galactose, fructose, lactose, maltose, mannitol, ribose, sucrose and so on. Each carbon source affects the growth rate and CO₂ production of *Lactococcus lactis* ssp. *lactis* C2, because of the different pathways that carbon sources are metabolized. The process of anaerobic metabolism involves to breakdown extracellular carbon source into pyruvate through glycolysis and then pyruvate converts into lactic acid through the enzyme L-lactate dehydrogenase (Hols et al., 1999).

2.3.2 Bacteria communication through chemical signals

Recent review articles (Stephan and Bassler, 2001; Ng and Bassler, 2009; Rutherford and Bassler, 2012) suggest that communication by chemical signaling between bacteria is widespread and important to regulation of microbial group growth. Quorum sensing is the process that allows microorganisms to communicate with each other through chemical signals. Quorum sensing enables microorganisms to share cell-density information and modify gene expression. In the review, of bacterial quorum sensing (Rutherford and Bassler, 2012) defined quorum sensing as a bacterial cell-cell communication process that involves the production, detection and response to extracellular signaling molecules called auto-inducers (AIs). Bacterial quorum
sensing processes included biofilm formation, virulence factor secretion, bioluminescence, antibiotic production, sporulation and competition for DNA uptake which is critical for bacteria survival (Ng WL, 2009). These processes seemed useless if they were performed by single bacteria, but quorum sensing controlled these processes when performed by groups of bacteria. Quorum sensing regulated many bacterial behaviors, which including symbiosis, virulence, antibiotic production, conjugation and biofilm formation (Stephan and Bassler, 2001).

In the review of bacterial quorum sensing, Rutherford and Bassler (2012) summarized that all types of quorum sensing systems could be divided into three basic principles. First, signal molecules (AIs) are produced. Then, AIs were detected by receptors in the cytoplasm or in the membrane (Rutherford and Bassler, 2012). Third, detection of AIs leaded to the activation of AI production by activating gene expression. In conclusion, all quorum sensing systems were related to production, detection and response to AIs. AIs increase in the growth environment with increased bacterial populations, and bacteria can monitor the concentration of AIs to alter gene expression.

Gram-negative and Gram-positive bacteria use different types of quorum sensing mechanisms to communicate with each other in the system. Gram-negative bacteria use small molecules called AIs to communicate. These AIs could be acyl-homoserine lactones (AHLs) or other molecules which production depends on S-adeno-sylmethionine (SAM) as a substrate (Rutherford and Bassler, 2012). However, Gram-positive bacteria differ from Gram-negative bacteria, because they use autoinducing peptides (AIPs) as signaling molecules. The signal molecules in this case were modified oligopeptides, which were secreted into the medium and accumulate at high cell density (Stephan and Bassler, 2001). Figure 5 shows some examples of autoinducers used by Gram-negative and Gram-positive bacteria.
Figure 5. Examples of autoinducers used by Gram-negative and Gram-positive bacteria.

Information obtained from: The languages of bacteria (Stephan and Bassler, 2001). A. represent examples of Gram-negative bacteria autoinducers. B. represent examples of Gram-positive bacteria autoinducers. C. 3(2H)-furanone is the LuxS-dependent autoinducer called AI-2.

2.3.3 Bacteria communications through physical signals

Early studies on the cell-cell communication through physical signals suggest, that microbial signaling may be occurring through these means. In 2011, Gemma published a review article on the experimental evidence that microorganism’s emission and their response to three physical signals: sound waves, electromagnetic radiation and electric currents (Reguera, 2011).

In 2004, Andrew et al., demonstrated that the cell wall of Saccharomyces cerevisiae (baker’s yeast) showed local temperature-dependent nanomechanical motion at a frequency of 0.8-1.6
kHz with average amplitude of 3.0±0.5nm by using an atomic force microscope (AFM). The motion of a typical yeast cell was recorded at temperature of 30°C. The motion was due to the active metabolic processes or mechanical resonance or Brownian motion (Pelling et al., 2004). By treating cells with sodium azide (NaN₃), which is a metabolic inhibitor to switch off ATP production in mitochondria, they concluded the cell motion was of metabolic origins, biologically driven, and required ATP from the disappearance of oscillatory motion in the sodium azide treated cells (Pelling et al., 2004).

In Japan, Matsuhashi et al. (1998) investigated how Bacillus subtilis and Bacillus carboniphilus responded to sound emission or production of sound waves under certain conditions. They reported that Bacillus carboniphilus requires carbon in the form of graphite or charcoal to response to the sound emitted by the speaker (Matsuhashi et al., 1998). The results suggested that B. carboniphilus could respond to continuous sine sound waves of 6-10,18-22 and 28-38 kHz in the promotion of colonies under non-permissive conditions of KCl stress and high temperatures (Matsuhashi et al., 1998). The sound production of three broad peaks by B. subtilis was observed at 8-10,18-22 and 27-43 kHz by using a sensitive pressure condenser microphone in the apparatus (Matsuhashi et al., 1998). The results of this research provided strong evidence that physical signals among microbial cells were important and suggested that the detected sound waves could function as growth-regulating signals between cells.

2.4 Bacteriophage development and the mechanism of infection

Many lactic acid bacteria are widely used by the food fermentation industry to make products like cheese, sour cream, yogurt, sauerkraut, pickles etc. Bacteriophage also can be called phage, which are viruses that attack bacteria cells (Marcó et al., 2012). Bacteriophages reproduce in host bacteria. After new bacteriophages are formed within the host cell enzymes are synthesized that cause of host cell to lyse, which in turn liberates the new bacteriophage. A new round of bacteriophage infection then ensues. In the food fermentation industry, the infection of bacteriophages into lactic acid bacteria was considered to be the most significant problem in the fermentation process, which often leads to starter culture failure (Dinsmore and Klaenhammer, 1995; Hicks et al., 2004). The infection of bacteriophage could prevent or slow the lactic acid production, which brought huge economic loss to the food fermentation industry (Dinsmore and
Klaenhammer, 1995). Bacteriophage can be easily introduced into the fermentation processing by: not fully sterilizing the milk, worker’s clothing, poor sanitation, ventilation and contamination in starter culture or medium.

Moreover, the number of bacteriophages that existed in the world was relative higher than the number of bacteria. It was estimated that the number of bacteriophages is between $10^{30}$-$10^{32}$ PFU, which outnumbers of the range of bacteria by 10 fold (Marcó et al., 2012). This is why it is important to study the mechanism of how bacteriophages infect bacteria and the interactions between the host and bacteriophages. Understanding these mechanisms will greatly benefit the food fermentation industry.

2.4.1 The mechanism of bacteria cell lyse by bacteriophage

The process of infection where the bacteriophage interacts with host cells to cause host cell lysis is composed of several steps. The first step is the adsorption of the bacteriophage onto the lactic bacteria surface. Bacteriophage specificity is also related to the amount, density and localization of receptors sites on the membrane wall (Rakhuba et al., 2010). The second step is the synthesis of bacteriophage components within the host cells, which include DNA/RNA and protein synthesis. Once the individual bacteriophage components are produced they are assembled in the host cell. The last event in the bacteriophage infection cycle is the lysis of infected host cell. Figure 6 shows a general bacteriophage replication cycle step by step. Shao et al. (2008) researched the relationship between bacteriophage adsorption rate and optimal lysis time. Their results indicated that a higher adsorption rate of bacteriophage coincided with a shorter the optimal lysis time (Shao and Wang, 2008).
2.4.2 Infection of *Lactococcus lactis* ssp. *lactis* C2 with c2, sk1 and ml3 bacteriophage

Bacteriophage c2, sk1 and ml3 are different in their morphology. Bacteriophage sk1 has a small isometric head (hexagonal shape) (Valyasevi et al., 1994). However, bacteriophage ml3 has a prolate head (elongated oval) (Valyasevi et al., 1990). Currently, no DNA similarities have been reported for bacteriophage having prolate and isometric heads (Forsman, 1993). Valyasevi et al.
(1990) noted that the bacteriophages receptor-binding site on the host cell membrane did not depend on the bacteriophage morphological shape.

Because of the different cell wall structure of gram-positive and gram-negative bacteria, the specific binding mechanism varies when bacteriophages bind to different bacteria. *Lactococcus lactis* ssp. *lactis* C2 is a typical spherical-shaped gram-positive bacteria, which is widely used in milk fermentation. Gram-positive bacteria have a thicker cell membrane composed of a peptidoglycan layer than gram-negative bacteria. Bacteriophage for these gram-positive bacteria will adsorb to peptidoglycan layer before it interacts with the cell membrane. Valysaevi et al. (1994) has analyzed the cell wall composition for neutral and amino sugars of *Lactococcus lactis* ssp. *lactis* C2 by gas-liquid chromatography (Valyasevi et al., 1994). Their results showed that the cell membrane was composed of rhamnose, galactose, glucose and N-acetylglucoamine in following concentration± SD: 234±20, 37.1±2.2, 66.0±5.3 and 73.5±6.2 mg/g, respectively (Valyasevi et al., 1994). Several previous researchers found that during the bacteriophage infection, bacteriophage was initially binding to the extracellular polysaccharides on the cell wall, which can be called the receptors (Valyasevi et al., 1990, 1991; Geller et al., 1993; Monteville et al., 1994; Valyasevi et al., 1994).

Bacteriophage sk1 receptors on *Lactococcus lactis* ssp. *lactis* C2, were partially blocked when rhamnose, glucose, galactose, N-acetylglucoamine and fructose were increased to 500mM. Blockage to sk1 adsorption was observed to be 64%, 26%, 26%, 5% and 8%, respectively, for each sugar (Valyasevi et al., 1994). These results suggested that rhamnose, glucose and galactose were possible components of the bacteriophage sk1 receptor on lactis C2 cell membrane. Valyasevi et al. (1994) used lectins from different bacteria to further verify the inhibition of bacteriophage adsorption by rhamnose, glucose, galactose, N-acetylglucoamine and fructose. Lectins are known as specific monosaccharide binding proteins. Lectins from concanavalin A can only bind to glucose. These results showed a 50% reduction in bacteriophage adsorption when concanavalin A was added to the medium. Rhamnose-specific lectin inhibited the bacteriophage adsorption by 83% at the highest lectin concentration of 1.20mg/ml (Valyasevi et al., 1994). To investigate the delay of bacteriophage infection, 0.6 M rhamnose, glucose and fructose was added to the medium prior to infect *lactis* C2 culture with bacteriophage sk1. In the control group without monosaccharide added, phage sk1 lysed the *lactis* C2 within 2h and complete lysis occurred before 5h (Valyasevi et al., 1994). Neither fructose nor glucose delayed
the lysis compared to the control group data. The results suggested that the bacteriophage sk1 receptor involved rhamnose in the cell wall.

Monteville et al. (1994) used a competitive inhibitory assay to determine the bacteriophage adsorption for seven phages included bacteriophage c2 and ml3. The inhibition of bacteriophage adsorption caused by rhamnose was from 64% to 86% (Monteville et al., 1994). The competitive inhibition of bacteriophage adsorption caused by glucose, galactose and N-acetylglucosamine were all lower than the value of rhamnose. In this research, bacteriophage c2 and ml3 was employed. The inhibition of bacteriophage c2 adsorption by Rhamnose, Glucose, Galactose and N-acetylglucosamine was 70±1.4%, 48±18%, 34±19%, and 22±2.8%, respectively (Monteville et al., 1994). Nevertheless, the inhibition of bacteriophage ml3 adsorption by Rhamnose, Glucose, Galactose and N-acetylglucosamine was 68±16%, 27±26%, 16±19% and 11±3.5%, respectively (Monteville et al., 1994). Only bacteriophage c2 and kh were observed to have delayed bacteria lysis when rhamnose was present. In conclusion, Monteville et al. (1994) suggested that rhamnose is the main receptor component on the host cell wall.

2.4.3 The effect of CaCl₂ solution on bacteriophage infection

The two vital requirements for bacteriophage to inject its DNA into a bacteria cell are calcium ions and adenosine triphosphate (ATP) (Sechaud et al., 1988). Not only the extracellular calcium ions are required for bacteriophage infection, but also intracellular ATP content could determine the extent of bacteriophage adsorption to host cells. In 1978, Watanabe et al. reported the intracellular ATP content of Lactobacillus casei in the glucose containing medium which were 2-3 μg/mg dry wt. cell in the early to middle stage of log phase and then decreased to 0.1μg/mg dry wt. cell or lower in the stationary phase due to the availability of glucose (Watanabe et al., 1979). The results showed that the process of bacteriophage injection and adsorption requires energy, which depends on the intracellular ATP content (Watanabe et al., 1979).

Kou et al. (1972) investigated the role of calcium ion in the proliferation of bacteriophage Xp12 of Xanthomonas oryzae. Their results showed that the normal proliferation of bacteriophage Xp12 required addition of calcium ions to the medium. The process could not be duplicated using manganese, magnesium and ferric ion to replace for calcium ions.

Valyasevi et al. (1990) found that rhamnose could inhibit the bacteriophage kh infection if additional calcium ions were added to the medium (Valyasevi et al., 1990). When rhamnose
without calcium ions addition, the percent of inhibition remained the same or very low level compared to the group where rhamnose with calcium ions was present.

Valyasevi et al. (1990) also investigated divalent cations include calcium, copper, zinc, manganese, magnesium and sodium as to their ability to make binding bridge between bacteriophage kh and the *Lactococcus lactis* subsp. *cremoris* cell wall. The results showed that bacteriophage kh adsorption to host cell wall required divalent cations and among them calcium, copper and zinc bound better than the other cations. Their conclusion suggested two functions for divalent cations in this phase of bacteriophage adsorption. The first function is that positive the charge on divalent cations can neutralize the repulsive negative charges on the host cell wall. The second function is to help release bacteriophage DNA to the host cell through calcium dependent gene regulation (Barbado et al., 2009).

Hicks et al. (2004) summarized the adsorption process as follows. After analyzing a peptide (n-terminal) that could be cleaved from the surface of lactic bacteriophage c2 using ficin that appeared to be from the f-protein, they noted that the peptide contained a large number of lysine, arginine, and aspartic acid amino acids. These amino acids would be key to the adsorption process. Hicks et al. (2004) also, observed that these bacteriophage peptides were extremely sticky and would cause *L. lactis* ssp. *lactis* C2 cells to form large complex helical clumps when grown in medium containing the peptides, suggesting that the peptides were coating the surface membrane of *lactis* C2. This observation helped explained why these peptides were fairly effective in inhibiting the infection of *lactic* C2 with c2 bacteriophage. Hicks et al. (2004) theorized that in the reversible step, which involves calcium, the calcium ion act as a salt bridge between the bacteriophage f-protein and the negatively charged *lactic* host membrane. The bacteriophage then forms an irreversible bond between the f-protein of the bacteriophages foot and a rhamnose moiety within the cell membrane or other moieties. He also, noted that some non-specific *lactis* C2 bacteriophage could bind to *lactis* C2 without infecting this host (unpublished data). Hicks, noted that even though the correct adsorption/bonding is formed between the non-specific bacteriophage and *lactic* C2 bacteria, no infection seemed to occur. Watanabe et al. (1984) also observed this situation when working with a mutant of *Lactobacillus casei* (Watanabe et al., 1984).

Evidently, once the bacteriophage has irreversibly absorbed to the host membrane a correct trigger moiety must be present and recognized by the bacteriophage before it can releases ATP
which causes the bacteriophages head to constrict and inject the phage DNA through the cell membrane. When bacteriophages inject their DNA, this is not necessarily a single event. When bacteriophages titer build up, many bacteriophages can be absorb and infect a single host cell (Hicks et al., 2004). Indeed, Cogan and Accolas (1995) published photomicrographs showing over a dozen bacteriophage attached to a host cell and none of the bacteriophage had DNA in their heads (Cogan and Accolas, 1995). Apparently, all were able to inject their DNA into the host cell.

2.5 Generations of acoustic emission study by Dr. Hick’s team

A number of AE studies on bacteria and bacteriophage have been conducted at Department of Animal and Food Science by Dr. C. L. Hick’s team since 2007. Dr. Hicks conducted joint research with Dr. J. Stencel of TriboFlow Separations in Lexington KY to design and make an acoustic emission-monitoring device. The AE-monitoring device has been changed and upgraded from the first generation to the current fourth generation device to fulfill the objectives of this research.

2.5.1 The first generation of acoustic emission-monitoring device

The first generation of acoustic emission-monitoring device, shown in Figure 7 was used to show that bacteria do emit AE. Two acoustic sensors were attached (silicon grease) using a rubber band to a thermally isolated graduated flask. The two sensors were connected to a computer to collect data. The graduated flask was wrapped with tygon tubing from the top to bottom. The tygon tubing was connected to a constant temperature water bath to maintain a temperature of 32°C. The outside of entire device was wrapped with several layers of 1cm bubble wrap for insulation. A sterilized magnetic stir bar was placed in the sterilized flask prior to growth medium being added and inoculated. Samples could be taken by stopping the AE collection, stirring the medium, and removing the sample, which was then used to measure optical density and cfu’s.

2.5.2 The second generation of acoustic emission-monitoring device

The second generation of AE-monitoring device is shown in Figure 8. Instead of tygon tubing to maintain constant temperature, a unique 5-inch delrin chamber was used to control the
environment temperature. The sensor was attached to one side of the delrin wall. The water flowing around the second’s generation device caused too much noise for practical use. Only limited data were collected from this device.

2.5.3 The third generation of acoustic emission-monitoring device

The third generation of AE-monitoring device was shown in Figure 9. A dual isolation chamber was manufactured to hold two separate measuring tubes. Both chambers in the compartment were identical and both of them could be opened from the top. In each of the chamber, two heaters were attached to wall as well as a thermocouple to maintain the correct environment temperature. In the compartment, a cell holder was manufactured from stainless steel and a squared glass tube was set in the middle of the cell holder. Two AE sensors were attached to the outer wall of the square tube on opposite sides. A stir plate was set under the bottom of each square tube. The outside of the compartment was covered with a 2.5 cm layer of black foam for insulation and the compartment was set on 2.5 cm of rubber to reduce external sounds and temperature variations.

2.5.4 The fourth generation of acoustic emission-monitoring device

The fourth generation was changed from squared glass tube to a cylinder made from PVC plumbing pipe. The most important change was sensor attached to the bottom of the cylinder tube to enclose the tube. This tube is described in detail in the material and methods section.

2.5.5 Acoustic emission research

In 2010, Wardani et al. used the third generation device to partially characterize two bacteriophages, sk1 and ml3, using this AE technique. The AE sensor used in this study collected data from 5-50 kHz. *Lactococcus lactis* ssp. *lactis* C2 was infected with bacteriophage sk1 and ml3, respectively, at 90 minutes after inoculation in M17 medium at 26ºC. Sound intensity was measured in attojoules and plotted as energy rate. By monitoring the control M17 medium and water, significance was set to determine peaks that exceeded a 3 sigma level. Their results showed that no acoustic peaks intensity exceeded 3 sigma without bacteriophage infection, however when sk1 and ml3 were present multiple acoustic peaks exceeded the 3 sigma level (Wardani et al., 2010). According to their report, the initial peaks for sk1 was observed at 33.2
min and the initial peaks for ml3 was observed at 40 min in the 26°C chamber (Wardani et al., 2010). These results suggested that bacteriophage sk1 and ml3 could be distinguished by their acoustic emission when infecting *Lactococcus lactis* ssp. *lactis* C2.

During 2013-2014, Cox (2014) determined AE produced by three strains of *Escherichia coli* (5024-parent, 8279-mutant and 8279-unrelated strain) using the fourth data collection generation device. Her final conclusion suggested that all three different strains of *E. coli* generate different AE’s, thus a unique fingerprint of sound was produced, which could contribute to their identification. Even though the mutant strain lack only one gene difference and a plasmid from the parent strain, there were 13 areas in the AE spectrum were sufficiently different that the areas were significantly different when the data was analyzed in 5 kHz units over a range from 100 to 500 kHz. In other areas of the AE spectrum which were non-significant, the researchers were impressed with how uniform the emissions were between the parent and mutant strains, which would be expected if the two bacteria were doing the exact same things. Cox (2014), suggested that many cellular metabolic functions were producing sufficient AE that they could be monitored using the 4th generation AE system.

*Figure 7. The first generation of acoustic emission-monitoring device (figures and photos obtained from Hicks photo library)*
Figure 8. The second generation of acoustic emission-monitoring device (figures and photos obtained from Hicks photo library)

Figure 9. The third generation of acoustic emission-monitoring device (figures and photos obtained from Hicks photo library)

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CHAPTER 3

MATERIALS AND METHODS

3.1 Biological and chemical supplies

All chemical supplies used in this research were purchased from Aldrich Chemical (St. Louis, MO) or Fishers Scientific (Pittsburgh, PA). M17 medium broth powder was purchased from Becton, Dickinson and Company (Sparks, MD). Table 2 shows all the items purchased, the brand name of the item, reference number, and lot number. Table 3 lists the composition of 37.25g M17 Difco™ medium that was used to prepare 1000 ml M17 broth using purified water.

Table 2. Biological and chemical supplies

<table>
<thead>
<tr>
<th>Item</th>
<th>Brand</th>
<th>Reference number</th>
<th>Lot number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Difco™ M17 broth</td>
<td>BD</td>
<td>218561</td>
<td>0125014</td>
</tr>
<tr>
<td>Difco™ Lactose</td>
<td>BD</td>
<td>215620</td>
<td>6254336</td>
</tr>
<tr>
<td>Bacto™ Agar</td>
<td>BD</td>
<td>214010</td>
<td>7116718</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>Fisher brand</td>
<td>C-7472152</td>
<td>12622</td>
</tr>
<tr>
<td>Skim milk powder</td>
<td>OXOID</td>
<td>LP0031</td>
<td>1193933</td>
</tr>
<tr>
<td>Cryogenic vial(2ml)</td>
<td>Fisher brand</td>
<td>02-281-284</td>
<td>06381283</td>
</tr>
<tr>
<td>Microcentrifuge tube(1.5ml-1.7ml)</td>
<td>Fisher brand</td>
<td>10-500-26</td>
<td>634828</td>
</tr>
<tr>
<td>Petrifilm™ Aerobic count plate</td>
<td>3M</td>
<td>----------------</td>
<td>2012-11KG</td>
</tr>
<tr>
<td>Syringe filter 0.45μm</td>
<td>Whatman</td>
<td>6901-2504</td>
<td>P693</td>
</tr>
</tbody>
</table>

*a Becton, Dickinson and Company*
Table 3. Composition of DifcoTM M17 broth (37.25g) made to 1000ml with purified water

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic digest of Casein</td>
<td>5.0g</td>
</tr>
<tr>
<td>Soy peptone</td>
<td>5.0g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>5.0g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.5g</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.5g</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>0.25g</td>
</tr>
<tr>
<td>Disodium-β-glycerophosphate</td>
<td>19.0g</td>
</tr>
</tbody>
</table>

- Adjust final pH 6.9±0.2
- Adjusted or supplemented as required to meet performance criteria
- Composition listed on the bottle

3.2 Stock of Bacterial strain and bacteriophages

*Lactococcus lactis* ssp. *lactis* C2 and bacteriophages c2, ml3, sk1 were obtained from the University of Kentucky Food Science culture library, which were initially obtained from T. R. Klaenhammer (North Carolina State University, Raleigh, NC). Both bacterial *Lactococcus lactis* ssp. *lactis* C2 stains and bacteriophages c2, ml3, sk1 were stored in microcentrifuge tubes at -80°C until used.

3.3 Preparation of medium for bacteriological and phage assays

M17 medium, skim milk, Bottom agar, Top agar and CaCl₂ solution were all prepared in the microbiological laboratory of the W.P. Garrigus building (University of Kentucky). All the materials and solutions involved in this research were autoclaved for 15 min at 121°C. The complete cycle in the autoclave machine was about 45 min each time.
3.3.1 Preparation of M17 medium

In 1975, Terzaghi and Sandine published that addition of 1.9% disodium-β-glycerophosphate (GP) into a complex medium could improve the growth of *Lactococcus* cultures. They first reported M17 medium to include: 5g Phytone peptone, 5g polypeptone, 2.5g yeast extract, 5g beef extract, 5g lactose, 0.5g ascorbic acid, 19g GP, 1ml 1.0 M MgSO₄·7H₂O and 1000ml distilled water (Terzaghi and Sandine, 1975). According to absorbance measurement and total counts, all strains of *Lactococcus lactis*, *Lactococcus cremoris* and *Lactococcus diacetilactis* grew better in M17 medium with GP than without GP (Terzaghi and Sandine, 1975). The buffer capacity was increased by the addition of GP, which benefit the culture growth.

M17 medium containing the materials listed in table 3 was prepared by following the manufacture’s instruction. Thus, 37.25g of the M17 powder was mixed into distilled water to prepare 1000 ml of medium. The solution was agitation to the boiling temperature and held for 1 minute to dissolve the broth powder completely. After the solution cooled down, 1g of lactose was added to 1000ml medium to prepare a 0.1% final lactose solution. The pH was adjusted to 6.9±0.2 (with 0.1 M KOH). The M17 medium was autoclaved for 15 min at 121°C. After sterilization, the medium was cooled and stored at 5°C until used for culture and/or bacteriophage growth. The lactose not only acted as a carbohydrate source in the medium, but was also helpful to isolate the bacterial mutants which lacked the ability to ferment lactose (Terzaghi and Sandine, 1975).

3.3.2 Preparation of skim milk

Skim milk powder (15g) was dissolved into 150 ml of deionized water with mixing. Skim milk solution also autoclaved for 15 min at 121°C and stored at 2°C until used.

3.3.3 Preparation of bottom agar and top agar

To prepare bottom agar 15g of bacto agar and 5g of lactose were added to 1000ml M17 medium, then 100ml 1M CaCl₂ solution was added. The pH was adjusted to 6.9±0.2 (with 0.1 M KOH) and the medium was autoclaved for 15 min at 121°C. Hot bottom agar was dispense (10ml) into Petri dishes, allowed to cool until solidified, inverted, and stored in a 5°C refrigerator.

Top agar was formulated by adding 4.5g bacto agar and 5g lactose into 1000ml M17 medium. Top agar was dispensed into 50ml test tubes and then autoclaved for 15 min at 121°C. Top agar
was stored at 5°C until used. Top agar was heated for 20 min in a steamer to liquefy it before use. The optimal dispense temperature for top agar was 45 °C.

3.3.4 Preparation of CaCl₂ solution
The molecular weight of CaCl₂ was 111 g/mole. Thus, 11.1g CaCl₂ was dissolved into 100ml distilled water to prepare a 1 M solution. CaCl₂ solution was used to prepare bottom agar.

3.4 Selection of C2 culture to make new stock culture
Starting culture was prepared by inoculating 1ml (1×10⁹ CFU/ml) stock *Lactococcus lactis* ssp. *lactis* C2 culture into 25 ml M17 medium in a sterile test tube. The mixture was incubated overnight for 16h in a 32°C water bath to prepare a fresh *L. lactis* ssp. *lactis* C2 culture. *L. lactis* ssp. *lactis* C2 culture was picked with a sterile loop and streaked across the surface of a Petri dish containing the solidified bottom agar. The inoculated plate was incubated at 32°C for 16 h. After 16 h, the streaked plate contained many individual colonies near the end of the streaking point that could be picked. Seven well-isolated colonies were picked separately by using a sterile loop to pick up most of a single colony and then inoculated in into a tube containing 25ml of sterile M17 medium. Tubes were incubated overnight for 16 h in a 32°C water bath. Tubes were labeled from 1 to 7 to differentiate the picked colonies.

Twenty-one sterile test tubes containing 10 ml of M17 medium were prepared, then 3 tubes were inoculated (1 ml) with one of the picked colonies of *L. lactis* ssp. *lactis* C2 culture. All 21 test tubes were incubated at 32°C in a water bath for 1h. After the incubation period, each set of 3 tubes were infected with either 0.2ml ml3, sk1 or c2 bacteriophage, respectively. All 21 test tubes containing the picked colony were infected with the various bacteriophage with the addition of the CaCl₂ solution. All tubes were observed to see how fast they cleared (lysed). Culture prepared from the number 7 colony was the first to clear (approx. 1 h after infection) when infected with all three bacteriophages. Thus, colony number 7 was selected for propagation because it was the most sensitive to all three bacteriophages.

3.5 Bacteria and bacteriophages propagation to make new stocks
New stocks of bacteria and bacteriophage were prepared by propagating selected organisms. Since *L. lactis* ssp. *lactis* C2 in tube number 7 was the most sensitive to all three bacteriophages, the strain in tube number 7 was choose for host culture propagation and for the propagation of
the three bacteriophages.

3.5.1 Bacteria propagation

M17 slants were prepared by adding 3ml of sterile melted bottom agar to a 10 ml sterile tube. All tubes were set at a 30 degree angle and allowed to solidify.

*L. lactis* ssp. *lactis* C2 culture from growth tube number 7 was streaked onto the M17 slants by using a sterilized inoculation needle. The inoculated M17 slants were incubated at 26°C for 48 h. Stock cultures of *L. lactis* ssp. *lactis* C2 culture were prepared by washing the surface of the M17 slants with 1ml of sterilized skim milk while agitating the surface with a sterile stab. A sterile Pasteur pipette was used to transfer the skim milk wash into a cryogenic vial (2ml). All vials were frozen and stored at -80°C until used.

3.5.2 Bacteriophages propagation

New stocks of *L. lactis* ssp. *lactis* C2 culture was used to make a new active culture. Active culture was prepared by inoculating 25 ml of sterilized M17 medium with a stock culture in a 50 ml test tube. This working culture was incubated for 16 h at 32°C in a water bath. After 16 h incubation, another sterilized test tube with 25 ml-sterilized M17 medium was inoculated with 1ml of active culture and incubated at 32°C for 1 h. At one hour the culture entering the log phase was infected with 0.025ml bacteriophage (either c2, sk1 or ml3) along with one drop (0.5ml 0.1M CaCl2 solution), mixed, and then incubated for an additional 4 h. Normally, It took about 3-4 hours for complete lysis to occur. A second culture inoculation done to increase bacteriophages numbers. Thus, an additional 0.5ml active culture was added to the mixture while maintaining the incubation temperature at 32°C and waiting until the second clearing to occur.

The cleared tubes contained lysed *L. lactis* ssp. *lactis* C2 cellular debris, a few mutant bacteriophage resistant bacteria and the newly propagated bacteriophage. To remove most of the debris and mutant cells the cleared M17 medium was filtered through a 0.45μm syringe filter. The filtered solution containing the bacteriophage was pipetted (~1.5 ml) into 2-ml micro-centrifuge tubes, frozen and stored at -80°C until used.

3.6 Enumeration of stock bacteriophages

The enumeration of stock bacteriophages was done followed the methods published by (Hicks
et al., 2004). *L. lactis* ssp. *lactis* C2 culture (1ml) was inoculated into 25ml-sterilized M17 medium and incubate for 16h in a 32°C water bath. Stock bacteriophage was serially diluted in distilled water blanks for enumeration. Initially, 0.01ml stock bacteriophage of ml3, sk1 or c2 was added to 99.99ml distilled water, respectively. A $10^4$ fold dilution was accomplished for all three bacteriophage. Then 1ml of this solution ($10^4$ fold dilution) was added to a tube containing 9 ml of distilled water to make $10^5$ fold dilution. This process was repeated to make successive dilutions for $10^6$ and $10^7$ fold dilutions. In the end, $10^4$, $10^5$, $10^6$ and $10^7$ fold dilutions for bacteriophage ml3, sk1, or c2 were prepared by this serial dilution process and vortexed to mix well. Sterilized top agar was heated for 20 minutes to liquefy and cooled to 45°C before use. Then 0.1 ml *L. lactis* ssp. *lactis* C2 culture, 0.1 ml diluted bacteriophage from different dilutions and 1 drop of CaCl$_2$ solution were added to 2.8 ml of top agar to make a top agar mixture (Table 4). Mixed top agar, was agitated quickly to avoid solidification and poured onto a bottom agar plate and covered, which was moved circularly to spread the top agar evenly across the bottom agar plate. All prepared plates were allowed to stand for a few minutes in order to solidify. Each agar plate was labeled with its type of bacteriophage and its dilution factor. All agar plates were incubated at 26°C for 6 hours. Plaques on each plate were counted after the incubation period.

**Table 4. The composition of top agar mixture to overlay on the bottom agar plate**

<table>
<thead>
<tr>
<th>Bacteriophage c2, ml3 and sk1 (ml)</th>
<th>Diluted bacteriophage from different dilutions factor 0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top agar</td>
<td>2.8</td>
</tr>
<tr>
<td>Active <em>Lactococcus lactis</em> ssp. <em>lactis</em> C2 culture 0.1</td>
<td></td>
</tr>
<tr>
<td>CaCl$_2$ solution</td>
<td>1 drop</td>
</tr>
</tbody>
</table>

3.7 Working bacteria culture preparation for acoustic emission monitoring

M17 medium was prepared by following the manufacture’s instruction and autoclaved for 15 min at 121°C. The day before the acoustic data collection, a working bacteria culture was prepared by adding 1ml of new stock *L. lactis* ssp. *lactis* C2 to 25ml M17 medium in a sterilized
test tube. The working culture was incubated overnight for 16h in a 32°C water bath. Over the 16h incubation period, the \textit{L. lactis} ssp. \textit{lactis} C2 cells in growth medium averaged $1 \times 10^9$ cfu/ml. This working culture was now ready for use in the acoustic emission monitoring system. This working bacteria culture was prepared prior to every acoustic emission experiment.

3.8 Latest generation of acoustic emission-monitoring device

The latest generation of acoustic emission-monitoring device was dramatically modified from the third generation of acoustic emission-monitoring device that was shown in Figure 9. The same environmental chamber was used (Figure 10), but a new tube & tube holder (Figure 11) was used. This acoustic emission-monitoring device was prepared under the direction of C. L. Hicks by Turners Machine Shop (Lexington, KY 40508) in consultation with TriboFlow Separations LLC (Lexington KY 40509). The acoustic emission-monitoring tube (Figure 12) was prepared from clear $\frac{3}{4}$ inch PVC plumbing pipe. Pipe dimensions were as follows: Tube length was 203.3 mm in length, OD of tubing ranged from 26.22 to 26.72 mm, ID ranged from 20.32 to 20.41 mm with a wall thickness ranging from 3.10 to 3.18 mm. At the bottom of the PVC tube a groove was machined into the tube starting 2 mm from the bottom of the tube to a circumference of 23.5 mm. An o-ring with the dimensions of: 23.88 mm OD and 18.68 mm ID and a wall thickness of 2.64 mm was snugly fit into the PVC groove using a small amount of vacuum grease. The R-6α sensor (Figure 13) could be twisted up into the o-ring to make a complete seal (Figure 12) that prevented the medium from leaking past the sensor.

Turners machine shop also machined a black plastic collar prepare from a slice (15 mm) off a solid block cylinder of HDPE (shown in Figure 14). The HDPE block was machined to contain a hole centered in the block that had an ID of 27.3 mm. Two grooves were machined into the inside face of this hole to a depth of 28.8 mm. Both grooves started approximately 2 mm from the bottom and top of the hole edges. Two o-rings that were 26 mm ID and 29.2 mm OD, and 3.2 mm thick were compressed into these two grooves. The PVC pipe could be twisted up into these o-rings for a snug fit. The HDPE block was further machined using the schematic shown in Figure 15, so that the LED emitter could be screwed into one side of the block and the photo detector monitor could be screwed into the adjacent side as shown in Figure 14.
3.8.1 Setup of apparatus before data collection

The environmental chamber was engineered with a temperature control system that could control the temperature within the chamber. Before each AE run the temperature controller was turned on approximately 2 h before the run was scheduled so that all of the components within the chamber would be at the correct temperature.

Prior to placing the PVC cylinder into the holder inside the chamber, a thin layer of silicon grease was applied to the edge of acoustic emission sensor to prevent the M17 medium from leaking into the sensor. Then the acoustic emission sensor was twisted into the bottom of cylinder tube. The O-ring placed at the end of PVC cylinder was also smeared with a thin layer of silicone grease to prevent leakage around the sensor. The PVC cylinder with sensor attached was placed vertically in the holder (Figure 11) in the chamber and sealed with a rubber stopper. The acoustic sensor was attached to a 40dB preamplifier model 1220A (PAC, Princeton Junction, NJ), which was in the compartment within the data collection chamber. The preamplifier transferred the collected data to an I/O board (PAC, Princeton Junction, NJ), within the computer, (Each board contained two channels that could receive a stream of data) that the data was received on. The acoustic emission data was collected and then analyzed using AEwin™ software (PAC, Princeton Junction, NJ).
Figure 10. The complete set up of acoustic emission-monitoring device (included the dual acoustic emission compartment, computer system and voltage meter)

Figure 11. Inside view of one of the environmental chambers. Arrow shows tube and tube holder.

Figure 12. Clear PVC tube containing an o-ring and R-6α sensor
The acoustic sensor used in this research was manufactured by Physical Acoustics Corporation described in the PCI-2 based AE system user’s manual (PAC manual, 2003). The acoustic sensor was a Model R6α-SNAD 52 and is shown in Figure 13. The R6α sensor cavity was machined from a solid stainless steel rod which enhances its ruggedness and stability. The top face of the sensor was made from ceramic (Figure 13B), which was in direct contact with the M17 medium within the PVC tube. The ceramic face was electrically isolated within the sensor and from the outside environment to ensure a low noise environment. The connector located on the side of sensor connects to the preamplifier. The frequency optimal response range of R6α sensor was between 35 and 100 kHz during operation, but some data was collected outside of this optimal range.

![Acoustic sensor](image)

**Figure 13. Acoustic sensor (α-serials Model R6α-SNAD 52)**

A: Bottom view of acoustic sensor  B: Top view of acoustic sensor
3.8.3 Modified acoustic emission cylinder tube with voltage measurement

An optical density measurement was made through the M17 medium using a LED (Nichia, NSPA510BS, Tokushima, Japan) and that had a peak wavelength of 580 nm and light range from 500 to 625nm. This peak frequency was close enough to the desired 600 nm used in spectrophotometers for cell growth monitoring that no spectral filter was used. The transmitted light was measured by a photo diode detector (AMS AG, Part number, TAOS TSL257S, Premstaetten, Austria). Both the LED and photo diode were mounted in a black HDPE block (Figure 14). Both the LED and photo diode were controlled by an electronic board (shown in Figure 16) manufactured by Reflectronics, Lexington KY (Part, PCB Series No 8) that delivered an output signal that was proportional to the measured light transmitted (Figure 14). The 4 to 20 mA output signal was transmitted across a 250 ohm resistor (part number MAS250R, Granger, Lexington, KY) to produce a 1 to 5 volt response (right side of Figure 16). The electronic board was calibrated so that when only M17 medium was placed in the PVC tube, a 5 volt reading would be measured. When the LED light was off only 1 volt would be read. Thus, when bacteria were present and absorbing or deflecting light the reading would be less than 5 volts but above 1 volt. The leads from a volt/ohm meter (black leads, Figure 16) were attached to both side of the resistor to record the voltage being measured (meter shown in Figure 17) across the resistor.

The clear PVC tube was wrapped above and below the HDPE collar with black PVC electrical tape purchased from a local hardware store to prevent outside light from getting to the photo diode detector (Figure 14). The PVC tube was then set into the acoustic emission compartment that isolated it from outside sound sources and controlled the environmental temperature. The chamber (described earlier) was insulated with 2.5cm black foam and sat on a rubber mat. The complete set up of acoustic emission-monitoring device was located in the food-engineering lab (room 189) which was on the first floor of the Charles E. Barnhart building. This room had a concrete pad for the floor that sat on 20 cm of gravel above the loam soil bed. Upon the concrete pad was a table which held a 15 cm layer of sand, topped with a 5 cm slab of granite. The environmental chamber set upon the granite slab. This was an excellent system to reduce extraneous noises coming through the soil/room/air to the environmental chamber.
Figure 14. Modified acoustic emission PVC tube.

Black HDPE ring containing the LED emitter and photo diode detector. PVC tube is wrapped with black electrical tape to prevent room light from being detected by photo diode. R-6α sensor was inserted into the bottom of the tube. The HDPE block was further machined using the schematic shown in Figure 15, so that the LED emitter could be screwed into one side of the block and the photo detector monitor screwed into the adjacent side as shown in Figure 14.
Figure 15. Schematic modified acoustic emission PVC tube.

Schematic showing the machined openings in the HDPE block that the LED emitter and photodiode detector were screwed into. Both the LED emitter and photodiode detector are shown screwed into these machined opening in Figure 14.

Drawing was produced by Dr. F. Payne, University of Kentucky.
Figure 16. The controller circuit board

The controller circuit board which controlled the current to the LED and the gain and photodiode detector signal conditioning returning from the photodiode. The 4 to 20 mA signal was dropped across the 250 ohm resistor (upper left of picture). The voltage across the resistor was measured with a volt/ohm meter shown in figure 17.
3.8.4 Acoustic emission data collection

Acoustic emission software allowed for the selection of the channels that the data was streaming into. Before data collection, a test trail collection was performed to establish the most appropriate threshold or dB level for the selected channel. The threshold dB level was adjusted to 21dB (generally). The acoustic sensor threshold level was always between 18-26dB. This eliminated most of the background noise. At the 21dB setting the background being recorded was approximately 2 hits/second. Channel 4 was selected to collect all data in this experiment. The hit represented the detection and measurement of an AE signal on a channel. The preamplifier was selected at 40dB and connected to sensor to reduce the effect of noise. The preamplifier was used to amplify the small electrical signal detected by sensor for further amplification.

Figure 17. Volt Ohm meter (VOM) used to read voltage measurement
After the complete set up of acoustic emission-monitoring apparatus, the PVC tube with the AE sensor was loaded with sterilized (50 ml) M17 growth medium. Once M17 was in the PVC tube, 2 ml of working bacteria culture was added by pipette, mixed well, and the acoustic emission data collection system was initiated on the computer. It took 90 min for the working culture to move through the lag phase into the log phase. Thus, at 90 min the data collection was paused and 0.05ml of the selected bacteriophage was added to the medium in the PVC tube to infect the culture along with 0.5ml CaCl₂ solution, then mixed. This whole infection procedure was less than 30 sec. The data collection was resumed for the next 2.5 h (total time was at least 4 h).

The acoustic emission data was detected when a sound waveform reached the acoustic sensor; this data was called a “hit”. The “hit” was the detection and measurement of an AE signal on channel 4. For every AE signal “hit”, the waveform components above the threshold level were extracted and recorded as an AE hit. The software recorded driving features including: time of hit, rise time, AE amplitude, AE duration, signal strength and absolute energy. The AEwin™ software has the capability to calculate, process and record the frequency derived from AE hit features in real time. The AEwin™ software takes this raw data and processes it using Fast Fourier Transform calculations to convert raw data to frequency-based data including: frequency centroid, peak frequency and 4 partial power frequency features (PAC manual, 2003).

The acoustic emission data was then exported from AEwin™ software to Excel 2011 for further data editing. The exported data was manipulated to only include columns in: time (in seconds), absolute energy, centroid frequency and peak frequency. Absolute energy is the true energy measurement of the AE hit and its units is in attojoules (aj) (PAC manual, 2003). The peak frequency is reported in kHz and is defined as the frequency at the point which the largest peak magnitude occurred during the record time period (PAC manual, 2003). The centroid frequency was also reported in kHz and is considered a real time frequency derived feature (PAC manual, 2003). The equation for centroid frequency was: SUM(magnitude* frequency)/ SUM(magnitude) (PAC manual, 2003). The centroid frequency represents the average AE frequency magnitude during the recorded time period for every data point. The acoustic emission data for different treatments and replications were combined in Quattro Pro X7 software and then analyzed using SAS 9.4 software, ultimately.

3.8.5 Optical density and voltage measurements during acoustic emission data collection

After inoculating working bacteria culture into M17 medium in the PVC tube, the acoustic
emission data collection was initiated. Acoustic emission data collection was paused at every 30 minutes to record the optical density and the voltage measurement. First the VOM meter was turn on to get the voltage measurement, then the data collection was paused to collect a 1 ml sample from the PVC tube. Prior to collecting the sample the medium within the PVC tube was stirred with a sterilized utensil for about 5 seconds. The 1ml of the sample was transferred to a crystal cuvette in the spectrophotometer (Model Spectronic™ 200E, ThermoFisher Scientific, Inc.). The optical density was determined at 600nm. The contents of the cuvette was poured back into the PVC tube after each optical density measurement and the system was resumed for acoustic emission data collection. This procedure took less than 45 sec.

3.9 Estimate growth of Lactococcus lactis ssp. lactis C2 concentration during the acoustic emission data collection

At every 30 minutes from 0h to 4 h, a 1 ml sample was taken from the PVC tube and serial diluted to 10⁴, 10⁵, 10⁶, 10⁷ and 10⁸. The 10⁶, 10⁷ and 10⁸ dilutions and pipetted onto the surface of duplicate aerobic Petrifilm™. A plastic spreader was placed on the top of Petrifilm™ aerobic count plate to disperse the solution uniformly. All the Petrifilm™ aerobic count plates were collected and incubated at 26ºC for over 24 hours. The bacteria cell colonies were counted on each labeled Petrifilm™ in order to estimate the L. lactis ssp. lactis C2 concentration during the growth and lysis phases.

3.10 Statistical analysis of acoustic emission data

Acoustic emission data from each run was exported from AEwin™ software (PAC, Princeton Junction, NJ) to Microsoft office excel 2011. The exported data was edited in excel to only include: time (seconds), absolute energy, peak frequency and centroid frequency. Time in seconds was converted to time in min to represent data in a time column. Because excel has a maximum data limited, the data from all treatments and replications were combined in QuatroPro software when the complete acoustic data sets were needed for analysis.

Acoustic emission data was statistically analyzed by using SAS 9.3 software. The collection of acoustic emission data from the standard L. lactis ssp. lactis C2 culture (treatment 4) was replicated two times. The collection of acoustic emission data from bacteriophage ml3 (treatment 1), sk1 (treatment 2) and c2 (treatment 3) were replicated at least three times, respectively.
In order to analyze the acoustic data before and after the infection point (90 min), the complete acoustic data from Qutropro was sorted by time in min using a SAS sort procedure. An example of this Sort Model is shown below to represent data from 90 - 240 min (1.5h-4h):

```
Data quattrofullAE;
input trt rep sec min AE Centroid PF;
cards;
;
Data sasinorder;
set quattrofullAE;
Proc sort;
by min;
run; Proc print
data=sasinorder;
run;
Data timecode;
set sasinorder;
If min ge 90 and min le 95 then time =1;
If min gt 95 and min le 100 then time = 2;
If min gt 100 and min le 105 then time = 3;
...
If min gt 235 and min le 240 then time = 30;
keep trt rep sec min time AE Centriod PF;
run;
Proc Print data= timecode;
run;
/*
```

Through the SAS sort procedure, a new sorted data sheet was compiled with the variable, timecode going from1-30 (representing the time from 90 to 240 min) with each unit representing a 5 min time range. Thus, the effect of treatment and time period of *L. lactis* ssp. *lactis* C2 bacteria could be statistically analyzed by using a SAS general linear model (GLM) and SS4 sum of squares. An example of the GLM-SS4 model that was run is shown below:
Data timecode;
input trt rep sec min AE Centroid PF time;
cards;
;
Proc Print;
Proc Corr;
Proc GLM;
Class trt rep time;
Model AE Centroid PF=trt time trt*time/ solution ss4;
lsmeans trt time trt*time/PDIFF stderr;
run;
/

Using the same design of statistical analysis, acoustic emission data from all treatments and replications were analyzed from 0-90 minutes, 90-120 minutes and 90-240 minutes. Significant different was considered when p-value was smaller than 0.05. Some trend information is given where the probabilities are greater than 0.05, but these a given so that the reader can use these at their own discretion.

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CHAPTER 4

RESULTS AND DISCUSSION

Acoustic information from *Lactococcus lactis* ssp. *lactis* C2 grown in M17 medium was compiled from 0 to 240 min, which included the lag, log, and beginning of the stationary phase. Controls were compared to other treatments where bacteriophage (c2, sk1, and ml3) were used to infect the host culture (*lactis* C2) at 90 min. The acoustic energy (AE) information collected contained the Absolute Energy (ABE) being generated, the average frequency being emitted (Centroid frequency, CF), and the intensity of the frequency (peak frequency, PF). These three parameters varied depending on the metabolic activity of the host culture, the stress being placed on the host culture (due to bacteriophage infections) and the actual sound of the bacteriophage DNA being injected into the host cells. Data for all of the activities were broken down from the main data set and presented in a structured manner so that the reader would be able to understand the complexity of the data and the analyses that was conducted to show the complexity of the acoustic energy being generated by the host bacteria and the bacteriophage.

4.1 Growth of Lactococcus lactis ssp. lactis C2

*L. lactis* ssp. *lactis* C2 enters the log phase from the lag phase at approximately 90 min (Figure 18). Figure 18, shows the change in Absorbance measured at 600 nm (A$_{600\text{nm}}$) for *lactis* C2 grown in M17 medium for the entire 240 min growth period. Note that as growth time passes 90 min, the A$_{600\text{nm}}$ increases rapidly as the culture enters the log phase. Then prior to 240 min the A$_{600\text{nm}}$ levels, indicating that little additional cell division was occurring and that *lactis* C2 had entered the stationary phase. This standard growth curve of *lactis* C2 was extremely typical of growth curves by lactic acid bacteria (LAB) (Hicks et al., 2004). Growth of *lactis* C2 was also determined by enumerating the growth (colony forming units, cfu) at 30 min intervals (Figure 18) and by measuring the change in voltage derived from the photo diode system (Figure 19). Figure 18 shows the average increase in cfu over the entire incubation period. Note that the rapid increase in cell numbers occurred from 90 min to just before 200 min. By the time the stationary phase was reached, the cfu went from an initial assay of $10^7$ cfu/ml to more than $10^8$ cfu/ml, a 1 log increase in cell numbers. No significant difference was observed between the
shape of the A$_{600nm}$ and CFU curves. Figure 19 shows the drop in voltage as cell density increased (more light being absorbed by cells) and compares this measurement with the A$_{600nm}$ data over the same time period. When the stationary phase was reached the voltage measurement went from 4.78 volts at 0 min and leveled at approximately 1.31 volts at 240 min. Note that voltage measurement decreased during the lag phase such that it was difficult to know that a lag phase even existed compared to the A$_{600}$ measurement. These observations suggested that the initial voltage measurement (0-90 min) derived from the photodiode was more sensitive to cell changes (possibly metabolic) than the A$_{600}$ measurement which is specific for the measurement of cell density or cell numbers (Jay et al., 2005). When the data was inverted (New voltage = 5- voltage) so that the curves would have the same shape, no significant difference was observed between the new voltage and A$_{600nm}$ data for measuring the increase in cell density during the log phase (90 to 240 min). All three methods (A$_{600nm}$, cfu, and voltage) appeared to be equal for tracking change in cell density. However, the voltage data appeared to be a better determinant of the activity occurring during the lag phase.

A fourth method that could be used to monitor cell numbers was the increase in total AE output as tracked by the AE software data collection system (Figure 20). Figure 20 shows the summation of the raw hit data as it was collected by the AE software. Notice that this curve is extremely similar to the A$_{600nm}$ curve. Earlier work, by Stencel (2010, Unpublished) showed that this curve was not statistically different than the A$_{600nm}$ curve. Since AE is a measurement of microbial noise or sound output, it would be extremely logical that the AE recorded would be equivalent to the number of bacteria and activity within a few millimeter of the sensor face.
Figure 18. Lactis C2 culture growth curve as measured by CFU and OD600nm

Figure 19. Absorbance and voltage measurement without bacteriophage infection
4.2 Monitoring bacteriophage activity during host growth and lysis

4.2.1 Absorbance measurements. *L. lactis* ssp. *lactis* C2 was used as the host bacteria for three different bacteriophages. These three bacteriophages were ml3, sk1, and c2. All three bacteriophages were able to cause the lysis of *lactis* C2. Working cultures of bacteriophage were developed by infecting *lactis* C2 and then harvesting the bacteriophage through a microspore filter. Ending titers (plaque forming units, pfu) of bacteriophage in the working culture are shown in Table 5.

Table 5. Enumeration of bacteriophage in working culture

<table>
<thead>
<tr>
<th>Phage Type</th>
<th>10⁵ dilution (pfu*)</th>
<th>10⁶ dilution (pfu)</th>
<th>10⁷ dilution (pfu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ml3</td>
<td>TNTC†</td>
<td>41</td>
<td>4</td>
</tr>
<tr>
<td>sk1</td>
<td>TNTC</td>
<td>43</td>
<td>4</td>
</tr>
<tr>
<td>c2</td>
<td>TNTC</td>
<td>20</td>
<td>2</td>
</tr>
</tbody>
</table>

* Plaque forming units/ml
† Too numerous to count

Both ml3 and sk1 stock averaged about 4 x 10⁷ pfu/ml whereas c2 average 2 x 10⁷ pfu/ml. To maximize the signal and quality of the data collected the bacteriophage were always added to the
lactis C2 culture at 90 min. At 90 min the host culture is just entering the log phase. By adding approximately $10^4$ (0.05 ml bacteriophage stock /50 ml) pfu at 90 min, infection of the host is immediate and complete lysis of all host cells will always occur prior to the time that the culture enters into the stationary phase. By adding the bacteriophage at this concentration several infection cycles can be tracked before complete lysis occurs or before the culture enters the stationary phase where cell replication stops. Normally two full lysis cycles can occur before the $A_{600\text{ nm}}$ falls dramatically. Lysis of lactis C2 was followed by measuring cell density ($A_{600\text{ nm}}$).

Figure 21 shows the average lysis curves for ml3, sk1, and c2 bacteriophage when the host lactis C2 was infected at 90 min and then compared with a standard growth curve. Note that while all three bacteriophage caused lysis, the timing of the lysis appeared to be slightly different, although there was no significant difference between the three curves. Bacteriophages ml3 and sk1 produced very similar lysis curves, whereas the c2 lysis curve was a bit more extended, which probably resulted from the stock culture being less numerous than the ml3 and sk1 cultures.

4.2.2 Comparison of Voltage and Absorbance measurements. Voltage reading taken at the same time as the absorbance measurement are shown in Figures 22, 23, and 24 for all three bacteriophages. Figure 22 compares the lysis curve derived from the voltage and that derived from $A_{600\text{ nm}}$ data for bacteriophage ml3. Figure 23 and 24 shows the same data for bacteriophages sk1 and c2, respectively. While both the $A_{600\text{ nm}}$ and voltage data show similar trends some subtle differences exist. Voltage measurements seem to lag $A_{600\text{ nm}}$ measurements. This observation is particular evident in Figures 23 and 24 at the 150 min observation, where the voltage measurement turns up well after the $A_{600\text{ nm}}$ measurement has turned down. Although the voltage measurement is derived from a photo diode receiving light from an LED, it is obvious that this light source is measuring something slightly different than what is being measured at 600nm. This trend was very apparent in Figure 24. Another subtle difference is the shape of the curves after lysis occurs. All $A_{600\text{ nm}}$ curves continue to decrease slightly after the main lysis event, but the voltage show a sharper break in the curve after the main lysis event. This is particularly evident in Figures 21 and 22 at the 180 min data point. The $A_{600\text{ nm}}$ data would suggest that some lysis is still occurring after the main lysis event, whereas the voltage data
Figure 21. Average absorbance of *Lactococcus lactis* ssp. *lactis* C2 with and without being infected by bacteriophage ml3, sk1 and c2.
Figure 22. Average absorbance and voltage measurement when Lactococcus lactis ssp. lactis C2 was infected with bacteriophage ml3.

Figure 23. Average absorbance and voltage measurement when Lactococcus lactis ssp. lactis C2 was infected with bacteriophage sk1.
Figure 24. Average absorbance and voltage measurement when *Lactococcus lactis* ssp. *lactis* C2 was infected with bacteriophage c2.

would suggest that once the main lysis event occurs, all lysis of cells was essentially finished. Logically, the voltage data seems to track lysis better than the absorbance data. *Hicks* (2010) summarized that after high phage titers build up lysis occurs not only from lytic enzymes released within the host cell but also from that being liberated into the medium. Thus, after significant lytic enzymes are released into the medium all cell lysis should end fairly abruptly. It is well known (Jay et al., 2005) that some mutant cells can resist lysis, probably by changing the hetropolysaccharide layer covering the cell membrane, which sterically blocks the action of the lytic enzyme from hydrolyzing the host cell membrane. However, the number of mutants surviving a lysis event is small and several hour of incubation are require before cell numbers increase enough to cause the absorbance to increase a second time (Jay et al., 2005). Thus, the continued decrease in the A\textsubscript{600nm} curve may be monitoring the continued hydrolysis of the cellular debris after the main lysis event. *Hicks et al.* (2004) observed that once the host cells had lysed that there was sufficient bacteriophage (DNA void bacteriophage shells) attached to the cellular debris to cause the debris to form large clumps of cellular debris. This resulted because of the sticky nature of the proteins coating the bacteriophage surface.
4.3 Acoustic Emission of *Lactococcus lactis* ssp. *lactis* C2 from 0 to 90 min

Acoustic emission data from 0-90 minutes were collected on all experimental treatments before the bacteriophage infection point which included all of the lag phase. In total, 11 replications were combined together for analyses. Background noise was very low after setting the software filter to 21 decibels (db). Background noise for absolute energy (ABE) and peak Frequency (PF) in M17 medium without culture added were as low as what Cox (2014) observed while monitoring background noise in TSB medium when she set the software filter at 21 dB. By setting the initial decibel threshold reading to 21dB, all data generated under this level were eliminated. This is a practice that is common in AE data collection (Corporation, 2003). Generally, the threshold level is set at a point where a small amount of noise can be seen to enter the data stream. Thus, when *lactis* C2 was added to the M17 medium the big increase in AE would be due the AE being produced by the culture. Various techniques were then employed to analyze these data to determine how the culture was affecting the AE being recorded.

The data were then sorted into 5 min intervals and each interval was numbered from 1 to 18, such that 1 equaled the interval from 0 to 5 min and 18 equaled the interval from 85 to 90 min. When the data was broken out in this manner the time interval became an independent variable when analyzing what was happening to ABE, CF, and PF.

4.3.1 Consistency of Acoustic Emission data. Data from the lag phases (0 to 90 min) of the 9 replications (lag phases of the treatments [treatments 1, 2, and 3] where bacteriophage were added at 90 min) were first compared to the lag phase of the control treatment (std) to see if there were any day to day differences in the data collection system. Figure 25 show the comparison for ABE. Note that while some small differences were apparent none were significant and the standard error was only 0.05, suggesting the variance between data sets were normal and no adjustments for different replications were needed.

A little more variation was noted for CF and PF data sets. The CF data set (Figure 26) showed no difference between the treatment 1 and the std control data set but some difference (p< 0.05) was observed between treatment 2 and treatment 3 with the control std data sets. However, all the differences were noted between the treatment groups (blue) which were run on successive days, showing that there was some shift or drift over time within the data collection system. This same shift was also noted within the PF data (Figure 27). After understanding that
a slight shift was occurring in some of the data due to day the experiments were run on, initial data evaluations were run with day as a covariant to see if some of the variation could be removed from the data sets. After viewing analysis with and without day as a covariant, no difference (p > 0.05) were observed, so all data analysis were conducted without using day as a covariant.

4.3.2 Absolute Energy emissions during lag phase. When time was broken out into 5 min intervals during the first 90 min during the lag phase, the overall ABE collected (Figure 28) appeared to be fairly random (not significant), but ranged from 0.4 to 0.59 attojoules (aj). While the overall average energy did not change, ten of the 18 ABE points were below 0.5 aj. Each data point represents an average of 686 data observations. Thus, once the culture was added to the medium the increase (p<0.0001) in sound (ABE) over the base line being emitted by lactis C2 was sustained at a fairly constant level as the culture prepared itself for cell division.

4.3.3 Changes in Centroid and Peak frequencies in the lag phase. As lactis C2 moved through the lag phase toward the log phase the CF increased (p < .0001) from 132.4 to more than 134 kHz (Figure 29). This shift in average frequency may result from changes in cellular activities as the cells prepare for cell division prior to the cells entering the log phase. After 25 min of incubation a metabolism event appeared to be occurring at an 18.33 min interval where the CF would drop to approximately 133 KHz for a short period, suggesting that the cells were completing metabolism cycle. This metabolic cycle may also be present in the ABE (Figure 28) data set, but seem to precede the CF data set by a few minutes.

Peak frequency also increased (p<.0001) with time, increasing from less than 8 kHz to more than 13 kHz (Figure 30). Although total energy (ABS, Figure 28) was roughly the same, the CF shift upwards to a higher frequency along with the sound intensity (PF) suggesting that certain activities were gearing up during this lag phase. These data suggest that activities occurring during the lag phase could be monitored using AE in ways that could not be monitored before. Peak frequency would be expected to increase if cell numbers increased near the AE sensor, which in the lag phase could happen if some of the cells were settling onto the face of the sensor, which was positioned at the bottom of the growth tube. However, Figure 30 shows the PF frequency increasing to approximately 14 kHz but then falling slightly. If cells were settling no
decrease in the signal would be expected. It is highly likely that the increase in PF is due to increase in metabolic activity.

![ABE LSMEAN FOR EACH TREATMENT IN 0-90 MINUTES](image)

- standard error=0.05, no difference in letters represent no significant difference

**Figure 25. Comparison of Absolute Energy (ABE) LSMEANs for each treatment from 0-90 min.**

![CF LSMEAN FOR EACH TREATMENT IN 0-90 MINUTES](image)

- standard error=0.1, differences in a,b,c represent significant difference
Figure 26. Comparison of Centroid frequency (CF) LSMEANS for each treatment from 0-90 min

- standard error=0.3, differences in a,b,c,d represent significant difference

Figure 27. Comparison of Peak frequency (PF) LSMEANS for each treatment from 0-90 min

Figure 28. Absolute Energy (ABE) LSMEANS for 5 minutes interval from 0-90 min
Figure 29. Centroid frequency (CF) LSMEANS for 5 minutes interval from 0-90 min

Figure 30. Peak Frequency (PF) LSMEANs for 5 minutes interval from 0-90 min
4.4 Statistic results of acoustic emission data from 0-240 minutes

After 90 min of incubation the culture entered the log phase. It was at the 90 min point the culture was infected with bacteriophage. Three different bacteriophages (ml3, sk1, and c2) were used to infect the \textit{L. lactis} ssp. \textit{lactis} C2 host culture. Infections by these three bacteriophage represented 3 different treatments which were also compared in some cases to a control (without bacteriophage) for the 4th treatment. The treatments where the host was infected with bacteriophage followed a lysis curve pattern as denoted in Figure 21, and the control followed a standard curve pattern as shown in Figure 18.

When data was analyzed where the bacteriophage had been used to infect the host \textit{lactis} C2 culture from the beginning of the acoustic data collection to the end, the bacteriophage effect (3 treatments) on least square means of ABE was 0.489, 0.429 and 0.485 with standard error 0.19 (Figure 31) for bacteriophage infections ml3, sk1, and c2, respectively. Ml3 may have been slightly different ($p<.077$) than sk1, but not c2. Also sk1 may have been slightly different ($p<.11$) than c2. While these means did not show normal significant differences, the differences in ABE might be used as a way to partly characterize these bacteriophages. Also, it should be noted that the ABE presented here are an average of both cellular and bacteriophage activity and are the sum of data over the 240 min incubation period.

The least square means of centroid frequency for the ml3, sk1, and c2 bacteriophages were 134.9, 133.4 and 133.5 with standard error 0.07, respectively which are shown in Figure 32. The average centroid frequencies for ml3 was significant higher ($p<.0001$) than sk1 and c2 bacteriophages, but sk1 and c2 were not significant different ($p=.66$).

The least square means of peak frequency for ml3, sk1, and c2 were 16.8, 11.5 and 10.4 with standard error 0.16 as presented in Figure 33. Peak frequency for ml3 was greater ($p<.0001$) than for sk1 and c2. Also, PF for sk1 was greater ($p<.0003$) than c2. These data suggest that these three different bacteriophage (ml3, sk1, and c2) could be distinguished due to their differences in ABE, CF, and PF. Thus, the AE data was sufficient to characterize these three bacteriophages even though the AE from the host bacteria was also present and the data was an average of the full 240 min incubation period.
Figure 31. Absolute energy (ABE) LSMEANS for each bacteriophage between 0-240 min.

Figure 32. Centroid Frequency (CF) LSMEANS for each bacteriophage between 0-240 min.
4.5 Acoustic emission data from 90-240 minute

4.5.1 Log phase growth. Since difference due to bacteriophage could be distinguished in the full data set, the lag phase data was removed from the data set. This analysis included only data after the host, *lactis* C2 was infected with bacteriophage ml3, sk1, or c2. It was hoped that, by removing the lag phase data, a more accurate characterization of the bacteriophage activity could be determined.

Analysis of the data from the infection point (90 min) to the end of data collection (240 min), showed that the bacteriophage affected the least square means of ABE, CF, and PF. The least square means of ABE for bacteriophages ml3, sk1 and c2 were 0.49, 0.43 and 0.48, respectively, with standard error 0.025. Slight differences (Figure 34) were observed between least square means of ml3 and sk1 (p < 0.08) and sk1 and c2 (p < 0.13) but no difference between ml3 and c2 (p = 0.86).

Centroid frequency for bacteriophages ml3, sk1, and c2 were 134.9, 133.4 and 133.5, respectively, with standard error 0.09 (Figure 35). Bacteriophage ml3 produced a higher CF than either sk1 of c2 (p < 0.0001), but no difference was observed between sk1 and c2 (p = 0.66).
Peak frequency for bacteriophage ml3, sk1, and c2 were 16.7, 11.7 and 10.5 with standard error 0.0002 (Figure 36). Bacteriophage ml3 produced a greater ($p < 0.0001$) PF than sk1 or c2 and sk1 produced a greater ($p < 0.0003$) PF than c2.

These data suggest that each bacteriophage has a fairly characteristic AE signal, with ml3 being the most different. Indeed ml3 produced the highest ABE, CF, and PF making ml3 easily distinguishable from sk1 or c2. Although sk1 and c2 had similar ABE and CF signals, they could easily be distinguished by the difference in their PF or intensity of sound being emitted.

**Figure 34.** Absolute energy (ABE) emitted for each bacteriophage between 90-240 min.
Figure 35. Centroid Frequency (CF) LSMEANS for each bacteriophage between 90-240 min

Figure 36. Peak Frequency (PF) LSMEANS for each bacteriophage in 90-240 min.
4.6 Effect of bacteriophage on *L. lactis ssp lactis* C2 from 90-120 min

Acoustic data from 90-120 min discerned how the host bacteria, *lactis* C2, was impacted after infection by the three bacteriophages. Analysis of data from 90 to 120 min comparing the treatments that contained the bacteriophage with the control (without bacteriophage) showed shifts in ABE, CF and PF.

Absolute energy values for bacteriophage ml3, sk1, and c2 verses the host *lactis* C2 were 0.56, 0.42, 0.46 (bacteriophage) and 0.43 (host), respectively, with standard error 0.06 (Figure 37). Bacteriophage ml3 produced a greater (p < 0.05) ABE than sk1 and c2. All other differences between bacteriophage and host were non-significant. After infection, bacteriophage ml3 increased (p < 0.08) the ABE during the first 30 min of infection from 0.43 to 0.56 over the control.

The least square means of the CF for bacteriophages ml3, sk1, and c2 were 134.3, 133.6, 133.4, respectively, compared to the host, *lactis* C2 at 134.5 with standard error of 0.2 as shown in Figure 38. Again ml3 CF was greater (p < 0.01) than sk1 and c2 CF's. Also the host, lactis C2 CF was greater (p < 0.006) than the sk1 and c2 CF's, but host, lactis C2 was not significant different (p = 0.66) from the ml3 CF.

The least square means for PF for bacteriophages ml3, sk1 and c2 were 16.5, 11.8, 9.4 kHz compared to the host lactis C2 of 15.1 kHz with standard error 0.4 (Figure 39). Bacteriophage ml3 produced a greater PF than sk1 or c2 (p < 0.0001) and the host, *lactis* C2 (p < 0.05). During the 90-120 minute period, the intensity of the PF for every treatment was shifted after bacteriophage infection. The least square means of PF for bacteriophage ml3 infection increased from 15.1 to 16.5, however bacteriophage sk1 and c2 decreased PF to 11.8 and 9.4 respectively.

These data from 90-120 min of ABE, CF and PF suggest that bacteriophage ml3 may cause less stress on the host *L. lactis* ssp. *lactis* C2 bacteria compared to bacteriophage sk1 and c2, because ml3 has CF and PF values that are closer to the *lactis* C2 values when infected. However, ml3 did produce a slightly greater ABE (P<0.08) than host, *lactis* C2.
Standard error 0.06

**Figure 37. Absolute Energy (ABE) LSMEANS from 90-120 min**

Standard error 0.2

**Figure 38. Centroid Frequency (CF) LSMEANS from 90-120 min**
4.7 Determination of bacteriophage cycles

It was presumed that the bacteriophages would produce a unique AE signature after the host cell lysed, and the newly liberated bacteriophage infected a new host cell. It was also presumed that the AE of infection could be unique to each bacteriophage type and that the point of each infection of the same bacteriophage would have the same AE characteristics (non-significant) but that these AE signatures would be different (significant) from those being emitted by the host cells. Also, all infection points would be at a specific time interval for each bacteriophage. To do this analysis, data from 90 min (infection point) to 240 min was selected, sorted as before into 5 min time periods with period 1 = 90 to 95 min and period 30 = 235 to 240 min. The data were then analyzed by bacteriophage type (ml3, sk1, and c2) and time period for ABE, CF, and PF. Once the least square means and significances for the means were generated, a visual observation of the significance table was made to determine if there were means that were non-significant at specific intervals.

4.7.1 Infection cycle of ml3.

At the point of infection bacteriophage ml3 was observed to have an average infection cycle of 30 min (taken from ABE data) or 31.7 min (taken from CF data), averaging 30.8 min. Since
each time period represents a 5 min time interval where the peak infection point was estimated the two averages are not exactly the same. The average ABE was 1.192 aj, CF was 132.9 kHz, and PF was 18.85 kHz. These AE values were an average of the values picked off the infection points. These average values would have limited host, *lactis* C2 AE information in them and would be considered to be of greater accuracy than those presented above. All AE values were averages from at least 4 infections points.

4.7.2 Infection cycle of sk1.

At the point of infection bacteriophage sk1 was observed to have an average infection cycle of 34.33 min (taken from CF data). It was much more difficult to pull an average infection point from the ABE data so no values were estimated from those data. The average ABE was 0.42 aj, CF was 133.2 kHz, and PF was 11.58 kHz. Again all AE values were an average of at least 4 infection points.

4.7.3 Infection cycle of c2.

At the point of infection bacteriophage c2 was observed to have an average infection cycle of 31.25 min (taken from ABE data) and 31.25 min (taken from CF data) for an average infection cycle of 31.25 min. The average ABE was 0.46 aj, CF was 133.9, and PF was 10.37. Again all AE values were an average of at least 4 infection points.

When the individual bacteriophage infection points were compared sk1 had a longer infection cycle at 32°C than ml3 or c2. Bacteriophage ml3 and c2 had infection cycles that were more similar. As the infection points moved through several cycles the later infection points became more diffused (spread over a longer time period). The sk1 infection points appeared to become more diffused quicker than ml3 and c2.

These values were somewhat in line with those observed by Wardani et al. (2010) who reported sk1 and ml3 to have a first infection peak at 33.2 and 40 min, respectively at 26°C. The lower temperature use in Wardani et al. (2010) research would be expected to prolong the infection cycle which was the case with sk1. The infection cycle of ml3 at 40 min would be longer than what would be expected from this research, unless temperature had a major impact on the number of bacteriophage being produced by ml3.
Another important observation was that as total lysis of the cells occurred and the medium was cleared (absorbance returned to near initial levels, Figure 21), the AE information could still be collected from the cellular debris. Evidently, as the last cells lysed, the new bacteriophage progeny could still bind irreversibly to some of the cellular debris and inject their DNA into the cellular debris. This was certainly noticeable as late as 185 min, well after the medium had cleared at approximately 150 min (Figure 21).

It was quite obvious that when the initial infection point (90 min was where bacteriophage was added to the host culture tube) was compared to the other infection points, that the AE that was being recorded was due to the sound of injecting the DNA from the head of the bacteriophage through the bacteriophage tail and through host, lactis C2 cell membrane. Evidently, when ATP is released and the bacteriophage shrinks its head to force the DNA out, AE was produced as the DNA rushes through the bacteriophage tail and cell membrane. The difference in AE would be sufficient to identify each of the three bacteriophages used in this study, and presumably all bacteriophages would be sufficiently different that they would also produce a distinct AE signature.

4.8 Effect of bacteriophage infection on host, lactis C2, stress

When the data was sorted by 5 min intervals such that time 1 = 90 to 95 min and time 7 = 120 to 125 min and analyzed for the effect of time on AE parameters, the effect of bacteriophage on host, lactis C2 cell stress became more apparent. These time periods between 90 and 125 min represent the period of time where host, lactis C2 is still increasing in cell numbers (prior to lysis phase). During this time the bacteriophage would have an initial infection point at 90 min and second infection point sometime between 120 and 125 min. After the second lysis cycle at approximately 150+ min cell numbers were rapidly declining. Thus, the time periods between 90 and 125 min were thought to be the most important for determining the stress that the host, lactis C2 would experience after being infected with bacteriophage. These initial analyses of the time sorted data include the data from all three bacteriophages. Figure 40 shows the shift in ABE after the bacteriophage has been added to the host, lactis C2, culture. Note that the ABE was 0.7 aj at the point of infection (90 min) but dropped (p<.0001) to 0.4 aj after the first 5 min of infection and stayed near that point for the following 15 min and then increased (p < 0.001) to

65
0.5aj for the remaining test periods. These data clearly show that the host, *lactis* C2 was under stress during this infection period.

![ABE LSMEANs for 5 min interval from 90-120 min](image)

**Figure 40. ABE LSMEANS for 5 min interval from 90-120 min**

Figure 41 shows the change in CF at the point of infection (90 min). Prior to the log phase the CF for lactis C2 was near or below the 133.5 kHz level. At point of infection the CF dropped to 132 kHz and then increased to approximately 134 kHz prior to lysis. Thus, the metabolic activity within the host, *lactis* C2 cells were shifted to a higher frequency once the infection had occurred. These data suggest that CF might be a helpful tool for monitoring bacteriophage infections.
Figure 41. Centroid Frequency LSMEANS for 5 min interval from 90-120 min

Figure 42 shows the change in PF after bacteriophage infection. Note that the PF initially dropped from 12.75 kHz to approximately 12 kHz and then increased to more (p< 0.0001) than 14 kHz in the end time periods. These differences might be interpreted by looking at what is going on in the cell. After infection the bacteriophage is taking control of cellular activities; thus as the cell shifts from cell division activities the intensity of sound (PF) decreases but once the cellular activities are shifted into manufacturing bacteriophage parts, the activity intensified the PF or the intensity of the sound. Thus, the curves observed in this study seem to be very logical.
Figure 42. PF LSMEANS for 5 min interval from 90-120 min

Figure 43 shows the CF analysis where all three bacteriophages are compared with the control curve (without bacteriophage, culture only) during the same time periods (90 to 120 min). While the CF increased slightly for the lactis C2 culture as it entered the log phase, the CF stabilized at a value of less than 135 kHz, and was fairly constant. However, when lactis C2 was infected with bacteriophage the CF shifted in various directions depending on bacteriophage type and had considerably more variability than the control curve. In general, ml3 bacteriophage produced a higher CF than sk1 or c2 bacteriophage, which was consistent with the earlier data. One would expect the AE from the growth of lactis C2 in the log phase to be more consistent than if it was infected with bacteriophage. Thus the data presented in Figure 43 would meet expectations.

Figure 43. Centroid Frequency LSMEANs for effect treatment and time from 90-120 min

Acoustic emission data appears to be a powerful tool to characterize some of the activities occurring within bacteria during the lag and log phases of growth and the stress caused by bacteriophage when they infect host cells. In addition, AE could be used to distinguish
bacteriophage types and possible understand the forces necessary to drive the DNA through the bacteriophage tail and through the host membrane.
CHAPTER 5

CONCLUSION AND FUTURE WORK

The AE derived from *Lactococcus lactis* ssp. *lactis* C2 grown with or without bacteriophage (ml3, sk1 and c2) infections showed different acoustic information through the entire 240 min data collection.

The average voltage and absorbance data with and without bacteriophage infection tracked the growth and lysis *L. lactis* ssp. *lactis* C2 and allowed for data analysis prior to infection, during infection, and after lysis. Tracking of cell growth (log phase) and lysis of *lactis* C2 was accomplished equally whether using voltage or absorbance measurements (no significant difference). However, voltage measurements were more accurate in tracking changes during the lag phase, whereas absorbance at 600 nm seemed to monitor cell division best. Both procedures were equal for tracking differences in cells as affected by the three different bacteriophages.

Acoustic emission from host cell growth from 0 to 90 minutes was always the same prior to bacteriophage infection for ABE. However, slight differences were observed for CF and PF which was presumed to be due to differences resulting from day, and/or temperature effects of the electronic boards, etc.

Statistical analysis of AE data from 0 to 240 min showed no ABE difference between bacteriophage ml3 and c2, but that ABE from either ml3 or c2 was significant higher than sk1. Also it was observed that no CF difference existed between bacteriophage sk1 and c2. But the CF from either sk1 or c2 was significant lower than ml3. Significant difference were observed in PF for each treatment with bacteriophage infection by ml3 exhibiting the highest PF at 16.8 kHz from 0 to 240 minutes. Acoustic data from 0 to 240 min, showed that bacteriophage ml3 produced the highest ABE, CF and PF signals.

Shifts in AE from 90 to 240 min showed that ABE, CF and PF data could easily distinguish ml3 from bacteriophage sk1 or c2, and likewise sk1 from c2. Thus, AE information could be used as a “finger print” to identify these bacteriophage.

Results from 90 to 120 min AE showed that ml3 produced a greater ABE than the other bacteriophage in the first 30 min of infection going from 0.43Aj to 0.56Aj over the control. Both
the CF from ml3 and host *lactis* C2 were greater than sk1 and c2 CF’s. But host *lactis* C2 was not significant different from ml3 CF. Bacteriophage ml3 produced a greater PF than sk1 or c2 and the host lactis C2. These data from 90-120 min of ABE, CF and PF suggest that bacteriophage ml3 may cause less stress on the host *L. lactis* ssp. *lactis* C2 bacteria compared to bacteriophage sk1 and c2 treatment, because ml3 had CF and PF values that were closer to the host values.

At point of infection bacteriophage ml3 was observed to have an average infection cycle of 30.8 minutes. Bacteriophage sk1 was observed to have an average infection cycle of 34.33 min and bacteriophage c2 was observed to have an average infection cycle of 31.25 minutes.

These AE data suggested that bacteriophage ml3, sk1 and c2 can easily be distinguished by the differences in ABE, CF and PF signals. The distinguishable difference produced by AE could provide a novel method to identify different bacteriophage and track certain metabolic and cell growth parameters. Further research is still needed to identify where the AE information is being generated and whether all bacteriophage are as different as those used in this study.
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