

OBSERVED DEFECTS OF SWISS CHEESE BASED ON THE MICROBIOME  
CONTRIBUTION TO THE PRODUCTION OF ORGANIC ACIDS

by

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of the requirements for the degree of

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## DEDICATION

This thesis is dedicated to a number of inspirationally wondrous people who have shaped my drive and perspectives throughout this adventure of life: To my father, Donald S. Campfield, who always supported and encouraged my inquiring mind, tenacious will, and constant desire to become the most exemplary version of myself. To my son, Lucian, who inspired me to advance my education and steadfastly endure sacrifices that have been vindicated by a happier life. To my Grandmother Shirley and my Aunt Melody who demonstrated numerous times that our outlook in life should not focus around the adversities we encounter but rather, the unpretentious fortitude with which we carry ourselves.

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## ABSTRACT

The United States Department of Agriculture downgrades on the order of 17% of all Swiss cheese produced in the United States due to defects. Many of these defects are related to improper eye formation, number, distribution, or size; leading to an industry loss of over \$69 million per annum. The microbiome in Swiss-type cheeses plays a significant role in eye development due to production of organic acids and gaseous emissions contingent on bacterial abundance and phenotype. The relationship between bacteria and the organic acids they produce leading to Swiss cheese defects can be correlated using Next-generation sequencing and high-performance liquid chromatography coupled with UV-Vis and mass spectrometry, respectively. From two processing facilities, Next-generation sequencing identified bacterial genera *Lactobacillus* and *Propionibacterium* to be associated with split/cracked cheese defects, and *Clostridium sensu stricto* 12, *Propionibacterium*, and *Lactobacillus* to be associated with irregular Eye formation/distribution (or collapsed eye formation) defects in Swiss cheese. Also identified through Next-generation sequencing was the genera “*Candidatus Berkiella*”, *Propionibacterium*, and *Lactobacillus* to be associated with blind defects in Swiss cheese. Chromatographic separation and identification of organic acids provided evidence that lower levels of acetic and propionic acids were found in the split/cracked cheese samples; lower abundance of acetic, lactic, propionic and butyric acids were found in blind cheese samples (while a higher abundance of citric acid was found); and lower concentrations of citric, acetic, and propionic acids were found in irregular eye distribution samples. From

these data, it can be concluded that Swiss cheese monitoring for bacteria in the genera *Lactobacillus*, *Propionibacterium*, *Clostridium sensu stricto* 12, and “*Candidatus Berkiella*” can be used as a predictor of three types of cheese defects before and during long storage times leading to inferior product resulting in losses to the processor while organic acid monitoring results proved to be inconclusive.

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## LIST OF ABBREVIATIONS

<i>16S rRNA</i>	<i>16S</i> ribosomal RNA gene
FDCA	Federal Food, Drug, and Cosmetic Act
FTIR	Fourier Transform Infrared Spectroscopy
HPLC	High Performance Liquid Chromatography
ISAT	Idaho Standards Achievement Test
LAB	Lactic Acid Bacteria
MS	Mass Spectrometry
NGS	Next-Generation Sequencing
NSLAB	Non-Starter Lactic Acid Bacteria
PAB	Propionic Acid Bacteria
QC	Quality Control

## CHAPTER ONE: INTRODUCTION TO SWISS CHEESE

### **1.1 Dairy Contributions to Health**

In recent years, consumers have become more aware and selective about how they obtain necessary nutrition causing dairy to be a focal point in these decisions.<sup>1</sup> Swiss cheese is a smart nutritional product due to having high protein and low carbohydrates; making it a popular choice among selective consumers. One ounce of Swiss cheese is packed with 8 g of protein, < 1 g of carbohydrates, 20–25% of daily calcium, 4–10% of daily vitamin A, and 8 g of fats.<sup>2</sup>

### **1.2 Economic Impact**

The National Agricultural Statistics Service reported the United States (U.S.) as having produced over 332 million lbs. of Swiss cheese in 2018, while the European Union countries produced over 22 billion lbs.<sup>3</sup> The state of Idaho's largest agricultural revenue producing industry is dairy; over 15 billion lbs. of milk is generated in Idaho, and on the order of 14 billion lbs. of that milk is used to make cheese. As of 2019, Idaho is ranked 3<sup>rd</sup> in the nation for dairy production.<sup>4</sup> Dairy generates jobs in other industries and tax revenue in all 50 states. An additional 2 million jobs are generated by an indirect economic ripple effect in addition to supporting over 2.9 million jobs in the direct business practices of farming, delivery and manufacturing. Peripheral industry jobs such as retail, transportation, construction and regulation contribute to an overall economic impact in the U.S. of more than \$628 billion.<sup>5</sup>

Jobs aside, the dairy industry provides nutrition curriculum for grades K–12, athletic support, youth programs and snacks during the Idaho Standards Achievement Testing period for children annually, providing millions of dollars in support of youth nutrition, education, and outreach programming. Dairy exportation is another significant contributor to the U.S. economy, yielding over \$5.5 billion in export sales; having increased more than 600% since 1995.<sup>6</sup> Annual revenue from cheese may be compromised due to the frequency of defects leading to quality downgrading of cheese. Each year, ~ 17% of the manufactured Swiss cheese in the United States is downgraded due to observed defects; resulting in an industry loss of over \$69 million.<sup>7</sup>

### **1.3 Observed Defects in Swiss Cheese**

For the purpose of this text, the terms “Swiss cheese” and “Emmental” are interchangeable. Swiss cheese includes any cheese made by the Swiss process as well as any other method to produce a product having the same physical and chemical attributes as cheese produced by the original Swiss process. While the production of Swiss cheese dates back to 500–5,000 B.C.,<sup>8,9</sup> there remain many production challenges that can result in a defective final product. Records and research confirm that observed imperfections in Swiss cheese has been problematic in local economies as well as overall product quality from the beginning, only making it into scientific journals with regard to methods for the improvement of cheese quality dating as far back as the 1920’s.<sup>10</sup> Consumers and food safety programs finally gave cheese regulatory attention with the passing of the Federal Food, Drug, and Cosmetic Act (FDCA) in 1938, establishing the quality standards and identity for food and consumer products such as Swiss cheese.<sup>11</sup>

### 1.3.1 Cheesemaking

Swiss cheese is produced by stirring a lactic acid bacterium such as *Streptococcus thermophilus*, (also referred to as “starter culture”) and rennet, which contains the active ingredient chymosin, into pasteurized, heated cow’s milk. Most cheese starter cultures contain strains of *Streptococcus thermophilus*, *Lactobacillus* spp. and *Lactococcus lactis*. These bacterial strains initiate the acidification of the milk resulting in coagulation of the milk protein, casein.<sup>12</sup> A propionic acid bacterium such as *Propionibacterium freudenreichii* ssp. *shermanii* is commonly added with or as part of the starter culture to ensure the formation of characteristic eyes and flavors in the cheese. The coagulated casein, referred to as cheese curds, are cut into pieces, stirred and poured into a mold containing holes. The cheese is then mechanically pressed inside the mold to remove excess solution whey from the matrix and form a solid block. Cheese blocks, which can weigh on the order of 1,200 lbs., are placed into a 22% brine bath at approximately 15 °C to remove any remaining whey by diffusion into the high salt content brine bath. The salinization process results in the formation of a surface barrier for the cheese block known as the rind. The desired rind thickness may require a few hours to several days to form, depending on the size of the cheese block.<sup>13</sup>

After production, Swiss cheese is stored in a refrigerated cooling room at a temperature of 7–13 °C for approximately 2 weeks, followed by a warming room with a temperature of 20–24 °C for three to six weeks, and a final curing room where it is held at 7 °C for four to twelve months (sometimes longer). The duration of the storage times allows for cheese ripening and final curing as desired by the manufacturer.<sup>14</sup> It is during these

times that defects are most often observed (during quality control (QC) inspections) in Swiss cheese.<sup>15</sup>

### 1.3.2 Defects

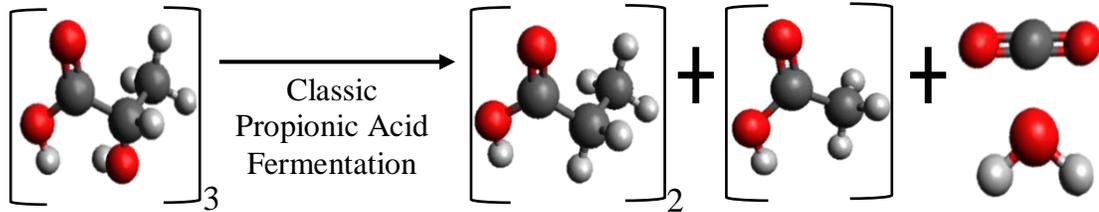
The observed defects examined in this text include but are not limited to irregular/collapsed eye distribution, splits and cracks, overset/gaseous, and blind samples. While there are many other types of defects that pertain to structure, finish, and cheese flavor, the studies presented here are limited to defects surrounding eye formation including distribution, size, shape, and occurrence. For reference, Table 1 lists attributes required to achieve grade-A Swiss cheese according to the U.S. Food and Drug Administration (FDA).<sup>16</sup>

**Table 1. Required Eye Characteristics: U.S. Grade-A Swiss Cheese**

<i>Eye Characteristic</i>	<i>Requirement</i>
<b><i>Diameter</i></b>	1-2 cm
<b><i>Size</i></b>	Relatively uniform
<b><i>Distribution</i></b>	Relatively uniform throughout block
<b><i>Shape</i></b>	Well-developed round or slightly oval
<b><i>Miscellaneous Eye</i></b>	Very slight: dull, rough and shell is acceptable

Swiss cheeses are known for their characteristic eyes or holes throughout the cheese matrix. Eye formation is primarily due to the growth and activity of *Propionibacterium freudenreichii* ssp. *shermanii* when lactate is metabolized in the warm room (> 21°C) during the ripening process.<sup>17</sup> The consumption of lactate peaks between days 28–35 from the start of manufacture, through a classic propionic acid fermentation process, resulting in the formation of propionate, acetate, CO<sub>2</sub> and water<sup>18</sup> (Figure 1). Propionate and acetate

contribute to the sweet and nutty flavors of Swiss cheeses, while the propionate and carbon dioxide are responsible for eye formation.



**Figure 1. Through propionic acid fermentation, *Propionibacterium freudenreichii* ssp. *shermanii* metabolizes lactate yielding propionate, acetate, water and carbon dioxide.**

#### Irregular/Collapsed Eye Formation

In addition to the gas producing bacteria required for proper eye formation, the Swiss cheese must contain fermentable substrates, appropriate elastic texture of the matrix, nucleation sites, and suitable environmental conditions such as pH, moisture, fat, calcium and salt content.<sup>19</sup> In the absence or overabundance of these factors, eyes will not be formed properly; appearing elongated and distorted instead of round or slightly oval. Severe cases yield irregular or collapsed eye formation as shown in Figure 2. This defect is primarily caused by spontaneous fermentation, overabundance of nucleation sites, abnormal moisture or pH throughout the cheese block, and/ or the presence of a gas causing bacteria such as clostridia.<sup>20</sup>



**Figure 2. Swiss cheese exhibiting irregular eye formation/distribution.**

Swiss cheese is very susceptible to *Clostridium* bacteria due to the anaerobic environment, increased ripening temperatures and the low salt content, allowing spores to germinate during ripening. Spores are often introduced to the milk used for Swiss cheese by fecal contamination of udders and the spores are able to withstand the temperature used during pasteurization.<sup>21</sup> These defects lead to misshapen eyes and can result in a soft-bodied cheese having poor matrix suitability; unable to withstand pressure while cooling after being subjected to the warm room. This poor matrix and temperature changes lead to eyes folding in upon themselves.<sup>22</sup>

### Blind

When a Swiss cheese is manufactured and exhibits decreased or absence of eyes, it is labeled as blind (Figure 3). Dairy propionibacteria contribute to the formation of eyes with an optimum growth temperature of approximately 30 °C.<sup>23</sup> While minimal growth

may take place at lower temperatures (14 °C), the warm room temperature should be as close to the optimum temperature noted or the propionic acid fermentation will be drastically reduced.



**Figure 3. Swiss cheese exhibiting a blind defect.**

During the cooking process, if the temperature is close to the lethal temperature of dairy propionibacteria (~ 62 °C) or if the temperature of the cheese block remains high during the pressing process, the number of active propionibacteria will be reduced resulting in a blind defect.<sup>24</sup> Additionally, too high of a salt concentration or too low of a solution pH will result in excessive acidification at the start of the ripening process, causing the growth of propionibacteria to slow or completely stop; resulting in reduced microbial activity and blind cheese.<sup>25</sup>

### Splits and Cracks

The observed splitting and cracking of Swiss-type cheeses is one of the least controlled defects in the dairy industry. Splitting and/or cracking generally occurs during

secondary fermentation and can appear as small cracks of only 1 cm in length to defects spanning the full length of 90 kg or larger cheese blocks (Figure 4).



**Figure 4. Swiss cheese exhibiting a split/cracked defect.**

In Swiss cheeses, the split/crack defect occurs during the curing process primarily in the final cold room (7°C). While slits and cracks may not affect the flavor of the cheese, it results in revenue loss due to downgrading and added difficulty in cutting by mechanical slicers, causing excess discarded product. Substantial research has been conducted to understand splits and cracks in cheese, but no consensus has emerged regarding causative conditions. Studies performed by Hettinga, et al. provided no evidence for correlation between the defect and excessive CO<sub>2</sub> or proteolysis in the cheese.<sup>26</sup> Park, et al., in 1967, reported no relationship between the defect and salt distribution, pH, proteolysis or moisture level, but did show that an increased incidence of splits and cracks occurred when certain propionibacteria strains were present in the starter culture used to make the cheese.<sup>27</sup> In their 2003 paper, White, et al. noted that although there is no relationship

between the split/crack defect and moisture, fat content, pH, protein degradation, or lactose content, the cheese produced in the summer months contained 2% higher moisture and had a greater incidence of splits.<sup>28</sup>

Even though consensus is lacking for definitive cause of split/crack defects it is understood that defects are associated with excessive gas production and/or an unstable cheese matrix that is unable to accommodate the gas produced. Secondary fermentation, however, is seemingly caused by gas production following desired propionic fermentation in the warm room.<sup>29</sup>

#### Overset/Gaseous

When a cheese block is labelled as overset, gassy, blown or having some other gas-related defect, there are several culprits that may be attributable to microbiology. These defects are characterized by excessive eyes or eyes of various shape and size (Figure 5) originating from aberrant microbial growth.<sup>30</sup> The two general classes of defect resulting from excessive gas production are early or late onset gasification.

Early gas production can introduce defects when too much gas is generated from time of initial cheese fermentation to approximately 3 weeks of ripening. Practices such as using unpasteurized milk or poor hygiene can result in the presence of coliforms such as *Enterobacter*, *Escherichia*, *Citrobacter*, and *Serratia* in the milk and cheese product. The presence of these coliforms have been consistently associated with early gas defects.<sup>31</sup>



**Figure 5. Swiss cheese showing overset/gassy defect.**

In the case of gas defects caused by late gas production, over gasification occurs from three weeks to as late as 6 months into ripening. Irregular, late gas production is associated with abnormal growth of propionic acid bacteria (PAB), butyric acid bacteria (such as *Clostridium* spp.), salt tolerant lactobacilli, and heterofermentative lactic acid bacteria (LAB), such as *Leuconostoc*. These bacteria will be discussed in greater detail in chapter 2. In addition to the previously mentioned bacteria, using a failed or expired starter culture, while in the presence of heterofermentative LAB, could result in eyes with overly large volume or excessive eye formation.<sup>32</sup> The growth of these bacteria is favored over the homofermentative starter culture bacteria when ripening takes place at 15 °C instead of 8 °C<sup>33</sup>, resulting in defects like those shown in Figure 5.

Many eye-formation defects have been observed in Swiss cheese samples and associated with bacteria that contribute to those defects or the organic acid concentrations

(both high and low) which result in those defects. As with any microbiome population, there are also symbiotic relationships between bacteria and organic acids produced.

## CHAPTER TWO: SWISS CHEESE MICROBIOME

### 2.1 Previous Research

Substantial research in the previous millennia has clearly demonstrated the significant role bacteria play in developing eye characteristics in Swiss cheeses. While bacteria are a necessity for Swiss cheese production and commonly used in starter culture recipes, bacteria are also identified as culprits for the previously discussed defects observed in cheese.<sup>34</sup> Bacterium survival is dependent on metabolites within the cheese as well as environmental factors such as temperature, salt and pH.<sup>35</sup> Given a sufficient environment to survive, dairy bacteria produce organic acids responsible for eye characteristics which include but are not limited to citric, acetic, lactic, propionic and butyric acids. However, the production of organic acids is not by itself diagnostic of an ideal cheese environment.

Many studies have been conducted that correlate the associations between bacteria and known defects observed in Swiss cheese, some of which are briefly reviewed here as they relate to the focus of this thesis. Some species of coliforms such as *Serratia*, *Enterobacter*, *Citrobacter* and *Escherichia*, have been linked to early gas defects<sup>36</sup> due to the production of CO<sub>2</sub> and/or H<sub>2</sub> as byproducts of lactose utilization which can be produced both aerobically (with air) or anaerobically (without air). Since H<sub>2</sub> is poorly soluble in the aqueous curd-phase of cheese making, the presence of very small quantities can lead to serious gas problems. Growth of these coliforms early in cheese production has been shown to contribute to early blowing or gas production defects as well as a reduction of desirable organic acids such as lactate and acetate.<sup>37</sup>

The presence of heterofermentative LAB, such as *Leuconostoc* or certain types of lactobacilli have been known to produce additional gas in cheeses due to metabolizing lactose, which produces the by-products lactate, acetate, CO<sub>2</sub>, and ethanol.<sup>38</sup> The growth of these bacteria is favored over the homofermentative starter culture bacteria when ripening takes place at 15 °C instead of 8 °C<sup>39</sup>, resulting in overset/gassy defects (Figure 5).

Clostridia, a type of butyric acid bacteria, ferments lactate to form acetate, butyrate, CO<sub>2</sub> and H<sub>2</sub>. This gas production, especially the H<sub>2</sub>, can result in the late blowing of cheeses. This late blowing is manifested by the appearance of cracks, abnormally shaped or excessively large eyes as well as blowholes.<sup>40</sup>

Classically, the study of cheese microbiology employed plating a homogenized sample on media followed by phenotypic representation. This selective condition was only useful in gaining information about specific strains of bacteria that grew well on media. This bias proved unsuitable for routine analyses since phenotypic characteristics are dependent on culture and environmental conditions.<sup>41</sup> Molecular techniques overcame many obstacles of phenotypic methods by characterizing nucleic acids, proteins, and fatty acids. Common molecular approaches include gel electrophoresis experiments and polymerase chain reaction (PCR), or real-time PCR applications, in combination with next-generation sequencing (NGS). The microbial profile of cheese dictates the product quality causing non-nucleic approaches to be deemed biased.<sup>42</sup> The results of this project support the correlation between bacterial populations present, and their production of organic acids, all of which contribute to the observed defects by way of Next-generation sequencing, NGS

(for bacterial analysis) and high-performance liquid chromatography, HPLC (for organic acid analysis).

Scientists have used techniques such as NGS, HPLC, mass spectrometry (MS), Fourier-transform infrared spectroscopy (FTIR), microbial plate counts/culturing and many for quantitative and qualitative analysis of bacteria and organic acids (separately), but there are very few (if any) studies that correlate numerous Swiss cheese defects with bacterial population and organic acid composition.

## **2.2 DNA Extraction**

### **2.2.1 Materials and methods**

Swiss cheese samples were obtained from two dairy processing facilities for the study of defects observed during ripening. The first processing facility, referred to as Site 1, provided “Good” Swiss cheese and “Blind” Swiss cheese samples in addition to “Irregular Eye Formation/Distribution”, “Overset”, and “Split/Cracked”. The second processing facility, referred to as Site 2, contributed Swiss cheese samples that were exhibited as having “Irregular Eye Formation/Distribution”, and samples observed to be “Overset” and “Split/Cracked”. Bacterial DNA was extracted from each block of Swiss cheese (>5 lbs.), triplicate samples were taken from locations where significant defect were observed. Sample preparation methods listed in the DNeasy PowerFood Microbial Kit Handbook were adapted for this process. Cheese samples were homogenized using a stomacher (Stomacher<sup>®</sup> 80 paddle blender) for 4 minutes and centrifuged to create a microbial pellet suitable for DNA extraction as well as separate liquid and fat to be removed from the sample. The pellet was processed using a Qaigen DNeasy PowerFood Microbial Kit (Lot: 160031156) per manufacturer protocol. DNA presence greater than 6.0

ng/ $\mu$ L ( $\pm 2.0$  ng/ $\mu$ L) was confirmed by a Thermo Scientific™ NanoDrop 2000c spectrophotometer.

### 2.3 Illumina Sequencing

Sequences from the *16S* ribosomal RNA (*16S rRNA*) gene is approximately 1550 base pairs in length and has been used extensively for the classification and identification of bacteria (and archaea) due to its universality in prokaryotes (Figure 6).<sup>43</sup>



**Figure 6. Bacterial 16s rRNA gene. Various sequence lengths are depicted by relatively sized regions.**

The *16S rRNA* gene is comprised of eight highly conserved regions and nine hypervariable regions across the bacterial domain. The hypervariable regions vary extensively in sequence among different bacteria.<sup>44</sup> In addition to this understanding, more conserved regions correlate to higher levels of taxonomy (i.e. phylum, order or family) and less conserved regions correlate to lower levels of taxonomy (i.e. genus).<sup>45</sup>

Using the *16S rRNA* gene for bacterial identification has the advantages of being inexpensive and enables phylogenetic comparison across multiple taxa with minimal worry regarding horizontal gene transfer. However, *16S rRNA* gene sequencing lacks accuracy at the species level and allows for PCR amplification biases.<sup>46</sup> Due to the decreasing cost of sequencing, many microbiome researchers have shifted to using more comprehensive methods such as whole genome shotgun metagenomics sequencing to classify complete functional microbiota characterization to include bacteria, viruses, and fungi.<sup>47</sup> Shotgun metagenomics is currently a more expensive method for sequencing but it does allow laboratories to study all genetic information for all organisms in complex samples.<sup>48</sup>

### 2.3.1 Materials and methods

DNA samples were packed with ice and shipped to the Idaho State Molecular Research Core Facility in Pocatello, Idaho where phylogenetic sequencing was conducted using an Illumina® MiSeq (s/n: M02404) for the prokaryotic *16S rRNA* gene. The polymerase chain reaction protocol used for gene amplification is summarized in Table 2. Equipment and reagents used for the PCR study are listed in Appendix A.

**Table 2. Thermal Cycle Program for PCR Amplification of 16S rRNA Genes from Swiss Cheese**

Temperature (°C)	Duration (min)	No. of Cycles
95	3.0	1
95	0.5	25
55	0.5	
72	0.5	
72	5.0	1
4	∞*	---

\* Amplicons are held at 4°C until analyzed.

Following high throughput sequencing, the high-quality sequences are filtered, trimmed, and combined into operational taxonomic units (OTUs), originating from 97% identity of the reads from the MiSeq instrument. All protocols and procedures used for the thermo cycler, followed the “Illumina *16S* Metagenomic Sequencing Library Preparation guide” provided by the manufacturer.

## **2.4 Bioinformatics**

After sample processing, the MiSeq system, equipped with MiSeq reporter software, provided analysis results. In accordance with the *16S* metagenomics workflow, organisms were classified from the V3–V4 amplicon regions using a database of *16S rRNA* data. Classifications were derived from the SILVA database. The workflow provided a classification output of reads at different taxonomic levels, including kingdom, phylum,

class, order, family, and genus.<sup>49</sup> This information was used to analyze the bacterial populations and densities at different taxonomic levels for each of the Swiss cheese samples and were then compared against the “Good” Swiss cheese samples.

## CHAPTER THREE: BACTERIAL HEAT MAPS

### 3.1 Intro to bacterial heat maps

Bacterial heat maps provide a graphical depiction of genus or species population using a system of color-coding to characterize abundance. Heat maps are used to visualize the variation in bacteria within a sample population by depicting the content of a dataset in a way that is easily interpreted.<sup>50</sup> in contrast to tabulation of extensive numerical data, which can be very difficult to decipher. Heat maps have been widely accepted for myriad applications including, website traffic analysis, company performance, and scientific data.<sup>51</sup> Heat maps will be used in this text to visualize bacteria present at different taxonomic levels in Swiss cheese samples.

### 3.2 Materials and methods

The taxonomic information resulting from the *16S rRNA* gene sequencing allowed construction of heat maps after the data was normalized to a percentage of each sample. For this project, using heat maps allowed for visualization of subtle differences between bacterial populations across samples of Swiss cheeses studied. Sample identities are located along the x-axis and the bacteria genus are listed along the y-axis. Colored areas in the body of the heat map show the density of bacteria per sample; higher densities are darker in color and lower density is lighter in color. The results are presented at different taxonomic levels and importance discussed.

### 3.3 Results/Discussion

#### 3.3.1 Phylum Level

Next-generation sequencing yielded information for the identification of 16 bacterial phyla (Figure 7). Bacteria concentration for triplicate samples were averaged to show the variation of phyla concentrations between the sample types.

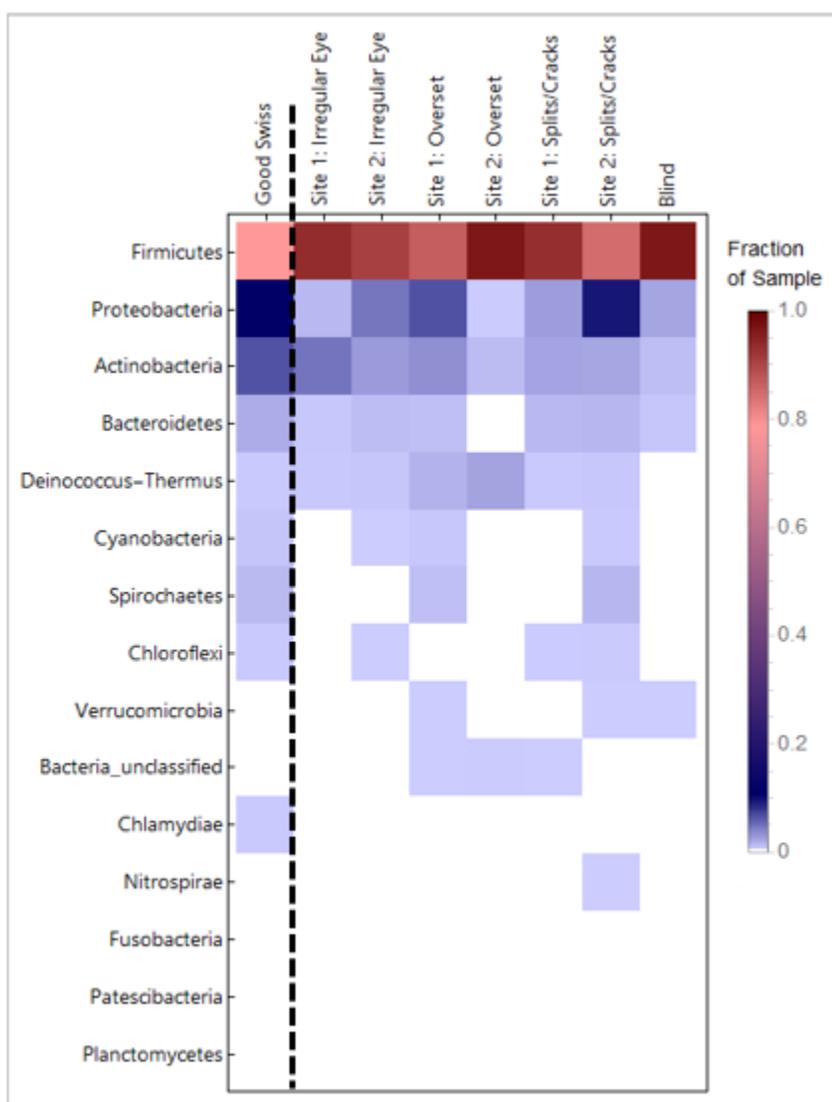
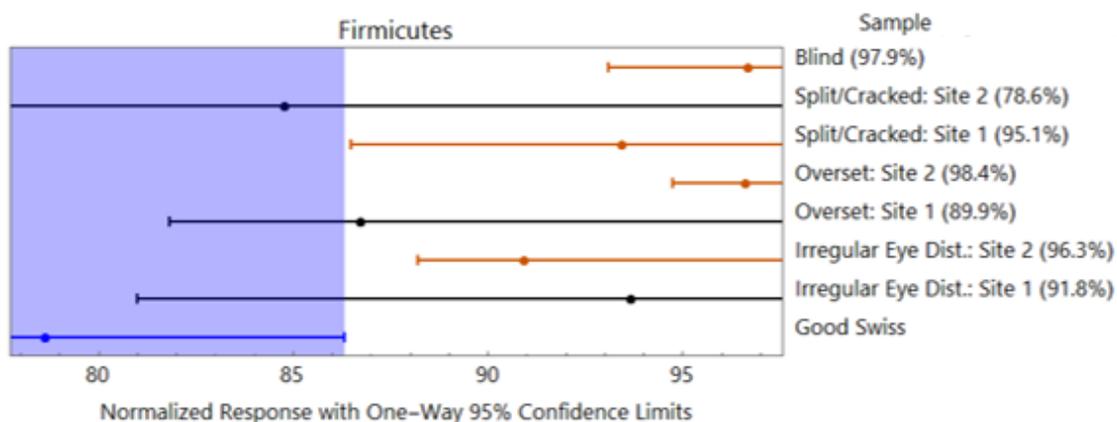


Figure 7. Phylum level heat map of bacteria in Swiss cheese samples.

### Firmicutes

From the heat map shown in Figure 7, the Firmicutes phylum is dominant across all cheese samples. While “Good” Swiss cheese samples contain an average of 78.64% ( $\pm 2.6\%$ ) Firmicutes bacteria, all defective cheese samples exhibit higher concentrations of Firmicutes bacteria ranging from 84–97%. A 95% confidence interval is a range of values having a 95% probability that those values will contain the true population mean. If two sample measurements have non-overlapping confidence intervals, it is with 95% confidence that that two samples are statistically different. Figure 8 shows the 95% confidence interval for statistically different bacteria when compared to the “Good” Swiss cheese sample, within the Firmicutes phylum for each sample of defective cheese.



**Figure 8. Statistically different bacteria at the 95 % confidence interval between defective cheese samples and “Good” Swiss cheese samples at the Firmicutes phylum.**

Split/Cracked, Overset, and Irregular Eye Distribution cheeses from Site 1 were not statistically different when compared to Good Swiss Cheese at the Firmicutes phyla.

### Proteobacteria

From the heat map in Figure 7, the overall concentration of Proteobacteria among cheese samples is lower ( $< 15\%$ ) compared to the Firmicutes bacteria ( $> 78\%$ ). At the 95%

confidence interval only four samples are statistically different when compared to the Good Swiss cheese: Irregular Eye Distribution (both locations), Overset cheese from Site 2, and Blind cheeses.

### Actinobacteria

This phylum of bacteria shows statistical difference between all cheese samples (when compared to Good Swiss cheese) except the Irregular Eye cheese sample from Site 1 and Overset cheese sample from Site 1. The defective cheese samples have a lower concentration of Actinobacteria compared to the amount Good Swiss cheese contains.

The following phyla were not statistically different when compared to “Good” Swiss cheese samples and will not be discussed further in this text: Bacteroidetes, Acidobacteria, Verrucomicrobia, Cyanobacteria, Chlamydiae, Planctomycetes, Fusobacteria, Nitrospirae, Patescibacteria, Chloroflexi as well as any unclassified bacteria.

Table 3 shows cheese samples that had a statistically higher (+) or lower (-) concentration of these three bacterial phyla (Firmicutes, Proteobacteria, and Actinobacteria), compared to the “Good” Swiss cheese samples.

**Table 3. Statistically Different Defective Swiss Cheeses Compared to Good Swiss Cheese at the Phylum Level**

<b>Bacterial Phylum</b>	<b>Sample</b>						
	Irr. Eye Site 1	Irr. Eye Site 2	Overset Site 1	Overset Site 2	Split/Crack Site 1	Split/Crack Site 2	Blind
Proteobacteria	-			-			
Actinobacteria		-		-	-		-
Firmicutes		+		+	+		+

Irr. =Irregular

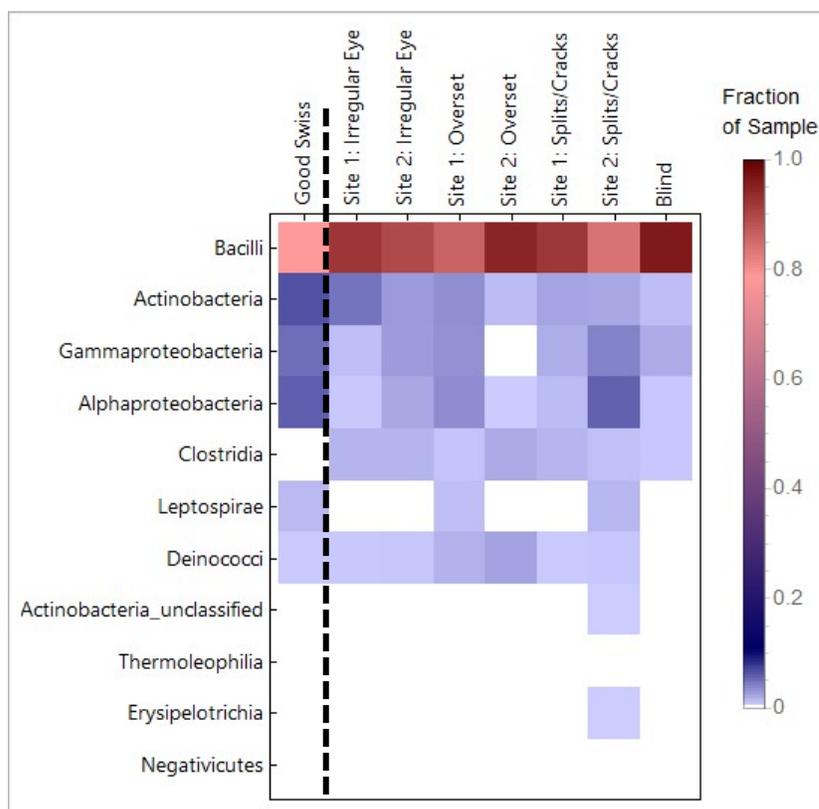
Overset cheese from Site 1 and Split/cracked cheese from Site 2 contained no statistically different bacterial populations from “Good” Swiss cheese. Cheeses exhibiting irregular eye formation (Site 2), overset characteristics (Site 2), split/cracked defects (Site

1), and blindness (Site 1) all exhibited statistically higher concentrations of Firmicutes bacteria and lower concentrations of Actinobacteria. Interestingly, cheese with irregular eye formation (Site 1) and an overset cheese (Site 2) showed lower concentrations of Proteobacteria. This proposes differing bacterial populations are contributing to the following defects: cheeses exhibiting an irregular eye defect, overset characteristics, and defects involving splits/cracks.

A “drill down” technique was then applied to the dataset to determine which bacteria in the defective samples were statistically different from bacteria found in the “Good” Swiss cheese samples. The “drill down” technique is a way to break the complex data down into progressively smaller parts to correlate a cheese defect to bacterial population. In this text, bacteria that were deemed statistically different at the phylum level composition were further analyzed at the class level. This process was repeated down to the genus taxonomic level. Any bacteria not deemed statistically significant (< 95% confidence level) were omitted from further analyses. All statistical confidence plots are available in Appendix B.

### 3.3.2 Class

The heat map in Figure 9 shows differences in bacterial concentration between cheese samples at the class taxonomic level. The heat map below encompasses bacterial classes deemed statistically different at the phylum level.



**Figure 9. Class level heat map containing bacteria from statistically different phyla.**

Bacilli is the most dominant bacterial class having a per sample concentration of over 75%. When compared to the “Good” Swiss cheese samples, the defective cheeses are comprised of lower percentages of Actinobacteria, Gammaproteobacteria, and Alphaproteobacteria. While many cheese samples have a lower concentration of bacteria from the Leptospirae class, it is not significant. Table 4 summarizes the evaluation of per-sample populations of bacteria corresponding to the heat map content in Figure 9.

**Table 4. Statistically Different Bacterial populations at the Class Level in Defective Swiss Cheeses v. “Good” Swiss Cheese**

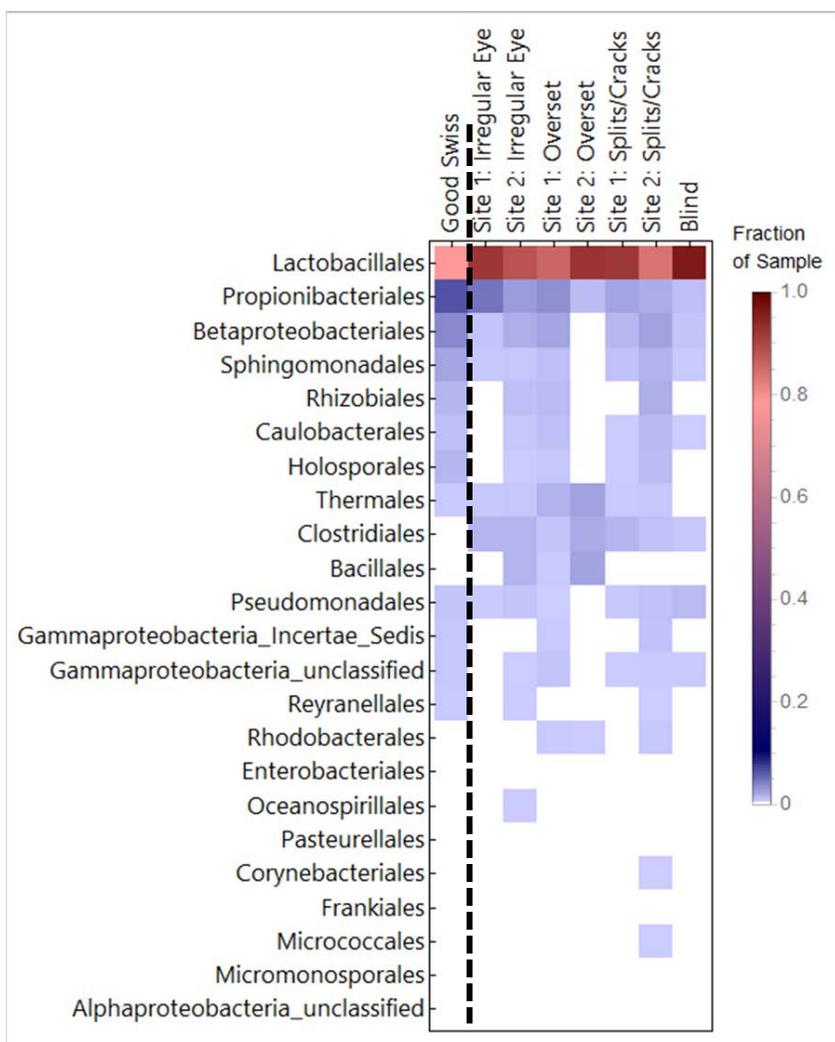
Bacterial Class	Sample						
	Irr. Eye Site 1	Irr. Eye Site 2	Overset Site 1	Overset Site 2	Split/Crack Site 1	Split/Crack Site 2	Blind
Alpha-proteobacteria				-			-
Gamma-proteobacteria				-			
Actinobacteria		-		-	-		-
Bacilli		+		+			+
Clostridia	+	+		+		+	

Irr. =Irregular

Statistical differences observed at the class level bacteria between “Good” Swiss cheese and defective samples included the following: Bacilli, Actinobacteria, Gammaproteobacteria, Alphaproteobacteria and Clostridia.

### 3.3.3 Order Level

At the order taxonomic level there are many subtle differences in bacterial concentration between the “Good” Swiss cheese compared to the defective cheese samples. Figure 10 provides the heat map of the orders of bacteria from statistically different classes analyzed previously.



**Figure 10. Order level heat map containing statistically different bacteria in Swiss cheese samples.**

Table 5 summarizes the heat map results below. Lactobacillales appears to be the most dominating order across all cheese samples, with per-sample concentrations ranging between 78–96%. This high percentage of Lactobacillales is expected due to being a member of the Firmicutes phylum, which was also the dominant bacteria present.

**Table 5. Statistically Different Bacterial Orders Between Defective Swiss Cheese & “Good” Swiss Cheese Samples**

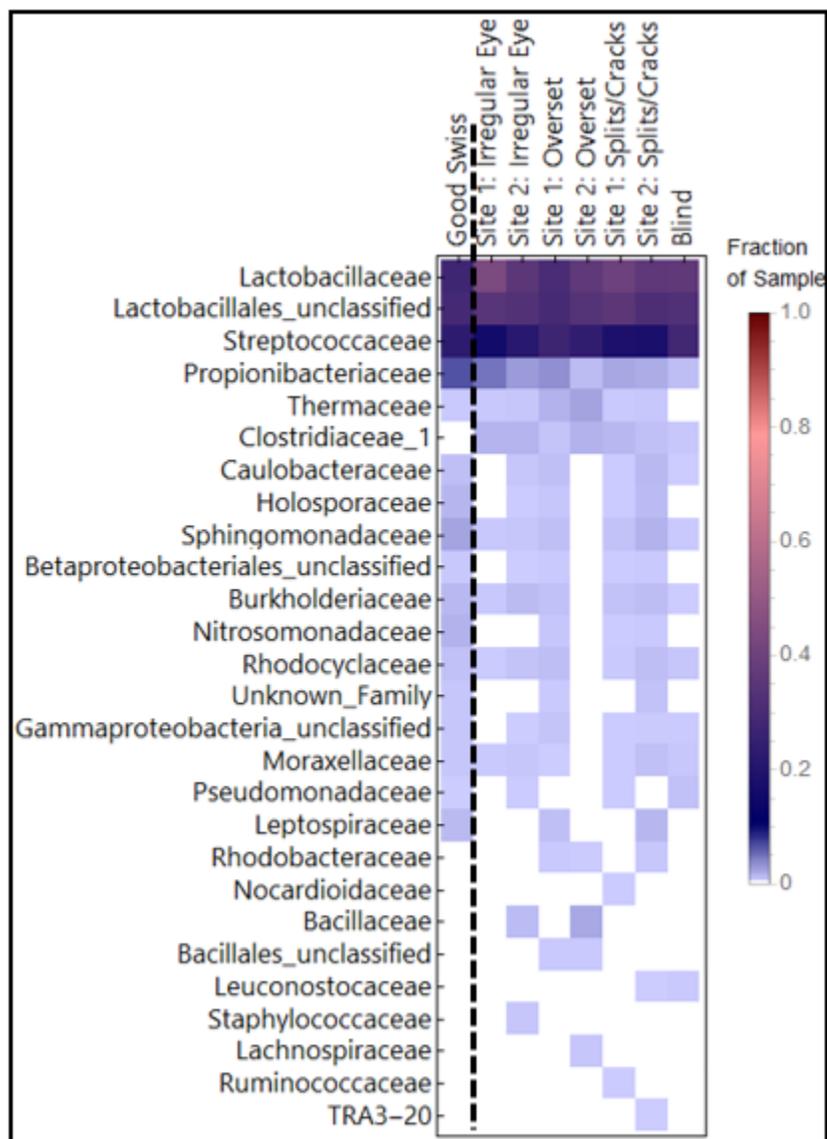
Bacterial Order	Sample						
	Irr. Eye Site 1	Irr. Eye Site 2	Overset Site 1	Overset Site 2	Split/Crack Site 1	Split/Crack Site 2	Blind
Rhodobacterales				-		+	-
Gamma-proteobacteria Incertae Sedis	-	-		-			
Gamma-proteobacteria Unclassified	-			-	-		-
Propionibacteriales		-		+			+
Bacillales				+			
Lactobacillales				+			+
Clostridiales	+	+		+		+	

Irr. =Irregular

Clostridiales bacteria is absent in the “Good” Swiss cheese sample but present in all other samples (also observed in the Clostridia class). Many concentrations of bacterial orders observed in the heat map are not statistically different from the “Good” Swiss cheese sample and will not be discussed further.

### 3.3.4 Family Level

The heat map created for the family level, Figure 11, is unlike the others seen thus far for taxonomic levels. Instead of one bacterial family dominating the samples, at first glance, there seem to be three families that dominate: *Lactobacillaceae*, *Lactobacillales* (unclassified) and *Streptococcaceae*.



**Figure 11. Family level heat map containing statistically different bacteria in Swiss cheese samples.**

The three leading families observed in the heat map above belong to the commonly dominating Firmicutes phylum encompassing approximately 30–50% of bacteria in each sample. Results from the heat map are summarized in Table 6 below.

**Table 6. Statistically Different Bacterial Families Between Defective Swiss Cheese & “Good” Swiss Cheese Samples**

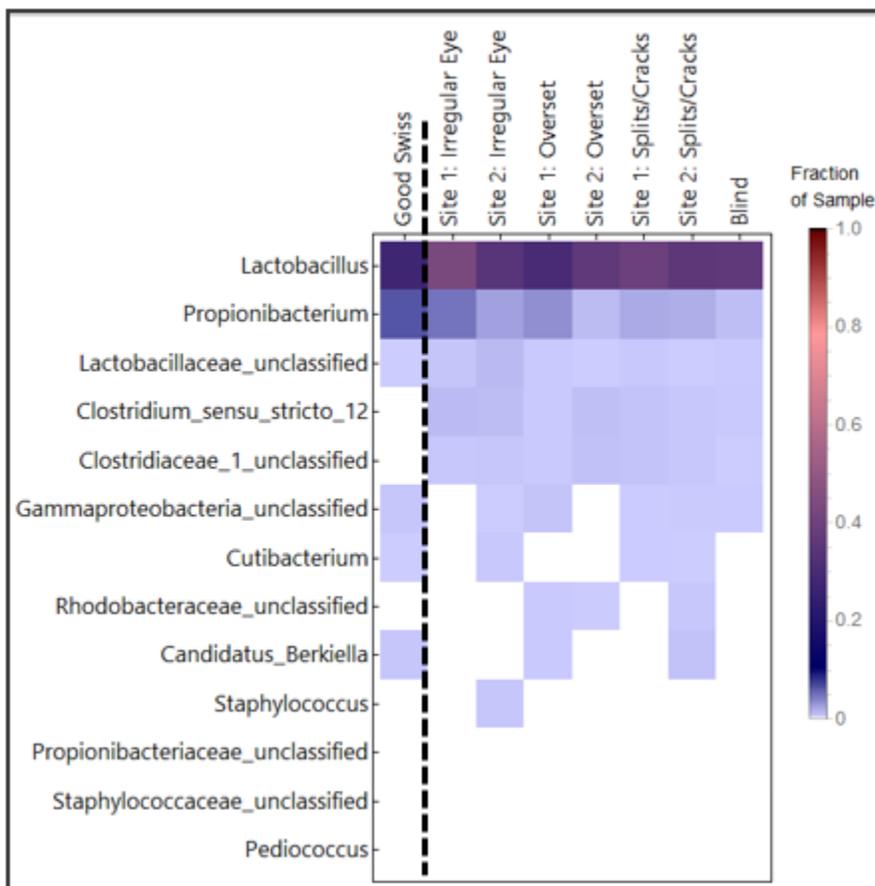
Bacterial Family	Sample						
	Irr. Eye Site 1	Irr. Eye Site 2	Overset Site 1	Overset Site 2	Split/Crack Site 1	Split/Crack Site 2	Blind
<i>Rhodobacteraceae</i>				-		+	-
<i>Unknown Family</i>	-	-		-			
<i>Gamma-proteobacteria (unclassified)</i>	-			-	-		-
<i>Propionibacteriaceae</i>		-		+			+
<i>Staphylococcaceae</i>				+			
<i>Lactobacillaceae</i>				+			+
<i>Clostridiaceae 1</i>	+	+		+		+	

Irr. =Irregular

The trend of “Good” Swiss cheese containing 0% of the Clostridia phylum while all defective samples contain at least 0.5% continues in the family level regarding the *Clostridiaceae 1* family. Cheese samples with an oversight defect from Site 2 are absent many bacteria observed in “Good” Swiss cheese and contain bacteria which are absent in “Good” Swiss cheese.

### 3.3.5 Genus

The genus level heat map returns to the pattern observed with only one bacterium dominating the samples; *Lactobacillus*, from the Firmicutes phylum (see Figure 12).



**Figure 12. Genera level heat map containing bacteria from statistically different families.**

The defective cheeses are lacking in *Propionibacterium* compared to “Good” Swiss cheese but continue to exhibit bacteria from the Clostridia phylum by ways of *Clostridium sensu stricto 12* and *Clostridiaceae 1* (unclassified) whereas “Good” Swiss cheese does not. An occurrence of *Rhodobacteraceae* (unclassified) was observed in 3 defect types but is absent in “Good” Swiss cheese samples. Tabulation of data contained in the heat map is provided in Table 7.

**Table 7. Statistically Different Bacterial Genera Between Defective Swiss Cheese & “Good” Swiss Cheese Samples**

Bacterial Genus	Sample						
	Irr. Eye Site 1	Irr. Eye Site 2	Overset Site 1	Overset Site 2	Split/Crack Site 1	Split/Crack Site 2	Blind
<i>Rhodobacteraceae</i> (Unclassified)						+	
“ <i>Candidatus Berkiella</i> ”	-	-		-	-		-
<i>Gamma-proteobacteria</i> (Unclassified)	-			-			
<i>Propionibacterium</i>		-		-	-	-	-
<i>Staphylococcus</i>		+					
<i>Lactobacillus</i>	+				+	+	
<i>Clostridiaceae 1</i> (Unclassified)		+				+	
<i>Clostridium sensu stricto 12</i>	+	+		+		+	

Irr. =Irregular

Table 7 summarizes statistically different bacterial genera in defective cheese samples compared to the “Good” Swiss cheese sample. While individual defects, samples and corresponding bacteria are addressed in detail in the following conclusion, there are a few noteworthy observations that can be made. Many defective cheese samples have decreased *Propionibacterium* populations which contribute to decreased eye formation throughout the cheese block. Some samples also present with increased populations of *Clostridium sensu stricto 12*, *Clostridiaceae 1* (Unclassified), and *Lactobacillus*, which are well understood and increased populations have been linked to the spoilage of Swiss-type cheeses. One cheese contained the presence of *Staphylococcus* not observed in any of the other cheese samples, including the “Good” Swiss cheese,

which may indicate a contamination of this cheese from an outside source during the manufacturing process.

### **3.4 Bacterial Heatmap Conclusion**

A drill down technique was used to determine if a bacterium at specific taxonomic levels should be considered significantly different (between “Good” Swiss cheese and other cheese samples exhibiting defects) and further analyzed or determined insignificant and omitted from further analysis in each cheese sample. This technique presented statistically significant bacteria at the genus level within cheese samples as they differed from those in the “Good” Swiss cheese sample. Bacterial populations across all cheese samples were compared to bacteria observed in “Good” Swiss cheese from Site 1. A correlation could be made between bacteria and cheese defects in samples from Site 1, while only providing suggestive correlation or trends observed in cheese samples from Site 2.

#### **3.4.1 Irregular Eye Formation/Distribution**

Cheeses exhibiting an irregular eye formation/distribution defect present with lower per-sample concentrations of “*Candidatus Berkiella*” and higher concentrations of *Clostridium sensu stricto* 12. Additionally, the sample from Site 1 contained lower concentrations of *Gammaproteobacteria* (Unclassified) and higher concentrations of *Lactobacillus*. The sample with this defect from Site 2 however, contained lower per-sample concentrations of *Propionibacterium* and greater concentrations of *Staphylococcus* and *Clostridiaceae* 1 (Unclassified) compared to the concentrations observed in “Good” Swiss cheese.

#### 3.4.2 Oversight

Cheese samples having an oversight defect from Site 1 did not contain any bacteria at the genus level deemed statistically significant compared to that of “Good” Swiss cheese. From Site 2, the oversight cheese showed higher concentrations of *Clostridium sensu stricto 12* while exhibiting lower per-sample concentrations of “*Candidatus Berkiella*”, *Gammaproteobacteria* (unclassified), and *Propionibacterium*.

#### 3.4.3 Split/Cracked

From Site 1 and 2, cheeses exhibiting a split/cracked defect presented with lower per-sample concentrations of *Propionibacterium* and higher concentrations of *Lactobacillus*. The cheese samples from Site 1 showing this defect contained a lower concentration of “*Candidatus Berkiella*” while the samples from Site 2 contained greater populations of *Rhodobacteraceae* (unclassified), *Clostridiaceae 1* (unclassified), and *Clostridium sensu stricto 12*.

#### 3.4.4 Blind

Cheese with a blind defect was only provided from Site 1. Compared to the “Good” Swiss cheese samples, these samples contained lower per-sample populations of “*Candidatus Berkiella*” and *Propionibacterium*.

## CHAPTER FOUR: ORGANIC ACIDS IN SWISS CHEESE

### 4.1 Organic Acid Contributions in Swiss Cheese

Common to Swiss-type cheeses are the organic acids citric, lactic, acetic, propionic, and butyric acids, which play significant roles in the formation of the characteristic eyes, as well as sensory characteristics such as flavor. The formation of eyes in Swiss cheese is largely due to propionic acid fermentation of lactate which produces propionic and acetic acids, and emits CO<sub>2</sub> gas. Eye formation and flavor profiles are formed while the cheese is ripening over many months to (sometimes) years. Flavor characteristics are initiated immediately after the addition of a starter culture, which assists in the acidification and coagulation of the milk creating a sour or bitter flavor. The sweet, nutty flavors created by lactate utilization begin taking place about 25 days after starter culture introduction. Most commonly used starter cultures include lactic acid bacteria (LAB), which ferment lactose into lactic acid, and heterofermentative starters, which ferment non-carbohydrate substrates, such as citrate, to produce the buttery-like flavors.

In addition to the LAB starter cultures used to make Swiss cheeses, there are nonstarter organisms indigenous to raw milk and consistently found in cheese processing facilities, which reintroduces the nonstarter organisms into the cheese after pasteurization proves lethal for most organisms. Nonstarter organisms contain nonstarter lactic acid bacteria (NSLAB) consisting of facultative heterofermentative lactobacilli. These lactobacilli include strains such as *Lactobacillus delbrueckii*, *Lactobacillus rhamnosus* and *Lactobacillus casei*. It has become common practice due to the length and cost of ripening

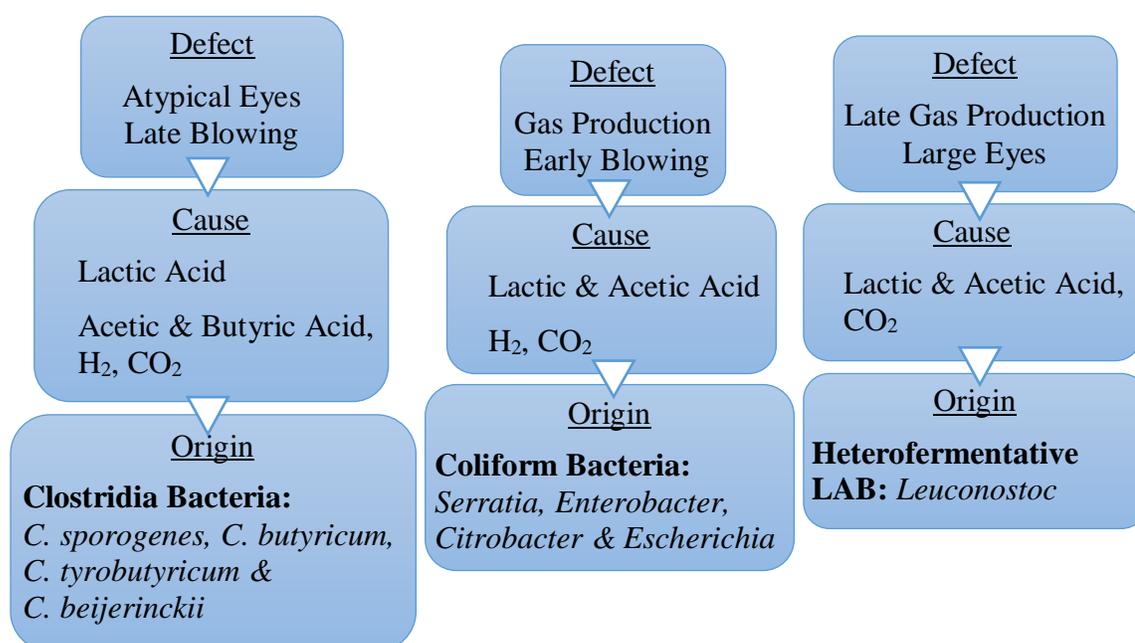
cheese to incorporate the use of specific strains of these NSLABs as an adjunct culture into the cheese recipe due to their acceleration of proteolysis (to shorten ripening times) and indirect contribution to the development of cheese flavors related to acidification by bacteria such as *Lactobacillus*.<sup>52</sup> Proteolysis and flavor development by lactic acid bacteria (starter and nonstarter) in cheese is created by metabolic and enzymatic activities on the milk fat, proteins, and carbohydrates. Starter lactic acid bacteria (SLAB) degrade large peptides that nonstarter lactic acid bacteria (NSLAB) use during proteolysis. NSLAB contribute to cellular lysis by creating bacteriocins and subsequently releasing enzymes that degrade small peptides into free amino acids required for the bacteria to thrive.<sup>53</sup> In Swiss cheese, many types of bacteria such as *Proteobacteria* ssp., *Clostridium*, and *Lactobacillus* produce polyamines (some of which are linked to food poisoning) via decarboxylation of amino acids that are later used in RNA and peptidoglycan synthesis.<sup>54</sup> The utilization of amino acids play a significant role on the propionic acid fermentation pathway<sup>55</sup>, for example, co-metabolism of aspartate and lactate result in a decreased propionate production and a decreased ratio between propionate and acetate. The salt-moisture ratio within the cheese matrix is deterministic if D-lactate is formed by NSLAB (high salt/moisture ratio) or L-lactate by SLABs (at low salt/moisture ratio). The metabolism of L-lactate is preferential in the propionic fermentation pathway to produce free fatty acids which contribute to the sweet and nutty flavors characteristics in cheese.<sup>56</sup>

#### 4.1.1 Problematic Organic Acids

A cooperative relationship exists between organic acids and the ability of bacteria within the Swiss cheese matrix to flourish. Lactic acid bacteria metabolize lactose to lactic acid which is utilized by propionic acid bacteria to produce propionic and acetic acids

alongside CO<sub>2</sub> and H<sub>2</sub>O.<sup>57</sup> These actions contribute to different microbial growth or hindrance throughout cheese ripening based on available starting substrates. A decreased quantity of organic starting material such as lactose or lactic acid, competitive bacteria such as starter lactic acid bacteria compared to nonstarter lactic acid bacteria (which create preferential and less preferential forms of lactate, respectively), or too few numbers of a starting bacteria can all cause disruption of the ecosystem within the microbiome of a cheese matrix.

Chapter 2 discussed bacteria that may cause defects related to eye characteristics in Swiss cheese and are summarized in Figure 13.



**Figure 13. Observed defects in Swiss cheese with associated acids, gases, and bacteria**

If found in excess, bacteria that are known to cause these defects include types of Coliforms, heterofermentative LAB and Clostridia bacteria. If present in a defective cheese sample, types of coliforms may produce a decreased quantity of lactic and acetic acids. Certain types of heterofermentative LAB, if present, may cause an increased quantity of

lactic and acetic acid, whereas if specific *Clostridia* bacteria are present within a sample, an increased quantity of acetic and butyric acids may be observed. All of these bacteria also produce CO<sub>2</sub> which greatly affects eye formation and both *Coliforms* and *Clostridia* bacteria produce H<sub>2</sub> gas which contributes to severe gas defects. Many defects observed in Emmental cheeses are contributed to an undesirable ratio between bacteria types leading to over and/or under abundance of organic acids relating to said defects.

## **4.2 High Performance Liquid Chromatography**

High-performance liquid chromatography (HPLC) is primarily an analytical separation technique that is customizable to accommodate a wide scope of analyses. Basic instrumentation can be coupled with numerous detectors to separate and purify chemical compounds with diverse polarities and molecular masses dependent on column selection and instrument parameters used during analysis. Scientists have employed the use of HPLC for quantitative and qualitative separation of mixture components, including organic acids in dairy products such as milk, whey, and multiple types of cheeses.<sup>58-59</sup> The HPLC methods customized for the qualitative and quantitative analysis of organic acids in Swiss cheese samples are described here.

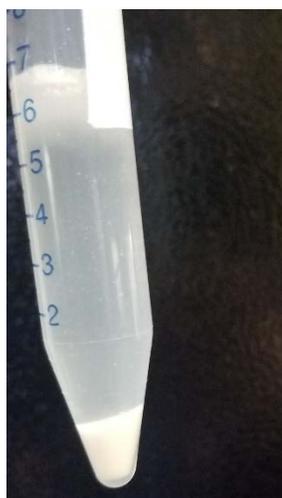
### **4.2.1 Method 1**

Modeled after the sample preparation described by Bevilacqua and Califano<sup>60</sup>, Swiss cheese samples were prepared in 8.0 mL of 0.5% (w/v) ammonium phosphate buffer with a pH of 2.2 using 2 g of diced cheese (< 2 mm diameter) and masticated by a Stomacher<sup>®</sup> 80 paddle blender for 1 minute (Figure 14) followed by 1 hr. of stirring on a magnetic stir plate.



**Figure 14. Cheese sample after being masticated by Stomacher 80 paddle blender.**

The solution was centrifuged for 5 minutes at 3500 rpm to separate precipitated proteins and fats from the solution (Figure 15).



**Figure 15. Precipitated fat and protein pellet from Swiss cheese sample.**

The aqueous layer, containing the organic acids of interest, was filtered twice using a 0.45  $\mu\text{m}$ , nylon syringe filter (Titan) before placing a 1.5 mL aliquot into an amber HPLC auto-sampler vial for analysis.

Sample analyses of organic acids in the cheese samples were carried out using a Dionex Ultimate 3000 HPLC system (Thermo Scientific™) with Chromeleon 7.2 software and was equipped with dual pumps, auto-sampler, and a diode array detector. Single sample analyses (one repeat injection) were performed using a gradient elution method

detailed in Table 8, with a mobile phase flow rate of 1.0 mL/min, UV-Vis detector monitoring at 214 nm, column temperature of 30°C ( $\pm 1^\circ\text{C}$ ), on a 150 x 4.0 mm C<sub>8</sub> column (Betasil) with a sample injection volume of 20.0  $\mu\text{L}$ .

**Table 8. HPLC Elution Gradient: Method 1**

<i>Time</i>	<i>Flow mL/min</i>	<i>%B</i>
<b>-1.00</b>	1.0	45.0
<b>0.00</b>	1.0	45.0
<b>1.75</b>	1.0	45.0
<b>4.00</b>	1.0	60.0
<b>8.00</b>	1.0	80.0
<b>30.00</b>	-Stop Run-	

Aqueous mobile phase (A) was 0.5% (w/v) (NH<sub>4</sub>)<sub>3</sub>PO<sub>4</sub> buffer prepared by diluting ammonium phosphate in 18.2 M $\Omega$  cm<sup>-1</sup> water, followed by pH adjustment to 2.2 with reagent grade phosphoric acid (Sigma Aldrich). Organic mobile phase (B) was 0.8% HPLC grade acetonitrile (VWR Analytical) with 0.1% phosphoric acid. All mobile phases were vacuum filtered using 9.0 cm, #40 ashless, Whatman filter paper. Lactate (1000  $\pm 4$   $\mu\text{g/mL}$ ), acetate (1000  $\pm 5$   $\mu\text{g/mL}$ ), citrate (1002  $\pm 4$   $\mu\text{g/mL}$ ), butyrate (1001  $\pm 5$   $\mu\text{g/mL}$ ), and propionate (1000  $\pm 6$   $\mu\text{g/mL}$ ) were purchased from Inorganic<sup>TM</sup> Ventures (Virginia) to use as reference standards.

#### 4.2.2 Method 2

A combination of two HPLC methods<sup>61-62</sup> was used to create a quantitative method for the analysis of organic acids in Swiss cheese samples. In 10.0 mL of 0.1% (v/v) formic acid, two grams of diced cheese (< 2 mm diameter) were reduced by a Stomacher<sup>®</sup> 80 paddle blender for four minutes followed by vigorous stirring for one hour on a magnetic stir plate. The solution was centrifuged for five minutes at 3500 rpm to pellet out the proteins and fats from the solution (Figures 14 and 15). The aqueous supernatant was

filtered twice by 0.45  $\mu\text{m}$  PTFE (Fisher brand) syringe filter before transfer into a 1.5 mL amber auto-sampler vial.

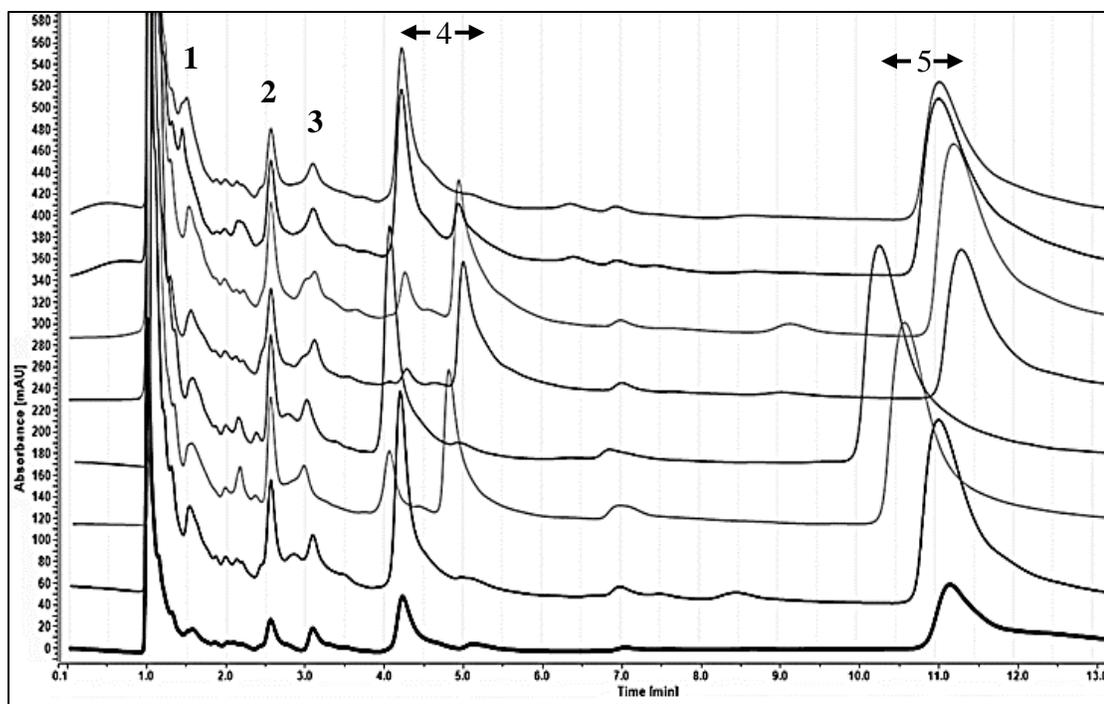
The HPLC system parameters mentioned previously were used with the following modifications: single sample analyses (one repeat injection per sample) were performed using a single mobile phase, isocratic method with a flow rate of 0.55 mL/min for 30 minutes, UV-Vis detector monitored at 220 nm, a column temperature of 55  $^{\circ}\text{C}$  ( $\pm 1$   $^{\circ}\text{C}$ ), on a 300 x 7.8 mm (9  $\mu\text{m}$ ) HPX-87H organic acid column (Aminex), with a sample injection volume of 20.0  $\mu\text{L}$ . The mobile phase consisted of 0.1% (v/v) formic acid in 18.2  $\text{M}\Omega\text{ cm}^{-1}$  water and was vacuum filtered using 9.0 cm, #40 ashless, Whatman filter paper. The following organic acids were obtained from commercial sources to be used as reference standards: sodium citrate (>99%) and sodium acetate (>99%) from Arcos Organics, sodium lactate (>98%) from Alfa Aesar, and sodium propionate (>98%) and sodium butyrate (>98%) from Tokyo Chemical Industry (TCI) America. Calibration curves for each organic acid have been made available in Appendix C. Calibration curves having a coefficient of determination (R-squared) value above 99% were developed for each of the organic acid standards, permitting quantitative analysis of these components from cheese samples.

Cheese samples were further analyzed by a single quadrupole mass selective detector (Bruker HCT Ultra PTM Discovery System ETDII) with the following parameters: ESI mode, capillary voltage of 4000 V, nebulizer pressure of 50.0 psi, dry gas flow of 11.0 L/min. and a drying temperature of 365 $^{\circ}\text{C}$ . The instrument averaged three scans targeting the 30–300 m/z ratios.

### 4.2.3 Discussion/Results

#### Method 1

Bevilacqua and Califano (4.2.1 Method 1) performed quantitative analysis of organic acids in dairy products including milk, yogurt, and Blue, Provolone, Port Salut, and Quartirolo cheeses, according to method 1; they did not analyze Swiss type cheeses. Using the previously discussed method, the resolution and separation of organic acids extracted from Swiss cheese was insufficient for quantitative determination but provided the proof of concept for qualitative screening. The chromatogram in Figure 16 shows the elution of organic acids from a sample of “Good” Swiss cheese (bolded) and defective Swiss cheese samples using method 1. The assignment of peaks to organic acids by UV–Vis identified peak 1 as citric acid, peak 2 as lactic acid, peak 3 as acetic acid, peak 4 as propionic acid, and peak 5 as butyric acid.



**Figure 16.** HPLC UV-Vis chromatogram ( $\lambda=220$  nm) of organic acids in cheese samples using a C8 column and phosphate buffer/acetonitrile mobile phase. Peak 1 = citric acid, peak 2 = lactic acid, peak 3 = acetic acid, peak 4 = propionic acid and peak 5 = butyric acid.

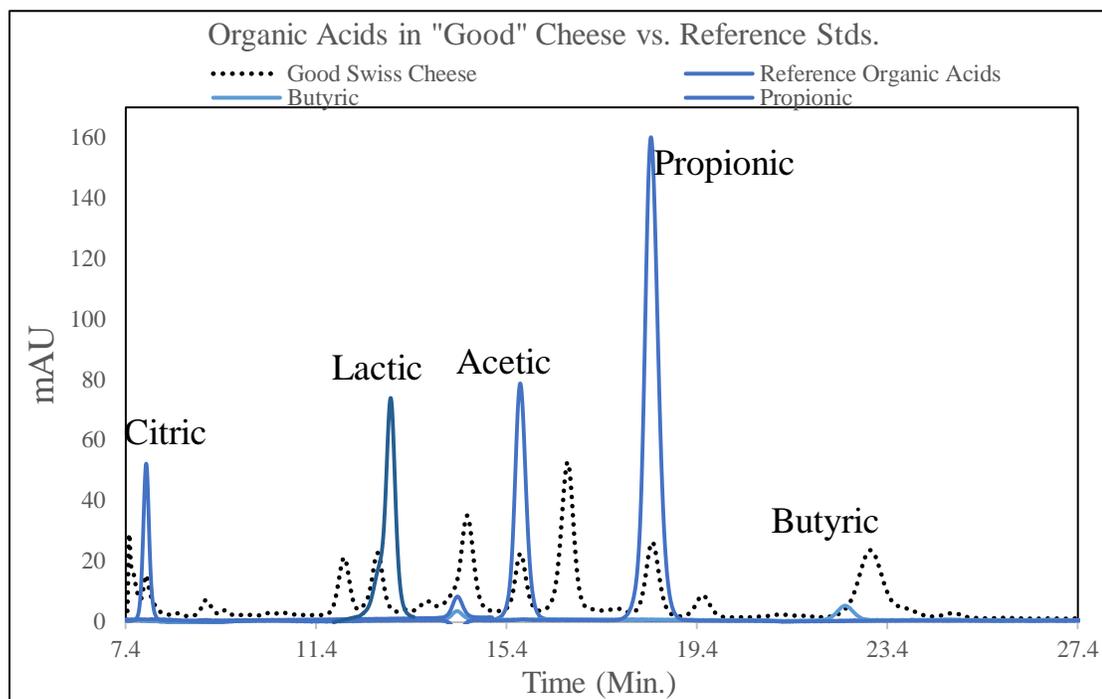
Refinement of the HPLC method included determination of optimal pH, flow rate, and mobile phase composition to obtain the best resolution for each organic acid. From a qualitative standpoint, general differences in acid ratios between samples can be observed between organic acid concentrations for citric and propionic acids at peak numbers 1 and 4 with minimal differences noted for lactic, acetic, or butyric acids at peaks 2, 3, and 5, respectively. Poor peak resolution and shifting retention time for each organic acid has prevented the HPLC–UV or LC–MS from providing a quick, reliable screening method for organic acids between “Good” Swiss cheese and defective Swiss cheese samples. The propionic and butyric acids at peaks 4 and 5 elute at various times; adding to the unreliability of this method under these conditions. The fluctuating elution times were likely caused by ambient temperature changes (which also affects pH) as well as miniscule

pH differences between eluent batches used during analysis. This method was not examined further.

### Method 2

Organic acid identification was verified using three techniques: comparison of UV–Vis elution times between organic acid reference standards and the organic acids in the cheese samples, the method of standard addition, and mass spectrometry. Supplemental information for the method of standard addition and mass spectrometry techniques are available in Appendices D and E, respectively.

**Site 1 Findings:** A chromatogram of organic acids from a sample of “Good” Swiss cheese was overlaid with organic acid reference standards (Figure 17) to identify organic acids within the “Good” Swiss cheese sample based on elution times using a UV–Vis detector ( $\lambda = 220$  nm). Table 9 lists organic acid standards with corresponding elution times.



**Figure 17.** Good Swiss cheese (•••) and organic acid standards (–) by LC-UV-Vis  $\lambda=220$  nm.

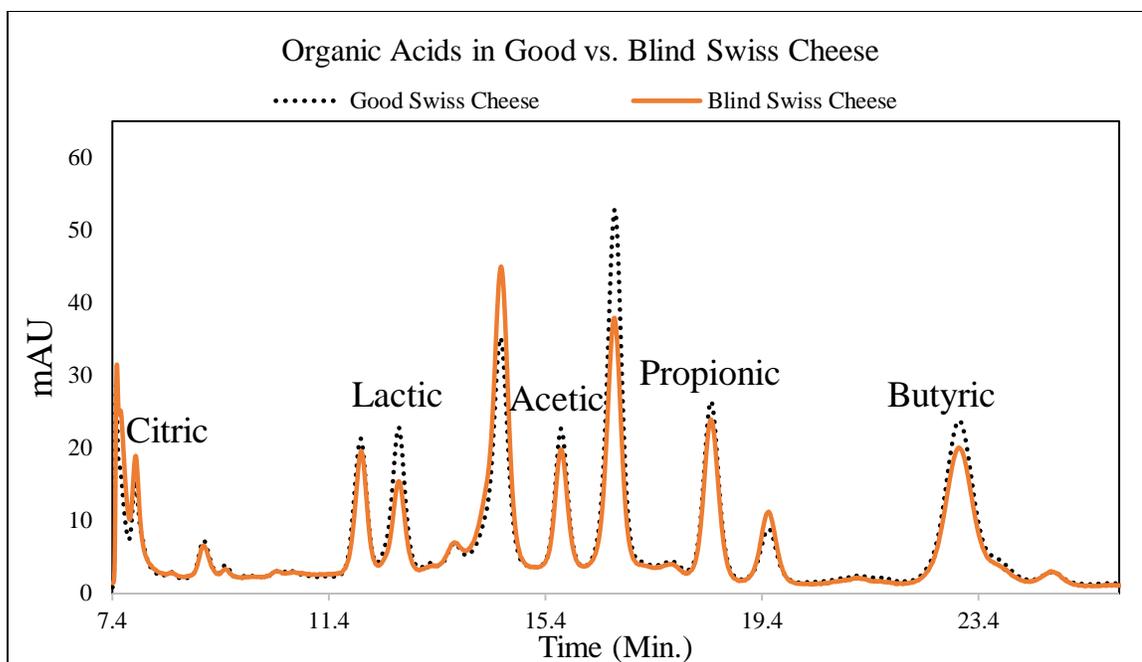
When positive organic acid identification could not be made using this technique, the method of standard addition was used in combination with mass spectrometry for component identification (See Appendix D and E).

**Table 9. HPLC Elution Times of Referenced Organic Acids**

<b>Organic Acid</b>	<b>Elution Time (min)</b>
Citric	7.72
Lactic	12.97
Acetic	15.70
Propionic	18.46
Butyric	22.45

A sample of “Good” Swiss cheese was provided from Site 1 and is compared to the Site 1 defective cheeses below. The organic acid differences are indicative of contributions to the exhibited defects within the cheese samples.

**Blind Defect:** Propionibacteria is a significant contributor to the formation of eyes in Swiss cheeses. When a cheese sample is labeled as blind, it is logical to expect a decrease in CO<sub>2</sub> which is accompanied by lower levels of propionic and acetic acids, and higher levels of lactic acid. Figure 18 presents the organic acids observed in a cheese sample exhibiting a blind defect and a sample of “Good” Swiss cheese for comparison.



**Figure 18.** HPLC chromatogram of organic acids present in “Good” Swiss cheese (••) and cheese with blind defects (—).

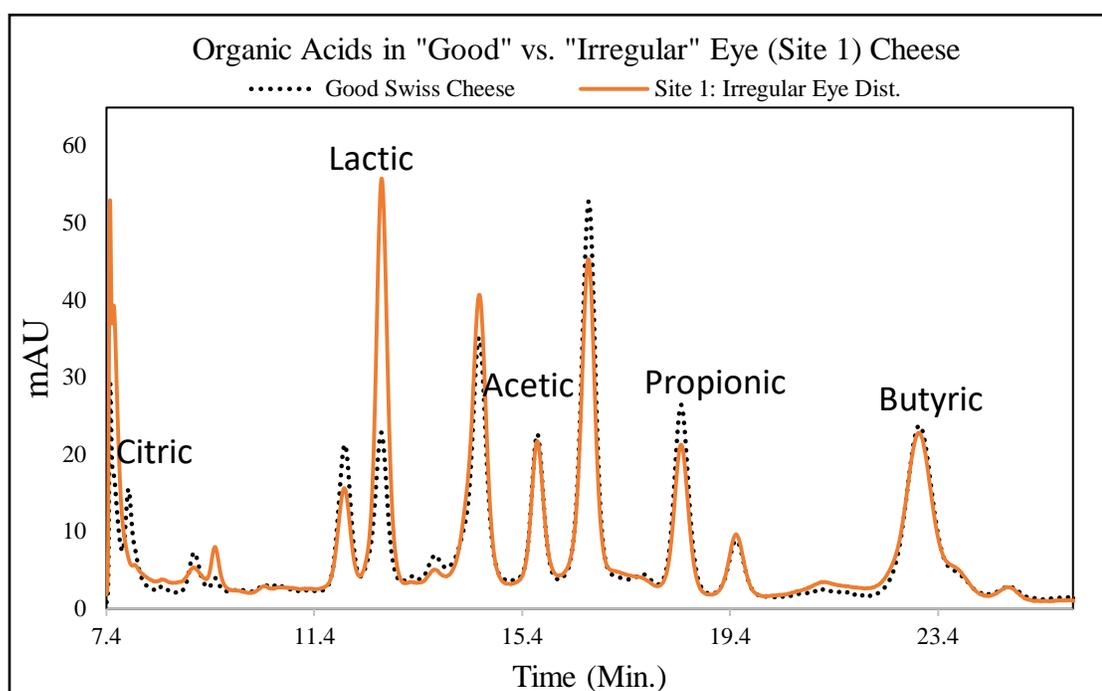
The chromatogram of organic acids from a blind Swiss cheese sample supports previous research reporting decreased quantities of propionic and acetic acids. The proportions of propionic, acetic, and lactic acids are suggestive of lower levels of propionibacteria in blind versus “Good” Swiss cheese samples. Table 10 summarizes organic acid concentrations observed in “Good” Swiss cheese and “Blind” Swiss cheese.

**Table 10.** Organic Acids Quantified in "Good" Swiss Cheese and Cheese with Blind Defects (Site 1)

Organic Acid	“Good” Swiss Cheese ( $\mu\text{g/mL}$ )	Blind Swiss Cheese ( $\mu\text{g/mL}$ )	Percent (%) Difference
Citric	0.090	0.104	14
Lactic	0.450	0.131	110
Acetic	0.893	0.723	21
Propionic	1.414	1.263	11
Butyric	1.995	1.546	25

The decreased quantity of lactic acid proposes lower activity or populations of lactic acid bacteria (LAB), based on the organic acid production or the presence of a competitive inhibitor bacteria. A competitive inhibitor bacterium such as *Propionibacterium shermanii* may be utilizing the lactose required by LAB disallowing the production of lactic acid if there is an unsuitable amount of lactic acid for the propionibacteria to metabolize.<sup>63</sup>

**Irregular Eye Defect:** Eye formation is paramount to the quality and success of good Swiss cheese. Figure 19 displays the organic acids observed in the sample of “Good” Swiss cheese and the defective Swiss cheese having “Irregular eye formation/distribution” from Site 1.



**Figure 19.** HPLC chromatogram of organic acids in “Good” Swiss cheese (•••) and cheese with irregular eye formation or distribution defects (—).

The most significant observation noted between these samples is the lack of citric acid and increased amount of lactic acid observed in the “Irregular eye formation/distribution”

sample. Organic acid quantities of the defective cheese sample and “Good” Swiss cheese sample are summarized in Table 11.

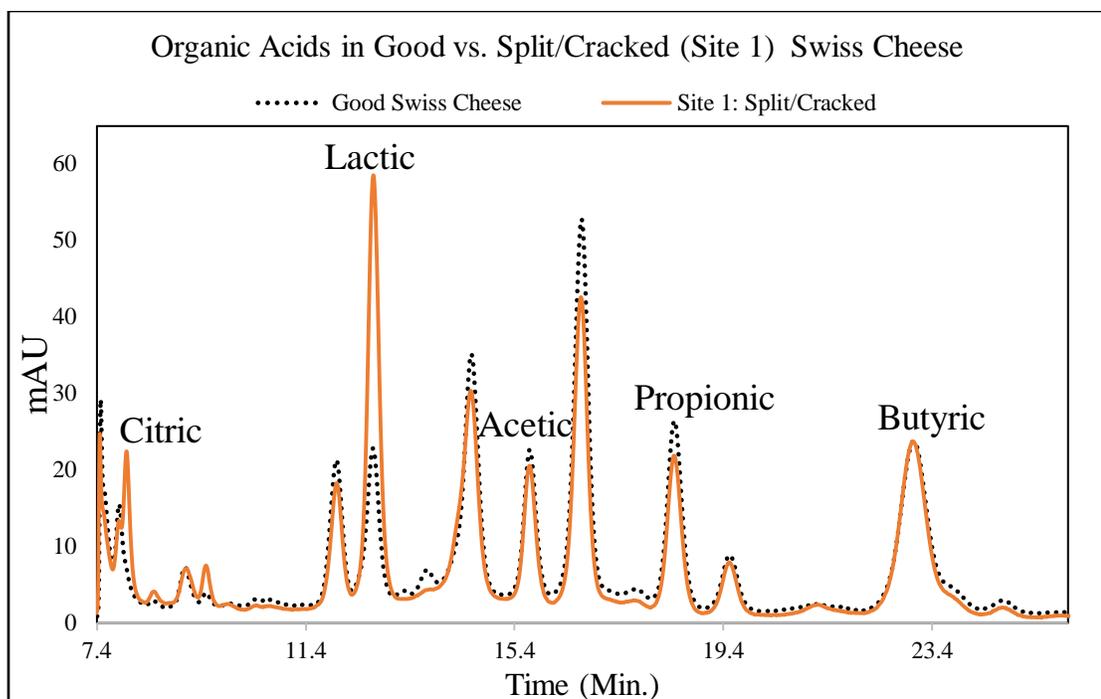
**Table 11. Organic Acids Quantified in "Good" Swiss Cheese and Cheese with Irregular Eye Formation/Distribution (Site 1)**

<b>Organic Acid</b>	<b>“Good” Swiss Cheese (µg/mL)</b>	<b>Irregular Eye Dist. (µg/mL)</b>	<b>Percent (%) Difference</b>
Citric	0.090	ND	N/A
Lactic	0.450	1.551	110
Acetic	0.893	0.856	4
Propionic	1.414	1.071	28
Butyric	1.995	1.917	4

ND = not detected; N/A = not available

The quantitative differences of organic acids indicate that a decreased quantity of citric and propionic acid, with a significantly increased concentration of lactic acid, correlate to the irregular eye formation/distribution defect in Swiss cheese. The excess lactic acid indicates the activity of the propionibacteria is less than expected or bacteria that produces lactic acid, such as LAB, is in excess. Acetic and butyric acids were similar between both cheese samples suggesting they do not contribute to the irregular eye defect in a significant manner.

**Split/Cracked Defect:** Split or badly cracked cheese is commonly due to secondary gas formation by propionibacteria or butyric acid bacteria and is associated with the split/crack defect after a curd loses its elasticity late in the ripening process.<sup>64</sup> These types of bacteria primarily metabolize lactic acid to form acetic and propionic acids (propionic acid bacteria) or acetic and butyric acids (butyric acid bacteria). Figure 20 displays a chromatogram of organic acids present in Split/Cracked Swiss cheese.



**Figure 20.** HPLC chromatogram of organic acids in “Good” Swiss cheese (•••) and cheese with Split/Cracked defects (—).

Site 1 Split/cracked Swiss cheese exhibits an excess of lactic and citric acids, while containing decreased concentrations of acetic and propionic acids (< 10% difference) compared to the quantities in “Good” Swiss cheese. The quantities of organic acids are shown in Table 12.

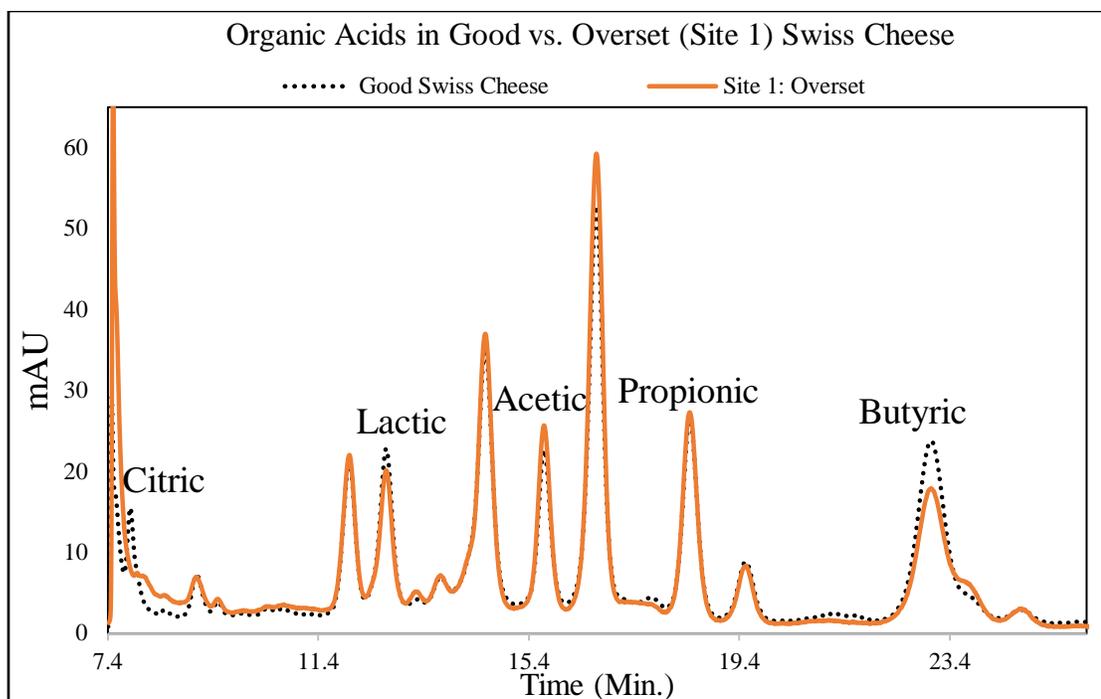
**Table 12.** Organic Acids Quantified in "Good" Swiss Cheese and Cheese with Split/Cracked Defect (Site 1)

Organic Acid	“Good” Swiss Cheese (µg/mL)	Split/Cracked Cheese (µg/mL)	Percent (%) Difference
Citric	0.090	0.203	77
Lactic	0.450	1.667	115
Acetic	0.893	0.820	9
Propionic	1.414	1.157	20
Butyric	1.995	2.044	2

The results of organic acid analysis suggest that decreased quantities of acetic and propionic acids, with an increased quantity of citric and lactic acids, correlate to the Split/Cracked defect in Swiss cheese. The chromatogram shown in Figure 20 is not

consistent with literature reports of secondary gas formation which takes place in the final cooling room after desired propionic acid fermentation has taken place.<sup>65</sup> If desired propionic acid fermentation had been achieved, a lower concentration of lactic acid should be observed; not an excess of 77% compared to the sample of “Good” Swiss cheese. While only a small increase of butyric acid is observed, it is understood that butyric acid produces H<sub>2</sub> gas which causes splits in cheese. This may indicate butyric acid bacteria is contributing to the split/cracked defect in this cheese sample.

**Overset/Gaseous Defect:** Overset or gaseous cheeses are resultant from unwanted microbial growth leading to excessive gas production. The deleterious microbial growth has been attributed to coliforms (early gas formation) or clostridia (late gas formation) bacteria. The chromatogram of overset cheese from Site 1 presented decreased quantities of citric and butyric acids compared to the sample of “Good” Swiss. cheese, with increased quantities of acetic and propionic acids (Figure 21).



**Figure 21. HPLC chromatogram of organic acids in “Good” Swiss cheese (•••) and cheese with Oversight/Gaseous defects (–) from Site 1.**

If the oversight defect in the Swiss cheese sample from Site 1 was due to late gas production from *Clostridium* bacteria, the acetic and butyric acid quantities should be increased (compared to the “Good” Swiss cheese sample). If the oversight defect was due to early gas production by *Coliforms*, the lactic and acetic acids would be lower in quantity than in “Good” cheese sample which would be accompanied by an increased production of CO<sub>2</sub> and H<sub>2</sub> gasses. Table 13 summarizes the quantitative organic acid results for “Good” and oversight Swiss cheese samples.

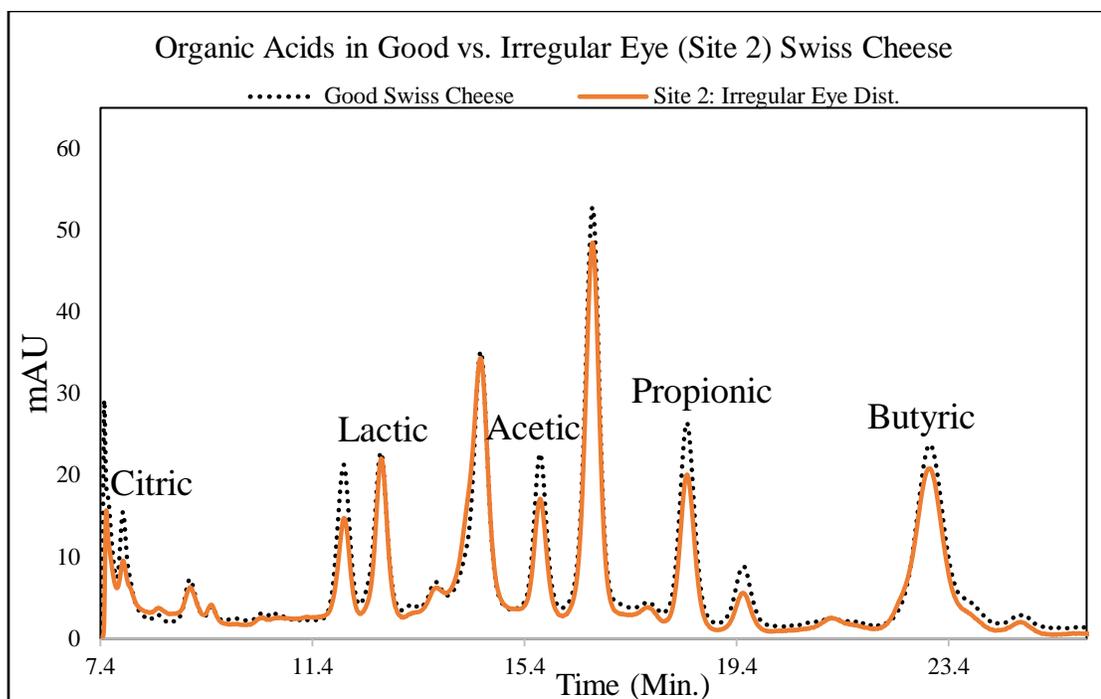
**Table 13. Organic Acids Quantified in "Good" Swiss Cheese and Oversight Cheese (Site 1)**

<b>Organic Acid</b>	<b>"Good" Swiss Cheese (µg/mL)</b>	<b>Overset Cheese (µg/mL)</b>	<b>Percent (%) Difference</b>
Citric	0.090	0.011	156
Lactic	0.450	0.427	5
Acetic	0.893	1.545	53
Propionic	1.414	1.606	13
Butyric	1.995	1.537	26

The observed organic acids in the oversight sample above do not follow previous associations in totality. There are increased quantities of acetic and propionic acids alongside decreased quantities of lactic and butyric acids. This does not follow late or early gas production alone.

**Site 2 Findings:** While Site 2 defective cheeses are compared to the "Good" Swiss cheese from Site 1, this serves as a suggestive observation of organic acids present in the defective cheese samples. A conclusive analytical analysis to correlation organic acids to defects would be more credible if the analysis was carried out using "Good" Swiss cheese samples from Site 2. The organic acid quantitative analysis was performed to assess any trend or correlation between the two sites, defects and organic acids.

**Irregular Eye Sample:** A sample of Swiss cheese from Site 2, having irregular eye distribution/formation, was analyzed for organic acid composition compared to Site 1 good Swiss cheese (Figure 21).



**Figure 22. HPLC chromatogram of organic acids in “Good” Swiss cheese (••) and cheese displaying irregular eye formation/distribution defects (—) from Site 2**

Present in this irregular eye formation cheese sample from Site 2 is a decreased amount of citric, acetic, propionic and butyric acids compared to the “Good” Swiss cheese sample from Site 1. Quantitation of the organic acids in the cheese samples is summarized in Table 14.

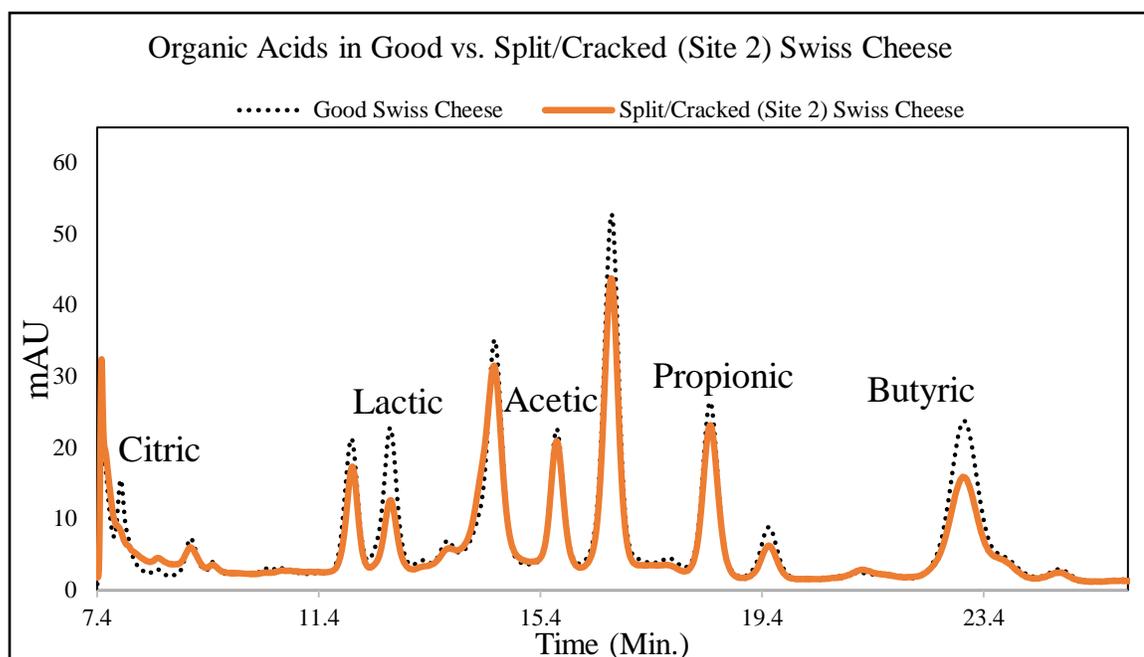
**Table 14. Organic Acids Quantified in "Good" Swiss Cheese and Cheese with Irregular Eye Formation/Distribution (Site 2)**

Organic Acid	“Good” Swiss Cheese (µg/mL)	Irregular Eye Dist. (µg/mL)	Percent (%) Difference
Citric	0.090	0.004	183
Lactic	0.450	0.293	42
Acetic	0.893	0.595	40
Propionic	1.414	1.030	31
Butyric	1.995	1.834	8

Irregular eye distribution/formation is a broad definition and encompasses any type of eye formation defects. It is logical to see variations in organic acid quantities between cheeses from both sites exhibiting similarly labeled defects. Although the cheese samples

from both Site 1 and Site 2 were labeled as having an “irregular eye distribution/formation” defect, the visual appearance of the defects were significantly different. The Site 1 defective cheese had an exterior that looked “wrinkled” with no obvious eyes present, while the cheese from Site 2 lacked even distribution of eyes throughout the matrix. Organic acid variation resultant from the activity of different bacteria would account for these defects to differ in presentation. The uneven eye distribution observed in the cheese sample from Site 2 alongside the decreased propionic acid, but similar lactic acid when compared to “Good” Swiss cheese, is indicative of decreased propionic acid bacteria activity, which may be caused by an expired starter culture, presence of bacteria inhibitory to propionibacteria, or less than optimum temperature during the ripening process. Both cheeses with an irregular eye defect exhibited decreased acetic, citric and propionic acid concentrations compared to the “Good” Swiss cheese sample.

**Split/Cracked Cheese:** Defective cheese from Site 2 showing Split/Cracked characteristics was analyzed for organic acid quantity compared to “Good” Swiss cheese from Site 1. This defective cheese sample contained decreased quantities of organic acids compared to the “Good” Swiss cheese sample shown in Figure 22.



**Figure 23.** HPLC chromatogram of organic acids in “Good” Swiss cheese (•••) and cheese with Split/Cracked defects (—) from Site 2.

When analyzing Swiss cheese with a split/cracked type defect (that is absent of eyes), it is expected to find lower quantities of propionic, acetic, and lactic acids. A summary table of the organic acid concentrations between “Good” Swiss cheese from Site 1 and defective Swiss cheese exhibiting splits and cracks from Site 2 is provided in Table 15.

**Table 15. Organic Acids Quantified in "Good" Swiss Cheese and Cheese with Split/Cracked Defect (Site 2)**

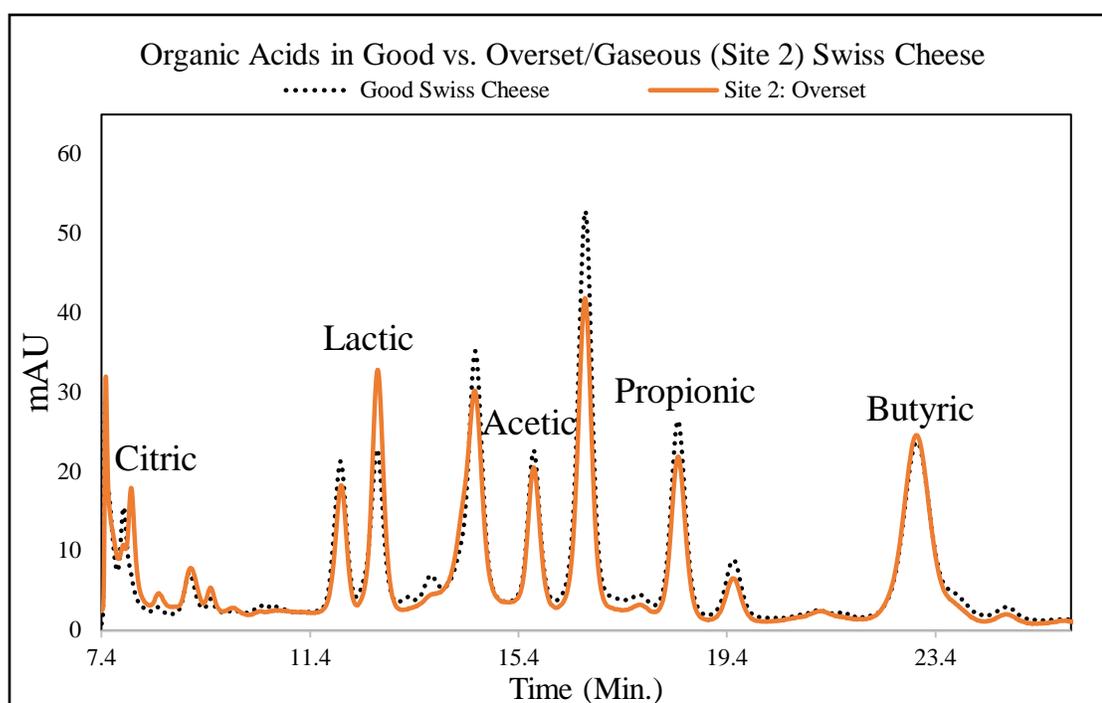
Organic Acid	“Good” Swiss Cheese ( $\mu\text{g/mL}$ )	Split/Cracked Cheese ( $\mu\text{g/mL}$ )	Percent (%) Difference
Citric	0.090	ND	N/A
Lactic	0.450	0.012	190
Acetic	0.893	0.819	9
Propionic	1.414	1.224	14
Butyric	1.995	1.296	42

ND = not detected; N/A = not available

The split/cracked cheese sample from Site 2 had few (if any) eyes present, correlating to decreased propionic and acetic acids. When comparing the three organic acid

quantities as a ratio (lactic: propionic: acetic) between the defective cheese and the “Good” Swiss cheese samples however, the degree to which the lactic acid is observed in a lower quantity in the defective cheese sample is suggestive of utilization by some other type of bacteria.

**Overset Cheese:** Cheese exhibiting an overset or gaseous defect from Site 2 was analyzed for organic acid quantities and compared to the “Good” Swiss cheese sample from Site 1. A chromatogram of these organic acids is displayed in Figure 23.



**Figure 24. HPLC chromatogram of organic acids in “Good” Swiss cheese (•••) and cheese with Overset/Gaseous defects (—) from Site 2.**

Overset/gaseous cheese from Site 2 contains increased quantities of citric and lactic acids, while exhibiting decreased quantities of propionic and acetic acids. Table 16 lists the quantities of organic acids in good Swiss cheese and cheese with an overset defect from Site 2.

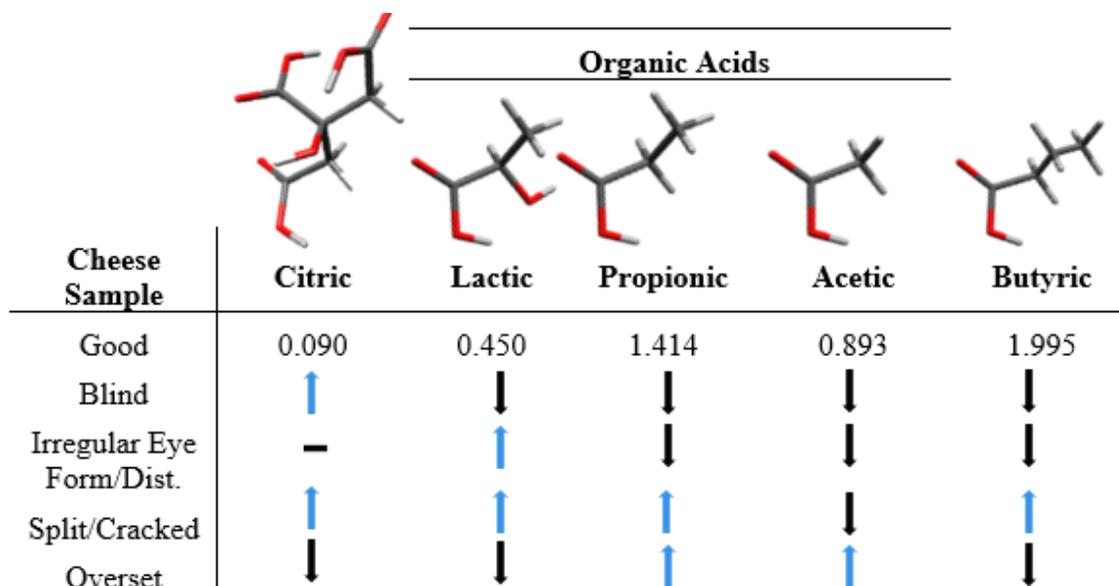
**Table 16. Organic Acids Quantified in "Good" Swiss Cheese and Overset Cheese (Site 2)**

<b>Organic Acid</b>	<b>"Good" Swiss Cheese (µg/mL)</b>	<b>Overset (Site 2) Cheese</b>	<b>Percent (%) Difference</b>
Citric	0.090	0.125	33
Lactic	0.450	0.780	54
Acetic	0.893	0.799	11
Propionic	1.414	1.154	20
Butyric	1.995	2.126	6

Defective cheese samples exhibiting overset from characteristics Site 2 comprises increased levels of lactic and citric acids compared to the "Good" Swiss cheese sample. The overset/gaseous cheese from Site 1 contained excess acetic and propionic acids, consistent with an excess of CO<sub>2</sub> gas being in the cheese matrix from propionic acid fermentation. The overset/gaseous cheese form Site 2 exhibits decreased amounts of propionic acid indicating another culprit may be responsible for this defect, or there may be fewer or less active propionibacteria within the cheese matrix.

#### **4.3 Organic Acid Conclusion**

When analyzing eye formation and distribution in a cheese sample, the most significant organic acid contributors studied include lactic, propionic, acetic, and butyric acids produced by propionic, lactic, and butyric acid bacteria. Figure 24 summarizes the data presented in Tables 10–13 for the organic acids quantified in cheese samples from Site 1.



**Figure 25.** A summary of Organic acids quantified in "Good" Swiss cheese and relative levels of organic acids studied in Site 1 cheeses exhibiting defects.

#### 4.3.1 Site 1 Organic Acid Conclusion

The organic acid chromatograms of "Good" cheese versus various defective Swiss cheese samples permitted quantification of organic acids that may correlate to the observed defects and bacterial population within the cheese matrix. It is worth noting that the following 3 cheese samples contained bacteria from the genus "*Candidatus Berkiella*": "Good" Swiss cheese (Site 1), Overset Swiss cheese (Site 1), and Split/cracked Swiss cheese (Site 2). The importance behind this bacterial identification demands further study due to the lack of studies on this genus, and the fact that it has only recently been cultured. "*Candidatus Berkiella*" currently contains only two known species; both of which are two intra-nuclear bacteria of freshwater amoebae which manipulate and inflict epigenetic changes to host cell functioning, such as vesicle trafficking. Due to limited knowledge surrounding "*Candidatus Berkiella*", no correlations can be made to organic acids. No other cheese samples in this study contained a "*Candidatus Berkiella*" population, and are therefore considered to be significantly different.

### Blind

A combined incidence of lower quantities of all organic acids noted, apart from citric acid, correlates to a cheese sample having blind defect characteristics. From chapter 2, blind defects in Swiss cheese correlate to cheese with a decreased *Propionibacterium* population resulting in decreased quantities of propionic and acetic acids in addition to decreased amounts of CO<sub>2</sub> which is primarily responsible for eye formation. At the order taxonomic level, blind cheese exhibits an increased *Lactobacillales* population which may hinder the activity of propionic acid bacteria, contributing to the decreased propionic and acetic acids in the blind Swiss cheese as well as contribute to the increased lactic acid quantity; leading to decreased eye formation. Additionally, the population of “*Candidatus Berkiella*” was shown to be significantly decreased from that of “Good” Swiss cheese but further studies are required to correlate the genus to organic acids found in Swiss cheese.

### Irregular Eye Formation/Distribution

An increased quantity of lactic acid by more than three times that found in “Good” Swiss cheese correlates to the increased *Lactobacillus* population in the cheese presenting irregular eye formation/distribution defect. Due to *Lactobacillus* being able to hinder the activity of PAB, decreased quantities of propionic and acetic acids in combination with the increased *Lactobacillus* population correlate to cheese presenting with an irregular eye distribution/formation defect. An abundance of lactobacillus may correlate to citric acid utilization by some species depending on the relative concentrations of galactose and pH within the cheese matrix. The significant *Clostridium sensu stricto 12* population observed is understood to produce butyric acid and H<sub>2</sub> gas in cheese, yielding atypical eye formation but no increase of butyric acid was observed and H<sub>2</sub> was not quantified in this project. The

irregular eye formation/distribution defect should be studied in greater depth to determine the contribution by the H<sub>2</sub> or butyric acid. This cheese sample contained a significantly decreased population of “*Candidatus Berkiella*”, but further studies are required to correlate this genus to the quantified organic acids.

#### Split/Cracked

Split/cracked defects in Swiss cheese can happen under different circumstances. The first circumstance is due to excessive gas production and/or a cheese matrix unsuitable to withstand the gas produced, while the second circumstance is due to secondary fermentation caused by gas production following desired propionic acid fermentation in the warm room. The cheese exhibiting the split/cracked defect from Site 1 contains decreased quantities of propionic and acetic acids which correlate to the cheese sample having a reduced population of *Propionibacterium*. The increased quantity of lactic acid correlates to the greater *Lactobacillus* population compared to the population observed in the sample of “Good” Swiss cheese. The Swiss cheese presenting with a split/cracked defect contained a decreased “*Candidatus Berkiella*” population and a higher citric and butyric acid quantities by 77% and 2% respectively, than was quantified in the “Good” Swiss cheese.

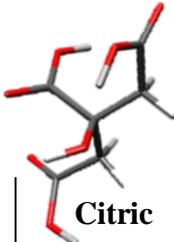
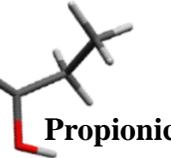
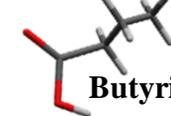
#### Overset

The sample of Swiss cheese exhibiting an overset defect from Site 1 contained no significantly different bacterial populations than that of the “Good” Swiss cheese. There was an increased quantity of acetic and propionic acids in this defective cheese with almost twice the quantity of acetic acid than was found in the “Good” Swiss cheese. A decreased quantity of citric acid by 156% and butyric acid by 26% was also noted in the overset

cheese. This defect exhibits high levels of acetic and propionic acid and low levels of citric and butyric acids. Based on these data, the activity of the bacteria responsible for producing the variation in organic acids observed from Site 1 remain speculative.

#### 4.3.2 Site 2 Organic Acid Conclusion

Figure 25 summarizes the organic acid concentrations in defective Swiss cheese samples from Site 2 (Tables 14–16) as well as the “Good” Swiss cheese from Site 1. While the organic acid data was analyzed in the same manner as cheese from Site 1, this data is not considered as reliable due to comparing cheeses from two different locations. Site 2 cheeses overall, demonstrated lower quantities of all organic acids compared to the “Good” Swiss cheese sample, apart from the overset cheese. Overset cheese had an increased quantity of butyric and lactic acids.

Cheese Sample	Organic Acids				
	 Citric	 Lactic	 Propionic	 Acetic	 Butyric
Good	0.090	0.450	1.414	0.893	1.995
Irregular Eye Form/Dist.	↓	↓	↓	↓	↓
Split/Cracked	—	↓	↓	↓	↓
Overset	↑	↑	↓	↓	↑

**Figure 26.** A summary of Organic acids quantified in "Good" Swiss cheese (Site 1) and relative levels of organic acids studied in Site 2 cheeses exhibiting defects.

### Irregular Eye Formation/Distribution

Cheese presenting with an irregular eye defect from Site 2 contained decreased quantities of propionic and acetic acids correlating to a decreased *Propionibacterium* population. Increased populations of *Staphylococcus*, *Clostridiaceae* (unclassified), and *Clostridium sensu stricto 12* were present while exhibiting a decreased population of “*Candidatus Berkiella*”. *Clostridium* is known to be associated with utilizing lactic acid during the ripening of cheeses and explains why lactic acid was not found to be in excess alongside the decreased *Propionibacterium* in this cheese sample. *Staphylococcus* is comprised of many species, some of which are linked to foodborne pathogens. All samples of cheese analyzed contained 0% of *Staphylococcus* except this sample, presenting with an irregular eye formation/distribution defect from Site 2. The result is indicative of an outside contamination source, rather than a mechanical production source, such as a contaminated starter culture or other ingredient.

### Split/Cracked

Cheese with a split/cracked defect from Site 2 contained decreased acetic and propionic acids correlating to a decreased *Propionibacterium* population. Similar to the split/cracked cheese from Site 1, this sample also contained an increased *Lactobacillus* population correlating to the significantly decreased citric acid quantity, but did not exhibit increased lactic acid compared to the sample of “Good” Swiss cheese. This is likely due to the increased spoilage bacteria population of *Clostridium sensu stricto 12* which can metabolize lactic and acetic acids to produce butyric acid, CO<sub>2</sub>, and H<sub>2</sub> gas. Increased populations of *Clostridiaceae* (unclassified), and *Rhodobacteraceae* (unclassified), which are also known spoilage bacteria were present in this defective cheese sample. This

split/cracked cheese sample from Site 2 was the only sample containing a significantly different *Rhodobacteraceae* population.

#### Overset

Cheese presenting with an overset defect from Site 2 contained decreased propionic and acetic acids and increased lactic acid correlating to a lower *Propionibacterium* population and increased butyric acid correlating to an increased *Clostridium sensu stricto* 12 population. The overset cheese from Site 2 and the split/cracked cheese from Site 1 are the only defective cheese samples to contain higher butyric acid quantities than that of the “Good” Swiss cheese. Additionally, this defective cheese sample contained decreased populations of “*Candidatus Berkiella*” and *Gammaproteobacteria* (unclassified). The organic acids quantified in this overset cheese sample were divergent from the similar defective cheese from Site 1 in every possible way. In cases where overset cheese from Site 1 was high in an acid, the overset cheese from Site 2 was low and vice versa. This indicates the overset defect across both sites was instigated and/or governed by different means.

## CHAPTER FIVE: CONCLUSION

Bacteria within cheese matrices produce organic acids and contribute to the overall product quality. In Swiss cheeses, atypical eye formation, which encompasses many observed defects, leads to downgrading of the cheese. All defective cheese samples were compared to the only “Good” Swiss cheese sample (Site 1) based on bacterial population and organic acid production. It is with confidence that correlations can be made between bacterial populations, organic acids produced and defective cheese samples from Site 1 and only speculative suggestion for the cheese samples from Site 2, since a suitable control was not available from this site.

### 5.1 Blind Defect in Swiss Cheese

Swiss cheese exhibiting a blind defect has an absence of or few eyes throughout the cheese block. Blind cheese contained lower per-sample populations of “*Candidatus Berkiella*” and *Propionibacterium* alongside decreased quantities of acetic, lactic, propionic, and butyric acids with an increased quantity of citric acid. This cheese sample contained a higher per-sample population of Lactobacillales, which are known to hinder Propionibacteriales. The decreased *Propionibacterium* correlates to decreased propionic and acetic acids (as well as decreased CO<sub>2</sub> but was not studied during this project). “*Candidatus Berkiella*” was only recently cultured and has not been significantly studied, therefore, no correlation to organic acids can be made at this time.

## 5.2 Irregular Eye Formation/Distribution Defect in Swiss Cheese

### 5.2.1 Site 1

Cheese presenting with this defect showed an increased per-sample population of *Lactobacillus* which correlates to an increased quantity of lactic acid and decreased quantities of propionic, acetic, and citric acids. An increased population of *Clostridium sensu stricto 12* is known to correlate to increased butyric acid and H<sub>2</sub> gas, yielding atypical eye formation but increased butyric acid was not observed for this cheese and H<sub>2</sub> was not quantified in this project. A further investigation of this bacterium and defect relating to H<sub>2</sub> is required.

### 5.2.2 Site 2

Cheese experiencing an Irregular eye defect from Site 2 contained a decreased per-sample population of *Propionibacterium* correlating to lower quantities of propionic and acetic acids. Additionally, this cheese showed greater populations of *Staphylococcus*, *Clostridiaceae* (unclassified) and *Clostridium sensu stricto 12*. The *Clostridiaceae* (unclassified) and *Clostridium sensu stricto 12* correlate to a lower quantity of lactic acid. The presence of *Staphylococcus* in only this cheese sample demands further study as some species are linked to foodborne pathogens and is indicative of an outside contamination source rather than a mechanical or production source (such as contaminated starter culture).

The differences between samples from both Sites indicate the “Irregular Eye Formation/Distribution” is driven by different means. This finding is not a surprise as various eye defects having encompassed by this label are vast.

### 5.3 Split/Cracked Defects in Swiss Cheese

#### 5.3.1 Site 1

A reduced population of *Propionibacterium* correlate to decreased propionic and acetic acids in this cheese while an increased quantity of lactic acid correlates to the increased per-sample population of *Lactobacillus*. This finding is supportive of the defect being driven by an unsuitable cheese matrix rather than excessive gas production in the warm room. This cheese also presented with increased quantities of citric and butyric acids which were not straightforwardly correlated to significantly different bacteria.

#### 5.3.2 Site 2

The Split/cracked cheese from Site 2 was similar to cheese from Site 1 regarding *Propionibacterium* and correlated acids. An increased population of *Lactobacillus*, depending on strains present, correlate to a decreased citric acid quantity. This cheese also contained an increased population of *Clostridium sensu stricto 12* correlating to decreased levels of lactic and acetic acids with increased butyric acid (this also indicates increased levels of CO<sub>2</sub> and H<sub>2</sub> gas within the matrix). A greater population of *Clostridiaceae* (unclassified) and *Rhodobacteraceae* were present in this sample but further studies are required to correlate organic acid contributions. This was the only cheese sample with an increased population of *Rhodobacteraceae*. These populations and organic acids indicate the defect was caused by excessive gas production within the cheese matrix.

### 5.4 Overset Defects in Swiss Cheese

#### 5.4.1 Site 1

Cheese having an overset defect from Site 1 contained no significantly different bacterial populations. Increased acetic and propionic acids and decreased citric and butyric

acids were found. Although suggestive, no bacteria can be correlated to this defect at this time and further study is required.

#### 5.4.2 Site 2

Overset cheese from Site 2 is correlated to lower per-sample populations of *Propionibacterium*, which relate to the lower propionic and acetic acids quantified. An increased *Clostridium sensu stricto 12* population correlates to increased butyric acid within the cheese. This cheese sample also contained a decreased population of *Gammaproteobacteria* (unclassified) which cannot be straightforwardly correlated to organic acids at this time. The presence of spoilage bacteria and increased butyric acid follow previous literature regarding gassy cheeses.<sup>66</sup>

The cheeses having the overset defect from Site 1 and Site 2 differed in every way. When cheese from Site 1 was high in an organic acid, cheese from Site 2 was low and vice versa. This is indicative of the defect across both sites being governed or instigated by different means.

### **5.5 Future Steps**

This project served as an introduction to correlating Swiss cheese defects to bacterial populations and organic acids produced, but exhaustive studies still need to be conducted. Due to the overpowering similarity in the *16s rRNA* gene amongst cheese samples, NGS only yielded results at the genus level with the parameters set forth by this project. A technique to provide strains of bacteria is required for a more thorough understanding of defect-bacteria-acid correlation. While some eye defects were correlated to contributors from two different sites, the analyses from Site 2 need confirmation with a “Good” Swiss cheese sample from Site 2.

Representative organic acids were studied based on previous literature and correlated to defects and bacterial populations, but were not exhaustive of all organic acids in Swiss cheese. The organic acid chromatograms showed additional acids, beyond the five studied, indicating their potential contribution to the specific defects; these unidentified organic acids require further investigation.

## APPENDIX A

**Supplementary 16S rRNA PCR Sequencing Equipment and Reagents**

**Table 17. 16S rRNA PCR and Sequencing Equipment**

<b>Manufacturer</b>	<b>Equipment/Model</b>	<b>Lot/Serial No.</b>
<b>Illumina</b>	MiSeq	M02404
MiSeq Reagent Kit: V2Cartridge 500-Cycles, ( Paired-End)		20374142
V2 Flow Cell		20365659
Nextera XT Index Kit		20048532
<b>Advanced Analytical Technologies Inc.</b>	Fragment Analyzer	3057
<b>Invitrogen</b>	Qubit 2.0 fluorimeter	1108003563
<b>Applied Biosystems by Life Technologies</b>	Veriti 96-Well Thermal Cycler 9902	2990230645
<b>BioRad</b>	Real-time PCR Detection System CFX96	785BR09333

**Table 18. 16S rRNA PCR Primer and reagents**

<b>Manufacturer</b>	<b>Reagent Name</b>	<b>Serial No.</b>
Roche	Kappa Hifi Hotstart ReadyMix	004792
MAGBIO	High Prep PCR	W1880001-1
TEKnova	10 mM Tris (pH 8.5)	T127518I1301
Decon Laboratories	Ethanol (200-proof)	2B7311
Fisher Scientific	DNA Grade Water	167096
<b>PCR Primers V4 Region</b>		
<i>16S</i>	TCGTCGGCAGCGTCAGATGTGTATAA	
<i>515F*</i>	GAGACAGGTGYCAGCMGCCGCGGTAA	
<i>16S</i>	GTCTCGTGGGCTCGGAGATGTGTATAA	
<i>806R**</i>	GAGACAGGGACTACNVGGGTWTCTAAT	

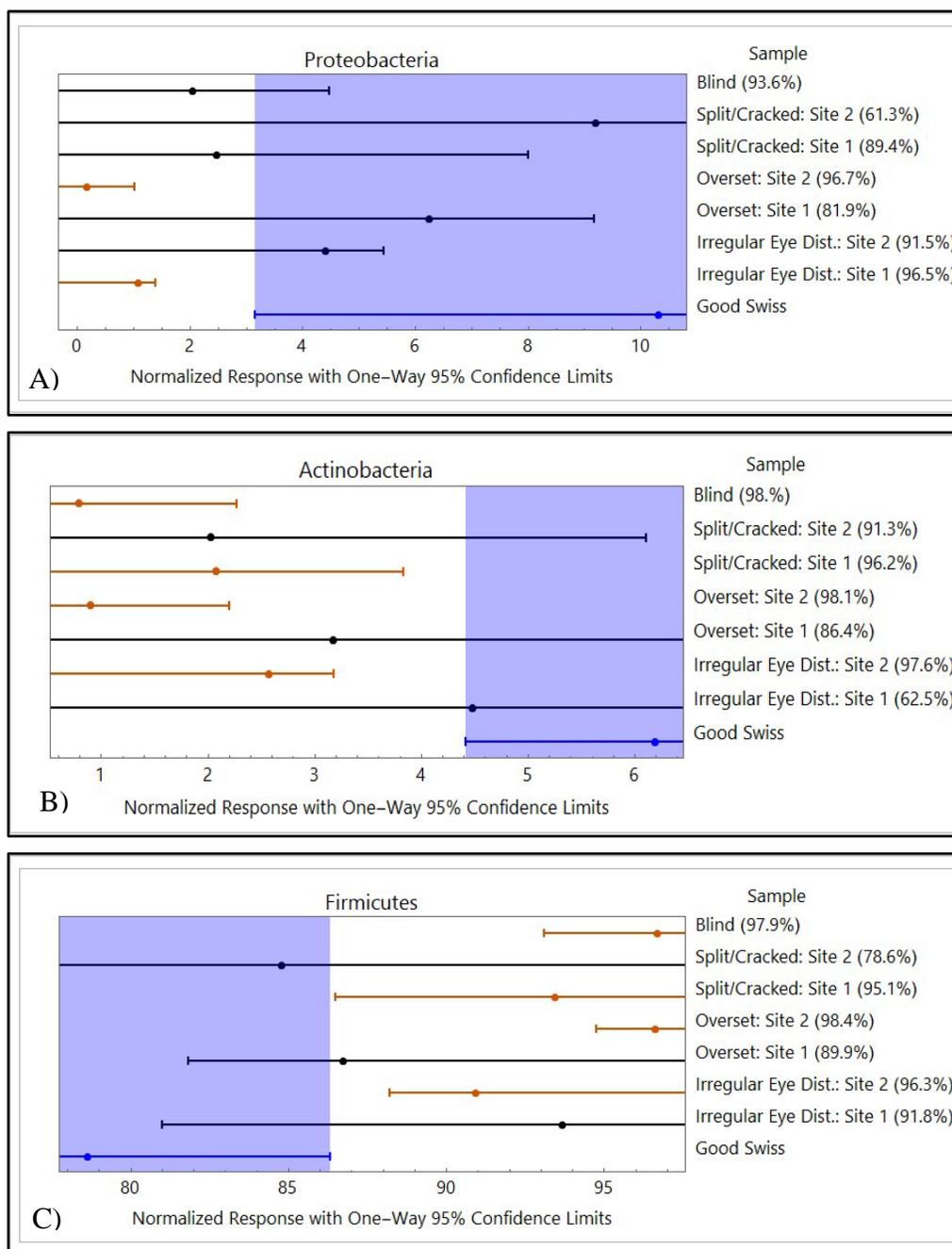
\*F corresponds to the primer in the forward direction

\*\*R corresponds to the primer in the reverse direction

## APPENDIX B

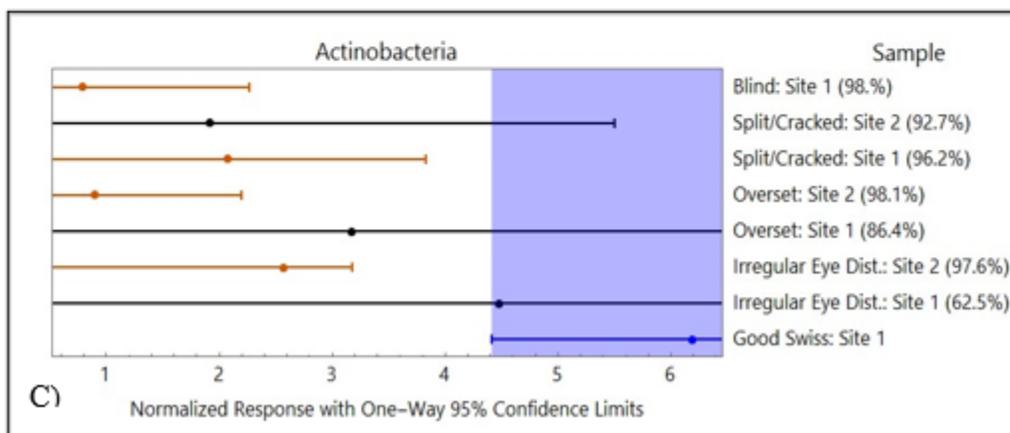
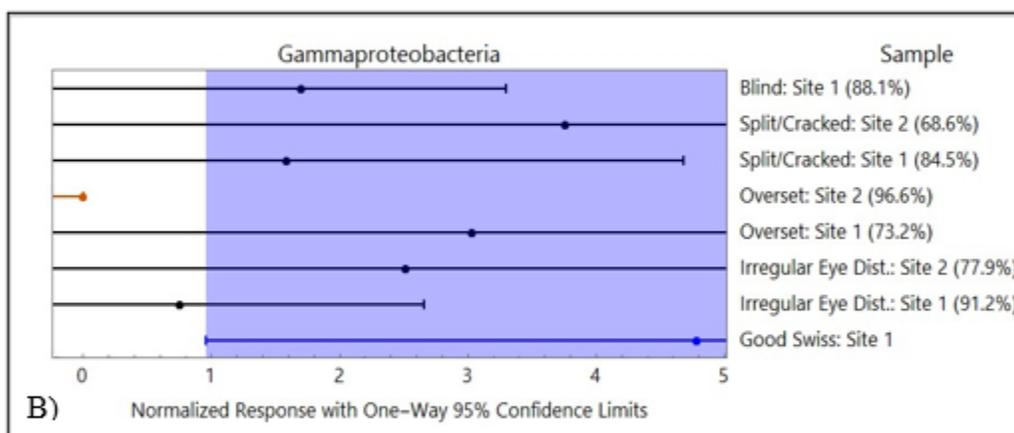
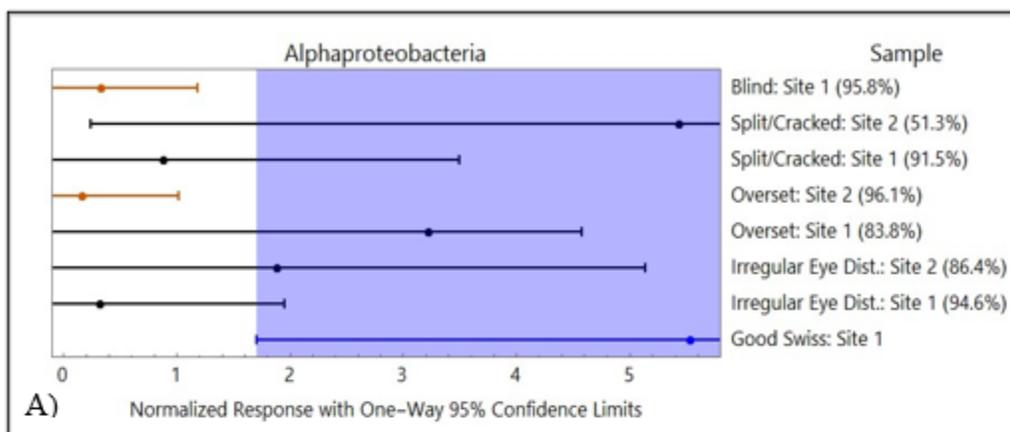
**Statistically Different Bacteria at the 95 % Confidence Interval**

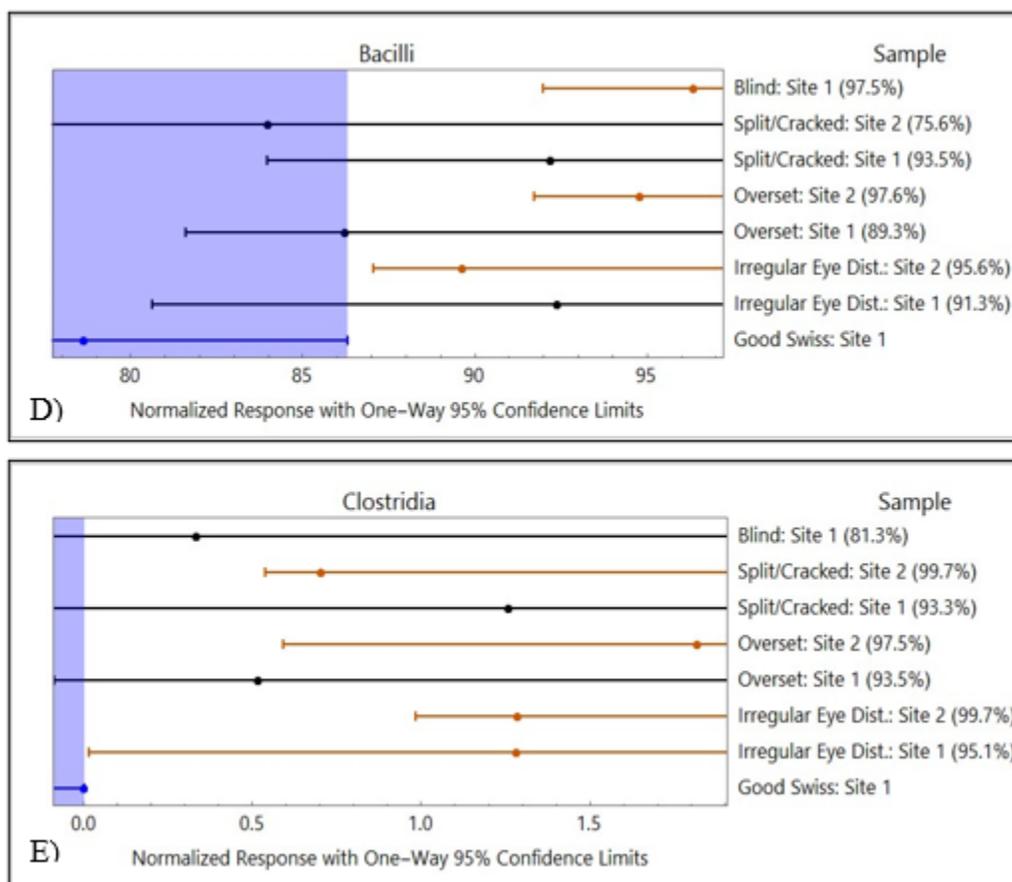
### B-1 95% Bacteria Confidence Intervals at the Phylum Level



**Figure 27.** Plots A-C show statistical differences of bacterial population between “Good” Swiss cheese and defective cheese samples at the phylum taxonomic level.

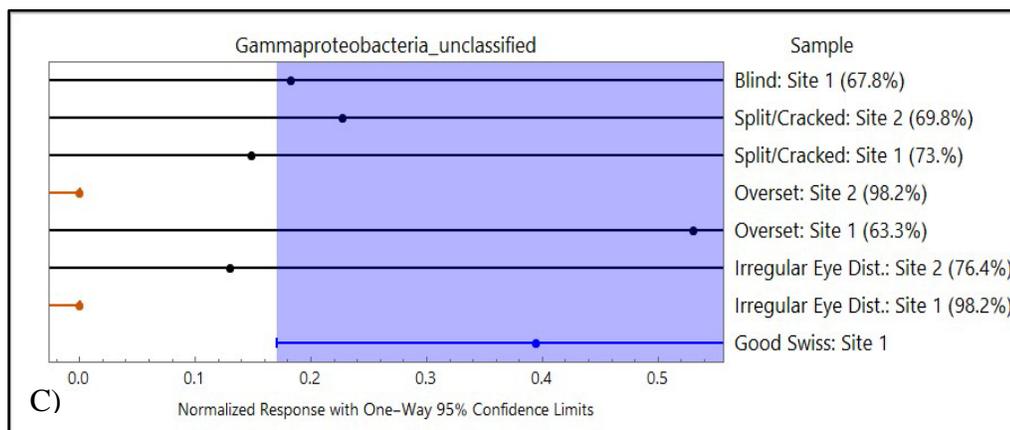
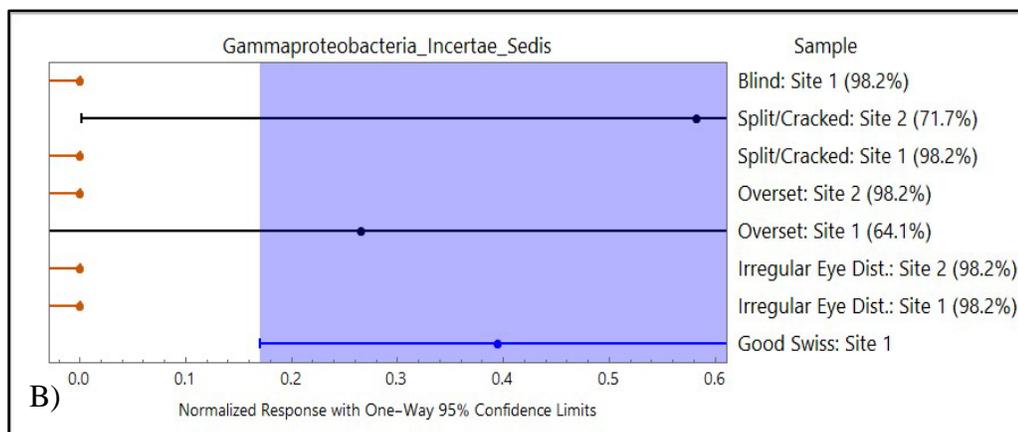
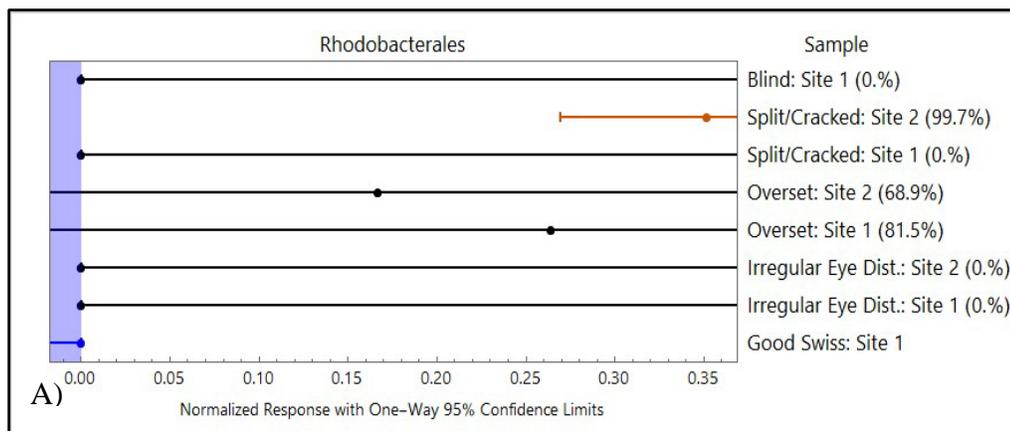
## B-2 95% Bacteria Confidence Intervals at the Class Level

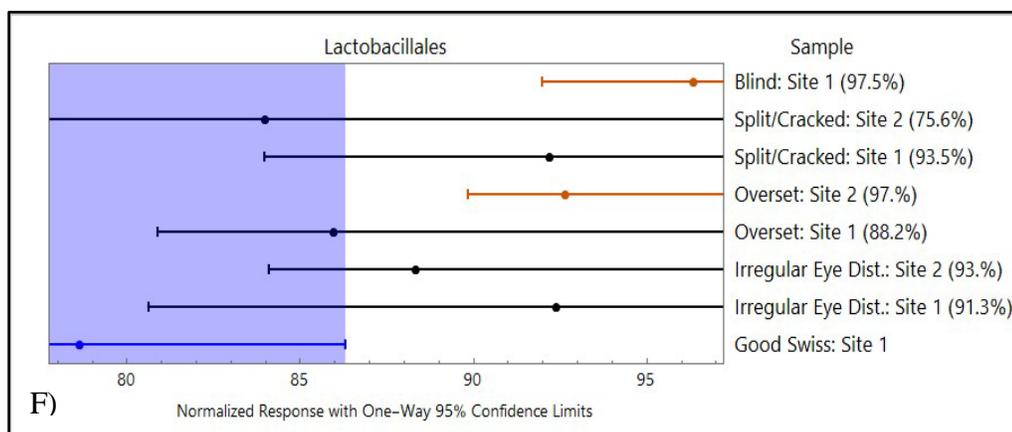
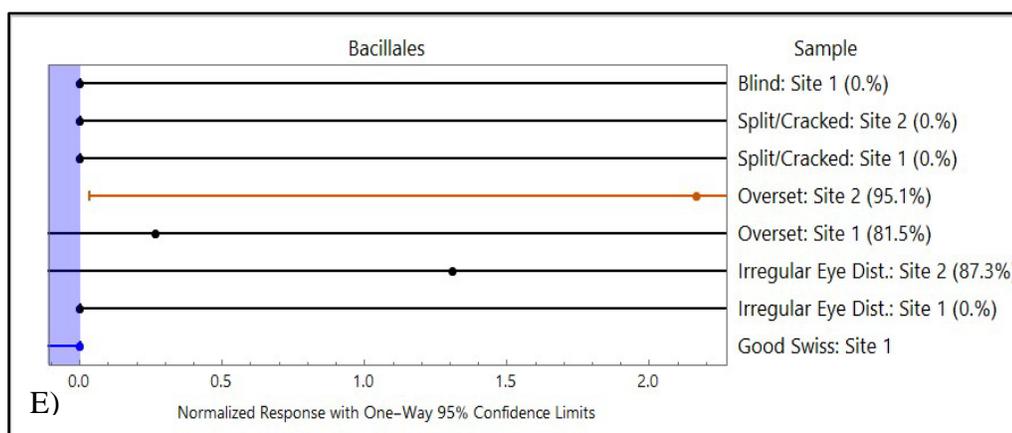
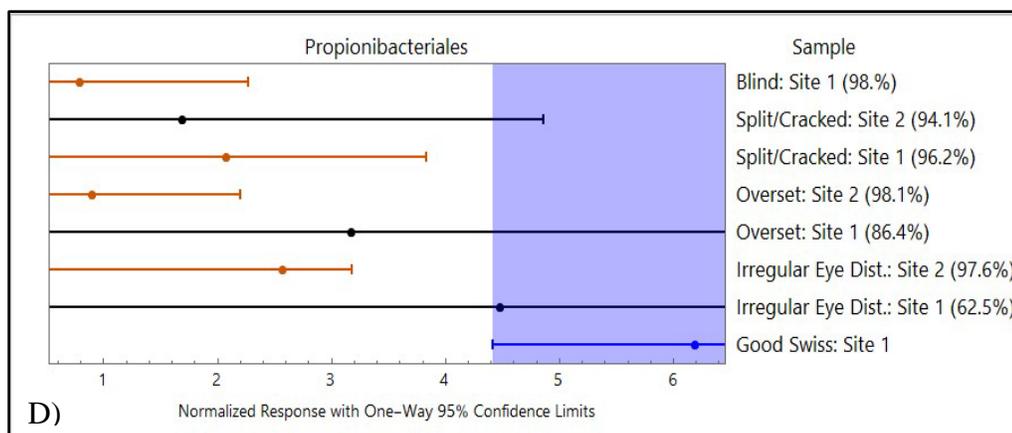


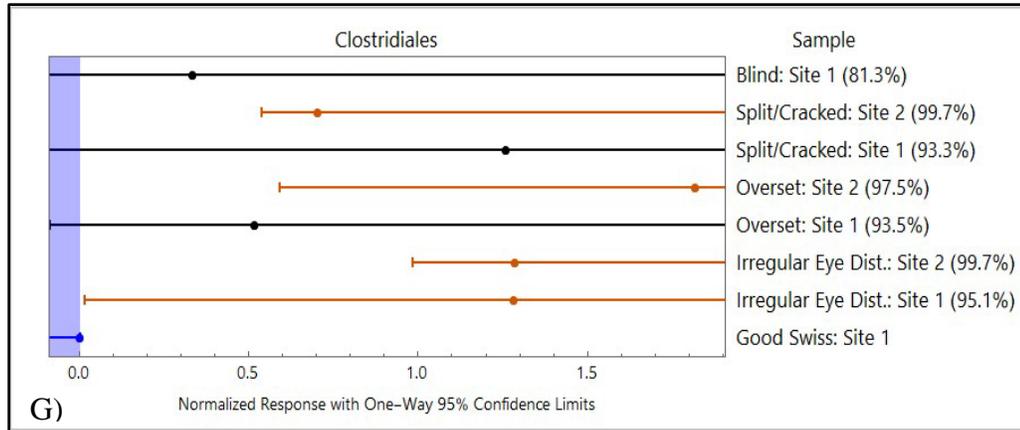


**Figure 28.** Plots A-E show statistical differences of bacterial population between “Good” Swiss cheese and defective cheese samples at the class taxonomic level.

### B-3 95% Bacteria Confidence Intervals at the Order Level

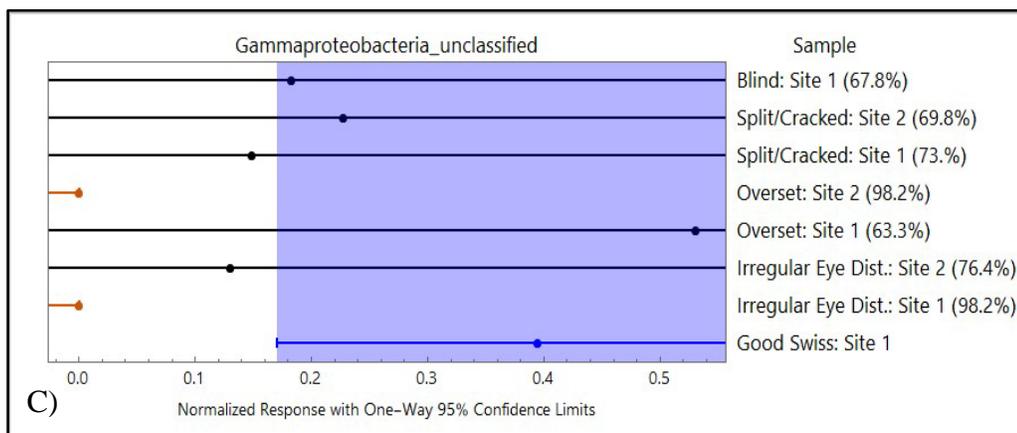
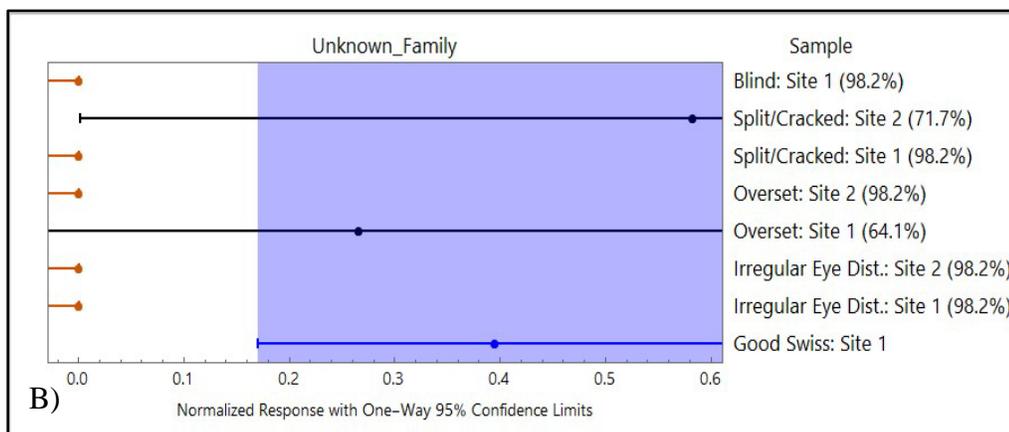
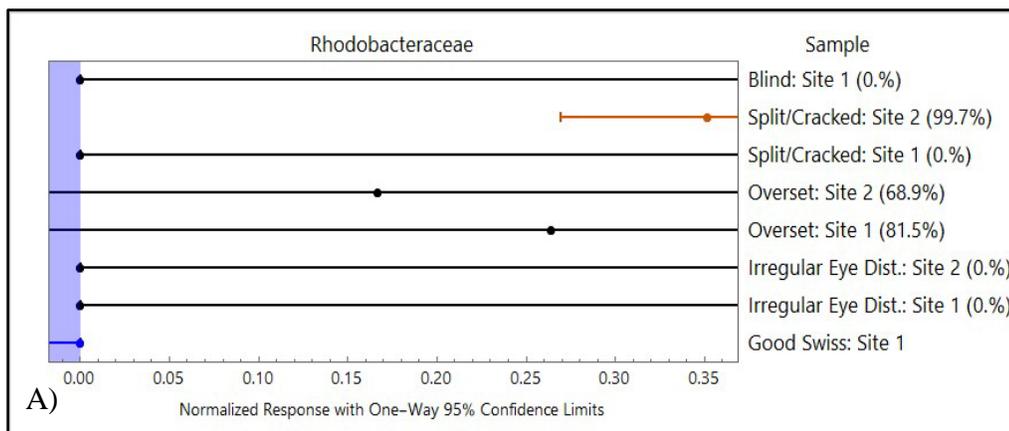


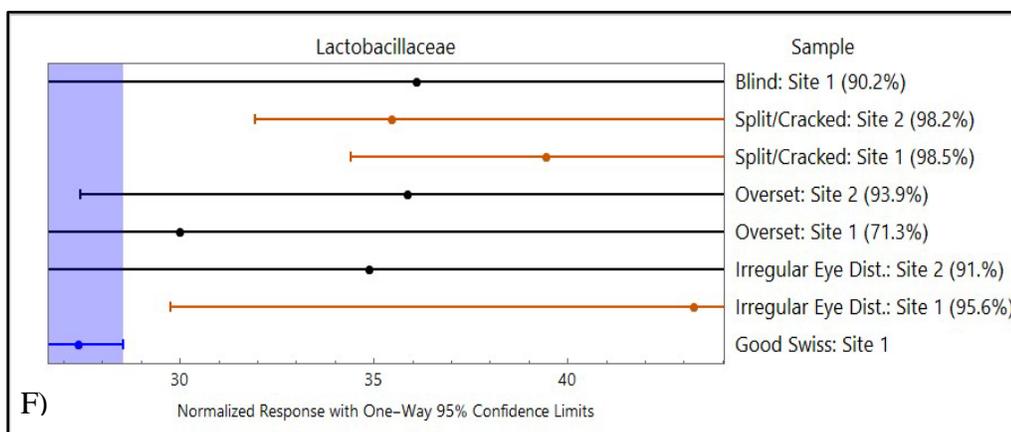
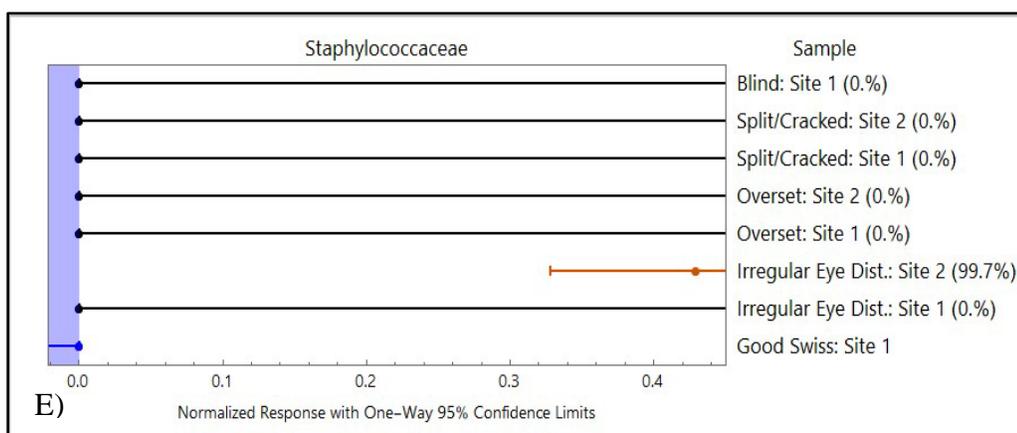
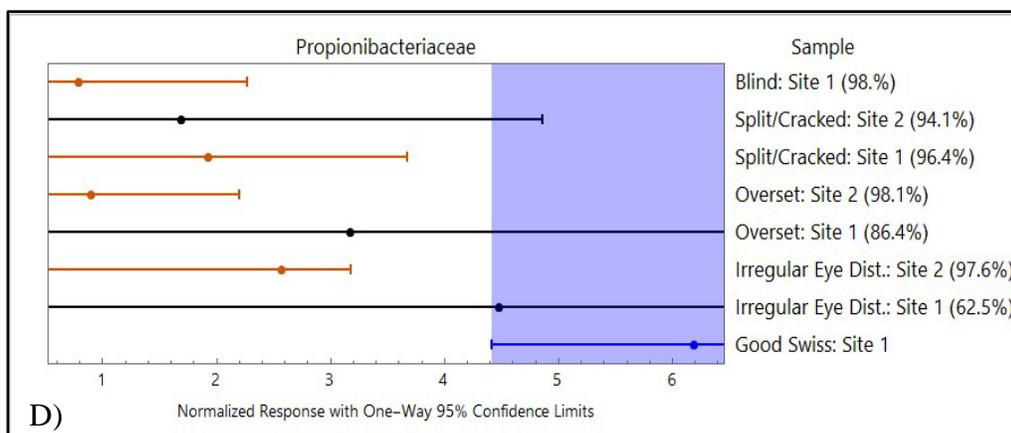


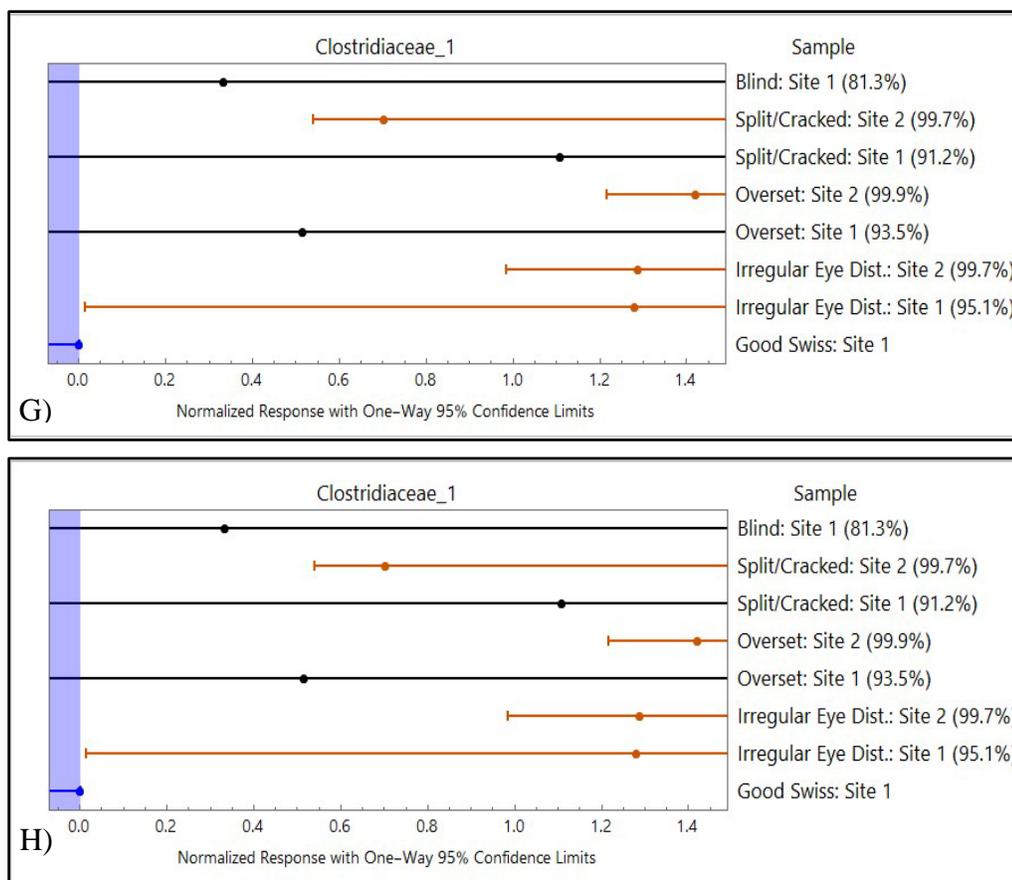


**Figure 29. Plots A-G show statistical differences of bacterial population between “Good” Swiss cheese and defective cheese samples at the order taxonomic level.**

### B-4 95% Bacteria Confidence Intervals at the Family Level

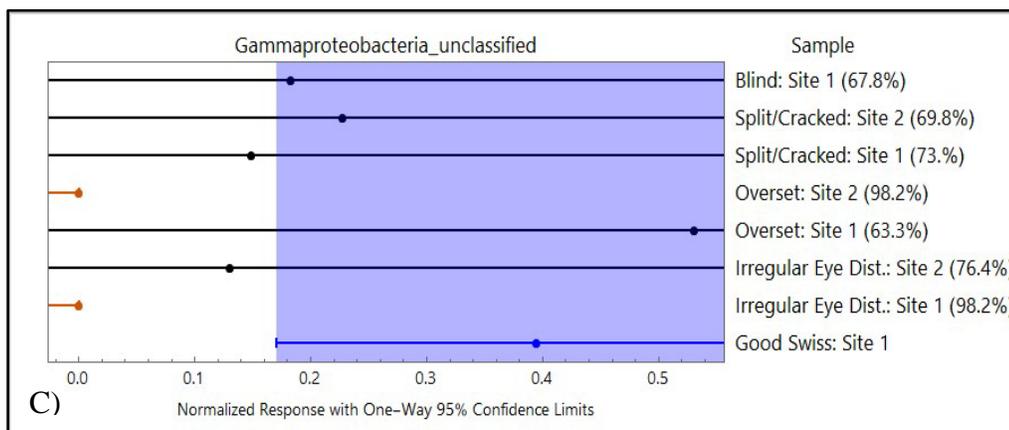
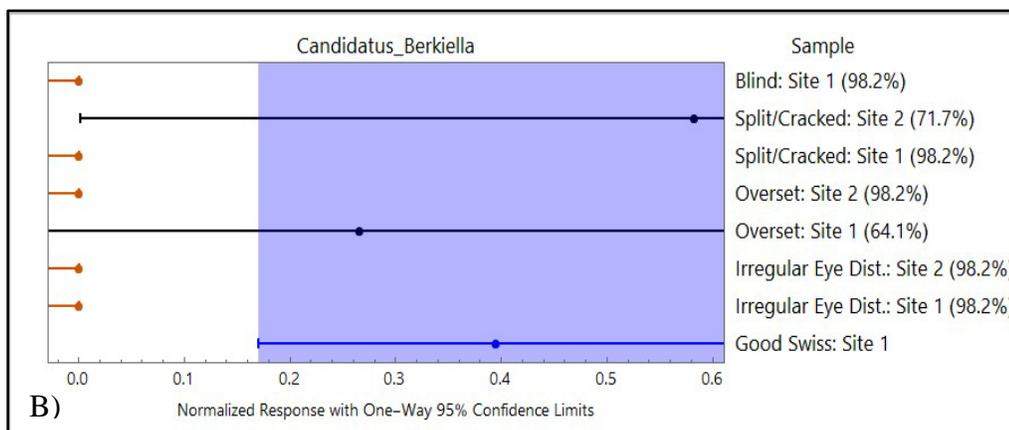
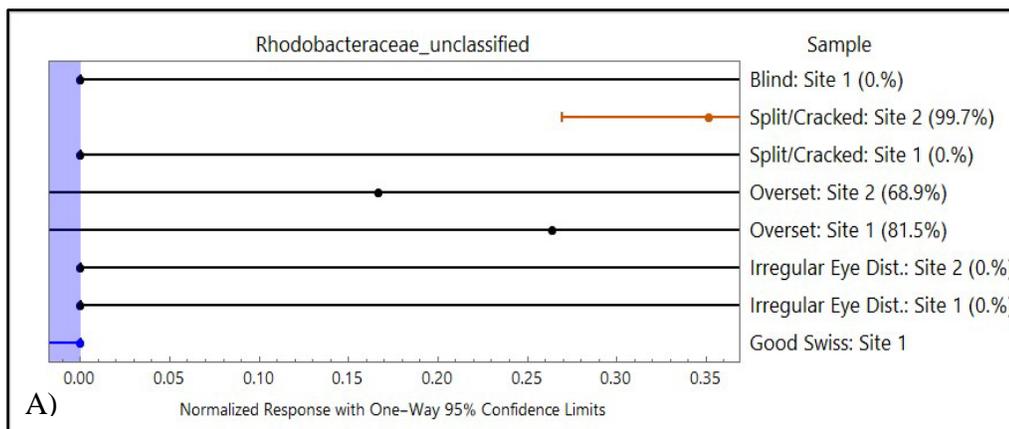


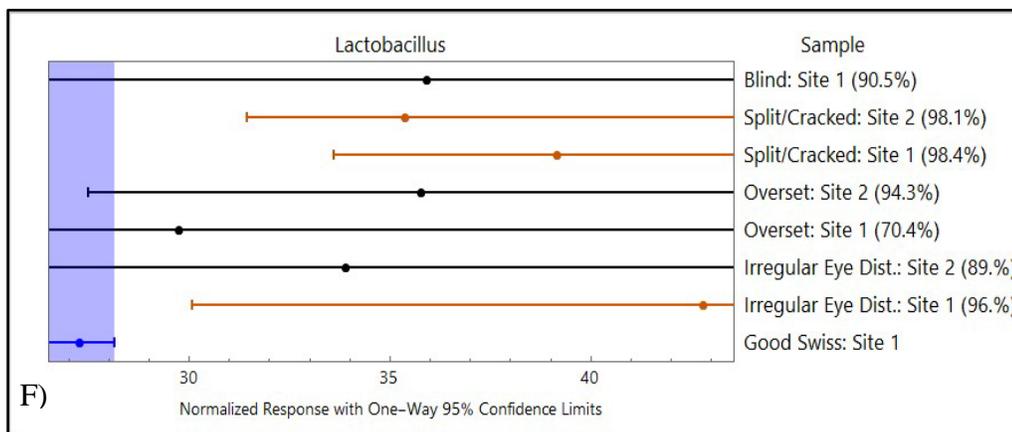
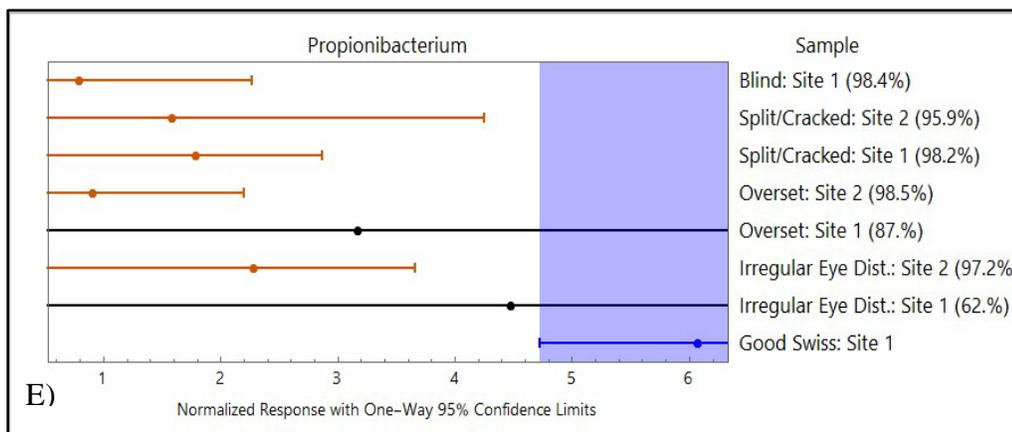
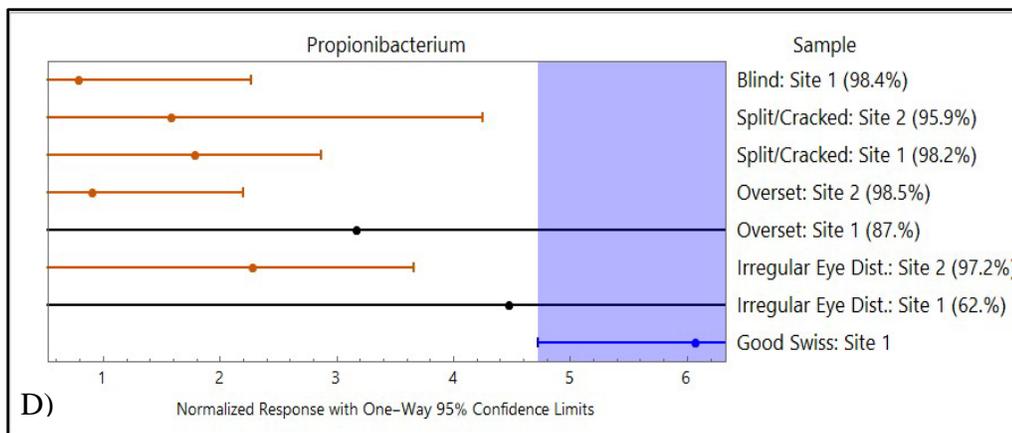


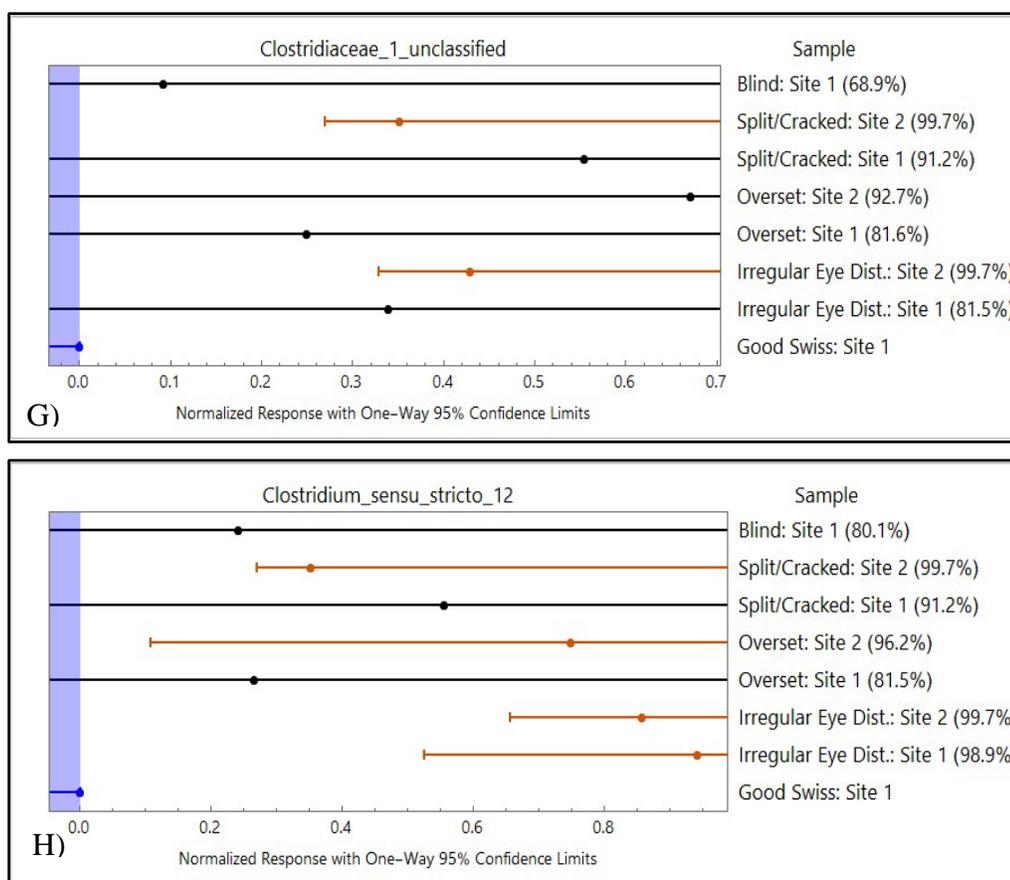


**Figure 30.** Plots A-H show statistical differences of bacterial population between “Good” Swiss cheese and defective cheese samples at the family taxonomic level.

### B-5 95% Bacteria Confidence Interval at the Genus Level



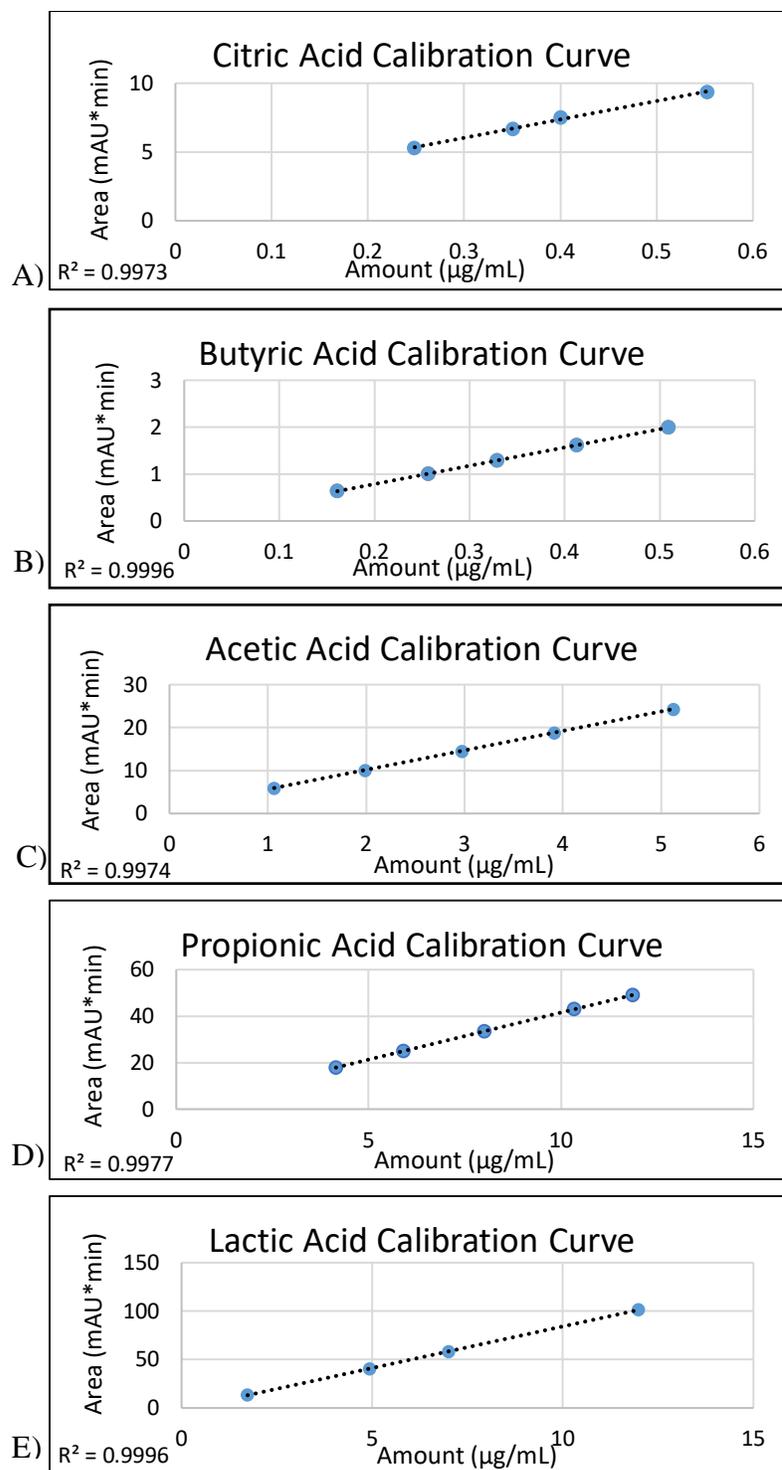




**Figure 31. Plots A-H show statistical differences of bacterial population between “Good” Swiss cheese and defective cheese samples at the genus taxonomic level.**

## APPENDIX C

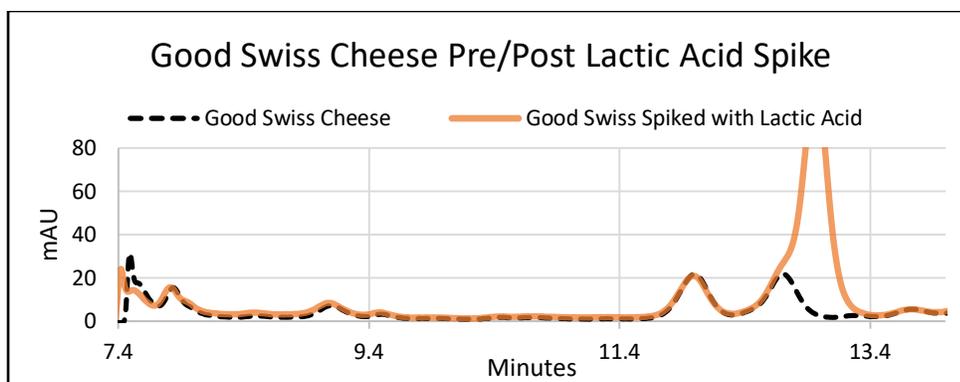
**Calibration curves for reference organic acid standards**



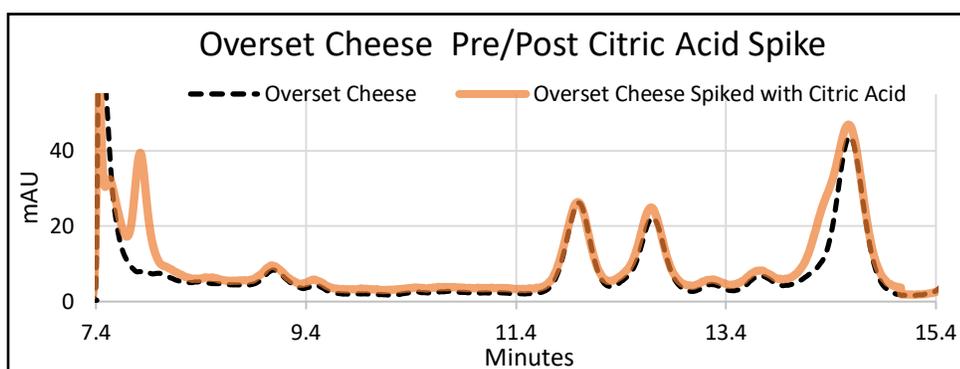
**Figure 32. Plots A-E are calibration curves for organic acids used as reference standards, aiding in quantification of organic acids in Swiss cheese samples.**

## APPENDIX D

**Method of standard addition for additional organic acid identification/confirmation**



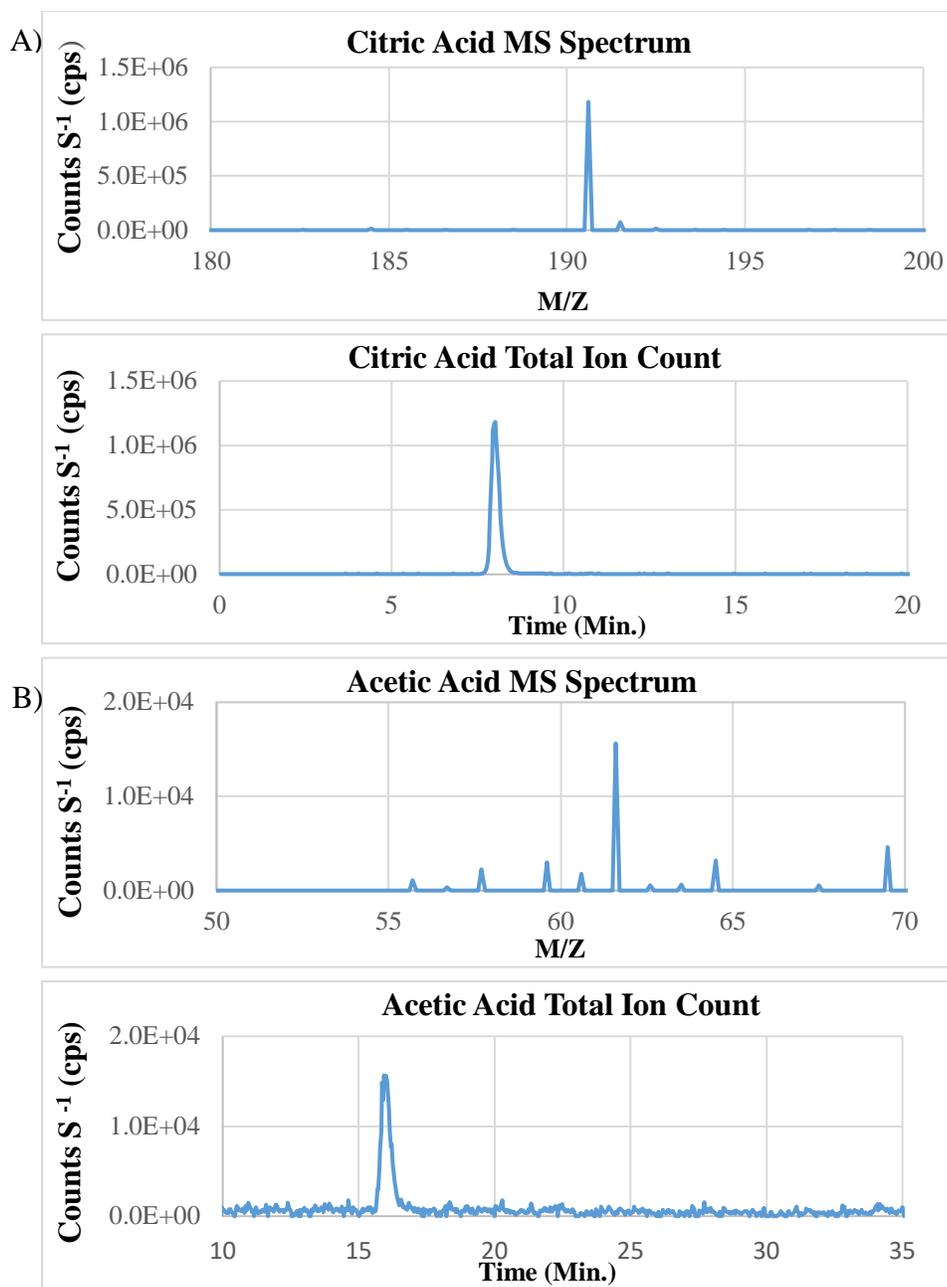
**Figure 33.** Magnified HPLC chromatogram of “Good” Swiss cheese sample spiked with lactic acid (13 min) to confirm identification.

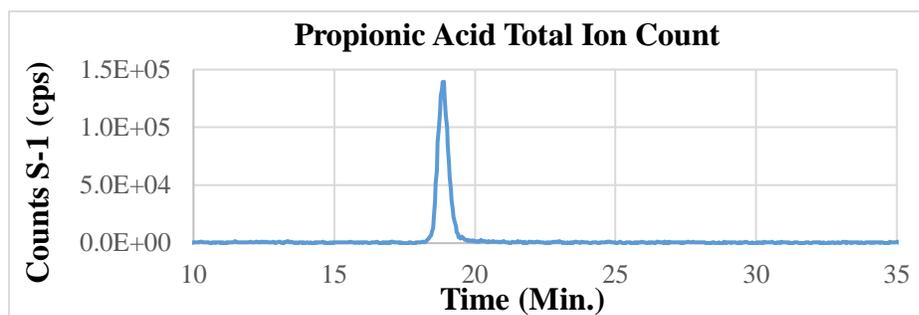
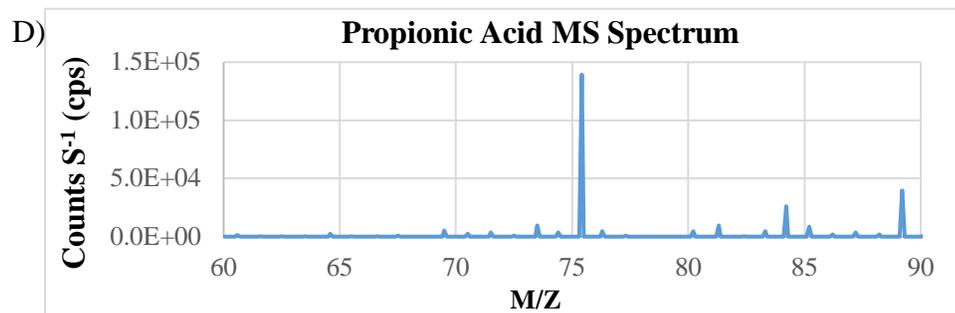
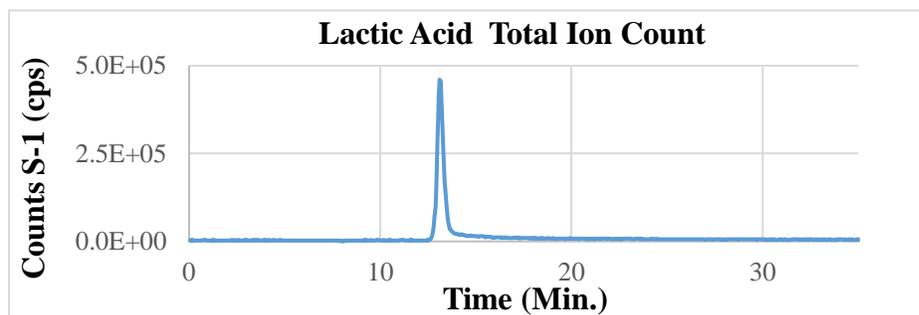
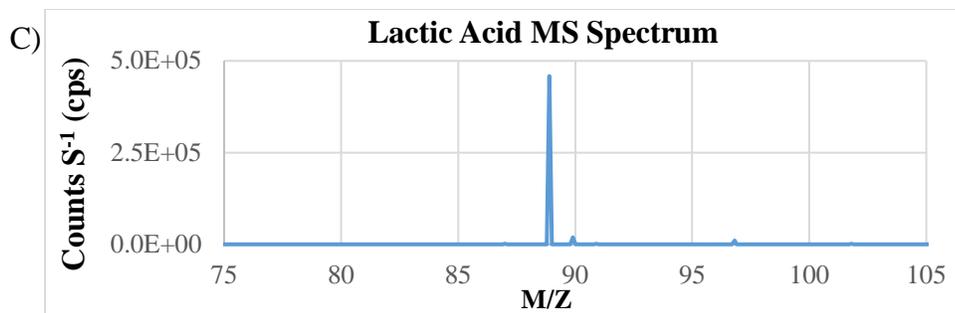


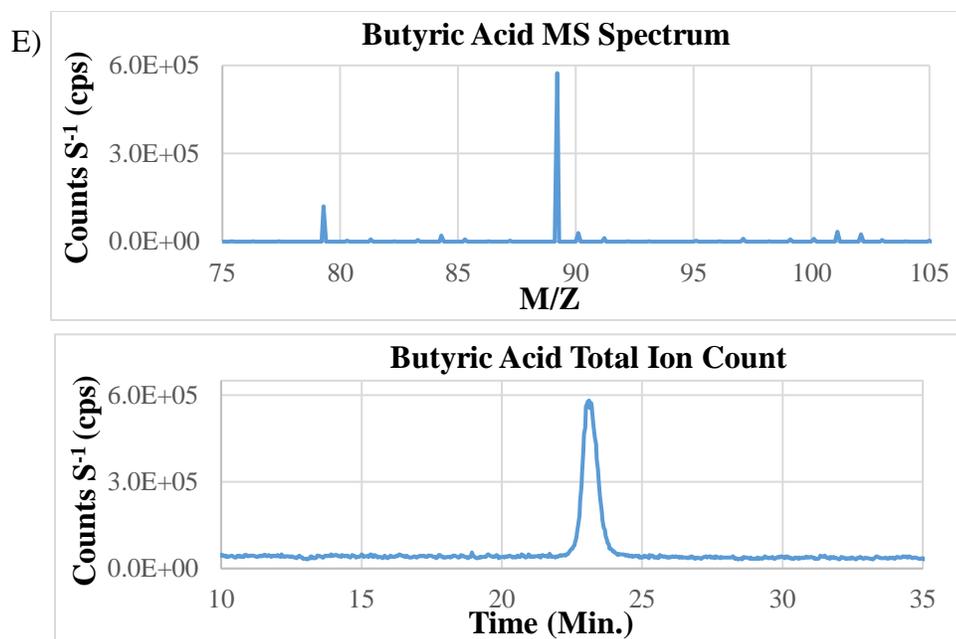
**Figure 34.** Magnified HPLC chromatogram of oversight cheese and oversight sample spiked with citric acid (7.5 min) to confirm identification.

## APPENDIX E

**Bruker MS spectra and total ion count chromatograms of organic acid reference standards.**







**Figure 35. Mass spectra and total ion count pairs (A-E) for organic acid identification/confirmation**

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