2017

Development of a Rapid Detection and Quantification Method for Yeasts and Molds in Dairy Products

Brandon Nguyen
University of Nebraska - Lincoln, bnguyen189@yahoo.com

DEVELOPMENT OF A RAPID DETECTION AND QUANTIFICATION METHOD FOR YEASTS AND MOLDS IN DAIRY PRODUCTS

by

Brandon Hoang Nguyen

A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Food Science and Technology

Under the Supervision of Professor Andréia Bianchini

Lincoln, NE

August 2017

DEVELOPMENT OF A RAPID DETECTION AND QUANTIFICATION METHOD FOR YEASTS AND MOLDS IN DAIRY PRODUCTS

Brandon Hoang Nguyen, M.S.

University of Nebraska, 2017

Advisor: Andréia Bianchini

A rapid quantitative PCR (qPCR) method was developed for the detection and quantification of fungi that are potentially present in dairy commodities. Genes of interest that were considered and used in the method development were the following: 18S rRNA, actin, beta-tubulin, and elongation factor 1-alpha. The following organisms were screened in this method development: *Galactomyces candidus, Debaryomyces hansenii*, *Yarrowia lipolytica*, *Penicillium roqueforti*, *Penicillium verrocosum* and *Cladosporium cladosporioides*. The developed method has a standard curve based on the organism, *Galactomyces candidus*, and the primers based on the elongation factor 1-alpha gene. Using this yeast and primers, this method can detect fungal counts above 10^2 CFU/mL. This method was compared to traditional plate counting on DRBC agar and currently available commercial methods (3M Rapid Yeast and Mold Petrifilms, Hygiena Qualicon BAX PCR Assay for Yeasts and Molds, and BIOTECON's FoodProof Yeast and Mold Quantification LyoKit -5'Nuclease- RP). The method comparison was performed at 4 set concentrations (10^6, 10^4, 10^2, and 0) in culture material and inoculated dairy samples. After this method comparison was performed in triplicate by two technicians, a survey testing 38 assorted dairy products (fluid milk, cottage cheese, yogurt, sour cream, and cheese) was performed. 16 of the 38 samples exhibited amplification using the developed

PCR method and with an estimated fungal presence in these samples between 10^2 and 10^5 CFU/mL. Of all the methods tested in the survey and the comparison work, the 3M Petrifilm provided the most consistent results that were comparable to the standard plate counting method on DRBC. The developed qPCR method was comparable in performance to the commercially available BIOTECON kit.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my mother Becky Gamblin for giving endless support throughout all stages in my life. Also, I would like each of my siblings, Alexander Gamblin, Ashley Gamblin, and William Gamblin, to know that I appreciate all of the phone calls, letters, and messages that they have made to me throughout my entire collegiate programs. I would also like to dedicate this thesis to my grandparents, Bounsam and Bounpheng Sisavanh. They both were strong support figures in my life and helped inspire my love of science and food. Although he has passed on, my grandfather's love and guidance has kept me motivated to pursue all of my dreams. I also want to thank my rest of my family for teaching and supporting me in everything throughout the years. To each of my friends (especially the "Bar Starz") that I have made throughout my years, I want all of you to know that I have the utmost gratitude for all of the moments that we have shared together and for enriching my life in many facets. I am very grateful for my primary advisor, Dr. Andréia Bianchini, for giving me the opportunity to work in her laboratory. I would also like to acknowledge Dr. Jayne Stratton and Dr. Heather Hallen-Adams for their contributions to this research project as well as my education. Finally, I would like to thank my lab mates Dr. Bismarck Martinez, Luis Sabillon, Rodrigo Mendoza, Ana Arciniega, Eric Oliver, Nabaraj Banjara, Esteban Valverde Bogantes, Rhaisa Crespo, and Amy Garrison for giving me constant support throughout my program.

TABLE OF CONTENTS

List of Tables ……....iv

List of Figures ……vi

Introduction……vii

1. CHAPTER 1 LITERATURE REVIEW……1

1.1 History of Dairy Consumption.……..2

1.2 Nutritional Significance of Dairy Consumption.……...3

1.3 Roles of Fungi in Dairy.……8

1.4 Quality and Shelf Life of Dairy Products.……...14

1.5 Fungal Detection and Quantification Methods……15

1.5.1 Currently Used Direct Counting Methods..……..15

1.5.2 Currently Used Plating Methods…..…….17

1.5.3 Currently Used "Rapid" Methods.……18

1.5.4 Development of "Novel" Technologies via Molecular Methods....…….19

1.5.5 DNA vs RNA……20

2. CHAPTER 2 MATERIALS AND METHODS……23

2.1 Fungal Cultures……24

2.2 Fungal Plate Count Method…….25

2.3 Primer Design for qRT-PCR and qPCR Protocols……..25

2.4 Rapid Method Development for the Quantification of Yeast and Mold…….27

2.4.1 RNA Extraction from Fungi…….28

2.4.2 DNA Extraction from Viable Fungi…….32

2.5 Development of PCR Protocols for Fungal Quantification…….35

2.5.1 Development of a qRT-PCR Protocol……..35

2.5.2 Development of a qPCR Protocol…….....36

2.6 Standard Curve Development for qPCR Protocols……..37

2.7 Inoculation of Dairy Products……..38

2.8 Validation of qPCR Method in Dairy Matrices……...39

2.9 Survey of Assorted Dairy Products and Fungal Quantification Methods……40

3. CHAPTER 3 RESULTS AND DISCUSSION…….…….41

3.1 Development of Primers for qRT-PCR and qPCR protocols……..42

3.2 Development of qRT-PCR Protocol for Fungal Detection……..48

3.3 Development of qPCR Protocol for Fungal Detection……53

3.4 Standard Curve Development for qPCR Protocol……..……..59

3.5 Validation of Developed Method in Dairy Matrices……...61

3.6 Survey of Assorted Dairy Products and Fungal Quantification Methods……68

4. CHAPTER 4 CONCLUSIONS……72

4.1 Development of Primers for qRT-PCR and qPCR protocols……..73

4.2 Development of qRT-PCR Protocol for Fungal Detection……..73

4.3 Development of qPCR Protocol for Fungal Detection……73

4.4 Standard Curve Development for qPCR Protocol ……..74

4.5 Validation of Developed Method in Dairy Matrices……...74

4.6 Survey of Assorted Dairy Products and Fungal Quantification Methods……74

5. CHAPTER 5 RECOMMENDATIONS FOR FUTURE RESEARCH…….76

References……..77

Appendix……87

LIST OF TABLES

Table 3.1 Primers Designed for qRT-PCR and qPCR Protocols…….43

Table 3.2 qPCR conditions for detection and quantification of fungi using DNA template and ef1-α primers……..47

Table 3.3 RNA quantification for RNA material extracted from fungal spore suspensions of known concentrations (i.e. CFU/mL), Hallen-Adams et. al (2007) extraction protocol (A), Qiagen Rneasy Mini Kit extraction protocol (B)……...52

Table 3.4 DNA Quantification for DNA material extracted from fungal spore suspensions of known concentrations (i.e. CFU/mL), via the Harju et al. (2004) protocol (A), and via QIAGEN Dneasy Plant Kit (B)…….54

Table 3.5 Ct values obtained for the EF1-α qPCR reaction using DNA extracted from live and dead cells of Galactomyces candidus……..57

Table 3.6 Comparison of the developed qPCR method to standard and commercially available rapid fungal detection and quantification methods with DNA extracted from Galactomyces candidus…….62

Table 3.7 Comparison of the developed qPCR method to standard and commercially available rapid fungal detection and quantification methods with DNA extracted from inoculated milk……...63

Table 3.8 Comparison of the developed qPCR method to standard and commercially available rapid fungal detection and quantification methods with DNA extracted from inoculated yogurt……...64

Table 3.9 Comparison of the developed qPCR method to standard and commercially available rapid fungal detection and quantification methods with DNA extracted from inoculated cottage cheese……...65

Table 3.10 Dairy Survey Results with 38 assorted dairy samples……68

LIST OF FIGURES

Figure 2.1 Two Approaches for the Rapid Method Development……....27

Figure 2.2 Positive Control Sample in BAX PCR Assay for Yeasts and Molds……..39

Figure 2.3 Negative Control Sample in BAX PCR Assay for Yeasts and Molds……40

Figure 3.1 Actin (act1) Primers (Primer Set 2) 12.5 ng cDNA template gradient PCR Quantification Curves (A) and Melting Curves (B)……..43

Figure 3.2 Redesigned-Actin (act1) Primers (Primer Set 4) 12.5 ng cDNA template gradient PCR Quantification Curves (A) and Melting Curves (B)……44

Figure 3.3 Elongation Factor 1-α (ef1a) Primers (Primer Set 5) 12.5 ng cDNA template gradient PCR Quantification Curves (A) and Melting Curves (B)……45

Figure 3.4 qRT-PCR Reaction results from *Galactomyces candidus* for Elongation Factor 1-α (ef1-α) primers with cDNA template concentration varying from 0 to 50 ng.

……49

Figure 3.5 Elongation Factor 1-α (ef1-α) primer qPCR results using the Harju et al. (2004) DNA extraction method and different intercalating dyes: Live fungal cells ranging from 10^6 to 10^1 CFU/mL using 300 µL of Reagent D (A), Dead fungal cells ranging from 10^6 to 10^1 CFU/mL using 300 µL of Reagent D (B), Live/Dead fungal cells at 10^6 CFU/mL using 500 µL of methylene blue (C), and Live/Dead fungal cells at 10^6 CFU/mL using 600 µL of Reagent D (D)……56

Figure 3.6 Standard curve determined for the EF1α qPCR using *Galactomyces candidus* as the model organism……..60

INTRODUCTION

Approximately 11% of dairy products available to market are lost at the retail level on an annual basis (Buzby et al. 2014). A majority of these losses can be attributed to microbial spoilage, however the associated incidence or economic costs incurred by losses caused specifically by yeast and mold contamination remains unknown due to commercial confidentiality agreements (Minervini et al. 2001). Developing a method that can determine the viable microorganisms present in any given food sample would be helpful for promoting food safety and food security. This technology would be especially important in dairy foods as their overall consumption is increasing worldwide. The dairy commodities that are most susceptible to fungal spoilage are as follows: conventionally pasteurized milk, yogurt, sour cream, cream, fresh cheeses, and sliced cheeses (Havranek and Hadžiosmanović 1996). Fungi play a significant role in dairy, whether their inclusion is deliberate or not. Yeasts and molds mostly cause organoleptic changes, desirable in some instances, but can pose a threat due to the production of secondary metabolites such as mycotoxins. The objective of this study was to develop a rapid detection and quantification method for fungi in dairy products to cut down on overall testing times on products with limited shelf lives and to maximize the value of the food produced.

Chapter 1. LITERATURE REVIEW

1.1 History of Dairy Consumption

Dairy consumption in humans dates back to approximately 11,000 years ago. Around this time in the Middle East, farming began replacing hunting and gathering. Most of the dairy was not consumed as fluid milk, but rather in the form of either yogurt or cheese (Curry 2013). Fermented dairy products such as yogurt and cheeses were the inadvertent product of several key factors: lack of modern sanitation, lack of temperature control, and limitations in storage technology (Hutkins 2006). The fermentation process was more an inevitable fate that all foods had due to the lack of preservation technology that we have today, an unintended consequence of the environment. The process of fermentation is carried out by microorganisms present in the milk. The microorganisms involved may produce beneficial metabolites or interact with the host/consumer in a positive manner via a probiotic effect (Stanton et al 2005). The fermentation of milk to produce yogurt and cheese allowed for greater consumption of dairy, primarily due to reducing the overall lactose present in the implicated food. There are some artifacts of ancient cheese making technology in Poland. Archaeologist Peter Bogucki discovered pottery dotted with tiny holes in a Stone Age site in central Poland in the 1970s. It had been speculated that these ancient perforated pottery pieces were used to strain cheese (Curry 2013). The same pieces of pottery were analyzed by geochemist Mélanie Roffet-Salque. Roffet-Salque assessed the residual fats that were imbedded into the clay, only to discover that the residues contained milk fats. This discovery provided evidence of early farmers using said pottery pieces to separate fatty milk solids from the whey portions (Roffet-Salque et al. 2013).

Lactose, the disaccharide composed of galactose and glucose, is typically indigestible for humans due to their inability to produce β-galactosidase, commonly referred to as lactase. Humans only recently gained the ability to produce lactase, the enzyme responsible for digesting lactose into galactose and glucose, throughout their lifetimes past childhood. This important genetic mutation allowed livestock agriculture to flourish as opposed to traditional hunting-gathering practices (Curry 2013). Lactase persistence beyond childhood is commonly associated with genetics and the geographical distribution of dairy farming (Scrimshaw and Murray 1988). Globally, there is a significant difference across populations that can or cannot digest lactose during adulthood. The highest rates of lactose tolerance are associated with those of Northern European descent (Bayless and Rosensweig 1966). The prevalence of lactose tolerance into adulthood in European populations could be attributed to positive selection over generations of dairy consumption, the sole source of dietary lactose (Bersaglieri et al. 2004). Symptoms associated with lactose intolerance vary among those afflicted with the condition but commonly can be described as follows: abdominal discomfort, bloating, diarrhea, and flatulence (Wilt et al. 2010).

1.2 Nutritional Significance of Dairy Consumption

Dairy can be used as an indicator of diet quality due to its high nutrient content (Fulgoni et al. 2007). Milk can be broken down into the following components: water, protein, fat,

and carbohydrate (primarily lactose), and ash. The percentages of each component will vary depending on what mammal produces the milk, however the proximate composition of cow's milk is as follows: 87.8% water, 3.3% protein, 3.3% fat, 4.7% carbohydrate (mostly lactose), and 0.7% ash (USDA 2009). Milk and other dairy products are a major source of macronutrients such as carbohydrates, protein, and fat. These commodities contribute on average 134 Calories of energy/capita/day, 8 grams of protein/capita/day, and 7.3 g of fat/capita/day (FAOSTAT 2012). The nutritional contributions of milk and dairy products are based on data collected globally; however, when the data is assessed based on continental regions, those numbers drastically change. Milk contributes approximately 2% of the dietary energy supply in Asia and Africa compared to the 8-9% in Europe, Oceania, and the Americas; 6-7% dietary protein supply in Asia and Africa compared to 19% percent in Europe; and 6-8% dietary fat supply in Asia and Africa compared to the 11-14% percent in Europe, Oceania, and the Americas (FAOSTAT 2012).

The most common milks consumed by humans are produced by cows, buffalo, goats, sheep, and humans (FAO 1972). The most commonly consumed xenobiotic milk by humans is from the cow. In 2010, cow milk alone accounted for 83% of all the milk produced globally for commerce (FAOSTAT 2012). The Holstein-Friesian breed is the most popular due to its high average milk production per head and efficiency of converting feed into protein (Fox 2008; Buchanan 2002). Cow's milk contains more proteins and minerals, particularly calcium and phosphorus, than human milk does. This difference is due to the nutrient requirements of a calf compared to a human baby. A calf

only takes 10 weeks to double its birth weight, compared to the 20 weeks needed for a human baby. Thus, a calf would require more nutrients than a human baby due to its higher rate of growth (Walker 1990).

The protein portion of cow milk is used as a standard in measuring protein quality due to its containing all of the essential amino acids, those amino acids that can only be obtained through dietary intake as they cannot be synthesized by human metabolism (Kanwar et al. 2009). Many human diets are deficient in certain essential amino acids. Wheat- and corn-based diets only contain 57-58% of the required levels of lysine, and cassava-based diets are deficient in leucine, valine, and isoleucine, only containing only 79% of required levels (WHO, FAO, and UNU 2007). Cassava is a staple in African, Asian, and Latin American diets with over 600 million people depending on it for their nutritional needs (FAO 2002). Supplementing staple-based diets with milk or other dairy products would help increase the bioavailability of the essential amino acids, which in turn would help improve overall nutritional and dietary quality.

The primary proteins found in cow's milk are whey and casein (Wijesinha-Bettoni and Burlingame 2013). With a high protein-digestibility-corrected amino acid score (PDCAAS) and the protein fraction being comprised of peptides and other bioactive factors, milk proteins may have specific effects on growth and recovery from undernutrition (Michaelsen et al. 2011). Casein is regularly accepted as a high quality source of amino acids for human growth. Whey has usually been just seen as a low-value byproduct of cheese production until recently (Bulut Solak and Akin 2012). Whey is the soluble portion of milk that is typically separated off from the solids portion

(predominantly casein protein) during the cheese making or casein manufacture operations in the dairy industry. The whey protein can be recovered by using technologies such as ultrafiltration and reverse osmosis to produce protein concentrates. These whey protein concentrates are then used to produce other products such as nutrition bars, powdered beverages, and/or sports meals (Korhonen 2009; Hernández-Ledesma, Ramos, and Gómez-Ruiz 2011). The first observed benefit of reutilizing whey protein is that it provides a good source of amino acids for growth. Other benefits associated with consumption of whey protein are as follows: protection against infection, immune enhancement, development of the gut, and a source of bioactive peptides (Kanwar et al 2009).

Fat is another macronutrient of interest when discussing milk. Milk fat is mostly comprised of triacylglycerols, which accounts for 97-98% of total lipids by weight and consist of fatty acids of varying length (C4 to C24) and levels of saturation. Whole cow's milk contains approximately 1.9 g of saturated fatty acids/ 100 g of milk. Oleic acid, a monounsaturated fatty acid, is the most abundant unsaturated fatty acid present in milk. There are 0.8 g monounsaturated fatty acids/ 100 g of milk. Polyunsaturated fatty acids account for 0.2 g / 100 g of milk (Haug, Høstmark, and Harstad 2007). Health concerns regarding fat intake has been of increased in public interest over time. These health concerns have influenced the development and marketing of modified milk products with varying fat contents such as skim milk or reduced fat milk. However, there is a need to balance reducing fat consumption in developed and high-income countries while increasing fat consumption in developing countries (FAO and WHO 2010).

Carbohydrates are another key macronutrient required in the human diet. The predominant form of carbohydrates in milk and milk products is lactose, a disaccharide which is composed of glucose and galactose. Besides providing energy, lactose and the other saccharides found in milk support growth, aid in the softening of stools, and enhance the absorption of water, sodium, and calcium (Hernández-Ledesma, Ramos, and Gómez-Ruiz 2011).

Cow's milk contains a wide variety of vitamins and minerals. Calcium, phosphorus, and potassium are the most abundant minerals present in milk. Calcium is needed to build and maintain strong bones and teeth, as well as for muscle control, and blood circulation (Kerstetter et al 2003). Phosphorus is a mineral that possesses a large variety of functions in the human body. It is responsible for the following: strong bone and teeth formation, filtering out waste in the kidneys, energy usage and storage, building blocks for DNA and RNA, and for the growth, maintenance, and repair of cells and tissues in the body (Gropper and Smith 2012). Potassium is another versatile mineral that has many roles in the human body. It is essential in maintaining fluid levels in the body, blood pressure regulation, nerve function, and muscle control (He and MacGregor 2008). The most abundant vitamins present in milk are Vitamin A, Vitamin D, Vitamin B12, Vitamin B2, and Vitamin B3 (FAO chapter 3 2013). The vitamin B complex is associated with the aqueous portion of milk, while Vitamin A and Vitamin D associate with the fat portion due to their solubility. Vitamin A is important in the human diet for its role in growth and development, maintenance of the immune system, and vision. Deficiency in Vitamin A can result in blindness and also increases risk of death (Sommer 2008). Vitamin D is

important for its role in bone metabolism and has some anti-inflammatory and immune-modulating properties. Vitamin D regulates calcium absorption in the small intestine and works with parathyroid hormone to mediate skeletal mineralization and maintain serum calcium homeostatis (Kulie et al. 2009). Vitamin B12 is a cofactor for two particular enzymes, methionine synthase and L-methyl-malonyl-coenzyme A mutase. It is necessary for the development and initial myelination of the central nervous system as well as maintenance of normal central nervous system function (Stabler 2013). Vitamin B2, commonly referred to as riboflavin, is an important cofactor in many redox reactions associated with human metabolism in the form of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Powers 2003). Vitamin B3, commonly referred to as niacin, serves as a precursor to coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). These coenzymes are required in many catabolic and anabolic metabolic processes in the human body, as well as cell signaling and DNA repair (Cox, Lehninger, and Nelson 2000).

1.3 Roles of Fungi in Dairy

Fungi are eukaryotic organisms that encompass yeasts and molds. Molds are typically characterized as being multicellular and filamentous, in contrast to how yeasts are unicellular and nonfilamentous. The reproduction of molds is done through the use of spores, whether they are asexual or sexual. Yeasts can only reproduce via sexual spores or through non-sporulation methods such as budding. The presence of fungi in many food systems, including dairy, is usually spoilage related. However, there are instances in

which the inclusion of fungal organisms is intentional for the production of particular products such as some mold ripened cheeses as well as yeast fermented beverages. Fermentation can be viewed as a controlled spoilage process, where microorganisms thrive in a food matrix of interest (i.e. milk, cheeses, etc.) to produce desirable metabolites that contribute to enhanced functionality, preservation, nutritional value, organoleptic properties, uniqueness, and economic value. The primary fungal cultures used for the production of fermented dairy products include *Penicillium roqueforti*, *Penicillium camemberti*, and *Saccharomyces kefyr* (Hutkins 2006).

Penicillium roqueforti is the mold responsible for blue veined mold ripened cheeses. Extensive proteolysis occurs through the production of extracellular proteinases, endopeptidases, and exopeptidases via the mold's metabolic processes. The resulting amino acids can then be further processed by various deaminases and decarboxylases that will release amines, ammonia, and other compounds that could possibly contribute to flavor. The flavor compounds that are typically associated with blue cheese are the end products of lipid metabolism. The *Penicillium roqueforti* mold produces lipases which will hydrolyze the triacyglycerides present in the milk that will eventually become short chain, volatile fatty acids such as butyric and caproic acids. The metabolism of free fatty acids via β-oxidation reactions will yield a variety of methylketones, the characteristic flavor and aroma compounds associated with blue cheese (Hutkins 2006).

Brie and camembert cheese are produced with *Penicillium camemberti*. This mold only grows on the surface of these particular cheeses, but similar proteolysis and lipolysis events happen as with blue mold cheeses. Further metabolism of the amino acids leads to

the formation of ammonia, methanethiol, and other sulfur compounds that are derived from sulfur-containing amino acids (Hutkins 2006).

Kefir is a fermented dairy product that is commonly consumed in the Middle East, Eastern Europe, and Central Asia. It is traditionally produced with kefir grains, which carry mixed populations of bacteria and yeasts. In the instances where yeasts are present, kefir can contain as much as 2% ethanol due to the fermentation of lactose. *Saccharomyces kefyr* can be used for the manufacture of kefir, but is not necessary (Hutkins 2006). High throughput sequencing shows that there is a diverse population of fungal genera present in kefir. The genera that have been found in kefir include *Saccharomyces*, *Candida*, *Kluyveromyces*, *Mycosphaerella*, *Mycoderma*, *Issatchenkia*, *Brettanomyces*, *Aspergillus*, *Fusarium*, *Pichia*, *Hansenula*, *Torula*, *Torulopsis*, *Malassezia*, *Dekkera*, *Penicillium*, *Kimbropezia*, *Bullera*, *Cryptococcus*, *Ganoderma*, *Heterobasidion*, *Teraosphaeria*, *Wallemia*, and *Zygosaccharomyces* (Marsh et al. 2013).

Spoilage of a given food-stuff could be defined as the result of undesirable changes caused by the growth and development of microorganisms. Fresh milk, which has a near neutral pH due to it mostly being water, is more likely to be affected with bacterial than fungal spoilage. Any bacteria present in this particular matrix will outcompete any fungi trying to grow at the same conditions. The conditions that encourage the growth of yeasts and molds in dairy products include low temperature, low pH, aerobic, and high carbohydrate content (Mayoral et. al 2005). Fungal spoilage causes many undesirable organoleptic changes such as: gas production, off-flavors, off-odors, proteolysis, and lipolysis (Horwood et al. 1987; Vivier et al. 1994); Maraz and Kovacs, 2014).

Yeasts can cause gas and off flavor production in cream and cottage cheese and rancidity or other flavor defects in butter (Walker and Ayres 1970; Frölich-Wyder 2003). *Geotrichum candidum* can cause spoilage in cream due to improper cleaning practices on the farm (Marth 1978; Craven et al. 2001). Yeasts such as *Pichia anomala* can thrive in yogurts, especially varieties that contain fruit or flavored syrups (Foschino et al. 1993). Some yeasts are included as starters in the manufacture of mold ripened cheeses for the development of texture utilizing their proteolytic and lipolytic capabilities (Pitt and Hocking 2009). *Geotrichum candidum* is typically included as part of the smear of surface ripened cheeses (Marcellino and Benson 1992). However, it can also be a spoilage organism in other varieties of cheeses (Gueguen 1988). *Debaryomyces hansenii*, *Kluyveromyces* species, *Saccharomyces cerevisiae* and *Candida* species are common on the surface of St. Nectaire, Camembert, and blue-veined cheeses and could play a role in texture and flavor development of these products (Addis et al. 2001).

Cheeses are very susceptible to mold spoilage and are typically kept at refrigeration temperatures. Retail packs are packed under vacuum or flushed with gas. These conditions will deter mold growth, except for molds that are psychrotolerant and can grow with relatively low oxygen. A common mold growth problem occurs during the maturation process of Australasian cheddar cheeses; this phenomenon is typically referred to as thread mold. Thread mold is caused by the growth of fungi in the folds and wrinkles of plastic film used in the packaging of the implicated cheeses (Hocking and Faedo 1992). Thread mold poses a serious threat to cheese manufacturers using a continuous forming system such as the Wincanton Tower (Pitt and Hocking 2009).

Cream cheese can be affected by the presence of heat resistant molds. *Byssochlamys nivea* forms very heat resistant ascospores that can survive the pasteurization process (Engel and Teuber 1991). The ascospores can germinate if the product is stored for prolonged periods of time or if it was inadequately cooled before storage. Some molds can grow in cheeses that contain mold inhibitors such as sorbate. *Penicillium* species, particularly *Penicillium roqueforti*, are capable of forming trans-1,3-pentadiene, causing a kerosene flavor defect, via decarboxylation of sorbate (Sensidoni et al. 1994). These *Penicillium* molds can also reduce sorbic acid into 4-hexanol and 4-hexanoic acid (Kinderlere and Hatton 1990).

Another concern regarding fungal growth in dairy products is the risk of mycotoxin production. *Penicillium roqueforti* can produce roquefortine and PR toxin. PR-imine was found in 50 of 60 samples of blue-vein cheese, but PR toxin was not found (Siemens and Zawistowski 1993). Roquefortine has been isolated in cheeses at varying levels ranging from 0.8 to 12 mg roquefortine /kg sample (Kokkonen et al 2005b). Another study had lower values detected ranging 0.08-1.47 mg roquefortine/kg sample (Finoli et al. 2001). Mycophenolic acid was isolated from moldy Manchego cheese (López-Dias et al. 1996). Ochratoxin A was reported in blue mold cheeses ranging from 0.25 to 3.0 µg/kg (Dall'Asta et al. 2008). Cyclopiazonic acid was detected in six samples of Italian Taleggio, but only confined to the rind of the cheese (Finoli et al 1999). Sterigmatocystin, which is produced by *Aspergillus versicolor*, was detected in the surface layer of hard cheeses in the Netherlands (Northolt et al. 1980). Despite the detection of these

mycotoxins in all of these varieties of cheeses, these levels were not considered to be of public health significance (Pitt and Hocking 2009).

Several genera of fungi were isolated from cheeses in a 2012-2013 survey, including, but not limited to: *Debaryomyces*, *Galactomyces*, *Yarrowia*, *Cladosporium*, *Aspergillus*, *Penicillium*, *Eurotium*, *Candida*, *Pichia*, and *Kluyveromyces* (Banjara et al. 2015). The most common fungi associated with the spoilage of dairy products belong to the following genera: *Cladosporium*, *Penicillium*, *Phoma*, *Candida*, *Yarrowia*, *Mucor*, *Geotrichum*, *Kluyveromyces*, *Rhodotorula*, *Pichia*, and *Moniliella* (Pitt and Hocking 2009)

Approximately 11% of dairy products available to market are lost at the retail level on an annual basis (Buzby et al. 2014). A majority of these losses can be attributed to microbial spoilage, however the associated incidence or economic costs incurred by losses caused specifically by yeast and mold contamination remains unknown due to commercial confidentiality agreements (Minervini et al. 2001). Commodities that are most susceptible to fungal spoilage are as follows: conventionally pasteurized milk, yogurt, sour cream, cream, fresh cheeses, and sliced cheeses (Havranek and Hadžiosmanović 1996). Fungi play a significant role in dairy, whether their inclusion is deliberate or not. When spoilage organisms are allowed to run rampant, food goes to waste. Based on the viability of yeasts and molds in these products, it is important that good manufacturing practices are followed.

1.4 Quality and Shelf Life of Dairy Products

Dairy products harbor a variety of microorganisms; most of the ones of concern are bacterial (FAO Milk and Dairy Chapter 6). Most dairy products are produced using milk that has gone through the pasteurization process, which controls a large majority of the bacteria of interest. The processing temperature and time of a typical batch pasteurization process is 63°C for 30 minutes. The parameters for pasteurization of milk are based on killing *Coxiella burnetii*, the most heat tolerant milk pathogen. The quality of Grade "A" pasteurized milk is as follows: bacterial counts are not to exceed 20,000 per mL or per g, coliform counts not to exceed 10 per mL, less than 350 milliunits/L and no positive results for drug residues (Grade "A" Pasteurized Milk Ordinance 2015). The Compendium of Methods for the Microbiological Examination of Foods shows that many dairy products should have standard counts of less than 10 CFU yeast and molds/g of product (Richter and Vedamuthu 2001)

Due to the limitations of current methods for detection and quantification, products are subject to retention for approximately 72-120 hours (3-5 days) before they can be released to market. For some products, this holding time is a significant portion of their shelf lives. The average shelf life of pasteurized fluid milk is anywhere from 10-14 days post-pasteurization. If the product is stored at 4°C or less, the product can last up to seven days past the posted "expiration" date. Sour cream, yogurt, and cultured buttermilk should have a shelf life of at least 30 days if the equipment and processing environment are maintained as specified in a producer's GMPs (Havranek and Hadžiosmanović 1996). Dairy products are considered to be "Ready to Eat" (RTE) products, foodstuffs that will

be directly consumed without any additional cooking and processing (Health Protection Agency 2009). As such, dairy products need to be processed with the utmost care and follow Good Hygienic Practices (GHPs) and GMPs. The greatest fungal safety concern for dairy products is the instance of mycotoxin being present in milk. Growth of *Aspergillus flavus* and *Aspergillus paraciticus* can lead to the production of Aflatoxin M_1, which is metabolically converted from the typically produced Aflatoxin B_1 by lactating mammals and secreted in the milk. Aflatoxin M_1 is a genotoxic carcinogen which is a significant risk to human health even at low concentrations (IARC 1993). The maximum limit for Aflatoxin M_1 in milk is 0.5 µg/ kg (FAO and WHO 1995a).

1.5 Fungal Detection and Quantification Methods

1.5.1 Currently Used Direct Counting Methods

Direct microscopic counting can be used to directly quantify the amount of yeast that is present in a given beverage sample with the ability to distinguish live and dead cells. The standard materials needed for this procedure are: Millipore disk filter holders for standard syringes, Millipore filters: AABG, 0.8 µm, black gridded; 25 mm diameter, disposable syringes, pipets, forceps, bibulous paper, microscope slides with 24 x 24 mm coverslips, and a fluorescence microscope with blue excitation capability, 10X eyepieces with Howard mold count or other eyepiece grid; 20x or 40x objective. Reagents used in this procedure are aniline blue (1% in 11.6 g/L K_2HPO_4, adjusted to pH 8.9 with K_3PO_4) and NaOH (25 g in 100 mL H_2O). Filter 10 mL of milk sample through the Millipore filter.

Use the Millipore disk filter holder that can attach to a standard syringe. The syringe should then be attached to the filter holder and pipette 10 mL of sample into the syringe. Press all of the sample through the filter, maintaining an air cushion of about 3 mL between the plunger and the sample. Remove the filter from the holder and place on a microscope slide, in such a way that the grids are parallel to the edges of the slide. Cover the filter with a drop of aniline blue solution and spread it over the filter with a glass rod or cover without touching the filter itself. Wait for 5 minutes before putting a coverslip over the filtered sample. Count the yeasts using the blue excitation microscope and count 3 squares of eyepiece grid in each field of filter not covered by gasket. Budding yeasts that have approximately equal-sized mother and daughter cells should be counted as two cells, differing sizes should only be counted as one cell. Count all yeasts located completely within an eyepiece square and all yeasts touching left and lower border of eyepiece square; do not count the yeasts touching the right and upper borders of the grid. The differentiation of live and dead cells is determined by the fluorescence observed. Dead cells will be more uniform in their fluorescence and be more granular than live cells. The following equation can be used to determine the yeast content of a given sample: $\frac{Number\ of\ yeasts\ counted}{Number\ of\ regions\ counted} \times \frac{working\ area\ of\ filter}{area\ of\ one\ region} \times \frac{1}{volume\ of\ liquid} = \frac{Number\ of\ yeasts}{mL}$ (Bacteriological Analytical Manual Chapter 18 2001).

1.5.2 Currently Used Plating Methods

Dilution plating can be used to determine the fungal load of a given food sample. The media that are typically used for the enumeration of fungi are Dichloran Rose Bengal

Chloramphenicol (DRBC) agar, Dichloran 18% Glycerol (DG18) agar, Plate Count agar (PCA) with an addition of 100 mg chloramphenicol/ liter of media, Malt agar (MA), Malt extract agar for yeasts and molds (MEAYM), and Potato dextrose agar (PDA). Many of these media contain antibiotics or can have antibiotics added to them to help reduce the bacterial populations present in a given sample of interest. Samples can either be prepared using a spread plate method or a pour plate method. Spread plate samples should be aseptically pipetted onto pre-poured solid DRBC agar plates and then the inoculum should be spread with a sterile rod spreader. The pour plate method can assess one mL portions of a given sample dilution. Place one mL of sample into a pre-labeled petri dish then add 20-25 mL of DG18 agar tempered to 45°C. Mix the contents by swirling the plates clockwise, then counterclockwise. Regardless of which plating method is used, the plates should be incubated at 25°C in the dark without inverting the plates. Plates should be counted after 5 days (120 hours) of incubation, if no growth is observed at 5 days, re-incubate for 48 hours. Plates containing 10-150 colonies should be counted. Yeasts can be counted up to 150 counts on a single plate without much difficulty; molds, however, might need to have a readjusted upper countable limit due to their colony size. Counts should be reported in terms of colony forming units (CFU)/g or CFU/mL based on the average counts on the replicate plates. The counts on these plates should be rounded to 2 significant figures. When no counts are observed, the reported yeast and mold count for that sample will be less than 1 times the lowest dilution used (Bacteriological Analytical Manual Chapter 18 2001).

1.5.3 Currently Used "Rapid" Methods

Several methods have been developed to reduce the overall time from sample to results. A rapid plating technology that can be used for determination of fungal counts in 48 +/- 2 hours is the 3M™ Petrifilm™ Rapid Yeast and Mold Count plate. This assay is an embedded culture medium system that includes antibiotics, a cold-water soluble gelling agent, and an indicator system for the enumeration of yeasts and molds. Yeasts will appear as small colonies with defined edges that appear to be three dimensional and have a uniform color ranging from pink-tan to blue-green. Molds will appear as large blue-green colonies with diffuse edges and a dark center. To inoculate the Petrifilm™, place the assay on a flat level surface, then lift the top film. Pipette 1 mL of sample onto the center of the bottom film, then replace the top film onto the sample. Use a flat spreader on the center of the film, press firmly on the center of the spreader to spread the inoculum evenly across the film. Remove the spreader and allow the plate to form a gel (approximately 1 minute). Plates will then incubate for 48 hours at 25°C. Yeast and mold results can be read and interpreted at that time, with an optional and additional incubation for 12 more hours to increase resolution. (3M™ Petrifilm™. 2013. Rapid Yeast and Mold Count Plates).

A molecular method in the form of the DuPont BAX system and its PCR assay for yeasts and molds can be used to get results in two days for enriched samples and same day results for direct testing samples. This method is based on the amplification of fungal DNA present in a sample. Samples can be enriched via the use of the PCR Assay for Yeast and Mold Supplement kit, with sample inoculum depending on the desired action

level. These enriched samples will incubate at 25°C for 44 hours prior to adding a DNA stabilizer, which is included in the supplement kit, and then homogenized prior to BAX PCR preparation. The BAX PCR assay utilizes a protease and lysis buffer that will help release any potential DNA in a given sample. Two heating steps are carried out on the samples in lysis buffer and protease. The first step takes place at 37°C for 20 minutes. The second step takes place at 95°C for 10 minutes. After the heating steps, the samples are placed into a cooling block for 5 minutes. 50 µL of the sample is added to PCR tubes in a cooling block, then resealed with optical caps. The sealed PCR tubes are loaded into the BAX cycler and the process begins. The assay's PCR takes about 3.5 hours before the results (positive, negative, or indeterminate) can be observed (DuPont Nutrition and Health. 2013. BAX System PCR Assay for Yeast and Mold.)

1.5.4 Development of "Novel" Technologies via Molecular Methods

Molecular assays, PCR in particular, have seen increased usage due to their specificity and sensitivity (Mayoral et al. 2006). These methods are predominantly used for the detection of pathogenic bacteria in food products, however this technology could be possibly used for the detection of fungi in food matrices (García et al. 2003). Traditional PCRs are visualized by gel electrophoresis and cannot provide quantitative data that could be used to approximate fungal load. However, the utilization of a quantitative PCR (qPCR), also referred to as real-time PCR, can possibly achieve this goal. Real time PCRs allow for amplification of a product to be observed throughout the course of an entire PCR via measurements of fluorescence values. Another possibility is the usage of

quantitative reverse transcriptase PCR (RT-qPCR) (Vaitilingom et al. 1998; Bleve et al. 2003). Reverse transcriptase PCR relies on extracting RNA that is then transcribed back into DNA, then amplified in a PCR reaction. The result of this process would demonstrate the expression of a gene of interest (Mayoral et al. 2005). These methodologies have been tested in a variety of food matrices including yogurt, cheese and milk (Vaitilingom et al. 1998; Bleve et al. 2003; Mayoral et al. 2006).

1.5.5 DNA vs RNA

DNA is the genetic basis of all living organisms and is present throughout an organism's life, even after death. DNA codes for RNA, which corresponds to how genes are expressed and code for specific proteins that assist in cell function. The idea that DNA codes for RNA which codes protein is often referred to as the "central dogma" of genetics. DNA is the standard nucleic acid used in most PCR reactions, however the use of RNA has been increasing since the development of reverse transcriptase PCR. RNA is very unstable in comparison to DNA and quickly degrades after cell death. Thus, RNA is a better viability marker than DNA, but is also more difficult to work with due to its increased sensitivity as well as inefficient RNA extraction protocols (Deepak et al. 2007). A streamlined DNA extraction protocol allows for rapid extraction of DNA from fungi (Harju et al. 2004). However, an issue with using DNA is the lack of differentiation of live and dead cells. The use of an intercalating dye such as BIOTECON's Reagent D can bind the DNA present in dead cells to eliminate potential PCR signals from the genetic material of dead cells (BIOTECON Diagnostics GmbH 2016).

Summary

The development of a rapid and cost-effective alternative fungal detection and quantification assay would help reduce the holding time needed for limited shelf life dairy products, which would increase their market availability. This alternative method could be done through the use of a reverse transcriptase quantitative PCR (RT-qPCR) or an ordinary quantitative PCR (qPCR). The detection of fungi that are potentially present in milk could be achieved through the use of specific primers designed using fungal housekeeping genes. Quantification of potentially present fungi could be accomplished by establishing correlations with fungal plate counts and measuring the threshold cycles associated with particular levels of inoculum. The proposed method seeks to reduce the time necessary for testing of dairy products, in order to make them more readily available for retailers and consumers.

Objectives

The goal of this research is to develop a rapid detection method for the quantification of fungi potentially present in a dairy matrix that can produce comparable results to validated methods currently used in the dairy industry. The objectives are as follows:

Objective 1: Development of specific primers for fungi commonly associated with dairy spoilage

Objective 2: Development of a protocol for quantification of viable yeasts and molds in dairy

Objective 3: Comparison of the developed PCR protocol in dairy matrices to traditionally used fungal detection methods (plate counting on DRBC agar) and commercially available rapid methods (3m Rapid Yeast Mold Plate Count Petrifilm, Hygiena Qualicon BAX PCR assay for Yeast and Mold, and BIOTECON Yeast and Mold Quantification Lyokit).

CHAPTER 2. MATERIALS AND METHODS

2.1 Fungal Cultures

The eight fungal strains used in this project were obtained from the Agricultural Research Service Culture Collection (NRRL, Peoria, IL, USA). The following strains were used: NRRL, *Debaryomyces hansenii* Y-1458; NRRL, *Galactomyces candidus*YB-223; NRRL, *Yarrowia lipolytica* Y-7751; NRRL, *Kluyveromyces marxianus* Y-876; NRRL, *Penicillium camembertii* 874; NRRL *Penicillium verrucosum* 5574; NRRL, *Penicillium roqueforti* 849; and NRRL, *Cladosporium cladosporioides* 3182. The lyophilized yeast and mold cultures obtained from the ARS culture collection were grown in yeast malt broth (YM Broth, Acumedia, Lansing, MI, USA) and potato dextrose broth (PD Broth, Acumedia, Lansing, MI, USA) for 48 and 96 hours at 25°C, respectively. An additional incubation was performed on culture transfers using the same conditions depending on the classification of the fungi (yeast or mold). Cultures were then stored in 15% glycerol at -80°C.

For each experiment, fungal cultures were thawed at room temperature, grown individually on potato dextrose agar (PDA, Acumedia, Lansing, MI, USA and BD Difco, Sparks, MD, USA) or in potato dextrose broth (PD Broth, Acumedia, Lansing, MI, USA) and incubated at 25°C for 72-120 hours. Cultures grown on agar plates had their biomass collected with a sterile loop and were transferred to 10 mL of sterile Butterfield's phosphate-buffered dilution water (stock was prepared using Potassium Phosphate, VWR, Radnor, PA, USA). Cultures grown in broth were centrifuged for 10 minutes at 4°C, 5,000x*g* (Model Sorvall ST 16R; Thermo Scientific, Langenselbold, Germany). The

resulting supernatant was discarded and the cells in the pellet were re-suspended in 10 mL of sterile Butterfield's phosphate-buffered dilution water.

2.2 Fungal Plate Count Method

As determined by different experiments, suspensions of fungal cells were serially diluted and then 100 µL of any given suspension were aseptically plated on pre-poured dichloran rose bengal chloramphenicol agar (DRBC agar , Acumedia, Lansing, MI, USA and BD Difco, Sparks, MD, USA). The fungal suspensions on the DRBC agar plates were then spread with a sterile L-shaped spreader. The DRBC plates were incubated without inversion at 25°C for 120 hours (5 days). Yeasts were counted up to 150 colonies on a single plate without much difficulty; molds, however, had a readjusted upper countable limit due to their colony size. Counts were reported in terms of colony forming units CFU/g or CFU/mL based on the average colony counts on the replicate plates. Final counts were rounded to 2 significant figures. When no counts were observed, the reported yeast and mold count for that sample was less than 1 times the lowest dilution used, and usually were < 10 CFU/mL.

2.3 Primer Design for qRT-PCR and qPCR Protocols

Primers for the amplification and detection of genetic material from dairy associated yeasts and molds were designed by aligning different gene sequences indicative of viability of those organisms. Four particular targets were selected for primer design: actin (act1), β-

tubulin (TUBB5), 18S rRNA, and elongation factor 1-alpha (EF1α). Sequences for the genes of interest were obtained from the GenBank/NCBI Nucleotide databases (National Center for Biotechnology Information, Bethesda, MD, USA). Gene sequences were obtained from four yeasts and molds that are prevalent in dairy products: *Galactomyces candidus*, *Yarrowia lipolytica*, *Penicillium roqueforti*, and *Cladosporium cladosporioides*. These sequences were compared and aligned by MUSCLE using MEGA6 software (Molecular Evolutionary Genetics Analysis Version 6.0). Consensus sequences for each gene were generated and primers were designed using various software and ordered from IDT (Integrated DNA Technologies, www.idtdna.com).

Primer evaluation was conducted to determine the following conditions: ability to amplify genetic material from fungal species associated with dairy, primer concentration, annealing temperature, and limit of detection/quantification The fungal species used for the evaluation were the following: *Galactomyces candidus*, *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Penicillium roqueforti*, *Penicillium verrocosum*, and *Cladosporium cladosporioides*. The optimal annealing temperature was determined through the use of gradient PCRs that had annealing temperatures ranging from 47°C to 57°C. Primers used for the PCR reactions were tested at the following volumes: 0.5 µL, 1.0 µL, 1.5 µL, and 2.0 µL. Each primer (forward and reverse) was used in a 1:1 proportion. The limit of detection and limit of quantification for the primers was determined by using set parameters and varying nucleic acid template amounts. After primer evaluation, the same process described was followed once again with only *Galactomyces candidus, Kluyveromyces marxianus,* and *Debaryomyces hansenii* to increase the specificity of the developed primers.

2.4 Rapid Method Development for the Quantification of Yeast and Mold

Two approaches were considered for the development of the rapid detection and quantification method for fungi. Figure 2.1 illustrates the two approaches used for the development of the rapid method.

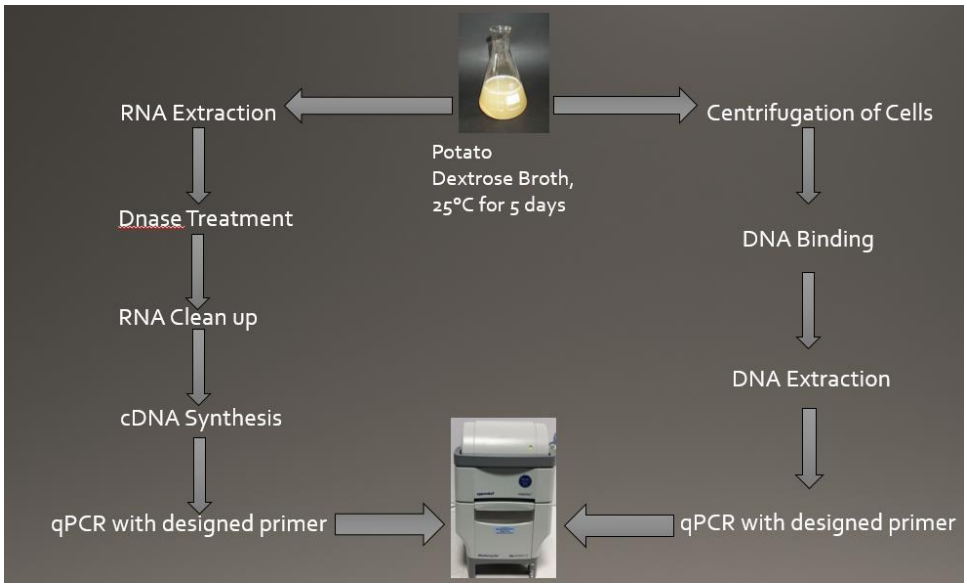


Figure 2.1 Two Approaches for the Rapid Method Development

The first approach considered was the use of RNA for detection and quantification from viable fungi. This RNA-based approach utilized several RNA extraction protocols: a Trizol reagent extraction described in Hallen et al. (2007), an extraction from a commercial kit (Qiagen Rneasy Mini Kit), and two modified extractions based on the Hallen protocol. The two modified protocols based on Hallen (2007) were performed with the following changes: 1) no Dnase treatment or RNA cleanup step, and 2) a hot phenol extraction to replace the Trizol reagent extraction. All extracted RNA samples

were converted to cDNA via reverse transcription prior to qPCR with the primers designed in 2.3.

The second approach considered for the rapid method development was the use of DNA. This DNA-based approach utilized three different DNA binding agents: methylene blue, propidium monoazide, and Reagent D (BIOTECON Diagnostics, Potsdam, Germany). Inclusion of the binding agents was intended to limit or remove the amplification of DNA from dead cells. Following the DNA binding step, several DNA extraction protocols were utilized: an extraction protocol as described by Harju et al. (2004), two modified variants of the Harju protocol, an extraction protocol from a commercial kit (Qiagen DNeasy Plant Kit), and a modified extraction procedure using the commercial kit. The two modified Harju DNA extraction protocols included the following additional treatments: 1) an additional heating step (5 minutes at 95°C) prior to lysis via cell disruptor and 2) a sonication step prior to lysis via cell disruptor. The modified commercial kit extraction protocol included a 5 minute incubation step, 2 minute bead beating step, and 5 minute incubation on ice prior to running the samples according to the manufacturer's protocol.

2.4.1 RNA Extraction from Fungi

Initially, the method described by Hallen et al. (2007), was used for the RNA extraction from fungi and that method is detailed here. For the RNA extraction as described by Hallen-Adams (2007), fungal cell suspensions were prepared as specified in 2.1, subsequently 1 mL of fungal cells were added to a baked ceramic mortar (baked at 200°

for 8 hours to destroy Rnase), along with 1 mL of Trizol reagent (Ambion, Life Technologies, Carlsbad, CA, USA). The samples were ground with a baked ceramic pestle. An aliquot of 1 mL of the resulting mixture was transferred to a micro-centrifuge tube. The samples were then incubated at room temperature for 5 minutes. Then, 200 µL of chloroform was added to each tube prior to incubation at room temperature for 3 minutes. Samples were centrifuged in a micro-centrifuge for 15 minutes at room temperature, 12,000x*g* (Eppendorf, Hamburg, Germany). After this centrifugation step, the aqueous portion was transferred to a new micro-centrifuge tube. To each tube 500 µL of "pine tree" CTAB (2% CTAB, 2% PVP K-30, 100 mM Tris-HCl pH 8.0, 25 mM EDTA, 2M NaCl, 0.05 mg/mL spermidine) was added to the aqueous portion and then incubated for 25 minutes at 65°C on a heating block (VWR, Radnor, PA, USA). After this incubation step, the tubes were filled with 24:1 chloroform: isoamyl alcohol and centrifuged for 10 minutes at room temperature, 12,000 rpm. The aqueous layer was collected and placed into a new micro-centrifuge tube. The new tubes were then filled with 500 µL 24:1 chloroform: isoamyl alcohol, then the micro-centrifuge tubes were respun for 10 minutes at room temperature, 12,000 rpm. The aqueous layer was transferred to a new micro-centrifuge tube. After the collection, 100 µL of 3M sodium acetate (NaOAC) and 500 µL isopropanol were added to the aqueous portion of the samples. Samples were stored for at least 10 minutes at -20°C for the precipitation of RNA (ribonucleic acid). Upon removal from incubation at -20°C, sample tubes were centrifuged for 10 minutes at room temperature, 12,000x*g*. The supernatant was removed from each tube and 1 mL of 75% ethanol was added. The tubes were then centrifuged for 5 minutes at room temperature, 7,500x*g*. The supernatant was removed and 20 µL of

Rnase-free water was added to each sample. The tubes were then incubated for 15 to 20 minutes at 65°C on a heating block to evaporate off excess ethanol from the previous step. RNA was quantified using a biophotometer (Eppendorf, Hamburg, Germany). After the initial quantification of RNA in samples, 100 µg of RNA is added to Rnase-free water for a total volume of 100 µL. 10 µL of 10x incubation buffer and 20 µL of 1 U/µL Dnase 1 (OPTIZYME Dnase 1, Fisher Scientific, Fair Lawn, NJ, USA) were added to the RNA solutions, followed by incubation for 15 minutes at 37°C. After the incubation, 4 µL of 0.2 M EDTA was added to each tube of RNA and then incubated once again for 10 minutes at 75°C. Dnase-treated RNA samples were cleaned up using a QIAGEN Rneasy mini kit as per the manufacturer's specifications (Rneasy Mini Kit, QIAGEN, Hilden, Germany). After the RNA cleanup was performed, RNA samples were quantified via biophotometer or a fluorometer (Qubit 2.0 Fluorometer, Invitrogen, Carlsbad, CA, USA) and stored at -80°C before reverse transcription and the quantitative PCR were performed.

Three other protocols were considered to obtain RNA from fungi. One RNA extraction procedure was a commercial kit from Qiagen (Qiagen RNeasy Mini kit). The extraction was divided into two distinct processes: an extraction step and a cleanup step. The manufacturer's instructions for the extraction and cleanup were followed without any deviations. The second RNA extraction procedure considered for method development was the previously described Hallen (2007) procedure without the Dnase treatment and RNA cleanup steps being performed. The exclusion of these two steps was considered to reduce the overall time needed to perform this protocol to make it more amenable to

same day testing (sample to results within 24 hours). The final RNA extraction procedure considered was a hot phenol extraction from Goswami et al. (2006). Fungal cell suspensions are centrifuged for 5 minutes at room temperature, 10,000 rpm. The supernatant was removed and the resulting pellet was resuspended in 1 mL of a heated (80° C) 1:1 mixture of extraction buffer (Tris-LiCl-EDTA-SDS): phenol. The resuspended pellet was transferred into a baked ceramic mortar and had an additional 1 mL of the heated 1:1 extraction buffer: phenol mixture added. The samples were ground with a baked ceramic pestle before they were decanted into a 5 mL tube. Then, 1 mL of chloroform was added to the tube and subsequently vortexed. The samples were spun for 30 minutes at room temperature, 2,500x*g*. The resulting aqueous layer was transferred to a new tube, had 300 µL of 8M LiCl added to it, and were incubated on ice for 2 hours. After the incubation on ice, the samples were spun for 30 minutes at 4°C, 12,000x*g*. The resulting supernatant was removed and the resulting pellet was washed with 3 mL of 2M LiCl. Samples were spun for 5 minutes at room temperature, 12,000x*g*. The samples had their resulting supernatants removed and the pellet was washed with 3 mL of 70% ethanol prior to being spun for 5 minutes at room temperature, 12,000x*g*. The supernatant was removed and the pellet was resuspended in 2 mL of DEPC water, 200 µL of 3M NaOAc, and 5.5 mL of 95% ethanol prior to incubation for 15 minutes at -80°C. After the incubation step, the samples were spun for 5 minutes at room temperature, 12,000x*g*. The supernatant was removed and the pellet was washed with 3 mL of 70% ethanol, before being spun for 5 minutes at room temperature, 12,000x*g*. The supernatant was discarded and the remaining pellet was dissolved into 25 µL of DEPC water. The dissolved pellet was transferred into a 1.5 mL micro-centrifuge tube and incubated at 65°C to evaporate

any excess ethanol from previous steps. RNA was quantified via biophotometer prior to Dnase treatment and RNA cleanup steps described in the original Hallen- (2007) protocol were performed without alterations. In each of the alternative RNA extraction methods, resulting RNA samples were quantified via biophotometer and stored at -80°C prior to the performance of reverse transcription and quantitative PCR.

2.4.2 DNA Extraction from Viable Fungi

Several strategies for DNA extraction were attempted for the rapid method development. These DNA extraction protocols included the following: 1) Bust n'Grab protocol from Harju et al. (2004), 2) two modified variants of the Harju protocol, 3) an extraction protocol using a commercial kit (Qiagen DNeasy Plant Kit), and 4) a modified protocol using the commercial kit. The DNA extraction protocol detailed in Harju et al. (2004) is a rapid extraction method that utilizes lysis buffer, cell disruption, freeze-thaw incubations, chloroform, ethanol precipitation, centrifugation, and suspension of DNA in TE buffer. The two modified variants of the Harju method performed were: 1) the use of an additional incubation for 5 minutes at 95°C prior to cell disruption and 2) sonication of the samples for 2 minutes prior to cell disruption. The commercial kit was used for two alternative DNA extraction protocols. The first protocol follows the manufacturer's instruction without any modifications. The second protocol using the commercial kit included a 5 minute incubation step at 95°C, a 2 minute cell disruption step, and a 5 minute incubation on ice prior to Rnase treatment. After the Rnase treatment was performed, the subsequent steps that followed were performed as per the manufacturer's

instructions. All of the DNA samples were then quantified via biophotometer and stored at -20°C prior to quantitative PCR. Based on the DNA concentration measured by biophotometer, the Harju protocol without modifications was chosen for all future DNA extractions due to its better performance compared to the other methods that were considered.

Several DNA binding agents were considered due to interest in only amplifying the DNA from viable cells. Three particular reagents were tested for their ability to bind DNA: propidium monoazide (Nocker et al. 2007), methylene blue (Nafisi et al. 2006), and Reagent D (Martinon et al. 2012). The DNA binding step was added prior to the cell lysis step of the original Harju protocol to allow for the dyes to intercalate into DNA obtained from membrane-compromised dead cells. When using the propidium monoazide dye, 1.25 µL of a 20mM stock solution was added to pelleted fungal culture, incubated in the dark at room temperature for 5 minutes, and exposed to light from a halogen lamp for 15 minutes. For binding with methylene blue, 300 µL of a 3 mM stock solution was added to pelleted fungal culture, incubated in the dark at room temperature for 10 minutes, and exposed to light from a halogen lamp for 5 minutes. The final reagent tested for the binding of DNA was Reagent D; 300 µL was added to pelleted fungal culture, incubated in the dark at room temperature for 10 minutes, and exposed to light from a halogen lamp for 5 minutes. Each of these binding steps were followed with a centrifugation step for 5 minutes at room temperature, 8,000xg, and then the reagents were removed prior to the addition of lysis buffer and performing all of the subsequent steps in the original Harju protocol without further modification. Based on the testing, Reagent D was chosen as the

binding agent due to its ability to correlate qPCR Ct values and fungal plate counts on DRBC agar.

The Harju (2004) protocol for DNA extraction and the Reagent D binding agent was chosen for the extraction of DNA from viable fungi for the rapid method development. Fungal cell suspensions were prepared as specified in 2.1. After fungal suspensions were prepared, a volume of 1.5 mL was transferred into a micro-centrifuge tube containing 0.3 g of glass beads. Cells were pelleted via centrifugation for 5 minutes at room temperature, 20,000xg. The supernatant was removed and 300 µL of Reagent D (BIOTECON Diagnostics, Potsdam, Germany) was added. The re-suspended cells in Reagent D were then incubated for 10 minutes at room temperature and in the dark. The samples were then subjected to a high-power halogen light bulb for 5 minutes to inactivate the Reagent D. After light exposure, samples were centrifuged for 5 minutes at room temperature, 8,000xg. The supernatant was removed after the centrifugation step. Then, 200 µL of lysis buffer (2% Triton x-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, and 1 mM EDTA pH 8.0) was added to the pellets and vortexed. This addition of lysis buffer was immediately followed with a 2 minute disruption step (Disruptor Genie, Scientific Industries, Bohemia, NY, USA). Following the disruption step, samples were placed at -80°C for 2 minutes, then 95°C for 1 minute. This tempering cycle was repeated twice, then samples were vortexed for 30 seconds. To each micro-centrifuge tube, 200 µL of chloroform was added, immediately followed with vortexing for 2 minutes. Samples were then centrifuged for 3 minutes at room temperature, 20,000xg. The aqueous portion was removed and placed into a new micro-centrifuge tube

that contained 400 µL of ice-cold ethanol. Samples were mixed by inversion, then incubated for 5 minutes at room temperature. After this incubation step, the samples were centrifuged for 5 minutes at room temperature, 20.000xg. The resulting supernatant was removed and then the remaining pellet was washed with 500 µL of 70% ethanol. This ethanol wash was followed with another centrifugation step for 5 minutes at room temperature, 20,000xg. The supernatant was removed and the remaining pellet was dried for 5 minutes at 60°C on a dry heating block. The dried pellet was re-suspended using 25 µL of TE buffer (10 mM Tris pH 8.0 and 1 mM EDTA pH 8.0). Re-suspended DNA was then quantified via the Qubit 2.0 fluorometer unit, and the DNA was stored at -20°C until samples were used for quantitative PCR.

2.5 Development of PCR Protocols for Fungal Quantification

2.5.1 Development of a qRT-PCR Protocol

Extracted RNA samples were removed from storage at -80°C and allowed to thaw at room temperature. Once thawed, RNA template was used for cDNA (complementary DNA) synthesis through the use of BIO-RAD's iScript cDNA synthesis kit as per the manufacturer's specifications (BIO-RAD, Hercules, CA, USA). The manufacturer's recommended cDNA synthesis program was carried out on a thermocycler (Model T100 Thermal Cycler, BIORAD, Hercules, CA, USA; Mastercycler, Eppendorf, Hamburg, Germany). The cDNA generated from the iScript assay was immediately used for quantitative PCR reactions run on a Realplex2 master-cycler (Eppendorf North America,

Hauppauge, NY, USA). Each PCR reaction contained the following reagents to a total volume of 25 µL per PCR reaction: 12.5 µL SYBR Green master mix, 2 µL of forward primer, 2 µL of reverse primer, 2 µL of cDNA template, and 6.5 µL of molecular biology grade water, plus 1 µL template cDNA. PCR reactions were pipetted into 96 well PCR plates, sealed, and spun down using the Sorvall ST 16R centrifuge for 5 minutes at 4°C, 100xg. After centrifugation, samples were placed in the Eppendorf Realplex2 master-cycler. The following parameters were used for the quantitative PCR: 520 nm SYBR filter, spectral calibration for background measurement, SYBR Green probes, and 25 µL sample volume. The conditions of the quantitative PCR reaction are as follows: 2 minute initial heating step at 95°C; denaturing, annealing, and elongation for 15, 15, and 20 seconds at 95°C, 52°C, and 68°C, respectively, repeated for 40 cycles; and a melting curve analysis that consisted of an initial heating step for 15 seconds at 95°C, cooling for 15 seconds at 60°C, a gradual ramped heating for 20 minutes to 95°C, and a final heating step for 15 seconds at 95°C. Samples run through this program were stored at -20°C after testing.

2.5.2 Development of a qPCR Protocol

Extracted DNA samples were removed from storage at -20°C and allowed to thaw at room temperature. Once thawed, DNA template was used in quantitative PCR reactions ran on an Eppendorf Realplex2 master-cycler as above.

2.6 Standard Curve Development for qPCR Protocol

Threshold cycle (Ct) values measured by the Eppendorf Realplex2 were compared to spread plate counts of the fungal culture concentration used for a given extraction. The assumption was that the more fungal material in suspension, the lower the Ct value and that a correlation would exist between the two indicators. Therefore, plate count values of a fungal culture were reported as log CFU/mL present in a given sample. That same sample was used for DNA or RNA extraction and the Ct value from the qPCR or qRT-PCR was recorded. Then, the log CFU/mL of a given inoculum level was compared to the Ct value associated with that same inoculum samples. A series of cell suspensions were tested to establish a pattern between amount of fungal material and their associated Ct values. The standard curve for a given fungus was determined by plotting the log CFU/ml present in a cell suspension versus the Ct value associated with that sample. The linear fit to the plotted values became the standard curve for an approximation if the quantity of fungi present in a sample.

2.7 Inoculation of Dairy Products

Whole fat fluid milk, cottage cheese, and yogurt were used for this portion of the project. Fungal cultures were grown and cell suspensions were prepared to inoculate dairy samples at different levels to evaluate the performance of the qPCR protocol developed and to observe the potential for a matrix effect. Fungal cultures for experiments were grown as specified in 2.1. The dairy samples were obtained from a local grocery store.

Fluid milk was left as is for inoculation with concentrated fungal suspensions. However, cottage cheese and yogurt samples had to be diluted in 1:1 ratio with Butterfield's phosphate-buffered dilution water to make the matrices more amenable for pipetting. Dairy samples were initially mixed in a 1:1 ratio with a high-level inoculum to produce the samples that contained the highest fungal concentration (10^6 CFU/g), then serially diluted (1 mL of inoculated dairy into 9 mL of non-inoculated dairy) in order to produce the subsequent dilution levels as desired. A small portion of the dairy matrices were retained to be tested for their naturally occurring fungal load.

2.8 Validation of qPCR Method in Dairy Matrices

To validate the developed quantitative method, it was compared to a series of standard and/or commercially available methodologies: conventional plating on DRBC agar plates as described in 2.2.; Rapid Yeast and Mold Petrifilm™ (3M); Hygiena Qualicon BAX System PCR Assay for Yeast and Mold (DuPont Nutrition and Health); and the BIOTECON Foodproof Yeast and Mold Quantification LyoKit – 5'Nuclease- RP (BIOTECON Diagnostics GmbH). The conventional plating was performed according to the methodology described in 2.2. The commercially available rapid methods were used following the instructions as provided by their respective manufacturer.

The Hygiena Qualicon BAX System PCR Assay for Yeast and Mold uses a proprietary internal positive control to determine the quality of a sample (positive or negative). The enriched protocol with an action limit of 25 CFU/g was used for this method comparison.

400 µL of sample was added to a BAX supplement tube and incubated for 44 hours at 25° C. After the incubation, samples were treated with 20 µL of DNA stabilizer and were subsequently disrupted for 15 minutes. Cluster tubes were prepared with the addition of 200 µL lysis reagent (comprised of lysis buffer and protease from the PCR Assay kit) and 20 µL of sample. Samples were heated for 20 minutes at 37°C, then heated once more for 10 minutes at 95°C. After the heat treatments, samples were cooled at 2-8°C for 5 minutes. 50 µL of sample lysate were added to PCR assay tubes for the BAX assay. The samples PCR tubes were loaded into a rack and placed in the BAX instrument for analysis. Figures 2.2 and 2.3 display a positive and negative control sample processed by this assay.

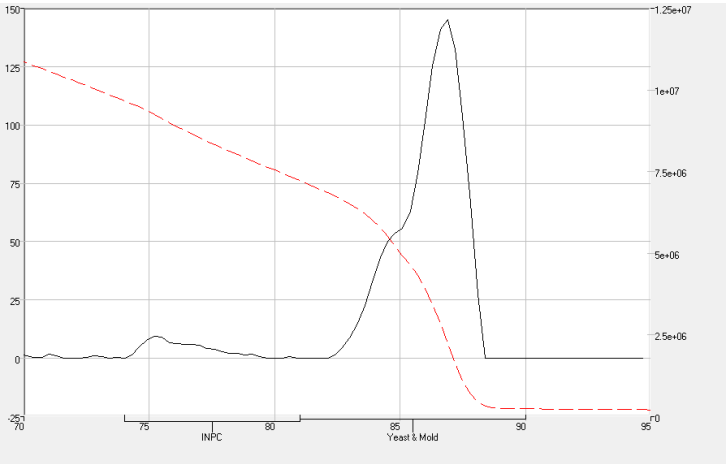


Figure 2.2 Positive Control Sample in BAX PCR Assay for Yeasts and Molds

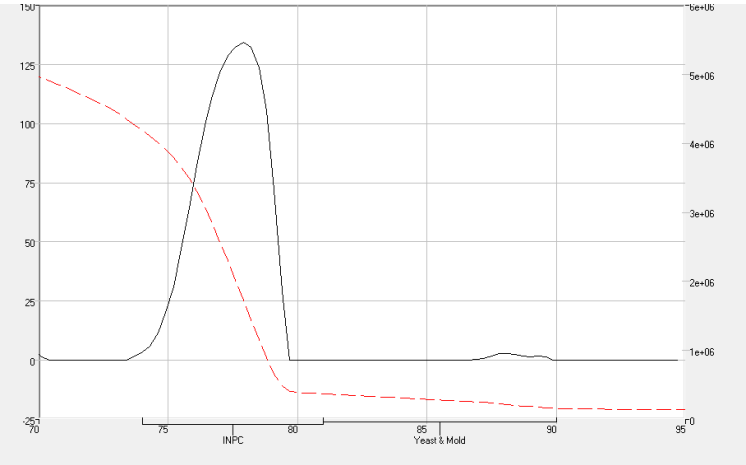


Figure 2.3 Negative Control Sample in BAX PCR Assay for Yeasts and Molds

The developed method was validated for culture material and inoculated dairy products by two technicians. The concentrations tested for the culture material and inoculated dairy were: 10^6 CFU/g, 10^4 CFU/g, 10^2 CFU/g, and 10^0 CFU/g. The culture used for this validation was *Galactomyces candidus* and the dairy matrices used in this validation were whole fat fluid milk, cottage cheese, and yogurt. The 10^0 concentration was used as a negative control for the culture material and to represent the naturally occurring fungal load present in a dairy matrix.

2.9 Survey of Assorted Dairy Products and Fungal Quantification Methods

In addition to the validation done using culture material and inoculated dairy products, a survey of assorted dairy products was carried out to compare the performance of the qPCR method to other methods for fungal quantification by one technician. For the survey, 38 samples of fluid milk, shredded cheese, yogurt, sour cream, buttermilk, and

cottage cheese were obtained from three different local grocery stores and were stored at room temperature for 48 hours prior to analysis. Shredded cheese, yogurt, cottage cheese, and sour cream samples were diluted with Butterfield's phosphate-buffered dilution water in a 1:1 ratio prior to testing (50 g sample to 50 g of diluent). Fluid milk and buttermilk samples were weighed as is (100 g sample) prior to testing. Once again, all the methods described in 2.8 were used for fungal quantification in each sample.

CHAPTER 3. RESULTS AND DISCUSSION

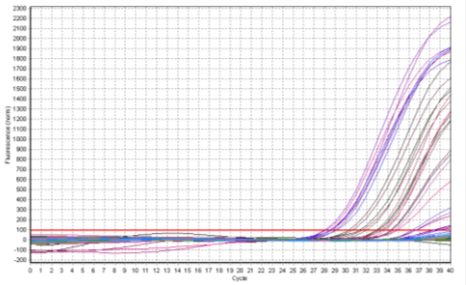
3.1 Primer Design for qRT-PCR and qPCR Protocols

Based on the methodology described for primer design (2.3), initially 3 sets of primers were designed based on fungal organisms that are considered to be relatively prevalent and abundant in dairy products which can be seen in Table 3.1 (Primer Sets 1-3).

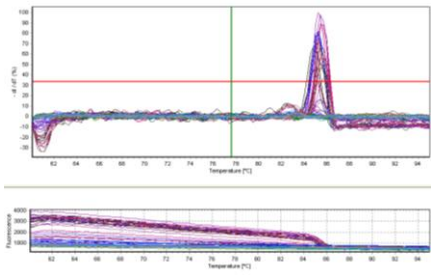
Table 3.1 Primers Designed for qRT-PCR and qPCR Protocols

Primer Set	Primer Target	Primer	Sequence (5' -> 3')	Size (BP)
1	18S rRNA	Fungal 18S-F	CGAGCGTCATTWCACCAC	174
		Fungal 18S-R	AATGAACGCTCGRACAGG	
2	Actin (act1)	Fungal actin-F	GAGGCYCCCRTCAAC	199
		Fungal actin-R	GGCCAGCCAKRTCRAB	
3	B-tubulin (TUBB5)	Fungal b-tubulin-F	GAGGGYGCYGARCTBRT	180
		Fungal b-tubulin-R	GGACCRSMNYKGAYR	
4	Redesigned-Actin (act1)	Fungal actin-F	CCACCATHTWCCCHGGTATT	100
		Fungal actin-R	TTTCTYTCKGGAGGAGCRATR	
5	Elongation Factor 1-α (ef1-α)	Fungal ef1-α -F	GWGGTAAYGTGYGGTGACTC	88
		Fungal ef1-α -R	CCWGGRTGGTTSAAGAYRATRA	

Preliminary qRT-PCR reactions were run with the initial set of primers listed in Table 3.1 using cDNA from 6 fungal organisms (*Debaryomyces hansenii*, *Galactomyces candidus*, Yarrowia *lipolytica*, *Penicillium roqueforti*, *Penicillium verrucosum*, and *Cladosporium cladosporioides*) to determine if good amplification could be achieved. In these reactions, the primer concentration was fixed at 2 µL forward primers and 2 µL reverse primers, 12.5 ng of cDNA template, and the temperature for annealing was varied. Based on the results obtained in these reactions, primer sets 1, 2, and 3 showed less than desirable results. These primers showed very little efficiency in detecting genetic material from yeast or mold cultures.



3.1 A



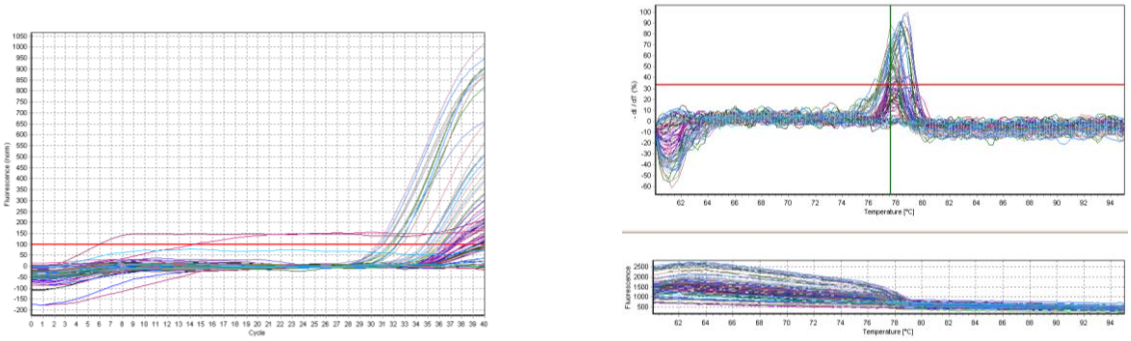
3.1 B

Figure 3.1 Actin (act1) Primers (Primer Set 2) 12.5 ng cDNA template gradient PCR Quantification Curves (A) and Melting Curves (B)

Figure 3.1 shows the results obtained for Actin (act1) primers. Similar results were obtained for the other two primer sets (18S rRNA and β-tubulin). Based on the results obtained while evaluating primer performance, the Actin (act 1) primer set was redesigned by basing the primer sequences on 3 more closely related organisms (*Kluyveromyces marxianus*, *Galactomyces candidus*, and *Debaryomyces hansenii*) and

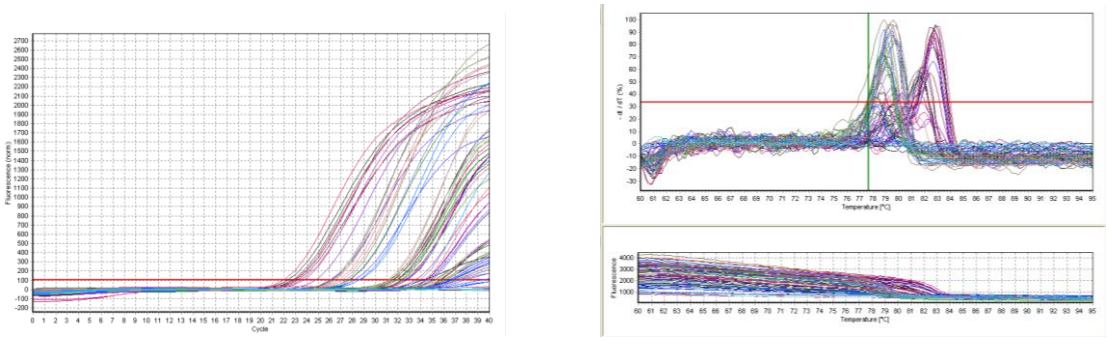
producing a smaller product (100 BP vs the original 199 BP). Additionally, one more gene was considered for primer design: Elongation Factor 1-α. These two sets of primers are shown in Table 3.1 (Primer Sets 4 and 5).

Primers for Elongation Factor 1-α (ef1a) and Redesigned-Actin (act1) provided amplification of all species used in the qRT-PCR reactions. However, the ef1a primers showed a better overall response, given the shape of the quantification curves and the lower/earlier Ct values associated with the amplification, than did the act1 primers (Figures 3.2 and 3.3). The Ct value corresponds to the cycle number (x-axis) when the fluorescence of the sample given by amplification of cDNA or DNA crosses the threshold of the equipment detector (red horizontal line in the graphs). The optimal temperature associated with both of these primers sets was approximately 52°C (Table 3.1) The primers for the expression of ef1α (set 5) had an optimal concentration of 2 µL of each primer (forward and reverse).



3.2 A 3.2 B

Figure 3.2 Redesigned-Actin (act1) Primers (Primer Set 4) 12.5 ng cDNA template gradient PCR Quantification Curves (A) and Melting Curves (B)



3.3 A 3.3 B

Figure 3.3 Elongation Factor 1-α (ef1a) Primers (Primer Set 5) 12.5 ng cDNA template gradient PCR Quantification Curves (A) and Melting Curves (B)

The redesigned actin primers and the primers for elongation factor 1-alpha gene were able to amplify genetic material from cultured yeasts and molds. The original act1 primer (primer set 2) was only able to amplify mold cDNA; however, the redesigned primer was also effective in amplifying yeast cDNA. A possible explanation for this newfound ability to quantify yeast cDNA in qPCR reactions could be that the initial set of primers (set 2) could been designed to amplify too large of a product compared to sets 4 and 5 (Table 3.1). The primers in set 4 and 5 were designed using sequences of three highly related yeast genera (*Kluyveromyces*, *Galactomyces*, and *Debaryomyces*) compared to the set 2 primers which were designed using four more distantly related genera (*Cladosporium, Penicillium, Yarrowia*, and *Galactomyces*). The efficacy of the primers included in set 4 and 5 could be attributed to the shorter products formed by them. Short products tend to be easier to quantify in a given reaction compared to longer products because they are more accessible in solution. Based on Figures 3.2 and 3.3, primer set 5 was more effective than primer set 4 due to better overall performance, more sigmoidal

curvature, and lower associated Ct values for samples of similar concentrations of template cDNA. Primer set 5 was chosen for all future work.

While evaluating the primers for their performance, the conditions needed to carry out the desired qRT-PCR and qPCR reactions had to be evaluated as well. As the ef1-α primers proved to be the most amenable to method development, the conditions to carry out the qRT-PCR and qPCR reactions were studied in an Eppendorf RealPlex 2 machine (Eppendorf, Hamburg, Germany). All temperatures used in the qRT-PCR and qPCR reaction were the program default ones, except the annealing temperature (52°C) which was determined through several gradient qPCR reactions for the ef1-α primers. Further alterations were made to the times for the annealing and elongation steps to produce qRT-PCR and qPCR conditions that provided more consistent amplification, which are present in Table 3.2.

Table 3.2 qPCR conditions for detection and quantification of fungi using DNA template and ef1-α primers.

	Pre-Incubation (1 Cycle)	**Amplification (40 Cycles)**	**Melting Curve**
PCR Protocol (DNA)	95°C for 2 minutes	95°C for 15 seconds	95°C for 15 seconds
		52°C for 25 seconds	37°C for 1 minute
		68°C for 25 seconds	Ramp up to 95°C (20 minutes)

Other studies have designed primers for the detection of fungi in dairy products. In Makino et al. (2010), primers were designed specifically to target yeast groups that potentially posed an opportunistic public health risk in dairy products. Comparatively, the primers in this study were

designed on the basis of prevalence and abundance of fungal organisms that were isolated from dairy products. The primers from Makino et al. (2010) were specifically designed to pick up target organisms did not amplify organisms that were from other groups. Cappa and Cocconcelii (2001) utilized Tr1 and Tr2 primers from Bock et al. (1994) to identify fungi from dairy products. The Tr1 and Tr2 primers are fungal specific primers designed to pick up a 581-bp fragment within the 18S rRNA. However, the primers were only used for qualitative work, as opposed to the intended quantitative work of this study. Tr1 and Tr2 primers were able to amplify DNA from two different fungi that were isolated from yogurt (*Penicillium chrysogenum* and *Cladosporium cladosporioides*). In Mayoral et al. (2005), primers were designed to amplify a 251-bp fragment of the 18S rRNA gene of yeasts. These primers were used on Kluyveromyces marxianus cultures of varying cell concentrations ranging from 106 CFU/mL to 101 CFU/mL and assorted yogurt samples. The primers and PCR reaction used in the Mayoral et al. (2005) study were able to detect the presence of yeast in two yogurt samples, while the other samples did not have any PCR products associated with them before and after incubation. In Vaitilingom et al. (1998), the primers designed for the detection of yeasts and molds were based on 3 sequences and 10 sequences, respectively, for ef1-α. The yeast primer produced a 657-bp product, while the mold primer produced a 498-bp product. Reverse transcriptase PCR (RT-PCR) was performed in the study, however it was measured by gel electrophoresis as opposed to using a real-time PCR machine to produce a qRT-PCR. Their primers were able to detect their designated fungal species tested (*S. cerevisiae*, *C. albicans*, *M. racemosus*, and *A. pullulans*). In Bleve et al. (2003), primers were designed based on the actin sequences of 16 different fungal species. These primers were able to amplify products from fungi, but not bacteria. These primers were used for qRT-PCR and were able to amplify fungi quantitatively.

The results from all of these studies show that designed primers and/or universal primers were able to amplify cDNA or DNA from fungi. The study done by Bleve et al. (2003) was the most similar to ours as it intended on using a quantitative measure to enumerate fungi in dairy matrices.

3.2 Development of qRT-PCR Protocol for Fungal Detection

Using the method as described by Hallen et al. (2007) for RNA extraction, further studies were conducted by varying the concentration of cDNA template and using the optimal reaction conditions (Table. 3.2). Figure 3.4 shows quantification curves associated with varying the cDNA template present in the SYBR Green reactions when ef1-α primers were used. These reactions were technical duplicates to test and to observe potential patterns due to cDNA template concentration. One pattern was observed from both sets of curves associated with this test, which was that the higher the concentration of cDNA template present in given reaction, the lower the Cr value. This observation was anticipated due to a relatively abundant amount of target nucleic acid present in the sample. According to results shown in Figure 3.4: Ct values ≤ 29 are the result of strongly positive reactions, which are indicative of abundant amounts of target nucleic acid in these samples; Ct values ranging from 30 to 37 correlate to moderate of target nucleic acid in the samples being analyzed; while Ct values from 38 to 40 are weak positive signals that could due to many issues such as minimal target nucleic acid present in the samples or from environmental contamination.

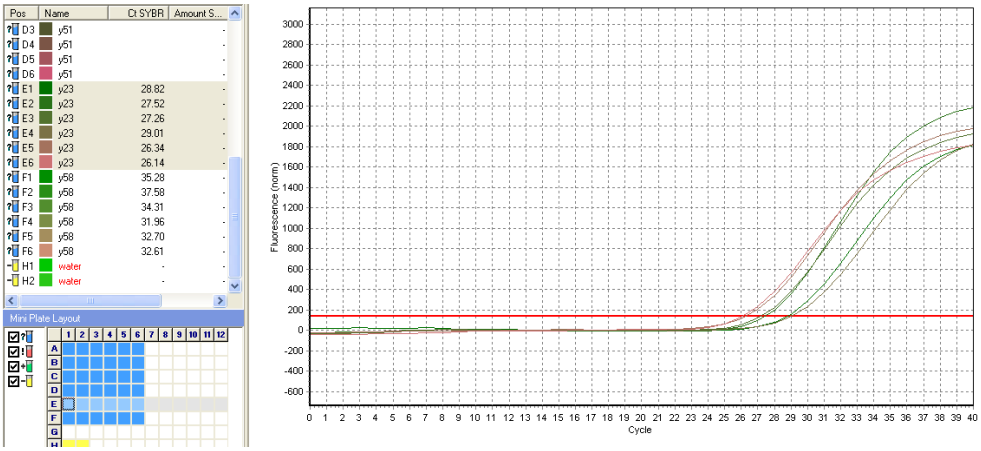


Figure 3.4 qRT-PCR Reaction results from *Galactomyces candidus* for Elongation Factor 1-α (ef1-α) primers with cDNA template concentration varying from 0 to 50 ng.

The qRT-PCR runs in Figure 3.4 were also ran with 6 different yeasts and molds (*Debaryomyces hansenii*, *Galactomyces candidus*, *Yarrowia lipolytica*, *Penicillium roqueforti*, *Penicillium verrucosum*, and *Cladosporium cladosporioides*).Both trials showed that the higher the concentration of cDNA added to each reaction, the lower the Ct value associated with that reaction, which would be essential for establishing a quantitative correlation between Ct values and amount of fungal specimen in dairy matrices. Another common feature of these reactions was that out of all the fungal samples tested, *Yarrowia lipolytica* had the least amount of amplification and quantification regardless of the cDNA concentration present. This lack of amplification and quantification could be the result of not matching as well to this set of primers compared to the other organisms tested.

Another observation was that even though both trials were prepared the same way, there was a slight difference in the Cts associated with the samples (second set of results not

shown). One set of experiments shows the majority of reactions having Cts ranging from 30 to 40 cycles, while the other set (Figure 3.4) showed the majority of the reactions having Cts ranging from 22 to 35 cycles. The same fungus (yeast or mold) was found in close proximity of one another, with slight decreases in Ct observed for each respective increase in cDNA template used in that reaction. However, the general pattern associated with the Cts for each organism was the same, despite the shift in the curves (i.e. samples that appeared earlier in one replicate would also appear earlier in the other replicate).

However, as further experiments were conducted to evaluate these shifts, and to attempt to resolve these run-to-run variations, the variability associated with the RNA extraction protocol became clear. One major issue with the RNA extraction was replicability. Even when using known sample concentrations, the amount of RNA quantified following extraction did not correlate well to its respective inoculum concentration (i.e. the RNA extracted and quantified from a suspension containing 10^6 CFU/mL of a particular organism was less than the RNA extracted and quantified from a suspension containing 10^4 CFU/mL of the same organism). Another issue observed was the lack of replicability on the amounts of RNA extracted and quantified from the same level of inoculum (i.e. two samples of 10^6 CFU/mL yielded two different quantities of RNA). Table 3.3 illustrates the lack of replicability in the RNA extraction protocol. Values reported indicate the amount of RNA quantified by spectrophotometer Eppendorf BioPhotometer Plus (Eppendorf, Hamburg, Germany). As Table 3.3 indicates, the amount of RNA obtained from different samples did not correlate well with concentrations of fungal material in those samples.

Table 3.3 RNA quantification for RNA material extracted from fungal spore suspensions of known concentrations (i.e. CFU/mL), Hallen et. al (2007) extraction protocol (A), Qiagen Rneasy Mini Kit extraction protocol (B).

RNA Quantified (µg/µL) from a given CFU concentration						
Organism	A	B	A	B	A	B
	10^6 CFU/mL		10^4 CFU/mL		10^2 CFU/mL	
Y-7751 (NRRL)	0.4710	12.4	0.0880	5.3	0.4468	N/A
Y-1458 (NRRL)	0.5678	6.0	0.9010	11.2	1.1560	N/A
YB-223 (NRRL)	0.3010	N/A	0.2670	N/A	0.4590	N/A
5574 (NRRL)	1.0485	N/A	0.7088	N/A	2.7322	N/A
849 (NRRL)	1.3180	N/A	0.2850	N/A	0.8490	N/A
3182 (NRRL)	0.7330	N/A	0.4580	N/A	0.1940	N/A

An alternative method was also used to quantify RNA after the extraction protocol, the Quant-it RNA assay kit (Thermo Fisher Scientific, Hamburg, Germany). The RNA material was evaluated with this assay kit and processed by a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Hamburg, Germany), and the results were similar to those obtained with the Eppendorf BioPhotometer Plus.

The primary reasoning for trying to use this RNA based methodology was that RNA is representative of viability of a given organism. Viability is an important diagnostic feature regarding food spoilage organisms as that determines whether or not a foodstuff(s) of interest, such as dairy commodities, will spoil. Using a RNA method for fungal quantification would allow for the distinction between live and dead cells in a given sample, as dead cells still remain after food processing operations, such as

pasteurization. Dead cells cannot contribute any quantifiable RNA in a sample because RNA quickly degrades after cell death. Thus, the viable cells would inherently be measured without the use of any additional reagents.

Several alternatives to the RNA extraction detailed in Hallen-Adams et al. (2007) were then performed to determine if the extraction method could be more replicable. These methods included the use of the hot phenol protocol from Goswami et al. (2006), an adaptation of the Hallen et al. (2007) protocol, and a commercial RNA extraction kit (RNeasy Mini Kit, QIAGEN, Germantown, MD, USA). Therefore, after attempting many strategies and the RNA extraction protocol continuing to prove to be too variable for optimization, resources and efforts were focused on DNA extraction associated with the use of a binding agent for DNA from non-viable cells.

Vaitilingom et al. (1998) discussed the difficulty of adapting RT-PCR to produce quantitative results (qRT-PCR) due to the variance in yield for the amplification and reverse transcription steps for different reactions. Bustin (2002) discusses the trends and potential problems with qRT-PCR, particularly the variance in reproducibility due to different equipment, technicians, reagents, etc. Bleve et al. (2003) were able to develop a reproducible qRT-PCR protocol for the detection of fungi in yogurt. However, they expressed concerns with the complexity and issues of sensitivity, reproducibility, and specificity that is typically associated with RT-PCR. The difference observed in the ability to produce a qRT-PCR between this study and the study performed by Bleve et. al (2003) agrees with the concerns made by Vaitilingom et al. (1998) and Bustin (2002).

3.3 Development of qPCR Protocol for Fungal Detection

DNA extraction was done using several methods, which are described in section 2.4.2., to see which one would provide the most consistency. Method evaluation was done based on the amount of DNA quantified by spectrophotometer Eppendorf Biophotometer Plus (Eppendorf, Hamburg, Germany) according to Barbas III et al. (2007).

Table 3.4 DNA Quantification for DNA material extracted from fungal spore suspensions of known concentrations (i.e. CFU/mL), via the Harju et al. (2004) protocol (A), and via QIAGEN Dneasy Plant Kit (B).

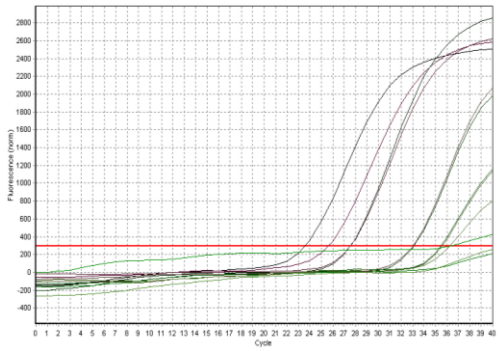
DNA Quantified (ng/µL) from a given CFU concentration						
Organism	A	B	A	B	A	B
	10^6 CFU/mL		10^4 CFU/mL		10^2 CFU/mL	
Y-7751 (NRRL)	42.5	5.2	6.9	2.9	0.5	N/A
Y-1458 (NRRL)	20.1	8.3	5.7	2.8	2.1	N/A
YB-223 (NRRL)	125.6	15.0	91.6	23.0	15.5	N/A
849 (NRRL)	59.8	0.3	17.6	0.8	14.4	0.6

Based on the results in Table 3.4 and DNA quantification for the other method variations tested, as well, the most consistency was observed with the original method described by Harju et al. (2004) (data not shown). As Table 3.4 indicates, DNA concentration is proportional to the amount of fungal material present during extraction, which was not necessarily observed with the RNA extraction protocols. This method proved to be a

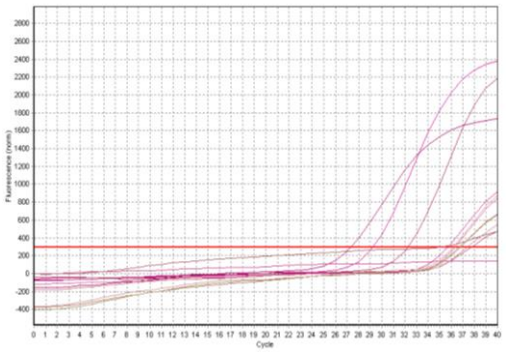
replicable alternative to the RNA extraction method from Hallen et al. (2007). This procedure was more consistent than the RNA extraction method as well as less time intensive. The only difference between the two protocols is the inherent lack of viability distinction with the DNA extraction method. This difference can however be overcome by the inclusion of an intercalating dye to bind any DNA from dead cells and minimize the amplification of any residual DNA that would otherwise provide false information (i.e. suggest higher counts of viable fungi present in the sample than what is actually present).

Attempting to improve the detection of only viable cells, different binding agents (Reagent D, propidium monoazide, and methylene blue) were all tested as well as different concentrations, which is described in section 2.4.2. Each of the considered binding agents (Reagent D, propidium monoazide, and methylene blue) work by binding any DNA from any dead cells present in a sample due to their ability to permeate the damaged cell membranes of dead cells, but not the intact membranes of live cells. Using the DNA extraction method proposed by Harju et al. (2004), the different binding agents and concentrations were tested by adding the binding step after cell harvesting, but prior to cell lysis. In Figure 3.5, qPCR curves were obtained from using the different binding agents in tandem with the DNA extraction method described in Harju et al. (2004). These curves show the difference in Ct values between live and dead fungal cells. Reagent D used at the concentration of 300 µL as recommended by the manufacturer (Biotecon Diagnostics, Potsdam, Germany) was more effective at differentiating live and dead cells than any of the other tested intercalating dyes (500 µL of methylene blue, 1.25 µL of

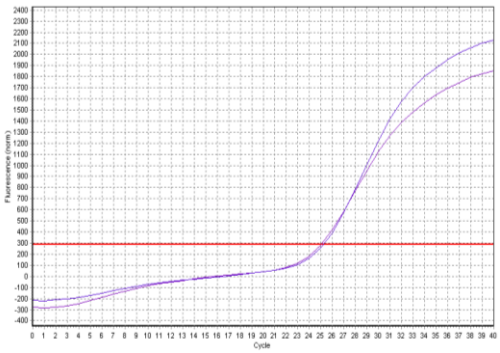
PMA, and 600 µL of Reagent D) (PMA data not shown). Based on these curves, Reagent D at 300 µL was chosen as the binding agent for viability differentiation as a separation of Ct values for live and dead cells could be observed. The 500 µL of methylene blue and 600 µL of Reagent D showed little to no separation in Ct values for live and dead cells of the same cell concentration.



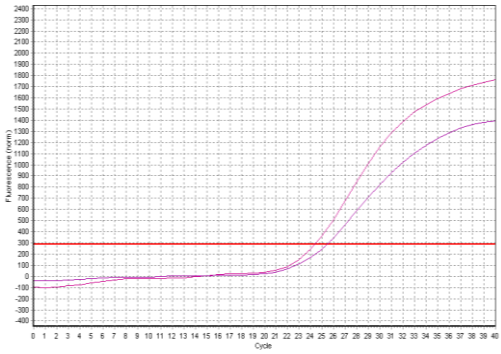
3.5 A



3.5 B



3.5 C



3.5 D

Figure 3.5 Elongation Factor 1-α (ef1-α) primer qPCR results using the Harju et al. (2004) DNA extraction method and different intercalating dyes: Live fungal cells ranging from 10^6 to 10^1 CFU/mL using 300 µL of Reagent D (A), Dead fungal cells ranging from 10^6 to 10^1 CFU/mL using 300 µL of Reagent D (B), Live/Dead fungal cells at 10^6

CFU/mL using 500 µL of methylene blue (C), and Live/Dead fungal cells at 10^6 CFU/mL using 600 µL of Reagent D (D).

Table 3.5 shows the difference on Ct values between live and dead cells of comparable CFU/mL concentrations. A general observation of the Ct values associated with live and dead cells is that there was a delay in Ct values observed for the DNA extracted from dead cells compared to the values obtained for live cells of the same concentration. (i.e. cell suspensions containing 108 CFU/mL of live cells showed up earlier than suspensions containing 108 CFU/mL of dead cells). As shown in Table 3.5, there is a 2 to 3 cycle delay from live to dead cells within the same cell concentration. Even though there was a difference in Ct values, one issue with the values obtained is that the delayed Ct values for dead cells still appear around the same times as live cells of lower concentration levels. Reagent D should be able to bind any DNA present in the concentrated dead cell suspension; however, it might have only done so in a limited capacity.

Table 3.5 Ct values obtained for the EF1-α qPCR reaction using DNA extracted from live and dead cells of *Galactomyces candidus*

Galactomyces candidus YB-223 (NRRL) Concentration and Viability	Ct Values (Replicate 1)	Ct Values (Replicate 2)	Plate Count Replicate 1 (CFU/mL)	Plate Count Replicate 2 (CFU/mL)
10^8 Live	24.26	24.91	1.40×10^8	1.35×10^8
10^8 Dead	27.31	30.73	<10	<10
10^6 Live	28.90	29.09	1.34×10^6	1.39×10^6

10^6 Dead	30.77	33.19	<10	<10
10^4 Live	32.91	31.99	1.38×10^2	1.33×10^4
10^4 Dead	30.64	34.63	<10	<10

In Nocker et al. (2007), propidium monoazide was used to differentiate live and dead cells present in the environmental samples tested. However, the results obtained in the study by Nocker et al. (2007) was only used for qualitative purposes (presence/absence) via denaturing gradient gel electrophoresis (DGGE). They were able to observe a difference across live and dead cells present in a sample by comparing the DGGE profiles with and without the use of the propidium monoazide. When the intercalating dye was used, samples containing 50% and 100% live cells were still able to be clearly visualized on the DGGE. The 10% live cell samples had a fainter band present. The non-spiked samples and the 0% to 1% live cell spiked samples using the dye did not produce a distinguishable product band. All of the spiked samples processed without propidium monoazide produced a distinct target band on the DGGE. Comparatively, in our study propidium monoazide was used to potentially bind DNA present from dead cells for qPCR. However, using propidium monoazide did not help discern differences in live and dead cells. Nafisi et al. (2006) used methylene blue to assess the stability and structural features of DNA. In our study, methylene blue was used as a potential binding agent, however no differences in Ct values were observed for live and dead cells at the same cell concentration. In Martinon et al. (2012), Reagent D and propidium monoazide were used as the binding agents for DNA from dead cells. They reported that qPCRs that included samples treated with either Reagent D or propidium monoazide had later Ct values for

samples of set cell concentrations than non-treated samples at the same cell concentrations. They concluded that PMA is more suitable for qPCR applications; however, this study was only able to observe reproducible live/dead differentiation with the use of Reagent D.

3.4 Standard Curve Development for qPCR Protocol

Using the method described by Harju et al. (2004) for DNA extraction, with the inclusion of Reagent D as the non-viable DNA binding agent, standard curves were built correlating the fungal concentration (log CFU/mL) as determined by plating on DRBC with Cts obtained from the qPCR reactions. The conditions used for the qPCR reaction to amplify extracted DNA with the ef1-α primers were the same ones established in Table 3.2. A typical reaction consisted of the following reagents: 12.5 µL of SYBR Green MasterMix, 2 µL of ef1-α forward primers, 2 µL of ef1-α reverse primers, 6.5 µL of molecular biology grade water (DNase and RNase free), and 2 µL of DNA template.

With the use of the ef1-α primers and conditions shown in Table 3.2, the qPCR reaction was used to develop a standard curve for Galactomyces candidus. The standard curve was developed by plotting the Ct values of DNA extracted from Galactomyces candidus dilution series against the log CFU/mL of those same dilution series, which was determined by DRBC plate counts. Figure 3.6 shows the standard curve that depicts the correlation between the log CFU/mL and the Ct associated with a particular sample. The equation of this curve is: $y = -2.54x + 43.14$, where x represents the log CFU of the

sample of interest and y represents the Ct of that particular sample. In following experiments, the qPCR provided the Ct value associated with a particular fungal load or concentration. The fungal concentration (log CFU/mL or log CFU/g) was then calculated using this curve.

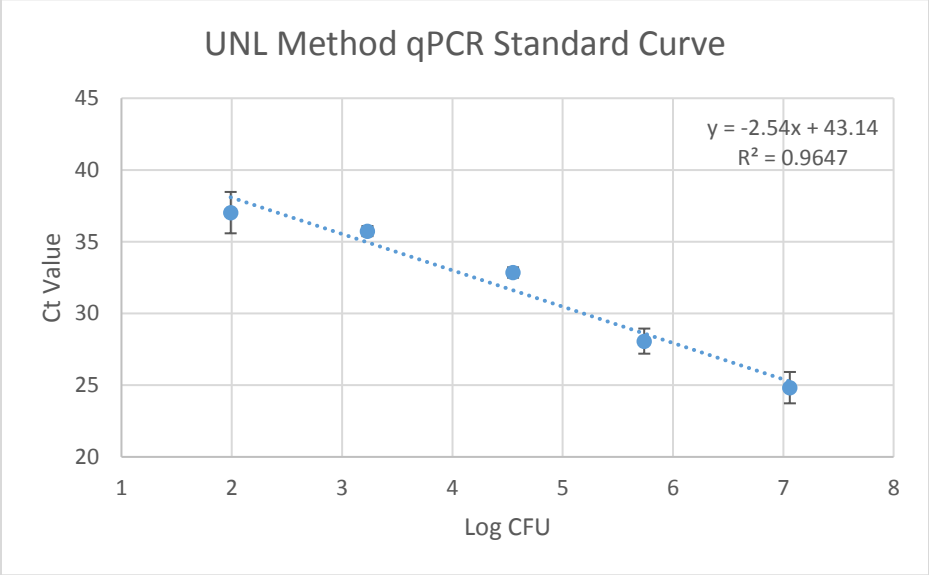


Figure 3.6 Standard curve determined for the EF1α qPCR using *Galactomyces candidus* as the model organism

Other qPCR studies developed standard curves using specific primers and the organisms that suited those primers. Bleve et al. (2003) produced a standard curve using actin gene primers by plotting Ct values against log CFU/mL, which was determined by standard plate count. The equation for their curve was **y= -1.86*x* + 27.80** with a R^2 value of 0.977. Makino et al. (2010) developed six different standard curves based on six specific sets of 26S rRNA primers by plotting Ct values against log CFU/mL, which was determined using a counting chamber. The equations for their six curves were: **y= -3.96*x* + 38.23**, **y=**

-3.49*x* + 32.31, **y= -3.70*x* + 36.26**, **y= -3.71*x* + 40.50**, **y= -4.03.*x* + 39.43**, **y= -3.54*x* + 39.86** with R^2 values ranging from 0.9992 to 0.9998. Martinon et al. (2012) developed three standard curves for their LUX primers by plotting Ct values as a function of log SGU, where SGU represents "signal generating units". The equations for their curves were: **y = -4.02.*x* + 35.84**, **y = -3.12*x* + 36.57**, and **y = -3.42*x* + 34.12** with R^2 values ranging from 0.993 to 0.999. All of the curves, including the one developed for *Galactomyces candidus* in this study, have data points that fall very closely to their fitted regression line. The differences in slope and y-intercept observed across all of the standard curves could be the result of many variables. Some of the variables that could potentially cause these differences are the organism(s) being assessed, the primers used, and the PCR reaction (reagents and processing conditions).

3.5 Validation of Developed Method in Dairy Matrices

Validation of the proposed method was done by comparing its performance with the results obtained by conventional plating on DRBC agar plates, 3M Rapid Yeast and Mold petrifilms (3M™ Petrifilm™, 2013.), Hygiena Qualicon BAX System PCR Assay for Yeast and Mold (DuPont Nutrition and Health. 2013.), and the BIOTECON Foodproof Yeast and Mold Quantification LyoKit – 5'Nuclease- RP (BIOTECON Diagnostics GmbH 2016).The validation was carried out using culture material and inoculated dairy products (fluid whole fat milk, yogurt, and cottage cheese). This validation used *Galactomyces candidus* YB-223 (NRRL), since a standard curve for quantification was available for this organism as shown in Figure 3.6. Culture material was serially diluted

to produce samples with varying fungal concentrations ranging from 10^6 to 10^2 CFU/mL. Additionally, inoculation of products with known amounts of fungi was performed in order to evaluate if the matrices would have any effect during the DNA extraction and qPCR reaction. The comparison of the methodologies in culture material and inoculated dairy matrices was performed by two technicians and in triplicate. The plate counts on DRBC was used as the "gold standard" for the comparison as it is listed as the standard method in the BAM (Bacteriological Analytical Manual).

Table 3.6 Comparison of the developed qPCR method to standard and commercially available rapid fungal detection and quantification methods with DNA extracted from *Galactomyces candidus*

Galactomyces candidus **(CFU/mL)**	**DRBC Counts (CFU/mL)**	**3M Rapid Yeast and Mold Petrifilm Counts (CFU/mL)**	**BIOTECON Fungal Quantification (CFU/mL)**	**UNL Method Quantification (CFU/mL)**	**BAX PCR Assay for Yeast and Mold (Positive or Negative)**
10^6	4.7×10^6	4.6×10^6	6.5×10^4	2.5×10^6	++++++
10^4	3.1×10^4	4.4×10^4	4.7×10^2	4.1×10^3	++++++
10^2	2.5×10^2	3.7×10^2	1.3×10^1	5.1×10^2	++++++
10^0	< 4	< 1	2.7×10^0	4.3×10^2	++++---

As shown in Table 3.6, the developed qPCR method was compared to the standard DRBC plate count method and the commercially rapid available methods in *Galactomyces candidus*. The method that was the closest to DRBC, was the Rapid Yeast and Mold Petrifilm from 3M, as the values were within 1 log. The BIOTECON method, which is qPCR based, underestimated the fungal load present by 1 to 2 logs, when samples containing 10^2 to 10^6 CFU/mL were used. The developed method was able to

quantify within 1 log for the samples containing 10^2 and 10^6 CFU/mL of *Galactomyces candidus*. The developed method was off by 1 log and 2 logs for the samples containing 10^4 and 10^0 CFU/mL, respectively. The BAX PCR assay showed positive results across all of the replicates performed by both technicians for the samples containing 10^2, 10^4, and 10^6 CFU/mL *Galactomyces candidus* and all but two of the 10^0 CFU/mL *Galactomyces candidus* samples. Positives appearing in the 10^0 CFU/mL samples could be due to possible enrichment of fungi present in those samples. In Tables 3.6, 3.7, 3.8, and 3.9, the BAX samples have six possible values which are denoted with either a "+" or a "-". These values designated whether the sample was comparable to the internal positive control for the BAX assay as described in section 2.8. The action limit for each BAX sample was 25 CFU/g.

Table 3.7 Comparison of the developed qPCR method to standard and commercially available rapid fungal detection and quantification methods with DNA extracted from inoculated milk.

Fluid Milk *Galactomyces candidus* (CFU/mL)	**DRBC Counts (CFU/mL)**	**3M Rapid Yeast and Mold Petrifilm Counts (CFU/mL)**	**BIOTECON Fungal Quantification (CFU/mL)**	**UNL Method Fungal Quantification (CFU/mL)**	**BAX PCR Assay for Yeast and Mold (Positive or Negative)**
10^6	2.7×10^6	3.6×10^6	1.1×10^3	2.1×10^5	++++++
10^4	2.5×10^4	5.4×10^4	9.8×10^5	3.7×10^1	++++++
10^2	1.0×10^2	3.5×10^2	7.8×10^5	N/A	++++++
10^0	3.0×10^0	2.5×10^0	8.1×10^5	3.7×10^1	++++++

As shown in Table 3.7, the developed qPCR method was compared to the standard DRBC plate count method and the commercially rapid available methods in inoculated milk samples. The method that was the closest to DRBC, was the Rapid Yeast and Mold Petrifilm from 3M, as the values were within 1 log. The BIOTECON method, which is qPCR based, underestimated the fungal load present by 3 logs, when samples 10^6 CFU/mL were used. The BIOTECON PCR overestimated the fungal load present by 1, 3, and 5 logs, when milk samples inoculated with 10^4, 10^2, and 10^0 CFU/mL were used. The developed method was off by 1 log for samples inoculated with 10^6 and 10^0 CFU/mL. The proposed method was off by 3 logs when milk samples inoculated with 10^4 CFU/mL of *Galactomyces candidus* was used and did not establish any quantification for samples inoculated with 10^2 CFU/mL. The BAX PCR assay showed positive results for each inoculation level in the milk samples as well as the un-inoculated milk samples. The positives in the un-inoculated milk samples are likely the result of enriching viable but non-culturable cells that are present.

Table 3.8 Comparison of the developed qPCR method to standard and commercially available rapid fungal detection and quantification methods with DNA extracted from inoculated yogurt

Yogurt *Galactomyces candidus* (CFU/g)	**DRBC Counts (CFU/g)**	**3M Rapid Yeast and Mold Petrifilm Counts (CFU/g)**	**BIOTECON Fungal Quantification (CFU/g)**	**UNL Method Quantification (CFU/g)**	**BAX PCR Assay for Yeast and Mold (Positive or Negative)**
10^6	3.0×10^6	3.2×10^6	7.2×10^3	2.1×10^5	++++++
10^4	3.3×10^4	3.3×10^4	3.2×10^5	1.7×10^3	+++++-
10^2	3.4×10^2	2.0×10^2	6.5×10^5	2.4×10^2	+++++-

10^0	< 2	< 2	4.4×10^1	1.9×10^1	++++--

As shown in Table 3.8, the developed qPCR method was compared to the standard DRBC plate count method and the commercially rapid available methods in inoculated yogurt samples. The method that was the closest to DRBC, was the Rapid Yeast and Mold Petrifilm from 3M, as the values were within 1 log. The BIOTECON method, which is qPCR based, underestimated the fungal load present by 3 logs, when samples containing 10^6 CFU/g were used. The BIOTECON PCR overestimated the fungal load present by 1, 3, and 1 logs, when yogurt samples inoculated with 10^4, 10^2, and 10^0 CFU/g were used. The developed method was within 1 log for the sample containing 10^2 CFU/g and was off by 1 log for samples inoculated with 10^6, 10^4, and 10^0 CFU/g were used. The BAX PCR assay showed positive results for all of the inoculated yogurt samples except for one replicate of the 10^4 and 10^2 CFU/g and all but two un-inoculated yogurt samples. The negative results for the un-inoculated yogurt sample could be indicative of no fungal presence in those particular samples.

Table 3.9 Comparison of the developed qPCR method to standard and commercially available rapid fungal detection and quantification methods with DNA extracted from inoculated cottage cheese

Cottage Cheese [*Galactomyces candidus*] (CFU/mL)	**DRBC Counts (CFU/mL)**	**3M Rapid Yeast and Mold Petrifilm Counts (CFU/mL)**	**BIOTECON Fungal Quantification (CFU/mL)**	**UNL Method Quantification (CFU/mL)**	**BAX PCR Assay for Yeast and Mold (Positive or Negative)**
10^6	2.7×10^6	2.9×10^6	1.5×10^5	3.0×10^4	++++++
10^4	2.3×10^4	2.8×10^4	2.0×10^5	8.3×10^2	++++++

10^2	2.6×10^2	3.1×10^2	3.8×10^4	4.5×10^1	++++++
10^0	< 2	< 2	8.8×10^4	N/A	+++++-

As shown in Table 3.9, the developed qPCR method was compared to the standard DRBC plate count method and the commercially rapid available methods in inoculated cottage cheese samples. The method that was the closest to DRBC, was the Rapid Yeast and Mold Petrifilm from 3M, as the values were within 1 log. The BIOTECON method, which is qPCR based, underestimated the fungal load present by 1 log when samples containing 10^6 CFU/g were used. The BIOTECON PCR overestimated the fungal load present by 1, 2, and 4 logs, when cottage cheese samples inoculated with 10^4, 10^2, and 10^0 CFU/g were used. The developed method was off by 1 log for the sample containing 10^2 CFU/g and was off by 2 logs for samples inoculated with 10^6 and 10^4 CFU/g were used. The un-inoculated cottage cheese samples could not be detected via the developed method. The BAX PCR assay showed positive results for all of the inoculated cottage cheese samples and all but one un-inoculated yogurt samples. The negative result for the un-inoculated cottage cheese could be indicative of no fungal presence in that sample or due to the sample being below the limit of detection.

Across all of the comparison work done in culture material and inoculated dairy products, a trend was observed for overestimation and underestimation with the two qPCR based methods (the developed method and BIOTECON's method) compared to the standard DRBC plating method. Any discrepancies observed in this comparison regardless of the medium tested (culture material or inoculated dairy) could be due to the presence of PCR inhibitors (proteins, lipids, carbohydrates, chelating agents, DNases, RNases, etc.), poor

sample handling and preparation, outliers caused by lack of amplification with some of the PCR reactions (developed method and BIOTECON), or the presence of fungi that could be viable but nonculturable (VBNC). The 3M petrifilms were the most comparable method to the DRBC "gold standard" as all of the values were within 1 log through all three replicates, regardless of the technician that performed the task. The BAX PCR assay for Yeast and Mold was used to obtain qualitative results (positive or negative). By comparing the performance of the developed method with the BAX method, it would indicate the value of the developed qPCR protocol to be used as a qualitative test. The BAX and the developed qPCR protocol had agreement in the detection of fungi in all of the samples used (culture and inoculated dairy) except in the instance of milk samples inoculated with 10^2 CFU/mL of *Galactomyces candidus*, where the developed method could not determine any quantification for that concentration. The BAX assay agreed with the DRBC standard for all samples with concentrations of 10^2, 10^4, and 10^6 CFU/mL or CFU/g. The samples that were not inoculated still showed positive results on the BAX in replicates where plate counts were determined as below the limit of quantification of the method (i.e. < 2 CFU/mL). This difference could be the result of the BAX enrichment step allowing injured cells to recover or the presence of fungi at levels below the limit of detection of the plating method.

Bleve et al. (2003) was able to detect as low as 10 CFU/mL with their qRT-PCR protocol via the standard curve developed in that study. This could not be achieved with the qPCR protocol developed in this study as the standard curve could not detect low concentrations of fungi within the 40 cycle amplification process used. They tested their assay in yogurt,

fruit juice, and fruit preserves. They noted lower assay sensitivity due to the extensive sample handling and processing required to process the yogurt in comparison to the other samples tested.

3.6 Survey of Assorted Dairy Products and Fungal Quantification Methods

A total of 38 samples were collected from three local supermarkets and tested using the developed method, standard DRBC plate count method, and the commercially available rapid methods. Table 3.10 displays the qualitative results from the Hygiena Qualicon BAX System PCR Assay for Yeast and Mold and the quantitative results from the DRBC plate counts, 3M Rapid Yeast and Mold Petrifilm plate counts, BIOTECON FoodProof Yeast and Mold Quantification LyoKit – 5'Nuclease- RP, and the developed qPCR protocol using EF1α primers. The samples correlate to a specific product type, which can be found the Appendix.

Table 3.10 Dairy Survey Results with 38 assorted dairy samples

Sample ID	DRBC Plate Counts (CFU/mL)	3M Rapid Yeast and Mold Counts (CFU/mL)	BIOTECON Quantification (CFU/mL)	UNL Method Quantification (CFU/mL)	BAX (Positive or Negative)
1	< 1	1.40×10^{1}	4.62×10^{2}	-	Positive
2	1.09×10^{2}	6.1×10^{1}	-	-	Positive
3	< 2	< 2	-	7.75×10^{3}	Negative
4	1.58×10^{6}	1.69×10^{6}	4.53×10^{1}	-	Positive
5	< 2	< 2	3.54×10^{4}	4.45×10^{2}	Negative
6	< 2	< 2	-	4.45×10^{1}	Negative
7	1.63×10^{2}	< 2	3.77×10^{3}	2.37×10^{3}	Positive
8	6.90×10^{5}	9.40×10^{5}	3.32×10^{12}	1.98×10^{5}	Positive
9	8.50×10^{5}	7.10×10^{5}	5.81×10^{5}	5.85×10^{2}	Positive

10	< 2	< 2	-	1.15×10^3	Positive
11	< 2	< 2	2.07×10^7	9.21×10^1	Negative
12	4.20×10^6	3.30×10^6	2.16×10^4	3.01×10^6	Positive
13	< 2	< 2	-	1.09×10^2	Negative
14	1.00×10^2	< 2	-	-	Positive
15	< 2	< 2	-	-	Negative
16	2.50×10^1	2.60×10^1	1.88×10^1	1.96×10^2	Positive
17	< 2	< 2	-	-	Negative
18	< 2	< 2	9.31×10^4	-	Positive
19	4.20×10^4	3.80×10^4	-	-	Positive
20	2.00×10^0	1.00×10^0	-	-	Positive
21	1.65×10^2	2.30×10^2	7.23×10^2	-	Positive
22	< 2	< 2	-	4.53×10^2	Negative
23	< 2	< 2	-	1.12×10^2	Negative
24	< 2	< 2	1.02×10^5	-	Positive
25	3.90×10^3	4.30×10^3	3.54×10^9	6.12×10^2	Positive
26	1.00×10^3	3.70×10^2	2.32×10^{12}	4.25×10^3	Positive
27	< 2	< 2	4.60×10^1	-	Negative
28	< 2	< 2	-	-	Negative
29	2.00×10^0	< 2	-	-	Positive
30	< 2	< 2	2.54×10^3	2.98×10^2	Negative
31	1.00×10^0	< 2	-	-	Negative
32	< 2	< 2	-	-	Negative
33	< 1	< 1	-	-	Negative
34	< 2	< 2	6.86×10^3	-	Negative
35	< 2	1.00×10^0	1.70×10^0	-	Positive

36	3.40×10^3	5.7×10^3	1.21×10^6	-	Positive
37	< 2	< 2	-	-	Negative
38	< 2	< 2	2.24×10^2	-	Positive

Out of the 38 samples, 21 were positive for the presence of yeast and mold according to the BAX PCR assay. The action level used for these samples was 25 CFU/g of sample. There were five particular positive BAX samples (2, 14, 19, 20, and 29) that exhibited no amplification on the other PCR platforms (BIOTECON and EF1α) and four particular positive BAX samples (10, 18, 24, and 38) that exhibited no growth on the plate count methods (DRBC and 3M Rapid Yeast and Mold Petrifilm). A possible explanation for this observation in the survey could be due to the enrichment step associated with the BAX PCR Assay for Yeast and Mold protocol allowing for any present injured cells to recover. 20 of the 38 samples tested were amplified enough to have fungal load quantifications on the BIOTECON PCR assay. The EF1α PCR was able to amplify enough DNA to have fungal load quantifications for 16 of the 38 samples used in the survey.

Among the 38 samples evaluated, 18 were able to be quantified using the plate counting methods (DRBC and 3M Rapid Yeast and Mold Petrifilm). While some samples were detected only via plate counts, others were detected only by quantitative PCR methods. There were 11 samples that were able to be plate counted, but had no associated quantitative PCR result (BIOTECON or EF1α). This lack of amplification on either PCR protocol for any of these samples could be the result of the fungi present in those samples not matching up well with the primers present in either reaction set (BIOTECON or EF1α).

There were several instances of overestimation and underestimation of the fungal load associated with a given sample when using the standard curve method opposed to traditional plate counting methods. Discrepancies in the fungal load quantified using the developed standard curve and the

fungal load determined via plate counts could potentially result from a variety of the following factors: amplification of dead DNA that would suggest higher counts than what is present, any fungi found in samples have sequence variation form the ef1-α primers used, present fungi could be viable but nonculturable (VBNC), or inhibition in the PCR due to the presence of proteins, carbohydrates, or lipids in the sample.

CHAPTER 4. CONCLUSIONS

4.1 Development of Primers for qRT-PCR and qPCR Protocols

Of the designed primers in this study, the primers corresponding to the elongation factor 1-α (EF1α) target were the most amenable to the qRT-PCR and qPCR applications. The other primers designed could be used as more specific primers depending on the target organism of interest or overhauled to target other organisms of high importance in the spoilage of food products.

4.2 Development of qRT-PCR Protocol for Fungal Detection

The use of RNA-based technologies for the rapid detection and quantification of yeasts and molds that are potentially present in dairy commodities was a possibility. However, at this time, the extraction and quantification are too variable and time consuming to allow the design of a practical rapid detection and quantification method that could be used by minimally trained personnel.

4.3 Development of qPCR Protocol for Fungal Detection

The method for rapid fungal detection and quantification was based on DNA extraction as opposed to RNA extraction. DNA based technology is more reliable and less time consuming than the RNA based technology. The DNA extraction listed in Harju et al. (2004) with the addition of BIOTECON's Reagent D was utilized to produce a replicable method that could be used for the detection of *Galactomyces candidus* in culture material

and in dairy matrices. This process is less time consuming than the RNA based method and was more reproducible, which is more amenable to process development and usage for minimally trained personnel.

4.4 Standard Curve Development for qPCR Protocol

A standard curve was developed for the EF1-α qPCR protocol using *Galactomyces candidus* as the model organism. The equation of the developed standard curve is: **$y = -2.54x + 43.14$** with an R^2 value of 0.9647. This equation was used to approximate the fungal load of a sample based on the Ct value associated with that sample.

4.5 Validation of Developed Method in Dairy Matrices

Using DRBC plating as the "gold standard", the 3M petrifilm plate counts were the most comparable at all inoculum levels in the culture material and dairy matrices. The current standard curve for the EF1-α qPCR and the BIOTECON PCR methodology currently overestimates or underestimates the fungal load present in a sample compared to the standard DRBC plate counts and the commercially available 3M Rapid Yeast Mold Petrifilms. The BIOTECON method works well for the 10^2 and 10^0 levels. The developed method works wells for the 10^6 and 10^2 levels. The BAX Yeast and Mold PCR assay is a good diagnostic tool that can be used to determine the quality of a sample, but it does not give an approximate load. The threshold that was tested in this study was 25 CFU/g, so any positive samples using the BAX assay contained at least 25 CFU/g fungal organisms.

4.6 Survey of Assorted Dairy Products and Fungal Quantification Methods

The developed EF1α PCR was able to amplify DNA and approximately quantify the fungal load of 16 samples out of the 38 tested samples. Plating on both standard DRBC and 3M Rapid Yeast and Mold Petrifilm was able to quantify fungi from 18 samples. The BIOTECON PCR was capable of amplifying DNA and estimating the fungal load of 20 samples. The BAX PCR assay for Yeasts and Molds was able to determine presence of fungi in 21 of the 38 samples used. The developed PCR method can be used to detect and estimate the potential fungal load present in a dairy sample depending on the fungi present in the sample. However, the method currently may either overestimate or underestimate the fungi present in a sample due to limitations in the method.

5. RECOMMENDATIONS FOR FUTURE RESEARCH

According to the observations made in this study, some recommendations can be made for future work regarding the development of rapid detection and quantification methods for fungi in food matrices:

1. Developing new primers that are able to detect and quantify more fungal species and consistently predict a given fungal load present in a sample at a given Ct range (i.e. samples containing 10^6 CFU/g will all have a Ct value of 18).
2. Further assessment of DNA binding agents (reagent, concentration, and processing time) to optimize live and dead cell differentiation.
3. Development of a standardized RNA extraction protocol that minimizes variance and reduces processing time.
4. Developing primers that are able to detect other organisms of interest in dairy such as *Coxiella burnetii* to assess milk quality and processing quality (i.e. pasteurization conditions).
5. Applying the developed qPCR fungal quantification method into other foodstuffs that are susceptible to fungal spoilage (i.e. grains).

References

3M™ Petrifilm™. 2013. Rapid Yeast and Mold Count Plates.

Addis, E., Fleet, G.H., Cox, J.M., Kolak, D. and Leung, T. 2001. The growth, properties, and interactions of yeasts and bacteria associated with the maturation of Camembert and blue-veined cheeses. International Journal of Food Microbiology. 69:25-36.

Bacteriological Analytical Manual, 2001. Chapter 18 Yeasts, Molds and Mycotoxins. 8th Edition.

Banjara, N., Suhr, M.J., Hallen-Adams, H.E. 2015. Diversity of Yeast and Mold Species from a Variety of Cheese Types. Current Microbiology, 70 (6): 792-800.

Barbas III, C.F., Burton, D.R., Scott, J.K., and Silverman, G.J. 2007. Quantitation of DNA and RNA. Cold Spring Harbor Protocols. Doi: 10.1101/pdb.ip47.

Bayless, T.M. and Rosensweig, N.S. 1966.A Racial Difference in Incidence of Lactase Deficiency. Journal of the American Medical Association. 197(12):968-972.

Bersaglieri, T., Sabeti, P.C., Patterson, N., Vanderploeg,T., Schaffner, S.F., Drake, J.A., Rhodes, M., Reich, D.E., and Hirschhorn, J.N. 2004. Genetic Signatures of strong recent positive selection at the lactase gene. American Journal of Human Genetics. 74(6):1111-1120.

BIOTECON Diagnostics GmbH. Reagent D. 2016.

Bleve, G., Rizzotti, L., Dellaglio, F., and Torriani, S. 2003. Development of reverse transcription (RT)-PCR and real time RT-PCR assays for rapid detection and

quantification of viable yeasts and molds contaminating yogurts and pasteurized food products. Applied and Environmental Microbiology, 69:4116-4121.

Buchanan, D.S. 2002. *Dairy animals: major Bosengal breeds. In* H. Roginski, J.W. Fuquay and P.F. Fox, eds. *Encyclopedia of dairy sciences, Vol. 2*, pp. 559–568. London, Academic Press, London.

Bulut Solak, B. and Akin, N. 2012. Functionality of whey protein. *Int. J. Health Nutr.*, 3(1): 1–7.

Bustin, S.A. 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. Journal of Molecular Endocrinology. 29: 23-39.

Buzby, J., Wells, H.F., Hyman, J. 2014. The Estimated Amount, Value, and Calories of Postharvest Food Losses at the Retail and Consumer Levels in the United States. USDA, Economic Research Service, February 2014.

Cappa, F. and Cocconcelli, P.S. 2001. Identification of fungi from dairy products by means of 18S rRNA analysis. International Journal of Food Microbiology. 69:157-160.

Cox, M., Lehninger, A.L., and Nelson, D.R. 2000 Lehninger Principles of Biochemistry. Worth Publishers.

Craven, H.M., Broome, M.C., Chandler, R.E. and Jensen, N. 2001. Dairy Products. In Moir, C.J., Andrew-Kabilafkas, C., Arnold, G., Cox, B.M., Hocking, A.D., and Jenson, I. (Eds) Spoilage of Processed Foods: Causes and Diagnosis. AIFST Inc. NSW Branch. 147-163.

Curry, A. 2013. The Milk Revolution. Nature, 500: 20-22.

Dall'Asta, C., Lindner, J.D., Galaverna, G., Dossena, A., Neviani,E. and Marchelli, R. 2008. The occurrence of ochratoxin A in blue cheese. Food Chemistry, 106:729-734.

Deepak, S.A., Kottapalli, K.R., Rakwal, R., Oros, G., Rangappa, K.S., Iwahashi, H., Masuo, Y., and Agrawal, G.K. 2007. Real-Time PCR: Revolutionizing Detection and Expression Analysis of Genes. Current Genomics. 8: 234-251.

DuPont Nutrition and Health. 2013. BAX System PCR Assay for Yeast and Mold.)

Engel, G. and Teuber, M. 1991. Heat resistance of ascospores of *Byssochlamys nivea* in milk and cream. International Journal of Food Microbiology. 12:225-234.

FAO. 1972. *Milk and milk products in human nutrition,* by S.K. Kon. FAO Nutritional Studies no.27, FAO, Rome.

FAO. 2002. *Partnership formed to improve cassava, staple food of 600 million people.* FAO news article.

FAO and WHO. 1995a. *Codex general standard for contaminants and toxins in food and feed*. Codex Alimentarius. CODEX-STAN 193-1995. Rome.

FAO and WHO. 2010. *Interim summary of conclusions and dietary recommendations on total fat & fatty acids.* From the Joint FAO/WHO Expert Consultation on Fats and Fatty Acids

FAOSTAT. 2012. FAO statistical database.

Finoli, C., Vecchio, A., Galli, A., and Franzetti, L. 1999. Production of cyclopiazonic acid by molds isolated from Taleggio cheese. Journal of Food Protection. 62:1198-1202.

Finoli, C., Vecchio, A., Galli, A., and Dragoni, I. 2001. Roquefortine C occurrence in blue cheese. Journal of Food Protection. 64:246-251.

Foschino, R., Garzaroli, C., and Ottogali, G. 1993. Microbial contaminants cause swellin and inward collapse of yoghurt packs. Lait 73:395-400.

Fox, P.F. 2008. Milk: an overview. *In* A. Thompson, M. Boland & H. Singh, eds. *Milk Proteins: from expression to food*, pp. 1–54. San Diego, CA, USA, Academic Press.

Frölich-Wyder, M.T. 2003. Yeasts in dairy products. In Boekhout, T. and Robert, V. (Eds). Yeasts in Food. Cambridge, UK. Woodhead Publishing. 209-237.

Fulgoni, V., III, Nichols, J., Reed, A., Buckley, R., Kafer, K., Huth, P., DiRienzo, D. and Miller, G.D. 2007. Dietary consumption and related nutrient intake in African-American adults and children in the United States: continuing survey of food intakes by individuals 1994–1996, 1998, and the National Health and Nutrition Examination Survey 1999–2000. *J. Am. Diet. Assoc.*, 107:256–264.

García, T., Mayoral, B., González, I., López-Calleja, I., Sanz, A., Hernández, P.E., and Martín, R. 2004. Enumeration of Yeasts in Dairy Products: A Comparison of Immunological and Genetic Techniques. Journal of Food Protection. Vol.67, No. 2: 357-364

Goswami, R.S., Xu, J-R., Trail, F., Hilburn, K., and Kistler, H.C. 2006. Genomic analysis of host-pathogen interaction between *Fusarium graminearum* and wheat during early stages of disease development. Microbiology. 152:1877-1890.

Grade "A" Pasteurized Milk Ordinance, Including Provisions from the Grade "A" Condensed and Dry Milk Products and Condensed and Dry Whey—Supplement I to the Grade "A" Pasteurized Milk Ordinance. Public Health Service/Food and Drug Administration. 2015.

Gropper, S.S. and Smith, J.L. 2012. Advanced Nutrition and Human Metabolism. Cengage Learning.

Gueguen, M. 1988, Moisissures responsables de defauts d'affinage en fromagerie (à l'exclusion des Mucoraceae). Microbiol. Aliments Nutr. 6:31-35.

Hallen, H.E., Huebner, M., Shiu, S-H., Güldener, U., and Trail, F. 2007. Gene expression shifts during perithecium development in Gibberella zeae (anamorph Fusarium graminearum), with particular emphasis on ion transport proteins. Fungal Genetics and Biology. 44 (2007): 1146-1156.

Harju, S., Fedosyuk, H., and Peterson, K.R. 2004. Rapid Isolation of Yeast Genomic DNA: Bust n'Grab. BioMed Central. 4:8

Havranek, J.L., Hadžiosmanović, M. 1996. Shelf life as a requirement for quality of milk products. Mljekarstvo, 3: 197-206.

He, F.J., and MacGregor, G.A. 2008. Beneficial Effects of Potassium on Human Health. Physiologia plantarum, 133(4): 725-735.

Health Protection Agency. Guidelines for Assessing the Microbiological Safety of Ready-to-Eat foods. London: Health Protection Agency, November 2009.

Hernandez-Ledesma, B., Ramos, M. & Gomez-Ruiz, J.A. 2011. Bioactive components of ovine and caprine cheese whey. *Small Ruminant Res.,* 101: 196–204.

Haug, A., Hostmark, A.T. and Harstad, O.M. 2007. Bovine milk of human nutrition – a review. *Lipids Health Dis.,* 6:25–41.

Hocking, A.D. and Faedo, M. 1992. Fungi causing thread mould spoilage of vacuum packaged Cheddar cheese during maturation. International Journal of Food Microbiology. 16:123-130.

Horwood, J. F., Stark, W., Hull, H. H. 1987. A fermented, yeasty 82engal defect in Cheddar cheese. Australian Journal of Dairy Technology, 42: 25-26.

Hutkins, R.W. 2006. Microbiology and Technology of Fermented Foods. Blackwell Publishing.

IARC. 1993. Vol. 56, *Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines, and mycotoxins*. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans

Kanwar, J.R., Kanwar, R.K., Sun, X., Punj, V., Matta, H., Morley, S.M., Parratt, A., Puri, M. and Sehgal R. 2009. Molecular and biotechnological advances in milk proteins in relation to human health. *Curr. Protein Pept. Sci.,* 10: 308–338.

Kerstetter, J.E., O'Brien, K.O., and Insogna, K.L. 2003. Low Protein Intake: The Impact on Calcium and Bone Homeostatis in Humans. Journal of Nutrition, 133(3): 855S-861S.

Kinderlere, J.L. and Hatton, P.V. 1990. Fungal Metabolites of sorbic acid. Food Addit.Contam. 7:657-669.

Kokkonen,M., Jestoi, M., and Rizzo, A. 2005. Determination of selected mycotoxins in mould cheeses with liquid chromatography coupled to tandem with mass spectrometry. Food Addit. Contam. 22:449-456.

Korhonen, H. 2009. Bioactive milk proteins and peptides: from science to functional

applications. *Aust. J. Dairy Technol.,* 64: 16–25.

Kulie, T., Groff, A., Redmer, J., Hounshell, J., and Schrager, S. 2009. Vitamin D: An Evidence-Based Review. Journal of the American Board of Family Medicine, 22:698-706.

Ledenbach, L.H., Marshall, R.T. 2009. Microbiological Spoilage of Dairy Products. In: W.H. Sperber, M.P. Doyle (Eds.), Compendium of the Microbiological Spoilage of Foods and Beverages, Springer, LLC.

López-Dias, T.M., Román Blanco, C., García Arias, M.T., García Fernández, M.C., and García-López, M.L. 1996. Mycotoxins in two Spanish cheese varieties. International Journal of Food Microbiology.30:391-395.

Maraz, A., Kovacs, M. 2014. Food Spoilage by Cold-Adapted Yeasts. In: Buzzini, P., Margesin, R. (Eds.), Cold- Adapted Yeasts, Springer.

Marcellino, N. and Benson, D.R. 1992. Scanning Electron and Light Microscopic study of microbial succession on Bethlehem St. Nectaire cheese. Applied and Environmental Microbiology. 58:3448-3454.

Marsh, A.J., O'Sullivan, O., Hill, C., Ross, R.P., and Cotter, P.D. 2013.Sequencing-Based Analysis of the Bacterial and Fungal Composition of Kefir Grains and Milks from Multiple Sources. PloS ONE, 8(7): e69371.

Marth, E.H. 1978. Dairy Products in Beuchat, L.R. (Ed) Food and Beverage Mycology. AVI Publishing Co. 145-172.

Martinon, A., Cronin, U.P., Quealy, J., Stapleton, A., and Wilkinson M.G. 2012. Swab sample preparation and viable real-time PCR methodologies for the recovery of

Escherichia coli, *Staphylococcus aureus* or *Listeria monocytogenes* from artificially contaminated food processing surfaces. Food Control, 24: 86-94.

Mayoral, M.B., Martin, R., Sanz, A., Hernandez, P.E., Gonzalez, I., and García, T. 2005. Detection of Kluyveromyces marxianus and other spoilage yeasts in yogurt using a PCR-culture technique. International Journal of Food Microbiology, 105: 27-34.

Mayoral, M.B, Martín, R, Hernández, P.E. González, I., and García, T. 2006. A Reverse Transcriptase PCR Technique for the Detection and Viability Assessment of *Kluyveromyces marxianus* in Yogurt. Journal of Food Protection, Vol. 69 No.9: 2210-2216.

Michaelsen, K.F., Nielsen, A.-L.H; Roos, N., Friis, H. and Mølgaard, C. 2011a. Cow'smilk in treatment of moderate and severe undernutrition in low-income countries. *In* R.A. Clemens, O. Hernell, K.F. Michaelsen, eds. *Milk and milk products in human nutrition,* pp. 99–111. Basel, Switzerland, S. Karger AG; Vevey, Switzerland, Nestle Nutrition Institute.

Minervini, F., Montagna, M.T., Spilotros, G., Monaci, L., Santacroce, M.P., Visconti, A. 2001. Survey on mycoflora of cow and buffalo dairy products from Southern Italy. International Journal of Food Microbiology, 69: 141-146.

Nafisi, S., Saboury, A.A., Keramat, N., Neault, J-F., Tajmir-Riahi, H-A. 2007. Stability and structural features of DNA intercalation with ethidium bromide, acridine orange and methylene blue. Journal of Molecular Sructure. 827:35-43.

Nocker, A., Sossa-Fernandez, P., Burr, M.D., and Camper, A.K. 2007. Use of Propidium Monoazide for Live/Dead Distinction in Microbial Ecology. Applied and Environmental Microbiology. 73(16): 5111-5117.

Northolt, M.D., van Egmond, H.P., Soentoro, P. and Deijll, E. 1980. Fungal growth and the presence of sterimatocystin in hard cheese. J. Assoc. Off. Anal. Chem. 63:115-119.

Pitt, J.I. and Hocking, A.D. 2009. Fungi and Food Spoilage. Springer

Powers, H.J. 2003. Riboflavin (Vitamin B-2) and Health. The American Journal of Clinical Nutrition, 77:1352-1360.

Richter, R.L. and Vedamuthu, E.R. 2001. Milk and Milk Products. In: Downes, F.P. and Ito, K (Eds). Compendium of Methods for the Microbiological Examination of Foods. American Public Health Association.

Roffet-Salque, M., Bogucki, P.I., Pyzel, J., Sobkowiak-Tabaka, I., Grygiel, R., Szmyt, M, and Evershed, R.P. 2013. Earliest evidence for cheese making in the sixth millennium BC in northern Europe. Nature, 493:522-525.

Sensidoni, A., Rondinini, G., Peressini, D., Maifreni, M., and Bortolomeazzi, R. 1994. Presence of an off-flavour associated with the use of sorbates in cheese and margarine. Ital. J. Food Sci. 6: 237-242.

Siemens, K. and Zawistoski, J. 1993. Occurrence of PR imine, a metabolite of *Penicillium roqueforti*, in blue cheese. Journal of Food Protection. 56:317-319, 325.

Sommer, A. 2008. Vitamin A Deficiency and Clinical Disease: An Historical Overview. Journal of Nutrition, 138: 1835-1839.

Stabler, S.P. 2013. Vitamin B_{12} Deficiency. New England Journal of Medicine, 368:149-160.

Stanton, C., Ross, R.P., Fitzgerald, G.F., and Van Sindern, D. 2005. Fermented functional foods based on probiotics and their biogenic metabolites. Current Opinions Biotechnology, 16:198-203.

USDA. 2009. *USDA national nutrient database for standard reference.*

Vaitilingom, M., Gendre, F., Brignon, P. 1998. Direct Detection of viable bacteria, molds, and yeasts by reverse transcriptase PCR in contaminated milk samples after heat treatment. Applied and Environmental Microbiology, 64:1157-1160.

Vivier, D., Rivemale, M., Reverbel, J.P., Ratomahenina, R., Galzy, P. 1994. Study of the growth of yeasts from Feta cheese. International Journal of Food Microbiology, 22: 207-215.

Walker, A.F., ed. 1990. *Applied human nutrition for food scientists and home economists*. Chichester, West Sussex, UK, Ellis Horwood.

Walker, H.W. and Ayres, J.C. 1970. Yeasts as spoilage organisms. In Rose, A.H., and Harrison, J.S. (Eds) The Yeasts Vol.3: 500-527. Academic Press.

WHO, FAO & UNU. 2007. *Protein and amino acid requirements in human nutrition*. Report of a Joint WHO, FAO and UNU Expert Consultation, WHO Technical Report Series 935.

Wijesinha-Bettoni, R. and Burlingame, B. 2013. Milk and Dairy Product Composition. In Muehlhoff, E., Bennett, A., and McMahon, D. (Eds). Milk and Dairy Products in Human Nutrition. FAO.

Wilt, T.J., Shaukat, A., Shamliyan, T., Taylor, B.C., MacDonald, R., Tacklind, J., Rutks, I., Schwarzenberg, S.J., Kane, R.L., and Levitt, M. 2010. Lactose intolerance and health. Evidence Reports/Techonology Assessments, No.192. Rockville, MD, USA. Agency for Healthcare Research and Quality

Appendix

Sample ID	Product
1	Vitamin D Milk (Missouri, USA)
2	Vanilla Yogurt (New York, USA)
3	All Natural Sour Cream (Missouri, USA)
4	Lowfat Small Curd Cottage Cheese (Arkansas, USA)
5	Low Moisture Part Skim Finely Shredded Mozzarella Cheese (Arkansas, USA)
6	Finely Shredded Mild Cheddar Cheese (Arkansas, USA)
7	Finely Shredded Swiss Cheese (Iowa, USA)
8	Shredded Gouda Cheese (Iowa, USA)
9	Shredded Colby Jack Cheese (Iowa, USA)
10	Finely Shredded Low Moisture Part Skim Mozzarella Cheese (Iowa, USA)
11	Finely Shredded Mild Cheddar Cheese (Iowa, USA)
12	All Nautral Sour Cream (Iowa, USA)
13	Plain Greek Yogurt (Iowa, USA)
14	Small Curd Cottage Cheese (Iowa, USA)
15	Low Fat Vanilla Yogurt (Iowa, USA)
16	2% Reduced Fat Ultra Filtered Milk (Illinois, USA)
17	Original French Vanilla Yogurt (Minnesota, USA)
18	Pure and Natural Sour Cream (Texas, USA)

19	Nonfat Plain Greek Yogurt (New York, USA)
20	Plain Nonfat Greek Yogurt (New York, USA)
21	Vanilla Yogurt (New York, USA)
22	Finely Shredded Low Moisture Part Skim Mozzarella (Wisconsin, USA)
23	Finely Shredded Cheddar Cheese (Wisconsin, USA)
24	Shredded Low-Moisture Part Skim Mozzarella (Illinois, USA)
25	Shredded Colby and Monterey Jack Cheeses (Illinois, USA)
26	Finely Shredded Reduced Fat Mild Cheddar (Illinois, USA)
27	Shredded Swiss Cheese (Illinois, USA)
28	Fancy Shredded Monterey Jack Cheese (Kansas, USA)
29	Shredded Colby/Jack Cheese (Kansas, USA)
30	Rich and Creany Sour Cream (Kansas, USA)
31	Lowfat Vanilla Yogurt (Kansas, USA)
32	Nonfat Plain Greek Yogurt (Kansas, USA)
33	Cultured Low Fat Buttermilk (Missouri, USA)
34	Sour Cream (Missouri, USA)
35	Small Curd Cottage Cheese (Missouri, USA)
36	Fancy Shredded Swiss Cheese (Kansas, USA)
37	Vanilla Greek Yogurt (Minnesota, USA)
38	Cream Top Whole Milk Vanilla Yogurt (New Hampshire, USA)