AN ABSTRACT OF THE THESIS OF

Kaitlyn Ruth Kornberg for the degree of Master of Science in Food Science and Technology presented on March 18, 2020

Title: Development of a High Throughput Method for Screening of Clean-Label Mold Inhibitors in Cheddar Cheese.

Abstract approved: ____

Christopher D. Curtin

Mold growth on cheddar-style cheese, though not a food safety issue, causes significant loss to the industry due to consumer rejection. For this reason, it is common for at-risk products, such as cheese shreds, to be coated with the mold inhibitor natamycin. While highly effective at extending shelf-life, preferences for clean-labels and organic foods is driving renewed interest in alternative mold inhibitors for the dairy industry.

The purpose of this study was to develop a high-throughput method and use this to quantify mold grow on a cheese-mimicking matrix as a means to screen candidate clean-label inhibitors.

In order to evaluate mold-growth at this scale, we adopted image-analysis using freely available ImageJ software (NIH) and the “read plate” plug-in, wherein changes in mold growth are measured using the lightness/darkness of pixels as a proxy for growth. Sensitivity of mold-growth detection was enhanced by incorporating methylene-blue dye into the cheese-mimicking

agar. In order to better apply results to the food science industry, the "Growthcurver" package from R statistical software was used to calculate lag time, the time-point at which the mold has reached 50% maximum growth. This metric allowed us to compare delays in growth to the control and quickly assess inhibitor efficacy.

To demonstrate the power of this approach, using a cheese-based agar, over eighty 96-well micro-titer cheese agar plates were prepared for inhibitor screening across the three proof of concept studies. Final results yielded analysis of 7,200 samples against varying concentrations of 7 inhibitors and combinations thereof. The results confirm the efficacy of natamycin, while alternative inhibitors caused less pronounced growth delays even at relatively high rates of application. Thyme oil and chitosan showed the greatest potential as alternative mold inhibitors. Thyme oil caused a delay in growth beyond the length of the experiment (168 hrs) for 5 of the 10 mold isolates. Chitosan showed a similar delay for 7 of the molds tested.

Future work should focus on analysis of additional inhibitor combinations and incorporate sensory analysis to evaluate their applicability for the dairy industry.

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Development of a High Throughput Method for Screening of Clean-Label Mold Inhibitors in Cheddar Cheese

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Kaitlyn Ruth Kornberg, Author

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TABLE OF CONTENTS

Page

1 INTRODUCTION 1

2 LITERATURE REVIEW 3

2.1 Cheese production 3

2.2 Microbial spoilage of cheese products 7

2.2.1 Bacterial impacts on cheese quality 8

2.2.2 Fungal spoilage 10

2.3 Strategies to mitigate microbial spoilage of food products 12

2.3.1 Physical treatments used in the dairy industry 14

2.3.2 Chemical and biological treatments 14

2.3.2.1 Organic acids and acidification 14

2.3.2.2 Microbial produced complex inhibitors 17

2.3.2.2.1 Natamycin 18

2.3.2.2.2 Nisin 19

2.3.2.3 Unpurified fermentates 20

2.3.2.4 Plant derived inhibitors 21

2.3.2.4.1 Essential oils 21

2.3.2.4.2 Complex extracts: Celery Powder 23

2.3.2.5 Other inhibitors 24

2.3.2.5.1 Lactoferrin and lactoperoxidase 24

TABLE OF CONTENTS (Continued)

2.3.2.5.2 Chitosan 26

2.3.3 Clean-label hurdle technologies for control of molds 26

2.4 Approaches to evaluate efficacy of mold inhibitors 28

2.4.1 Experimental Design: challenge testing on product or lab analog 28

2.4.2 Quantification of mold growth 29

2.4.3 Classical microbiological methods 30

2.4.3.1 Analytical proxies for mold biomass 30

2.5 Summary and research objectives 31

3 MATERIALS AND METHODS 33

3.1 Chemicals 33

3.2 Mold culture preparation and maintenance 33

3.3 Preparation of inhibitor treatments 34

3.4 Shredded cheese shelf life study 34

3.5 Cheese agar 36

3.6 Radial Growth on proposed cheese mimicking matrix 37

3.7 Preliminary image analysis trials 37

3.8 Image analysis to distinguish levels of mold growth 38

3.9 Proof of concept experiments 38

3.10 Analysis with ImageJ/“Growthcurver” 40

TABLE OF CONTENTS (Continued)

4 Results 43

4.1 Efficacy of natamycin for extension of shredded cheese shelf life 43

4.2 Evaluation of proposed cheese-mimicking matrix 45

4.2.1 Radial hyphal growth of molds on cheese-agar 45

4.2.2 Improvement of mold visualization using methylene blue dye 47

4.3 Evaluation of image-analysis as a tool to monitor mold growth on cheese agar 49

4.3.1 Changes in grayscale spectra of images act as a suitable proxy for mold growth .. 49

4.3.2 Quantitative analysis of mold growth based upon grayscale pixel count 51

4.3.3 Generation of mold growth curves based upon grayscale pixel count 55

4.3.4 Proof of concept – inhibitor screening experiment 58

4.3.4.1 Evaluating methodological sources of variation 68

4.4 Repeatability of screening experiment 70

4.4.1 Potential for combinatorial treatments 72

4.5 Challenges with testing of clean-label inhibitors 75

4.5.1 Inhibitor induced color change 75

5 Discussion 79

5.1 Conventional strategies to protect shredded cheese from mold spoilage are highly effective 79

5.2 Image analysis can be used to screen for mold inhibitors in a cheese-mimicking matrix 80

TABLE OF CONTENTS (Continued)

5.3 Clean label inhibitors show promise as label-friendly alternatives to natamycin 84

5.4 Conclusions and future directions 86

6 References 88

7 Appendix 1: Supplementary figures 102

LIST OF FIGURES

Figure Page

1. Schematic of conventional cheese making production ……………………...…….4

2. Schematic of current preservation technologies……………………….………....13

3. Development of mold growth on shredded cheddar cheese with or without the addition of natamycin ……………………...……………….……….....44

4. Mold radial growth on standard lab media (PDA) and cheese agar in response to natamycin ………………..……………………………..……….46

5. Mold radial growth on cheese agar and cheese agar with the addition of 0.0001% methylene blue ……………………..……..…………...………………………….48

6. Grey scale spectra of *Penicilium* and *Geotrichum* molds grown on cheese agar and blue cheese agar after 72 hours of growth …….……………………..…….……50

7. Evaluation of image analysis for distinction of mold growth levels ……..…….…….52

8. Evaluation of the ImageJ "ReadPlate" plugin for analysis of white and blue mold growth …………………………………………….…………..…..54

9. Time lapse photo series and growth curves plotted by "Growthcurver" R package …………………… ………..………..………..……56

10. Differential responses of molds A, B, and C to increasing concentrations of thyme oil… ……………………..……...………………………....59

LIST OF FIGURES (Continued)

Figure Page

11. Heats maps of principle proof of concept experiment on chitosan, clove oil, thyme oil, and natamycin …………………………..……………...….62

12. Evaluating the coefficient of variation for controls of the proof of concept experiment …………………………………………..…….…..…..…..65

13. Coefficient of variation on lag time for molds A, B, and C……………………...…67

14. Evaluation region of interest diameter for impact on lag time estimates………..….69

15. Repeatability of inhibitor screening experiments……………………………….…..…71

16. Plots of lag times for a subset of combinatorial data…………………..……………73

17. The impact of Microgard® on color changes in blue cheese agar over 168 hour……………………………………………………………..…...…76

18. The impact of lactoferrin on color changes in blue cheese agar over 168 hours……….……………………………..…………………...…78

A1. Time lapse photo series of time-to-mold on bags of shredded cheese….....………102

A2. Heats maps of secondary proof of concept experiment on chitosan, thyme oil, and combinatorial treatments…….………………………………103

LIST OF TABLES

Table Page

1. Common spoilage fungi isolated from cheese……………………………..…….. 11

2. Ten mold isolates used in proof of concept experiments ……………………..…. 34

3. Inhibitor treatments, carrier solutions, and stock concentrations used in proof of concept screening experiments…………….….. 34

4. Treatments for shredded cheese shelf life study ………………..…..……...… 35

5. Treatments for first proof of concept experiment…………………………..……..41

6. Additional, combinatorial treatments for second proof of concept experiment……………………………………………...………….42

7. Treatments for third proof of concept experiment………………...……….…42

8. Different metrics for assessing growth of *Geotrichum* and *Penicillium*……… 57

1 INTRODUCTION

Among U.S. consumers, cheese is a vital component of the food system, impacting economic, social, and cultural ideas. As cultural shifts turn to industrial agriculture to meet rising food needs, food safety and quality become paramount to maintain large-scale food production. Despite good manufacturing practices, there are still major obstacles to reduce mold spoilage, pathogenic bacteria, and other microbial concerns. In the food industry, mold and fungal spoilage create serious financial damage, with 5-10% of all product loss due to fungal contamination (Garnier et al. 2017). Because cheese is a value-added product with additional labor, ingredient, and production costs, its spoilage significantly impacts creameries, particularly those operating on a smaller scale. Cheese-associated molds are typically non-mycotoxin producing organisms and are not a food safety concern, but contribute significantly to consumer rejection. Producers often turn to manufactured, commercial, and chemical preservatives due to the acid tolerant, psychotropic, and resistant nature of the fungi (Gross and Robbins 2000; Hassan et al. 2016).

In order to control mold spoilage in cheese, producers rely largely on natamycin, produced by *Streptomyces natalensis,* a non-dairy associated soil bacterium. Natamycin is typically applied to the surface of shredded cheese in combination with an anti-caking agent. Manufactured preservatives, such as natamycin, have a high efficacy against molds but, as preservatives, are often seen as unfavorable by the consumer (Asioli et al. 2017).

A 2017 Institute of Food Technologists (IFT) study showed that 68% of consumers are willing to pay more for a “clean label product”, while 53% indicated omitting certain ingredients is more important to them than including beneficial ones. In addition, a nearly 8% increase in “all natural” and “no artificial or additive ingredients” claims illustrates the popularity of the clean-label trend

(IFT 2017). This clearly demonstrates the need for cheese processors to reevaluate current practices to meet a changing market. Organic producers often rely on the naturally occurring antimicrobial properties of lactic acid bacteria found in the starter culture, as natamycin cannot be labeled as organic under the USDA guidelines (Dalié et al. 2010). These cultures are much more variable than natamycin with regards to their inhibition of mold growth, resulting in poor shelf life stability. Research into alternative, naturally occurring, anti-mycotic food additives has yielded promising results, but none have been applied widely on a commercial level across the dairy industry. Furthermore, most research has focused on efficacy of potential inhibitors in non-dairy matrices and is limited by current low-throughput methods for analysis of food products. This creates barriers for trialing larger numbers of inhibitors and combinations thereof.

As a result, this study focused on development of a high throughput technique using image analysis to quantify mold growth and screen naturally derived anti-mycotics against a panel of dairy mold isolates on a cheese-mimicking matrix. Using this technique, we demonstrated that grayscale pixel intensity, measured over time using high-resolution images, can be used as a proxy to quantify mold growth. When combined with the "Growthcurver" package and R statistical software, the lag time can be generated. For the purposes of this work, lag time is defined as the time at which molds reached 50% of growth, allowing for comparison of growth delay relative to controls. Mold isolates showed differential responses to treatments, with chitosan and thyme oil showing the greatest overall potential based upon the number of molds inhibited. Initial trials of combinatorial treatments show an additive effect on some molds and no impact on others, highlighting the importance of mold selection in light of differential responses to inhibitor treatments. Overall, using this new image analysis technique, potential mold inhibitors can be trialed on a large-scale to replace natamycin in dairy matrices.

2 LITERATURE REVIEW

2.1 Cheese production

In an effort to understand sources of microbial contamination, the history of cheese can provide a point of reference. Cheese production has a long history closely tied to the rise in agriculture, with estimates placing the origin of cheese production at 10,000 years old (Fox et al. 2017).

This long history has spawned many different types of cheese and production styles, ultimately seen in the microbial diversity of conventional cheese making today. Traditional cheese making did not relay on inoculated cultures and instead was influenced by the equipment choice (wood, clay, etc.), season, cow feed, climate, and more (Gaglio et al. 2016; Giello et al. 2017). In raw milk, hay feed favors *Streptococcus thermophilus, Lactococcus lactis,* and *Pseudomonas* sp. while silage has been shown to favor *Lactococcus lactis* and *Acinetobacter* sp. (Giello et al. 2017). These microbiota ultimately influence cheese production and have led to the wide array of cheese made today under conventional practices (Yeluri Jonnala et al. 2018). Mold-ripened cheeses have similarly been influenced by traditional cheese making techniques, with French cheese-makers establishing ideal conditions for wild inoculation of *Geotrichum candidum* on bloomy rind cheeses (Marcellino et al. 2001) and others favoring conditions for *Penicillium roqueforti* (Donnelly 2014). Even, today, for some “designation of origin” cheeses, wood tools are required, ultimately because they provide a diverse microbial inoculum, including some molds (Lortal et al. 2020).

In conventional cheese making practices, specific organisms are inoculated rather than harbored in the cheese making/milking equipment. Because most cheese makers in the U.S. operate using modern equipment, this work will focus on contemporary cheese making practices. See Figure 1 for a general schematic of cheese production in a large-scale dairy plant.

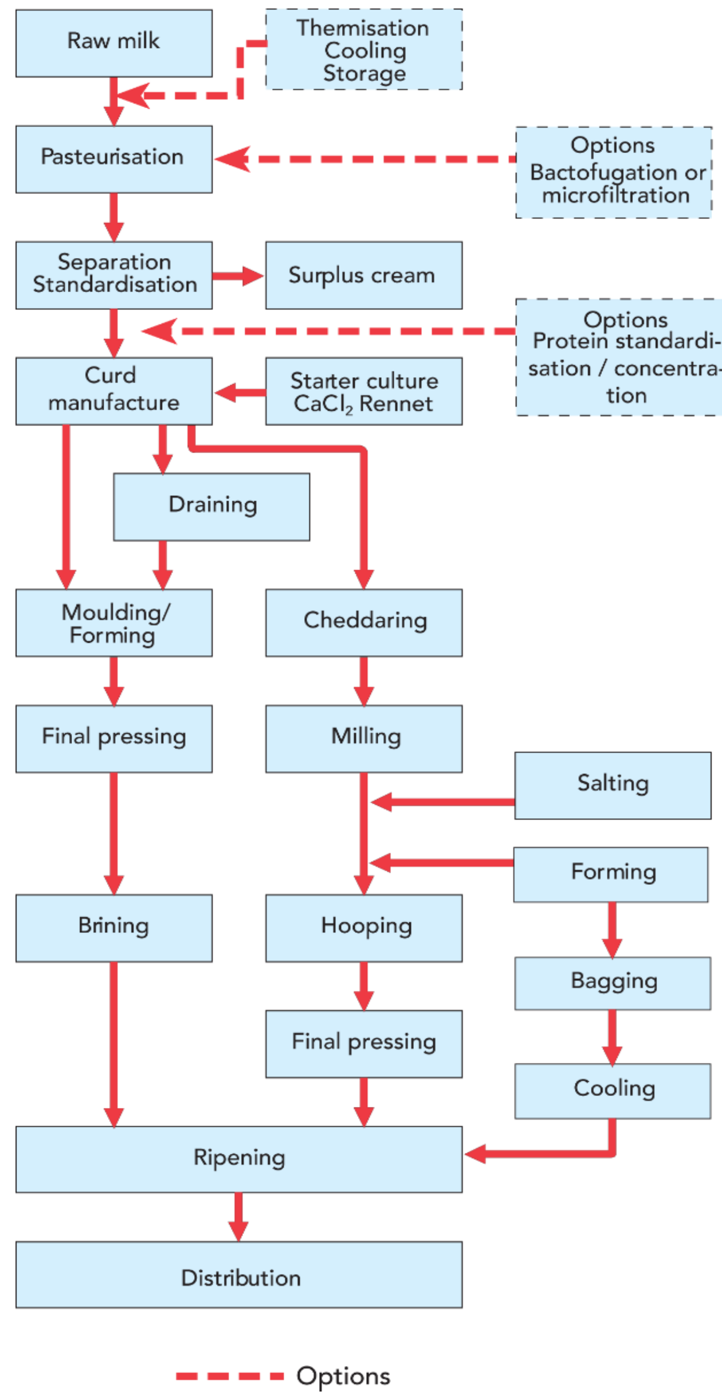


Figure 1: Schematic of conventional cheese making production. Adapted from the Tetrapak Cheese Processing Handbook.

Today, the cheese-making process begins at a food production plant equipped with modern, stainless steel equipment. Typically, raw milk is received at the production facility and is kept raw, heat treated, or pasteurized depending on the cheese style. Milk is then standardized using a microfiltration process or centrifugation to separate cream and skim milk (Kumar et al. 2013; Bylund et al. 2015) and components are recombined to create the desired fat level. This milk may be concentrated to increase solids and remove water (McNair et al. 2018a, El-Gazzar and Marth 1991), helping to mitigate later challenges of whey disposal.

Cheese-milk is then inoculated with a starter culture/acidifying agent to reduce the pH of the milk, followed by the addition of rennet, which begins the coagulation and curd formation process. Rennet is typically animal-derived and consists of the enzyme chymosin, though can be derived from plant-based sources, (Garg and Johri 1994) with recent technology allowing for creation of rennet from genetically modified bacteria, yeasts, and even fungi (Zumrut 2018). There is some evidence to indicate the source of rennet may impact textural properties of the cheese (García-Gómez et al. 2019), though it ultimately contributes to the breakdown of the casein micelle and subsequent curd formation (Garg and Johri 1994; Horne and Lucey 2017). Cheese producers vary several parameters to meet product specifications, including; the culture mixture, fermentation time, fermentation temperature, and humidity conditions. However, most cheeses contain a mixture of mesophilic, thermophilic, and adjunct cultures (Stadhouder 1991; Parente et al. 2017). Because these bacteria have different ideal conditions, they will grow and develop at different points in the fermentation process with the cheese maker manipulating environmental conditions to promote growth of the desired bacteria and subsequent flavor metabolites. Mesophilic bacteria will begin their growth cycle between 25-40° C, while thermophilic bacteria can survive at higher temperatures and can continue to grow in conditions up to 50°C (Bylund et al. 2015). These

bacteria typically bring flavors to the final product through the breakdown of protein (casein) and fat (Kindstedt 1993), while adjunct cultures, often *Lactobacillus* sp.*,* vary in their ideal temperature and add flavor to cheese through production of specific metabolites (Kindstedt 1993; Hong et al. 1998).

Upon inoculation, cheese is typically allowed to ferment until the pH has reached the desired level for the cheese style and ideal flavor development, then the curds are cut and the whey is drained. Processors may choose to reserve the whey for further processing into powders or discard it after treatment in a waste facility (Bylund et al. 2015).

This project focuses on cheddar cheese due to its popularity in the United States, with over 3.8 billion pounds produced in 2018 (Statista 2018). In order to make cheddar, after the initial curd cutting and removal of whey, it undergoes a cheddaring process to give it the characteristic cheddar flavor and texture (El-Gazzar and Marth 1991, Singh et al. 2003). In this process, the cheese is pressed into blocks, typically 40 lbs, and stacked such that the weight from the blocks forces the expulsion of more whey. The blocks are rotated until the optimal water activity has been reached and if desired, placed in an aging chamber for further flavor development. These blocks are then processed into smaller pieces, shredded, or sliced for retail (Bylund et al. 2015).

Other cheese styles may be shaped into rounds or blocks characteristic of their origin. Hard cheeses will undergo additional aging. Mold ripened cheeses, such as camembert, brie, and reblochon, will be inoculated with white molds such as *Geotrichum candidum* and *Penicillium camemberti*, (Oise Irlinger et al. 2015) and ripened in a humid environment. Others may be inoculated with *Penicillium roqueforti* to create blue cheese and give the cheese its characteristic flavor. Despite being intentionally inoculated for their desirable flavor characteristics (Hymery et al. 2014), for other cheese styles these molds are considered spoilage organisms (Wendorff et al. 1993). Because

of the complexity of cheese microbial communities, controlled inhibition poses a unique challenge for cheese makers. They must promote the growth of desired bacteria/molds, while inhibiting spoilage organisms, thus making selective inhibition a key component of cheese production.

2.2 Microbial spoilage of cheese products

In cheese, there are concerns of both pathogenic spoilage and quality spoilage. Pathogenic spoilage, largely associated with *Listeria monocytogenes*, *Escherichia coli,* and *Staphylococcus aureus* (Zottola and Smith 1991; Abdalla et al. 1993, Choi et al. 2016), present a public food safety risk due to their ability to cause severe illness in humans (Choi et al. 2016). Recalls are often associated with fresh or soft cheeses, with 2020 having a recall on a fresh cheese due to *E. coli* contamination and a semi-soft string cheese due to a *Listeria* risk (FDA 2020).

Mold growth on cheese is rarely hazardous to human health with reports indicating 1.8%-12.4% of molds samples from cheese having the ability to produce mycotoxins. Indeed, although cheese is a relatively strong substrate for mold growth, it is a poor substrate for mycotoxin production with variable and negligible amounts reported (Bullerman 1981; Dobson 2017). Some cheese associated fungi, including *Candida parapsolosis,* have been cited as a potential infectious agent, though no known infection due to consumption has been documented for this particular fungi (Banjara et al. 2015) nor have any human mycotoxin poisonings from cheese consumption (Garnier et al. 2017).

Regulators attempt to mitigate the risk of raw milk cheeses using the FDA established 60-day rule, wherein raw milk cheeses are considered safe after a 60-day aging period. However, this rule has recently been called into question with minimal evidence indicating its efficacy (D'Amico et al. 2008; Brooks et al. 2012). There is some work indicating that pathogenic bacteria may survive on cheese in pasteurized milk and raw milk, even after consideration of the FDA 60-day aging rule

(D'Amico et al. 2008), though there is little information on the impact of this procedure on molds. Because of this, equipment sanitation is key and pathogenic bacteria pose a significant health risk in cheese production.

Quality spoilage is not a food safety concern, but an issue of consumer rejection due to off flavors, textural issues, or poor visual appearance of the cheese. Quality issues can arise from both non-pathogenic bacteria and from mold growth. The following sections focus on cheese spoilage, with emphasis on mold spoilage, due to the creation of visual rejection by consumers.

2.2.1 Bacterial impacts on cheese quality

The fermented nature of cheese and use of starter and adjunct cultures makes controlled bacterial growth an essential component of cheese making. Many production organisms, such as lactic acid bacteria, are often accompanied with non-starter lactic acid bacteria (NSLAB) that can result in a quality issue. Some strains may cause undesirable gas formation or other off flavors during ripening (Somers et al. 2001). Contamination by NSLAB can also promote biofilm formation and result in the development of calcium lactate crystals in the cheese (Somers et al. 2001; Kubota et al. 2008, Agarwal et al. 2006).

Clostridium tyrobutyricum, a non-pathogenic bacterium, is also detrimental to cheese producers. It is associated with "late blowing" in hard cheeses, the result of spores germinating and producing gas, causing cracking and off-flavors. This ultimately leads to rejection of the cheese, causing product and financial loss (Klijn et al. 1995).

Psychrophilic bacteria, such as *Pseudomonas* sp., also create significant product spoilage through gelation of milk and development of a bitter taste (Morales et al. 2005). These bacteria survive readily at refrigerated temperatures and have the ability to persist even if proper temperature-

controlled storage is maintained (Chen et al. 2011). Psychrophilic bacteria often form biofilms in raw milk with some research indicating they can alter phenotypes through phase variation (de Oliveira et al. 2015). In this process, bacteria mitigate the stresses of a rapidly changing environment through regulation of protein expression. Psychrophilic bacteria have demonstrated an ability to break down milk fat and protein (Kilcast and Subramamiam 2011) which interferes with the expected microbial breakdown by cheese starter culture and can produce undesirable flavors. Bitter aftertaste, characteristic of these bacteria, results from the decomposition of β-casein that is no longer attached to the casein micelle due to the low temperature storage (Lemieux and Simard 1991).

Propionibacterium, typically represented by *Propionibacterium freudenreichii,* creates an additional quality concern. This bacteria can be challenging because it is desirable in Swiss style cheese but presents as a defect in other products, with unintentional inoculation by wild *Propionibacterium* causing uneven “eyes” and other quality issues (Fröhlich-Wyder et al. 2017).

Even in the absence of non-starter microbes, cheese can be spoiled by failed starter cultures. Today, this phage contamination in bulk starter culture is still a major area of concern for many producers. Phages can have detrimental impacts on quality and product loss, often resulting in stuck fermentations, wherein inoculated bacteria are no longer able to acidify/produce the desirable flavor metabolites. When contaminated with phages, starter cultures fail to acidify the cheese-milk at the expected rate, if at all (Johnson 2017). This typically results in the loss of the milk and the loss of the bulk starter culture itself. Most processors using a bulk starter culture technique attempt to diversify with a wider range of bacteria to minimize the impact of phage contamination, though this is not always successful. However, because these issues are found before the cheese leaves the production facility, they do not impact consumers, though may compromise future cheese

production if equipment is not sanitized properly after these issues are discovered (Somers et al. 2001). Although somewhat more expensive, many dairy companies have moved to purchasing concentrated starter culture to avoid these losses (Johnson 2017). Commercial starter cultures are typically sold freeze dried and a new culture package is added to each batch of cheese/yogurt so there is no risk of cross contamination.

Because bacteria present a clear quality issue, this poses a serious obstacle for processors, making sanitization an essential component of cheese making.

2.2.2 Fungal spoilage

Mold spoilage, as mentioned previously, is not a large food safety concern, but an issue of quality and consumer rejection, posing a serious challenge for cheese manufacturers.

Mold spoilage can originate from pre or post-consumer locations and are common even in low water activity cheeses, where bacteria typically struggle to grow (Vanderzant et. al., 1985). They often enter the food matrix through inadequately sanitized equipment or as airborne contaminants (Grande-Tovar et al. 2018). At the plant level, contaminants may enter the product through air, equipment, brine, or even packaging, though air appears to be the most common source. There is additional opportunity for contamination at the farm level, with mold spores originating from cow feed (Hymery et al. 2014). It is unclear if these ultimately impact the cheese microbiota, though their survival is likely influenced by the processors choice to make raw, pasteurized, or heat treated cheese (Doyle et al. 2017). Cheddar cheese, the focus of this research, is prone to mold spoilage due to its relatively high water activity of 0.956-0.965 (Schmidt and Fontana 2008, Hickey et al.) which contributes to the ideal growth conditions for mycotic activity. For a list of common cheese molds and yeasts, see Table 1.

Table 1: Common spoilage fungi isolated from cheese.

Genus	Yeast/Fungi	Species	Cheese Type	Citation
Penicillium Sp.	*Filamentous fungi*	*Penicillium brevicompactum* *Penicillium Commune* *Penicillium palitans* *Penicillium solitom* *Penicillium roqueforti* *Penicillium glabrum* *Penicillium expansum* *Penicillium chrysogenum* *Penicillium bialowiezense*	Soft, Semi-hard, hard cheeses	1,3,3,4,5
Aspergillus Sp.			Semi-hard cheeses	1,2,4
Cladosporium Sp.		*Cladosporium sphaerospermum*	Fresh cheese	1,2
Mucor Sp.		*Mucor racemosus*	Semi-hard cheeses	1,2,5
Geotrichum Sp.		*Geotrichum candidum*	Semi-hard cheese	2.3,5
Galactomyces Sp.		*Galactomyces geotrichum*	Fresh cheese	1
Phoma Sp.		*Phoma pinodella*	Soft cheese (cream cheese)	1
Trichosporon Sp.		*Trichosporon asahii*	Fresh Cheese	1
Meyerozyma Sp.	*Yeast*	*Meyerozyma guilliermondii*	Soft cheese (cream cheese)	1
Rhodotorula Sp.		*Rhodotorula mucilaginosa*	Fresh Cheese	1
Yarrowia Sp.		*Yarrowia lipolytica*	Semi-hard cheese	1

[1]Garnier et al. (2017) [2] Hymery et al. (2014) [3] Kure et al. (2004) [4]Banjara et al. (2015)[5](Barrios et al. 1998)

When assessing alternative preservatives, it is important to note the most common spoilage cheese molds for target treatment. Yeast also pose some risk to cheese spoilage, typically assoicated with *Candida* sp. in cheddar cheese (Hocking, Alisa Faedo 1992). However, mold spoilage is more widely reported and is thus the focus of this work. Mold formation on cheese is typically associated with *Penicillium* sp. and a select few other molds. Barrios, Medina, Lopez, & Jordano (1998) found, upon the analysis of 52 cheeses, that 63% contained *Penicillium* sp., 27% contained *Mucor* Sp., and 17% contained *Geotrichum* Sp. This mirrors work at Universitè de Brest in France, which cited *Galactomyes geotrichum, Mucor racemosus, Penicillium commune,* and *Yarrowia lipolytica* as the main cheese spoilage organisms (Garnier et al. 2017). Overall, *Pencicillium* sp. appears to be the most frequent mold spoilage organism and thus presents the main concern for mold inhbition.

In addition to the more common, aerobic molds, thread molds pose an additional concern, as they can grow in anaerobic, vacuum packed cheese. The most commonly isolated thread mold species from cheese are *Cladosporium cladosprioides*, *Penicillium commune*, *C. herbarum, and Penicillium glabrum* (Hocking, Faedo, 1992).

Based on the large diversity of molds and their contribution to consumer rejecton, it is unsuprising that cheese producers have ongoing interest in preservation strategies and new technologies.

2.3 Strategies to mitigate microbial spoilage of food products

The demand for food safety has historically been the driver of food preservation technologies. Even prior to the onset of modern day agriculture and food production plants, humans used physical technologies to modify and preserve foods for storage across variable weather (Hayashi 1989; Mustapha and Lee 2017). The most well-known include treatments such as heating, salting,

smoking, canning etc. These focus primarily on manipulating the physical composition of the food product to extend shelf-life through fermentation, drying, or protein denaturation (Danyluk and Worobo R.W. 2012; Mirmoghtadaie et al. 2016). Today there are a large number of thermal and non-thermal physical treatment technologies used in the food industry (Figure 2).

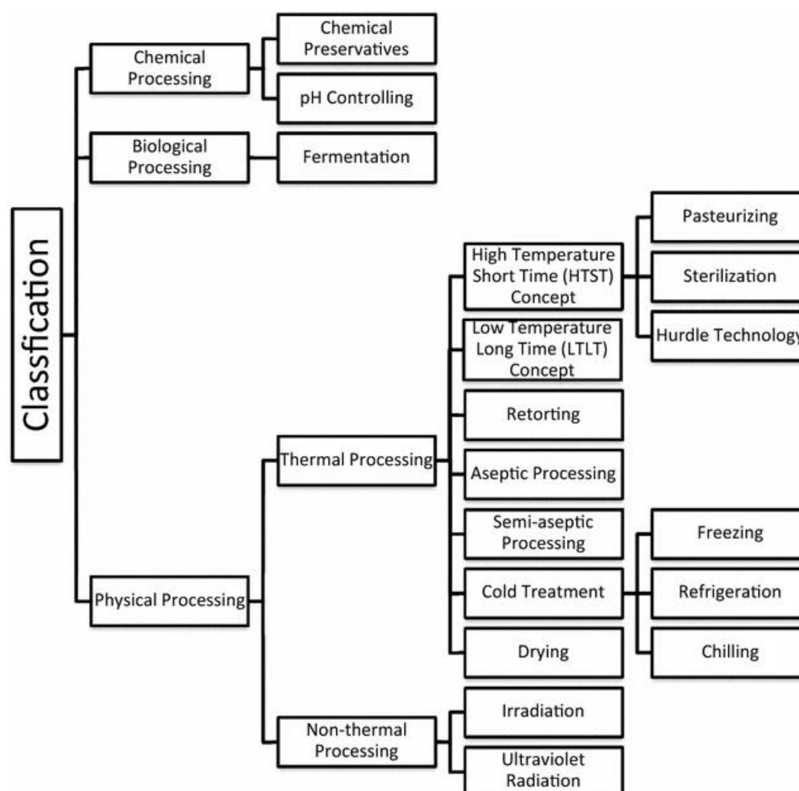


Figure 2: Schematic of current preservation technologies. Adapted from Amit et al. (2017).

Physical treatments are often combined with chemical preservatives that are helpful to maintain food stability for longer periods of time. These combinatorial approaches are effective because they place multiple “hurdles” in the way of microbial growth (Singh and Shalini 2016). In light of the movement to clean labels in the food industry, this section focuses on biological, chemical, and plant-based treatments that are naturally-derived. In this context the hurdle concept will be critically important to ensure preservative replacements are effective.

2.3.1 Physical treatments used in the dairy industry

Throughout millennia, individuals have used a variety of technologies to improve the shelf life and taste of food, with methods evolving over time. Because of the perishable nature of cow's milk, the shelf life has traditionally been elongated by processing it into cheese or yogurt. The onset of pasteurization, famously invented by Louis Pasteur in the 1860s (Steele 2000), greatly improved food safety in dairy and is still used to preserve fluid milk, among other beverages. Modified atmospheric packaging (MAP) used on shredded cheese, produce, and snacks, has also greatly improved shelf life by removing oxygen from the packaging container and replacing it with a mixture of nitrogen and carbon dioxide. Because mold spoilage organisms are typically aerobic, the removal of oxygen greatly improves longevity of the product (Xiao et al. 2017; Ceylan et al. 2017; Solomakos et al. 2019) and has led to the promotion of convenience cheese products. Most recently, there have been developments trialing high pressure processing (Batty et al. 2019) for use of bloomy rind cheeses, though impact on texture proved undesirable.

2.3.2 Chemical and biological treatments

With new developments in food preservation technologies, researchers have turned to additive agents in addition to physical treatments, as a means to prolong shelf life. This often includes biocontrols, which produce organic acids or other antimicrobial compounds. Fermentates have also gained popularity, typically produced from culturing milk or clean-label substrates (Ribes et al. 2017; Hossain et al. 2017). With these advances, there are more opportunities to create long-term shelf stable foods and meet consumer demands for a clean label.

2.3.2.1 Organic acids and acidification

Historically, acid fermentation (microbial production of organic acids) has been a very successful preservation technology. Organic acids generally function by increasing the proton concentration

and lowering the external pH to gain shelf life (Lucera et al. 2012). Their natural occurrence in beer, bread, wine, vinegar, yogurt, and cheese all show utility in food preservation. Today, various organic acids are added as chemical preservatives in foods as diverse as animal feed and apple juice (Anyasi et al. 2017).

Sorbate, a common organic acid used in the bread industry (Suhr and Nielsen 2004; Guynot et al. 2005; Degirmencioglu et al. 2011) has shown mixed success as an alternative to propionate, which bakeries are attempting to replace. Because spoilage molds in breads are often of overlapping genus/species to those in cheese, these are viable options for exploration.

Aside from microbially-derived organic acids, some dairy products are acidified using other naturally acidic additives. Several types of cheese, including queso fresco and paneer are acidified using lemon or vinegar without the addition of rennet. Because this acidification provides some natural mold inhibition, scientists have looked to improve functionality in mozzarella by combining lemon with the brine and a gel solution at 500, 1000, and 1500 ppm. Even under temperature abuse conditions, this showed an increase in shelf-life without reduction of the cheese microflora (Conte et al. 2007). In addition to the mentioned bio-controls, several other, less prominent fermentates have been explored for their anti-mycotic activity. For example, phenylactic acid (PLA) has shown antifungal activities in lab media against *Aspergillus* sp., *Penicillium* sp., and *Fusarium* sp., when isolated from bread. When lab media was adjusted to pH 4, PLA showed significant inhibition, indicating that it may function well in many food matrices (Lavermicocca et al. 2003).

Organic acid producing bacteria may also increase shelf life through means other than acidification. Lactic acid bacteria (LAB) in particular, are well known for their antimicrobial

properties, used as a naturally-occurring bio preservative (Garnier et al. 2017c; Özogul and Hamed 2018; Bekhit et al. 2019).

Some evidence indicates that inhibition by LAB may be a product of out-competing spoilage bacteria for nutrients and LAB's ability to produce specific inhibitory metabolites (Dalié et al. 2010).

The interest in LAB as a preservative has spiked with trends in clean labels (Leroy and De Vuyst 2004) and can generally be seen divided among four genera, *Lactococcus, Lactobacillus, Leuconostoc, and Pediococcus* (Dalié et al. 2010). This preservative technology has been investigated in a series of food matrices, largely dairy and baked goods (Cheong et al. 2014; Lynch et al. 2014; Le Lay et al. 2016) with mixed success. Researchers in Cork, Ireland tested the efficacy of *Lactobacillus amylorvorus* against the common cheddar cheese spoilage mold, *Penicillium expansum*. This species of LAB has previously shown additional success against *Penicillium roqueforti, Aspergillus fumigatus,* and *Fusarium culmonrum.* These molds were tested in a milk media and cheddar cheese, with LAB demonstrating synergy with propionic bacteria. (Lynch et al. 2014; Fernandez et al. 2017). Lactic acid bacteria and its constituents have also shown success in a model cheese system (Leyva Salas et al. 2017, Garnier et al. 2019), indicating potential viability in shredded cheeses.

LABs are already commercially viable, with several companies providing clean label inhibitors for purchase, including HOLDBAC® YM-C Plus (Fromagex, 2012). Because of the clear inhibitory potential of LAB, it is worth exploration in additional dairy matrices.

In addition to LAB, *Propionibacterium* have shown potential in terms of biocontrol, beyond their contribution of propionic acid and are commonly used in bread as an inhibitor (Lennox and

McElroy 1984, Guynot et al. 2005; Samapundo et al. 2017). In cheese*,* *Propionibacterium* are used to manufacture Swiss, creating the characteristic holes through carbon dioxide production and imparting the distinctive Swiss flavor (Defects et al. 1974; Fröhlich-Wyder et al. 2017). Combinations of *Propionibacterium jensenii* with *Lactobacillus paracasei* strains (SM20, SM29, and SM63) have also shown fungal inhibition in yogurt and cheese against several *candida* sp. (Fernandez et al. 2017), with positive anti-fungal activity noted in lab media research (Ho et al. 2009). This indicates synergy of *Propionibacterium* and *Lactobacillus* as bio-preservatives and warrants further investigation. In addition to their antimicrobial benefits, these bacteria can easily grow in a dairy based medium, aiding with potential labeling issues. Lastly, because they can be maintained in a liquid form or dried into a powder, they can be added to a food system in several forms and could be applied with the shredded-cheese anti-caking agent with equipment already present in shredded cheese plants (Schwenninger and Meile 2004).

2.3.2.2 Microbial produced complex inhibitors

In addition to biocontrols, advances in food technology have promoted the use of fermentates, essentially the addition of microbial metabolites to extend the shelf life of food.

The prototypical case of inhibition by biological metabolites is seen for penicillin, produced by the common mold, *Penicillium.* Emerging from the work of Alexander Fleming (Hassan and Hassan 2016), this fermentate has changed the course of antibiotic treatments for human bacterial infections (Gaynes 2017). The invention of penicillin led to additional advancements in classes of anti-mycotics such as azoles and polyenes, used to treat common yeast/fungal diseases (Bodey 1992; Ashbee and Gilleece 2014). Natamycin, an example of a polyene (Atta et al. 2015), is now commonly used in the food industry. Bacteriocins, an additional preservative option, are a subset of antimicrobial peptides produced by bacteria which interact with other, vulnerable bacterial

membranes leading to cell death through formation of pores in the cell (Venema et al. 1995). Bacteriocins are composed of two categories, antibiotics which use the Tol protein system (a protein complex) or the Ton system (a transport mechanism) to penetrate the outer membrane of bacteria (Danese et al. 2004; Yang et al. 2014). A common example of a bacteriocin is nisin, used largely in products such as processed cheese (Delves-Broughton 1996). Because these compounds can be produced by common fermentation/food bacteria, researchers have begun to explore them as potential clean label mold inhibitors. However, few have been trialed extensively and seldom in cheese or dairy products. With potential to create naturally derived anti-mycotics, bio-controls such as nisin, cultured dextrose, and other antimicrobials should be explored as a means to meet consumer demands.

2.3.2.2.1 Natamycin

Natamycin, a polyene derived from the soil bacteria *Streptomyces natalensis*, has shown high efficacy in food systems and has become ubiquitous with modern cheese production/large scale dairy. For convenience and high-risk products, natamycin provides the shelf life expected by U.S. consumers (Davidson et al. 2005).

For shredded cheese, the compound is applied in a powder form, combined, anecdotally, with potato starch or cellulose as an anti-caking agent and occasionally xanthan gum as a carrier. With a reported minimum inhibitory concentration ranging from 0.125-20 ppm (Cong et al. 2007; Sradhanjali et al. 2018) and anecdotal industry use at 10 ppm, natamycin is readily incorporated at low, non-toxic doses.

Despite the heavy literature on natamycin, its mode of action remains unclear. Mold inhibitors typically function through disruption of cell wall membranes, compromising permeability and prohibiting reproduction of filamentous fungi and related species. Initial research showed

natamycin binding to ergosterol, a sterol, and altering the state of the cell wall by making it ‘leaky’ (Pedersen 1992). However, recent research indicates that natamycin may bind to ergosterol without disrupting the structure of the membrane (Te Welscher et al. 2008). As mentioned earlier, its efficacy in cheese is associated with its ineffectiveness against bacteria, largely caused by their lack of sterols (Pedersen, 1992) and allowing for the natural cheese ripening process.

The prevention of mycotic growth without bacterial inhibition makes natamycin an ideal solution for ripened cheeses. Despite its efficacy, natamycin lost significant consumer support and has even been banned from prominent retail chains, including Whole Foods. Previous research has shown it can cause food poisoning-like symptoms (Newcomer et al. 1960) though this study’s relevance has since been brought into question, as it used extremely high research doses ranging from 200-1000 mg, administered through an oral tablet. Based upon cheese consumption data, as detailed by the World Health Organization report of 2006, a person would likely consume 0.1 mg/kg bodyweight or about 6.8 mg/day (2006). Furthermore, according to USDA guidelines, natamycin is not considered organic, putting organic dairy producers at a disadvantage for shelf life longevity.

In order to reassess current technologies to replace natamycin, future areas of research should include biocontrols and their metabolites, organic acids, and essential oils. Challenges lie in finding an equivalent inhibitor without increasing costs or developing a negative organoleptic impacts. In the next section, emerging additive preservation technologies are discussed.

2.3.2.2.2 Nisin

Produced by fermentation with *Lactococcus lactis*, the bacteriocin nisin is typically used as a bio preservative against gram positive and gram-negative bacteria, with common uses including dairy and meat products (Gharsallaoui et al. 2016). Additional innovations in biotechnology and pharmaceuticals (Shin et al. 2016) have increased its usage. Nisin most likely functions by

impacting the plasma membrane and murein synthesis (Henning et al. 1986), a polymer that forms a barrier on the outer layer of the plasma membrane. However, nisin, similar to natamycin, has recently lost favorability with consumers due to perceptions of unnatural origins.

2.3.2.3 Unpurified fermentates

Cultured dextrose is generally produced using the simple sugar dextrose and fermenting with *Lactococcus lactis* in combination with *Propionibacterium freudenreichii*, a bacterium typically associated with the production of Swiss cheese. Though not as widely used as its nisin counterpart, it can be found in cheese sauces, salad dressings, and other condiments. An example of a cultured dextrose product that is commercially available is Microgard® (DuPont). Literature (Martinez, Rodrıguez and Suarez 2016 in Mills, Ross, & Hill, 2017) validates the effiacy of this product, with success in chocolate and dairy products. Minimal publicly available research has been performed on this inhibitor, though several patents indicate its antimicrobial properties in cheese based products. For example, a patent cited in 2010 indicates cultured dextrose has shown success at a formulation rate of only 0.5% in cheese sauce, with minimal off flavors over the 24 month study (Gamay et al. 2010). This may indicate the success of cultured dextrose in more traditional cheese and dairy based products, particularly if paired with hurdle technologies.

In baked goods, cultured dextrose has also been explored as an alternative to propionate with success against *Penicillium* sp., with results potentially translating to dairy products (Samapundo et al. 2016). Because cultured dextrose uses bacteria often found in the cheese-making process, it may help to overcome labeling challenges and makes it a strong candidate for an alternative inhibitor to natamycin.

In bread, cultured corn syrup, citric acid, cultured wheat solids, and cultured dextrose have all shown significant ability to inhibit *Aspergillus tritici* and *Aspergillus amstelodami* in pound cake,

with those cultured in lower pH media showing greater inhibitory effects (Samapundo et al. 2017). Because these bread molds are also commonly found as spoilage molds in dairy, it is worthwhile to investigate these fermentates in dairy matrices.

2.3.2.4 Plant derived inhibitors

2.3.2.4.1 Essential oils

Essential oils, derived from herbs and other plants, have a long history of use as antimicrobials. Many historical foods eaten prior to refrigeration technologies incorporated large amounts of spices for preservation during winter months. Research indicates essential oils have a similar impact, though there are organoleptic and application concerns that must be addressed prior to commercial use (Embuscado 2015).

Essential oils exhibit antifungal properties through a variety of mechanisms, generally by interacting with the fungal cells walls and disrupting cell formation. Because ergosterol is an essential component of fungal cell membranes, many essential oils that bind to ergosterol disrupt the permeability of the membrane. Additionally, essential oils can block the formation of β-glucans, causing additional disruption of cell wall integrity and formation. They may also work inside the cell by inhibiting the electron transport chain within cell mitochondria or inhibiting efflux pumps that remove toxins (Nazzaro et al. 2017). Additional research indicates they may be successful due to their high antioxidant activity, flavonoid concentration, and ability to reduce oxidative rancidity (Embuscado 2015). In conventional food practices, essential oils are usually paired with a hurdle technology to reduce organoleptic influences.

Some of the most widely recognized antimicrobial oils include clove and thyme oil, though their applications in cheese are fairly limited. Some research on eugenol (clove) and thymol (thyme) in

Arzúa-Ulloa cheese indicates they may be successful against *Penicillium citrinum,* with eugenol showing complete inhibition at 200 µg/L (Vásconez et al., 2000).

Microcellular foam starch (MFS) has also been used as a medium to deliver essential oils to cheese with success of several extract types. Han et al. (2014) placed MFS in a paper sachet with rosemary/thyme oils and placed this in a bag of shredded mozzarella inoculated with *Listeria Monocytogenes.* The sachet (containing 500 mg MFS, 1% or 10% w/w thyme and/or rosemary oils) showed significant inhibition of the bacteria. However, the addition of the oils created undesirable sensory characteristics when tested on a limited sensory panel. Furthermore, because this study focused on bacteria, the information is not as readily applied to mold inhibition.

Other starch-based films have been successful at inhibiting mold growth when combined with essential oils. Using linalool (basil), carvacrol (oregano), and thymol (thyme) at rates of 1%, 3%, and 5% (w/w) scientists were able to create films for application on cheddar cheese inoculated with *Aspergillus niger.* Using these films, colony count sizes showed a significant reduction (Han et al. 2014). In an additional effort to utilize essential oils in combination with a waste stream, scientists have begun research on the use of whey protein to carry these antimicrobial constituents. In one study, whey protein films were created with 1.0%-4.0% oregano, rosemary, or garlic essential oils and tested against *E. coli*, *S. aureus*, *S. enteritis*, *L. monocytogenes*, and *L. plantarum*. Those containing oregano essential oil at a 2.0% level were most successful at inhibiting mold growth with garlic showing inhibitory impacts at higher levels of 3.0% and 4.0%.

These results are promising, as film technology becomes more advanced. However, whey protein films are somewhat limiting due to their inability to withhold moisture. Thus, these films must be incorporated with lipids or other hydrophilic components, adding challenges to certain food matrices (Seydim and Sarikus 2006).

In addition to starch films, studies have shown success using protein-based gelatin film for dried fish preservation in combination with oregano extract, rosemary extract, or chitosan. Because this study was combined with high pressure processing (HPP), there are some limitations to dairy applications (Batty et al. 2019). In dried fish, researchers found success of anise oil at 4% on inhibition of *Aspergillus flavus* and *Penicillium* Sp., both common cheese molds. (Matan 2012).

Some essential oils have shown successful inhibition at rates as low as 0.5µl/g-20 µl/g (Burt 2012). However, challenges with organoleptic properties and volatility must be managed.

Overall, the previous literature on essential oils focuses primarily on non-dairy matrices. In addition, nearly all research includes the use of hurdle technologies, primarily edible films, as a means to initiate a slow release of essential oils and reduce organoleptic impacts. Because of this, the true, individual impact of essential oils is not widely known and requires more exploration for use in dairy. Preliminary results, however, look promising and warrant future exploration.

2.3.2.4.2 Complex extracts: Celery Powder

Additional plant-based sources have also shown promising use in the food industry as antimicrobials. Celery powder is well known for its use as a natural nitrate source in deli meat and sausage production, with antioxidant properties providing an additional barrier to spoilage (Ahmed Mohamed Mousa et al. 2015).

Celery powder in meat production is typically used as an alternative to chemically synthesized nitrates for inhibition of *Clostridium Botulinum* (Golden et al. 2014). In terms of bacterial inhibition, celery seeds function by interrupting active transport, disrupting oxygen uptake, or oxidative phosphorylation through changes in the electron carrier (Yarbrough et al. 1980). Research from China indicates the essential oil form may have antifungal properties (Liu, Liu,

Xie, & Mu, 2017) while additional research suggests similar functionality of celery seeds (Llewellyn et al. 1981). However, the majority of screening studies focus on use in the meat industry for bacterial inhibition. With minimal research conducted on mold inhibition, future investigations are required to determine the efficacy of celery powder as a clean-label inhibitor in cheese.

2.3.2.5 Other inhibitors

2.3.2.5.1 Lactoferrin and lactoperoxidase

There is also additional potential to derive inhibitors from the milk itself through use of the natural lactoperoxidase system in milk and its inherent fungistatic properties (Andersson et al. 2000). Because this system is naturally occurring in milk, labeling concerns are more readily overcome. Lactoferrin is also naturally present in the human exocrine system and breast milk (Fernandes and Carter 2017), making it safe for human consumption (Reiter and Härnulv 1984) and potentially explaining the purpose of its antimicrobial properties. In human milk, lactoferrin claims responsibility as the primary antifungal (Andersson et al. 2000) and has the added benefit that is it very heat resistant, withstanding the normal pasteurization of bovine milk and human milk for infant feed. Because of this, in combination with its label-friendly properties, lactoferrin makes an ideal inhibitor.

Lactoferrin and the lactoperoxidase system are both utilized as a preservation method in countries where refrigeration is not feasible. Lactoferrin functions through the chelation of iron and thus can only inhibit organisms that contain a significant amount of this metal, somewhat limiting their inhibition potential. Lactoferrin, in particular, is generally more effective against bacteria than yeasts and molds, meaning timing of application is crucial during cheese production in order to prevent death of desired organisms (Lucera et al. 2012).

The lactoperoxidase system contains three basic components, lactoperoxidase, thiocyanate, and hydrogen peroxide. By increasing concentrations of thiocyanate and hydrogen peroxide, the enzyme creates antimicrobial constituents that help fight pathogens in the mammary glands of certain animals and potentially in food systems (De Wit and van Hooydonk 1996).

Because of emerging information on lactoferrin's fungistatic properties, researchers are beginning to investigate its use in food products and supplements. One group utilized whey protein isolate as a film medium, adding lactoferrin, lactoferrin hydrosolate, and lactoperoxidase to inhibit *Penicillium commune* at concentrations of 5.6, 29, and 59 mg per 8.5 cm diameter, 0.3 mm thick film. Minimal inhibition concentration (MIC) for lactoferrin and lactoferrin hydrosolate were found to be 10 mg/ml with additional success of variations of component combinations (Min and Krochta 2005). Furthermore, a Canadian research group isolated *Penicillium* Sp. from water bottles and found inhibition of spores for 9 and 21 days at 60 and 300 μg/ml of lactoferrin respectively (Liceaga-Gesualdo E.C.Y. Li-Chan Skura 2000).

Lactoferrin is limiting in that it is expensive to produce and generally reserved for infant feed/clinical trials. However, in the movement to utilize whey waste, researchers have discovered the use of whey proteins as an antioxidant and lactoferrin source. The success of whey protein for microbial inhibition may be due to the presence of lactoferrin in combination with other whey components. Scientists from Ireland created an inhibitory solution for tomatoes using a 0.0-5.0% solution of delectated whey proteins (DWP). The results showed a 1.5 and 1.0 log reduction in total aerobic and yeasts & mold counts respectively, at a 3.0% application rate. At this rate, the tomatoes showed no organoleptic issues, though higher concentrations did produce off flavors (Ahmed et al. 2011).

Though expensive, because it is required in small amounts, this inhibitor has potential use if combined with hurdle technologies and thus is worth further exploration.

2.3.2.5.2 Chitosan

Chitosan is a polysaccharide derived from crustacean shells or fungi. Similar to essential oils, chitosan is not widely used in dairy, with most investigative research focusing on use in films. Instead, it is generally associated with edible packaging/films in the fruit and vegetable industry, though these films have not been widely applied on a commercial scale (Jianglian 2013). This inhibitor functions by interrupting the negatively charged components of macromolecules on the cell wall surface, thus changing the permeability of the plasma membrane.

Research in dairy indicates that at a rate of only of 0.075% in cheese milk, chitosan can reduce coliforms, *Pseudomonas*, and yeasts and molds without impacting LAB (Altieri et al. 2005). Similarly, a study on Karish cheese used a solution of 0.5% chitosan and 1.0% chitosan with acetic acid to extend shelf life up to 18 days and sensory properties by 3 days (Avalia and Pubvet 2012). Ricotta, flor di latte, mozzarella, and other pasta filata cheeses have shown success with chitosan applications (Di Pierro et al. 2011), indicating it may provide an option as an alternative, label-friendly inhibitor.

2.3.3 Clean-label hurdle technologies for control of molds

Hurdle technologies allow producers to use small amounts of naturally perceived inhibitors that may be more costly or less effective and combine them with other methods to maintain the same expected shelf life. In addition to reducing costs, the combination of label friendly inhibitors can help the industry overcome the organoleptic challenges of preservation technologies. Particularly a concern for essential oils, through utilization of multiple inhibitors, concentrations can be

lowered to promote efficacy while reducing off-flavors (Rasooli 2014). Investigating hurdle technologies may help overcome some of the challenges facing processors as they reformulate to include these antimycotics.

An emerging technology, edible films, is gaining research strength to be used as packaging or as a contamination barrier. Films are generally made of polysaccharides, pectin, or hemicelluloses and have significant environmental benefits, due to their biodegradable and edible properties (Gagri et al. 2004). Food films are typically investigated as an antimicrobial carrier, creating a bio protective layer against the environment. Because films are a form of active packaging, mold inhibitors can be incorporated to form a slow-release antimicrobial cover, allowing for significant shelf-life extension (Suput et al. 2015).

In a lab setting, films are typically made by spreading the prepared mixture onto a nonstick surface, allowing for formation, and removal of the film (Vásconez et al. 2009). They may also be created using a conveyor belt and continuous casting method (Dutta et al. 2009) or through extrusion (Nur Hanani, Beatty, Roos, Morris, & Kerry, 2012). The application of films through dipping/spraying makes them easy to incorporate into a process line or in combination with the anti-caking agent currently applied to shredded cheese. With films sprayed in thin layers undetectable to consumers, they make an ideal antimicrobial medium. Edible films are on the forefront of food packaging research and their applications are yet to be fully realized. Initial research shows potential in a variety of food matrices (Cong et al. 2007; Vásconez et al. 2009; Grande-Tovar et al. 2018; Mujtaba et al. 2019), including dairy, thus, their exploration may help overcome challenges of the clean label trend.

2.4 Approaches to evaluate efficacy of mold inhibitors

Emerging preservation technologies are expansive with clear, potential applications in the dairy industry for control of mold spoilage. However, there are several challenges that must be overcome in order to evaluate large numbers of potential inhibitors and combinatorial treatments.

2.4.1 Experimental Design: challenge testing on product or lab analog

In research, both food matrices and lab media have widespread use as screening methods for new microbiological inhibitors, with each having distinct benefits. Lab media is the common choice, providing standardization critical to enable comparison of results across the scientific community. However, research on lab media has the caveat that the data cannot be directly applied to the food industry. Differences in pH, nutrition, surface exposure, water activity, and packaging all play a role in the efficacy of an inhibitor and growth rates of molds (Imani Rad et al. 2017; Van de Vel et al. 2019; Shannon et al. 2019). Research on food matrices, though ideal from an application standpoint, is labor intensive and by nature subject to the variability of food products. Recent research (Garnier et al. 2017) indicates that inhibitors have different levels of anti-mycotic activity when grown in a cheese matrix or on potato dextrose agar (PDA). Similarly, false inhibition of lactic acid bacteria (LAB) has been noted on de Man, Rogosa, and Sharpe (MRS) agar due to the incorporation of acetate and its known antimicrobial properties (Delavenne et al. 2011). Thus, research on laboratory media, though more repeatable, can produce ambiguous results when applied to food production. Because of this, food-focused research is ideal when it can be carried out in a food mimicking matrix.

Many researches have attempted to create more applicable laboratory medias by directly incorporating food products such as wine (Oro et al. 2019) into laboratory media or developing cheese-based broth (de Carvalho et al. 2015) to screen for inhibiton potential in essential oils.

A newly described method utilized a 24 well plate technique to create small cheeses and test a variety of bacterial isolates for their capacity to inhibit mold growth (Garnier et al. 2018). Using this method, researchers created mini-cheeses from ultra-filtered, pasteurized, and standardized retentate, essentially an ultra-concentrated pre-cheese. This pre-cheese contained 244g/kg dry matter (24.4% solids as compared to 13% solids in standard milk) and was inoculated with starter culture and rennet prior to being poured into plates. This approach, based on Hannon et al., 2006, has the benefit of removing whey prior to making the cheese, by creating a highly concentrated milk. Additional methods have developed mini cheeses in 96 well plates using custom made curd-stirrers, developing cheeses as small at 1.7 ml (Bachmann et al. 2009). This allows for easy replication, high throughput sampling, and an easily modified cheese recipe. However, hand stirring the curds is fairly labor extensive, making it time consuming to prepare many plates. Furthermore, analysis of deep well plates requires additional, specialized equipment.

Work from Tufts university (Wolfe et al. 2014; Cosetta and Wolfe 2020) accomplished this with the development of cheese agar. Researchers were able to successfully recreate the microbial community of bloomy rind cheeses in a 96 well micro-titer plate. Using this method, research can be performed using a large number of replicates, in a reproducible format and the results can be more directly applied to the dairy industry.

2.4.2 Quantification of mold growth

In order for molds to grow, spores must first have access to proper nutrients, including water, oxygen, and environmental conditions. Spores may be lying dormant and be triggered to grow through activation of nutrient receptors, indicating to the organism the optimal environmental conditions have been reached (Chandler 2017). If optimal environmental conditions are maintained, molds will eventually show visual, hyphal growth (Smith and Grula 1981). The

classical microbiology methods are focused on visual growth or measurement of cell mass, though some methods focus on cell counts. Because mold cells can elongate and vary in size, unlike bacteria cells, the method of detection can substantially impact results.

2.4.3 Classical microbiological methods

Common classical methods include the use of enumeration through standard plate counts (SPC), radial growth, and time to detection (Kosegarten et al. 2017, Vlaemynck; Gachon and Saindrenan 2004). All of these methods are limited in their labor, material costs, or throughput. However, these are all focused on direct counts or observations of the mold, rather than using biomass or other metrics as a means to measure growth. SPC is beneficial because it allows for counting of colonies, which may be valuable for food safety research, in which colony counts are necessary to determine the bacterial limit for safe consumption (Hayes et al. 2001). Radial growth measures changes in colony size after hyphal growth has become visible, information that may be useful for understanding the impact of an inhibitor on growth delay (López-Malo et al. 1995). Lastly, visual detection indicates first sign of mold growth, without regard to amount or size of colonies. This is ideal for shelf life, where consumer rejection is the primary concern. Thus, depending on the end goal of the research, different classical microbiology methods may be selected.

2.4.3.1 Analytical proxies for mold biomass

More recent methods have focused on biomass or DNA as a means to measure growth, rather than a direct cell count. Research into high-throughput fluorescent staining has gained popularity, with Figueroa-López (2014) developing an assay in a 96 well plate, using fluorophore to bind to the chitin as a proxy for measuring fungal biomass of *Fusarium verticillioide.* However, this method is limited in its use of lab media rather than a food-mimicking matrix. In dairy, 1536 well plates have been used to assess cell viability (Rohman and Wingfield 2016) in which cells are dyed with

resazurin, subsequently reduced to resorufin and fluorescing to a bright pink. This method focuses on fluorescing materials in the cell wall and thus illustrates total cell mass rather than cell count. Additionally, qPCR is often used for fungal detection (Rodríguez et al. 2012) and uses DNA as a means to measure growth, based on the content of the cell's nucleus (Hunter et al. 2010). Because mold cells elongate with hyphal growth, these metrics are not necessarily correlated.

Garnier et. al. (2018) used their 24 well plate method with miniaturized cheese to test LAB fermentation products (Garnier et al. 2019) for their potential as mold inhibitors, using visual scoring as a means to quantify mold growth. Though ideal in its use of a food matrix, visual scoring adds an element of subjectivity.

With advances in computer technologies, movement to image analysis has gained traction and spurred innovations in software. Currently, the majority of software is based on automated colony counting rather than measurement of growth (Lamprecht et al. 2007, Hartmann et al. 2018, Stolze et al. 2019). Emerging developments by Biosense (Denmark) have shown success of mold growth quantification through their instrument, the OCellScope, with new research using this technique to screen for bioactive peptides in milk (McNair et al. 2018). This method is both quantitative and high throughput, using pixel intensity and their trademark software to identify pixels which contain growth and count the individual pixels containing this growth.

2.5 Summary and research objectives

The current use of natamycin in the dairy industry requires significant reevaluation due to consumer concerns. In order to meet consumer demands for clean labels and provide an alternative for organic producers, it is essential to find naturally perceived inhibitors for dairy systems. With the wide array of potential inhibitors, including bio-controls, fermentates, organic acids, lactoferrin, plant-based inhibitors, and films, there is significant potential to overcome these

barriers and create an extended shelf life product. However, the large number of potential inhibitors necessitates a high throughput method to evaluate their efficacy on a cheese-mimicking matrix.

Thus, our specific research objectives were to:

1) Develop a quantitative, high throughput method for screening inhibitors on a cheese-mimicking matrix using image analysis.
2) Screen candidate clean-label and combinatorial treatments for their efficacy in slowing the growth of dairy-relevant mold isolates.

3 MATERIALS AND METHODS

3.1 Chemicals

Clove Oil, thyme oil, tween 80, and peptone were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetic Acid and DMSO were purchased from Fisher Scientific (Hampton, NH, USA). Chitosan (149 kDA, 97% deacetylation) was purchased from Primex (Siglufjordur, Iceland). Xanthan gum was purchased from MP Biomedicals (Irvine, CA, USA). Natamycin was purchased from MedChem Express (Monmouth Junction, NJ, USA). Celery juice powder was purchased from The Sausage Maker (Buffalo, NY, USA). Potato dextrose agar (PDA) was purchased from EMD Millipore (Burlington, MA, USA). Bacteriological agar was purchased from VWR (Rador, PA, USA). Lactoferrin was donated by Glanbia (Twin Falls, ID, USA). Microgard® was donated by Dupont (Wilmington, DE, USA)

3.2 Mold culture preparation and maintenance

All ten mold isolates (Table 2) used in this study, with the exception of mold G, were isolated and provided by a local creamery, herein referred to as our industry partner. Mold G was isolated from a brie cheese, purchased from Trader Joe's (Corvallis, OR, USA). All molds were identified using a third-party laboratory (Certified Laboratories, Melville, NY, USA). The mold isolates were maintained on PDA throughout the duration of the experiment and incubated at room temperature (~22°C). Every 4-6 weeks, molds were re-streaked on PDA by scraping spores from the previous plate onto fresh media.

All spore suspensions were aseptically prepared in 0.1% peptone water (Sigma Aldrich, St. Louis, MO, USA) using a hemocytometer and adjusted to $5*10^3$ CFU/ml (Garnier et al. 2018). Spore suspension concentrations were confirmed by standard plate count (SPC) method, through dilution and plating on PDA, followed by inoculation for 72 hours at 22°C.

Table 2: Ten mold isolates used in proof of concept experiments.

Mold Isolate	**Identification**
Mold A	*Penicillium enhinulatum*
Mold B	*Penicillium* sp.
Mold C	*Penicillium aurantiugriseum*
Mold D	*Penicillium* sp.
Mold E	*Penicillium roqueforti*
Mold F	*Penicillium* sp.
Mold G	*Geotrichum candidum*
Mold H	*Penicillium* sp.
Mold J	*Penicillium* sp.
Mold K	*Penicillium* sp.

3.3 Preparation of inhibitor treatments

Stock concentrations of all inhibitor treatments were prepared as described in Table 3 and diluted for application using a two-fold serial dilution series in the appropriate carrier solution.

Table 3: Inhibitor treatments, carrier solutions, and stock concentrations used in proof of concept screening experiments.

Inhibitor	**Carrier**	**Concentration of Stock Solution**
Natamycin	DMSO	800 ppm
Clove oil	0.05% Tween80	16,000 ppm
Thyme oil	0.05% Tween80	16,000 ppm
Chitosan	1.0% acetic acid	2% w/v
Celery juice powder	Sterile water	3% w/v
Microgard®	Sterile water	0.75% w/v
Lactoferrin	Sterile water	10% w/v

3.4 Shredded cheese shelf life study

In order to assess the efficacy of natamycin in shredded cheese, a shelf-life study was employed. Because mold spoilage is an issue of visual concern, we measured time to spoilage through visual assessment of mold growth, with the shelf life defined as the first day of visible hyphal growth. Shredded cheese was provided by our industry partner with or without the natamycin/anti-cake coating. The natamycin coating was dosed to achieve a rate of 10 mg/kg. In order to stimulate

mold growth, a cocktail of spores from molds A-K (equal proportion) was prepared and diluted to achieve a high level (10^4 CFU/bag), a medium level (10^3 CFU/bag), and a low level (10^2 CFU/bag) of inoculation. In order to ensure mold growth was visible, spores were inoculated by transferring 1.0 ml of the mold cocktail in the center of the clear window in the bags of shredded cheese. See Table 4 for a full list of treatments.

Table 4: Treatments for shredded cheese shelf life study.

Treatment	Opened	Inoculation level (spores/bag)	Natamycin (+/-)
1	No	0	+
2	Yes	0	+
3	Yes	0*	+
4	Yes	opened and inoculated with the low level of spores	+
5	Yes	opened and inoculated with the medium level of spores	+
6	Yes	opened and inoculated with the high level of spores	+
7	No	0	-
8	Yes	0	-
9	Yes	0*	-
10	Yes	opened and inoculated with the low level of spores	-
11	Yes	opened and inoculated with the medium level of spores	-
12	Yes	opened and inoculated with the high level of spores	-

*Peptone blank

All treatments were performed in triplicate (n=60). Shredded cheese bags were stored under refrigerated conditions (7°C) for the duration of the experiment (272 days). In order to mimic

consumer abuse, bags of shredded cheese were opened (with the exception of treatment 1) and placed at room temperature (22°C) for 30 minutes each day under a sterile, laminar flow hood. This was reduced to three days a week after 30 days, and then halted after 118 days. Pictures were taken each day that bags underwent the "abuse" conditions, using a light box (Finnhomy®, China) and phone camera (LG G4) attached to a tripod, to determine visible time-to-mold. Upon sufficient and visible mold growth, bags were discarded.

3.5 Cheese agar

Cheese agar (Wolfe et al. 2014), was selected and adapted as a reproducible, cheese mimicking matrix. All cheese used in this study was provided by the Oregon State University (OSU) creamery. Briefly, cheddar cheese blocks were shredded and frozen, freeze dried (Hull Corporation Model 651M-9WDF20 or VirTis Console 4.5), and ground using a mortar and pestle. For final preparation of agar, 100 g freeze dried cheese, 50 g xanthan gum, 17 g bacteriological agar, and 1000 ml water were combined, blended for 60 seconds (Oster blender), and autoclaved for 15 min at 250 °F (121 °C) on a liquid cycle. For enhanced mold visualization of some cheese agar, 0.0001% methylene blue (MB) was added after autoclaving, herein referred to blue cheese agar.

For high throughput experiments, cheese agar was pipetted into 96 well micro-titer plates. Initially, due to its high fat nature, cheese agar proved challenging to pipette. To mitigate this, the optimal temperature for consistent pipetting was determined. Subsequently, an aluminum reservoir (B&P Scientific Inc.) was preheated to 90° and 80°C cheese agar was poured into the reservoir which was kept on a heating block (Eppendorf, Hauppauge, NY, USA) to maintain optimal pipetting temperature. All plates were prepared by transferring 150 µl of CA into each well using an 8-channel pipette (Integra, Plainsboro Center, NJ, USA).

3.6 Radial Growth on proposed cheese mimicking matrix

To determine efficacy of cheese agar as a replacement for lab media and the impact of MB on growth, a time-lapse study was carried out. 20 ml of 1) cheese agar, 2) PDA, 3) cheese agar with the addition of MB, and 4) PDA with the addition of MB, were poured into petri dishes. Plates were left untreated or spread with a final dosage of 10 ppm natamycin. Petri dishes were then spotted in triplicate with $5*10^3$ CFU/ml *Penicillium* Sp. and *Geotrichum candidum*. Petri dishes were carried out in duplicate. The initial inoculation diameter was measured by spotting 10 µl of 0.01% methylene blue onto cheese agar or PDA, in triplicate, and measuring the average diameter using ImageJ. Images were captured every 24 hours over 7 days, taken using the SPImager light box (S&P Robotics Inc, Toronto, CA, USA). Upon completion of the experiment, ImageJ Software was used to measure the diameter of each colony at the given time point. To account for variation in growth patterns, the largest and smallest diameter were averaged to generate the colony diameter. Subsequently the colony diameters were averaged to get a single value for each treatment at each time point.

3.7 Preliminary image analysis trials

Because early stages of mold growth were difficult to visualize on initial image analysis experiments, methylene blue was trialed to improve image sensitivity in conjunction with pixel intensity. Micro-titer plates prepared with cheese agar and blue cheese agar were spotted with 10 µl of $5*10^3$ CFU/ml *Geotrichum candidum* and *Penicillium* Sp. After 48 hours of growth at room temperature (22°C), images were taken and assessed using ImageJ software.

Briefly, using ImageJ, a circle was drawn over the entirety of each well in the micro-titer plate and the pixel spectra/pixel intensity was measured within that circle. Grey scale spectra is defined as a measure of a pixel's lightness/darkness on a gray scale ranging from 0 (complete blackness) to

255 (complete whiteness). The pixel intensity is defined as the count of pixels at each value on the gray scale (0-255). Thus, as assessment of the spectra indicates the level of lightness/darkness of the area and changes in lightness/darkness should correspond to changes in mold growth.

3.8 Image analysis to distinguish levels of mold growth

Upon confirmation that the gray scale spectra shifts with changes in mold growth, the ImageJ plugin "ReadPlate" was assessed for further analysis of the data. The "ReadPlate" plugin utilizes the gray scale spectra to calculate the raw integrated density (RID) in the region of interest (ROI). The RID is defined as the sum of all gray scale values in the ROI and can thus illustrate a more complete picture of mold growth. For example, because high values on the gray scale are associated with whiteness (255) and low values with blackness (0), a high RID indicates a light/white area and a relatively lower RID indicates a dark/black area of growth.

With this principle in mind, a brief experiment was carried out to determine if RID values correspond to different levels of mold growth and to further analyze the utility of MB for enhanced image sensitivity. Micro-titer plates were filled with cheese agar and blue cheese agar, as previously described in section 3.6. In order to generate wells with different levels of growth, entire rows of wells (n=12) were inoculated with final dosages of 10^{-1} CFU/well-10^{4} CFU/well of *Geotrichum candidum* (n=12), with inclusion of two rows (n=24) of agar-only blanks. Plates were incubated at room temperature (~22°C) and images of plates were taken after 48 hours. Upon completion, grayscale images were analyzed using the "Readplate" plugin with an 80 pixel diameter to avoid well-edge effects.

3.9 Proof of concept experiments

Experiment 1: Blue cheese agar was dispensed into sixteen 96-well micro-titer plates. Inhibitor treatments of clove oil, thyme oil, chitosan, and natamycin (as a positive control), were prepared

in six different treatment levels using a two-fold dilution series. See Table 5 for a list of all treatments. Upon preparation of plates, 15 µl of each treatment were dispensed in quadruplicate wells and allowed to dry in a laminar flow hood under sterile conditions. Following treatment, 10 µl of each of 10 mold suspensions (See Table 2) were inoculated to achieve a final dosage of 50 CFU/well. Plates were then fitted with a lid, sealed with Parafilm, and incubated at room temperature (22°C) for the duration of the experiment. High resolution photos were taken every 12 hours over 7 days (168 hours total) using the SPImager light box and camera set up described in section 3.6. Images were used to measure RID at each time point as a proxy for mold growth, using the absolute value of the blanked sample. Because shifts in spectra can be positive or negative, when "blanked" through the subtraction of the agar background, the resulting value may be positive or negative, thus, the absolute value of the blanked sample was used. Upon analysis, there was no difference ($p<0.05$) in blanks based on plate location (data not shown). Thus, blank wells were averaged prior to subtraction from sample wells for all further analysis. Using the "Growthcurver" package (Sprouffske and Wagner 2016) in conjunction with R statistical software, growth curves were fitted to each well and the lag time metric estimated. "Growthcurver" defines lag time as the time point at which the measured growth has reached 50% of its maximum. Lag time was used to measure differences in growth delay in response to inhibitors. Because the software attempted to extrapolate the lag time if it extended beyond the length of the experiment, any lag times over the 7 day time frame were changed to 168 hours, the final time point of the study.

Experiment 2: Thirty-six 96-well micro-titer placed were prepared with blue cheese agar, including the same treatments as described in Experiment 1. In addition, combinatorial treatments of thyme/chitosan at 12 levels were included to evaluate potential additive effects of treatments.

For additional treatments, see Table 6. Following treatments, the experiment was carried out as described in Experiment 1 over a course of 168 hours.

Experiment 3: twenty-two 96-well micro-titer plates were prepared with blue cheese agar and treated with a two-fold dilution series of candidate clean label inhibitors celery power, lactoferrin, Microgard® and combinations thereof. See Table 7 for a complete list of treatments. Experiment was carried out as described in experiment 1.

3.10 Analysis with ImageJ/"Growthcurver"

At each 12 hour time point in experiments 1-3, ImageJ software was used to measure pixel density of each well using the previously captured high resolution images. Using the "ReadPlate" Plugin, a template was overlaid over the 96 well micro-titer plate and the software captured RID of each well within a diameter set by the user. Because no color modifications were made to the image, in this study, the "gray" scale was selected and the diameter was set at 80 pixels, for improved consistency. Subsequently, all RID data was blanked by subtracting the average untreated wells (carriers only) from each sample and converting the new sample number to an absolute value. This calculation is summarized in the formula below.

$$Converted\ sample\ Value = ABS\ [original\ sample - (average\ blank\ value)]$$

Sample values were then used to fit growth curves and calculate lag times using the "Growthcurver" Package from R statistical software (3.6.1). Lag times subsequently were tested using ANOVA and Tukey's post hoc test in comparison to control growth (no inhibitor treatment).

Table 5: Treatments for first proof of concept experiment.

Inhibitor	Final Treatment Level
Clove Oil (ppm/well)	1600.0
	800.0
	400.0
	200.0
	100.0
	50.0
Thyme Oil (ppm/well)	1600.0
	800.0
	400.0
	200.0
	100.0
	50.0
Chitosan (%/well)	0.02
	0.01
	0.005
	0.0025
	0.00125
	0.000625
Natamycin (ppm/well)	80.0
	40.0
	20.0
	10.0
	5.0
	2.5

Table 6: Additional, combinatorial treatments for second proof of concept experiment.

Treatment	Chitosan (%/well)	Thyme Oil (%/well)
1	0.005	200.0
2	0.01	200.0
3	0.02	200.0
4	0.005	400.0
5	0.01	400.0
6	0.02	400.0
7	0.005	800.0
8	0.01	800.0
9	0.02	800.0
10	0.005	1600.0
11	0.01	1600.0
12	0.02	1600.0

Table 7: Treatments for third proof of concept experiment.

Inhibitor	Final Treatment Level
Microgard® (%/well)	0.3
	0.15
	0.075
	0.0375
	0.01875
	0.009375
Lactoferrin (%/well)	1.0
	0.5
	0.25
	0.12
	0.06
	0.03
Celery Powder (%/well)	0.3%
	0.15
	0.075
	0.0375
	0.01875
	0.009375

4 RESULTS

4.1 Efficacy of natamycin for extension of shredded cheese shelf life

While natamycin is widely used to protect cheese from mold spoilage, relatively few studies have directly shown the efficacy of natamycin on shredded cheese. Therefore, an experiment was carried out to evaluate the shelf life of shredded cheese with and without natamycin under simulated conditions of consumer abuse. Shredded cheese coated in anti-caking agent that did, or did not, contain a standard application of natamycin was provided by an industry partner, thereby allowing for testing of products packaged and made under standard conditions of a large cheese-processing plant. Results are summarized in Figure 3, while representative images used to generate time-to-mold data are shown in Appendix 1: Figure A1.

There was no occurrence of visible mold spoilage on shredded cheese in unopened packages throughout the 272 days of this experiment, regardless of the presence of natamycin. The opened controls (blank and carrier) showed no statistical differences in shelf life to one another (for either natamycin ($P>0.05$) or untreated cheese ($P>0.05$), demonstrating that the peptone used to inoculate with mold had no effect. While three levels of mold spore inoculation were used, there was no difference in time-to-mold between these treatments with ($P>0.05$) or without ($P>0.05$) natamycin. This suggests a relatively low saturation point of 10^2 CFU/bag, beyond which additional spores did not affect shelf life of this cheese.

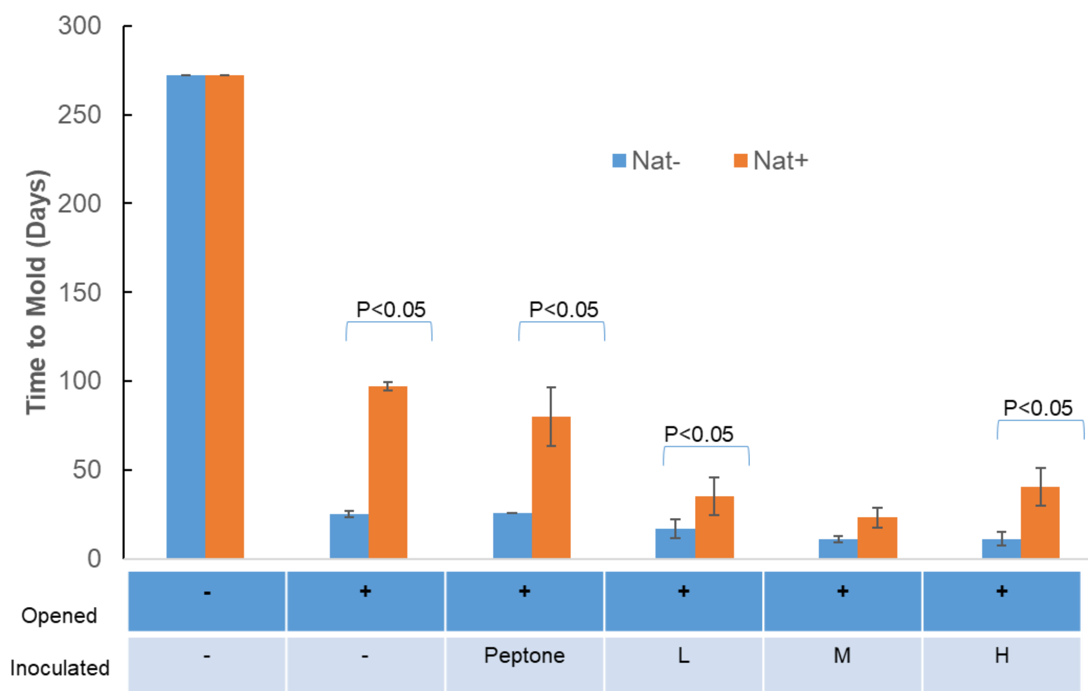


Figure 3: Development of mold growth on shredded cheddar cheese with or without the addition of natamycin. Bar graph depicts the average time taken for detection of visible mold growth and error bars represent the standard deviation between replicates. Cheese was inoculated with a cocktail of the ten mold isolates to replicate a low level (L) of contamination (10^2 CFU/bag), a mid level (M) of contamination (10^3 CFU/bag) and a high level (H) of contamination (10^4 CFU/bag) alongside controls bags, which were left unopened, opened and untreated, or opened and inoculated with a peptone blank (carrier solution). Significance values indicate natamycin treatments which were significantly longer than their untreated counterparts.

Natamycin treatment significantly increased time-to-mold for the controls, the highest level of inoculation, and the lowest level ($p<0.05$). At the medium level of inoculation, despite a mean increase in shelf life of 12 days, the results were not statistically significant ($p=0.62$). The time lapse photo series (Appendix 1: Figure A1) further illustrates these results with all natamycin-treated cheese showing a delay in visible mold growth, with marked delays for opened controls, the highest level of spore inoculation, and the lowest level of spore inoculation.

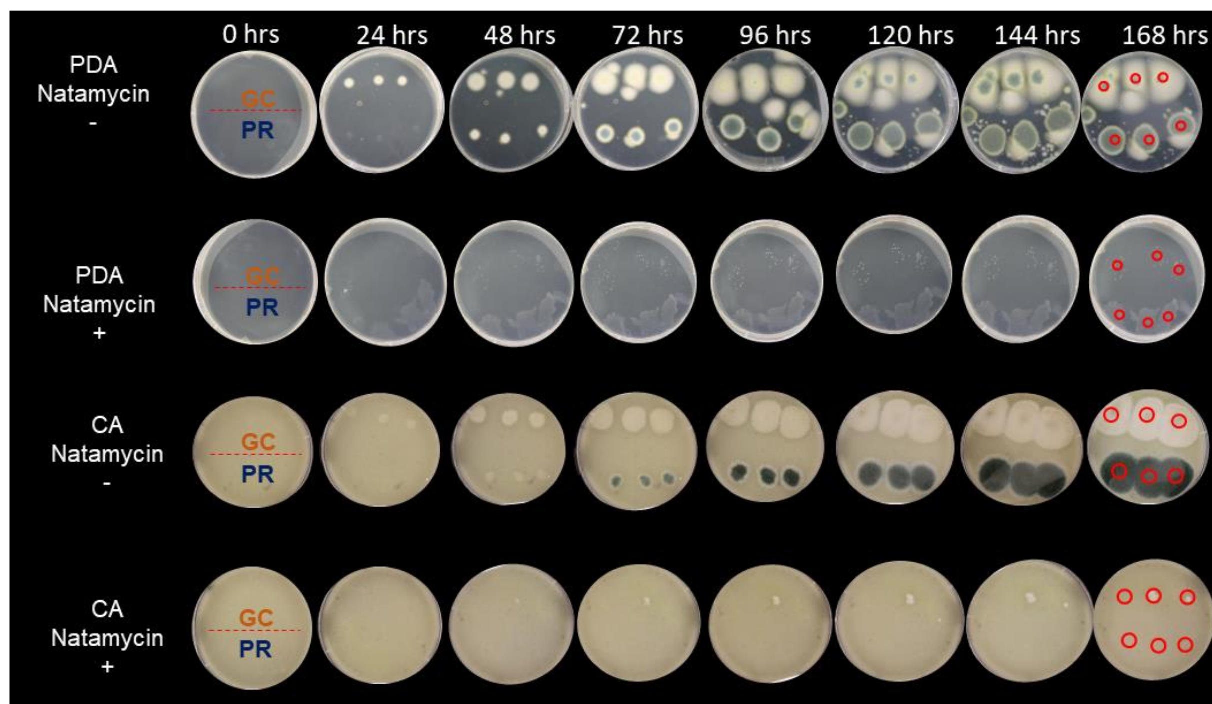
Although shredded cheese may be the most industry-applicable context for evaluation of mold inhibitor efficacy, this methodology is limited in its throughput. With 60 bags of shredded cheese, we were only able to test the efficacy of a single inhibitor. Thus, in order to test a broad range of potential anti-mycotics, it was necessary to implement a high-throughput method to increase sample number.

4.2 Evaluation of proposed cheese-mimicking matrix

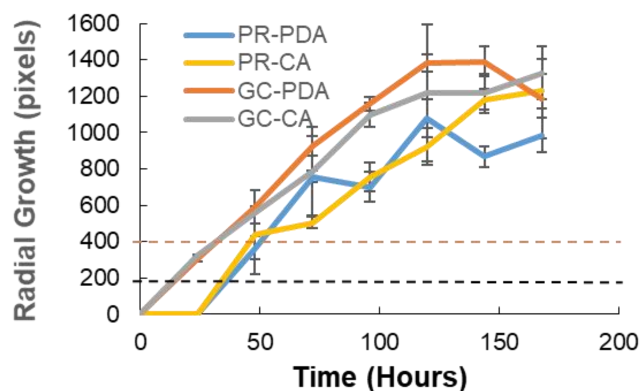
4.2.1 Radial hyphal growth of molds on cheese-agar

High-throughput screening methodologies require standardization of a test matrix, often based upon widely used laboratory media. For mold-growth, a commonly used media formulation is potato dextrose agar (PDA). However, previous research (as described in section 2.5), indicates that standard laboratory media formulations may overestimate inhibitor efficacy. Recent studies have developed more realistic matrices for studying microbiology of cheese, based upon miniaturizing the cheese-making process (Garnier et al. 2018) or using freeze-dried cheese in a solidified agar gel (Wolfe et al. 2014). In order to evaluate the latter as a cheese-mimicking matrix and assess differential mold responses to media type, a radial growth experiment was carried out (Figure 4) with natamycin used to elicit an inhibitor response.

A



B



C

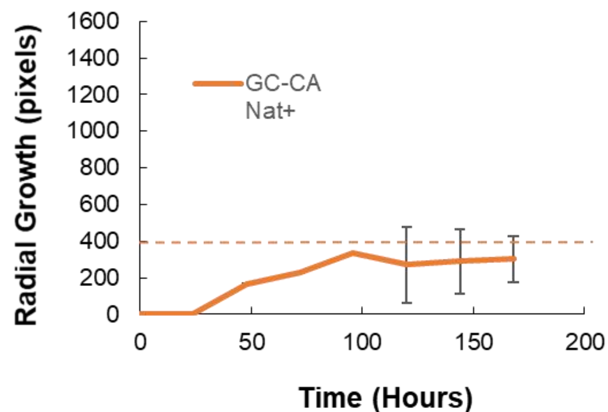


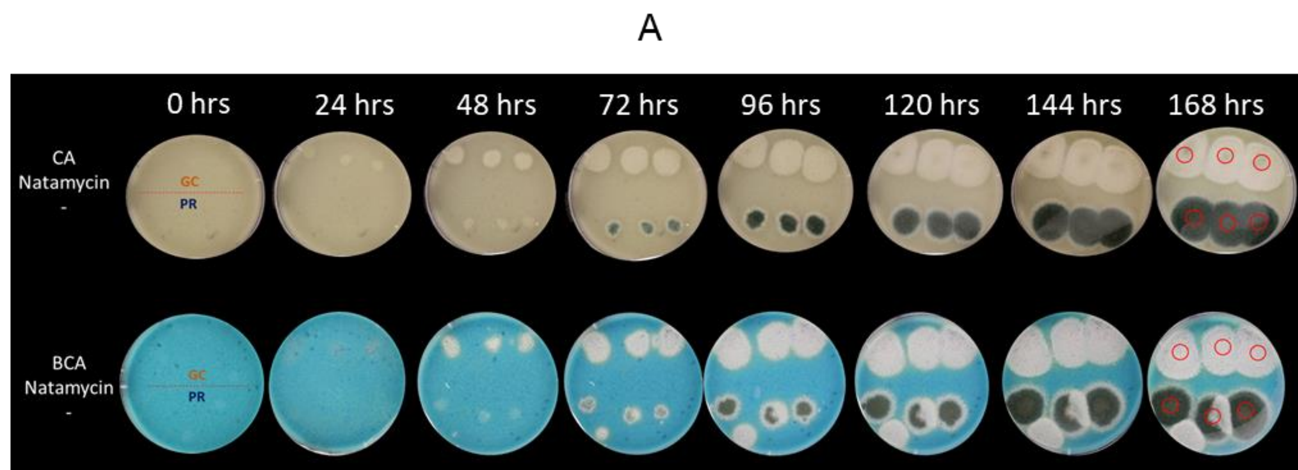
Figure 4: Mold radial growth on standard lab media (PDA) and cheese agar in response to natamycin. Photo series (A) depicts a representative petri dish inoculated with *Geotrichum* (GC) and *Penicillium* (PR) over the 168 hour experiment. Line graphs without natamycin (B) and with natamycin (C) depict growth curves based upon colony diameter +/- standard deviation, as measured using ImageJ. Orange dotted line represents initial inoculation diameter on CA and black dotted line represents the initial inoculation diameter on PDA.

As evident in the time-lapse photo series depicted in Figure 4A, when no inhibitor was present PDA and CA allow for similar rates of mold growth. Indeed, there were no statistically significant differences when comparing radial growth on cheese agar to that on PDA (Figure 4B) at each point ($P>0.05$) with the exception of *Penicillium* at 144 hours ($p>0.05$). Addition of natamycin drastically influenced growth of *Geotrichum* and *Penicillium* (Figure 4A). On PDA, *Geotrichum* showed initial signs of colony formation, but these pinprick colonies did not expand and therefore no radial growth of hyphae was measured. A visible smear in the regions where *Penicillium* spores had been applied became visible within 24h, but there was no visible hyphal radial growth. By contrast, small *Geotrichum* colonies were detected on cheese agar plates treated with natamycin from 48hrs (replicate 2) and 120h (replicate 3), where subsequent radial expansion was observed over time (Figure 4C).

4.2.2 Improvement of mold visualization using methylene blue dye

Early stages of mold growth were difficult to visualize on the tan cheese agar in the radial growth experiment (Figure 4) and preliminary image analysis trials in 96-well plates (results not shown), thus prompting exploration to modify cheese agar for improved visualization. Methylene blue (MB) dye was added to cheese agar at a rate of 0.0001%, as this rate produced sufficient blue color to visualize growth. Upon addition of MB, a radial growth experiment was carried out to determine impact on mold growth/visualization (Figure 5).

A



B

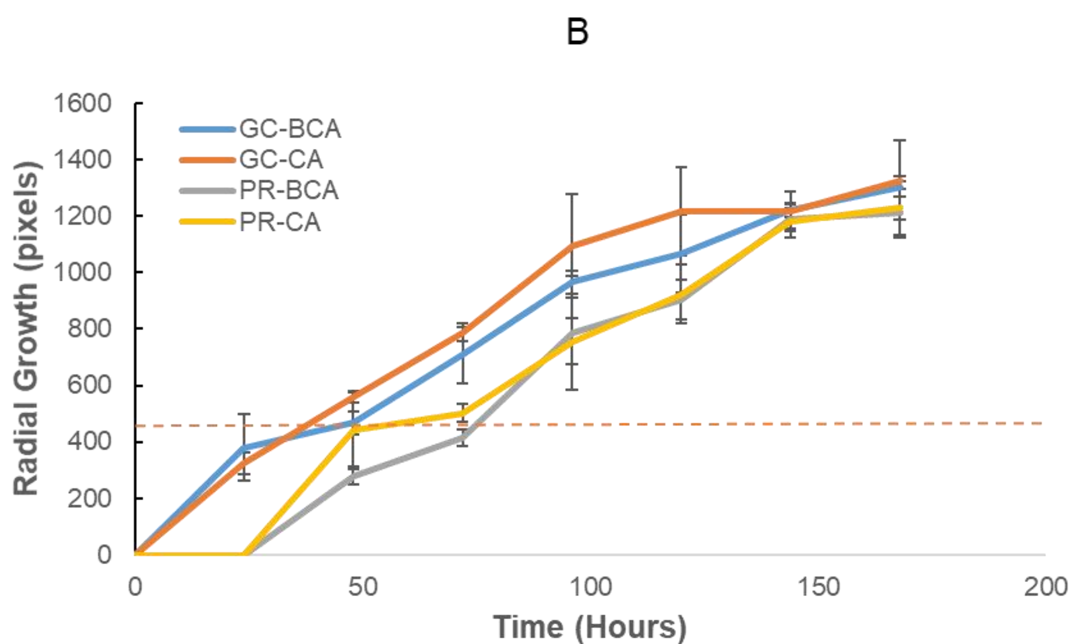


Figure 5: Mold radial growth on cheese agar and cheese agar with the addition of 0.0001% methylene blue. Time lapse photo series (A) depicts mold growth of representative petri dish replicates inoculated with *Geotrichum* (GC) and *Penicillium* (PR) over the 168 hour experiment. Line graph (B) depicts mean colony diameter +/- standard deviation for *Geotrichum* or *Penicillium* on cheese agar or blue cheese agar. Orange dotted line represents initial inoculation diameter on cheese agar.

Visually, there was no apparent impact of methylene blue on the timing of mold colony formation or extent of hyphal spread (Figure 5A). On both media, *Geotrichum* colonies were visible at 24 hours, while *Penicillium* colonies could be seen at 48 hrs. Radial growth curves on blue cheese agar did not differ from those on standard cheese agar at any time point ($P>0.05$). This indicates that MB, at a rate of 0.0001%, is a suitable option for improved visualization on cheese agar without inhibiting mold growth.

4.3 Evaluation of image-analysis as a tool to monitor mold growth on cheese agar

4.3.1 Changes in grayscale spectra of images act as a suitable proxy for mold growth

While label friendly mold inhibitors have been screened in a wide variety of food matrices, there is still a need to improve high throughput processes. Having shown that blue cheese agar is an appropriate matrix for mold growth, we evaluated the utility of image analysis as a proxy for visual assessment of colony formation in 96-well plates. At this scale, there is limited opportunity to observe hyphal spread by measuring colony radius, indeed the diameter of the wells was roughly equivalent to the dispersal of inoculum drops on petri dishes. Therefore, we used ImageJ to extract the grayscale ‘spectra’ of cheese agar and blue cheese agar on which mold growth was visible and compared these spectra to untreated media (Figure 6).

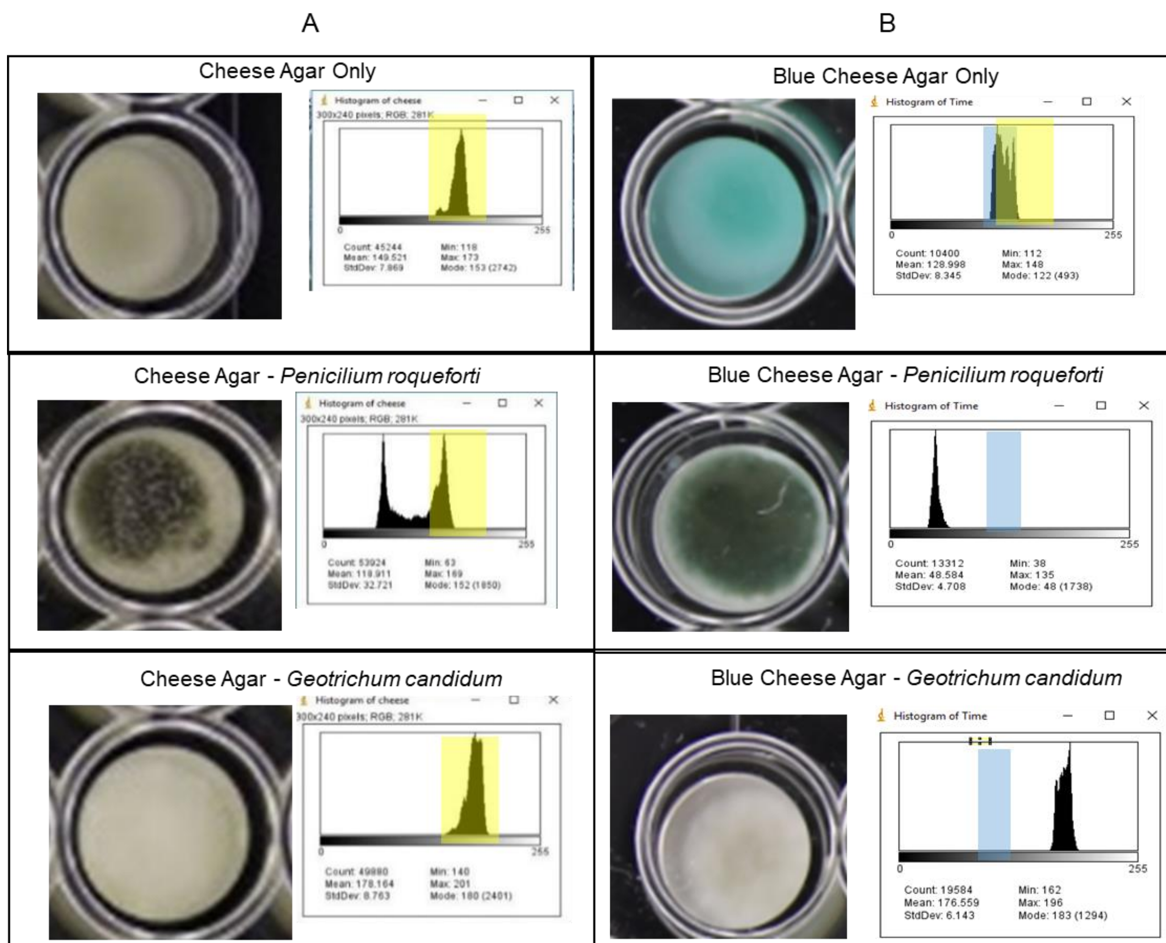


Figure 6: Grey scale spectra of *Penicillium* and *Geotrichum* molds grown on cheese agar and blue cheese after 72 hours of growth. Histograms, derived using ImageJ, are shown alongside individual wells on cheese agar (A) or blue cheese agar (B). The spectra represents the count of pixels at a given value (0-255) on the gray scale in the region of interest (ROI). Overlaid yellow squares highlight cheese agar background spectra and overlaid blue squares highlight the blue cheese agar background.

As described in section 3.7, the gray scale spectra is defined as the level of darkness/lightness of an individual pixel on a scale of 0 (black)-255 (white). The spectra (Figure 6) shows the pixel intensity (count of pixels at a given position along this grayscale). With the inoculation of *Penicillium* on cheese agar (Figure 6A), there is clear visual growth and shift of the spectra to a lower grayscale value. The additional peak on the spectra reflects the partial growth of the mold and captures visible areas of cheese agar. *Geotrichum* was less easily visualized, as the mold growth is of a similar color intensity to the agar background. This was consequently seen in the spectra, which was only shifted minimally towards a higher grayscale value on the histogram.

Upon the addition of MB (Figure 6B) the background spectra of the agar was shifted to a lower point on the grayscale. Clear visual distinction of growth in wells inoculated with *Penicillium* and *Geotrichum* corresponded with high pixel counts at grayscale values distinct from the agar background. For *Penicillium,* the grayscale value was substantially lower than blue cheese agar while *Geotrichum* growth resulted in a distinct shift to a lower grayscale value.

4.3.2 Quantitative analysis of mold growth based upon grayscale pixel count

Following analysis of the gray scale spectra, we then sought to evaluate whether image analysis data could distinguish between wells that visually differed in the extent of mold growth. To achieve this, different rows of 96-well plates containing cheese agar or blue cheese agar were inoculated with *Geotrichum* spore suspensions to achieve final inoculation densities from 10^4 CFU/well-10^{-1} CFU/well (Figure 7).

Data was extracted from grayscale images of each plate using the “ReadPlate” ImageJ plugin and presented in Figure 7. As mentioned in section 3.8, raw integrated density (RID) is defined as the sum each pixel’s gray scale value in the ROI, and thus can be used to illustrate a complete picture of the gray scale spectra.

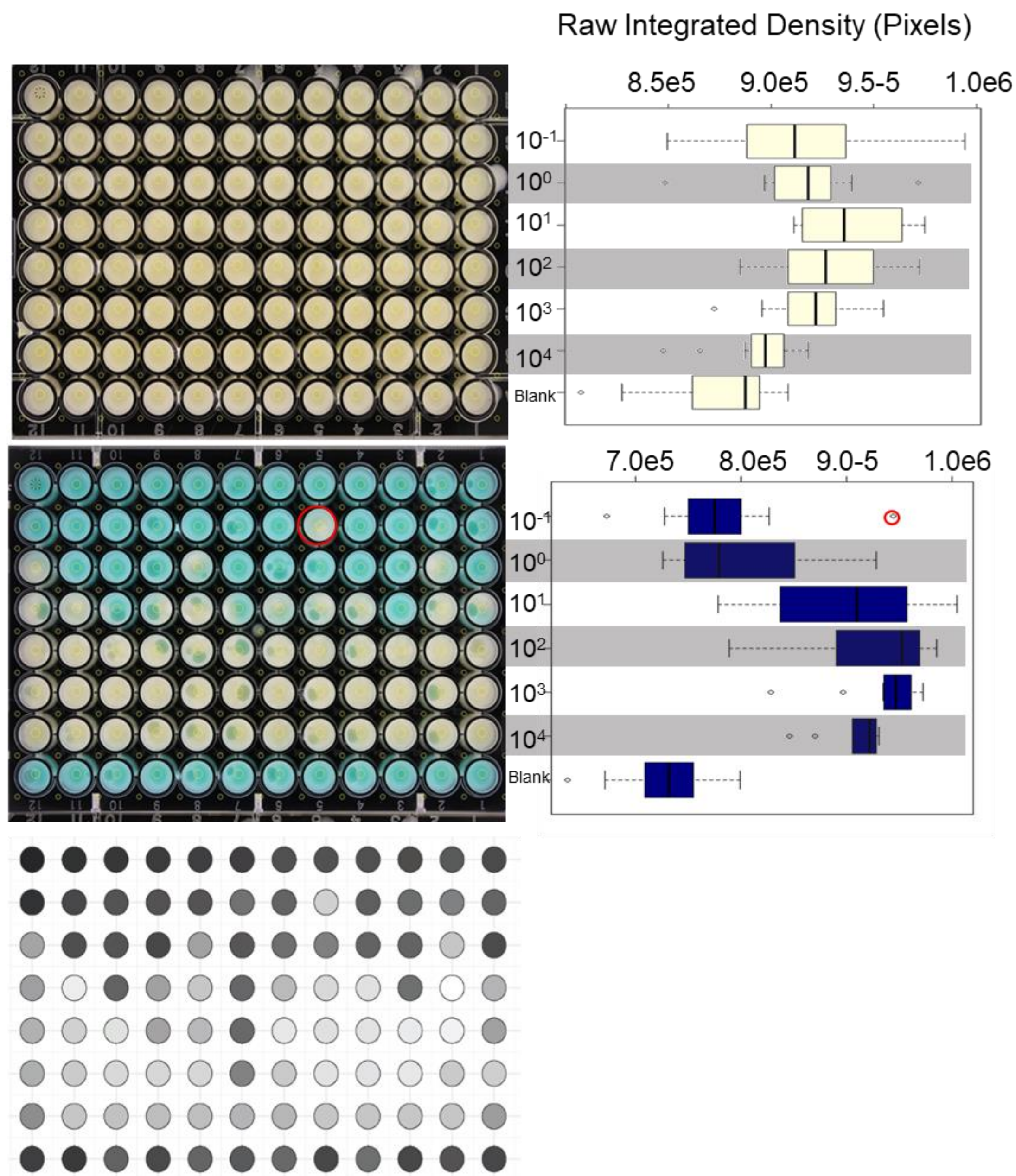


Figure 7: Evaluation of image analysis for distinction of mold growth levels. Cheese agar plate (A) and blue cheese agar plate (B) were inoculated with *Geotrichum* mold and are shown after 48 hours of growth. Plates were incubated at 22°C for the duration of the experiment. Overlaid yellow circles are indicative of the 80 pixel diameter used to generate data from ImageJ (NIH) software. Associated boxplots depict RID at relative concentrations. Data from blanks (rows A and H) were combined for boxplot generation. The accompanying heat map (C), overlaid over a 96 well plate, depicts RID on a gray spectra scale, with white representing a high RID and black representing a low RID.

As described previously (section 4.2.2), the addition of MB greatly enhances visibility of mold growth on cheese agar. RID for *Geotrichum* on cheese agar (Figure 7A) shows no statistical differences between wells inoculated at any spore density relative to blank wells ($P>0.05$), consistent with visual assessment of the image – no mold growth is visible. However, on blue cheese agar (Figure 7B) there were statistically significant results when comparing inoculated wells to blank wells ($P<0.05$). More variation in RID was seen between replicate wells at intermediate inoculation levels ($10^0 – 10^2$) than high inoculation levels ($10^3 – 10^4$), evident by skewed boxplot distributions, which corresponds with visual assessment of the blue cheese agar plate image. Furthermore, a clear outlier in the 10^{-1} row (Figure 7, red circle) can be seen as an outlier in the boxplot. To further illustrate correspondence between the RID output and image, RID is shown as a heat map (Figure 7C) overlaid on a 96 well plate. A similar plot is shown in Figure 8 for a plate image generated in a subsequent experiment, with both white and blue mold growth to demonstrate the efficacy of this approach on molds of different colors.

The plate image (Figure 8A) and corresponding heat map (Figure 8B) indicate similar results for *Geotrichum* (Mold G) as seen in Figure 7, with increased RID values corresponding to a lighter color on the heat map where visible growth has occurred. As anticipated, *Penicillium* Sp. (Mold H) shows a darkening on the plate and corresponding low RID value on the heat map. Variable growth of an additional *Penicillium* isolate (Mold J) on the plate is also reflected in the heat map, with a light gray intensity. With this assessment, it is evident that RID/image analysis corresponds with visual assessment of mold growth and can be used as quantitative proxy.

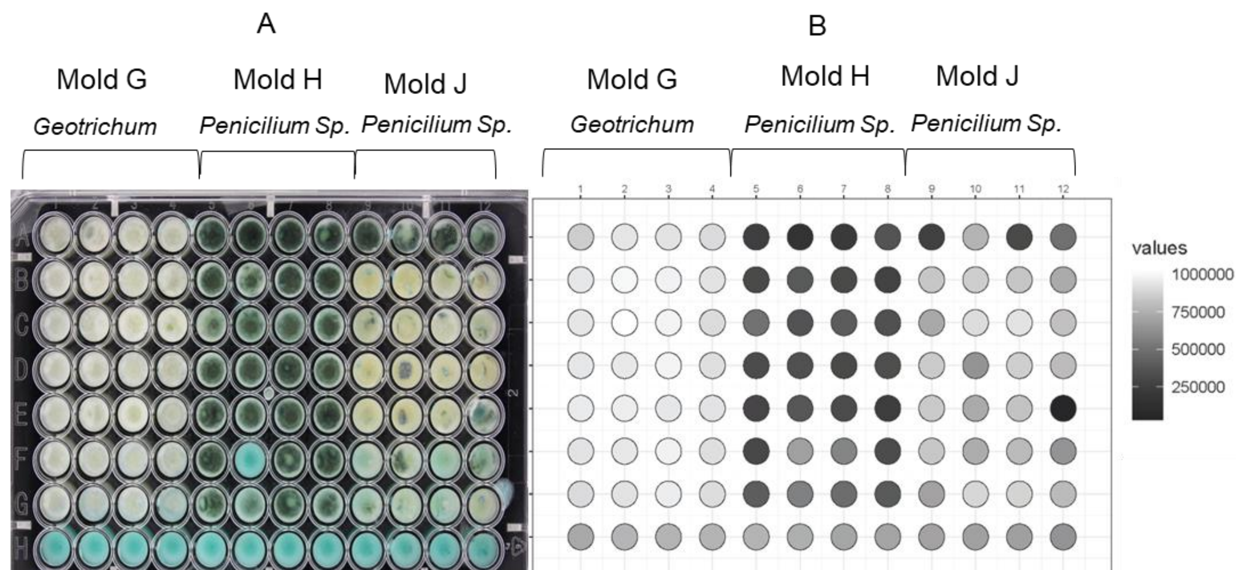


Figure 8: Evaluation of the ImageJ "ReadPlate" plugin for analysis of white and blue mold growth. Plate was inoculated with three mold isolates, a white mold (Mold G) and two blue molds (Molds H and J) and is shown after 96 hours of growth. The Plate was incubated at 22°C for the duration of the experiment. Rows B-H were treated with varying concentrations of clove oil. The plate image (A) illustrates visual growth of the three molds and accompanying heat map (B) depicts RID across the plate on a gray spectra scale with white representing a high RID and black representing a low RID.

4.3.3 Generation of mold growth curves based upon grayscale pixel count

To assess the application of image analysis to measure mold growth over time, the R package "Growthcurver" was utilized. The package was applied to RID values for a time-lapse image series of multi-well plates for two mold isolates grown on blue cheese agar. In addition to plotting growth curves, this package estimates lag time, defined as the time point at which 50% of the maximum data value has been reached. In this case the data was RID values, which, as shown in previous section, are representative of mold growth. Lag time presents an ideal metric for comparing mold growth in respect to inhibitors, as molds such as *Geotrichum* and *Penicillium* grow at very different rates. (Figure 9, Table 8).

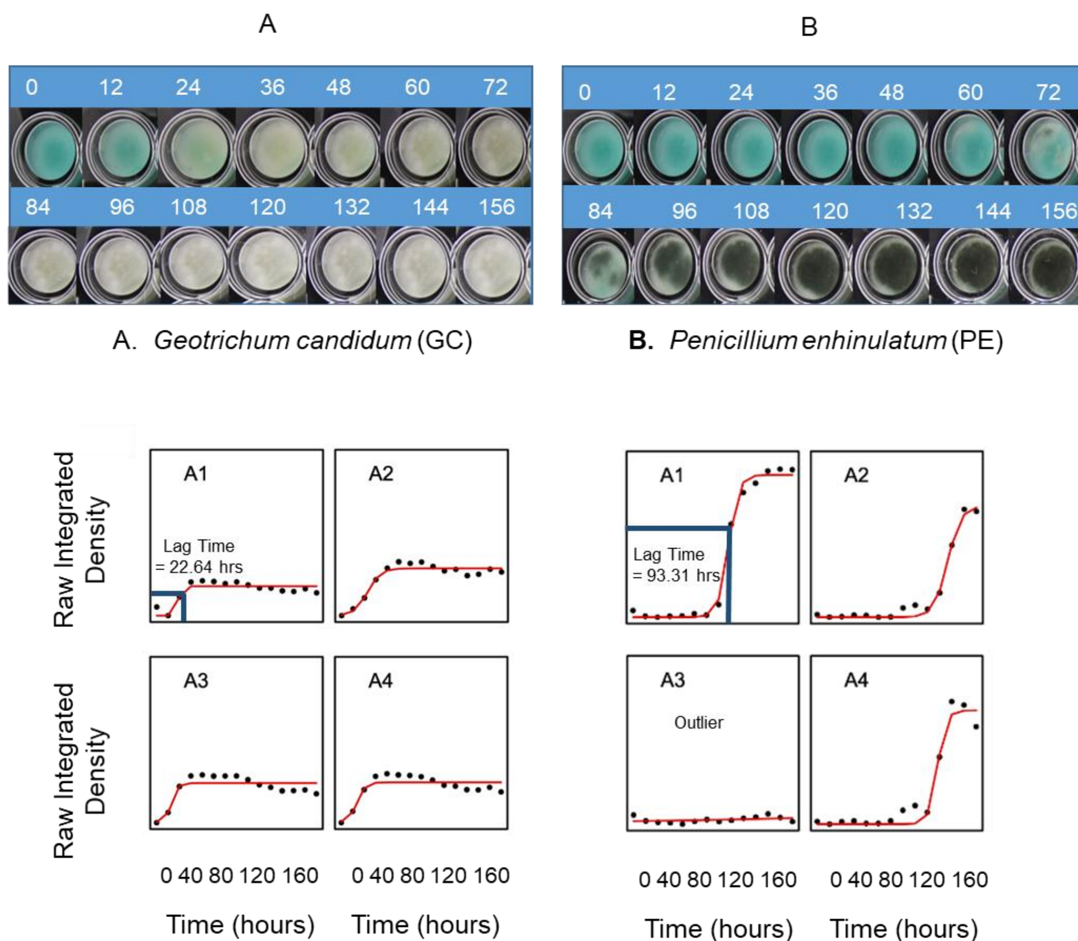


Fig 9: Time lapse photo series and growth curves plotted by "Growthcurver" R package. Photo series depict *Geotrichum candidum* (A) and *Penicillium enhinulatum* (B) colony development over the 168 hour experiment. Growth curves are based upon RID values for quadruplicate wells inoculated with Geotrichum (C) and *Penicillium* (D). Lag times for first replicate have been overlaid over the plot and outliers noted.

Table 8: Different metrics for assessing growth of *Geotrichum* and *Penicillium*

Mold	Median Lag Time (hours)	Standard Deviation of Lag Time	Final Median RID	Standard Deviation of RID	Average Generation Time	Standard Deviation of Generation Time
Geotrichum candidum	19.5	5.5	1.7×10^5	3.7×10^4	3.8	1.4
Penicilium enhinulatum	112.9	13.6	4.6×10^5	2.5×10^5	3.0	1.0

Geotrichum candidum (Figure 9A), for example, is a relatively fast growing mold with a median lag time approximately 90 hours shorter than that estimated for the blue mold, *Penicillium enhinulatum.* The estimated lag time for *Penicillium enhinulatum* corresponds with visual assessment of the representative time lapse photo series (Figure 9B). Mold growth appears on the blue cheese agar around 60-72 hours, then appears to be half-covered by hyphal growth at 84 hours, and fully covered by 108 hours. Similarly, in the representative series for *Geotrichum candidum* the media appears relatively free of mold growth at 12 hours but diffusely covered by 24 hours, and completely covered by 36 hours. Relative variation in lag-time estimates across replicate wells was greater for *Geotrichum* than for *Penicillium*. This is seen visually in differences in growth curves and increased RID/standard deviation (Table 8) for *Penicillium* (4.6×10^5 +/-2.5 x 10) relative to *Geotrichum* (1.7×10^5+/-3.7 x 10). Some growth curves for *Geotrichum* show an increased initial slope compared to that of other replicates. A contributor to this noise was the low frequency of images captured during the early stages of *Geotrichum* growth. It is also worth noting that final RID (blank-subtracted, absolute values) for *Geotrichum* were much lower than for *Penicillium*, which compresses the dynamic range of the growth curves. Final RID values for *Penicillium* were significantly more variable than for *Geotrichum*,

suggesting it may be a less reliable parameter for comparison of mold growth in response to inhibitors. This is further illustrated in the growth curves with well A1 for *Penicillium* (Figure 9B) showing a higher end RID than other replicates. The other parameter estimated, generation time, was similar for both molds in terms of value and extent of variability between replicates. Because what we see visually in terms of mold growth is a composite of spore germination, vegetative growth, and hyphal extension, it is difficult to relate generation time to the representative image series.

4.3.4 Proof of concept – inhibitor screening experiment

To test the broader applicability of image-analysis based mold growth quantitation for high-throughput screening, a proof-of-concept experiment was performed using candidate clean-label inhibitors chitosan, thyme oil, and clove oil, with natamycin included as a positive control. For each inhibitor, a two-fold dilution series was applied to blue cheese agar, and then all wells were inoculated with 50 spores of the ten different test fungi. Time-lapse photograph series were analyzed in bulk using “Readplate” and “Growthcurver”, resulting in growth curves for every well on every 96-well plate. An example of growth curves generated for a thyme oil treated plate inoculated with three mold isolates is shown in Figure 10, along with derived lag-time estimates.

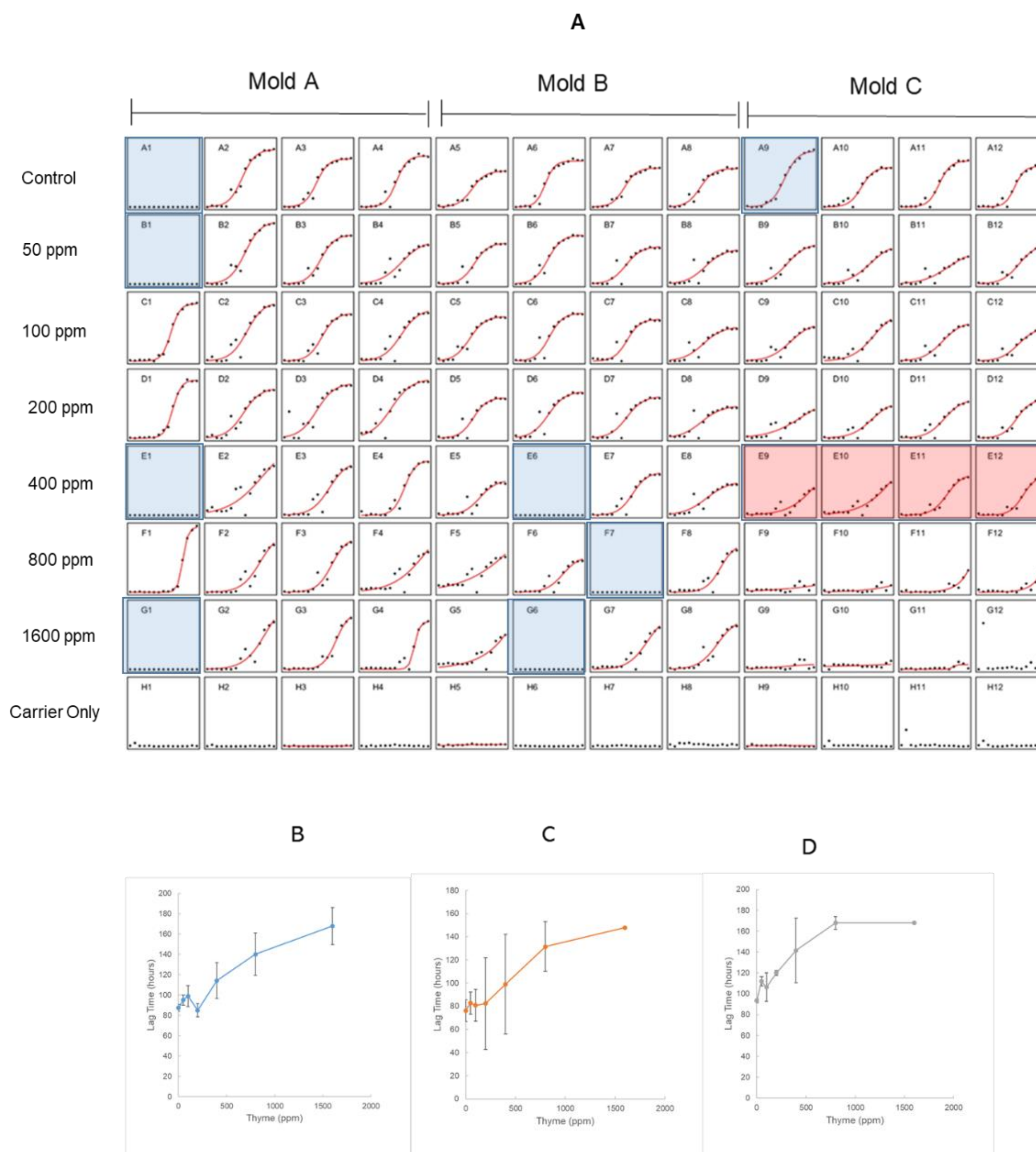


Figure 10: Differential responses of molds A, B, and C to increasing concentrations of thyme oil. Growth curves (A) for molds A, B, and C, as drawn by "Growthcurver" package using RID as a proxy for mold growth. Plate was treated with increasing concentrations of thyme oil in quadruplicate. Accompanying line graphs depict mean lag time for mold A (B), mold B (C), and mold C (D) with error bars representing standard deviation. Potential failed inoculations, noted as outliers, are outlined in blue. Examples of biological variation are outlined in red.

Growth curves, as seen in Figure 10, show a visual decrease in slope with an increasing concentration of thyme oil. This is indicative of increased inhibition with an increase in thyme oil treatment level and is matched quantitatively with increases in lag time seen in Figure 10B-D. Furthermore, growth curves and generated lag times allow for visualization of variation between replicates. For example, wells highlighted in red are all inoculated at a concentration of 400 ppm thyme oil yet show lag times ranging from 111-168 hours. This is consequently seen in the high standard deviation (Figure 10D). An example of potential failed inoculations can be seen in Figure 10A, blue squares. Notably, the lack of growth in well A1 is likely due to a failed inoculation rather than true inhibition, as no inhibitor is present in that replicate. This brings into the question the growth seen throughout column A, with inhibition seen at 400 ppm and 1600 ppm, but not the mid-level application point of 800 ppm.

Wells E6, F7, and G6 were also removed from analysis as outliers, due to their appearance on the plate as failed inoculations, though stochastic effect cannot be ruled out completely as an explanation for the lack of growth. At the end of the experiment, only blue cheese agar was visible in these wells, making failed inoculation more likely than stochastic growth, as there likely would be some amount of mold growth if this were the response of an inhibitor treatment. Wells A9 and E4 were removed due to bubbles present in the agar that potentially interfered with analysis.

Molds A and B showed a statistically significant increase in lag time at the three highest application rates, while mold C showed a similar delay at the three highest application rates ($p<0.05$). The growth curves of this plate thus also highlight the differential responses of molds to inhibitors and demonstrates that growth curves provide both a visual and quantitative tool when combined with lag time for analyzing growth.

To compare inhibitor efficacy and evaluate the utility of this screening approach, lag time was used as a metric to analyze results. Median lag-times are summarized in a heat map (Figure 11) of the four trialed inhibitors against ten mold isolates, clustered according to similarity of overall mold-response to the panel of inhibitors. In other words, columns of the heat-map represent each mold isolate, and those mold isolates that cluster together were nominally sensitive or tolerant to the same inhibitors.

Based upon hierarchical clustering, there appeared to be two groups of molds. Cluster one comprised of Molds K, H, and E and cluster two comprised of molds A, B, C, D, F, and J. Cluster one molds appeared to be more sensitive to inhibitors than the cluster two molds. Mold G was not inhibited by any of the alternative label-friendly mold inhibitors trialed in this experiment ($P>0.05$), and therefore was not clustered with any other molds.

As anticipated, natamycin was universally effective against all mold types ($P<0.05$). At even the lowest concentration, the lag time was increased beyond the final point time point of the study (168 hours). Though all lag times were greater than 168, this does not necessarily indicate that the molds were completely inhibited. For example, Mold G showed some growth at lower treatments of natamycin, however, growth never reached 50% of the control and thus still indicates an extended lag time.

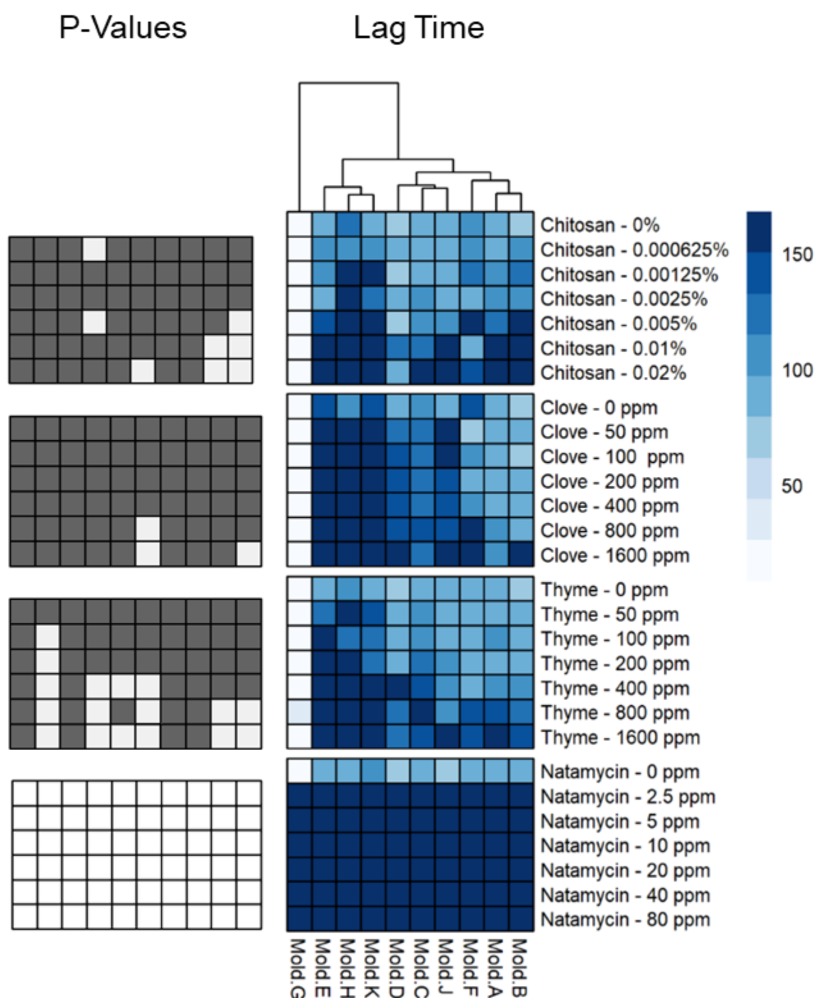


Figure 11: Heats maps of principle proof of concept experiment on chitosan, clove oil, thyme oil, and natamycin. Clustered heat map depicts lag times (hours) of inhibitor treatments. Dark blue indicates a long lag time and light blue/white indicates a short lag time or no inhibition. Clustering highlights similarity in mold growth and differential responses to inhibitor treatments. Lag time p-values indicate statistical significance, calculated using Tukey's post-hoc test and are in corresponding positions to lag time heat map. White represents statistically significant results while black represents a p-value of one, or non-statistically significant results.

Apparent increases in lag-times for each mold in response to the clean-label inhibitors were not necessarily significantly different. For example, chitosan treatment substantially increased median lag times for all molds except G and D at the highest application rate (0.02%), yet results were only significant ($P<0.05$) for three molds (A, B, and C). Molds A and B were most convincingly inhibited by chitosan; mold A showed an increase at the two highest application rates, ($P<0.05$, lag time increase of 37-73 hours) while mold B showed a statistically significant increase at the three highest treatments levels ($p<0.05$) with an increase of 90 hours. Lag times for Mold K were significantly increased at 0.000625% and 0.005% chitosan ($P<0.05$), but not at other levels. Mold C showed a significant increase at an application rate of 0.02% and 0.01% chitosan ($p<0.05$), increasing lag time by up to 74 hours.

As seen previously in Figure 10, variation between replicates at high treatment levels make interpretation of significance values less clear. Molds A, B, E, H, and J also showed a 50% delay in mold growth, at a minimum of the highest level of chitosan application, though they were not of statistical significance ($p>0.05$).

Clove oil treatment led to an increase in lag time for several molds, though to a lesser extent than chitosan, and with fewer significant differences. Mold C showed a significant increase in lag time at 400 ppm, 800 ppm and 1600 ppm application rates ($p<0.05$) with increases in lag time ranging from 24-47 hours, as also noted in Figure 10. Molds A and B showed similar increases at 800 ppm and 1600 ppm ($p<0.05$). At the highest concentration, Molds A, B, D, and J showed a 50% or more increase in lag time compared to the controls, though not necessarily statistically significant. Of the three tested clean-label inhibitors, thyme oil caused significant increases in lag times for the broadest range of molds. At the three highest application rates for mold K, thyme oil significantly increased lag time ($p<0.05$) with up to a 73 hour increase in lag time. Similarly, at

the five highest application rates, mold E ($p<0.05$) showed an increased lag time of up to a 76 hour difference. Mold C demonstrated statistical increases in lag time at 400 and 800 ppm application rates ($p<0.05$), though not 1600 ppm, which may be attributed to variation between replicates at higher concentrations of inhibitors. Mold D similarly showed increased lag times at 400 ppm and 1600 ppm applications of thyme oil ($p<0.05$), though not at the intermediate rate of 800 ppm. Both molds A and B showed an increase at the two highest treatment levels, consistent with their clustering in the heat map. Molds A, B, C, D, E, F, and K all showed at least a 50% increase in lag time at the two highest concentrations of thyme oil, with some molds showing extended lag times at lower concentrations, however, these results were of mixed significance.

Evaluating biological sources of variation

Based upon results of preliminary experiments, and clear visual evidence that inhibitors affected mold growth, it was surprising to find so few statistically significant growth delays in response to the clean-label inhibitors. Consequently, several potential areas of experimental variation were explored. First, variation in estimated lag-time of each mold under control conditions was assessed by plotting the coefficient of variation (CV) (Figure 12).

For all molds, the lag time CV for untreated controls was less than 20%, with seven of the ten molds showing a CV less than 10%. Those molds with a higher CV (molds H, F, and G) were associated with no statistical significance

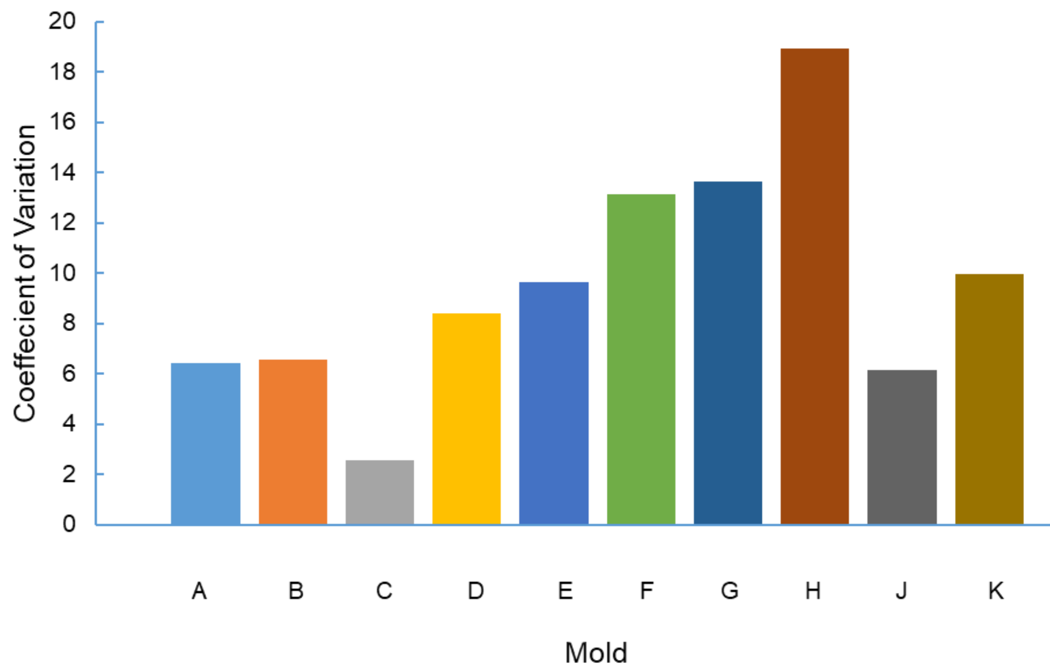


Figure 12: Evaluating the coefficient of variation for controls of the first proof of concept experiment. Bar graph depict the coefficient of variation for each mold across all controls (n=16)

As the treatment concentrations increased and each mold approached the growth/no growth interface, it is reasonable to expect more variation in their lag times. Thus, in order to further investigate the lack of statistical significance on molds with a clear increase in lag time, the CV was investigated for a subset of molds treated with chitosan (Figure 13).

As seen in Figure 13, Molds A, D, and F all show an increase in CV with an increase in inhibitor concentration. This begins to explain a lack of statistical significance seen even for mold isolates with a large mean increase in lag time in response to inhibitor treatments. However, some mold isolates, such as Mold E (Figure 13C) show an initial increase in CV then a sharp decrease at the two highest treatment levels, with a CV of 0%. In this case, the growth/no growth barrier is approached at a mid-level treatment, resulting in increased variation. Molds were then completely inhibited at the higher concentrations of treatments, thus reducing the CV.

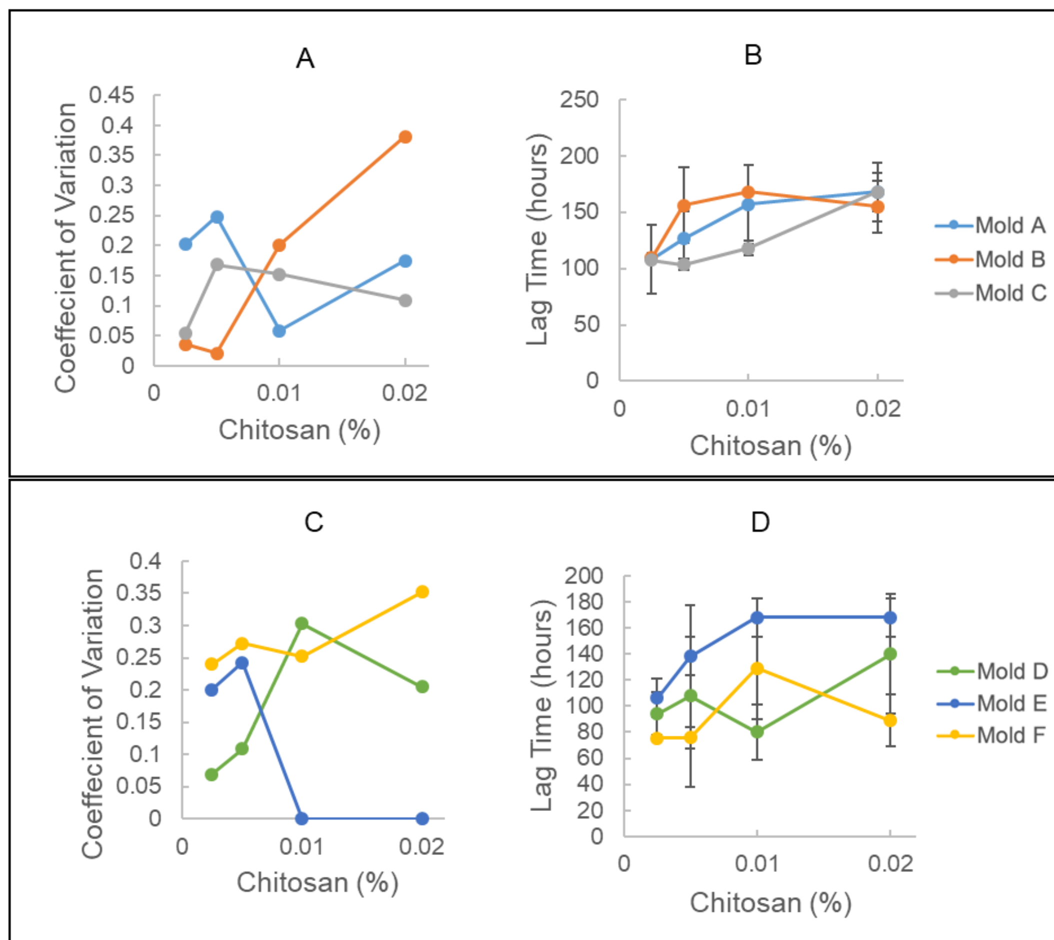


Figure 13: Coefficient of variation on lag time. Line graphs depict the CV on lag times for mold isolates A-C treated with chitosan (A) and associated lag times (B). CV on lag time for molds D-E (C) and their respective lag times (D) are also depicted.

4.3.4.1 Evaluating methodological sources of variation

A visually notable feature of mold growth in response to the clean-label inhibitors was that within a given well, there would be areas with dense growth and areas of sparse growth. During method validation on growth in the absence of inhibitors this “patchiness” was typically minimal and only present in one or two sequential images. To mitigate well edge effects, the region of interest defined for “ReadPlate” analysis was a circle of 80 pixels in diameter, centered in each well. To evaluate whether this was a source of variation between replicates, RID was recalculated for a representative set of plates using a larger pixel diameter of 250 pixels and analyzed to determine if increasing ROI better accounted for “patchiness” to reduce variation (Figure 14).

Upon initial assessment of the growth curves, there is minimal to no visible difference of curves between pixel diameter selection. All growth curves visually match the time-lapse photo series. Differences in lag time estimates using 80 and 250 pixel diameters are summarized in a histogram (Figure 14A). The histogram forms a standard bell curve with only one of the 96 samples, or 1.04% falling outside two standard deviations of the mean. This indicates that increasing the pixel diameter (ROI) does not improve data consistency and an 80 pixel diameter is appropriate. Furthermore, calculations of lag times at 80 pixels indicate controls with lag times ranging from 88-96 hours and use of 250 pixel demonstrates lag times ranging from 85-90 hours, with no replicates falling outside two standard deviations of the mean. These small discrepancies between ROIs further demonstrates that this was not a major contributor to perceived variation

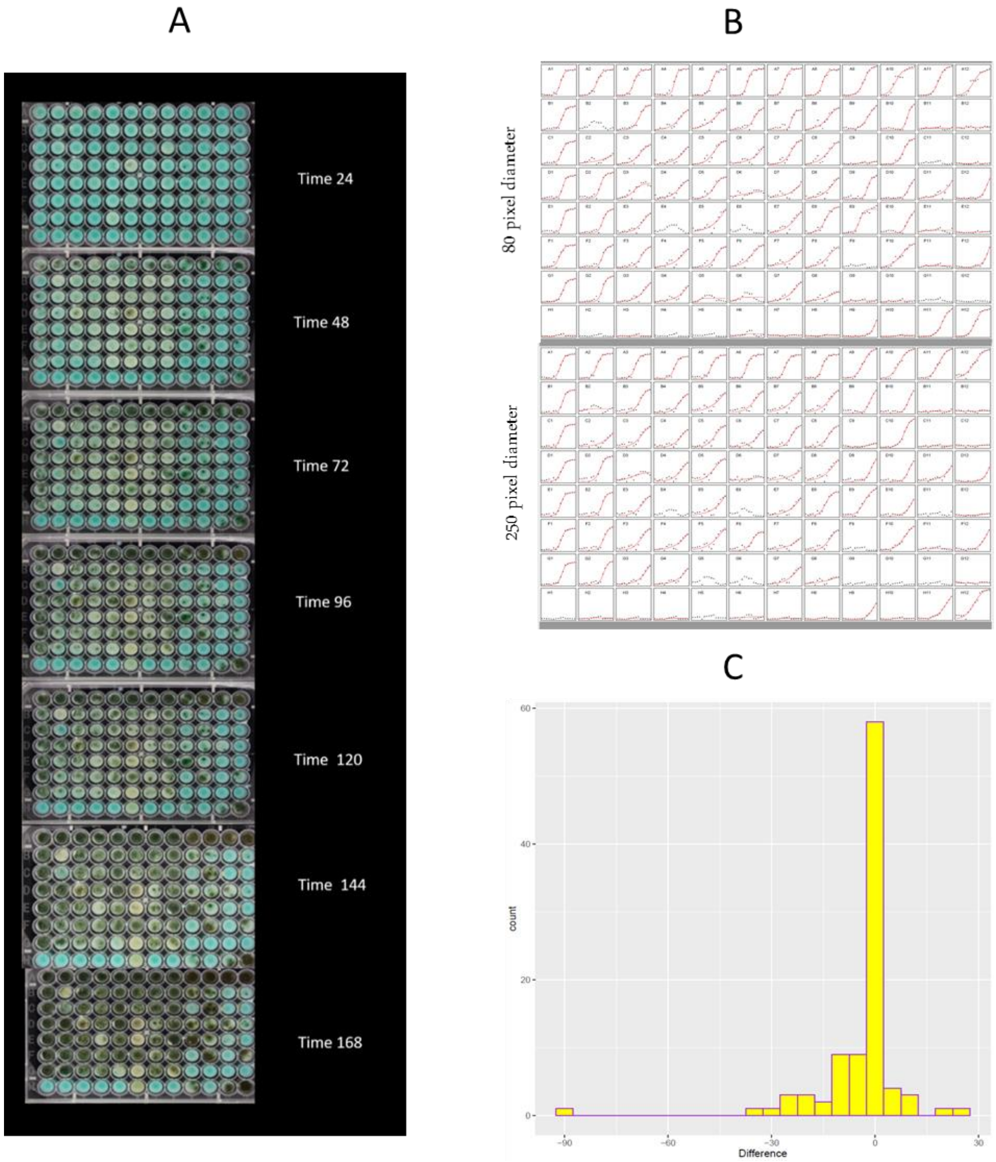


Figure 14: Evaluation of region of interest diameter for impact on lag time estimates. The above plot depicts the time photo series (A) and growth curves, as drawn by the "Growthcurver" software, using an 80 pixel or 250 pixel (B) diameter. The associated histogram (C) depicts differences in lag time as calculated using the two pixel diameters.

4.4 Repeatability of screening experiment

A second inhibitor screening was carried out using the image analysis technique to examine repeatability. Inhibitors from the preliminary experiment were included with the addition of a parallel experiment containing a chitosan and thyme oil combinatorial series.

In order to analyze consistency between experiments, linear regression analysis was performed (Figure 15) across controls and inhibitor treatments.

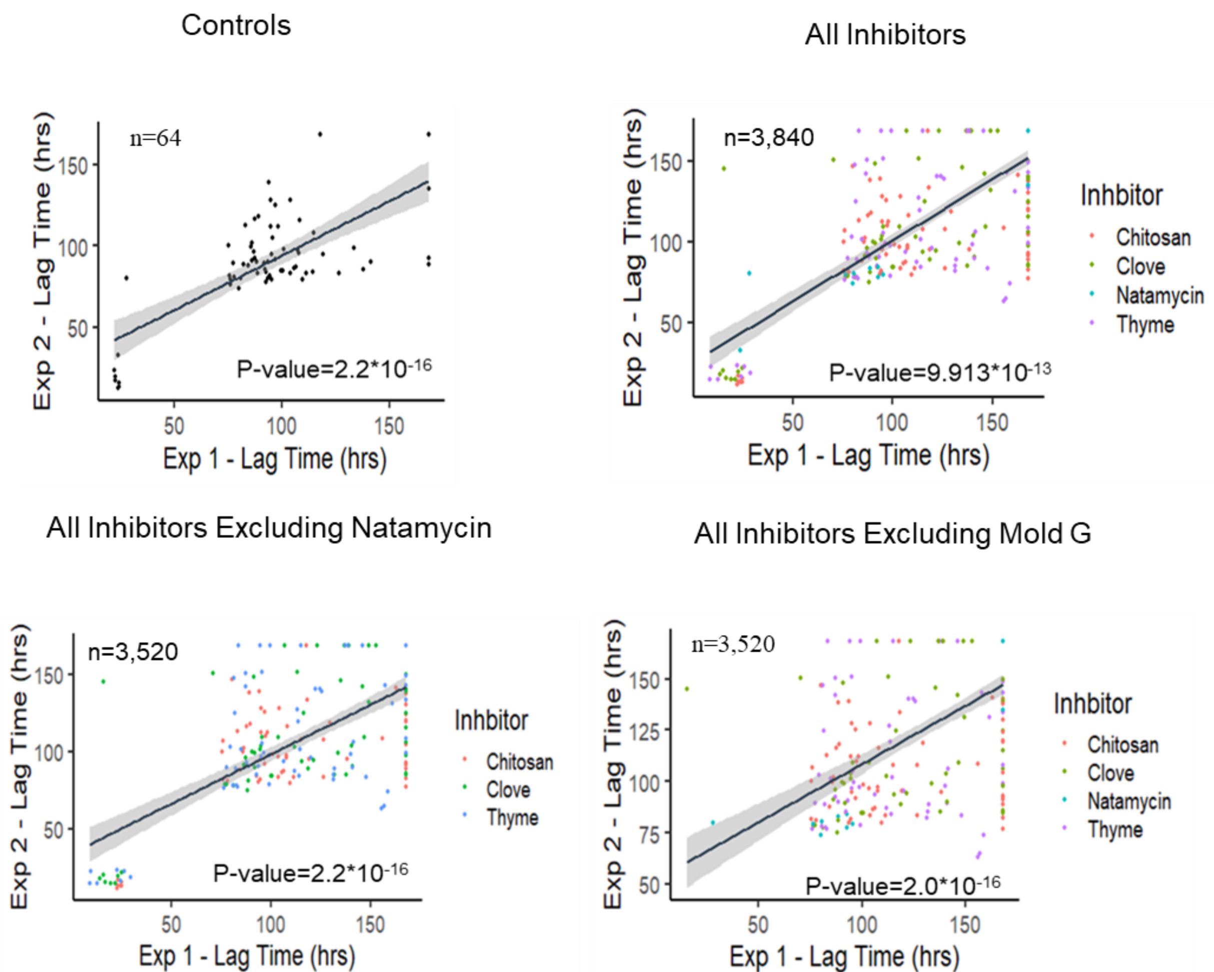


Figure 15: Repeatability of inhibitor screening experiments. Linear regression analysis was performed on lag times of the two screening experiments for controls (A), all inhibitors, (B) all inhibitors excluding natamycin (C), and all inhibitors excluding *Geotrichum.*

Linear regression on controls (Figure 15A) reveals a significant correlation between experiments ($P<0.05$), indicating that, despite variation, control growth is repeatable.

Analysis of all treatment replicates further indicates significant correlation ($p<0.05$) between experiments (Figure 15B). Due to the known high efficacy of natamycin, we explored analysis including only alternative mold inhibitors (Figure 15C). Upon exclusion of natamycin, linear regression still demonstrates a significant correlation among lag times between experiments ($P<0.05$). Lastly, because of the very short lag times of Mold G, linear regression was performed on all replicates excluding Mold G, under consideration that it may be anchoring the data. Upon analysis, this still resulted in a statistically significant correlation ($p\text{-value}<0.05$) among the results for the two experiments. Thus, despite initial appearances of variability, the combination of analysis on ROI, mold patchiness, control lag times, and experiment repeatability indicate these fluctuations are due to variations in the data and are not an artifact of the methodology.

4.4.1 Potential for combinatorial treatments

In an effort to better evaluate the impact of combinatorial treatments, a parallel experiment including combinatorial treatments was included, as mentioned in section 4.4. A subset of molds that underwent combinatorial treatments are depicted in Figure 16. Because inhibitor treatments did not have the additive effect anticipated an example, “expected’ plot was created for easier comparison (Figure 16A). For a heat map of all data, see Appendix 1: Figure A2.

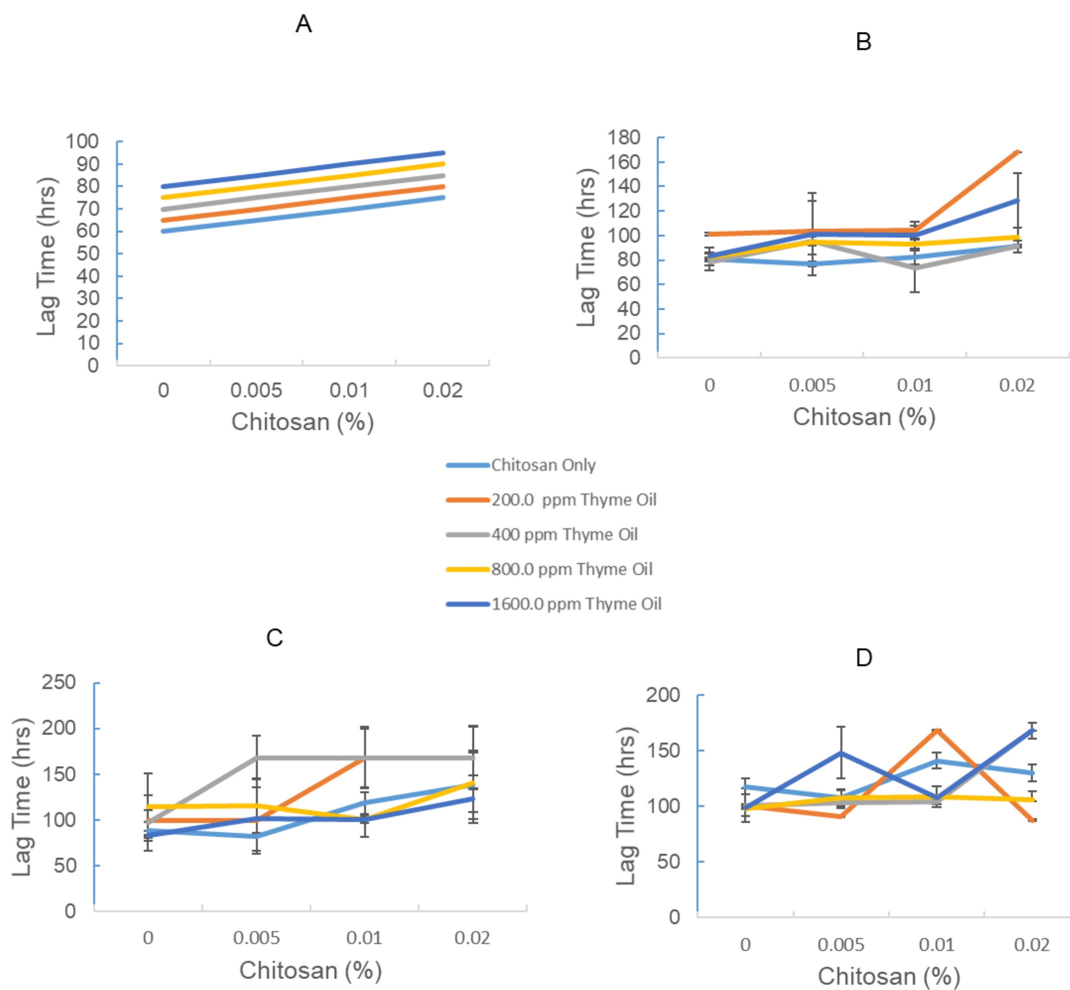


Figure 16: Plots of Lag times for a subset of combinatorial data. Plots depict an example/expected line graph of combinatorial treatments (A), included due to the unexpected results of the combinatorial data. Lag times of molds A (B), mold B (C), and mold D (D) are also depicted. Example molds were selected due to unique differences seen in heat map clustering.

Combinatorial treatments on Mold A (Figure 16B) indicate some additive effect with the addition of 1600 ppm thyme, as lag times are higher than the chitosan only treatment. However, mold A does do not appear to increase lag time, with the exception of treatments including 200 ppm or 1600 ppm thyme oil. Mold B (Figure 16C), does demonstrate an additive effect, with all combinatorial treatments showing an increase in lag time. However, the high standard deviation brings the combinatorial impact into question. Mold D also shows some additive effect (Figure 16D), with treatments of 200 and 400 ppm showing an increase in lag time. Unexpectedly, treatments of 800 and 1600 ppm do not appear to have an additive effect on this mold. Additional molds, including Mold J showed clear inhibition by combinatorial treatments with an increase in lag time at 7 of the treatment levels ($p<0.05$). Similarly, mold H showed inhibition at 10 treatment levels ($p<0.05$). This begins to illustrate differential responses of mold inhibitors to different treatments and application rates.

In general, molds A, B, C, E, and H all showed a significant increase at the highest combinatorial treatment ($p<0.05$). Surprisingly, 0.01% chitosan/200 ppm thyme and 0.02% chitosan/800 ppm thyme showed the greatest inhibition potential, despite being a mid-level of application. Mold B shows an increase from 72 hours to 168 hrs at treatments of 0.01% chitosan/200 ppm thyme Oil and 0.02% chitosan/200 ppm thyme Oil ($p<.05$), with increases in lag time of 38-96 hours.

Mold F showed an increase from 98 hrs to 168 hours across 4 of the combinatorial treatments, however, these results were not necessarily statistically significant. Mold F did show a lag time of 168 hours at the highest level of the chitosan only treatment, indicating combinatorial treatments may not be necessary for this mold isolate.

Combinatorial treatments also appeared highly effective against molds D and H. On mold D, all applications including 400 ppm or less thyme showed a lag time >168 hours, while on mold H

0.02% chitosan/200 ppm thyme and all treatments including 400 ppm thyme showed a lag time of 168 hours. Surprisingly, applications including a thyme oil concentration at 400 ppm or lower appeared more effective against these molds than those treatments including an application rate of 800 ppm or 1600 ppm. Molds A and C also showed inhibition with combinatorial treatments, increasing lag time to 168 hours with an application of 0.02% chitosan/400 ppm thyme and 0.02% chitosan/1600 ppm thyme. However, mold C did demonstrate the same level of inhibition with the application of 0.02% chitosan only.

Because of the large fluctuations in lag time responses, interpreting inhibition of additional molds is too ambiguous to make generalized conclusions. Some mold isolates show clear increased inhibition with combinatorial treatments, however, several molds, for example molds E and J, show little change in lag time or are too variable of data for statistical significance.

4.5 Challenges with testing of clean-label inhibitors

4.5.1 Inhibitor induced color change

Unexpectedly, some inhibitors (Microgard®, lactoferrin, and celery powder) imparted a color change on the blue cheese agar that occurred prior to mold growth of corresponding controls. Visual analysis indicated (Figure 17, Figure 18) this color change was not due mold growth and the inhibitors displayed anti-mycotic properties. However, because an inhibitor only, no mold control was not included, these samples could not be properly analyzed.

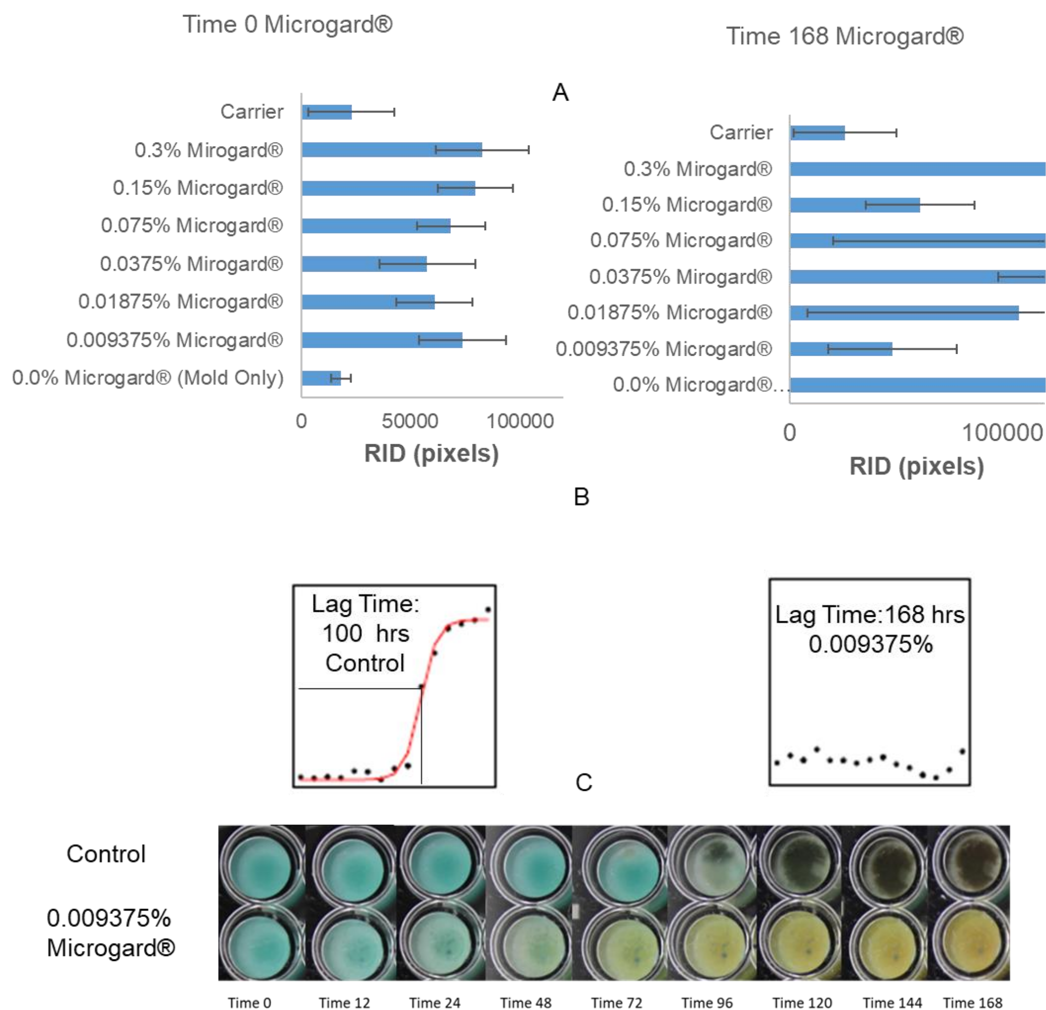


Figure 17: The impact of Microgard® on color changes in blue cheese agar over 168 hours. The plot above depicts the observed RID across inhibitor levels for the first and final time points (A) and the growth curves of a control and low treatment level replicate (B). The associated time lapse photo series (C) demonstrates the color change as seen over time with the application of 0.009375% Microgard®.

For example, Microgard® (Figure 17) shows a slow color change from the blue agar to a yellow color beginning at 24 hours and continuing throughout the length of this experiment. This yellow color does not align with the initial stages of *Penicillium* sp. mold growth, which are typically white in color and begin at 72 hours. This color change is seen quantitatively in the change in RID with an increase in values across the length of the experiment. Similarly, in the growth curves, this is seen when “Growthcurver” is unable to fit a growth curve at even the lowest treatment level. Visually confirmed by the photo series in Figure 17C, the color change is not attributed to mold growth. In this case, the RID does accurately reflect the lag time, however, because the impact on lag time was not consistent across inhibitor types, this became difficult to interpret. For example, in the presence of other color-imparting inhibitors (Figure 18), such as lactoferrin, an unreasonably short lag time is drawn.

Lactoferrin, as seen in Figure18C, shows a color change at about 72 hours of growth. However, similar to Microgard®, this color change does not align with the expected color/general appearance of mold growth, indicating an impact by the inhibitor itself. This change in RID can also be seen in the bar graphs (Figure 18A) and subsequently, the lag times (Figure 18B). The control replicate picture draws a lag time of 88 hrs while the lowest treatment level draws a lag time of only 26 hours. In the case of lactoferrin, the color change generally caused “Growthcurver” to draw a shorter lag time than visually matched the growth on the wells. Ultimately, because of this inconsistency in lag time among these treatments, they were excluded from analysis until future experiments can be modified to include the proper controls.

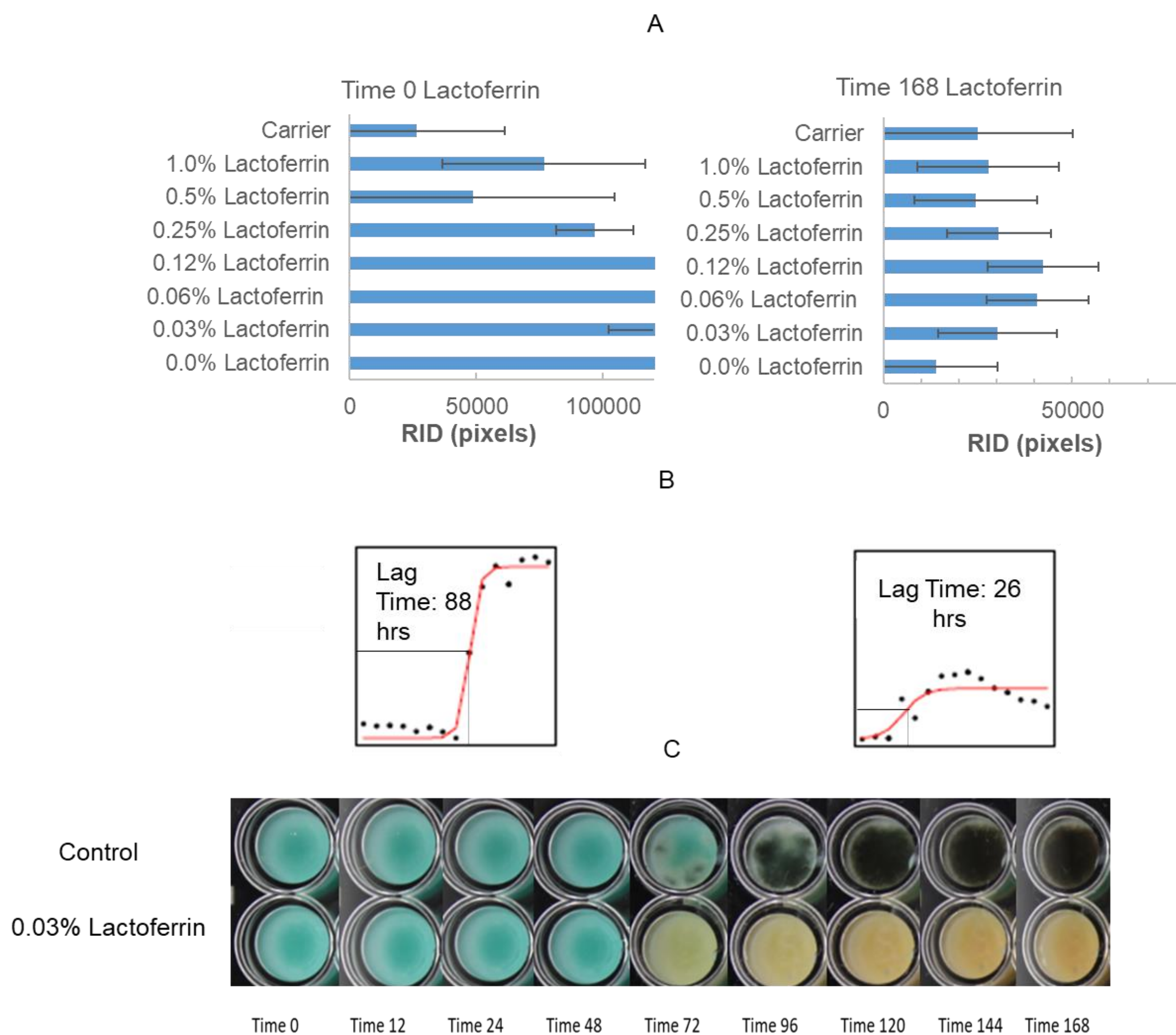


Figure 18: The impact of lactoferrin on color changes in blue cheese agar over 168 hours. The observed RID values at the initial time point and final time point (A) can be seen in the bar graphs above. The growth curves for the control and lowest lactoferrin treatment (B) help to visualize changes in lag time across inhibitor treatments. The associated time lapse photo series (C) provides visual confirmation of the color change.

5 DISCUSSION

5.1 Conventional strategies to protect shredded cheese from mold spoilage are highly effective

Changing market demands are causing food processors to reevaluate preservation technologies as the demand for clean labels rises (IFT 2017). Cheese, due to its perishable nature and high water activity (Hickey et al.; Schmidt and Fontana 2008) is particularly prone to mold spoilage. In convenience cheese products, such as shredded and sliced cheeses, this risk is elevated, as mold may be pushed from the rind through the cheese during the shredding/cutting process. This increase in surface area and oxygen exposure ultimately promotes the aerobic nature of most molds (McGinnis and Tyring 1952). Currently, with an increase in convenience product sales, producers are incorporating natamycin into their product to meet shelf life demands, thus making this inhibitor ubiquitous with the U.S. shredded cheese industry (Berry 1999). Though several studies have demonstrated efficacy of natamycin in a variety of applications (Elayedath and Barringer 2002; Kallinteri et al. 2013; Leyva Salas et al. 2017b; Costa et al. 2018), minimal work has been done on the shelf life of shredded cheese. Thus, a shelf life study was performed on bags of shredded cheddar with or without the addition of natamycin.

In our study, we observed a complete lack of mold growth in unopened bags, both with and without natamycin. This is likely explained by the industry partner's choice to flush packages with nitrogen prior to packaging cheese. Most of the filamentous fungi that spoil cheese are aerobic and thus cannot grow without the presence of oxygen. The literature (Felfoul et al. 2017, Solomakos et al. 2019) indicates variants of this modified atmospheric packaging (MAP) are successful at prolonging shelf life in several food applications. MAP typically involves removing all oxygen from the packaging environment and replacing it with a combination of CO_2 and N_2, with 100%

nitrogen showing complete inhibition of mold growth (Felfoul et al. 2017), thus the nitrogen flush by our industry partners was highly successful at prolonging shelf-life.

Mold growth occurred on control cheese bags, which were opened but not inoculated with the mold cocktail. This may be due to exposure of cheese to mold spores at the facility during processing that did not germinate until exposed to oxygen. Though all bags were opened under sterile conditions in a laminar flow hood, we cannot exclude cross contamination between controls and inoculated bags.

In all other treatments, natamycin positive bags showed an increase in shelf life compared to natamycin negative bags, confirming the high efficacy of natamycin against the inoculated molds (largely *Penicillium* sp.). Surprisingly, an increase in concentration of spore inoculations did not correlate to a decrease in shelf-life, indicating there may be a point of saturation at which an increase in spore concentration no longer impacts visible time to mold. However, this assessment is not matched as clearly in the literature, with Baert et al. (2008) indicating mold innoculum size has a greater impact on lag time for slower growing molds, but has less effect on fast growing molds. Because the slow growing *Penicillium* sp. and fast growing *Geotrichum candidum* molds were included in the mold cocktail, the growth and subsequent detection of the fast-growing mold likely overtook the detection of its delayed counterparts.

5.2 Image analysis can be used to screen for mold inhibitors in a cheese-mimicking matrix

Though natamycin clearly has a high efficacy in shredded cheese, the demand for clean-label inhibitors necessitates investigation into alternative preservation technologies. In an effort to quantitatively screen for inhibitors, we evaluated image analysis for measurement of mold growth in a cheese mimicking matrix. Researchers have reported discrepancies in MICs using different

lab medias and food matrices (Imani Rad et al. 2017; Van de Vel et al. 2019; Shannon et al. 2019). In the context of dairy, Garnier et al. (2018) reported that inhibitor MICs were typically lower in cheese than in a lab media, though this varies based on mold selection. To ensure that results of our screening method would be applicable to use on cheese, we adopted cheese agar (Wolfe 2014), which has been used to accurately recreate the microbial community of cheese and can be adapted to different cheese types. As noted on cheese agar and reported by, Garnier, there were no differences in growth rates of *Penicillium* sp. However, there were differences in growth of *Geotrichum candidum*. In a preliminary comparison we observed differential response to natamycin treatment for *Geotrichum* mold on cheese agar and lab media, with formation of pinprick colonies, but no radial growth on PDA and complete inhibition of 3/6 replicates on cheese agar with minor radial growth of the remaining three replicates.

In addition to use of a testing matrix more comparable to a real-world product, effective screening requires that inhibitors be applied at a range of concentrations to a representative panel of spoilage organisms, thus necessitating a high through-put method. Previous research has focused on fluorescent techniques (Figueroa-López et al. 2014), staining chitin in fungal walls without staining bacteria. Other researchers have focused on image analysis techniques to increase throughput of samples. The majority of work focuses on high throughput techniques to measure colony counts rather than the rate of growth. For example, software such as CellProfiler™ (Lamprecht et al. 2007) uses high-resolution images in combination with an algorithm to identify and count areas of growth. ImageJ (NIH) has also been used to count yeast colonies on cannabis flowers (Stolze et al. 2019), though this had a 12% error rate when compared with manual counting. Hartmann et al. (2018) developed a medium-throughput automated image analysis technique to determine lag time of bacterial growth under contamination conditions of cheese. In

order to increase throughput, a motorized platform was used to accommodate four plates. Upon obtaining images of colony growth every ten minutes, images were further analyzed for lag time in Matlab. However, this study was limited by throughput, lack of a food-mimicking matrix, difficulties distinguishing growth from the background, and a requirement of image modification. The recently developed OCellsScope (Biosense, Denmark), aimed to increase throughput with use of a multi-well automated microscope in combination with an algorithm to assess and count pixels which contained cell growth. The emerging technology of OCellsScope has been used to screen for bioactive peptides in fermented milk (McNair et al. 2018). Garnier et al. (2018) utilized a 24 well plate technique to measure growth, creating miniaturized cheeses to better replicate the food matrix. However, the use of subjective, visual scoring limits this approach.

In this work, micro-titer plates were combined with ImageJ and the “Readplate” plugin to capture raw integrated density of mold growth over time from high-resolution photos. Subsequently, the R “Growthcurver” package was used to calculate lag time using raw integrated density as a proxy for mold growth, while proof of concept experiments reveal biological variation in response of molds to inhibitors, this method has future utility in determination of MICs and improved investigation of antimicrobial compounds, with the ability to screen thousands of samples.

Due to the inherent variation of microbial populations under stress conditions, statistical analysis proved challenging with lag time as the main metric. Potential explanations for this include the variable and lengthy lag time of some mold isolates in the absence of inhibitors, and the relatively short time frame of the experiment. For example, as mentioned in section 4.3.2, mold G, the fastest of the molds, ranged in lag time from 22-24 hours while Mold F, one of the slower growing molds, ranged in lag time from 80-140 hours. Additionally, the large variation among replicates posed challenges for analysis, as mentioned previously in Figure 10, this inherent variation makes

statistical assessment challenging as molds may show a practical increase in lag time, but not a statistically significant delay due to large standard deviations.

Analysis was further challenged by the relatively short time frame of the experiment. Slow growing molds, even without the presence of an inhibitor, may not have reached full growth until beyond the midpoint of the experiment. Thus, any subsequent delay beyond 168 hours may not be fully captured in the time frame of the experiment and, consequently, the statistical analysis. Future experiments should thus be carried out for longer periods of time to assess slow-growing molds. Lastly, as concentrations of inhibitors increase, mold isolates begin to verge on the barrier of growth or no growth. Because only a single, viable spore is required for germination and subsequent hyphal growth, populations with only a few successful individuals will show a mildly delayed lag time. In other replicates, with a slightly different population of spores, growth may be completely inhibited, resulting in an extended lag time. Thus, differences seen in a very small portion of the population can cause a large impact on standard deviation and statistical analysis. An example of this is seen in Figure 13, where molds show an increase in CV with an increase in application rate until the point of inhibitor saturation. Individual responses to a stressor in a population may be variable as the level of stress or treatment concentrations increase. Interpretation of this variable response may be aided by probabilistic statistics. This stochastic phenomena in microbiology is well demonstrated (Palou and Ló Pez-Malo 2005) and has been incorporated into biological modeling systems, typically used for the prediction of the growth/no growth barrier of food safety relevant bacteria (Ratkowsky and Ross 1995; McMeekin et al. 2000). Future work may explore additional approaches to better account for stochastic variation as molds approach the growth/no growth interface. This may include the percent increase in lag time or the investigation of other statistical methods, such as a rank-sum test.

5.3 Clean label inhibitors show promise as label-friendly alternatives to natamycin

Using our high throughput image analysis methodology, we were able to trial three clean label candidate inhibitors (chitosan, clove, and thyme), and one combinatorial treatment (chitosan/thyme oil). We focused on a topical application of inhibitors, as it mirrors the current use of natamycin in the industry. However, previous work has largely focused on incorporating these inhibitors with hurdle technologies in films, packaging, or in combinatorial treatments, making comparisons challenging. Some commercially available options, such as Microgard®, are designed for inclusion directly into the cheese milk, an option that may be less suitable for shredded cheese. Chitosan, clove, and thyme oil were chosen as trial options due to their extensive research in literature (Devlieghere et al. 2004; Gutierrez et al. 2008; Matan 2012; Liu et al. 2017).

Chitosan has widely reported success as a bacterial and mold inhibitor (Vásconez et al., Conte et al. 2007, Lucera et al. 2012). Altieri et al. (2005), found efficacy of chitosan against bacteria with inclusion of 0.075% directly in the cheese making process, while we found success with topical application of only 0.02% by volume against molds. Other researchers have shown success using chitosan in films against yeast and molds in cheese (Duan et al. 2007a, Fajardo et al. 2010) and in brine in Halloumi cheese (Mehyar, Ghadeer Nabulsi, Anas Saleh). Fajardo et al. (2010) included up 5% in chitosan films for application on cheese, much higher than the 0.02% used in our experiment. However, because there is no indication of the rate of release in films, it is difficult to compare these concentrations. Nonetheless, this research indicates that chitosan may have practical, topical applications at lower rates than previously tested in films.

Thyme oil is similarly supported in the literature as an antimicrobial, with strong inhibition qualities due largely to its phenolic content (Skandamis et al. 2002). However, its impact has mostly been evaluated for bacteria (Liu et al. 2017). Researchers have demonstrated the

antimicrobial activity of thyme oil in sachet release systems in bagged, shredded cheese with volatile concentrations up to 450 ppm or 10% of the sachet (Han et al. 2014). In our study, we saw inhibition at concentrations as low as 400 ppm. Other studies have shown more varied results, with bacteria inhibited at ranges of 75-1100 ppm and molds inhibited at much lower at ranges of 80-97 ppm (Fani and Kohanteb 2017). Vázquez et al. (2001) trialed rates of 100-200 ppm, showing some delay in *Penicillium* sp. at these rates.

Though it is clear thyme essential oil has potential use as an inhibitor, its aromatic qualities pose potential sensory challenges. The detection threshold of thyme oil has been reported at 50-1200 ppm in cottonseed oil (Farag et al. 1989), while in sunflower oil it has been reported at 124 ppm (Bitar et al. 2008). Though the sensory threshold is varied, it is clear it must be evaluated specifically for a cheese matrix before use in the industry.

In our experiment clove oil showed some inhibition potential, though was effective across a lower range of molds than that seen with thyme or chitosan. The literature is more varied in regards to inhibitory success of clove oil with some researchers (Pinto et al. 2009) reporting MICs against bacteria of 0.08-.64 ppm and minimal fungicidal concentrations (MFC) of 0.16-1.25 ppm, much lower than that seen from our results (400 ppm-1600 ppm). On the other hand, Wang et al. (2018) observed inhibition against bacteria at 700 ppm, more closely matching the fungicidal results found in our study.

Lastly, using our new image analysis technique, we trialed a combinatorial treatment of thyme oil and chitosan. We found some additive impact of thyme oil and chitosan against six of the ten mold isolates tested. Though little work has been performed on cheese, several publications indicate the additive effect of these inhibitors in other food matrices, such as smoked eel (El-Obeid et al. 2018)

and sausage (Vafania et al. 2019), with general reports of synergy in films as well (Mujtaba et al. 2019).

Quantifying mold growth on blue cheese agar does have some limitations. Two inhibitors evaluated, lactoferrin and Microgard®, unexpectedly caused a color change in the agar. It is unclear if this is due to their inclusion as a topical agent rather than directly in the cheese milk, due to interactions with methylene blue, or interactions with the cheese-mimicking matrix itself. Nonetheless, this must be considered in future studies and proper controls included.

5.4 Conclusions and future directions

Overall, using this new image analysis technique, we were able to screen a wide variety of potential mold inhibitors for their anti-mycotic potential to determine their efficacy in a cheese mimicking matrix. Because efficacy of inhibitors is matrix dependent, this provides an industry-applicable format for screening anti-mycotics while maintaining a high throughput and relatively low cost. Upon completion of the three proof of concept experiment, we were able to trial over 7,200 samples with nearly 110,000 data points taken across time points. Thus, there is great potential for this experiment to be used in examining additional, clean label mold inhibitors.

Due to challenges with statistical analysis, future studies may focus on including more replicates to better account for variation or assess probabilistic statistics for analysis. Ideally, similar, future experiments will be carried out over a longer time to better account for slow growing molds. Due to the unanticipated color change of some inhibitors, there is a need to include an inhibitor only control for blank subtraction, as well as already included inoculation blanks.

Future work may also focus on including additional clean label inhibitors and combinations thereof. This may include biological isolates such as lactic acid bacteria and *Propionibacterium*,

fermentates, or commercially available options. An interesting area of study may include combining inhibitors with the use of other hurdle technologies, such as films applied by electrostatic spraying onto the 96 well plate.

Lastly, work should include a follow up experiment for promising clean-label inhibitors on shredded cheese bags and subsequent sensory analysis.

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7 APPENDIX 1: SUPPLEMENTARY FIGURES

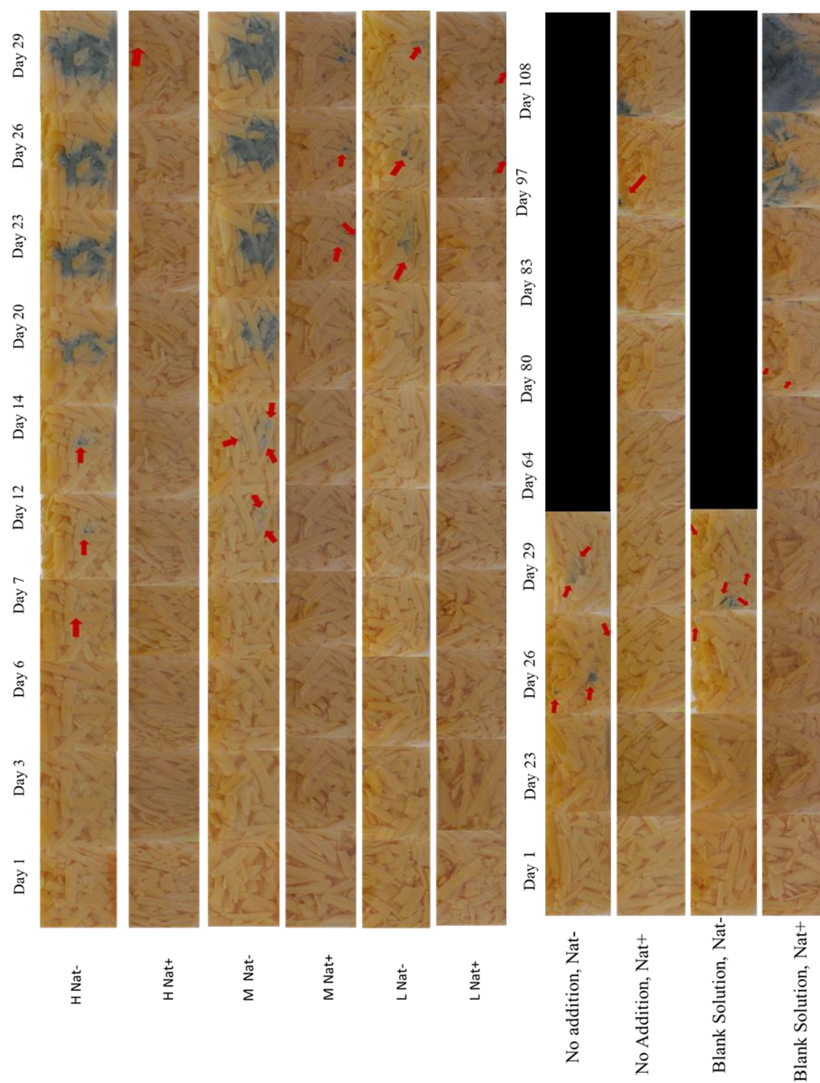

Figure A1: Time lapse photo series of time-to-mold on bags of shredded cheese. Photo series depicts one of three replicates, the unopened bags were not included, as they did not develop mold during the length of the experiment. Image time points were selected to include first time point of visual mold detection and the immediately preceding time-point. Overlaid red arrows indicate location of first detectable visual mold spoilage. After molds showed clear visual growth, shredded cheese bags were discarded

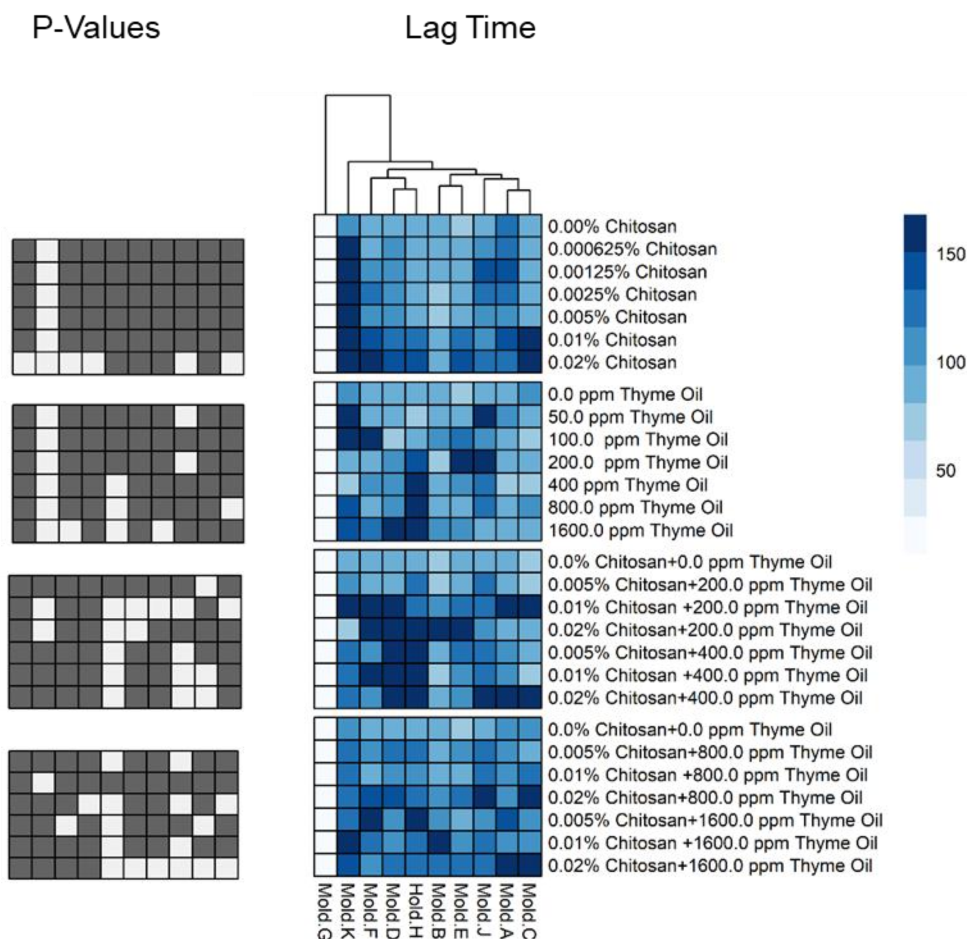

Figure A2: Heats maps of secondary proof of concept experiment on chitosan, thyme oil, and combinatorial treatments. Clustered heat map depicts lag times (hours) of inhibitor treatments. Dark blue indicates a long lag time and light blue/white indicates a short lag time or no inhibition. Clustering highlights similarity in mold growth and differential responses to inhibitor treatments. Lag time p-values indicate statistical significance, calculated using Tukey's post-hoc test. White represents statistically significant results while black represents a p-value of one, or non-statistically significant results.