AN ABSTRACT OF THE THESIS OF

Kristen Jensen for the degree of Master of Science in Food Science and Technology presented on June 1, 2022

Title: Development of High-Throughput Screening Methods to Determine Efficacy of “Clean-Label” Antifungals for Application on Cheese

Abstract approved:

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Cheese has been produced since ancient times and continues to be an important nutrient-dense staple in many diets around the world. Cheese quality is of high importance to consumers, and it is important for cheese producers to establish process controls to prevent and control cheese spoilage. Fungal spoilage is of particular concern to producers, as visible mold growth on cheese is strongly rejected by consumers.

Currently, the most commonly used fungal preservative in the cheese industry is natamycin. While very effective, products on which it has been applied cannot be labeled as organic. Furthermore, the general trend towards “clean-label” foods means that cheese producers are seeking alternatives to natamycin.

To facilitate discovery of an organic and ‘clean-label’ mold inhibitor, a high-throughput screening method was developed which was subsequently used to screen potential inhibitors. This screening method utilizes analysis of time series images of 96-well cell culture plates containing the application of

different mold inhibitors inoculated with relevant fungi to determine delays in mold growth. Antifungal activity against cheese-relevant spoilage fungi was discovered for each of the inhibitors screened: chitosan, clove essential oil, and thymol.

A second high-throughput antifungal screening method was then developed, based upon fluorescent staining of mold biomass as a proxy for mold growth. The two high-throughput screening methods were compared, finding that the fluorescent detection method was a more robust method for determining antifungal activity of candidate mold inhibitors. This method was used to complete a combinatorial screening of clove essential oil and thymol combined with chitosan. Increased efficacy was found with inclusion of these bio-active substances into chitosan, and findings were validated on commercial cheese. The *in vitro* experiments (utilizing the fluorescent screening assay) related well to *in vivo* studies on cheese. Test solutions of thymol and clove essential oil combined with chitosan that displayed efficacy in the screening experiment translated to a delay in mold growth when applied to cheese. However, no alternative treatment performed as well as natamycin.

Future work can utilize these screening methods to continue screening of potential mold inhibitors. Expansion into novel and developing areas of antifungals relevant to cheese such as bioprotective cultures and/or bio-active peptides would be a suitable application to utilize the screening methods developed in these studies. Further combinatorial treatment analyses, like

those performed in this study, would offer an increased likelihood of finding a treatment solution with equivalent efficacy to natamycin, due to possible synergistic or additive antifungal activity from different compounds.

Development of High-Throughput Screening Methods to Determine Efficacy of “Clean-Label” Antifungals for Application on Cheese

by
Kristen Jensen

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented June 1, 2022
Commencement June 2022

Master of Science thesis of Kristen Jensen presented on June 1, 2022

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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.

Kristen Jensen, Author

ACKNOWLEDGEMENTS

The author expresses sincere appreciation to Dr. Christopher Curtin for his guidance and support throughout the research and writing processes. His knowledge, kindness, and curiosity were invaluable to the completion of this work. Additionally, to Dr. Joy Waite-Cusic for her constant assistance and much needed emotional support.

CONTRIBUTION OF AUTHORS

Dr. Christopher Curtin, Dr. Joy Waite-Cusic and Kaitlyn Kornberg were involved in the experimental design for Chapter 2. Experiments were carried out by Kaitlyn Kornberg with data and statistical analysis performed by Kristen Jensen. Dr. Christopher Curtin, Dr. Joy Waite-Cusic and Kristen Jensen were involved in the experimental design for Chapter 3. Kristen Jensen carried out experiments and performed data and statistical analysis for this Chapter. Kristen Jensen drafted manuscripts in Chapters 2 and 3, with revisions by Dr. Christopher Curtin and Dr. Joy Waite-Cusic.

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1.1 Cheese Production Process

The production of all types of cheese involves relatively the same general process (Figure 1.1), with modifications implemented to create different varieties of cheese. To begin the cheese making process, milk is first pasteurized and standardized. In some traditional cheese making processes, raw milk is used. In either case, the milk is then either acidified by cultures of lactic acid bacteria and subsequently coagulated with the use of rennet or coagulated with the use acid with or without heating. The coagulum is then dehydrated by cutting, stirring, and heating and curds are separated from the whey. Additional processes happen at this step that are variety specific. Some varieties are salted and added to molds which are pressed to drain whey. Ripened cheeses are aged to allow flavors and texture to develop.

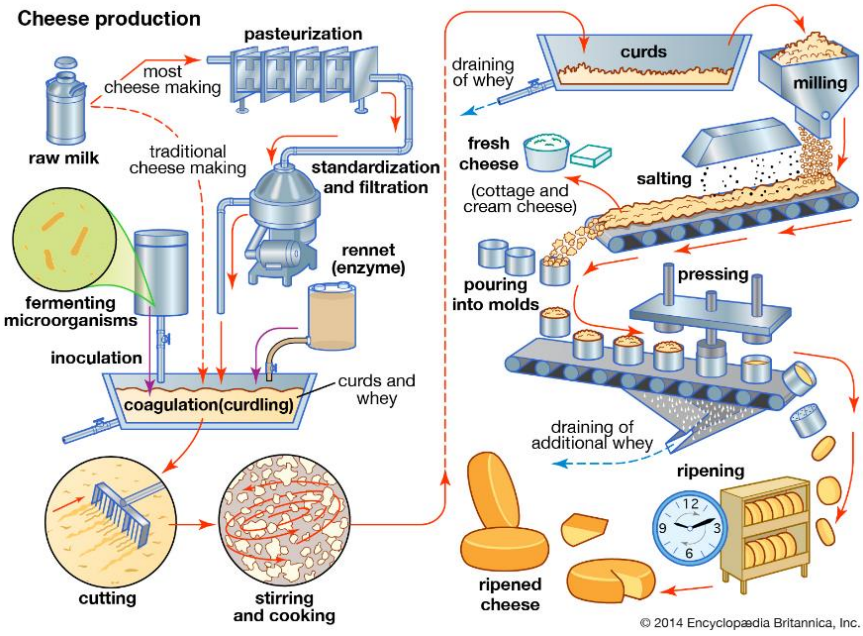

Figure 1.1 Cheese Production Process. Reprinted from Encyclopedia Britannica Inc.

1.2 Types of Cheese

According to CODEX Alimentarius, Cheese may be classified according to (1) Firmness, (2) Principal Ripening, and (3) Milk Fat Content. Firmness may be designated as either: Extra Hard, Hard, Firm/Semi-Hard, or Soft according to the moisture level (%) on a fat-free basis. Principal ripening may be designated by: Ripened, Mold Ripened, Unripened/Fresh, or In Brine. Ripened cheese is cheese that is not ready for consumption shortly after manufacture but is held at specified conditions (like temperature and time) allowing for biochemical and physical changes that create distinct organoleptic properties (Tetrapak, 2015). Ripening is normally reserved for rennet cheeses (Fox, 2017) and is further classified according to the type of organism responsible for the production of metabolites creating such changes, like mold. Unripened cheeses, most commonly those produced by acid coagulation (Fox, 2017), are ready for consumption shortly after manufacture. Milk Fat Content may be declared as: High Fat, Full Fat, Medium Fat, Partially Skimmed, or Skim according to the fat content on a dry basis. Types of cheeses according to their principal ripening and means of coagulation are presented in Figure 1.2.

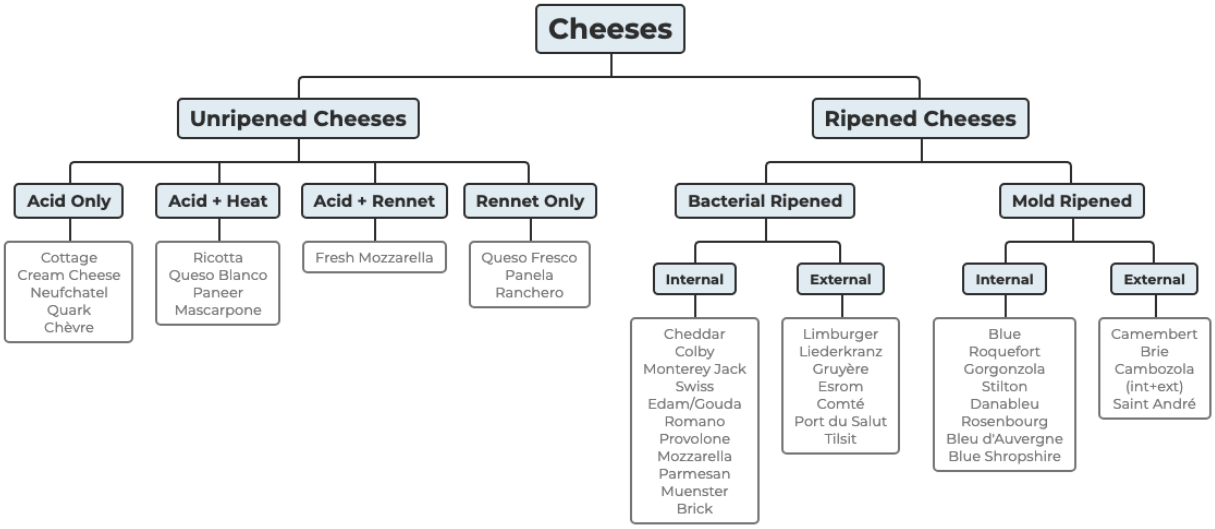

Figure 1.2 Cheese Types. Reprinted from Cheese Science Toolkit (https://www.cheesescience.org/cheese_types/)

1.3 Spoilage Challenges to Cheese Producers

Food spoilage is a major issue present in the food industry today. Up to one third of all food is spoiled before consumption, representing about 1.3 billion tons of food waste per year (Gustavsson et al., n.d.). Microbiological spoilage of foods includes deterioration of food brought on by fungi (yeasts and molds) as well as bacteria. Cheese producers utilize a variety of methods to assess the degree of spoilage and employ many technologies to mitigate the extent of spoilage.

1.3.1 Economic/Environmental Impact of Food Waste

Dairy foods are the third highest food group in terms of dollar value of food lost at the retail and consumer level in the United States, with 17% of the total value lost - behind only meat, poultry, and fish at 30%, and vegetables at 19% (Busby et al., 2014). It is important to note that some dairy foods contribute much more proportionally to these food losses. Extended shelf-life products

like dried milk powders and UHT fluid milk contribute much less to food waste compared with conventionally pasteurized fluid milk, cheese, and cultured products (Martin et al., 2021). The implications of food spoilage are not only profound from an economic loss standpoint for consumers and food companies, but also regarding an environmental impact, and food and nutritional insecurity (Papargyropoulou et al., 2014, Gunders et al., 2017). Preventing and mitigating the amount of microbial spoilage of cheese, hence extending the shelf life, is extremely important to alleviate costs to producers, retailers, and consumers and helps to reduce the environmental impact of cheese production/consumption (Lu & Wang, 2017).

1.4 Fungal Spoilage

Dairy products are typically less susceptible to fungal spoilage than other products like fresh produce, due to pasteurization and storage at refrigerated temperatures. Additionally, some dairy products like sour cream, yogurt, and buttermilk are fermented and may contain competitive microbiota and a lower pH that may reduce fungal spoilage (Garnier et al., 2017). However, fungal spoilage is still a major problem in the dairy industry. While yeast may contribute to fungal spoilage, most comes from growth of filamentous fungi like molds. Up to 100 mold species have been identified in dairy product spoilage. Those responsible for spoilage after commercial cheese production are diverse and belong to many genera: *Acremonium, Alternaria, Aspergillus, Aureobasidium, Botrytis, Cladosporium, Epicoccum, Eurotium, Exophiala, Fusarium, Gliocladium, Lecanicillium, Mucor, Penicillum, Rhizopus,* and

Wallemia. In one study on commercial cheeses, *Penicillium* spp. were found to be the most frequent spoilage mold present, occurring in 63% of cheeses sampled, followed by *Mucor* spp. (27%) (Hymery et al., 2014). Another study also found *Penicillium* spp. to be the most frequent spoilage mold present, occurring in 45% of all cheeses sampled, followed by *Aspergillus* spp. in 11% (Banjara et al., 2015). *Penicillium, Aspergillus,* and *Mucor* are the most frequently reported spoilage genuses with 37, 9, and 4 species isolated from cheese products, respectfully (Garnier et al., 2017).

Fungal spoilage in dairy products can manifest in many different forms, e.g., visible fungal growth, production of metabolites causing off odors/flavors, as well as visible changes in color/texture (Garnier et al., 2017). Spoilage molds such as *Penicillium, Fusarium,* and *Aspergillus* spp. can also produce mycotoxins posing a risk to human health. However, no cases human food poisoning have been attributed to moldy cheese, most likely due to the large amounts required to be consumed to lead to a mycotoxin intoxication. Research also suggests that cheese is not a conducive growth medium for production of mycotoxins by molds (Hymery et al., 2014).

1.4.1 Sources of Fungal Contamination

Fungal contamination of cheese can occur at many different points in the cheese production process. For most cheeses, milk is pasteurized before use in cheese production. Most yeast and molds are not heat-resistant and are subsequently killed during pasteurization. Accordingly, mold spoilage is often due to airborne spores dispersed in the dairy plant air contaminating the milk

after pasteurization or the cheese during any point in the cheese making process (Garnier et al., 2017). Each step in the cheese production process introduces a potential point for fungal contamination. For example, brine from the brining process has been found to introduce molds like *Penicillium commune* (Marín et al., 2015). One study demonstrated counts of 10^9 cfu/cm^2 inside a brining tank (Bokulich & Mills, 2013). Further down the cheese production process, removing cheese from vats using pressurized air was found to be the source of a major fungal contamination in a dairy by *Geotrichum candidum* (Kure et al., 2004). Finally, adding inclusions like fruit can also introduce potential contamination sources of yeasts and molds (Filtenborg et al., 1996).

1.5 Control of Fungal Spoilage

1.5.1 Good Manufacturing Practices and Physical Methods

Various practices should be implemented in order to prevent fungal spoilage and to control the degree of proliferation of mold growth. Food safety plans are mandatory for all food manufacturing environments and consist of an analysis of all hazards present as well as risk-based preventive controls to mitigate the identified hazards (FDA Food Safety Modernization Act, 2011). Preventive controls for fungal contamination may include process controls and sanitation controls. Also included are Current Good Manufacturing Practices (cGMPs) which outline requirements for hygiene practices and safe food handling.

Many physical methods of controlling fungal spoilage exist and should be utilized during manufacturing. These methods can be found in Table 1.1. Unfortunately, these methods only remain effective until the consumer opens the package. After opening, the cheese may be contaminated by spores in the environment. Additionally, poor storage conditions such as those that give spores access to oxygen or ideal growth temperatures (25-30°C) will accelerate growth.

Table 1.1 Physical Methods to Control Fungal Spoilage

Method	Concept	Reference(s)
Milk Treatments: Pasteurization UV inactivation Cavitation High Pressure Processing Pulsed Electric Fields	Fungal spore reduction in milk lowers the number of spores that are able to germinate and proliferate into visible growth in cheese	 FDA, 2017, TetraPak, 2015, Vadillol et al., 1987 Can et al., 2014, Ricciardi et al., 2020, Koutchma et al. 2009 Badve et al., 2013, Sun et al., 2021 Datta & Deeth, 1999, Sun et al., 2021 Bendicho et al., 2002
Air Filtration	Spore reduction in the air lessens contamination	Kure & Skaar, 2019, Kure, 2004
Refrigeration	Temperature for optimal growth of molds is 25-30°C; storing cheese at refrigeration	Huang et al., 2011, FDA, 2021, Dix & Webster, 1995

	temperatures of 4°C will delay mold growth	
Modified Atmosphere Packaging	Fungi associated with cheese spoilage are obligate aerobes; replacing air inside packaging with carbon dioxide and nitrogen will deter growth	Farber, 1991, Messer, n.d.
Waxes and Protective Coatings	Provides a physical barrier to prevent further contamination as well as an oxygen barrier deterring growth	Bucio et al., 2021, Zvomuya, 2011
Cheese treatments: UV	Subjecting cheese to UV light immediately before or after packaging inactivates fungal spores that are present on the cheese surface	Can et al., 2014, Ricciardi et al., 2020, Koutchma et al., 2009

1.5.2 Use of Preservatives

1.5.2.1 Weak Organic Acids and Salts

The most common weak organic acids that are used as cheese preservatives are: sorbic acid, benzoic acid, and propionic acid. The salts of these acids are also used: potassium sorbate, calcium sorbate, sodium benzoate, potassium benzoate, calcium benzoate, and sodium propionate (Garnier et al., 2017). These additives are mixed into the cheese, rather than being applied to the cheese surface. Weak organic acids and their salts display inhibition against both bacteria and fungi. The mode of action against fungal and bacterial growth relies on the pH of the cheese and the pKa of the acid. If the pH of the cheese is below the pKa of the acid, the undissociated acid (protonated) will flow into bacterial or fungal cells. Once inside the cell (pH ~7) the acid will dissociate into its unprotonated form. The accumulation of protons inside the

cell causes the intracellular pH to drop. A gradient is formed into the cell by dissociation of the acid, causing more weak acid diffusion into the cell followed by its subsequent dissociation. This continues until the pH gradient across the cellular membrane is destroyed, effectively killing the cell (Konuk and Ergeden, 2017).

1.5.2.2 Natamycin

Natamycin is a polyene macrolide antibiotic and antifungal agent used widely in the food industry, especially in dairy products, for the prevention of mold and yeast growth (Chen et al., 2008). In the United States, according to the FDA, Natamycin can be applied on cheese as an antimycotic not exceeding 20 mg per kg (20 ppm) in the finished product (CFR - Code of Federal Regulations Title 21). Natamycin is produced by aerobic fermentation of the bacterium *Streptomyces natalensis.* a gram-positive spore-forming bacteria found in soil (Davidson & Zivanovic, 2003).

The mechanism of action of natamycin and other polyene macrolide antibiotics against yeasts and molds is based on the inhibition of amino acids and glucose transport through fungal cell membranes due to its specific binding to sterols, principally ergosterol in fungal cell membranes (Boothe, 2015). It has no antibacterial activity, due to the lack of sterols present in bacteria, and can be used without interrupting the bacterial ripening processes of cheese (Stark & Tan, 2003). Natamycin is active at very low concentrations, with most molds inhibited at 0.5–6.0 mg/L, although some species require higher concentrations (Delves-Broughton, 2011). In vitro

studies on natamycin often produce different results when applied to an *in vivo* sample. For example, on PDA, 2.5 mg/L natamycin completed inhibited mycelial growth of *Scletotinia sclerotiorum* but the concentration had to be at least 50 ppm to inhibit growth when applied to carrot samples (Ojaghian et al., n.d.).

1.5.2.3 Bioprotective Cultures

Many dairy producers seeking to opt out of chemical preservatives have reformulated their products to include the use of bioprotective cultures. These cultures are living organisms that are deliberately added to food products with the intention to control unwanted bacterial/fungal growth without adversely affecting the sensorial properties of the food (Martin et al., 2021).

Bioprotective cultures control growth of unwanted organisms mainly through displacement, competition for nutrients, acidification and production of metabolites. The use of bioprotective cultures, mainly Lactic Acid Bacteria (LAB), have been studied widely in cheese. LAB produce weak organic acids like lactic and acetic acid that lead to further acidification of the cheese as well as disruption of fungal cell membranes (Salas et al., 2019). Additionally, some LAB are known to produce bacteriocins which can inhibit the growth of some genetically similar bacterial species (Savadogo et al., 2006).

Mold ripened cheeses also employ a type of bioprotective culture. By inoculating the outer surface of cheese with a desirable fungal species (e.g. *Penicillium camembertii* on Camembert) that then grows on the rind, space is taken up and competition for nutrients occurs with other fungal species that

have the chance to contaminate the cheese. Additionally, molds often produce antifungal proteins that inhibit the growth of different fungal species (Delgado et al., 2016). Not only can ripening molds potentially prevent the growth of mycotoxin-producing molds, though not of particular human health concern, but they can reduce the potential for unsightly mold growth that may be concerning to consumers (Delgado et al., 2016). For example, P. camembertii and G. candidum, often used in mold ripened cheeses, are white in color and create a rind that is accepted by consumers.

1.5.3 Importance of 'Clean-Label' Alternatives to Natamycin

Consumers in the late 1970s and early 1980s began becoming concerned about the additives that were in food products. By the 1990s the focus moved to the desire to consume 'natural' foods. To this day, most consumers prefer to purchase products with few additives. This consumer demand has led to a removal of artificial colors, flavors, and preservatives in many food products (Brockman and Beeran, 2011). Such foods that are free of additives with negative connotations are often deemed to be 'clean-label'. Similarly, single ingredients that have a positive connotation or are thought of as 'natural' may also be referred to as a 'clean-label' ingredient.

Although natamycin is a naturally occurring substance and holds GRAS status by the FDA (CFR Title 21, Section 172.155), it has lost some favorability from consumers in recent years. Whole Foods has banned the use of natamycin except for in waxes of cheese rinds (*Food Ingredient Quality Standards: Whole Foods Market*, n.d.) and other grocery store chains like

Natural Grocers, and Raley's Supermarkets have banned its presence altogether (Dalié et al., 2010; *Grocery - Things We Won't Carry and Why: Natural Grocers*, n.d.; *Raley's One Market Banned Ingredients - Raley's Family of Fine Stores*, n.d.). Additionally, foods that incorporate natamycin cannot be labeled as organic according to the USDA (Dalié et al., 2010). For these reasons, organic cheese producers and natural grocery store chain cheese suppliers would benefit from a label-friendly alternative to natamycin.

1.5.4 Clean Label Alternatives to Natamycin

Research suggests that many potential 'clean-label' and organic alternatives to natamycin exist, but have yet to be validated for application on cheese. Some of these alternatives include edible films, essential oils, biologically active peptides, bioprotective cultures, and fermentation products. Each of these potential additives displays antifungal activity based on unique mechanisms of action. Edible films act as physical barriers to oxygen and can be used to deliver additives with antifungal properties like essential oils (Costa et al., 2018). Biologically active (bio-active) peptides are short amino acid fragments of proteins that display biological activities, some of which include anti-fungal effects (Pan et al., 2019). Bioprotective cultures are specific microbial strains which are deliberately added to foods to inhibit the growth of unwanted organisms by acidification and production of metabolites (Salas et al., 2018). Fermentation products are produced from fermented starch or milk powders which may contain bioprotective cultures, organic acids, and other metabolites that deter mold growth. The fermentation

products of culturing starch or milk powders are commercially available and demonstrate efficacy against yeast and molds, with increased efficacy as pH decreases (Samapundo et al., 2017).

1.5.4.1 Chitosan

Chitosan is a polysaccharide obtained by the deacetylation of Chitin and in recent years has been utilized to create edible films. Chitosan has demonstrated antifungal activity but largely depends on the degree of acetylation, molecular weight, derivatization, and preparation method used (Verlee et al., 2017). Other 'clean label' fungal inhibitors like essential oils and bioprotective cultures have been studied in combination with chitosan with some success (Hasheminejad et al., 2019, Salas et al., 2017).

1.5.4.2 Essential Oils

Essential oils have been utilized since the Middle Ages for their antifungal properties. Aromatic plants produce essential oils to protect them from many biological pests, including fungi. They are generally extracted from plants by distillation (Bakkali et al., 2008). Essential Oils are classified as GRAS by the FDA (FDA). The antifungal activity of many essential oils against foodborne fungal species has been widely reported. For example, thymol, the major component of thyme essential oil, has displayed inhibition against *Aspergillus spp*. and *Cladosporidium spp*. (Abbaszadeh et al., 2014). Clove Essential Oil has displayed antifungal activity against *Aspergillus flavus* with results varying on lab media versus on an actual food matrix (Omidbeygi, 2007).

1.5.5 Hurdle Technology Concept

To mitigate fungal spoilage most effectively in cheese, combinations of preservation methods (‘hurdles’) should be applied. This concept is commonly referred to as ‘Hurdle Technology’. The application of hurdle technology is especially important in the context of potentially less-effective mold inhibitors. Not all hurdles are created equal - the ‘higher’ the hurdle, more fungal spores will be required to overcome it in order for the mold to continue growing (Leistner & Gorris, 1995). Natamycin is particularly ‘high’ hurdle as it is an extremely effective mold inhibitor. Unless a ‘clean-label’ mold inhibitor is found with similar efficacy to Natamycin, additional hurdles must be applied for the same shelf-life to be achieved.

1.6 Screening of Mold Inhibitors

1.6.1 Classical microbiological methods for screening antifungals

Various classical microbiological methods can be utilized when screening compounds for antifungal activity such as disk diffusion, standard plate counts, measurement of hyphal diameter, and simply tracking time until visible mold growth is noted (Fernandez et al., 2017, Kuorwel et al., 2012, Cheong et al., 2014). These methods are supply and labor intensive, drastically limiting the number of potential inhibitors able to be screened within a reasonable time period. Utilizing high-throughput methodologies to screen antifungals that do not rely on manual counting, measuring, or assessing the amount of mold growth are vital when screening a high number of potential inhibitors or combinations thereof.

1.6.2 Lab Media vs. Food Application

In vitro screening of antifungal agents can be an efficient approach for high-throughput methods. It is important to note differences in activity of antifungal agents that may occur between microbiological lab media and actual food matrices. Differences in composition, including pH, buffering capacity, water activity, and sugar concentration can affect the ability and rate of mold growth (Beuchat, 1983; Calhoun & Galgiani, 1984; Membré et al., 1999). Additionally, certain additives in lab media can inhibit fungal growth. For example, using the de Man, Rogosa, and Sharpe (MRS) agar medium has been shown to strongly impact the expression of antifungal activity by LAB since MRS contains acetate, reinforcing LAB antifungal activity (Delavenne et al., 2012). In the lactate-tryptone-yeast extract medium commonly used to grow Propionibacterium, the presence of cyclic and linear peptides and diketopiperazines have shown antifungal activity (Lind et al., 2007). Garnier et al. (2018) found that the minimum inhibitory concentrations of natamycin for spoilage fungi were generally slightly lower when using model cheeses compared to potato dextrose agar (PDA). To maximize the chances of finding an efficient antifungal compound, food-based media screening should be utilized. In our case, since we are interested which antifungal compounds will perform well in a cheese system, we utilized a cheese-based agar.

1.6.2.1 Cheese Agar

Various studies relating to the microbiology of cheese have utilized cheese agar. Masoud and Jakobson (2003) used a cheese agar medium to create growth conditions relevant to surface ripened cheeses to assess the pigmentation produced by *Coryneform* bacteria. Larsen et al. (2002) utilized a blue cheese-based agar to assess mycotoxin production of different *Penicillium* molds on cheese. Neviani et al. (2009) developed a cheese agar containing grated Parmagiano Reggiano ripened cheese to recover minority bacterial populations. Wolfe et al. (2014) used a cheese curd agar medium to create an *in vitro* reconstruction of microbial communities of cheese rinds. Overall, cheese agar is a reproducible cheese mimicking model for *in vitro* experiments. It can be used to replace the cost and labor intensiveness of *in vivo* experiments performed on actual cheese.

1.6.3 High-Throughput Screening Methods

Screening many potential mold inhibitors for efficacy against cheese relevant molds can be very labor intensive, time consuming, and can fail to produce clear and quantitative results. Utilizing a high-throughput screening methodology can mitigate these issues and allow for testing of more inhibitor concentrations and combinations. Various high-throughput screening methods have been developed to screen anti-fungal activity. Kjeldgaard et al. (2021) developed two high throughput methods of screening the antifungal activity of *Bacillus spp*. The first method is an agar-based screen in 48-well microtiter plates that relies on visual scoring of each well. The second method

utilizes liquid broth media and uses a spectroscopy reading at 600 nm to quantify mold growth.

Other high-throughput screening methods developed by Inglin et al. (2021) for detection of antifungal activity by *Lactobacillus spp*. also rely on visual assessment. Garnier (2018) similarly studied antifungal activity of LAB by visual assessment of mold inhibition on a cheese-mimicking matrix.

High-throughput screenings can be used as an initial determination of which mold-inhibitor candidates should be followed up with *in vivo* experiments. This helps reduce labor-intensive *in vivo* screenings by selecting only mold inhibitor concentrations/combinations that show potential with *in vitro* experiments.

1.7 Research Objectives

The research objectives of this thesis were to:

1. Develop screening methods to efficiently screen organic, ‘clean-label’ additives that show promise as cheese antifungal preservatives
2. Refine high-throughput screening method(s) to increase the number of additives able to be screened
3. Screen additives, including combinations thereof to find applications that results in inhibition of cheese relevant fungi
4. Validate that additives displaying antifungal activity with rapid screening method work on actual cheese under normal storage conditions

2. Image-analysis methodology for high-throughput screening of clean-label mold inhibitor efficacy on cheese-based agar

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For submission to Journal of Dairy Science Communications

2.1 Introduction

In the dairy industry, fungal spoilage is typically not of food safety concern, but instead an issue of food waste and consumer rejection due to the production of off flavors, textural issues, or poor visual appearance of food products (Garnier et al., 2017).

Fungal contamination of cheese may occur at any step during production because mold spores are ubiquitous in the environment and are common airborne contaminants (Garnier et al., 2017). Mold growth on cheese is often controlled using packaging strategies that minimize oxygen exposure like with vacuum sealing or modified atmosphere packaging (Solomakos et al. 2019). However, when packaging is opened by the consumer, oxygen is no longer excluded, and mold growth typically follows. This situation is the most problematic for products that are multi-use and have high surface area, such as shredded cheese.

To combat mold growth in these products, shredded cheese is commonly coated with an anti-caking powder (e.g., potato starch, cellulose) containing up to 20 mg/L natamycin (United States Food and Drug Administration, 2019). However, natamycin is not allowed in organic food products (Organic Materials Review Institute) and is perceived negatively by some consumers due to increased desire for ‘clean-label’ products. (Brockman and Beeran, 2011).

Efforts to identify clean-label mold inhibitors have largely focused upon essential oils and antagonistic microorganisms (Salas et al., 2017). Some

essential oils and their components inhibit food relevant fungi, for example thymol (the major component of thyme essential oil) (Abbaszadeh et al., 2014) and Clove Essential Oil (Omidbeygi, 2007). While screening mold inhibitors, researchers have reported discrepancies in MICs when 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 species. For these reasons, it is imperative to perform screening experiments on the food-matrix of interest. Garnier et al. (2018) utilized mini-cheese in 24-well plates to screen bioprotective cultures of Lactic Acid Bacteria, manually scoring visible fungal growth to evaluate antifungal activity.

In this study, our objective was to develop a screening methodology with enhanced throughput, using cheese-mimicking agar in 96-well plates and quantitative image-analysis.

2.2 Materials and Methods

2.2.1 Mold culture preparation and maintenance

Four mold strains (*G. candidum*, *P. echinulatum*, *P. palitans*, and *P. roqueforti*) were previously isolated from commercial cheese products and were identified using a third-party laboratory (Certified Laboratories, Melville, NY, USA). The mold isolates were maintained on Potato Dextrose Agar (PDA; EMD Millipore, Burlington, MA) with incubation and storage at 22°C.

Every 4-6 weeks, molds were re-streaked on PDA by transferring spores to fresh media.

Mold spore suspensions were prepared by scraping hyphal mold growth with a sterile scalpel into 0.1% peptone water (Sigma Aldrich, St. Louis, MO, USA). Spore density estimated using a hemocytometer (SKC, Inc.) and adjusted to approximately 5 x 10^3 spores/mL. Spore suspension density was confirmed by standard serial dilution and spread plating 100 µL of each spore suspension on PDA with enumeration after incubation at 22°C for 72 hours.

2.2.2 Methylene Blue Cheese (MBC) agar preparation

Cheese agar was prepared in a manner similar to that presented by Wolfe (2014) with modifications. Cheddar cheese was produced by the Oregon State University Creamery (Corvallis, OR). Cheddar cheese blocks were shredded, frozen (-20°C), and freeze dried (Model 651M-9WDF20, Hull Corporation, Warminster, PA or VirTis Console 4.5, SP Industries, Warminster, PA). Freeze-dried cheese was ground using a mortar and pestle. Final cheese agar formulation included 100 g freeze-dried cheese, 50 g xanthan gum (MP Biomedicals, Irvine, CA), and 17 g bacteriological agar (VWR, Rador, PA) per liter of water. Ingredients were homogenized using a blender (Oster, Boca Raton, FL) and sterilized by autoclaving at 121°C, 15 min. For enhanced mold visualization, methylene blue (MB; Bio Basic, Markham, Ontario, Canada) was added after autoclaving to achieve a final concentration of 0.0001%. Methylene Blue Cheese (MBC) agar was tempered to 80°C using an aluminum reservoir (V&P Scientific, San Diego,

CA, USA) on a heating block (Eppendorf, Hauppauge, NY, USA) prior to pipetting into 96-well plates (150 µL/well).

2.2.3 Antifungal efficacy using MBC Agar

Antifungals with potential utility in cheese products were selected based on communication with dairy industry partners (Table 2.1). Antifungal solutions were prepared using appropriate solvent and dilution from stock solutions to working solutions using a 2-fold dilution series (in solvent). A volume of 15 µL of the antifungal solution was dispensed onto the surface of the MBC agar previously solidified in the 96-well plate and dried in the laminar flow hood. Individual wells were inoculated with 10 µl of previously described spore suspension (approximately 50 spores/well). The plate was sealed, wrapped in Parafilm, and incubated at 22°C for 7 days with imaging every 12 hours.

Table 2.1 Antifungals evaluated for their potential to inhibit fungal growth on Methylene Blue Cheese (MCB) agar.

Antifungal	Source	Solvent	Stock Solution	Treatment Range (per volume of agar)
Natamycin	MedChem Express	DMSO	800 mg/L	2.5-80 mg/L
Clove oil	Sigma Aldrich	0.05%	16,000 mg/L	50-1600 mg/L

	(St. Louis, MO)	Tween 80		
Thyme oil	Sigma Aldrich	0.05% Tween 80	16,000 mg/L	50-1600 mg/L
Chitosan (149 kDA, 97% deacetylation)	Primex (Siglufjordur, Iceland)	1.0% acetic acid	20,000 mg/L	62.5-2000 mg/L

2.2.4 Image capture and data analysis

Incubated 96-well plates were photographed using an SP Imager light box (S&P Robotics, Inc., Toronto, Canada) using the following settings: 5184 x 3456 pixels, autofocus, ISO 100, lens aberration correction, auto white balance. Photographs were imported into Image J software (National Institutes of Health, Bethesda, MD) and converted to grey scale (0 = black; 255 = white). Pixel spectra and pixel intensity were measured and analyzed using the “ReadPlate” plugin using a 250-pixel diameter. Pixel intensity is an integer from 0 (black) to 255 (white) and indicates the level of lightness/darkness of the area. 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 provides a quantifiable measurement of mold growth for both green molds (decreased RID) and white molds (increased RID). This change in RID is used to quantify mold growth. The absolute value of the change in RID between inoculated and uninoculated wells (n= 4) was used for all statistical analyses. Statistical analyses were performed using R-studio (version 3.6.1) and the “Growthcurver” package (Sprouffske & Wagner, 2016) was used for fitting growth curves for each well. Lag time (time to 50% maximum RID) was

selected as the metric to compare efficacy of antifungal treatments using ANOVA with Tukey's post hoc test. Since the "Growthcurver" package attempts to extrapolate lag time values if they are extended beyond the length of the experiment (7 days), any lag times over this time frame were capped at 168 hours.

2.3 Results and Discussion

2.3.1 Analysis of mold growth on Methylene Blue Cheese Agar in 96-well plates using Image J

MBC agar supported growth of all molds in this study. Photographs of *Penicillium roqueforti* and *Geotrichium candidum* on MBC agar after 7 days of growth at 22°C are shown in Figure 2.1. The corresponding greyscale spectrum next to each well shows the pixel intensity (count of pixels at a given position along this greyscale) of the 250-pixel diameter zone of analysis for the uninoculated control and the shift due to mold growth. Clear visual distinction of growth in wells inoculated with *P. roqueforti* and *G. candidum* corresponded with grayscale values distinct from the agar background. For *P. roqueforti,* the grayscale value was substantially lower (darker) than blue cheese agar while *G. candidum* growth resulted in a distinct shift to a higher grayscale value (lighter). Figure 2.1 also demonstrates the necessity of addition of Methylene Blue to cheese agar, as cheese agar without methylene blue has less of a magnitude of difference in pixel intensities to wells inoculated with *G. candidum.* Addition of Methylene Blue to a final concentration of 0.0001% allows pixel intensities within the well, and

subsequently RID values, to be more distinctly different from white mold growth such as with *G. candidum*.

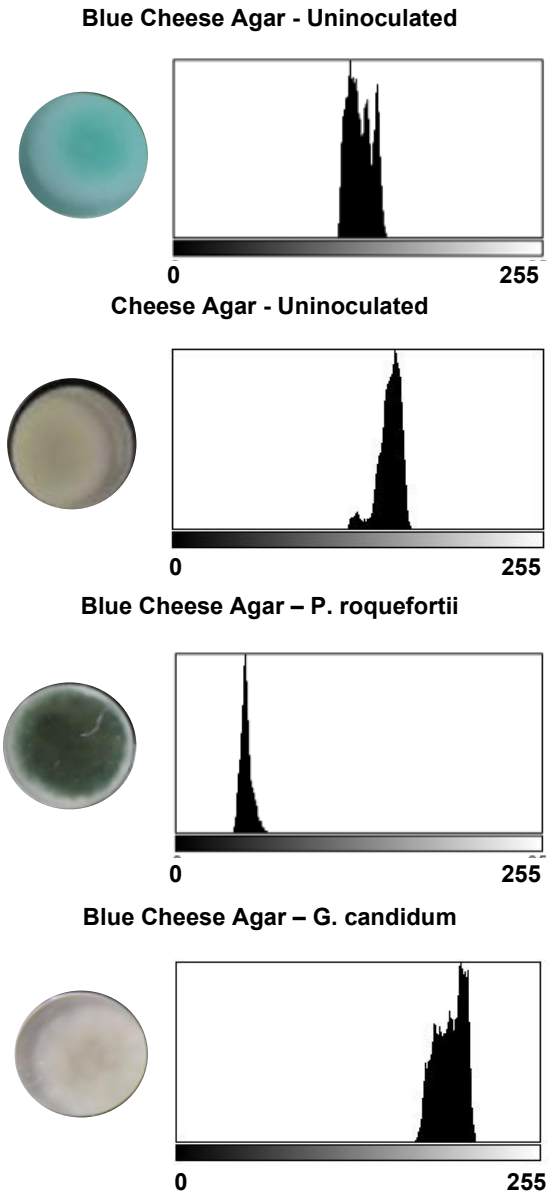

Figure 2.1 Grey scale spectra of Methylene Blue Cheese (MBC) Agar control (uninoculated), Cheese Agar (no methylene blue) control (uninoculated), MBC inoculated with *Penicillium roqueforti,* and MBC inoculated with *Geotrichum candidum* after incubation at 22°C for 72 hours. Histograms, derived using ImageJ, are shown alongside individual wells. The spectra represent the count of pixels at a given value (0-255) on the grey scale in the region of interest (ROI). Overlaid blue squares highlight the grey scale range of the uninoculated control (MBC agar).

Previous studies by Garnier et al. (2018) described and utilized a cheese-based agar medium for evaluating mold growth using a 24-well plate format and visual scoring. We further modified this approach to a 96-well format and incorporated methylene blue to facilitate improved throughput and objective data collection and analysis by taking advantage of the ImageJ software and "Readplate" plugin. This approach supported the clear detection of mold growth from high-resolution photos.

2.3.2 Generation of mold growth curves based on grayscale pixel count

Hyphal growth of *P. echinulatum* and *G. candidum* on MBC agar over time is shown in Figure 2.2A. *P. echinulatum* is visible on MBC agar around 60-72 hours, hyphal growth expands to cover half the well at 84 hours, and the well is fully covered by the mold after 108 hours. Similarly, in the representative series for *G. candidum* the media appears relatively free of mold growth at 12 hours but diffusely covered by 24 hours, and completely covered by 36 hours. These two molds grow at significantly different rates, with *G. candidum* being representative of a relatively fast-growing white mold with a mean lag time of 22.9 hours and *P. echinulatum* representing a slower growing green mold (mean lag time 85.2 hours). Grayscale pixel analysis of time-lapse images confirmed that image analysis corresponded with visual assessment (Figure 2.2B).

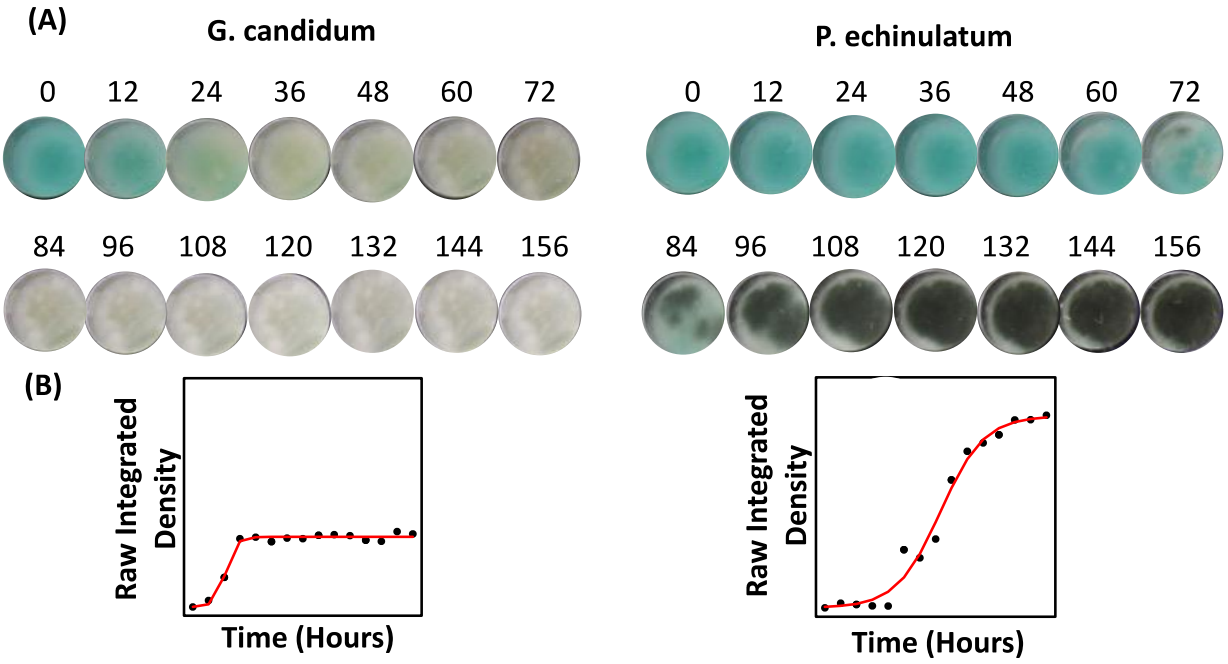

Figure 2.2 Growth behavior of *Geotrichium candidum* and *Penicillium echinulatum* on Methylene Blue Cheese (MBC) agar with incubation at 22°C for 7 days. The top panel (A) is a time lapse photo series with images captured every 12 hours. The bottom panel (B) are respective growth curves plotted by "Growthcurver" R package. Growth curves are based upon RID values for individual wells.

Final RID (subtracted from uninoculated wells, absolute values) for *G. candidum* were much lower than for the *Penicillium* strains, which compresses the dynamic range of the growth curves. Additionally, final RID values for the *Penicillium* strains were more variable than for *G. candidum*, suggesting this metric may be a less reliable parameter for comparison of mold growth in response to inhibitors. The other parameter estimated, generation time, was similar for all 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. To compare inhibitor efficacy and evaluate the utility of this screening approach, lag time was used as a metric to analyze results.

2.3.3 Screening antifungals based on significant increases in lag time across four mold strains

Using the combination of MBC agar in a 96-well format and image analysis, we were able to trial three clean label candidate inhibitors (chitosan, clove essential oil, and thymol). We focused on a topical application of inhibitors, as it mirrors the current use of natamycin in the cheese industry. Chitosan, clove essential oil, and thymol 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). Concentrations of the four antifungals (clove oil, thymol, chitosan, and natamycin) that displayed fungal inhibition are summarized in Table 2.2.

Table 2.2. Summary of Growth Curve metrics for each fungal strain (n=4) and minimum concentrations of Chitosan, Clove Essential Oil and Thymol that displayed inhibition. Statistically significant differences ($P<0.05$) in lag

time between the control (without antifungal) and each antifungal treatment were used as a proxy for inhibition and were determined by a One-way ANOVA followed by Tukey's post-hoc test.

Mold	**Growth kinetics on MBC agar***			**Minimum inhibitory concentrations derived from curve-fit "lag-time" parameter**			
	Lag Time (h)**	**Maximum Raw Integrated Density (x1000, arbitrary units)****	**Generation time (h)****	Natamycin (mg/L)	Chitosan (mg/L)	Clove EO (mg/L)	Thymol (mg/L)
G. candidum	22.9 ±1.3	220.4±19.0	4.9±1.3	2.5	-	-	-
P. echinulatum	85.2 ±3.9	530.7±46.7	10.6±0.8	2.5	2000	1600	800
P. palitans	91.1 ±8.1	446.8±39.2	10.7±1.6	2.5	1000	200	200
P. roquefortii	93.2 ±1.8	438.9±77.6	13.8±4.8	2.5	1000	800	400

*Growth parameters estimated from quadruplicate control wells, presented as mean +/- standard deviation
**Lag Time, Max RID, and Generation Time correspond to t_mid, k, and t_gen calculated by the Growthcurver R-Studio package

Natamycin was universally effective against all mold types, increasing lag time beyond the control (0 mg/L) at all concentrations (2.5, 5, 10, 20, 40, and 80 mg/L) ($P<0.05$). 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 hours, this does not necessarily indicate that the molds were completely inhibited.

Apparent increases in lag-times for each mold in response to the clean-label inhibitors varied by compound and dosage across the various molds. Chitosan treatment increased lag times for all molds except *G. candidum* at the highest application rate (2000 mg/L) ($P<0.05$), with lag times increased for *P. palitans* and *P*. *roquefortii* at a lower dosage of 1000 mg/L. Clove essential oil at 1600, 200, and 800 mg/L increased lag time for *P*. *echinulatum, P. palitans,* and *P. roqueforti*, respectively. Thymol at 800 and 1600 mg/L increased lag time for all molds except *G. candidum*, with lower levels of 200 and 400 mg/l effective against *P. palitans* and *P. roqueforti*, respectively. Essential oils as well as bioprotective cultures have been studied in combination with chitosan (Hasheminejad et al., 2019, Salas et al., 2017), a polysaccharide obtained by the deacetylation of Chitin. Chitosan alone has also demonstrated antifungal activity but largely depends on the degree of acetylation, molecular weight, derivatization, and preparation method (Verlee et al., 2017). Researchers have shown success using chitosan in films against yeast and molds in cheese (Duan et al. 2007, Fajardo et al. 2010) and in brine in Halloumi cheese (Mehyar et al., 2017). Fajardo et al. (2010) included up 5% in chitosan films for application on cheese, much higher than the 2% 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. Previous studies on chitosan have also widely reported success as a bacterial inhibitor (Vásconez

et al. 2001, 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% (2000 mg/L) by volume against molds.

Thyme essential oil, of which thymol is the major component, 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 mg/L or 10% of the sachet (Han et al. 2014). In our study, we saw inhibition of thymol at concentrations as low as 200 mg/L. Studies on thyme essential oil have shown more varied results, with bacteria inhibited at ranges of 75-1100 mg/L and molds inhibited at much lower at ranges of 80-97 mg/L (Fani and Kohanteb, 2017). Vázquez et al. (2001) trialed rates of 100-200 mg/L, showing some delay in *Penicillium spp*. at these rates.

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

In our experiment clove oil showed inhibitory potential, though was relatively less effective against molds compared to thymol due to higher concentrations being required for 2 of the target molds. The literature is more varied regarding inhibitory success of clove oil, with some researchers (Pinto et al. 2009) reporting MICs of 0.16-1.25 mg/L, much lower than that seen from our results (400 mg/L-1600 mg/L).

G. candidum was sensitive to natamycin; however, it was not inhibited by any of the alternative mold inhibitors trialed in this experiment ($P>0.05$). *G. candidum* is notoriously resistant to antifungals (Pottier et al., 2007) but in one study did show resistance to clove and thyme oil (on Czapek Yeast Autolysate Agar) when used at over twice the maximum concentration that was utilized in this experiment (Foltinova et al., 2019). The *Penicillium* strains clustered based on their sensitivity to clove essential oil and thymol.

2.4 Conclusion

The R-Studio “Growthcurver” package was utilized to calculate lag time using change in raw integrated density as a proxy for mold growth on MBC agar. While the proof-of-concept experiment revealed biological variation in response of molds to inhibitors, this method has future utility in determination of MICs and improved investigation of antifungal compounds, with the ability to screen thousands of samples. Using this new image analysis technique, we were able to screen a 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 antifungals while maintaining a high throughput and relatively low cost.

3. Relative efficacy of clean-label antifungals to inhibit filamentous fungi on cheese: A comparison of high-throughput screening methods

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For submission to Journal of Dairy Science

3.1 Introduction

Food waste is a substantial problem in the dairy industry, with most waste coming from microbiological spoilage deteriorating products through the growth of either fungi (yeasts and molds) or bacteria. Dairy foods are the third highest food group succumbing to food spoilage in terms of dollar value lost at the retail and consumer level in the United States, with 17% of the total value lost - behind only meat, poultry, and fish at 30%, and vegetables at 19% (Buzby et al., 2014). The implications of spoilage are not only profound regarding economic loss, but also in terms of environmental impacts, and food/nutritional insecurity (Papargyropoulou et al., 2014, Gunders et al., 2017). In the cheese industry, preventing and mitigating the amount of microbial spoilage of cheese, hence extending the shelf life, is extremely important to alleviate costs to producers, retailers, and consumers and helps to reduce the environmental impact of cheese production/consumption (Lu & Wang, 2017).

Natamycin is a polyene macrolide antibiotic and antifungal agent used widely in the dairy industry, especially topically on cheese products, for the prevention of mold and yeast growth (Chen et al., 2008). In the United States, according to the FDA, natamycin can be applied on cheese as an antimycotic not exceeding 20 mg per kg (20 mg/L) in the finished product (CFR - Code of Federal Regulations Title 21). However, it cannot be used in ‘organic’ cheese products and has lost consumer acceptance in recent years (Brockman and Beeran, 2011). Currently, there is no alternative antifungal product that is

thought to be ‘clean-label’ or that can be utilized in ‘organic’ cheese that is as effective as natamycin.

Research suggests that various potential natamycin alternatives exist but have yet to be validated for topical usage on cheese. These potential mold inhibitors include: edible films, essential oils, bio-active peptides, fermentation products, and bio-protective cultures (Costa et al., 2018, Khorshidian et al., 2018, Samapundo et al., 2017, Salas et al., 2018). Essential oils alone (Pandey et al., 2017) and in combination with chitosan (Kucukkaya et al., 2020, Martinez et al., 2018) have been previously studied for their antifungal properties in food applications. Fungal preservatives tend to vary in their effectiveness depending on mold species, with a range of .002 to >.01 mg/cm^2 natamycin found to be effective across a diverse set of cheese spoilage molds on microbiological media (Garnier et al., 2017). Thus, with numerous potential inhibitors to screen at varying concentrations across many relevant cheese spoilage fungi, traditional methods of determining antifungal activity would be laborious. Additionally, combinatorial antifungal treatments have been shown to be more effective, often resulting in additive or synergistic antifungal activity, further increasing the number of tests required for screening (Spitzer et al. 2016). For example, chitosan, when utilized as an edible film, has demonstrated increased efficacy as an antifungal with inclusion of bio-active compounds like essential oils (Costa et al., 2018).

Fluorescent staining has been used in various contexts, outside of the food industry, to determine fungal cell counts or biomass with use of the fluorescent dye, Calcofluor White. Calcofluor White binds to chitin, found in the cell wall of fungi. One study utilized image analysis of epifluorescence microscopy in conjunction with fluorescent staining to determine fungal biomass in leaves. Leaf samples were stained and resulting epifluorescent images with contrast between the fungal hyphae and background were used to calculate the percentage of the total sample that was taken up by fungal biomass (Schonholzer and Zeyer, 1995). Stagoj et al. (2004) developed a similar method to determine cell numbers of yeast by fluorescent staining centrifuged yeast cells after cultivation and measuring resulting emission (460 nm) at an excitation wavelength of 360 nm. Cell numbers per well were determined by use of a standard curve. Another method developed by Alvelo-Maurosa et al. (2016) utilized image-based analysis of micrographs and Image J imaging software to determine vegetative and sporulating cell counts after dual staining with Calcofluor White and Syto 9.

Research suggests that screening mold inhibitors on lab media produces different results than when screened on food matrices. Garnier et al. (2018) found lower minimum inhibitory concentrations for antifungals plated on cheese as compared to laboratory media. This suggests using cheese as the growth medium for screening is advantageous for finding effective concentrations with real-world applications on cheese products.

In this study we evaluated the utility of image-analysis based screening on cheese-agar (Jensen and Kornberg, in preparation) for a wider range of mold species, alongside a fluorescence-staining based assay. For molds that display color variation, or when seeking to screen at a single time-point, the fluorescent assay was found to more robustly estimate mold growth and enable detection of growth inhibition. The fluorescence-based screening assay was then used to determine antifungal activity of combinatorial treatments based upon essential oils and chitosan. Combinations identified as more inhibitory than each treatment alone were validated as topical treatments on cheddar cheese.

3.2 Materials and Methods

3.2.1 Chemicals and Laboratory Media

All chemicals and laboratory media were obtained from Sigma Aldrich (St. Louis, MO) unless otherwise stated. Natamycin was purchased from MedChem Express (Monmouth Junction, NJ). Xanthan gum was purchased from MP Biomedicals (Irvine, CA).

3.2.2 Mold culture maintenance and preparation of spore suspensions

Fungal species used in this study are listed in Table 3.1. *Penicillium echinulatum*, *Penicillium palitans,* and *Penicillium roquefortii* were isolated by a commercial cheese manufacturer from contaminated products and identified by Molecular Epidemiology Inc. (Seattle, WA). *Aspergillus niger, Geotrichum candidum, Mucor hiemalis,* and *Penicillium camembertii* were purchased from Carolina Biological Supply Company (Burlington, NC, USA).

All molds were maintained on (Potato Dextrose Agar) PDA at ambient temperature and transferred monthly onto fresh media by scraping and re-streaking of spores.

Table 3.1 Mold species and strains used in antifungal screening assays.

Species and Strain	Strain origin	Commercial Cheese Applications	Cheeses with Reported Spoilage Defects	Reference(s)
Aspergillus niger	Purchased	None	Blue-veined cheese	*Moubasher et al. 1978* *Hassanin et al. 1993*
Geotrichum candidum	Purchased	Camembert, Brie	Buffalo, goat, and sheep cheese	*Montagna et al. 2004*
Mucor hiemalis	Purchased	None	Buffalo, goat, and sheep cheese Hard and semi-hard cheese	*Montagna et al. 2004*
Penicillium camembertii	Purchased	Camembert, Brie, Langres, Coulommiers, Cambozola	Hard and semi-hard cheese	*Aran et al. 1987*
Penicillium echinulatum	Isolated by Cheese Manufacturer	None	Hard and semi-hard cheese Semi-soft cheese	*Lund et al., 1995, Kure et al., 2001, Aran et al., 1987, Kure et al., 2000*
Penicillium palitans	Isolated by Cheese Manufacturer	None	Fresh unripened cheese Hard and semi-hard cheese	*Garnier et al. 2017*
Penicillium roqueforti	C Isolated by Cheese Manufacturer	Blue cheeses, Gorgonzola, Stilton	Buffalo, goat, and sheep cheese Hard and semi-hard cheese Mold ripened cheese Semi-Soft cheese	*Montagna et al., 2004* *Hocking et al., 1992* *Lund et al., 1995* *Kure et al., 2001* *Aran et al., 1987* *Kure et al., 2000*

Adapted from “Diversity and Control of Spoilage Fungi in Dairy Products: An Update” by L. Garnier, F. Valence, and J. Mounier, 2017, *Microorganisms, 5*(42), p. 5.

Spore suspensions of each mold were prepared by carefully scraping the surface of mature hyphal growth on PDA with a sterile scalpel, and transferring collected spores into sterile 0.1% peptone water. Suspensions were adjusted to ~ $5x10^3$ spores/mL following enumeration by hemocytometer (Hausser Scientific, Horsham, PA) counting at 400x magnification using Leica ICC50 W microscope (Wetzlar, Germany). Each suspension was spread-plated onto PDA in duplicate to confirm spore concentration and adjusted with sterile peptone as required.

3.2.3 Cheese Agar

For anti-fungal screening experiments, cheese-agar was prepared as described by Wolfe et al. (2014) with minor modifications. Cheddar cheese (Kroger Medium Cheddar) was purchased from Fred Meyer (Corvallis, OR), shredded, frozen (-20°C), and freeze dried (Model 651M-9WDF20, Hull Corporation, Warminster, PA or VirTis Console 4.5, SP Industries, Warminster, PA). 100 g freeze dried cheese was combined with 50 g xanthan gum, 17 g bacteriological agar, and water to reach 1000 mL and blended (Oster blender). This mixture was then autoclaved for 20 minutes at 121°C on a liquid cycle. Where image-analysis was to be performed, methylene blue was added to reach a final concentration of 0.0001% in the agar. Cheese Agar has a water activity of 1.00 and a pH of 6.05. This compares to typical water activity values of .956-.965 (Schmidt and Fontana, 2008) and pH values of 5.1-5.3 (Lawrence et al., 2004) for cheddar cheese.

Cheese agar was held at 70°C on a heating block (Eppendorf, Hauppauge, NY, USA) in an autoclaved aluminum reservoir (V&P Scientific, San Diego, CA, USA), and 150 μL of agar was added to each well of a 96-well plate using an 8-channel pipette (Integra, Plainsboro Center, NJ, USA) fitted with wide bore pipette tips (Integra, Plainsboro Center, NJ, USA).

3.2.4 Mold inhibitor screening 96-well plate setup

Solutions of thymol and clove essential oil were prepared by addition into a carrier solution of 0.5% Tween 80 to achieve desired concentrations of 16,000 mg/L, 8,000 mg/L, 4,000 mg/L, and 2,000 mg/L. The chitosan (149 kDa, 96% deacetylation) solution was prepared by addition into 1.0% acetic acid to a concentration of 20,000 mg/L. Solutions containing thymol/clove essential oil in combination with chitosan were prepared by direct addition of thymol/clove essential oil into chitosan solution to achieve the same concentrations. When 15 µL of these solutions is added to 150 µL of cheese agar, the concentrations based on amount of agar then equal 1600 mg/L, 800 mg/L, 400 mg/L, and 200 mg/L respectively. The chitosan solution of 20,000 mg/L results in a final concentration of 2000 mg/L based on amount of agar. The natamycin solution was prepared in methanol to achieve 80 mg/L (8 mg/L based on agar). Application rates of treatments in terms of surface area can be found in Table 3.2. All solutions were heated for 10 minutes at 50°C.

Table 3.2 Antifungal concentrations on cheese agar and cheddar cheese based on volume and surface area.

Antifungal	Antifungal Stock Solution Concentration (mg/L)[a]	Antifungal concentration in cheese agar by volume (mg/L)[b]	Antifungal concentration in cheese agar concentration by

			surface area (mg/cm^2)[c]
Natamycin	80	8	0.00375
Clove EO/Thymol	2000	200	0.09365
Clove EO/Thymol	4000	400	0.1875
Clove EO/Thymol	8000	800	0.375
Clove EO/Thymol	16000	1600	0.75
Chitosan	20000	2000	0.9375

[a]15 μL and 82.91 μL of stock solution was added to the surface of the cheese agar (96-well) and cheddar cheese (24-well) in each well, respectively.
[b]Volume per well was 150 μl and ~825 μl for cheese agar and cheddar cheese, respectively.
[c]Agar surface area per well was .32 cm^2 and 1.77 cm^2.

To prepare test plates, 15 μL of each inhibitor solution and corresponding blank (carrier solution) was transferred to designated wells using a 96-channel pipette (Integra, Plainsboro Center, NJ, USA), into either a blackened 96-well plate (BrandTech Scientific Inc., Essex, CT) containing cheese agar or a clear 96-well plate (VWR International, Radnor, PA) containing methylene blue-cheese (MBC) agar. Solutions were allowed to dry for 20 minutes under a sterile laminar flow hood before addition of spore suspensions. Inocula preparations of each test species were transferred onto surface of cheese agar into designated wells using a 12-channel pipette (Integra, Plainsboro Center, NJ, USA) to achieve a density of approximately 50 spores/well. All tests were carried out in quadruplicate wells (n=4). Blackened 96-well plates used for fluorescent staining were sealed with a breathable film (Diversified Biotech, Inc., Dedham, MA) to facilitate sacrificial staining of partial plates and to minimize growth variability around the plate. Clear 96-well plates used for image analysis were incubated with lids sealed

using parafilm in order to minimize contamination from opening plates for photographing. All plates were incubated at 27°C for up to 6 days.

3.2.5 Image Analysis of Mold Growth

An image analysis procedure was adopted from Jensen and Kornberg (in preparation). Methylene blue was added to cheese agar at a concentration of 0.0001% to enhance visualization of mold growth. Incubated clear 96-well plates were photographed using a Canon EOS Rebel DSLR Camera inside a SP Imager light box (S&P Robotics, Inc., Toronto, Canada) using the following settings: 5184 x 3456 pixels, autofocus, ISO 100, lens aberration correction, auto white balance. Photographs were imported into Image J software (National Institutes of Health, Bethesda, MD). Pixel spectra and pixel intensity of the gray color channel were measured and analyzed using the "ReadPlate" plugin using a 250-pixel diameter for each well. Pixel intensity is an integer from 0 (black) to 255 (white) and indicates the level of lightness/darkness of the area. 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 provides a quantifiable measurement of mold growth for both green molds (decreased RID) and white molds (increased RID). The change in RID from un-inoculated wells (absolute value) of Methylene Blue Cheese agar (MBC agar) is used to quantify mold growth.

3.2.6 Fluorescent Staining of Mold Hyphae on Cheese Agar Surface

A fluorescent staining procedure was modified and adapted from the procedure developed by Stagoj et al. (2004) for liquid cultures. Briefly, 60 µL of a 1N solution of Sodium Hydroxide was added to each well along with 15 µL of Calcofluor White (1g/L, with 0.5 g/L Evans Blue) and plates were incubated for 1 hour at 27°C. Staining solution was removed and wells washed with 100 µL distilled water, taking care not to disturb agar surface. Fluorescence was read at an excitation wavelength of 365 nm and an emission of 435 nm in SpectraMax M2 plate reader (Molecular Devices, San Jose, CA), using the following settings; Emission Auto-cutoff, 30 flashes per read, well-scan (9-points per well).

Since chitosan is produced by the deacetylation of chitin, the compound being stained by Calcofluor White, un-inoculated wells of chitosan were stained to determine any possible impact on RFU measurement. There was no evidence that the means from the untreated, un-inoculated cheese agar wells were different from the un-inoculated wells treated with chitosan ($p=1.00$, One-way ANOVA followed by Tukey Post-Hoc Test).

3.2.7 Validation of mold inhibitor efficacy on cheese

Cheese slices (Medium Cheddar Farmstyle cut, Tillamook) were purchased locally (Fred Meyer, Corvallis, OR) and sterilized under UV light (Air Science LLC, Fort Meyers, FL) for 20 minutes on each side. Following sterilization, a 1.5 cm diameter sterile borer was used to cut the cheese and the resulting small circular pieces (~1.77 cm^2, ~.825 cm^3) of cheese were placed in sterile

24 well plates (VWR International, Radnor, PA). Cheese pieces were either left untreated (negative control) or treated with solutions of interest (Table 3.2), as well as 8 mg/L Natamycin (positive control). Volume of treatment was 82.97 µl to keep concentration based on volume and surface area consistent with the fluorescent staining screening experiment. The cheese used for our spoilage experiment had a water activity (METER Group AG, Munchen, Germany) of .9354, possible lowered by purchasing pre-sliced. 4 replicates given for each test, which were allowed to dry for 20 minutes under laminar flow before mold inoculation. Resulting water activity of cheese pieces were determined for each test after drying. Molds were inoculated onto the cheese by placement of a 10 µL droplet of a suspension of $5x10^3$ spores/mL, depositing approximately 50 spores onto each piece. 4 un-inoculated, non-treated replicates were included to ensure complete sterilization of cheese slices was achieved. The 24-well plates were sealed with parafilm and stored at 8°C to mimic typical consumer refrigeration conditions. Plates were monitored daily for visual mold growth.

3.2.8 Data Analysis

All statistical analyses performed consisted of One-way ANOVAs followed by Tukey or Dunnett's post-hoc tests to determine significant differences. A sample size of four was achieved for each test (n=4). Natural log (ln) was used to transform non-normally distributed RFU and RID values. All analyses were performed in R-Studio.

3.3 Results

3.3.1Comparison of image analysis and fluorescent staining for quantitation of mold growth on cheese agar in 96-well plates

Each mold species evaluated grew robustly on cheese agar within the assay period, exhibiting dark or light hyphal spread within wells that was visible between 24 and 72 hours of inoculation (Figure A.1). Summation of pixels according to darkness/lightness, as described in our previous work (Jensen and Kornberg, submitted), did not yield growth signal (ln RID) that agreed with visual scoring of growth for some mold species (Figure 3.1, Figure A.1). LOESS growth curve fits of ln RID values for *G. candidum*, *M. hiemalis*, and *P. echinulatum* demonstrate an increase in growth that levels off over time, corresponding with visual observation. However, for *A. niger*, *P. camemberti*, *P. palitans,* and *P. roqueforti*, shifts in ln RID either increased then decreased, decreased then increased, or produced no growth at all (resulting in a flat growth curve). Instances in which RID trends did not reflect what can be seen visually were caused by simultaneous presence of both light and dark hyphal growth. This occurred for *A. niger*, *P. camemberti*, *P. palitans*, and *P. roqueforti* after 104, 72, 72, and 80 hours, respectively (Figure A.1). Since the RID of the MBC agar is in between RID values of dark and white wells, when dark and white hyphal growth are present simultaneously, they effectively cancel out to equal the RID of the background MBC agar.

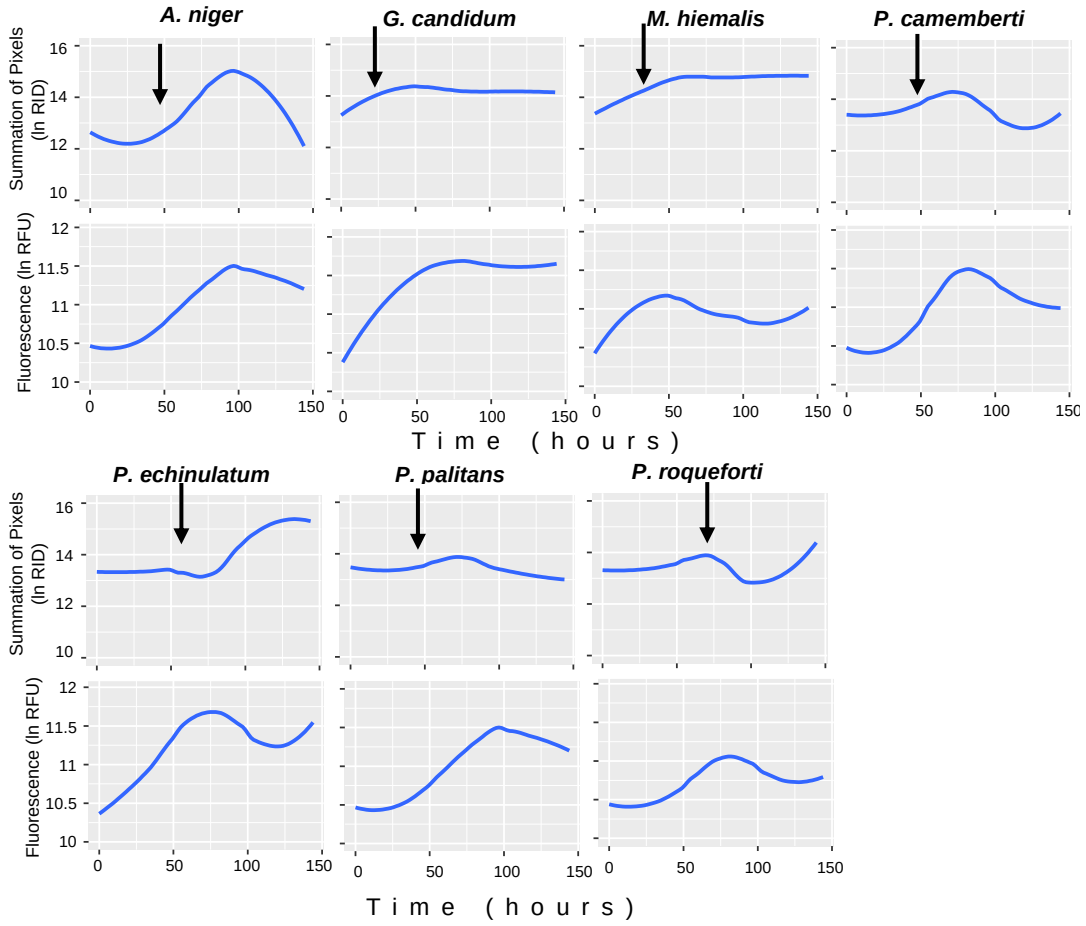

Figure 3.1. Growth of molds on cheese agar captured using image analysis and fluorescent staining assays. Grayscale images of wells were converted to raw integrated density (RID) values with natural log-transformation prior to subtracting background (un-inoculated, non-treated wells containing MBC agar). Relative Fluorescence Units (RFU) of Calcofluor-white stained hyphae were also natural log-transformed. Baseline fluorescence of un-inoculated cheese agar averaged 10.28 (10.00 to 10.50, 95% CI). Each black circle is a measurement of a single well with each treatment replicated in four wells. LOESS line of best fit is shown in blue, with grey shaded area representing 95% confidence intervals. Arrows indicate the time point at which wells were visually scored as positive for mold growth (see Table 3.3).

On the other hand, for all mold species tested it was possible to observe growth signal by fluorescent staining of mold biomass. Furthermore, for three of the tested species an increase in ln RFU was apparent at earlier time-points than it was possible to score growth visually (Table 3.3). For example,

all four wells of *P. echinulatum* were manually scored for presence of hyphal growth at 56 hours, whereas a significant increase in ln RFU occurred at 32 hours ($P<0.05$). By comparison, a significant change in ln RID was not detectable until 96 hours for this mold species.

While generating growth signal for a wider range of mold species, and providing enhanced sensitivity relative to visual scoring, fluorescent staining of mold biomass requires destructive sampling of wells. Therefore, its application is best suited to single time-point screening. Comparison of RFU values over time (Figure A.1) identified that for all molds there were significant differences ($P<0.05$) relative to time of inoculation at 48-96 hrs.

Table 3.3. Comparison of time (in hours) to detection in mold growth on cheese agar by visual analysis, image analysis, and fluorescent screening assays. Time points for image analysis and fluorescent screening assays were the first timepoint at which there was a statistically significant difference from time 0 based on the average pixel intensity (ln RID) and average relative fluorescence (ln RFU), respectively.

Mold species	Time (hours) to detection of mold growth		
	Visual analysis	Fluorescent Staining Assay*	Image Analysis*
A. niger	48	48 (0)	80 (+32)
G. candidum	24	24 (0)	24 (0)
M. hiemalis	32	24 (-8)	24 (-8)
P. camemberti	48	56 (+8)	72 (+24)
P. echinulatum	56	32 (-24)	96 (+40)
P. palitans	48	48 (0)	N/A**
P. roqueforti	72	48 (-24)	N/A**

*Significant increase from Time 0 based on ANOVA with a Tukey's post-hoc test ($P<0.05$)

** No significant increase noted ($P>0.05$, one-way ANOVA)

3.3.2 Single Time-point Proof of Concept Experiment using Fluorescent Staining Approach

In order to determine whether single time-point fluorescence-based screening method could detect partial, as well as complete mold inhibition, a proof-of-concept experiment was performed. Natamycin was applied in varying concentrations to 96-well plates and inoculated with all mold species, and plates processed during the 48-96hr period identified as suitable for detection of mold growth. At 8 mg/L, natamycin was completely inhibitory to all assayed mold species, with no difference in ln RFU relative to uninoculated wells detected at any time-point ($P>0.05$).

Lower concentrations of natamycin (2 - 4 mg/L) were partially inhibitory for all mold species except *M. hiemalis*, which displayed ln RFU values for these natamycin concentrations that were equivalent to untreated controls ($P>0.05$) at all three time-points. Partial inhibition by lower natamycin concentrations was indicated by ln RFU values that were significantly different from controls ($P<0.05$) at 48 or 72 hours, but not at 96 hours. Significant inhibition was noted for 4/7 molds at 2 and 4 mg/L natamycin at 48 hours, and 3/7 and 5/7 molds for 2 and 4 mg/L, respectively, at 72 hours. Additionally, *A. niger*, *P. camemberti*, *P. palitans*, and *P. echinulatum* displayed increased ln RFU values in untreated control wells from 48 to 72 hours, indicating that growth was still in progress during this time. A screening time point of 72 hours was selected as resolution to detect partial inhibition was similar to 48 hours, with more established hyphal growth present at 72 hours.

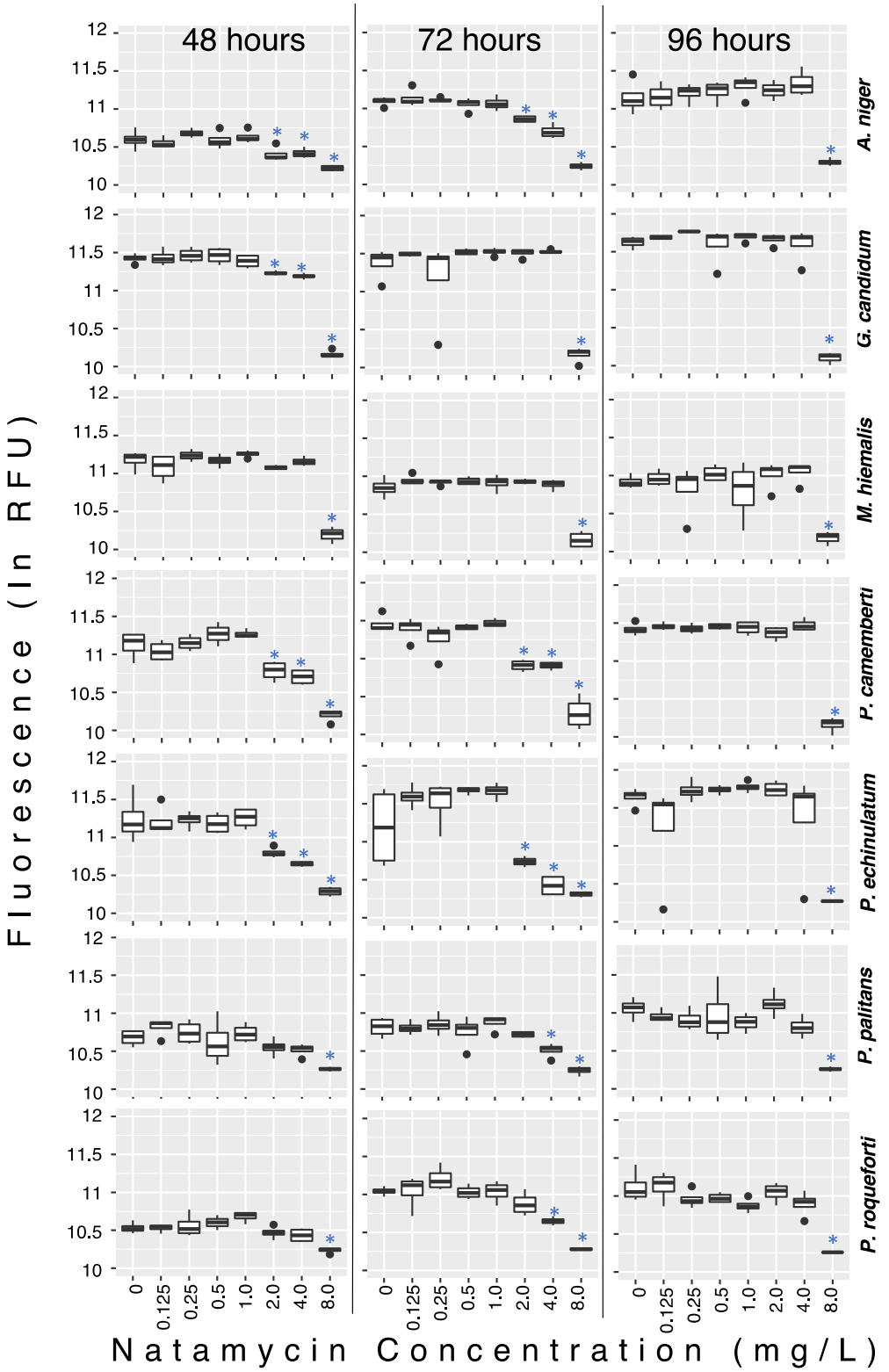

Figure 3.2 Evaluation of fluorescent staining assay to determine relative antifungal activity of natamycin dose (0.125 -8.0 ppm) on surface of cheese agar. Cheese agar was incubated for up to 96 hours at 27ºC prior to

fluorescent staining. Each test performed in quadruplicate (n=4) with data reported as average fluorescence (ln RFU). Average fluorescence of uninoculated cheese agar was 10.26 (10.14 to 10.37, 95% CI). Significant difference from untreated control (Dunnett's post-hoc test, $P<0.05$) indicated by asterisk.

3.3.3 Screening of potential combinatorial Antifungal Treatments

We previously found that thymol and clove essential oil, as well as chitosan, were able to inhibit mold growth on cheese agar (Jensen and Kornberg, in preparation). Results of a single time-point screen of combinatorial treatments based upon these inhibitors, applied to the surface of cheese agar, are shown in Figures 3.3 & 3.4. Response to thymol and clove essential oil alone and in combination with 2000 mg/L chitosan varied by mold species. *M. hiemalis* growth was not inhibited by any of the treatments, as ln RFU values were not significantly different ($P>0.05$) to those of untreated controls. Chitosan inhibited growth of all other mold species ($P<0.05$), and any additive or synergistic effects of combinatorial treatments would allow essential oils to be applied at lower concentrations when combined with chitosan, relative to application on their own.

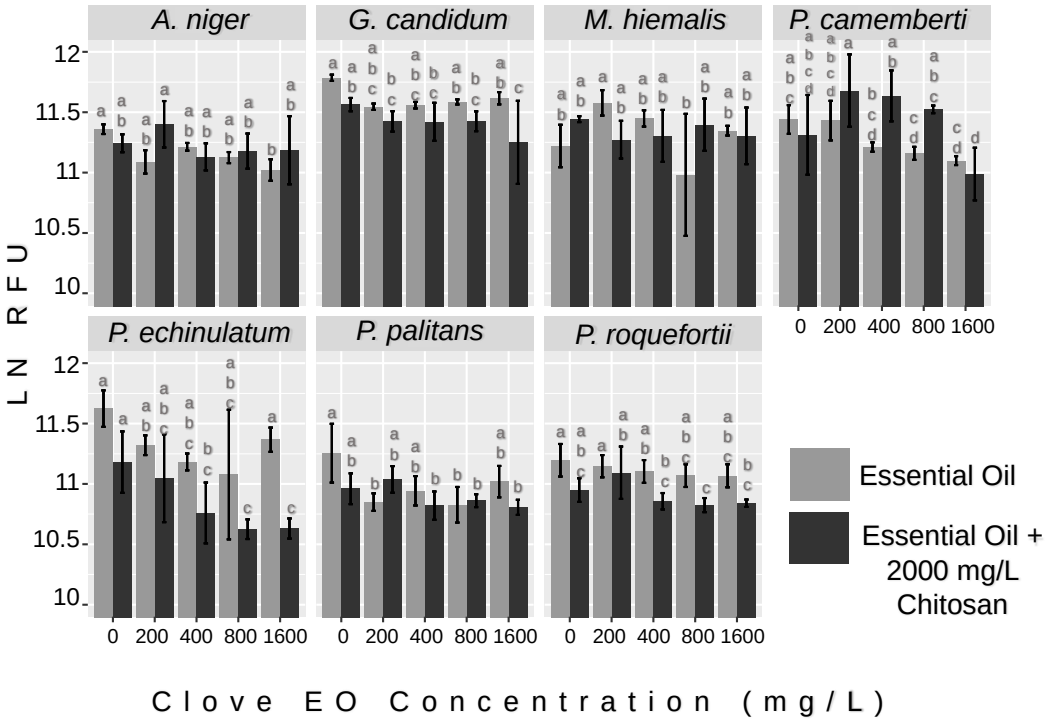

Figure 3.3 Inhibition of mold growth in response to varying concentrations of clove essential oil, with and without addition of chitosan expressed by ln RFU of calcofluor-white stained biomass on surface of cheese-agar in blackened 96-well plates. Significant differences (One-way ANOVA followed by Tukey post-hoc test) between treatments and positive controls indicated by different letter codes ($P<0.05$). Ln RFU of uninoculated cheese agar was 10.51 (10.42 to 10.59, 95% CI).

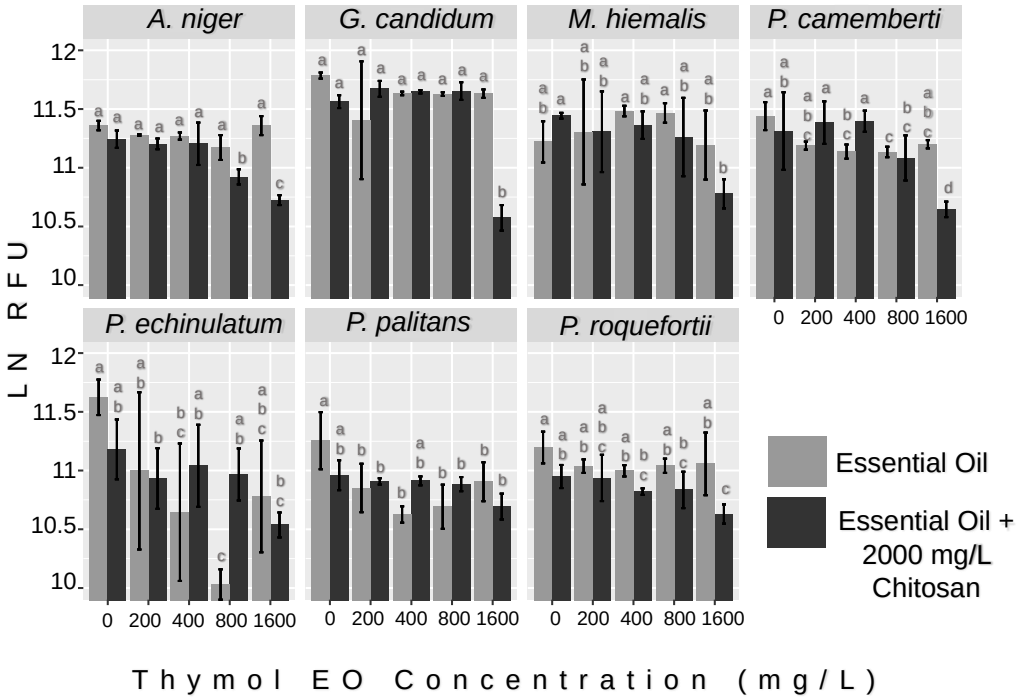

Figure 3.4 Inhibition of mold growth in response to varying concentrations of thymol, with and without addition of chitosan expressed by ln RFU of calcofluor-white stained biomass on surface of cheese-agar in blackened 96-well plates. Significant differences (One-way ANOVA followed by Tukey post-hoc test) between treatments and positive controls indicated by different letter codes ($P<0.05$). Ln RFU of uninoculated cheese agar was 10.51 (10.42 to 10.59, 95% CI).

Clove Essential Oil at 800 mg/L was only found to be inhibitory by itself towards *P. palitans*, but an additive inhibitory effect was found when it was applied in combination with chitosan. This combination displayed inhibition to not only *P. palitans,* but additionally to *G. candidum*, and *P. echinulatum*. Similarly, 1600 mg/L of Clove Essential Oil displayed inhibition against *A. niger* and *P. camemberti,* while combining the essential oil with chitosan was found to be inhibitory to all assayed mold species except *M. hiemalis*.

For thymol alone, 800 mg/L showed efficacy against the *Penicillium* species of *camemberti*, *echinulatum*, and *palitans*, while 800 mg/L combined with chitosan was inhibitory to not only these species but to *P. roqueforti* as well

as *A. niger*. 1600 mg/L thymol was only effective against *A. niger* and *P. camembertii* but addition of 2000 mg/L chitosan displayed antifungal efficacy to all mold species except *M. hiemalis*. Since concentrations of 800 mg/L and 1600 mg/L for thymol and clove essential oil combined with 2000 mg/L chitosan resulted in the highest number significant differences (decreases) from the control across the highest number of molds these tests were chosen to validate for efficacy on actual cheese.

3.3.4 Validation of mold inhibitor efficacy on cheese

All assayed mold species grew on the cheese surface under simulated consumer refrigeration conditions, varying in the amount of time before hyphal growth was visible (Figure 3.5). For untreated controls, this time varied from 9 days (*P. echinulatum*) to 38 days (*A. niger* and *P. camemberti*), much later than in the screening assay. Cheddar cheese treated with 8 mg/L natamycin did not support development of visible hyphal growth throughout the duration of the experiment for any of the assayed mold species. Each of the combinatorial treatments (800 or 1600 mg/L of clove EO/thymol with chitosan) caused a delay in hyphal growth. In some cases the delay was relatively short (5-7 days for the 1600 mg/L Clove with 2000 mg/L chitosan treatment against *P. palitans*), however 800 ppm Clove EO with 2000 mg/L chitosan induced a delay of 22 days for growth of *G. candidum*. Notably, for *M. hiemalis*, the combinatorial treatments were found to delay hyphal growth through the 42-day duration of the experiment, performing as well as natamycin. *A. niger* and *P. camemberti* experienced delayed growth of

controls (not growing until 38 days) that limited ability to discern delays induced by natamycin or combinatorial treatments.

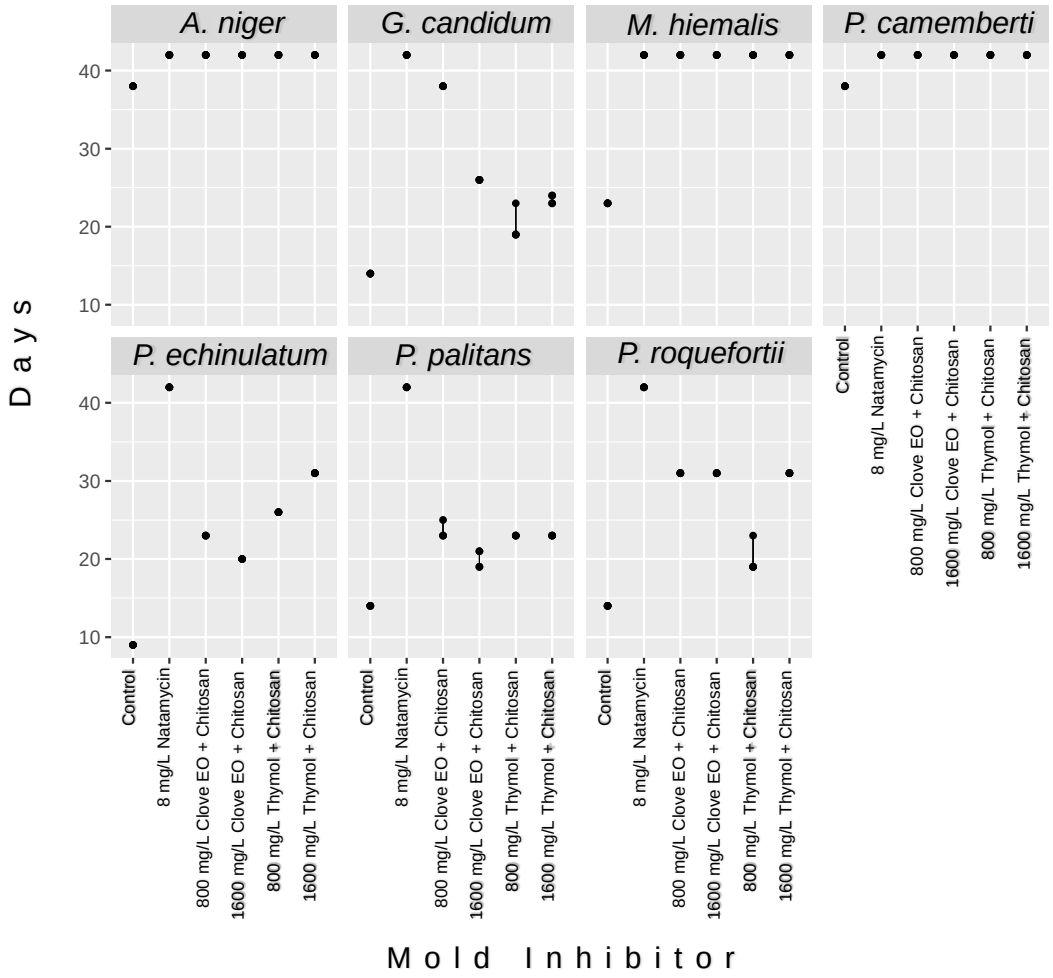

Figure 3.5 Range plot of days until appearance of visual mold growth on cheese after application of Mold Inhibitors, with storage under simulated consumer refrigeration conditions (8°C). Experiment was carried out for 42 days.

3.4 Discussion

Various methods have been developed to screen for antifungal compounds, typically involving the use of liquid cultures grown in laboratory media (Medina et al., 2012, Chadha & Kale, 2015). In this study, our objective was to validate a method for screening on solid cheese-mimicking agar, that could be utilized

in a high-throughput format. We found that fluorescent staining of hyphal growth afforded advantages over imaging and image-analysis (Jensen and Kornberg, in preparation), particularly for molds that displayed variation in production of pigments. Filamentous fungi produce a range of pigmented compounds such as melanins, phenazines, flavins, quinones, and carotenoids (Gmoser et al., 2017), moreover many factors can affect pigment production like carbon/nitrogen source, vitamins/minerals, temperature, pH, amount of oxygen/aeration, light, stress, and irradiation (Pomberio-Sponchiado et. Al, 2017). While imaging has limitations, the fluorescent assay requires additional test plates if measurements at multiple time points are desired since Calcofluor White is lethal to fungal growth. For this reason, further efforts to address RID limitations may be advantageous. In order to mitigate issues presented from pigment variation on RID measurements, future research could focus on determining completely inhibitory concentrations instead of finding delays in growth from creation of growth curves. For example, Fowler et al. (2018) found success with determining inhibitory concentrations of drugs against *Mycobacterium tuberculosis* cultivated in 96 well plates with the use of AMyGDA software, a Python package. This package uses backlit photos of plates with transparent microbiological agar to determine presence of growth by quantifying the number of dark pixels inside the well.

Using the fluorescent staining assay, we were able to detect complete inhibition of hyphal growth on cheese agar for all tested mold species by 8

mg/L natamycin, and partial inhibition of most species at lower concentrations. Most food-spoilage fungi have reported inhibitory concentrations of natamycin less than 10 mg/L (Stark & Tan, 2003). It is important to note that inhibitory concentrations vary depending on mold species and growth medium, with indications that lower minimum inhibitory concentrations are exhibited by molds when grown on cheese vs. laboratory media (Garnier et al., 2018). Application rates of natamycin vary in the cheese industry, depending on cheese type and perceived potential for exposure to mold spores, but are typically around 10 mg/L (Dupont). Our method, on cheese-mimicking agar, appears to be well-suited to estimating realistic topical applications of mold inhibitors on cheese.

Alternative clean-label inhibitors are generally less effective at inhibiting spoilage molds than natamycin (Seydim et al., 2020) and may only cause a delay of hyphal spread rather than complete inhibition (Kringel et al., 2020). Assays designed to detect partial and complete inhibition have greater chance to identify treatments that could potentially be used to extend shelf-life. In our proof-of-concept study, we found partial inhibition for concentrations of 2-4 mg/L natamycin across mold species, but ability to detect this inhibition was diminished by 96 hours. For the fastest growing mold, *M. hiemalis*, partial inhibition was not detected, most likely due to any delays in mold growth being undetectable due to rapid growth. For *G. candidum,* another rapid growing mold, ability to detect partial inhibition was diminished by 72 hours for this same reason.

Various clean label inhibitors show potential for use on cheese including chitosan, a polysaccharide compound that has been widely studied for its antimicrobial properties, with effectiveness largely depending on degree of deacetylation, molecular weight, derivatization, and preparation method (Verlee et al., 2017). Bio-active compounds like essential oils have also widely been studied for their antifungal effects, including applications in foods as preservatives (Pandey et al., 2017). Effective concentrations for essential oils have been reported from 40 to 3500 mg/L (D'agostino et al., 2019); with concentrations reportedly being organoleptically detectable as low as 50 mg/L this may pose limitations to applicability in cheese (Farag et al., 1989). Based upon detection of growth-delay (lag-time) by image analysis, we previously found that both clove essential oil and thymol could exert partial inhibition upon *P. echinulatum*, *P. palitans*, and *P. roqueforti* within the concentration range of 200-1600 mg/L. Using fluorescent detection of growth inhibition at a set time-point, we observed similar results with clove essential oil and thymol for *P. palitans* and *P. echinulatum*. However, in this study, *P. roqueforti* did not display a significantly decreased ln RFU in response to either inhibitor. Despite there being an increase ($P<0.05$) in lag time, the magnitude of this difference was slight (~40 hours for Clove EO, ~45-75 hours for thymol). Since we screened at 72 hours, some sensitivity to detect partial inhibition could have been lost due to tests catching up to the amount of growth in the control set. A relatively low lag time increase could also be why Jensen and Kornberg (in preparation) found levels of 1000 - 2000 mg/L chitosan effective

against *P. roqueforti*, *P. palitans*, and *P. echinulatum* (20-75 hours), but in this study we did not find 2000 mg/L chitosan to be effective against these same molds.

Previous studies on chitosan have demonstrated its viability as an antifungal for food applications, with consistently increased efficacy with inclusion of essential oils (Costa et al., 2018). We found 800 and 1600 mg/L thymol and Clove Essential Oil combined with 2000 mg/L chitosan to be most effective across the highest number of molds. No treatment solutions were found to be inhibitory against *M. hiemalis*, possibly due to this mold growing rapidly and reaching its maximal ln RFU within 48 hours (Figure 3.1).

In our experiments, inclusion of both thymol and clove essential oil (at 800 and 1600 mg/L) combined with chitosan (2000 mg/L) demonstrate efficacy for use on cheese as antifungal preservatives, delaying time to visual detection of growth for all assayed fungal species that grew on cheddar cheese. This delay in mold growth indicates the decreased ln RFU proxy for mold biomass experienced for these treatments in the previous screening experiment is an accurate sign of antifungal activity.

Inclusion of thymol into emulsions of chitosan has previously been studied for preservation of refrigerated pork, with success found with shelf-life extension. However, this research focused on inhibition of bacteria rather than fungi (Liu and Liu, 2020). Thymol, when used alone, was found to be inhibitory in ranges from 150 to 550 against foodborne fungal pathogens when applied to PDA (Abbaszadeh et al., 2014). However, in that case, thymol was prepared

in 5% DMSO while we used 0.05% Tween 80 in this study. Some research suggests that DMSO causes an additive antifungal effect to fungal preservatives, lowering MICs (Randhawa et al., 2007).

Clove essential oil has also been studied for its antifungal properties, with increased efficacy observed with encapsulation into chitosan when applied to PDA. It is hypothesized that this was due to the controlled release of volatiles from the essential oil as well as from the inhibitory effect of Chitosan alone (Hasheminajad et. Al, 2019). Other research suggests that encapsulation is helpful for bioactive compounds, like thymol and clove essential oil, due to protection from oxidation, volatilization, and interactions with the food matrix (Christaki et al., 2021).

When applied to cheese, all inhibitor solutions increased the water activity of the substrate. In order to mitigate for potential loss of shelf life due to higher water activity, salt or sucrose could be added to test solutions in the future to decrease water activity of the solution itself (Rahman, 2009). When applied as a film, chitosan has not shown to significantly affect the water activity of cheeses (Al-Nabulsi et al., 2020). Natamycin exists as a white powder and in the cheese industry is mixed with water and applied as a surface treatment by spraying or immersion, or is included into wax rinds. For shredded cheeses, anticaking agents like powdered cellulose or potato starch are combined with natamycin to evenly coat the cheese (Delves-Broughton et al., 2005). Our results indicate that high-throughput combinatorial screens can be performed on cheese agar, mimicking topical application on cheese, to predict shelf-life

extension. Shelf-life extension of up to 3 weeks were achieved by clean-label mold inhibitors in this study. Our approach, using fluorescent staining of hyphal mold biomass provides the ability to screen additional inhibitors and discover moderately effective treatments.

3.5 Conclusion

The fluorescent staining of mold biomass assay for antifungal screening provides a high-throughput format for determining efficacy of emerging mold inhibitors for use on cheese. Compared to the established image analysis screening method developed by Jensen and Kornberg (in preparation) the fluorescent staining method improves throughput by only requiring a single time point for taken measurements, compared to taking photographs at regular time intervals for a week. Additionally, the mold pigmentation does not affect fluorescent measurements like is apparent with image analysis.

4. General Conclusions and Future Outlook

This thesis determined the viability of using two novel high-throughput antifungal screening methods to find emerging ‘clean-label’, ‘organic’ mold inhibitors in hopes of finding effective alternatives to natamycin. The development of an image analysis screening method in Chapter 3 found antifungal activity with chitosan, as well as well with thymol and clove essential oil. The diversity and number of molds used for screening was expanded upon in Chapter 4, which sought to compare the use of the image analysis screening method to another novel antifungal screening method based on fluorescent detection of mold. A drawback of the image analysis

screening method was discovered with completion of experiments in Chapter 4: pigmentation of mold produces irregular results for certain molds.

Chapter 4 not only sought to prove the concept of using fluorescent detection of mold growth as a way to screen emerging mold inhibitors, but also to increase the throughput of such a screening assay. With the use of the image analysis screening method developed in Chapter 3, all data collected was taken at regular time intervals for 1 week. Due to the fluorescent staining procedure's tests being sacrificial, since the stain kills the mold upon contact, the screening method was developed to be completed at a single time point. This increased the through-put of the method and allows for more inhibitors and combinations thereof to be screened in a given time period.

The fluorescent staining method developed in Chapter 4 was also used to determine the efficacy of combinations of antifungals. Combinatorial antifungal treatments are not commonly used in the context of food but are very common for battling human fungal diseases. Combinations of previously studied antifungals demonstrated enhanced efficacy compared with singular use. When validated on cheese under refrigeration conditions, treatments of thymol and clove essential oil translated to a delay in time to visible mold growth.

Our work drew upon previous research done to develop more rapid assessments of antifungal activity. However, prior to these studies, screening methods mostly relied on visual detection and assessment of mold growth – a proxy that is subjective and unable to be quantified or statistically assessed.

Additional work in this area could focus on additional emerging areas of ‘clean-label’ antifungals like bioprotective cultures or bio-active peptides. The two antifungal screening methods from this study could be used to assess antifungal activity of treatments of interest.

‘Clean-label’ and preservative-free ingredient labels are emerging desires from consumers not only of cheese, but of all foods. The methods from our studies could be used to screen alternative mold inhibitors for any food. Our experiments used cheese microbiological agar to mimic growing conditions of the actual food product in question, but other growing mediums could be utilized that would mimic the growing conditions of the food in question.

An important follow-up to any mold inhibitor screening is the assessment of sensorial impacts that an effective mold inhibitor may impart to the cheese. Even if a mold inhibitor is extremely effective at controlling mold growth, if it imparts undesirable aromas or flavors to the food product it will have limited practical application. Sensory analysis should be performed before commercialization of a food containing a new mold inhibitor.

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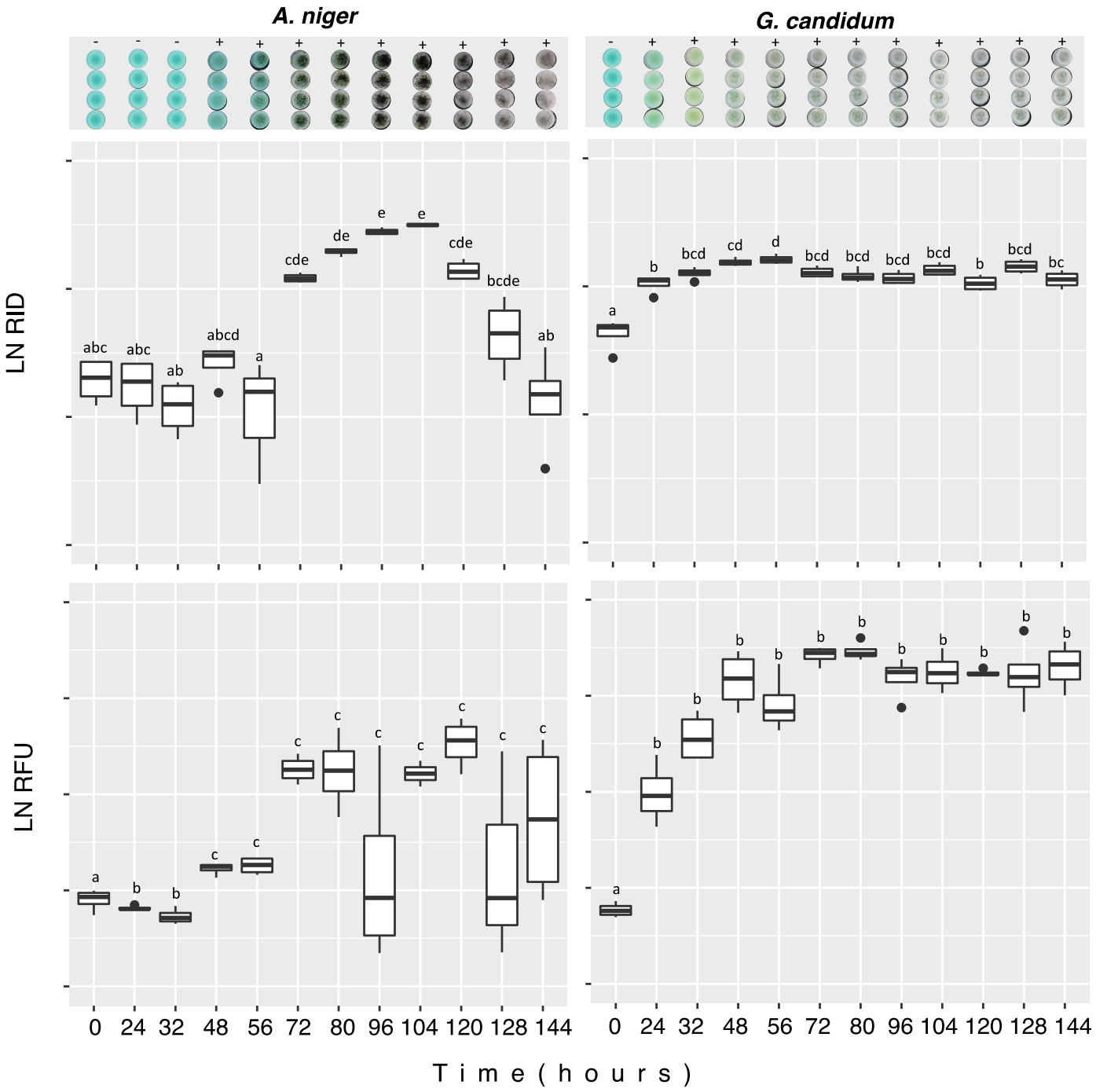
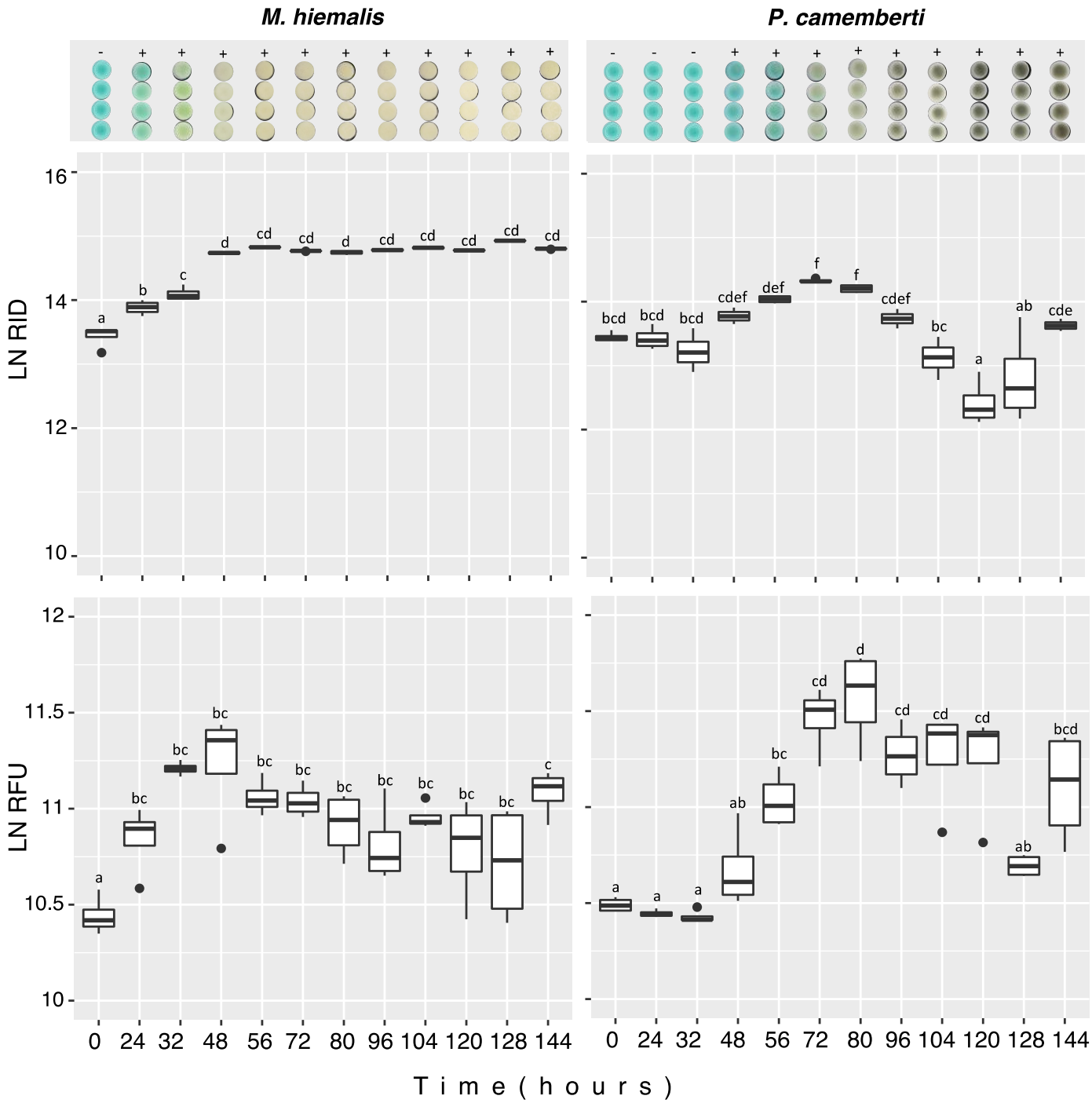
Appendix

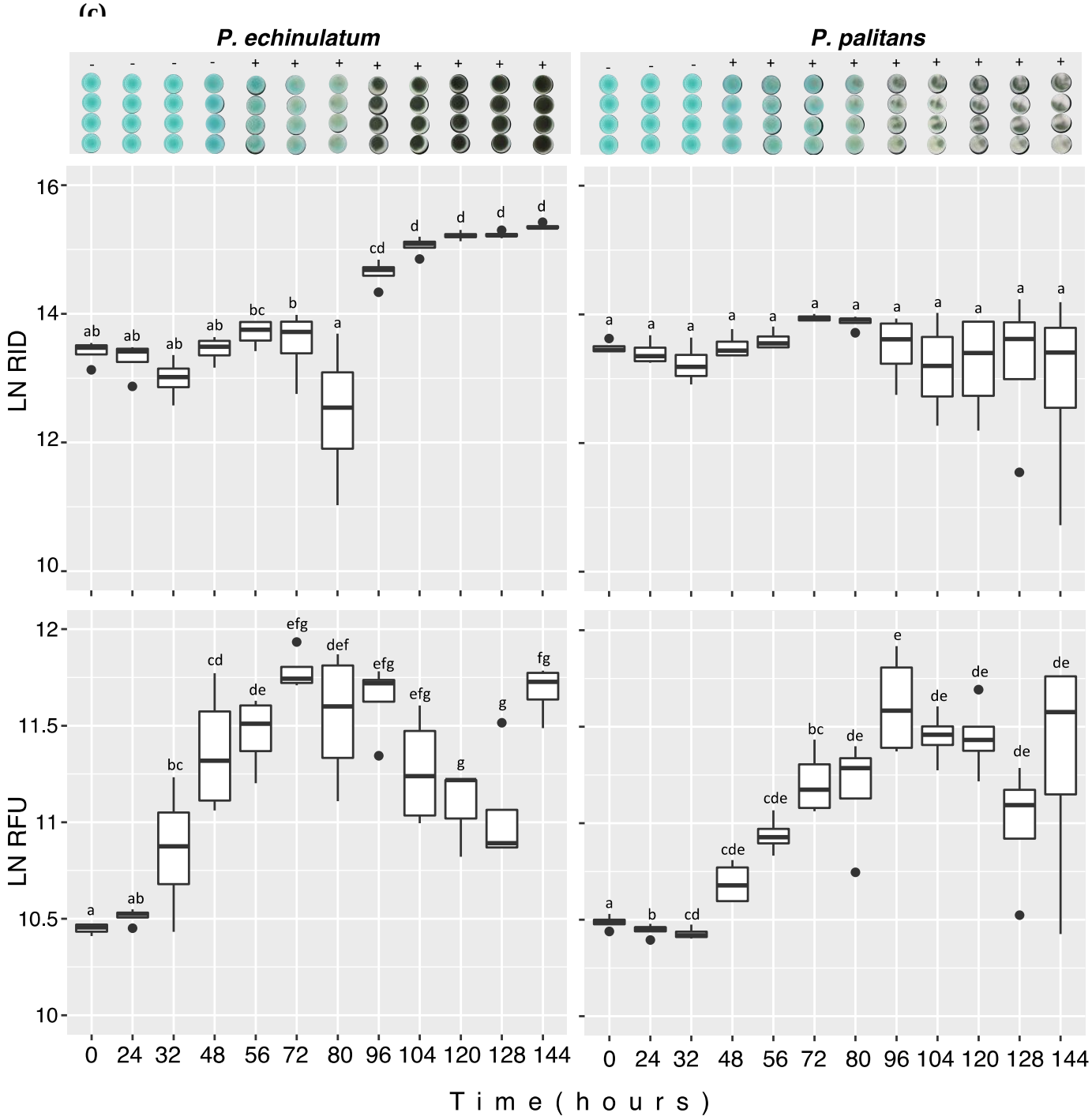
(a)


A. niger
G. candidum
LN RID
LN RFU
0 24 32 48 56 72 80 96 104 120 128 144
Time (hours)

(b)


M. hiemalis
P. camemberti
LN RID
LN RFU
Time (hours)
0 24 32 48 56 72 80 96 104 120 128 144
16
14
12
10
11.5
11
10.5
a b c d cd cd d cd cd cd cd cd
bcd bcd bcd cdef def f f cdef bc a ab cde
a bc bc bc bc bc bc bc bc bc bc c
a a a ab bc cd d cd cd cd ab bcd

(c)


P. echinulatum
P. palitans
LN RID
LN RFU
Time (hours)
0 24 32 48 56 72 80 96 104 120 128 144

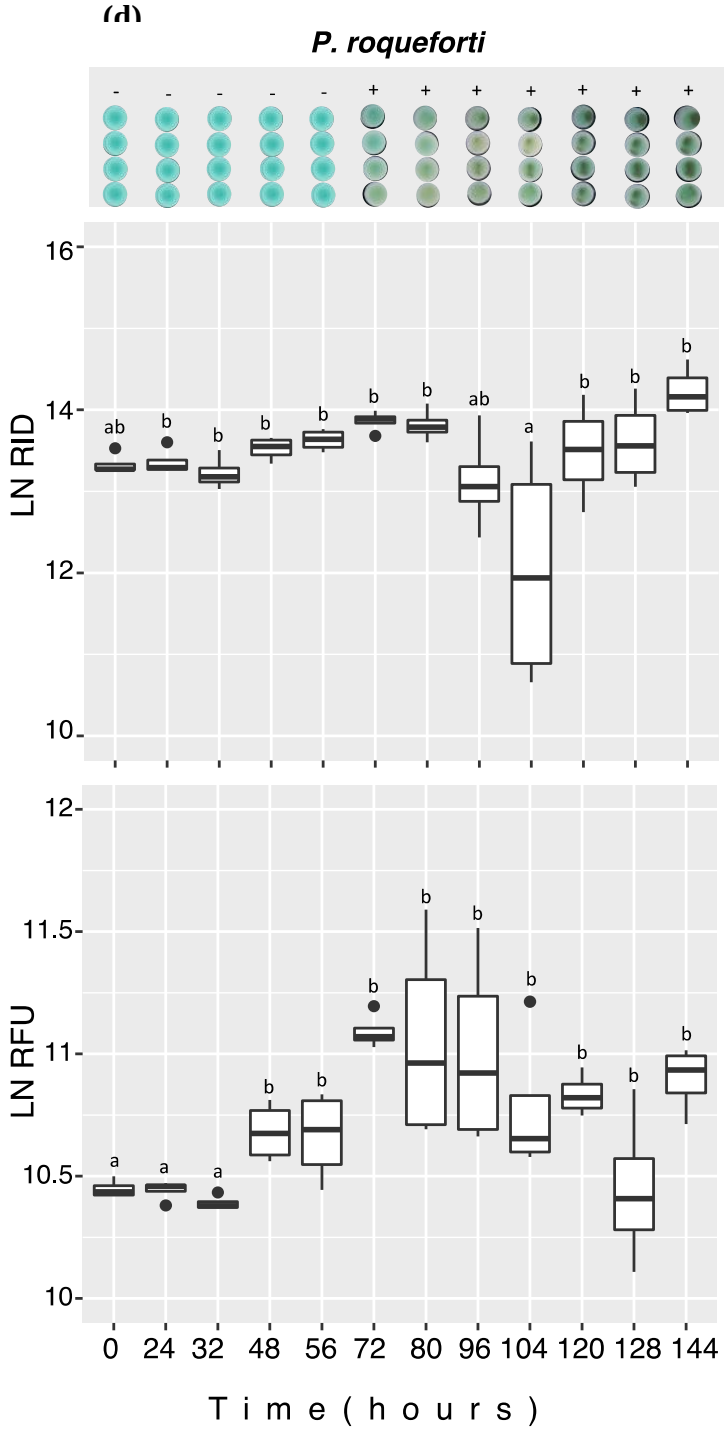


Figure A.1 Boxplots and corresponding images of time series data from Figure 1 for (a) *A. niger* and *G. candidum* (b) *M. hiemalis* and *P. camemberti* (c) *P. echinulatum* and *P. palitans* and (d) *P. roqueforti*. Means not sharing any letter are significantly different by the Tukey post-hoc test at 5% level of significance.

Table A.1 Water activity of cheddar cheese pieces (1.77 cm^2, .825 cm^3) after application of mold inhibitors (82.91 μL).

Mold Inhibitor Solution Applied	**Water activity (a_w)**
None (Control)	0.9354
8 mg/L Natamycin in Methanol	0.9544
400 mg/L Clove EO, 2000 mg/L Chitosan	0.9574
800 mg/L Clove EO, 2000 mg/L Chitosan	0.9583
800 mg/L Thymol, 2000 mg/L Chitosan	0.9566
1600 mg/L Thymol, 2000 mg/L Chitosan	0.9585