THERMAL INACTIVATION AND THE EFFECT OF PRESERVATIVES ON THE SURVIVAL OF OSMOPHILIC FUNGI IN A LOW WATER ACTIVITY MODEL CONFECTIONERY FOOD

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Master of Science in Food Science & Technology

by

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August 2016

ABSTRACT

Osmophilic fungi are capable of growing under normal fungal water activity limits of 0.85. They cause spoilage in confectionery and bakery food products and have the potential to survive mild heat treatments. Preservatives and heat treatments can prevent fungal spoilage from occurring.

Osmohilic molds, *Aspergillus pseudoglaucus* and *Aspergillus fischeri* were inoculated into confectionery model foods adjusted to 0.70, 0.75 and 0.80 a_w with either no preservative, 0.1% potassium sorbate or 0.002% natamycin. These products were incubated at 23°C for six months. Results showed that preservatives were not necessary to inhibit growth at or below 0.75 a_w. Both natamycin and sorbate were effective at preventing growth at 0.80 a_w.

Thermal death kinetics were also evaluated for both mold species. *A. pseudoglaucus* exhibited increased heat resistance at 0.7 a_w with D-values of 4.9, 1.6 and 0.83 min at 78, 80 and 82°C respectively as well as z-values of 5.2, 12.0 and 8.1°C for 0.7, 0.75 and 0.8 a_w. *A. fischeri* exhibited extreme heat resistance at 0.75 a_w with a D_{90} of 34.8 min, D_{92} of 17.1 min and D_{94} of 7.65. z-values for 0.7, 0.75 and 0.8 a_w were 7-7.1, 5.8-6.0, 6.3-7.1°C depending on the strain.

BIOGRAPHICAL SKETCH

Elizabeth Claire Buerman, daughter of Karen and Gary Buerman, was raised in Newark, New York. She attended Cornell University for a degree in Interdisciplinary Studies with a concentration in Food Microbiology. After receiving her Bachelor of Science in 2012, Elizabeth moved to Englewood Cliffs, NJ. She worked as a Category Microbiologist with Unilever in the Research and Development Department. She was a member of a cross-functional team that produced many new, preservative free spreads under “I Can’t Believe It’s Not Butter” and “Country Crock” brand names. In 2014 Elizabeth returned to Cornell University to work under Dr. Olga Padilla-Zakour; studying osmophilic molds in low water activity foods.

To Winston Woolly Mammoth and Grigor:

For always being number one

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Olga Padilla-Zakour, for mentoring and teaching me over the past two years. I am extremely appreciative of the opportunity to learn so much at Cornell. I would also like to thank Dr. Randy Worobo, my minor advisor, for sharing his expertise of Food Microbiology.

So many members of the Cornell Community were invaluable during the past two years of research. A special thanks to the members of the Padilla-Zakour and Moraru labs for letting me take over multiple benches. As well as to the members of the Worobo lab for letting me join lab dinners! An extra special thanks to Sheena Hilton for helping design some of my methods as well as Abby Snyder and Claire Zoellner for double checking my math! And thank you to Stephen Perry for helping with all of the statistical modeling. An extra-extra special thanks to Grigor Lynch for proof reading and editing so many papers he understood nothing about. And Winston Woolly Mammoth for being fluffy.

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CHAPTER 1

CONTROL OF FUNGI IN CONFECTIONERY AND BAKERY PRODUCTS: A REVIEW

ABSTRACT

Osmophilic fungi are capable of growing in low water activity products and causing economic losses in the confectionery and bakery food sector. Many methods can be used as hurdles to synergistically inhibit spoilage. These include lowering the water activity, extending heat treatments, adding preservatives and adjusting the packaging.

INTRODUCTION

Bakery and confectionery products can range in water activities (a_w) of 0.20 for hard candies and up to 0.80 a_w for creams. The definition of "confectionery" can cover cakes, candies, chocolates, fillings and icings. In this review, bakery products and confectionery products will be included together (*17*). Products above 0.60 a_w are at risk for yeast and mold spoilage. Yeast and mold spoilage cause visible growth, product bloating, or slime production – making them unacceptable for consumers whether at retail or in the home. Confectionery products with water activities below 0.90 a_w will inhibit the growth of pathogenic bacteria, except *Staphylococcus aureus,* however, between 1 and 5% of the total bakery volume are spoiled by fungi, based on product and season (*34,29*). Methods such as thermal processing, lowering water activity, adding preservatives, and more recently, using modified packaging all inhibit mold spoilage and extend shelf life. However, there is limited information available specifically for confectionery products with low water activities. Most of the information available is based on experiments in microbiological media rather than actual food products; or does not consider water activities at

which osmophilic fungi can grow. Increasingly consumers are looking for clean label products with limited or no preservatives. Hurdle methods, such as lower water activity and thermal processing combined synergistically to completely inhibit spoilage, must be put into place to extend shelf life, reduce spoilage and reduce economic losses in the bakery and confectionery sector.

Table 1.1. Water Activity (a_w) range of selected bakery products

Product	**a_w**
Crackers	0.20-0.30
Chocolate coated doughnuts	0.82-0.83
Soft cookies	0.50-0.78
Bread	0.96-0.98
Gummies and jellies	0.50-0.75
Jams	0.80-0.85

(Adapted from Smith, J.P., D. Phillips Daifas, W. El-Khoury, J. Koukoutsis, and A. El-Khoury 2004 and Ergun, R., R. Lietha, and R. W. Hartel 2010)

PRESERVATIVES

Organic acid preservatives and their salts such as benzoic acid, sorbic acid, or propionic acid are commonly added to a wide range of food products (*23*). Potassium sorbate is somewhat more effective at slightly higher pH levels (generally up to 6 pH for sorbate and only up to 4 pH for benzoate) and thus is more commonly used in bakery products in the range of 0.1 to 0.3%. Sorbate added in the range of 0.05 to 0.2% can be effective in confectionery products depending

on water activity (*5*). It can be added to formulas directly or used in packaging to decrease post process mold growth. Under acidic conditions benzoic acid and sorbic acid are in their undissociated form and can enter cell membranes. They then lead to an accumulation of protons within the cell from the lowered internal pH and reduced ATP production leading to delayed growth and sporulation (*23*). The concentration of sorbate needed for inhibition is fungal strain and product medium dependent (*14*). Sorbate is less effective in high fat products due to its reduced solubility in lipids. Sorbate has also been shown to sometimes increase thermal treatment effectiveness but not necessarily with heat resistant molds such as *Aspergillus fischeri.* However, it can reduce recovery of injured cells after heat treatments (*5*). Sorbate is commonly used in dried fruit, juices, syrups, preserves, baked goods, and sugar confectionery products to extend shelf life (*21*).

Propionic acid is frequently used for breads because it is less likely to interrupt the fermentation and rising process. Propionic acid does not inhibit yeast nor most bacterial growth, although it does prevent *Bacillus* spoilage which can be a problem in bread products (*29*). It is also more effective in acidic conditions. It works similarly to sorbate and benzoate by entering the cell membrane, acidifying the cell, interfering with the proton gradient and transport systems and thus interrupting ATP production (*10*). Propionic acid can also add a strong, unpleasant smell to products (*27*).

An alternative to traditional, chemical preservatives is to use fermented preservatives produced by bacteria. Natamycin is produced by fermentation of *Streptomyces natalensis* and is a small polyene macrolide antibiotic with antifungal properties (*31*). It has a molecular mass of 665.75 and a formula of $C_{33}H_{47}NO_{13}$ (*22*, *31*). Natamycin has been used for decades in the preservation of cheeses and sausages; inhibiting growth of yeasts and molds. Most food spoilage

molds are inhibited by less than 10 ppm of natamycin with a few exceptions up to 60 ppm. It has low solubility in aqueous solutions but due to its low minimum inhibitory concentration, this is generally not an issue. It is stable at neutral pH values, but not below pH 4. Natamycin had generally been assumed to work by binding to ergosterols in fungal cell walls causing ions to leak out of the membrane leading to cell death, as other polyene antibiotics do (*31*, *33*). Most fungi that are resistant to natamycin have limited ergosterols (*31*). Ergosterols are to fungi as cholesterol is to mammals and are involved in membrane permeability, transportation and sporulation (*18*). However, more recent studies have shown that natamycin does not affect membrane permeability but rather inhibits growth by interfering with vacuole fusion. It has been hypothesized that natamycin disrupts protein re-arrangements by binding to ergosterols in early vacuole fusion steps (*33*). It has also been show to disrupt uptake of nutrients, disrupt endocytosis in fungi, and disrupt hyphal growth (*35*).

While the typical use for natamycin is in the fermented foods industry, it has been proven to successfully inhibit fungal spoilage in fruits, juices, bakery products, and confectionery fillings such as cream fillings and icings through methods such as dipping, spraying and directly adding to formulations (*31*). Natamycin is "Generally Recognized as Safe" by the Food and Drug Administration in the United States for use in non-standard of identity cheese, yogurts, cottage cheese, sour cream, cream cheese, dressing, tortillas and bread (*7*).

Table 1.2. Preservative activity

Preservative	pH range	Heat stable	FDA maximum level	Activity
Propionate	<5	Heat stable	0.3% in flour used in baked goods (*4*)	Molds and *Bacillus subtilis* (*29*)
Sorbate	< 6	Heat stable	0.1% (*28*)	Yeast, mold and some bacteria (*4*)
Benzoate	< 4.5	Heat stable	0.1% (*4*)	Yeast, mold and bacteria (*4*)
Natamycin	4.5-9	Heat sensitive	0.02% in cheese (*7*)	Yeast and mold (*31*)

OSMOPHILIC FUNGI

Osmophilic yeasts and molds represent a large portion of confectionery and bakery products' microbial spoilage and are the primary source of microbial spoilage in the intermediate water activity range (*29*, *16*). Molds in particular have the ability to survive the heat processing that is regularly used in confectionery production (*34*). Osmophilic molds can produce dormant, heat resistant ascospores through sexual recombination. These spores have thick walls and higher cytoplasm viscosity that protect them (*8*). The heat processing temperatures and times used for confectionery foods can act as a heat shock, which activate the spores and can initiate germination. Less resistant molds that may not survive thermal processing can still cause contamination post-process by improper cooling which causes formation of condensation or exposure to the environment. Good hygiene and sanitation in factories is necessary, but studies have found up to 10^3 CFU/m^3 levels of mold in bakery facilities, depending upon processing conditions and ingredients used in formulations (*6*). Packaging can be used to combat post-

process contamination but poses issues with condensation formed with warm products. Aseptic handling can be used but is very expensive. UV, Infrared radiation, microwave heating, low dose irradiation, and pulsed light have all been evaluated as potential treatments for bakery products. Currently some of these methods show promising results but still have issues such as causing condensation, high cost or have not considered potential sensory effects (*29*).

Aspergillus fischeri and *Aspergillus pseudoglaucus*, previously known as *Neosartorya fischeri* and *Eurotium repens* respectively, are osmophilic fungi that are capable of surviving heat treatments and growing at low water activities. These name changes have been implemented recently starting in 2014 to simplify genus and species names within mycology. Previously, names were based primarily on morphology rather than phylogenetic information (*26*). *A. fischeri* and *A. pseudoglaucus'* survival has been evaluated in fruit products, juices, buffers, and agars but not in confectionery products. Most of these experiments start with a heat shock step that activates dormant ascospores and allows them to germinate while inactivating vegetative cells that cannot survive such extreme temperatures. The heat shock step is typically at a lower temperature than that which the D-value is evaluated. A D-value is the amount of time, typically in minutes, to cause a 90% reduction in microorganism level at a particular temperature. A z-value is the temperature change necessary to decrease the D-value by 1 log (*8*). These thermal inactivation curves have found that the media used for heat inactivation can have a significant effect on inactivation or survival. For example, Kotzeikidou (1997) examined survival of *N. fischeri* and other heat resistant molds, recovered from spoiled canned tomato paste, in both tomato juice and phosphate buffer. Under all conditions, survival was greater in the juice with 16% soluble solids (*19*). Beuchat (1986) evaluated *A. fischeri* and other heat resistant molds in five different fruit fillings composed of different fruits at 88°C and 91°C as well as in a challenge

study at room and refrigerated temperatures. Results varied between *N. fischeri* strain as well as fruit filling. Challenge study results showed visible growth at room temperature within a few days, but visible growth at 10°C after more than two months (*2*). Temperature, food composition, and mold strain clearly have significant effects on spoilage rates.

Organic acids and preservatives can greatly decrease heat inactivation time. Rajashekhara et al. (2000) found that grape juice components offered more protective benefits to *N. fischeri* than mango juice. They also found that the addition of lactic acid, malic acid, citric acid, sodium benzoate or potassium sorbate to either mango or grape juice greatly reduced the amount of time needed to inactivate ascospores at 85°C. Lactic, malic and citric acids contributed to time reductions similarly. Sorbate and benzoate combined to a total 0.1% concentration of preservatives in juice proved to be the most effective additive, compared at equivalent concentrations, in order to decrease heating time by half and cause a 3 log reduction (*24*). Another study focused on *N. fischeri's* survival in apple juice, papaya juice and pineapple juice at high temperatures (80°C, 85°C, 90°C). Salomão and others (2007) found that inactivation rates were slowest in apple juice, due to low citric acid content, and fastest in pineapple juice, which is a more acidic juice. This study confirmed that high heat treatments are necessary for fungal reduction if no other hurdle methods are added (*25*). These juice studies were important to identify the correct time and temperature combination in juice production to allow the highest sensory and nutritional qualities during processing. However, this information cannot be directly transferred to a bakery or confectionery process.

While some studies have focused on *A. fischeri's* extreme heat resistance, very few have focused on the heat resistance of fungi previously categorized as *Eurotium*. Although they are less heat resistant, they have the potential to survive mild treatments and grow at very low water

activities. Splittstoesser and others (1989) were one of the few considering the heat resistance after encountering a spoilage outbreak of grape jams and jellies. The heat shock activation step of the tested organism - *E. herbariorum,* a close relative to *A. pseudoglaucus,* was compared between water and grape juice, with only slight differences in recovery (less than one log), unlike experiments with *N. fischeri* which showed higher recovery in juice. D-values were also evaluated. The D_{70} in 5°Brix juice was 2.5 min and doubled to 5.0 min at 65°Brix grape juice. The high level of sugar is believed to have a protective effect on ascospores. The D_{70} in 5°Brix juice with 0.05% sorbate at pH 3.5 was very similar to the D_{70} in 5°Brix juice without preservative. The addition of fumaric acid demonstrated no differences on the D_{70}-value: 1.4 min for the control versus 1.4 min with 1000mg/L fumaric acid (*30*).

POST PROCESS CONTROL

Even after thermal processing, contamination can still occur. Preservatives can be added to the formulation of the product, sprayed onto the outside, or added to packaging to minimize contamination concerns. As consumers become more sensitive to clean label products other factors such as pH, water activity, and temperature should also be considered as potential hurdles. Combinations of these conditions have been studied on both media and in foods. Gock et al. (2003) examined germination times and growth rates of seven types of osmophilic fungi that have been recovered from spoiled food including molds that were previously considered *Eurotium* on Petrislides with media adjusted to target pH (4.5, 5.5, 6.5, 7.5) and water activity (0.70, 0.74, 0.78, 0.82, 0.86, 0.89, 0.92). Inoculated Petrislides were incubated at 25°C, 30°C and 37°C and colonies were evaluated regularly. In general, germination occurred more quickly at lower pH values and higher water activities. *Aspergillus* species (*Eurotium spp.*) evaluated

showed the ability to germinate and grow at water activities as low as 0.70 a_w (*12*). Suhr and Nielson (2004) evaluated growth of eight molds frequently found in bakery spoilage on media adjusted to typical rye bread water activity, preservative levels and pH levels. Propionate (0.3%) was effective at inhibiting growth for all molds except for *Penicillium roqueforti* and *Eurotium rubrum* unless under high pH (4.8) or water activity (0.97) conditions. Follow up experiments, by Suhr and Nielson, demonstrated that sorbate and benzoate were more effective than propionate. Both of these two previous studies, carried out on agar, reveal the importance of water activity, pH and preservative type and concentration (*32*). However hurdle methods are best examined in model foods. In 2002 Guynot et al. evaluated the effect pH (6.0 and 7.5), water activity (0.80, 0.85, 0.90), and preservatives (sodium benzoate, calcium propionate, potassium sorbate) on room temperature incubated sponge cake inoculated with osmophilic *Aspergillus* (*Eurotium*) molds. The preservatives, with sorbate as the most effective, were more effective at the lower pH (6.0) and water activity values (0.80 and 0.85 a_w). When Guynot et al.'s results were compared to studies of hurdle methods in non-food products, they found that preservatives were less effective in real food (*14*). In a follow up study, Guynot et al. (2005) used a bread analogue at pH 4.5 and 5.5 to evaluate their previous results of preservative effectiveness in acidic conditions more closely. Fresh baked bread was inoculated with the same *Eurotium* molds used previously and incubated at room temperature. As expected, mold growth increased with increasing water activity, but 0.3% potassium sorbate inhibited growth at 0.90 a_w. Sorbate (0.03%) was effective at reducing growth at or below 0.85 a_w in the 4.5 pH bread analogue as well as at 0.80 a_w at 5.5 pH. The equivalent concentrations of propionate and benzoate were less effective than sorbate under all conditions evaluated (*16*).

Char et al. (2007) considered the interaction of potassium sorbate, pH and water activity in an Argentine confectionery product composed of evaporated milk, sugars and vanillin. Water activity was adjusted to 0.80 and 0.85, pH was adjusted to 5.5 or 6.0, and potassium sorbate was added in concentrations from 0.0-0.2%. Product was inoculated with *E. chevalieri* a known osmophilic spoilage mold found repeatedly in food products. Sorbate (0.2%) was insufficient to inhibit growth at 0.85 a_w and 6.0 pH. However 0.15% sorbate combined with 0.80 a_w regardless of pH inhibited growth. Sorbate (0.1%) when combined with pH 5.5 and 0.8 a_w was also effective (*3*).

MODIFIED ATMOSPHERE PACKAGING

Methods using packaging to prevent spoilage can avoid the use of unwanted preservatives. Modified atmosphere packaging involves packaging the product with a wrapper within which the atmosphere inside has been altered with gases to increase shelf life. Generally the wrapper is impermeable to prevent gases and water vapor from transferring between outside and inside environments. Usually carbon dioxide combined with nitrogen in varying ratios is used to reduce aerobic bacterial and fungal growth (*29*). Different fungal species have varying susceptibilities to CO_2 environments, especially if low concentrations of oxygen are still available within the package. This emphasizes the importance of removing oxygen before sealing (*11).* Currently this method is more popular in Europe than in the United States. Modified atmosphere packaging has been able to extend shelf life in some instances from days to weeks. This method is also more environmentally sustainable than freezing products. However there are still some challenges to this method as it allows conditions for the pathogen *Clostridium botulinum* to grow (*29*). Examples of shelf life extension in bakery products have

been demonstrated by Abellana et al. (2000) with sponge cake at a neutral pH, with varying water activities between 0.75 and 0.90. Cake was inoculated with osmophilic *Aspergillus* (*Eurotium*) strains isolated from spoiled products and packaged under 11 different variations of CO_2 to O_2 ratios. CO_2 (100%) inhibited visible mold growth for over 30 days regardless of O_2 levels. CO_2 (100%) combined with water activities below 0.85 were effective at completely inhibiting growth. However, using 100% CO_2 in packaging is not realistic as it has the potential to form a vacuum that crushes product. It can also become soluble in water and form carbonic acid - producing an unappealing flavor change. The authors of the sponge cake study came to a conclusion that O_2 levels below 1% and CO_2 levels at 60% would be ideal for modified atmosphere packaging of intermediate moisture bakery products held at room temperature (*1*). Combining results of potassium sorbate in sponge cake and modified atmosphere packaging of sponge cake, Guynot et al. (2004) evaluated shelf life of sponge cake prepared with 0.0 – 0.2% potassium sorbate, 0.80-0.90 a_w, 6.0-7.5 pH and packed with 0-100% CO_2. Each variable played a significant role in predicting fungal growth at room temperature. Lower pH (6.0) and water activity (0.80) allowed for lower concentrations of (0.0%) CO_2 and (0.05%) potassium sorbate to inhibit growth. CO_2 (70%-100%) at 0.90 a_w allowed potassium sorbate to be completely removed from the formulation at pH 6.0. As expected, sorbate was less effective at neutral pH. Higher levels of (30-70%) CO_2 and (0.05-0.2%) sorbate were necessary to control fungal growth; with higher levels of CO_2 and sorbate necessary at higher water activities. The results showed that preservative levels can be lowered or completely removed when hurdles of low water activity and modified atmosphere packaging with high levels of CO_2 are combined to reach a shelf life of at least 28 days at room temperature (*15*).

ACTIVE PACKAGING

Active packaging uses a similar approach, but rather than adding gases within the products' environment, it encloses the product within a package containing an antimicrobial. A few of the numerous preservatives considered for active packaging include: sorbate, benzoate, propionate, nisin, and triclosan (*20*). A few studies have considered the effect of cinnamon essential oil added to packaging to reduce mold growth in bakery products. Films containing between 5 and 10% cinnamonaldehyde have been found to be effective in extending shelf life and as an alternative to adding preservatives (*13*, *20*). Active packaging can also use an oxygen absorber to remove oxygen from the inner environment to reduce growth, similar to modified atmospheric packaging but with fewer opportunities to crush the product from the formation of a vacuum. Or, ethanol can be added however it is absorbed by the product; increasing alcohol content and can impart an unwanted flavor (*13*). Active packaging shows promise for shelf life extension, but is still very expensive (*11*).

CONCLUSIONS

In conclusion, there are many opportunities for future studies involving the bakery and confectionery sector due to the wide range of products with varying attributes, especially concerning methods to inhibit fungal spoilage at low water activities. Heating medium, even when keeping soluble solids and pH equivalent, can affect how long a thermal process must take to be effective at reducing spoilage. Preservative can be less effective in food matrices than in similar agar analogues. There are many opportunities for packaging methods to work synergistically with formulation and processing methods to extend shelf life.

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CHAPTER 2

EFFECT OF WATER ACTIVITY AND PRESERVATIVES ON SURVIVAL OF OSMOPHILIC FUNGI IN A LOW WATER ACTIVITY MODEL CONFECTIONERY FOOD

ABSTRACT

Fungal spoilage on bakery and confectionery foods can cause retailers and consumers to reject the products, causing economic loss. Hurdle methods must be combined to inhibit osmophilic fungal growth. Low water activity (a_w) is frequently used in confectionery products as a hurdle, but osmophilic molds have the potential to grow as low as 0.60 a_w. The objective of this study was to consider water activity levels at 0.70, 0.75, and 0.80 a_w combined with no preservative, traditional preservative potassium sorbate and fermented preservative natamycin in a confectionery model food made from evaporated milk and fructose. The inoculated model was incubated for six months at 23 ± 2°C. Known contaminants, *Aspergillus pseudoglaucus* and *Aspergillus fischeri,* that are capable of surviving heat processing and growing at low water activities were used. Low levels of potassium sorbate and natamycin; 0.1% and 0.002% respectively, were added.

A significant four-way interaction between fungal type, preservative type, water activity and week in challenge study was found. No visible growth was seen in products at 0.70 a_w or 0.75 a_w. *A. pseudoglaucus* showed visible growth at 0.80 a_w with no preservative but was inhibited at 0.80 a_w by both 0.1% sorbate and 0.002% natamycin (20 ppm). While *A. pseudoglaucus* and *A. fischeri* counts stayed stable or declined slightly with the addition of sorbate at all water activities, levels declined more quickly at 0.80 a_w with natamycin. These results can provide guidelines for future product development within confectionery products.

INTRODUCTION

Spoilage in bakery and confectionary products can cause large economic losses, estimated between 1 and 5% of total products (*5*). Most spoilage organisms cannot survive the cooking step. However molds in the *Penicillium* and *Aspergillus* families, some of which can grow at low water activities, can re-contaminate these products post process (*12*). *Aspergillus pseudoglaucus and Aspergillus fischeri,* previously known as *Eurotium repens* and *Neosartorya fischeri* respectively, are especially adept at surviving and growing in low water activity products (*10*). The re-naming process for the *Aspergillus* genus and species has been implemented since 2014 in an effort to simplify nomenclature based primarily on phylogeny rather than morphology (*10).* Osmophilic spoilage molds can enter the facility through ingredients like fruits and nuts or be present in the processing environment (*3*). To combat this, many formulas contain preservatives such as potassium sorbate or sodium benzoate. However, many consumers are looking for clean ingredient labels when making purchases, which make combining several hurdles necessary to inhibit mold growth such as lowering water activity and adding low levels of preservatives.

Potassium sorbate and sodium benzoate are common preservatives found in many types of foods and inhibit the growth of molds and yeast, particularly under acidic conditions, at pH less than 5. An acidic environment is necessary for the potassium sorbate to be in its actively antimicrobial, undissociated form (*9*). Sorbate enters through the cell membrane, decreases cell pH and interrupts ATP production causing a fungistatic state (*8*). The wide range of products containing potassium sorbate includes tomato products, semi-dried fruits, fruit juices, fruit preserves, wine, baked goods, and confectionery fillings (*7).* Sorbate can be applied as an ingredient in the product, sprayed onto the product or included in packaging (*11*). However,

health conscious consumers have been reluctant to purchase products containing traditional preservatives.

A fermented preservative, natamycin, is produced by fermentation of *Streptomyces natalensis* and is a fungal inhibitor but is not active against bacteria. It is commonly used in cheeses and sausages, typically applied on the surface and has been studied less commonly in juice, fruits, and raw meats. Natamycin works by binding to ergosterol in the fungal cell wall and interfering with vacuole fusion. It has been hypothesized that natamycin disrupts protein re-arrangements by binding to ergosterols in early vacuole fusion steps (*13*). It has also been shown to disrupt uptake of nutrients, disrupt endocytosis in fungi, and disrupt hyphal growth (*14*). Natamycin is "Generally Regarded as Safe" or GRAS by the FDA for use in cheeses (*4*).

Previous studies have evaluated the efficacy of potassium sorbate, sodium benzoate, and propionic acid and water activity in confectionary or bakery foods, but have not considered natamycin. Guynot et al. (2002) and Char et al. (2007) found that lower pH and lower water activity are necessary to keep sorbate levels low while still preventing visible fungal growth in bread analogues or dulce de leche (*1*, *5*). Potassium sorbate was found to be more effective against growth at pH 6.0-7.5 and 0.80-0.90 a_w and that very low amounts of preservative can enhance fungal growth rather than inhibit (*5*).

It is important to understand the implications of post-process contamination or survival of osmophilic molds in intermediate to low water activity confectionary products. The objective of this study was to compare no preservative, traditional preservative, and fermented preservative hurdle methods combined with varying low water activity confectionary model foods over a six-month shelf life study at room temperature.

MATERIALS AND METHODS

Cultures. Single colonies of *Aspergillus pseudoglaucus* and *Aspergillus fischeri* were obtained from the Food Microbiology Laboratory at the New York State Experiment Station (Geneva, NY) and plated onto Malt Extract Agar (Fluka Analytical, St. Louis, MO) with 20% Dextrose (Fisher, Fair Lawn, NJ). Cultures were incubated at 30 ± 2°C for 30 days.

Spore suspension preparation. Ascospore presence after 30 days of incubation was confirmed by microscopy. Ascospores were harvested by adding sterile water with 0.01% Tween 80 (Baker Analyzed, Philipsburg, NJ) to individual plates and scraping the wet surface with a sterile knife. The ascospores and mycelia were filtered through sterile cheesecloth into a sterile bottle to isolate ascospores only. The suspensions were divided into 1 mL sterile tubes and kept frozen at 0 ± 2°C until use.

Confectionery Model. Commercially available evaporated milk containing milk, dipotassium phosphate, carrageenan, and vitamin D3 was used. D-Fructose (Fisher, Fair Lawn, NJ), and only in the case of 0.7 a_w sodium chloride (Fisher, Fair Lawn, NJ) was added to reach target water activity levels of 0.70, 0.75, and 0.80 when inoculated with fungal spore suspensions. Fermented preservative samples had Natamax (Dansico, New Century, KS) added to achieve a 20 ppm natamycin level. Traditional preservative samples had potassium sorbate (Hoechst, Irving, TX) added to achieve a 0.1% level. The food model (60 g) was aseptically weighed out in sterile centrifuge tubes, in triplicate, in preparation for inoculation.

Physicochemical parameters. Water activity measurements were made by an Aqua Lab Dew Point Water Activity Meter 4TE (Decagon, Pullman, WA). pH was determined with an Oakton pH 5 Acorn Series pH meter (Oakton Instruments, Vernon Hills, IL). Total soluble solids

were determined with an Abbe refractometer (Leica Inc., Buffalo, NY). All analyses were measured four separate times.

Inoculation. *A. pseudoglaucus* and *A. fischeri* spores were heat shocked at 65 ± 1°C for 30 minutes to activate ascospores and inactivate remaining vegetative cells. *A. pseudoglaucus* (0.05mL) and 1 mL of *A. fischeri* was added to each 60 g tube of model confectionary food in triplicate to achieve the target inoculation level of 3-4 logs. After 30 minutes of mixing, 5 g of inoculated product were weighed out and diluted in 45 grams of 0.1% Peptone water (BD, Sparks, MD). Contents were stomached in a Seward Stomacher 400 circulator (Seward, UK) for 30 seconds at 230 rpm. Appropriate dilutions were spread plated on Rose Bengal agar containing 0.01% chloramphenicol (BD, Sparks, MD) in duplicate. Plates were incubated for 7 days at 30 ± 2°C. Inoculated tubes were incubated at 23°C for 26 weeks, except when visible growth was observed in all three samples, and plated frequently, following the same methods as day zero.

Statistical analyses. Analyses of variance (ANOVA) and pairwise tests were performed using R version 3.2.3, with packages lsmeans 2.23, lme4 1.1-12, lmerTest 2.0-30 and ggplot2 2.1.0, based on a model correcting for baseline inoculations and comparing logarithmic changes in growth over time (R Core Team, Vienna, Austria). Differences were considered significant at a probability (P) value of 0.05.

RESULTS AND DISCUSSION

Physicochemical measurements. The model product was characterized by a_w, total soluble solids and pH as shown in Table 2.1 for *A. pseudoglaucus* product and Table 2.2 for *A. fischeri*.

Table 2.1. Measured a_w, total soluble solids and pH values of evaporated milk and fructose mixture used to determine shelf life of *A. pseudoglaucus* [a].

Nominal a_w	**a_w**	**Total soluble solids content (°Brix)**	**pH**
0.70	0.700±0.001	72.6±0.3	5.5±0.05
0.75	0.751±0.002	69.1±0.4	6.1±0.2
0.80	0.800±0.002	64.6±1	6.1±0.1

[a] Values are the average ± standard deviation (n=12)

Table 2.2. Measured a_w, total soluble solids and pH values of evaporated milk and fructose mixture used to determine shelf life of *A. fischeri*[a].

Nominal a_w	**a_w**	**Total soluble solids content (°Brix)**	**pH**
0.70	0.701±0.002	73.0±0.7	5.5±0.07
0.75	0.749±0.002	70.2±0.5	6.0±0.1
0.80	0.799±0.003	65.6±0.3	6.0±0.08

[a] Values are the average ± standard deviation (n=12)

Influence of a_w and preservatives on Shelf life. See Figure 2.1 for shelf life results.

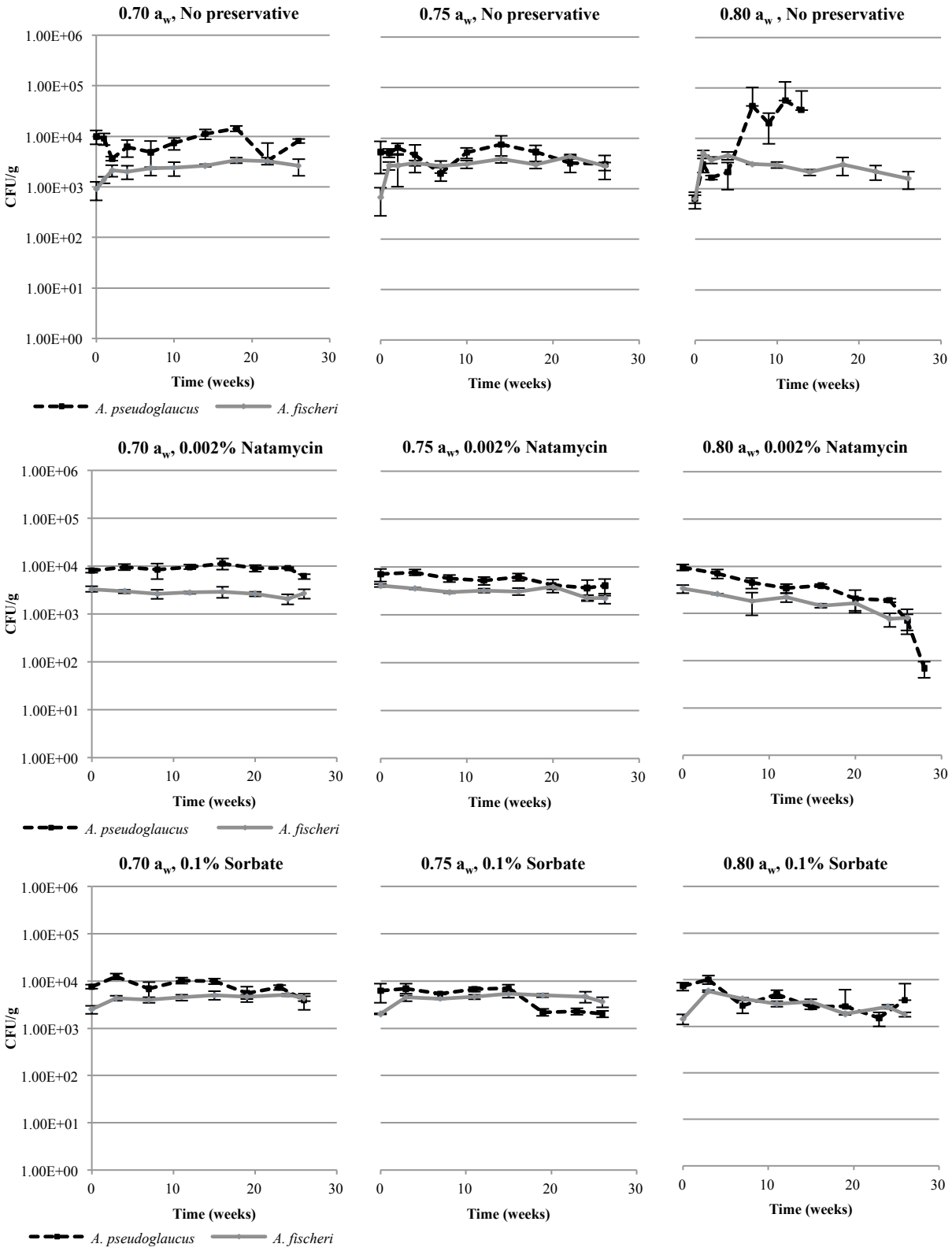


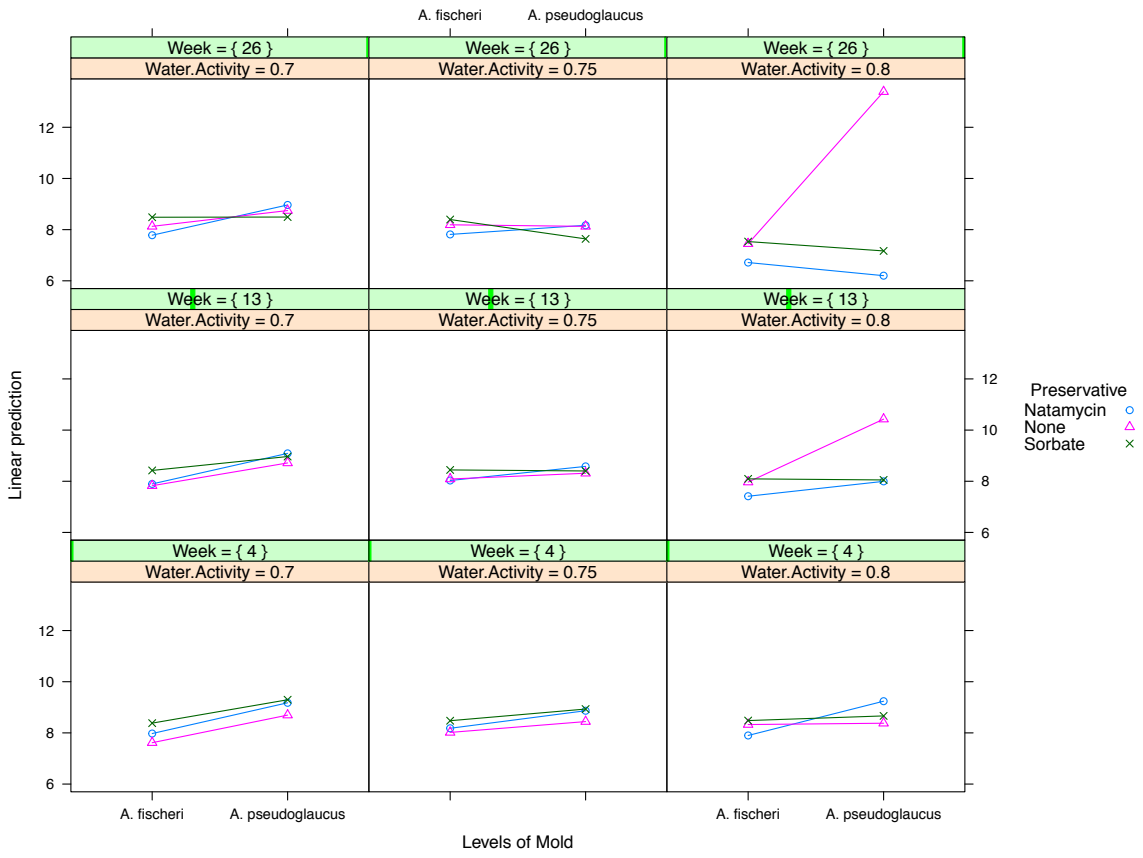
Figure 2.1. Shelf life results. Error bars represent standard deviation

Pairwise and ANOVA tests show a four way interaction between time in shelf life, water activity of model product, preservative type, and mold inoculated. Based on the data, we chose to create a statistical model specifically evaluating fungal growth changes over time with corrections made for varying initial inoculation levels. At weeks 4, 13, and 26 there were no significant differences between all preservative levels in the 0.75 a_w *A. pseudoglaucus* group. At week 4, there were no differences between any of the 0.80 a_w *A. pseudoglaucus* groups, but by week 13 there was a significant difference ($p<0.01$) between no preservative and the preservative groups which continued to week 26. There was visible growth in the 0.80 a_w *A. pseudoglaucus* no preservative method but there was no visible growth over 26 weeks with either sorbate or natamycin additives. There were significant differences ($p<0.01$) between all three groups inoculated with *A. pseudoglaucus* at 0.80 a_w by week 26. Testing was extended several weeks for 0.80 a_w natamycin *A. pseudoglaucus* samples to show the reduction of recoverable cells whereas 0.80 a_w sorbate *A. pseudoglaucus* samples stayed stable. Regarding the 0.70 a_w *A. pseudoglaucus* groups; there was a significant difference between no preservative and sorbate at week 4, but no difference between any of the 0.70 a_w *A. pseudoglaucus* groups at weeks 13 or 26.

There were also no significant differences between any of the 0.75 a_w groups inoculated with *A. fischeri*. At week 4, there was a significant difference between no preservative and the sorbate group in 0.70 a_w but no differences between 0.70 a_w no preservative and natamycin nor between 0.70 a_w sorbate and 0.70 a_w natamycin. By week 13, the difference between 0.70 a_w no preservative and 0.70 a_w sorbate continued and there was also a difference between 0.70 a_w natamycin and 0.70 a_w sorbate. This was similar to results in the *A. pseudoglaucus* inoculated product. However, by week 26 there were no significant differences between any of the 0.70 a_w *A. fischeri* groups. The 0.80 a_w groups all started with no significant differences, but by week 13

and continued through week 26 there were significant differences between the 0.80 a_w natamycin and sorbate groups. There was no visible growth of *A. fischeri* within any of the sample tubes at any water activity or preservative. See Figure 2.2 for diagram summarizing statistical differences.

Figure 2.2 Statistical differences between linear prediction model variables.



These results are comparable to the Guynot et al. (2002) study of Spanish cake analogues prepared at water activities between 0.80 and 0.90 and at pH values between 6.0 and 7.5. They found that a hurdle effect with low water activity (0.8) and lower pH (6.0) combined with 0.3% sorbate slowed *Aspergillus* (*Eurotium)* growth the most. Guynot et. al also found that *A.*

pseudoglaucus (*E. repens*) growth was inhibited at 0.03% but other related species were not. However, Guynot et al. (2005) extended the study at lower pH values in bread (4.5-5.5) and found that 0.03% potassium sorbate slowed growth of all the molds tested at 4.5 pH and was most effective at lower water activities (at or below 0.85 a_w) or at 5.5 pH at 0.80 a_w (*6*). This emphasized the fact that product components and attributes have a large effect on level of preservative needed to extend shelf life.

These results are also similar to Char et al.'s (2007) study of Argentine dulce de leche prepared at water activities from 0.80 to 0.90, pH at 5.5 and 6, and varying potassium sorbate levels from 0.0% to 0.2%. Their evaluation found that *E. chevalieri* was less likely to grow at lower water activity and lower pH. When these two hurdles were combined, potassium sorbate levels could be reduced to 0.1% to inhibit growth at room temperature for three months (*1)*. Our results also showed 0.1% sorbate to be effective.

Our observations were also confirmed by Dagnas et al. (2014) who found that water activity, fungal species and medium all have significant impact on colony growth rates. They inoculated *E. repens, A. niger and Penicillium corylophilum* onto malt extract media adjusted to different pH and water activities and incubated the plates at different temperatures. Different fungal species did not all fit the same model formed. *E. repens* results did not match that found in literature, emphasizing the effect of food components outside of variables tested. *E. repens* grew faster at lower a_w and was more sensitive to pH than *A. niger and Penicillium corylophilum* (*2*).

CONCLUSIONS

The results from our study can be used for product development of new confectionery foods. The combination of water activity, total soluble solids and preservative levels evaluated can provide a guide for shelf life expectations. It also indicates that fermented preservatives can be more effective at 0.80 a_w, based on our results finding mold survival stable with the addition of sorbate but decreasing with natamycin. Low water activities at or below 0.75, in products with similar attributes can also prevent spoilage mold growth of the tested osmophiles without the need of any additional preservatives.

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CHAPTER 3

THERMAL RESISTANCE OF OSMOPHILIC FUNGI IN LOW WATER ACTIVITY CONFECTIONERY MODEL FOODS

ABSTRACT

The public is increasingly searching for preservative-free and "natural" food options. Confectionery products with low water activities are typically considered mold resistant, however there are several osmophilic fungi that can cause considerable economic loss by reducing shelf life. Until now, very few studies considering low water activity tolerant molds in food products containing all nutrients essential for mold growth; sugars, carbohydrates, proteins and fats, but not preservatives, have been published. The objective of this study was to evaluate the effect of water activity on the thermal tolerance and survival of *Aspergillus pseudoglaucus* and *Aspergillus fischeri*. The decimal reduction time (D-value) and the temperature needed to cause a 1-log change D-value (z-value) in a confectionary model comprised of evaporated milk and fructose adjusted to 0.70, 0.75 and 0.80 water activities was determined by creating thermal death time curves. Experiments were performed in triplicate. *A. fischeri* exhibited greater thermal tolerance than *A. pseudoglaucus*. A water activity and temperature interaction was observed, however it was not linear. *A. fischeri* exhibited greatest thermal tolerance in 0.75 a_w at 90°C (11.5-34.8 min), in 0.80 a_w or 0.75 a_w depending on the strain at 92°C (4.3-17.1 min) and in 0.75 a_w at 94°C (2.4-7.7 min). *A. pseudoglaucus* exhibited greatest thermal tolerance in 0.70 a_w at 78°C (4.9 min), 80°C (1.7 min) and 82°C (0.8 min). The data from this study will be useful for determining limits for thermal processing of low water activity confectionary products to control the growth of osmophilic fungi.

INTRODUCTION

Economic losses in the bakery and confectionery sector due to spoilage by low water activity tolerant microorganisms have been reported in the 1 to 5% by volume range (*16*). Consumer preference for preservative-free products and short ingredient decks has added to the difficulties to control mold growth that lead to these economic losses. Previous studies have shown that bakery factories can have a high level (10^3 CFU/m^3) of spore contamination, depending on ingredients and time of year, which can compromise the expected shelf life of products, particularly post-process (*4*). Confectionery foods typically have low water activities (a_w), from 0.20 to 0.80, which make them less susceptible to microbial spoilage because molds cannot grow below 0.60 a_w and most molds and bacteria cannot grow below 0.85 a_w (*2, 18*). However, osmophilic fungi, which are able to grow below 0.85 a_w, can still cause visible spoilage that makes the product undesirable to consumers and retailers. They are also able to grow on products with targeted low water activities but have been cooled improperly causing condensation (*18*).

Aspergillus pseudoglaucaus and *Aspergillus fischeri,* formerly known as *Eurotium repens* and *Neosartorya fischeri,* form heat resistant sexual ascospores with thick cell walls that can survive heat processing or contaminate products post-heat step and have the potential to cause spoilage (*5,13*). These names were reclassified in 2014 as a result of the mycological community simplifying names based on phylogenetic information rather than morphological data (*13*). Ascospores are dormant cells that have the potential to survive for long periods of time before experiencing heat shock, possibly from the product's thermal treatment, and germinating in a product (*1*). *Aspergillus spp.* are frequently the cause of bakery product contamination in low water activity products (*3*). They have also been recovered from spoiled products like grape

jams, canned fruits and juices (*9*). Particularly heat resistant fungi are able to survive typical confectionary heat steps at or above 93°C (*18*). *A. fischeri* has been found to survive at boiling temperatures in water (*9*).

The heat tolerance of ascospores can vary based on the strain, water activity, soluble sugar content, acid presence and preservative presence in the product (*10, 15*). A protective effect due to high sugar content and low water activity has been observed to influence thermal death time (*3, 15*). Due to these different tolerances based on medium, it is imperative to study the heat resistance of representative osmophilic molds in varying confectionery model foods.

The objective of this study was to evaluate the effect of water activity on the thermal tolerance and survival of *Aspergillus pseudoglaucus* and *Aspergillus fischeri* in a confectionery model food. The decimal reduction time (D-value) at several temperatures to produce a 1 log change in D-value (Z-value) was determined in an evaporated milk and sugar model at 0.70, 0.75 and 0.80 a_w.

MATERIALS AND METHODS

Cultures. Single colonies of *Aspergillus pseudoglaucus* and *Aspergillus fischeri* strains NFM1 and PN17 were obtained from the Food Microbiology Laboratory at the New York State Experiment Station (Geneva, NY) and plated onto Malt Extract Agar (Fluka Analytical, St. Louis, MO) with 20% Dextrose (Fisher, Fair Lawn, NJ). NFM1 was isolated from spoiled cherry pastry filling and PN17 from a concord grape drink. Cultures were incubated at 30 ± 2°C for 30 days.

Spore suspension preparation. Ascospore presence after 30 days of incubation was confirmed by microscopy. Ascospores were harvested by adding sterile water with 0.01% Tween

80 (Baker Analyzed, Philipsburg, NJ) to individual plates and scraping wet surface with a sterile knife. The ascospores and mycelia were filtered through sterile cheesecloth into a sterile bottle to isolate ascospores only. The suspensions were divided into 1 mL sterile tubes and kept frozen at 0 ± 2°C until use.

Confectionery Model. Commercially available evaporated milk containing milk, dipotassium phosphate, carrageenan and vitamin D3 was used. D-Fructose (Fisher, Fair Lawn, NJ), and only in the case of 0.70 a_w sodium chloride (Fisher, Fair Lawn, NJ) was added to reach target water activity levels of 0.70, 0.75 and 0.80.

Physicochemical parameters. Water activity measurements were made by an Aqua Lab Dew Point Water Activity Meter 4TE (Decagon, Pullman, WA). pH was determined with an Oakton pH 5 Acorn Series pH meter (Oakton Instruments, Vernon Hills, IL). Total soluble solids were determined with an Abbe refractometer (Leica Inc., Buffalo, NY). All analyses were measured four separate times, per batch.

Thermal tolerance determination. Spore suspensions were removed from the freezer and thawed at ambient temperature. Upon thawing, the suspensions were heat shocked for 30 minutes to ensure germination of heat resistant ascospores and inactivation of remaining vegetative cells. *A. pseudoglaucus* was heated at 65°C and *A. fischeri* was heated at 75°C which were determined to be optimum temperatures based on earlier experiments. Heat shocked spore suspension (0.15 mL) was added to 11 g of product and stirred for 15 minutes to stabilize at target water activity. Inoculated product (0.3 mL) was added to 2 inch 2 mm polyethylene bags (Uline, Chicago, IL), which had been sterilized by 26.8 J/cm^2 by pulsed light, and sealed with an impulse sealer (Uline, Chicago, IL). Sealed bags were placed between pre-heated aluminum coupons and submerged into a water bath adjusted to the target temperature. Samples were

removed from water bath at selected times based on temperature and water activity. After heat treatment, the samples were cooled on ice, cut open, and diluted appropriately. Samples were spread plated on Rose Bengal Agar with 0.01% chloramphenicol (Difco, BD, Sparks, MD) to aid in enumeration and incubated at 30 ± 2°C for one week (*5*). Colonies were enumerated and plotted on survival curves. D-values were calculated by using the negative reciprocal of the log transformation of survival curve slopes. z-values were calculated by using the negative reciprocal of the slope of the linear association between the logarithmic D-values and temperature. All experiments were performed in triplicate.

Statistical analyses. Analyses of variance (ANOVA) were performed using R version 3.2.2 (R Core Team, Vienna, Austria). Differences were considered significant at a probability (P) value of 0.05.

RESULTS AND DISCUSSION

Physicochemical measurements. The model product was characterized by a_w, total soluble solids and pH as shown in Table 3.1.

Table 3.1. Measured a_w, total soluble solids, pH values and formulations of evaporated milk and fructose mixture used to determine the D- and z-values of *A. fischeri* and *A. pseudoglaucus*[a].

Nominal a_w	**a_w**	**Total soluble solids content (°Brix)**	**pH**	**% Evaporated Milk**	**% Fructose**	**% Sodium Chloride**
0.70	0.702±0.001	72.9±0.3	5.6±0.1	33	65	2
0.75	0.750±0.002	70.3±0.3	6.1±0.1	36	64	-
0.80	0.803±0.003	64.8±0.5	6.0±0.1	47	53	-

[a] Values are the average ± standard deviation (n=4)

Influence of varying a_w on thermal tolerance. The D- and z-values of *A. fischeri* and *A. pseudoglaucus* in the model product at 0.70, 0.75 and 0.80 a_w are presented in Tables 3.2, 3.3 and 3.4. All thermal destruction curves showed linear declines on logarithmic scales, as expected at these high temperatures. Previous studies have exhibited logarithmic death at high temperatures in juices (*7*).

Table 3.2. D- and z-values of *A. pseudoglaucus* in evaporated milk and fructose mixture with three a_w values [A].

a_w	D-value (min) at given temperature (°C)[B]			z-value (°C)
	78	80	82	
0.70	4.87±0.58[a]	1.64±0.01[c]	0.83±0.03[e]	5.2
0.75	1.72±0.17[b]	0.89±0.10[d]	0.80±0.03[e]	12.0
0.80	1.63±0.33[b]	1.15±0.01[d]	0.52±0.01[e]	8.1

[A] Values are the average ± standard deviation (n=3)

[B]Values not sharing a common superscript letter are associated to significantly different D-values ($P < 0.05$) based on *ANOVA* and pair-wise tests.

An ANOVA test was performed with *A. pseudoglaucus* data to compare interactions between a_w, temperature and resulting D-values, which showed significant interactions. *A. pseudoglaucus* demonstrated greatest thermal tolerance at 0.70 a_w at 78°C (4.87 min), 80°C (1.64 min) and 82°C (0.83 min). In general, the heat tolerance of *A. pseudoglaucus* increased as the water activity decreased. See Figure 3.1.

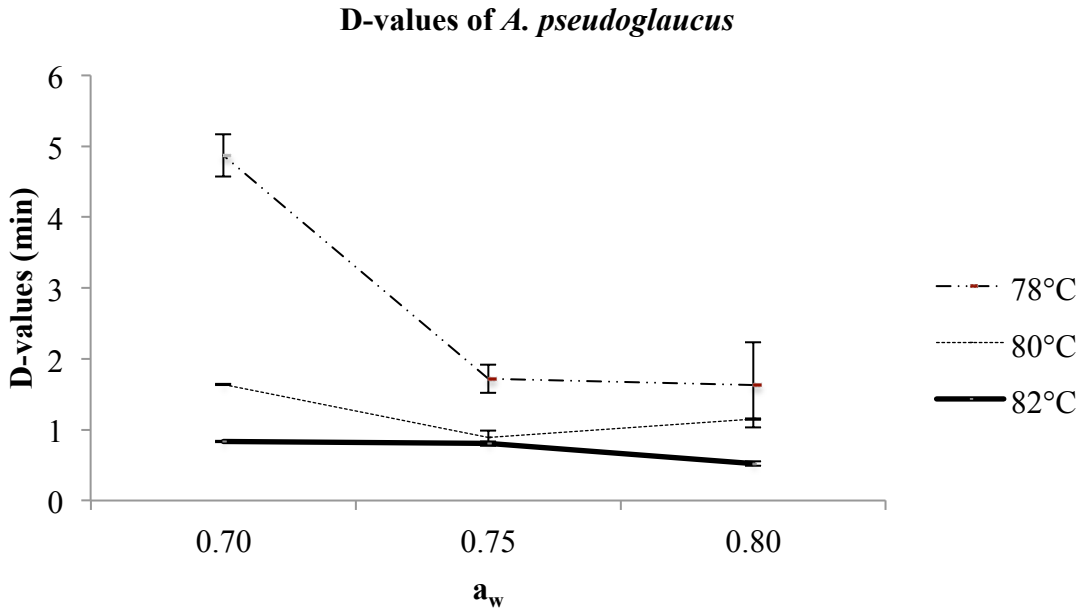

Figure 3.1. Interaction between $a_{w,}$ temperature and D-values of *A. pseudoglaucus.*
Error bars show standard deviation

Pairwise tests were performed to compare significant differences between individual points. There was no significant difference between D_{78}, D_{80} or D_{82} between 0.75 and 0.80 $a_{w.}$ nor between any of the D_{82} points. The rest of the points had significant differences in pairwise tests with D_{78} between 0.70 and 0.75 a_w, D_{78} between 0.70 0and 0.80 a_w, D_{80} 0.70 and 0.75 a_w, all very significant ($p<0.01$). *A. pseudoglaucus* behaved as expected and had similar D-values when compared to previous studies. Kocakaya Yildiz and Coksoyler (2002) found *A. chevalieri* to have a D_{80} value of 5.5 and a D_{83} value of 3.77 min when heated in apricot juice (*6*). *Aspergillus* strains previously considered *Eurotium* have also been found to exhibit increasing heat tolerance in grape juice highlighting the effect of media on heat resistance. Splittstoesser and others (1989) found that the D_{70} values of *A. herbariorum* doubled from 2.5 min in 5°Brix to 5 min in 60°Brix

grape juice (*17*). We observed similar results with D_{78} more than doubling between 0.80 a_w (64.8 °Brix) and 0.70 a_w (72.9°Brix).

To examine the influence of water activity on survival of *A. fischeri,* the D-values at each temperature were compared to water activities in an ANOVA which showed significant interactions. The interactions observed were complex and not linear. *A. fischeri* MF1 pairwise tests between water activities and temperatures showed significant differences at all points except at 94°C between all water activities and at 92°C between 0.70 a_w and 0.75 a_w as well as between 0.75 a_w and 0.80 a_w, but there was a significant difference at 92°C between 0.70 a_w and 0.80 a_w. *A. fischeri* MF1 exhibited greatest thermal tolerance in 0.75 a_w at 90°C (11.54 min), in 0.80 a_w at 92°C (4.30 min) and in 0.75 a_w at 94°C (2.36 min) as shown in Figure 3.2. Regarding z-values, no trend was observed. This is different than what has been observed in previous studies, in different media, most frequently fruit products. Salomão (2007) found general trends of decreasing z-values with increasing heat resistance. However, these previous experiments were carried out in juices at lower temperatures (*12*). Based on these unexpected results, a second strain, *A. fischeri PN17* was subjected to the same methods as a comparison. *A. fischeri* PN17 showed even more pronounced resistance at the 0.75 a_w as shown in Table 3.4 and Figure 3.2. *A. fischeri* PN17 exhibited greatest thermal tolerance in 0.75 a_w at 90°C (34.81 min), in 0.75 a_w at 92°C (17.09 min) and in 0.75 a_w at 94°C (7.65 min). Pairwise tests between water activities and temperatures for PN17 showed significant differences at all points except at 94°C between 0.70 a_w and 0.75 a_w as well as between 0.70 a_w and 0.80 a_w.

Table 3.3. D- and z-values of *A. fischeri* NFM1 in evaporated milk and fructose mixture with three a_w values [a].

a_w	D-value (min) at given temperature (°C)			z-value (°C)
	90	92	94	
0.70	5.01±0.44[a]	2.29±0.06[d]	1.34±0.12[f]	7.0
0.75	11.54±0.48[b]	3.43±0.42[d,e]	2.36±0.16[f]	5.8
0.80	9.29±0.25[c]	4.30±0.18[e]	2.16±0.23[f]	6.3

[a] Values are the average ± standard deviation (n=3)

Table 3.4. D- and z-values of *A. fischeri* PN17 in evaporated milk and fructose mixture with three a_w values [a].

a_w	D-value (min) at given temperature (°C)			z-value (°C)
	90	92	94	
0.70	27.42±1.15[a]	13.79±0.64[d]	7.49±0.26[g]	7.1
0.75	34.81±3.18[b]	17.09±0.46[e]	7.65±0.35[g]	6.1
0.80	22.65±2.34[c]	11.13±0.18[f]	6.21±0.34[g]	7.1

[a] Values are the average ± standard deviation (n=3)

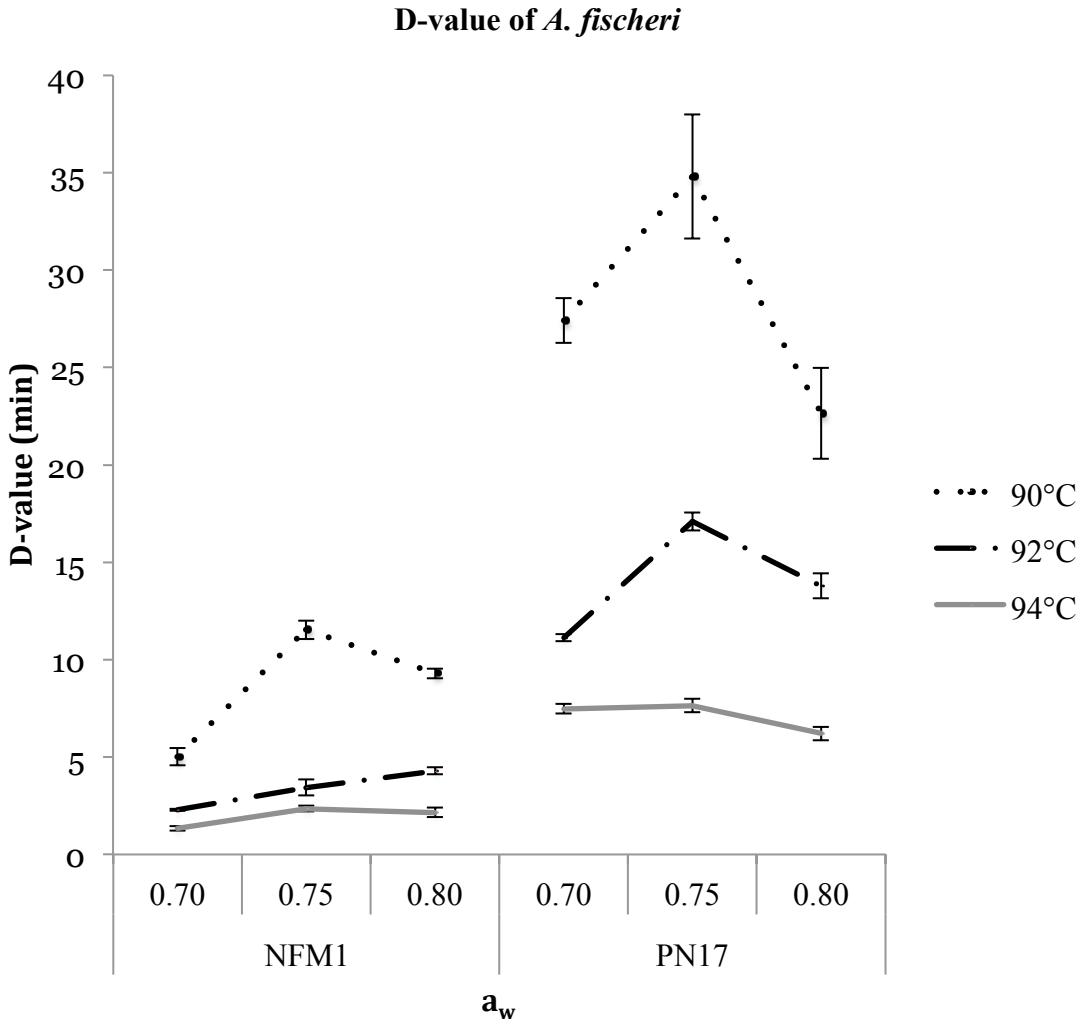

Figure 3.2. Interaction between $a_{w,}$ temperature and D-values of *A. fischeri* NFM1 (strain 1) and PN17 (strain 2) [a]

[a] Error bars show standard deviation

Heat resistance depends on the fungal strain, which we believe explains the wide variability between D-values of *A. fischeri* strains MF1 and PN17 in these studies (*12*). Salomão et al. (2007) observed non-logarithmic death of *N. fisheri* in apple, papaya and pineapple juices at temperatures between 80 and 90°C. The z-values calculated from their study ranged from 5.0

to 5.9°C which are similar to our values between 5.8 and 7.1°C in a product with at least six times as much soluble solids (*12*). Beuchat (1986) also saw wide variability between strains of *A. fischeri* heated in Blueberry, Cherry, Peach, Raspberry and Strawberry fruit fillings at 82.0, 85.0, 86.5, 88.0 and 91.0°C. Comparing z-values between strains in the fruit fillings show up to a 5.0°C difference (*1*). Our calculated z-values between strains were very similar but D-values differed by as much as 22 minutes at 0.70 a_w.

Future studies will be necessary to explore the difference in *A. fischeri* strains and how they affect survival in varying media, especially low water activity products. It is clear from our study that they can have a significant effect on survival during processing. The non-linear trend between water activity and D-value points should be explored more thoroughly. We originally hypothesized that the cultures were pre-adapted to 0.75 a_w originally. However, PN17 was isolated from a grape drink with a high water activity and showed greater heat resistance at 0.75 a_w than the NFM1, isolated from cherry filling. Water activity of the malt extract agar that cultures were grown on was also checked and reported to be at 0.98 a_w at inoculation and at 0.97 a_w after a 30 day incubation period. Ascospores were also grown on malt extract agar without the addition of 20% dextrose for 30 days. Their survival at 0.70, 0.75 and 0.80 a_w was comparable to the ascospores grown with 20% dextrose. None of these hypotheses can explain the phenomenon.

Good hygienic practices should always be followed in food production. However, there is not much information about how to reduce fungal spoilage in low water activity products. While our results found very high temperatures and long hold times necessary to reduce *A. fischeri* contamination, previous experiments showed that *A. fischeri* is not able to cause visible growth in the same model products. *A. pseudoglaucus* was able to cause visible growth in the 0.80 a_w

product held at ambient temperatures. In comparison, D_{60}-values for common mold spoilage organisms such as *A. niger*, *Penicillium citrinum* and *P. roquefortii* are less than one minute in 0.1M citrate buffer at pH 4 (*14*). And D_{56}-values of *A. niger* in beer have been found to be less than one minute as well (*11*). Therefore, we would recommend following the D- and z-values relevant to *A. pseudoglaucus* in the future when controlling fungal growth in low water activity products through thermal treatments. Alternative options include limiting oxygen availability to fungal organisms through modified atmospheric packaging or adding preservatives to the formulation.

CONCLUSIONS

Future studies should be focused on studying the behavior of *A. fischeri* at 0.75 a_w during heat treatment. *A. pseudoglaucus* exhibited expected behavior with increased heat resistance at 0.70 a_w. Due it it's ability to grow at low water activities, we would recommend following heat processes targeted to reduce *A. pseudoglaucus* spoilage. Following the evaluated *A. pseudoglaucus* D-values as guidelines we would recommend the following treatments for a 3-D inactivation.

Table 3.5. Calculated 3-log reductions for *A. pseudoglaucus* at suggested temperatures[a].

Temperature (°C)	**a_w**	**Time (min)**
70	0.70	524
	0.75	25
	0.80	49
80	0.70	3
	0.75	3
	0.80	4
90	0.70	0.08 (5 sec)
	0.75	0.6 (36 sec)
	0.80	0.2 (12 sec)

[a] Values were calculated using formula $D_T = D_o * 10^{\frac{T_o - T}{z}}$ (*8*)

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