USE OF ANTIMYCOTICS, MODIFIED ATMOSPHERES, AND PACKAGING TO AFFECT MOLD SPOILAGE IN DAIRY PRODUCTS

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Dissertation submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Food Science and Technology

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November, 24 1998
Blacksburg, Virginia

USE OF EXTRINSIC MECHANISMS AND FACTORS TO AFFECT MOLD SPOILAGE IN DAIRY PRODUCTS

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Abstract

The effects of natamycin, oxygen scavengers and a 25% CO_2:75% N_2 modified atmosphere on the growth of *Penicillium roqueforti* in shredded cheddar and mozzarella cheese stored at 10°C for 0, 60, 120, or 180 day was studied. Microbiological and sensory testing was assessed on 0, 7, 14 and 21 days after opening. Carbon dioxide decreased ($P<0.05$) as O_2 ($P>0.05$) and N_2 ($P>0.05$) increased throughout storage. Cheddar and mozzarella cheeses were stored for 180 and 60 days, respectively without significant ($P> 0.05$) increases in yeast and mold populations. Fungal populations increased significantly ($P< 0.05$) after packages were opened. Differences in yeast and mold (YM) counts during storage and once the packages were opened were independent of natamycin application and presence, O_2 scavengers and inoculated *Penicillium roqueforti* for both types of cheeses.

Growth of *Penicillium roqueforti, Aspergillus niger, Geotrichum candidum* and *Neosartorya fischeri* were evaluated in atmospheres of 0:30:70, 0.5:29.5:70, 1:29:70, 2:28:70, and 5:25:70, O_2:CO_2:N_2 over a 5-day period. Spores were cultured on antibiotic-supplemented potato dextrose agar (pH 5.6, a_w 0.95) and incubated at 25°C. All four molds germinated and grew at 0.5:29.5:70. Extent of mycelia growth diameter (mm) increased significantly ($P<0.05$) as oxygen concentration increased from 0.5% to 5%. All growth was inhibited at 0:30:70, but germination and growth occurred once cultures were exposed to 20.9% atmospheric O_2, indicating that a modified atmosphere containing no residual O_2 is fungistatic.

Yeast and mold growth was seen in ultra-pasteurized (UP) extended shelf-life fluid milk stored at (7.2°C). Ten half-pint, pint, quart and half gallon filled cartons were randomly selected from all UP products available. Samples, pulled at random on day 0, 15, 30, 45, and 60, were plated on Yeast and Mold Petrifilm™. Forty-seven percent of the UP products stored for 45 days tested positive for mold. Fungal growth was apparent down the side and along the bottom of the 5th panel. Contamination was traced to the presence of yeast and mold spores in paperboard cartons. Pinholes were present in the polyethylene coating and wicking occurred at the unskived 5th panel. Fungi of similar origin and fatty acid profile were isolated from UP milk products and the paperboard cartons.

Acknowledgments

There are several individuals that I wish to acknowledge. First, I would like to thank my husband, Alan G. Grove, for his constant love and support. Without you, none of this would have been possible. I would also like to express my deepest thanks to my parents, Charles G. and Juanita D. Moler for their endless encouragement and constant support. Dad I wish you were here to see me receive my degree.

I am very appreciative of the guidance, wisdom, and support provided by my graduate committee, Dr. Joe Marcy, Dr. Cameron Hackney, Dr. Susan Duncan, Dr. Rusty Bishop, and Dr. Gerry Jones. I also want to thank Harriet Williams, Walter Hartman, John Chandler, Joe Boling and Diane Bourne for their skillful technical assistance and friendship over the years.

I would also like to thank Dr. William Etgen for his encouragement and support during my undergraduate education. You taught me to believe in myself and for that I will always be eternally grateful.

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INFLUENCE OF MODIFIED ATMOSPHERE PACKAGING ON FUNGAL SPOILAGE IN DAIRY PRODUCTS

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ABSTRACT

Fungi make up a large group of microorganisms that affect the food supply because of their ability to invade and decompose foods and beverages. Fungi have relatively versatile environmental requirements. They are obligate aerobes which grow in wide pH (2 to 9) and temperature (10 to 35°C) ranges at a minimal water activity (a_w) of 0.85 or less. *Aspergillus* and *Penicillium* spp. dominate the fungal flora of contaminated dairy products. *Aspergillus* and *Penicillium* species also dominate the fungal flora in factories and warehouses in which semi-hard cheeses are produced and stored. Additional isolates include *Cladosporium* and *Eurotium* species as well as *Geotrichum candidum* and *Phoma* species. Modified atmosphere packaging (MAP) has been used successfully to limit fungal growth and extend the shelf-life of dairy foods. A review of MAP research in relation to mold growth in dairy foods is presented.

Key Words: Fungi, Modified Atmosphere Packaging

Yeasts and molds have been used favorably by the dairy industry in the processing of cheese and fermented products (Marth and Yousef, 1991). However, fungal contamination has been implicated in the spoilage and decomposition of milk and processed dairy foods (Fente-Sampayo et el. 1995). Contamination can occur during processing or packaging.   Post-processing contamination is typically associated with unclean equipment, dirty packaging materials or poor air quality. Initially, contaminated products may have no visible signs of growth but fungi may be isolated upon mycological examination. Fungal growth occurs in various forms which include slimy films, white mycelium growth, free floating masses and highly colored sporulating spots (Rhodes, 1978). When mold growth renders dairy products unfit for sale or consumption, the dairy industry as well as consumers suffer economically from the loss.

This paper will present a general review of fungal classification and discuss fungi typically isolated from dairy products. Modified atmosphere packaging (MAP) has been used successfully to limit fungal growth and extend the shelf-life of dairy foods. A summary of MAP research findings is presented.

Fungal classification

Yeast and filamentous fungi belong to the kingdom *Fungi*. The division *Eumycota* is the class of fungi of most importance to the food and beverage industries. *Eumycota* may be further divided into five subdivisions, *Ascomycotina, Basidiomycotina, Chytridiomycotina, Zygomycotina* and *Deuteromycotina*. The first four subdivisions are considered true fungi based on their sexual spore production. The fifth subdivision is classified as *Deuteromycetes* or *Fungi Imperfecti* because no known sexual stage exists (Beneke and Stevenson, 1987; Alexopoulos et al., 1996). Most *Deuteromycetes* are the imperfect forms of *Ascomycetes* and *Basidiomycetes*. *Ascomycetes, Zygomycetes* and *Deuteromycetes* tend to occur most often in food (Mossel et al., 1995; Pitt and Hocking, 1997).

Microbiologists typically separate true fungi into yeasts or molds based on colony appearance and microscopic examination. Molds are typically filamentous and are primarily classified according to the morphology of the hyphae, sexual and asexual spores (Barnett and Hunter, 1987). Yeasts are mainly single cells, which reproduce by budding. They lack defined morphological characteristics, therefore classification is based on physiological characteristics.

Growth requirements

Fungi have relatively versatile environmental requirements. They are obligate aerobes, which grow in a wide pH (2 to 9) range and at a minimal water activity (a_w) of 0.85 or less (Beuchat, 1978; Mislivec, 1978). Minimum fungal growth temperature is between 0-5°C, optimum is 25-30°C, and maximum is 40-45°C (Christensen, 1987). Mossel et al. (1996) provides more information on growth temperatures of specific fungal contaminates. Fungi do not grow well in fresh moist foods due to competition from bacteria (Beneke and Stevenson, 1987; Beuchat, 1983). However, under less optimum environmental conditions, such as lower pH, reduced water activity, and refrigerated temperatures, fungi may thrive.

Common dairy spoilage molds

Fresh, sliced, blocked and shredded cheeses are frequently spoiled by yeasts and molds. *Aspergillus* and *Penicillium* spp. dominate the fungal flora of contaminated cheese products (Bundgaard-Nielsen and Nielsen, 1995). Lund et al. (1995) identified 371 cheese spoilage fungi; *Penicillium* species accounted for 91% of these fungal contaminates. In a similar study, Hoekstra et al. (1998) found that *Aspergillus* and *Penicillium* species dominated the fungal flora in factories and warehouses in which semi-hard cheeses were produced and stored. Marth and Yousef (1991) cited a study by Gaddi that identified 144 mold cultures isolated from 14 varieties of cheeses. *Penicillium* spp. accounted for 69% of the isolates, 9 % were *Aspergillus* spp., 8% *Scopulariopsis* spp., 3% *Mucor* spp. and 2% *Cephalosporium* spp. Additional isolates include *Cladosporium* and *Eurotium* species (Bundgaard-Nielsen and Nielsen, 1995; Hoekstra et al., 1998) as well as *Geotrichum candidum* and *Phoma* spp. which were recovered through surface sampling (Hoekstra et al., 1998). Morphological structures of these and various other spoilage molds are well illustrated in Mossel et al. (1996), and Barnett and Hunter (1987).

Aspergillus spp. are found in the air and soil and are associated with living and decaying plants and animals. The conidiophores are upright and vesicles develop at their terminal ends. Phialides are formed at the apex and conidia (phialospores), which are one-celled spheres, and attach to one another in a chain-like fashion. A brilliant display of color is associated with certain species of *Aspergillus*. This pigmentation is commonly used to characterize particular species (Raper and Fennell, 1965). *Aspergillus penicillioides,* and *A. versicolor* were isolated from cheese factories and warehouses in a study by Hoekstra et al. (1998).

Penicillium spp. are commonly isolated from the air and soil. Conidiophores rise from the mycelium usually as single units. They branch at the apex and end in a group of phialides. Phialides are the reproductive structures of *Penicillium* spp. The conidia are borne from the phialides or conidiogenous cells. They form a chain-like structure and may be either hyaline (transparent) or brightly colored (Pitt, 1979). *Penicillium roqueforti* and *Penicillium camemberti* are typically used to produce mold-ripened cheeses. However, they have also been isolated from cheese that has spoiled prematurely. *Penicillium bevicompactum, P. chrysogenum, P. commune, P. corylophilum.* and *P. discolor* were also encountered in cheese factories and warehouses (Hoekstra et al., 1998).

Geotrichum spp. are very common in the soil. Mycelium is white and septated and there are no conidiophores present. The conidia are formed by segmentation of the vegetative hyphae. Conidia (arthrospores) are one-celled, hyaline, cylindrical tubes with truncated ends. *Geotrichum candidum,* more commonly know as "machinery mold" or "dairy mold", has been isolated on harvesting and packaging equipment. Its enumeration has been used as a rapid method for assessing plant sanitation (Marth, 1987). *Geotrichum candidum* is frequently associated with the molding of foods that contain lactic acid as well as ripe fruits and vegetables (Beneke and Stevenson, 1987; Brackett, 1987; Splittstoesser, 1987). It has been isolated from the surface flora of mold-ripened cheeses like Camembert (Marth and Yousef, 1991)

Modified atmosphere packaging

Modified atmosphere packaging (MAP) maybe defined as the packaging of a perishable product where the gaseous environment, within the package, is altered to a composition that is equivalent to something other than air. Most fresh foods respire or contain organisms that respire. The oxygen present is consumed and carbon dioxide and water vapor are released. The environment is thus altered or modified from its original composition (Brody, 1996; Ooraikul and Stiles, 1991).

Modified atmosphere packaging has numerous advantages, but there are also disadvantages. It can potentially increase the shelf-life by 50%, reduce economic loss due to spoilage, increase distribution areas which reduces cost, increase quality, and maintain product integrity better than vacuum packaging. The disadvantages include increased costs associated with gases, packaging equipment, and refrigerated temperatures. Ideal gas composition may vary from product to product (Farber, 1991).

The technique involves packaging products in high barrier films that are flushed with carbon dioxide (CO_2), oxygen (O_2), and nitrogen (N_2). The gases are mixed in predetermined proportions

depending on the nature of the packaged product. A detailed list of MAP gases can be found in Farber et al. (1990). Carbon dioxide and N_2 are commonly used for dairy product packaging. Nitrogen is an inert, tasteless gas with low water solubility. There is no antimicrobial activity directly associated with N_2. It is used to displace the oxygen and to prevent the package from collapsing (Farber, 1991).

Carbon dioxide is a dense gas which aids in displacing air in the package (Anonymous, 1981). It has been shown to exhibit both bacteriostatic and fungistatic effects (Alves et al., 1996; Rosenthal et al., 1991). Microbial growth which is limited under CO_2 enriched environments returns to normal when O_2 is replenished. A detailed understanding of the specific mechanisms of CO_2 is not well known. A historical review of these scientific theories is discussed in Daniels et al. (1985) and Dixon and Kell (1989). Briefly, theories include, but are not limited to, O_2 displacement, pH adjustments, cellular penetration, inhibition of cell division and alteration of the metabolic process. Although there are different ideas concerning the mode of action, the overall result is an increase in the lag phase and a decrease in the growth rate during the logarithmic phase.

Fungi are usually regarded as obligate aerobes and, in the absence of oxygen, spores will not germinate and growth will not occur (Mislivec, 1978). Zero percent oxygen exhibits fungistatic, but not fungicidal properties (Nielsen et al., 1989). Therefore once oxygen is made available, germination and growth will occur. However, fungi are generally tolerant of low oxygen concentrations. Griffin (1972) noted that soil fungi were relatively insensitive to severe reductions in oxygen concentration. When germination or linear growth rates were evaluated, there was little change noted until the oxygen concentration reached 4% in the gas phase. Below 4% there was a noticeable difference between species, and nearly all linear growth declined at 1% oxygen. *Penicillium roqueforti*, used in the production of blue cheese, is also capable of growth at very low oxygen levels (Pitt, 1979). Studies by Magan and Lacey (1984) showed that field and storage fungi were sensitive to low O_2 (0.14%) concentrations when a_w and temperature were decreased. However, *Penicillium* spp., especially *Penicillium roqueforti*, was remarkably tolerant of low O_2 concentrations at 14°C.

Ellis et al. (1993b) concluded that spore counts greater than 10^1 spores $\bar{g}^1$ would reduce the shelf-life of high moisture products packaged in >5.0 % residual O_2. Paster (1990) cited work by Miller and Golding (1949) that concluded the amount of dissolved oxygen in the media required for normal growth was low, and *Aspergillus* and *Penicillium* spp. growth was only affected when oxygen level were <0.5%. *Aspergillus flavus* exhibited more extensive growth at 10-20% O_2 than at 5% (Ellis et al, 1993a,b). *Aspergillus niger* grew in MAP products packaged in <1% O_2 (Smith et al., 1986).

Although fungi can tolerate low O_2 concentrations and increased CO_2 concentrations, growth is delayed considerably, especially at low a_w and temperature. The combined effects of these parameters are more effective in controlling fungal growth then any one of these factors used alone.

MAP applications in dairy foods

Modified atmosphere packaging is one technique that has been successfully used to improve the shelf-life of fresh, sliced and shredded cheeses (Fedio et al., 1994; Maniar et al., 1994; Mannheim and Soffer, 1996; Yoder et al., 1995). Alves et al. (1996) showed that modified atmospheres containing 100% CO_2 or 50/50 CO_2: N_2 significantly increased the shelf-life of sliced cheese when compared to air. Yeast and mold growth was limited at 50% CO_2 and completely inhibited under 100% CO_2. Atmospheres containing 100% N_2 had little inhibitory effect on growth of yeasts and molds when compared to air. Mozzarella cheese did not exhibit physical or chemical changes under MAP storage; however sensory degradation did occur.

Effect of MAP containing 2-18% O_2 and 5-23% CO_2 mixed in N_2, on survival and growth of *P. commune* in cheese stored at 10°C was studied by Haasum (1995). The longest shelf-life was in the presence of low concentrations of CO_2. Sporulation and growth rates were longer at a concentration of <10% CO_2 than at 12-23% CO_2. In a later study by Haasum and Nielsen (1996) it was determined that storage periods >56 days with atmosphere containing <20% CO_2 resulted in sublethal injury of the conidia of *P. commune* which increased lag times and retarded growth rates. The authors concluded that low levels of CO_2 (<20%) were most beneficial in reducing the growth of *P. commune* after the package was opened.

Controlling yeast and mold growth is a real concern for manufacturers of Parmesan and Romano cheese. Yoder et al. (1995) evaluated the effect of reducing a_w of Parmesan and Romano cheeses to 0.88 and 0.84, respectively, prior to packaging with 25% CO_2/75% N_2. Sarantopoulos et al. (1995) successfully stored (25°C) Parmesan cheese at 32% moisture for 124 days under a modified atmosphere (MA) containing a high concentration of CO_2 without significant chemical changes. Final yeast and mold (YM) counts were 5.00 x 10^4 CFU/g. Cheese was acceptable from a sensory standpoint up to 98 days.

Cottage cheese is a fresh, unripened product that is produced from pasteurized skimmed cows' milk (Kosikowsi, 1977). Standard shelf-life of cottage cheese is estimated to be 10-21 days (Bishop and White, 1985). Growth of contaminating yeasts and molds cause undesirable changes in flavor, odor, texture, and appearance (Chen and Hotchkiss, 1993). Modified atmosphere packaging has been utilized to successfully extend the shelf-life of cottage cheese. Maniar et al. (1994) determined that cottage cheese flushed with CO_2 and N_2 maintained satisfactory sensory characteristics after 28 days of MAP storage at 4°C.

Rosenthal et al. (1991) examined the effect of 67.1% CO_2: 6.6% O_2: 26.3% N_2 on the shelf-life of cottage cheese stored at 4°C. Modified atmosphere cheeses were compared to controls that were stored in air. Yeast and mold growth was inhibited during the 67 days of MAP storage. Neither cheese flavor nor texture was effected by MAP storage. After the MAP storage period, cheese samples were exposed to air. Yeast and mold counts increased significantly over the next 28 days indicating that CO_2 exhibited a fungistatic effect on yeast and mold growth.

Flushing the package headspace with pure CO_2 also prevents fungal spoilage (Mannheim and Soffer, 1996). Yeast and mold counts were lower for cottage cheese flushed with CO_2 than without. Carbon dioxide provided protection against yeast and mold growth which extended the

shelf-life (8°C) by 150% with no effect on sensory characteristics.   Fedio et al. (1994) determined that the growth of yeasts and molds was strongly affected by modifying the storage atmosphere. Yeast and mold counts in cottage cheese packaged in air increased from 10^3 to 10^7 over 28 storage days. Fungal growth (10^4) was suppressed in cottage cheese packaged in 100% N_2. Yeast and mold counts declined in cottage cheese packaged in 50% CO_2: 50% N_2 and 100% CO_2. These results differ from those of Rosenthal et al. (1991) who determined that gassing with N_2 had little inhibitory effect on growth of yeasts and molds in cheese.

Influence of temperature

Exposure of food to different storage temperatures can affect the spoilage rate of the product under normal and modified atmospheres. Coppola et al. (1995) studied the effect of three different storage temperatures (4°, 7° and 13° C) on the shelf-life of mozzarella cheese. No significant change in microbial count was seen at 4° or 7°C; however samples stored at 13°C showed signs of increased growth.

Carbon dioxide is highly suitable at refrigeration temperatures but it is much less effective at nonrefrigeration temperatures (Brody, 1989; Farber, 1991; Rosenthal et al., 1991). Temperature abuse plays a significant role in determining the shelf-life expectancy of a MAP product.

Chemical preservatives & antimycotics

Chemical preservatives like sorbate and propionate have been used with some success to extend the shelf-life of dairy products; however they may cause undesirable off-flavors (Liewen, 1992; Marth and Yousef, 1991). Marth et al. (1966) determined that some *Penicillium* species were capable of growth in the presence of sorbates even when it was applied at concentrations greater than 3000 ppm.

The antifungal agent, natamycin (pimaricin) has been applied to cheese surfaces to inhibit mold growth and extend the shelf-life (Fente-Sampayo et al., 1995). Natamycin is a polyene antifungal antibiotic produced by *Streptomyces natalensis.* Its mechanism of action entails binding to cell membrane sterols, primarily ergosterol, which causes them to leak (Pedersen, 1992). Bacteria are unaffected by natamycin since their cell membrane lack sterols. The level of fungal resistance to natamycin is low. Sensitivity of 26 mold strains, primarily *Penicillium* and *Aspergillus* sp., isolated from cheese warehouses was not affected after several years of continuous use of natamycin (de Boer and Stolk-Horsthuis, 1977).

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IMPROVED QUALITY OF SHREDDED CHEDDAR AND MOZZARELLA CHEESE THROUGH THE USE OF ANTIMYCOTICS, OXYGEN SCAVENGERS AND MODIFIED ATMOSPHERE PACKAGING

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ABSTRACT

A retail package system was used to study the effects of natamycin, oxygen scavengers and a 25% CO_2:75% N_2 modified atmosphere on the growth of *Penicillium roqueforti* in shredded cheddar and mozzarella cheese stored at 10°C for 0, 60, 120, or 180 days (cheddar only). Quality was assessed by microbiological and sensory tests 0, 7, 14 and 21 day after opening. Carbon dioxide concentration decreased ($P<0.05$) as O_2 ($P>0.05$) and N_2 ($P>0.05$) concentrations increased throughout the storage period. Cheddar cheese packages averaged 25.04:0.43:74.53 (CO_2:O_2:N_2) at day 0 and 4.39:2.01:93.60 after 180 day of storage. Mozzarella packages averaged 24.76:0.61:74.66 at 0 d and 5.25:1.59:93.16 after 120 d. Cheddar and mozzarella cheeses were stored for 180 and 60 days, respectively without significant ($P> 0.05$) increases in fungal populations; however yeast and mold populations increased significantly ($P< 0.05$) after cheddar and mozzarella packages were opened. Differences in yeast and mold (YM) counts during storage and once the packages were opened were independent of natamycin application and presence, O_2 scavengers and *Penicillium roqueforti* for both types of cheeses. Sensory of cheddar and mozzarella cheeses declined ($P<0.25$) as the number of days a package was open increased. This decline coincided with increases in the fungal population.

Key Words: modified atmosphere, natamycin, oxygen scavenger, shredded cheese, *Penicillium roqueforti*

INTRODUCTION

The demand for high quality, convenient, and innovative fresh foods is on the increase. This buying trend has encouraged dairy processors to introduce new packaging styles for traditional products in order to be more competitive in the dairy case. Many cheese processors now offer several varieties of shredded cheeses at the retail level.

In 1996, consumption of American and Italian cheeses rose by 1.4% and 3.8%, respectively to 12 lbs. and 10.8 lbs. (Gorski, 1998). Production of natural cheddar cheese equaled 1,195,705

metric tons in 1997 (Anonymous, 1999). Cheddar cheese is the most common type of cheese produced in the United States. Named for the village of Cheddar in Somerset, England, it is characterized as a hard, ripened cheese made from cow's milk with a smooth, firm and reasonably pliable body. Good quality cheddar cheese has a moderate, slightly nutty, "cheddar" flavor (Bodyfelt et al., 1988). It is sold as mild, medium, sharp, or extra sharp depending on the length of time it has been aged. Mozzarella cheese production equaled 1,016,534 metric tons in 1997 (Anonymous, 1999). It is characterized as a soft, unripened cheese that is typically made from cow's milk with a smooth, rubbery texture. Good quality mozzarella cheese has a very mild flavor (Bodyfelt, et al., 1988).

Cheese is spoiled by mold growth more often than any other dairy commodity. *Penicillium* species are the dominant fungi associated with cheese spoilage (Lund et al., 1995). Mold contamination can occur during ripening or packaging. Proper cleaning and disinfecting of processing facilities is important to prevent such contamination. Resistance of yeast and molds to commercial disinfectants was studied by Bundgaard-Nielsen and Nielsen (1995). Resistance was dependent on the strain and species and active agent in the disinfectant. *Penicillium roqueforti* var. *roqueforti* demonstrated resistance to quaternary ammonium, quaternary ammonium + aldehydes, isopropanol, some chlorine compounds, ampholyte and formaldehyde.

Modified atmosphere packaging (MAP) is one technique used to improve the shelf-life of sliced and shredded cheeses (Alves et al., 1996; Fedio et al., 1994; Mannheim and Soffer, 1996; Rosenthal et al., 1991; Sarantopoulos et al., 1995; Yoder et al., 1995). MAP may be defined as the packaging of a perishable product where the gaseous environment within the package is altered to a composition that is equivalent to something other than air. Most fresh foods respire or contain organisms that respire. The oxygen present is consumed, and carbon dioxide and water vapor are released. The environment is thus altered or modified from its original composition (Brody, 1996; Ooraikul and Stiles, 1991).

Carbon dioxide (CO_2) and nitrogen (N_2) are commonly used in MAP shredded cheese (Farber et al., 1990). Carbon dioxide is dense gas which aids in displacing air in the package (Anonymous, 1981). Nitrogen is an inert, tasteless gas with low water solubility. It is used to displace the oxygen and to prevent the package from collapsing.

Carbon dioxide exhibits both bacteriostatic and fungistatic effects (Alves et al., 1996; Rosenthal et al., 1991). Growth is limited in CO_2 enriched environments but normal growth rates resume when O_2 is introduced. However, the specific mechanism of these effects is not well known. A historical review of these scientific theories is discussed in Daniels et al. (1985) and Dixon and Kell (1989). Briefly, theories include, but are not limited to, O_2 displacement, pH adjustments, cellular penetration, inhibition of cell division and alteration of the metabolic process. Carbon dioxide retards the growth of gram-negative, aerobic, psychrotrophic bacteria, and molds while allowing facultative anaerobic bacteria like *Lactobacillus* and *Streptococcus* species to grow.

Alves et al. (1996) showed that modified atmospheres containing 100% CO_2 and 50/50 CO_2: N_2 significantly increased the shelf-life of sliced cheese when compared to air. Yeast and mold growth was limited at 50% CO_2 and completely inhibited under 100% CO_2. The effect of MAP storage on the growth of *Penicillium commune* isolated from Danish cheese was studied by

Hassum and Nielsen (1996). Storage periods >56 days with atmosphere containing <20% CO_2 resulted in sublethal injury of the conidia of *P. commune* which resulted in increased lag times and retarded growth rates. It was determined that low levels of CO_2 (<20%) were most beneficial in reducing the growth of *P. commune* after opening the package.

Most molds are absolute aerobes and, in the absence of oxygen, spores do not germinate and growth will not occur (Mislivec, 1978). The growth of many yeasts is also drastically reduced in an oxygen-free atmosphere (Mislivec, 1978; Abe and Kondoh, 1989). In the presence of sufficient O_2, mold growth is likely to occur. In general, 2-3% residual O_2 can be measured in the headspace of most MAP shredded cheese products. Oxygen scavengers have been used to reduce the O_2 level in several MA products (Abe and Kondoh, 1989).

The term "oxygen scavenger" is used to describe chemical additives, introduced into the package, that decrease the level of O_2 in the package headspace. The materials react chemically with oxygen or add carbon dioxide to the package atmosphere. Glucose oxidase is one type of scavenger. Glucose binds with O_2 to form gluconic acid and hydrogen peroxide. Catalase reduces hydrogen peroxide to water and oxygen (Richardson and Hyslop, 1985). One half mole of oxygen is lost in each cycle.

Natamycin (pimaricin) is a polyene antifungal antibiotic produced by *Streptomyces natalensis.* It has an empirical formula of $C_{33}H_{47}NO_{13}$ and a molecular weight of 665.74 (Food Chemical Codex, 1996).   Natamycin is an off-white powder that is practically insoluble in water. Approximately 5ppm natamycin has been shown to inhibited mold growth on cheese (Bullerman, 1977; de Ruig and Van der Berg, 1985; Fente-Sampayo et al., 1995; Lück and Cheesman, 1978).

Natamycin binds the cell membrane sterols, primarily ergosterol, which in turn causes the membrane to leak (Kobayashi and Medoff, 1977; Pedersen, 1992; Stock, 1981). Bacteria are unaffected by natamycin since their cell membrane lacks sterols. The level of fungal resistance to natamycin is low. Twenty-six mold strains, primarily *penicillium* and *aspergillus* sp., isolated from cheese warehouses were still sensitive to natamycin even after several years of continuous use (de Boer and Stolk-Horsthuis, 1977).

Objectives of the project

Limited research is available on the effectiveness of antimycotics and oxygen scavengers in preventing mold growth on shredded cheeses. The objectives of this study were; (1) determine the combined effectiveness of antimycotics, oxygen scavengers and modified atmosphere packaging in preventing the growth of *Penicillium rogueforti* on shredded cheddar; (2) determine if there was a difference between methods of natamycin application; (3) determine the packaging conditions which optimally limits the growth of *Penicillium roqueforti* while maintaining a safe and acceptable product.

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MATERIALS AND METHODS

Blocks of cheese

Two hundred fifty four-kg of 30-day-old cheddar cheese and 10-day-old mozzarella cheese was provided by Alto Dairy, Waupun, WI. Individual blocks of cheddar and mozzarella cheese, weighing 9080 g and 4540 g, respectively, were vacuum packaged and shipped via refrigerated carrier. Blocks of were stored in a walk-in cooler (5° C) over night, then shredded, and packaged the following day.

Two identical experiments were performed with cheddar and mozzarella cheese. Each experiment consisted of nine different treatment groups; each treatment required 27.24 kg of cheese. Three blocks of cheddar (9080 g) or 6 blocks of mozzarella (4540 g) were shredded and packaged per treatment. Due to space restrictions within the pilot plant, only one block of cheddar or mozzarella could be shredded, inoculated and packaged at a time.

Inoculum

Penicillium roqueforti, a common contaminate of cheese, was selected as the inoculum for this study because of its resistance to many cleaning agents (Bundgaard-Nielsen and Nielsen, 1995). Midwest® blue mold powder stock (SKW Bio-Systems, Waukesha, WI) was determined to contain 2.5×10^8 spores/g.

Oxygen scavenger

Cellulose, an anti-caking agent used in the packaging of shredded cheese, served as the carrier for the oxygen scavenger. Powdered cellulose (Qual Flo™) and powdered cellulose, with dextrose and enzymes (Flow Am® 200) were supplied by Qualcepts, Minneapolis, MN. Glucose oxidase was the active "scavenging" component in Flow Am® 200. Both types of cellulose are designed to be applied at 2% of the product weight. Thus depending on the treatment either 545 g of Qual Flo™ or Flo Am® 200 was applied to 27.24 kg of cheese in each experimental block.

Natamycin

Natamycin from two suppliers (Delvocid™ from Gist-brocades, Menomonee Falls, WI and Natamax™ from Pfizer Inc., Milwaukee, WI) containing 50% natamycin (pimaricin) and 50% lactose were thoroughly mixed together in order to eliminate differences between products. The Delvocid™/Natamax™ mixture was applied to the cheese and the cellulose in this experiment.

Manufactures recommend that shredded cheese contain between 4-6 ppm of natamycin. Directions stated that a 2500 ppm suspension, containing 50% natamycin, should be sprayed onto shredded cheese at a rate of 6.0 mL/kg. The Code of Federal Regulations (CFR) however, places

restrictions on the concentration of natamycin that may be applied to cheese. Code 21CFR172.155(c)(1), referring to natamycin (pimaricin), states the additive may be applied by dipping or spraying, using an aqueous solution containing 200 to 300 parts per million of additive (FDA, 1996). Therefore a 600-ppm solution, containing 50% natamycin (300-ppm), was applied to the cheese at a rate of 25 mL/kg. The 600 ppm solution was made by bringing 2.4 g of the Delvocid™/Natamax™ mixture up to 4 L with distilled H_2O. Natamycin is not soluble in water therefore a magnetic stirrer was used to keep natamycin suspended in the aqueous solution during spray application.

A spray apparatus, constructed from 8.0 mm stainless steel piping with an in-line ball valve, was used to apply the 600 ppm natamycin suspension to the cheese. The apparatus delivered 375mL/min (6.25 mL/sec) of Delvocid™/Natamax™ solution with the aid of a light volume transfer pump at 552 kPa (80 psi). Spray nozzles were obtained from Spraying Systems Co. Charlotte, N.C and stainless steel fogger tips were provided by Gist-brocades, Menomonee Falls, WI. Each nozzle had a 38 cm diameter spray pattern, thus two nozzles sufficiently covered the 76 cm polypropylene tank that was used to tumble the cheese during treatment. The 600 ppm natamycin suspension was sprayed over 9080 g of cheddar cheese for 36-37 sec. (~228 ml) and for 18-19 sec. (~114 ml) over 4540 g mozzarella cheese.

Two of the treatments required that natamycin be sprayed onto the cellulose and dried, before being applied to the cheese. Since the CFR does not regulate the concentration of natamycin that can be applied to cellulose, a 2500 ppm solution (1250 ppm natamycin) was used. This solution was made by placing 2.5 g of the Delvocid™ /Natamax™ mixture (50% natamycin) in a 1 L graduated cylinder and filling to volume with distilled H_2O. The 2500 ppm Delvocid™ /Natamax™ suspension, which contained 1250 ppm natamycin, was sprayed onto a predetermined amount of cellulose at a rate equivalent to the manufactures recommendation for cheese, 6.0 mL/kg. For example, a single treatment (27.24 kg of cheese) would require 545 g of cellulose. Instead of applying 164 mL of 2500 ppm the Delvocid™ /Natamax™ solution to the cheese, the solution was applied directly onto 545 g of cellulose. The natamycin treated cellulose was stirred, then covered with slotted foil and allowed to air dry in a laminar flow hood for two days. Cellulose was stirred periodically to facilitate drying.

Preparation of inoculum

The blue mold powder stock was diluted by placing 2 g 2.5×10^8 spores/g into 998 g of cellulose, Qual-Flo™. To insure the best possible distribution of *P. roqueforti,* the inoculum was mixed into the cellulose with the aid of a planetary type pan mixer. The mixing bowl and tool, both of stainless steel construction, were sanitized with a 200 ppm chlorine solution. Although this type of mixer visits all parts of the stationary pan, mixing was stopped every five minutes to scrape the edges of the bowl into the center. Total mixing period was twenty minutes. The final concentration of the "*P. roqueforti* stock cellulose" was 50×10^4 spores/g cellulose. Using the planetary mixer, 3.1g 50×10^4 *P. roqueforti* stock cellulose was mixed with 1250 g of Qual Flo™, Flow Am® 200, Qual Flo™ plus natamycin or Flow Am® 200 plus natamycin, depending on the treatment. Enumeration of cellulose resulted in 10^3 spores/g. Inoculated cellulose was applied to shredded cheese at a rate of 2%; therefore, 182 g or 91 g was delivered onto 9080 g of cheddar or

4540 g of mozzarella cheese, respectively. Final enumeration resulted in 10-100 spores/g of shredded cheese.

Shredding and packaging of cheese

Processing equipment and cheese contact surfaces were sanitized prior to use with 200 mg/L (ppm) XY-12 liquid sanitizer provided by Klenzade, Division of EcoLab Inc., St. Paul, Minnesota. Air quality can contribute to overall product contamination therefore air samples were collected throughout the processing area using a Biotest Air Sampler RCS from Biotest Diagnostics Corp. (Denville, New Jersey) (Hickey et al., 1993). Air was sampled for two minutes using yeast and mold airstrips, which were incubated at 25-28°C for 5 days, then counted. The manufacture recommends that yeast and mold counts be <100 CFU/m^3.

Cheese blocks were sliced into small (~12x10x5 cm) pieces and feed into a bench model Paxton-Hallde RG-7 commercial food preparation machine (Paxton Corporation, Shelton, CT) with the aid of a FLEXI push feeder. A 4.5 mm raw food shredding blade was used to achieve the desired shred size. Shredded cheese was placed in a tumbler that was constructed from a 113 L cylindrical polypropylene tank with a cover. The tank resided horizontally on rollers which were supported by steel rods. A belt driven electric motor spun the rollers, which rotated the tank at a rate of 6.5 rotations per min. The amount of shredded cheese that was processed at one time was restricted to 9080 g of cheddar and 4540 g of mozzarella due to limited space in the tumbler.

Cellulose (182 g) was metered onto the 9080 g cheddar cheese (91 g/4540 g mozzarella cheese) and tumbled for 5 min. Natamycin was applied to specific treatments with the aid of the spray apparatus described above. The apparatus was inserted through a 15.24 cm hole that was cut into the tumbler cover. The spray apparatus, which delivered 375 mL/min of 600 ppm Delvocid™ /Natamax™ suspension (50% natamycin), was sprayed over 9080 g of cheddar cheese for 36-37 sec. (~228 mL) and for 18-19 sec. (~114 mL) over 4540 g mozzarella cheese. The cheese was tumbled for 10 min to insure the natamycin properly coated the cheese surface.

A KOCH vacuum packaging machine, Model X-200 (KOCH Supplies Inc., Kansas City, MO) was used to modify the atmosphere and seal the 227 g packages of cheese. Air was replaced with 75% N_2 and 25% CO_2 (Brammer Welding, Roanoke, VA). A gas blender (Smith Equipment, Watertown, South Dakota) was used to deliver the proper ratio of nitrogen:carbon dioxide (75:25). Samples of this mixture were analyzed on a Fisher-Hamilton Model 29 (Fisher Scientific Co., Pittsburgh, PA) gas partitioner throughout the packaging process to insure the proper ratio was entering the bag. Cheese (227 g) was packaged in 15 x 23 cm (6 x 9 in) BDF bags (Cryovac, Duncan, SC). The bags, made from a multilayered, coextruded polylefin formulation, had an oxygen transmission rate of 4.0 cc/m^2/24 hr and a water vapor transmission rate of .75 gms/100 sq. in/24hrs (Cryovac).

A rapid package integrity tester built from 1.27 cm thick Lexan™ (polycarbonate) was used to insure the integrity of the package seal, thus allowing leakers to be rejected from the study. A single 227 g package of cheese was placed on the bottom of a 17 x 25 x 25 cm (7 x 10 x 10 in) vacuum tight polycarbonate box. The underside of the lid supported a depth gauge, which was

mounted perpendicular to the lid surface. Vacuum was applied causing the bag to expand. The depth gauge caliper was pushed up by the bag and the needle registered the expansion in the package. If the needle on the depth gauge remained stationary, this was an indication of a good seal. However, if the needle moved in a counter clockwise direction, due to deflation, then the package was regarded as a leaker and the contents were removed and repackaged.

Packages of MA cheeses were placed in open bins and stored in a 10°C walk-in cooler that had a relative humidity of 27%. Four storage periods (0, 60, 120, 180 days) were targeted for cheddar cheese while mozzarella was examined over the course of three storage periods (0, 60, 120). Once the bags of cheese were opened, testing occurred at day 0, 7, 14, and 21. A detailed description of the testing scheme is described below.

Cheese profile

Water activity, pH, fat content and moisture measurements were performed on the shredded cheddar cheese according to *Standard Methods for the Examination of Dairy Products (*Hickey, et al., 1993). A modified Babcock was used to determine the fat content; pH was measured using the Quinhydrone System. A Decagon CX-1 Water Activity System (Decagon Devices, Inc. Pullman, WA.) and an IR-100 Moisture Analyzer (Denver Instrument Co. Arvada, CO.) were used to determined water activity and moisture content, respectively.

Headspace gas analysis

Low levels of residual oxygen are common in the package headspace because it is difficult to completely evacuate all the air from inside the bag. Headspace gas analysis was performed on two packages per treatment immediately after packaging to determine initial headspace gas composition. Gas was drawn from the package with a 1.0 mL gas-tight syringe with a luer lock needle attachment and PrecisionGlide 23 gauge needles (Becton Dickinson & Co., Rutherford, NJ).

Gas analysis was performed on a Fisher-Hamilton Model 29, (Fisher Scientific Co., Pittsburgh, PA) gas partitioner. A 30% Di(2-ethylhexyl) sebacate (60/80 mesh) on chromosorb PAW-DMCS column and a molecular sieve 13X (45/60 mesh) column were used in series. Helium served as the carrier gas at a flow rate of 40 mL min^{-1}. The column temperature was ambient and the cell temperature was 70°C. Peaks were recorded and analyzed using a Hewlett Packard integrator (Palo Alto, CA., Model 3396A). Blood gas (5.13 % CO_2: 14.93 % O_2: 79.95 % N_2) was used as the standard. Changes in gas composition were monitored on two packages per treatment throughout the storage period of 0,60,120, 180 days for cheddar and 0, 60, 120 for mozzarella cheese.

Natamycin analysis

Natamycin concentration on the shredded cheese was determined using the International Dairy Federation's protocol for Cheese and Cheese rind, the Determination of Natamycin Content, Method of Spectrometry & HPLC-UV (Anonymous, 1987). The assay had limit of detection of 1

µg/ml (ppm). The procedure was used to confirm the natamycin concentration of the 300 and 1250 ppm stock solutions before application, and to determine that the concentration of natamycin of the shredded cheese.

Yeast and mold analysis

Yeast and mold were enumerated by placing an 11g sample of cheese and 99 mL phosphate/magnesium chloride solution (Marshall, 1993) into a Seward 400 Stomacher bag (Scientific Products, London, UK, S8254-1). Samples were stomached for two min, and 1 mL of the dilution was plated on Petrifilm™ Yeast & Mold (YM) film (3M Company, St. Paul, MN) in duplicate (Beuchat et al., 1990; Beuchat et al., 1991). Inoculated Petrifilm™ were incubated at 25°C and counted after 5 days.

Sensory analysis

The "*In/Out*" method, (Munoz, et al., 1992) was used to determine if the cheese was in or out of "specifications" for a saleable product. The panel consisted of four to five people with sensory experience in dairy foods. The panel evaluated eight samples per session including a freshly shredded sample of cheddar and mozzarella cheese, which served as a reference/standard. Each sample was discussed to determine if it was "in" or "out" of specifications with the final decision being a majority rule.   There were no defined specifications or guidelines and there was no training or product orientation. Consequently, each panelist made a decision based solely on their individual experience and familiarity with the product.

Experimental design

A balanced design which included the first four treatments groups (I,D,NS; I,D,S; I,I,NS; I,I,S) was established along with five control groups (Fig. 2.1). Treatments were coded according to variables assigned with in each group. The first variable indicates if the cheese was inoculated (I) with *Penicillium roqueforti* or not inoculated (NI). The second variable describes the application of natamycin application as direct (D) or indirect (ID). Direct means that natamycin was applied directly to the cheese, indirect means that natamycin was applied to the cellulose, then to the cheese. The third and final variable indicates if the powdered cellulose contained an oxygen scavenger (S) or not (NS).

The first control (NI-N-S) was not inoculated nor treated with natamycin but it contained an oxygen scavenger in the cellulose. The second control (I-N-NS) was inoculated with 10-100 spores/g of *Penicillium roqueforti*, but it was not treated with natamycin and it did not contain an oxygen scavenger in the cellulose.   The third control (NI-N-NS) was designed to mimic current industry practices. Neither inoculum nor natamycin was applied and no oxygen scavengers were present in the cellulose.

The testing scheme was designed around four MAP storage periods (0, 60, 120, and 180 days) and four days (0, 7, 14, 21) after opening. On storage day 0, 12 samples from the first seven treatment groups were randomly selected from the refrigerated storage facility. To

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determine shelf-life after opening, ten of the twelve samples were opened, the bags were rolled down from the top then paper clipped and returned to the refrigerator for evaluation over the next twenty-one days. A gas sample from the remaining unopened packages was analyzed to determine the proportions of gas in the modified atmosphere. The packages were then opened and 11 g per package was weighed out for microbial testing. A gram of cheese from each package was weighed out and extracted to determine natamycin concentration. Packages from each treatment group were then combined for sensory evaluation. Seven days after opening, two packages that were opened previously were randomly selected from each treatment and sampled for microbial and sensory evaluation. Analyzes were repeated 14 and 21 days after the packages had been opened.

Upon completion of all tests for storage day 0, two more controls were developed to evaluate the effect of inoculum on the yeast and mold count. Natamycin was sprayed directly onto the cheese for both treatment groups. One group received cellulose without an oxygen scavenger (NI-D-NS) and the other group with an oxygen scavenger (NI-D-S). No inoculum was added to the cheese in either of these treatments. This control was designed to be analyzed at storage day 0, 60 and 120, with microbial and sensory testing occurring 0, 7, 14, and 21 days after opening.

Statistical analysis

Raw data was entered into spreadsheet file (Microsoft® Excel 97 for Windows 95, Microsoft Corporation, Redmond, WA.) then transferred to SAS® program for analysis (SAS Institute Inc., Cary, NC). The raw spore count data were transformed to Log [(spore + 1) dilution factor]. The split plot design was analyzed using analysis of variance (ANOVA). Differences in treatment groups were considered significant when $P < 0.05$.
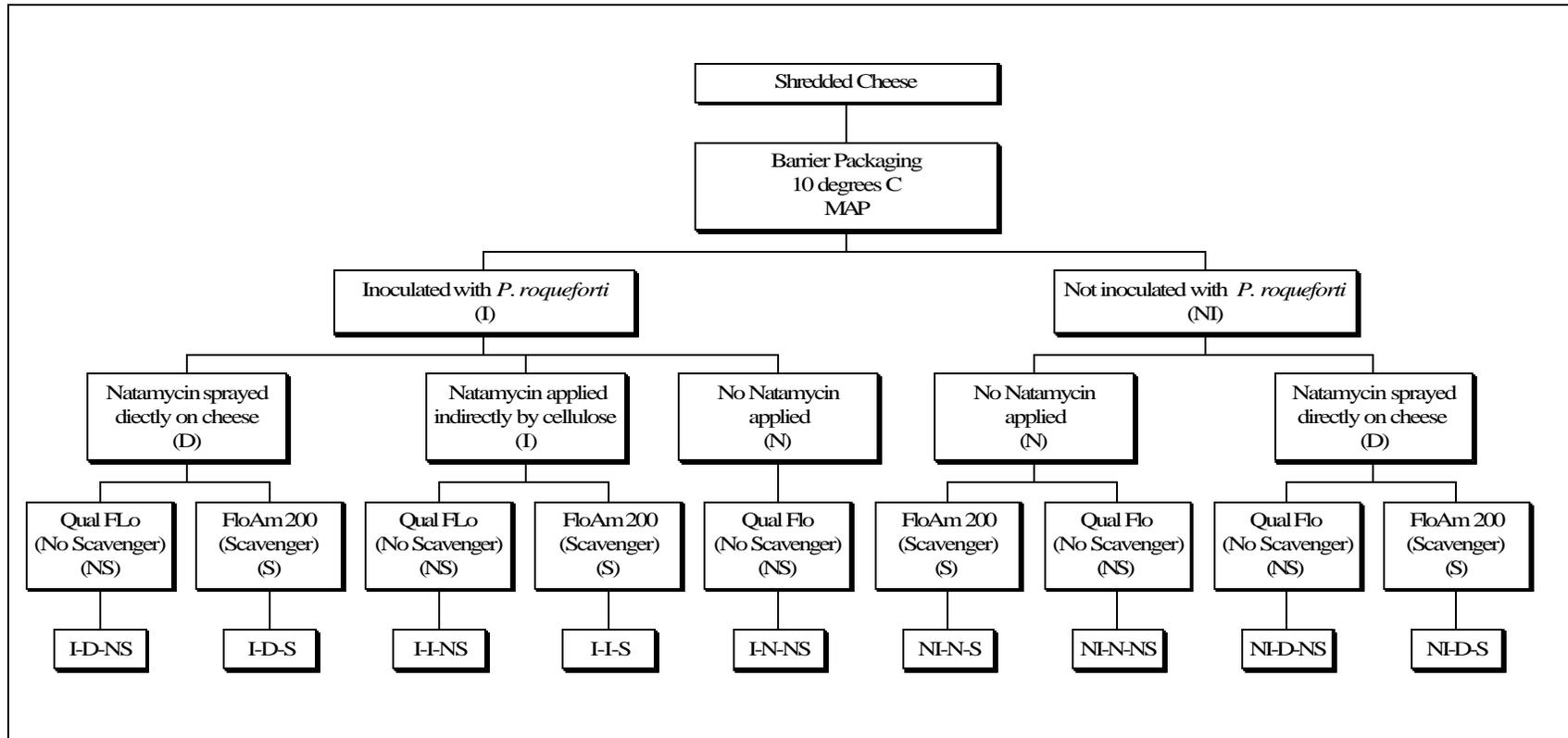
Fig. 2.1- Scheme for investigating the application and effect of natamycin, presence of oxygen scavengers and modified atmosphere packaging on the growth of *Penicillium roqueforti* in shredded cheddar and mozzarella cheese stored at 10°C.

The first four treatments (I-D-NS, I-D-S, I-I-NS, I-I-S) were analyzed to determine the affect of natamycin application and the presence of an oxygen scavenger on YM count. Treatments I-D-NS and I-D-S were compared to NI-D-NS and NI-D-S to determine the affect of the inoculum on YM count. Additional analysis combined all treatments to determine significant interactions.

The experimental design and sensory analysis results of shredded cheddar and mozzarella cheese is located in Appendix C.

RESULTS AND DISCUSSION

Cheese profile

Fat content of cheddar and mozzarella cheese averaged 32.23% ± 0.25 and 22.27% ± 0.25 (wet basis), respectively while pH averaged 5.07 ± 0.06 and 5.23 ± 0.15 respectively at the time of packaging. Water activity and moisture content measured 0.944 ± 0.005 and 36.04 ± 0.55, respectively for cheddar cheese and 0.95 ± 0.005 and 44.43 ± 0.52, respectively for mozzarella cheese. After 180 d, water activity of cheddar cheese dropped insignificantly (P>0.05) to 0.927 ± 0.004 while moisture remained constant at 36.47 ± 1.19. Due to poor taste and heavy mold growth, data collection on mozzarella cheese was terminated after 120 d of storage. Mean water activity (0.93 ± 0.004) did not change significantly (P>0.05) over the 120 d storage period and moisture content remained constant at 44.26 ± 0.55.

Air samples

Yeast and mold air samples were taken in the processing facility and the cooler to determine air quality at the time of packaging and initial storage. Counts in the processing area averaged 146 CFU/m^3, which is higher than the recommended level of <100 CFU/m^3. Natural contamination in the air may have contributed to total fungal counts for both inoculated and uninoculated cheeses. Air samples from the 10°C cooler showed no signs of contamination.

Natamycin levels

The concentration of natamycin applied to the surface of the cheddar and mozzarella cheese averaged 5.4 ± 0.5 µg/ml (ppm) and 5.4 ± 0.2 µg/ml (ppm), respectively, across all experimental blocks (Table 2.1). No significant (P>0.05) difference in concentration was evident when the two methods of application were compared. Natamycin (300 ppm) sprayed directly on the cheddar and mozzarella cheese averaged 5.5 ± 0.5 µg/ml and 5.5 ± 0.2 µg/ml, respectively. Natamycin (1250 ppm) applied to the cellulose and distributed on the cheese by tumbling had a mean of 5.2 ± 0.3 µg/ml for both cheddar and mozzarella cheeses. Both application methods were successful in applying the manufactures recommended level (4–6 µg/ml) of antimycotic to the shredded cheese surface. It is worth noting the indirect method (cellulose treated with natamycin in advance) was

easier to apply at the time of packaging. Additional moisture associated with the direct application made the cheese sticky and harder to handle.

Table 2.1-Mean natamycin concentration (μg/ml) of shredded cheddar and mozzarella cheese at time of packaging. (n=2)

Treatment [1]	Shredded Cheddar		Shredded Mozzarella	
	Mean (μg/ml)	SD[2]	Mean (μg/ml)	SD
I-D-NS	5.7	1.1	5.1	0.1
I-D-S	5.6	0.5	5.3	0.2
NI-D-NS	5.5	0.2	6.1	0.2
NI-D-S	5.2	0.3	5.6	0.1
Mean - Direct	5.5	0.5	5.5	0.2
I-I-NS	5.2	0.3	5.2	0.3
I-I-S	5.3	0.3	5.1	0.3
Mean - Indirect	5.2	0.3	5.2	0.3
Mean - All Treatments	5.4	0.5	5.4	0.2

[1] Treatment: I-D-NS = inoculated (*P. roqueforti*), natamycin applied directly to cheese, no scavenger in cellulose; I-D-S = inoculated, natamycin applied directly to cheese, O_2 scavenger in cellulose; NI-D-NS = no inoculum, natamycin applied directly to cheese, no scavenger in cellulose; NI-D-S = no inoculum, natamycin applied directly to cheese, O_2 scavenger in cellulose; I-I-NS = inoculated, natamycin applied indirectly to cheese via cellulose; I-I-S = inoculated, natamycin applied indirectly to cheese via cellulose, O_2 scavenger in cellulose.
[2] Standard deviation

Headspace gas composition

Headspace gas composition within each treatment was significantly (P<0.05) affected during storage of 180-days for cheddar (Table 2.2) and 120-days for mozzarella cheese (Table 2.3). Equilibrated modified atmospheres were not established within the barrier film. Carbon dioxide levels decreased significantly (P<0.05) and N_2 levels increased significantly (P<0.05) in all treatments. Oxygen concentrations increased over the 120 and 180-day storage periods but changes were not significant (P>0.05). Headspace gas composition of MAP shredded cheddar cheese averaged 25.04:0.43:74.53 (CO_2:O_2:N_2) at the time of packaging (Table 2.2). At storage day 60, CO_2 concentration declined to 16.86% while O_2 and N_2 content increased to 1.00% and 82.20%, respectively. This trend continued through storage day 120 when the average gas composition reached 6.98:2.53:90.49 (CO_2:O_2:N_2). Carbon dioxide levels fell to 4.39%, O_2 averaged 2.01% and N_2 measured 93.60% after 180-days of storage.

Initial headspace gas composition of MAP shredded mozzarella cheese averaged 24.75:0.61:74.66 (CO_2:O_2:N_2) on day 0 (Table 2.3). After 60 days of storage, CO_2 concentration declined to 15.04% while O_2 and N_2 content increased to 0.97 and 84.00%, respectively. Just like the trend seen in cheddar cheese, CO_2 continued to decline while O_2 and N_2 increased over the next 60 days. By storage day 120, headspace gas composition measured 5.25:1.59:93.16 (CO_2:O_2:N_2). The large drop in CO_2 is partially due to transmission across the bag but CO_2 is also soluble in the lipid phase and therefore can be physically absorbed into the cheese (Anonymous, 1981; Farber, 1991).

Table 2.2-Mean headspace gas composition of modified atmosphere packaged (MAP) shredded cheddar cheese packaged in multilayer high barrier film and stored at 10°C over a period of 180 days. (n=2)

T[1]	MAP	0 X	0 SD[2]	60 X	60 SD	120 X	120 SD	180 X	180 SD	0-180 X	0-180 SD
I-D-NS	% CO_2	24.73^c	0.83	16.68^b	1.22	5.70^a	2.02	2.50^a	0.42	**12.40**	**9.51**
	% O_2	0.11^a	0.15	0.65^a	0.26	4.44^a	5.12	3.45^a	0.62	**2.16**	**2.76**
	% N_2	75.16^a	0.68	82.66^b	0.96	89.86^c	3.09	94.05^c	0.21	**85.43**	**7.79**
I-D-S	% CO_2	25.00^d	0.18	17.59^c	1.17	8.13^b	0.94	3.80^a	0.63	**13.63**	**8.83**
	% O_2	0.34^a	0.41	0.86^a	0.58	2.54^a	1.54	1.16^a	0.36	**1.22**	**1.09**
	% N_2	74.67^a	0.59	81.55^b	1.74	89.34^c	0.59	95.03^d	0.28	**85.15**	**8.28**
I-I-NS	% CO_2	23.83^d	0.78	16.79^c	0.82	8.06^b	1.05	4.05^a	0.15	**13.18**	**8.23**
	% O_2	0.23^a	0.13	0.22^a	0.31	2.74^a	2.11	1.03^a	0.37	**1.05**	**1.37**
	% N_2	75.95^a	0.91	83.00^b	0.51	89.20^c	1.06	94.91^d	0.52	**85.76**	**7.57**
I-I-S	% CO_2	24.50^d	0.86	16.22^c	0.06	7.69^b	0.38	4.26^a	0.62	**13.17**	**8.41**
	% O_2	0.37^a	0.11	0.83^a	0.22	1.53^a	0.91	3.36^a	1.02	**1.52**	**1.33**
	% N_2	75.13^a	0.97	83.00^b	0.10	90.78^c	0.53	92.38^c	0.39	**85.32**	**7.36**
NI-N-S	% CO_2	25.30^d	0.76	19.21^c	0.41	8.62^b	0.51	4.46^a	0.74	**14.4**	**8.86**
	% O_2	0.98^a	0.12	0.00^a	0.00	1.11^a	0.18	2.47^a	1.51	**1.14**	**1.10**
	% N_2	73.72^a	0.63	81.27^b	1.09	90.28^c	0.34	93.07^c	0.77	**84.58**	**8.19**
I-N-NS	% CO_2	25.37^d	0.59	17.78^c	0.34	7.77^b	0.53	3.87^a	0.22	**13.70**	**9.02**
	% O_2	0.69^a	0.09	0.44^a	0.09	1.45^a	0.78	1.45^a	0.04	**1.01**	**0.57**
	% N_2	73.94^a	0.69	81.78^b	0.43	90.78^c	1.31	94.67^d	0.18	**85.3**	**8.63**
NI-N-NS	% CO_2	25.89^c	0.17	20.64^b	0.66	6.83^a	0.55	7.75^a	0.39	**15.28**	**8.78**
	% O_2	0.35^a	0.13	0.48^a	0.68	2.24^a	2.00	1.16^a	0.11	**1.05**	**1.13**
	% N_2	73.77^a	0.05	78.88^b	0.02	90.94^c	1.45	91.10^c	0.50	**83.67**	**8.11**
NI-D-NS	% CO_2	24.89^c	0.61	13.95^b	2.16	3.17^a	0.90	-	-	**14.00**	**9.78**
	% O_2	0.51^a	0.04	3.06^a	3.65	2.99^a	2.72	-	-	**2.18**	**2.42**
	% N_2	74.60^a	0.57	83.00^b	1.49	93.84^c	1.82	-	-	**83.81**	**8.70**
NI-D-S	% CO_2	25.84^b	0.07	12.85^a	4.01	6.83^a	5.80	-	-	**15.17**	**9.24**
	% O_2	0.33^a	0.04	2.44^a	2.84	3.75^a	1.72	-	-	**2.17**	**2.14**
	% N_2	73.83^a	0.03	84.71^a	1.17	89.42^a	7.52	-	-	**82.65**	**7.92**
ALL	**% CO2**	**25.04**	**0.78**	**16.86**	**2.63**	**6.98**	**2.26**	**4.39**	**1.56**		
	% O2	**0.43**	**0.28**	**1.00**	**1.53**	**2.53**	**2.00**	**2.01**	**1.17**		
	% N2	**74.53**	**0.89**	**82.20**	**1.73**	**90.49**	**2.51**	**93.60**	**1.46**		

[1] Treatment: I-D-NS = inoculated (*P. roqueforti*), natamycin applied directly to cheese, no scavenger in cellulose; I-D-S = inoculated, natamycin applied directly to cheese, O_2 scavenger in cellulose; I-I-NS = inoculated, natamycin applied indirectly to cheese via cellulose; I-I-S = inoculated, natamycin applied indirectly to cheese via cellulose, O_2 scavenger in cellulose; NI-N-S = no inoculum, no natamycin, O_2 scavenger in cellulose; I-N-NS = inoculated, no natamycin, no scavenger in cellulose; NI-N-NS = no inoculum, no natamycin, no scavenger in cellulose; NI-D-NS = no inoculum, natamycin applied directly to cheese, no scavenger in cellulose; NI-D-S = no inoculum, natamycin applied directly to cheese, O_2 scavenger in cellulose.
[2] Standard deviation
[a,b,c,d,] Tukey's studentized range test. Means with different letters in each row are significantly different (P<0.05).

Table 2.3 -Mean headspace gas composition of modified atmosphere packaged (MAP) shredded mozzarella cheese packaged in multilayer high barrier film and stored at 10°C, over a period of 180-days. (n=2)

T^1	MAP	MAP Storage (days)							
		0		60		120		0-120	
		X	SD	X	SD	X	SD	**X**	**SD**
I-D-NS	% CO_2	24.97 [a]	0.34	15.19 [b]	0.23	5.02 [c]	0.71	**15.06**	**8.93**
	% O_2	0.60 [a]	0.26	1.65 [a]	1.14	0.84 [a]	0.20	**1.03**	**0.72**
	% N_2	74.65 [c]	0.22	83.15 [b]	0.91	94.14 [a]	0.91	**83.98**	**8.76**
I-D-S	% CO_2	25.73 [a]	0.83	13.03 [b]	0.81	4.33 [c]	0.34	**14.36**	**9.64**
	% O_2	0.53 [a]	0.07	0.90 [a]	0.14	0.67 [a]	0.13	**0.70**	**0.19**
	% N_2	73.74 [c]	0.90	86.07 [b]	0.67	95.00 [a]	0.47	**84.94**	**9.56**
I-I-NS	% CO_2	23.67 [a]	0.35	13.09 [b]	2.85	3.51 [c]	0.03	**13.41**	**9.10**
	% O_2	0.93 [a]	0.27	2.73 [a]	3.03	3.72 [a]	3.53	**2.46**	**2.44**
	% N_2	75.44 [c]	0.08	84.18 [b]	0.18	92.78 [a]	3.17	**84.13**	**7.88**
I-I-S	% CO_2	24.18 [a]	2.42	14.79 [b]	0.81	5.24 [c]	0.01	**14.74**	**8.55**
	% O_2	0.61 [a]	0.10	0.79 [a]	0.05	1.20 [a]	1.70	**0.87**	**0.81**
	% N_2	75.21 [c]	2.32	84.42 [b]	0.87	93.56 [a]	1.70	**84.40**	**8.32**
NI-N-S	% CO_2	25.89 [a]	1.56	18.07 [b]	1.42	5.46 [c]	2.04	**16.47**	**9.31**
	% O_2	0.62 [a]	0.20	0.42 [a]	0.01	1.89 [a]	1.42	**0.97**	**0.96**
	% N_2	73.50 [c]	1.76	81.52 [b]	1.14	92.66 [a]	0.61	**82.56**	**8.67**
I-N-NS	% CO_2	23.95 [a]	2.15	17.84 [b]	0.16	4.62 [c]	1.04	**15.47**	**8.91**
	% O_2	0.41 [a]	0.30	0.33 [a]	0.47	0.91 [a]	0.06	**0.55**	**0.37**
	% N_2	75.63 [c]	1.86	81.82 [b]	0.31	94.48 [a]	0.98	**83.98**	**8.64**
NI-N-NS	% CO_2	24.39 [a]	0.63	16.47 [b]	0.78	5.95 [c]	0.99	**15.60**	**8.30**
	% O_2	0.86 [a]	0.68	0.43 [a]	0.06	0.93 [a]	0.45	**0.74**	**0.44**
	% N_2	74.76 [c]	0.05	83.09 [b]	0.72	93.12 [a]	1.43	**83.66**	**8.25**
NI-D-NS	% CO_2	25.35 [a]	0.56	13.83 [b]	0.80	7.62 [c]	0.07	**15.60**	**8.06**
	% O_2	0.35 [a]	0.17	0.58 [a]	0.20	3.11 [a]	3.39	**1.35**	**2.04**
	% N_2	74.31 [c]	0.38	85.59 [b]	0.60	89.27 [a]	3.32	**83.06**	**7.14**
NI-D-S	% CO_2	24.71 [a]	0.10	13.01 [b]	1.89	5.52 [c]	0.67	**14.41**	**8.70**
	% O_2	0.61 [a]	0.06	0.87 [a]	0.41	1.07 [a]	0.05	**0.85**	**0.28**
	% N_2	74.68 [c]	0.16	86.12 [b]	1.48	93.41 [a]	0.62	**84.74**	**8.47**
ALL	**% CO2**	**24.76**	**1.20**	**15.04**	**2.19**	**5.25**	**1.30**		
	% O2	**0.61**	**0.28**	**0.97**	**1.10**	**1.59**	**1.69**		
	% N2	**74.66**	**1.12**	**84.00**	**1.80**	**93.16**	**2.06**		

[1] Treatment: I-D-NS = inoculated (*P. roqueforti*), natamycin applied directly to cheese, no scavenger in cellulose; I-D-S = inoculated, natamycin applied directly to cheese, O_2 scavenger in cellulose; I-I-NS = inoculated, natamycin applied indirectly to cheese via cellulose; I-I-S = inoculated, natamycin applied indirectly to cheese via cellulose, O_2 scavenger in cellulose; NI-N-S = no inoculum, no natamycin, O_2 scavenger in cellulose; I-N-NS = inoculated, no natamycin, no scavenger in cellulose; NI-N-NS = no inoculum, no natamycin, no scavenger in cellulose; NI-D-NS = no inoculum, natamycin applied directly to cheese, no scavenger in cellulose; NI-D-S = no inoculum, natamycin applied directly to cheese, O_2 scavenger in cellulose.
[2] Standard deviation
[a,b,c,d] Tukey's studentized range test. Means with different letters in each row are significantly different (P<0.05).

Oxygen scavengers present in the cellulose did not affect the O_2 concentration in shredded cheese packages. Initial headspace O_2 concentrations were very low. At day 0, cheddar cheese packaged with O_2 scavengers averaged 0.34 (I-D-S), 0.37 (I-I-S), 0.98 (NI-N-S) and 0.33 (NI-D-S) while mozzarella cheese averaged 0.53 (I-D-S), 0.61 (I-I-S), 0.62 (NI-N-S) and 0.61 (NI-D-S). Oxygen concentrations in these packages were not significantly (P>0.05) different from cheddar and mozzarella cheeses packaged without O_2 scavengers, 0.11 (I-D-NS), 0.23 (I-I-NS), 0.69 (I-N-NS), 0.35 (NI-N-NS), 0.51 (NI-D-NS) and 0.60 (I-D-NS), 0.93 (I-I-NS), 0.41 (I-N-NS), 0.86 (NI-N-NS), 0.35 (NI-D-NS), respectively.

Over the 180 day (cheddar) and 120 day (mozzarella) storage periods, O_2 concentrations in packages containing O_2 scavengers were not significantly (P<0.05) different from packages that did not contain O_2 scavengers. After 180 days of storage, cheddar cheese packaged with O_2 scavengers averaged 1.16 (I-D-S), 3.36 (I-I-S), and 2.47 (NI-N-S) while cheddar cheese packaged without O_2 scavengers averaged 3.45 (I-D-NS), 1.03 (I-I-NS), 1.45 (I-N-NS) and 1.16 (NI-N-NS). Mozzarella cheese packaged with O_2 scavengers averaged 0.67 (I-D-S), 1.20 (I-I-S), 1.89 (NI-N-S) and 1.07 (NI-D-S) while mozzarella cheese packaged without O2 scavengers averaged 0.84 (I-D-NS), 3.72 (I-I-NS), 0.91 (I-N-NS), 0.93 (NI-N-NS) and 3.11 (NI-D-NS) after 120 days of storage. These results show that oxygen scavengers are not necessary if initial headspace O_2 concentration is minimized.

Yeast and mold count

The affect of natamycin application, oxygen scavengers, length of MAP storage period, and number of days a package was open were analyzed by comparing mean log yeast and mold (YM) counts from treatments I-D-NS, I-D-S, I-I-NS, and I-I-S.

Method of natamycin application, direct (D) vs. indirect (I), did not significantly (P >0.05) affect mean log YM counts in cheddar or mozzarella cheeses. Yeast and mold population on cheddar cheese averaged 3.32 ± 2.2 CFU/g with direct application and 3.22 ± 2.2 CFU/g with indirect application. Mozzarella cheese contained 4.34 ± 2.5 CFU/g and 4.33 ± 2.5 CFU/g with direct and indirect application, respectively.

Length of MAP storage period affected cheddar and mozzarella cheeses differently (Fig. 2.2). Mean YM log populations isolated after 0, 60, 120, and 180 days of MAP storage were not significantly different (P>0.05) for cheddar cheese. However, MAP storage period had a significant (P<0.05) affect on YM log population of mozzarella cheese. Yeast and mold log counts increased insignificantly (P>0.05) from 3.73 ± 2.61 CFU/g to 4.32 ± 2.59 CFU/g between 0 and 60 days of storage, respectively. A significant (P<0.05) increase occurred between 0 (3.73 ± 2.61 CFU/g) and 120 (5.15 ± 1.99 CFU/g) days MAP storage. Results show that MAP shredded cheddar and mozzarella cheeses can be stored up to 180 and 60 days, respectively without a significant increase in mold growth.

Yeast and mold counts on shredded cheddar and mozzarella cheeses increased (P<0.05) after the packages were opened (Fig. 2.3). There was no difference (P>0.05) in YM population on cheddar cheese between day 0 (1.26 ± 0.27 CFU/g) and 7 (1.62 ± 0.89 CFU/g). However counts

increased significantly (P<0.05) over the next two weeks with day 14 and 21 averaging 3.67 ± 0.10 CFU/g and 6.51 ± 0.13 CFU/g, respectively. Mean log YM population on mozzarella cheese increased (P<0.05) from 1.67 ± 0.74 CFU/g on day 0 to 2.88 ± 1.35 CFU/g on day 7. Increased growth (P<0.05) continued throughout the study, day 14 averaged 5.22 ± 0.96 CFU/g and day 21 averaged 7.56 ± 0.33 CFU/g.

Oxygen scavengers had no affect (P>0.05) on mean log YM counts. Cheddar and mozzarella cheeses packaged with an O_2 scavenger averaged 3.38 ± 2.4 and 4.18 ± 2.5, respectively. Cheeses packaged without an O_2 scavenger averaged 3.17 ± 2.1 CFU/g of cheddar and 4.49 ± 2.5 CFU/g of mozzarella. The lack of an affect is a direct result of low O_2 concentrations upon packaging and no significant increase in O_2 levels throughout the storage period.

Since there was no significant difference between the two methods of natamycin application, a single variable was designed to indicate the "presence of natamycin". Presence or absence of natamycin was analyzed for significance across all 9 treatments. Results indicate the presence of natamycin alone did not reduce (P>0.05) YM counts. Cheddar cheese containing 5.5 ± 0.5 ppm of natamycin had a mean YM population of 3.32 ± 2.2 CFU/g which was not significantly different (P>0.05) from cheese without natamycin, 3.0 ± 2.1 CFU/g. Mozzarella cheese exhibited similar results, cheese with 5.5 ± 0.2 µg/ml natamycin averaged 3.94 ± 2.3 CFU/g and 4.38 ± 2.4 CFU/g without natamycin. Results disagree with the findings of Bullerman (1977) and Fente-Sampayo et al. (1995) who showed total inhibition of fungi was achieved with 5 ppm of natamycin. These results were achieved on blocks of cheese which have a lower surface to volume ration than shredded cheese. Since natamycin is not completely soluble in water and shredded cheese has a larger surface to volume ratio, higher residual concentrations maybe required for natamycin to be effective on shredded cheese.

Inoculation of cheese with *Penicillium roqueforti* did not contribute to increased YM counts. The overall spore loading of inoculated cheese, 3.33 ± 2.3 (cheddar) and 4.42 ± 2.4 CFU/g (mozzarella) was higher then uninoculated cheese, 2.98 ± 2.0 (cheddar) and 3.93 ± 2.2 CFU/g (mozzarella) but differences were not significant (P>0.05). Results indicate that natural contamination in the air contributed to total fungal counts on both inoculated and uninoculated cheeses. A positive relationship between fungal distribution in the air, and fungi on the surfaces of processing rooms, ripening chambers, and the cheese has been demonstrated previously (Fente-Sampayo et al., 1995).
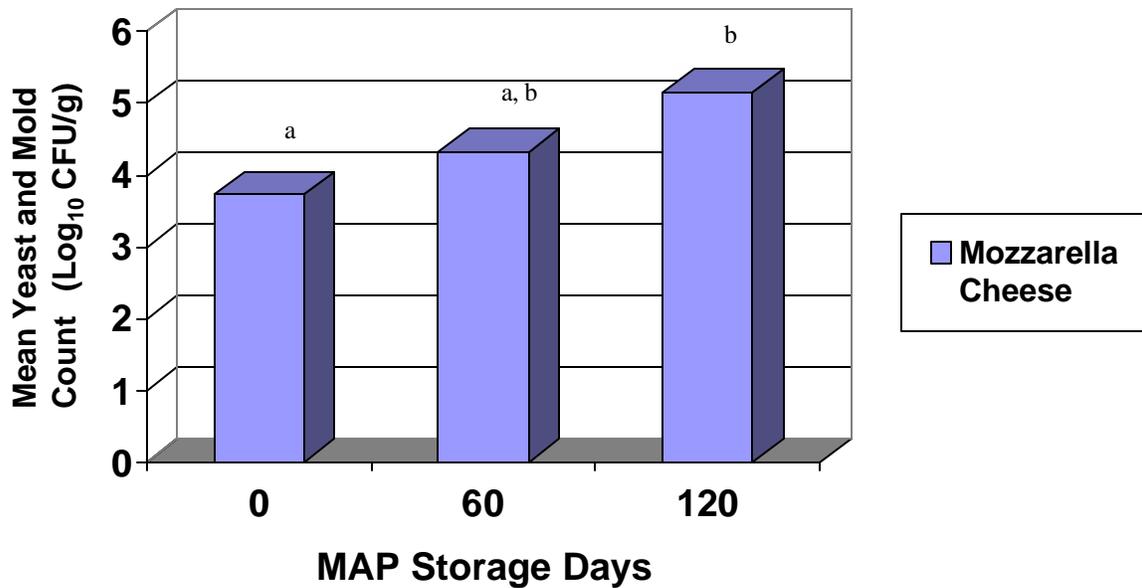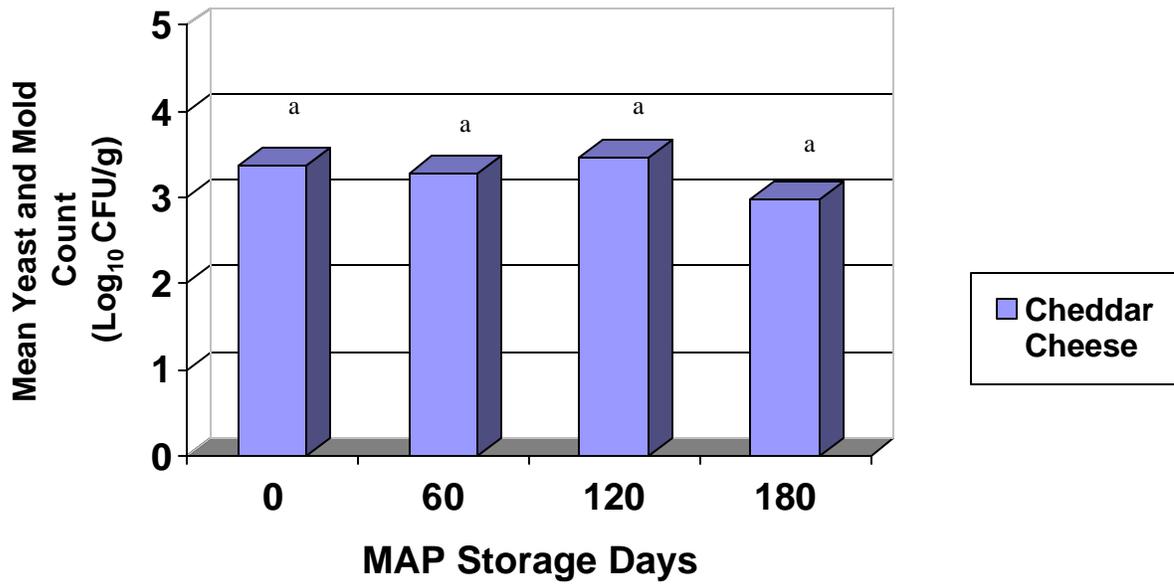
Fig. 2.2- Effect of MAP storage period (days) on growth of *Penicillium roqueforti* in MAP shredded cheddar and mozzarella cheeses stored at 10°C. Mean CFUs (n=32) with a different letter differed significantly (P<0.05).

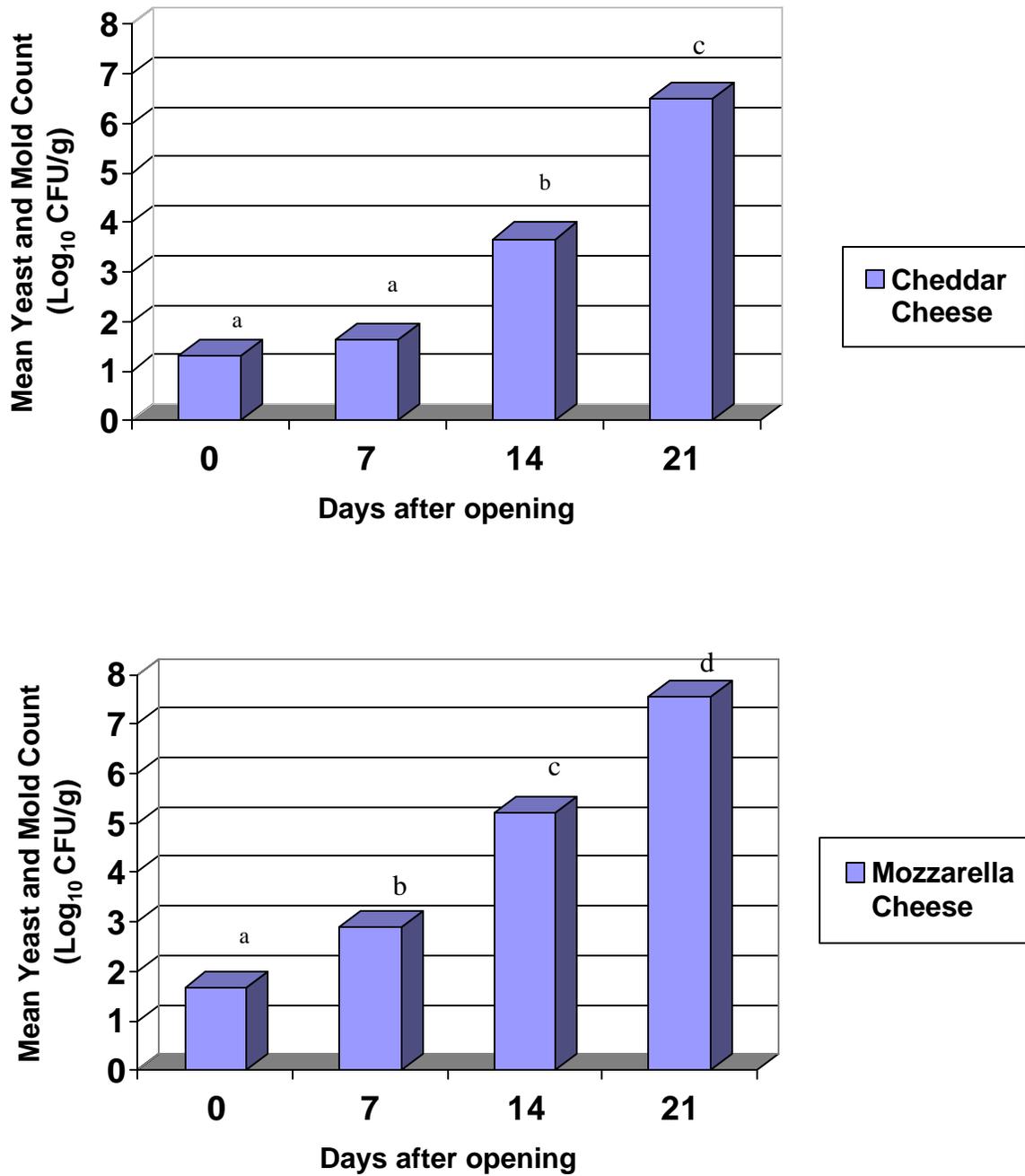**Fig. 2.3- Effect of length of time (days) a package has been open on growth of *Penicillium roqueforti* in MAP shredded cheddar and mozzarella cheeses stored at 10°C.** Mean CFUs (n=32) with a different letter are significantly (P<0.05) different.

CONCLUSIONS

Maintaining MA with limited headspace oxygen was key to achieving a 60-day and 180-day storage period for mozzarella and cheddar cheese, respectively. This was accomplished using oxygen barrier bags with adequate seams and seals. All bags were checked for leaks prior to storage and leakers were re-packed. Under the conditions of this experiment, natamycin residues of 4–6 μg/ was not effective in preventing yeast and mold growth on shredded cheddar or mozzarella cheeses. Further research is warranted to determine if natamycin is effective when applied at higher concentrations. Shredded cheddar cheese was stored for seven days after opening without a significant increase in YM population. Microbial quality of cheddar and mozzarella cheese deteriorated significantly after being open for 14 and 21 days. Oxygen scavengers did not contribute to reduced YM counts in cheddar or mozzarella cheeses. These results do not justify the additional cost associated with adding an oxygen scavenger to the cellulose of MAP shredded cheese if the air in the package is purged prior to sealing. The proportion of samples that were "*In*" specifications of a saleable product declined as YM population increased.

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INFLUENCE OF MODIFIED ATMOSPHERES CONTAINING LOW AMOUNTS OF RESIDUAL OXYGEN ON GROWTH OF *PENICILLIUM ROQUEFORTI, ASPERGILLUS NIGER, GEOTRICHUM CANDIDUM* AND *NEOSARTORYA FISCHERI*.

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ABSTRACT

Growth of *Penicillium roqueforti, Aspergillus niger, Geotrichum candidum* and *Neosartorya fischeri* in atmospheres containing 0:30:70, 0.5:29.5:70, 1:29:70, 2:28:70, 5:25:70, $O_2:CO_2:N_2$ were studied over a 5-day period. Spores were cultured on antibiotic-supplemented potato dextrose agar (pH 5.6, a_w 0.95) and incubated at 25°C. All four molds germinated and grew at 0.5:29.5:70. Extent of mycelia growth (mm) increased significantly ($P<0.05$) as oxygen concentration increased from 0.5% to 5%. All growth was inhibited at 0:30:70, but germination and growth occurred once cultures were exposed to 20.9% atmospheric O_2, indicating that a modified atmosphere containing no residual O_2 is fungistatic.

Key Words: modified atmosphere, oxygen, mold,

INTRODUCTION

Many food products are packaged in the presence of a modified atmosphere (MA), oxygen, carbon dioxide and nitrogen to prevent the growth and proliferation of microorganisms. Modified atmosphere packaging (MAP) may be defined as the packaging of a perishable product where the gaseous environment within the package is altered to a composition that is equivalent to something other than air. Most fresh foods respire or contain organisms that respire. The oxygen present is consumed, and carbon dioxide and water vapor are released. The environment is thus altered or modified from its original composition (Brody, 1996; Ooraikul and Stiles, 1991).

Carbon dioxide will inhibit microbial and mold growth (Rosenthal et al., 1991) but is not effective against CO_2 producing yeast (Brody, 1989). Packaging to essentially eliminate O_2 from the atmosphere in contact with the product is also useful in controlling mold growth. However, it is difficult to completely evacuate all the air from inside the bag, resulting in 0.5-3% or higher residual O_2 in the package headspace (Abe and Kondoh, 1989; Ahvenainen, 1992).

The percent of residual O_2 that can be present in the MA of a highly perishable product like shredded cheese is a topic that is not well defined in the literature. Brody (1989) suggested that O_2 levels must be reduced to <5% in order to achieve a measurable effect on the growth of organisms.

Soil fungi were shown to be relatively insensitive to severe reductions in O_2 concentration (Griffin, 1972). Germination and linear growth rates of these fungi showed little change until the O_2 concentration was reduced to 4% in the gas phase. Below 4%, there was a noticeable difference among species and growth declined at 1% O_2. Magan and Lacey (1984) examined the ability of field and storage fungi to grow in atmospheres containing 0.14-21% O_2 and 0.03-15% CO_2 on wheat extract agar. It was determined that many fungi were tolerant of low (0.14%) O_2 concentrations when grown at high (0.98) water activity.

Ellis et al. (1993b) concluded that spore counts greater than 10^1 spores g^1 would reduce the shelf-life of high moisture products packaged under MA containing >5% residual O_2. *Aspergillus flavus* exhibited more extensive growth at 10-20% O_2 than at 5% (Ellis et al., 1993a,b). Paster (1990) cited work by Miller and Golding (1949) that determined the amount of dissolved O_2 in the media required for normal growth of fungi was low, and *Aspergillus* and *Penicillium* spp. growth was affected only at <0.5% O_2. *Aspergillus niger* grew in bakery products packaged in <1% O_2 (Smith et al., 1986). *Neosartorya fischeri*, a heat-resistant mold implicated in the spoilage of fruit juice, is known to grow at O_2 levels as low as 0.10% (Nielsen et al., 1989). *Penicillium, Geotrichum,* and *Aspergillus* spp. have been isolated from various dairy and food products (Marth, 1987; Bundgaard-Nielsen and Nielsen, 1995).

Penicillium roqueforti and *Geotrichum candidum* are common flora on mold ripened cheese (Marth and Yousef, 1991). Lund et al. (1995) found that *Penicillium* species was the dominant fungi associated with cheese spoilage. *Penicillium roqueforti* is also known for its resistance to many cleaning agents (Bundgaard-Nielsen and Nielsen, 1995). *Penicillium roqueforti* has been shown to be one of the more tolerant species when exposed to very low O_2 levels (Magan and Lacey, 1984; Pitt, 1979). Spoilage by *Geotrichum candidum*, more commonly referred to as "machinery mold" or "dairy mold", is often linked to dirty processing equipment (Eisenberg and Cichowicz, 1978; Beneke and Stevenson, 1987).

The objective of this study was to determine the influence of various headspace oxygen concentrations (0, 0.5, 1, 2, and 5.0%) dispersed in CO_2 and N_2 on the germination and growth on dairy spoilage molds including *Penicillium roqueforti, Aspergillus niger,* and *Geotrichum candidum. Neosartorya fischeri* was included as a control organism since the literature states that it can grow at 0.10% O_2. Temperature, pH and a_w were held constant at the most favorable conditions for growth.

MATERIALS AND METHODS

Organisms

Penicillium roqueforti and *Geotrichum candidum* were supplied by Systems Bio-Industries Inc., Waukesha, WI. *Aspergillus niger* and *Neosartorya fischeri* were obtained from American Type Culture Collection, Rockville, MD. Lyophilized cultures were rehydrated according to manufacturers directions.

Substrate

Potato dextrose agar (Difco, Detroit, MI) with antibiotics (PDAA) containing 100 ppm each of chlortetracycline-HCL and chloramphenicol was used as the basal media throughout the study (Beuchat, 1979). Each 100 x 15 mm petri plate contained approximately 20 mL of PDAA, pH 5.6, a_w 0.97. Plates were stored under 100 % N_2 for 24 hr prior to inoculation to reduce the dissolved O_2 content in the agar (Nielsen et al., 1989.)

Preparation of inoculum

Spores were suspended in sterile 99 mL phosphate/magnesium chloride (PMC) dilution water (Marshall, 1993). Three 100-mm PDAA plates per organism were inoculated using the spread plate method with 0.1 mL of dilution. Plates were incubated at 25°C for 10 d. Conidia and ascospores were harvested by flushing each plate with 10 mL of sterile PMC dilution water and gently rubbing the surface with a sterile bent glass rod (Nielsen et al., 1988). The suspensions were pipetted into a sterile test tube. This procedure was repeated twice more, combining the three suspensions. Spore suspensions were vortexed for 2 min, allowed to settle, then vortexed an additional 2 min. Suspensions then were decanted through sterile glass wool into a sterile tube and centrifuged at low speed for 10 min. The PMC dilution water was decanted off and spores were resuspended in 10 mL of malt extract broth (Difco, Detroit, MI).

Uniformity of spore inoculation was achieved by pipetting 1.5 mL of the suspension into a 2.0 mL cryogenic screw-cap tube. Tubes were frozen under liquid nitrogen for 24 hrs, then placed in a subzero (-80°C) storage unit. Spore load was determined by thawing multiple tubes and surface plating on PDAA. Based on the count obtained (10^6 spores mL^{-1}), serial dilutions were prepared to achieve a final concentration of 10^2 spores mL^{-1} (Ellis et al., 1993b.)

Antibiotic supplemented PDA plates were inoculated via spread plate method with 0.1 mL (10^2 spore mL^{-1}) of suspension, resulting in ca. 10^1 viable spore mL^{-1}. Ten PDAA plates, two per O_2 concentration, were inoculated for each organism. One non-inoculated plate was also placed in the atmosphere chamber to serve as a control. All experiments were done in triplicate.

Incubation

Nine PDAA plates, two inoculated plates per organism and one uninoculated control, were placed in each gas tight atmospheric chamber. Nitrogen was flushed at a rate of 0.60 L min^{-1} for 40 min to reduce the O_2 level in the chambers to ca. 1.0%. The chambers were then flushed with the respective gas mixture at a flow rate of ca 0.60 L min^{-1} for 30 min. Flow rate was then reduced and maintained at ca 0.07-0.08 L min^{-1} during the 5 day incubation period at 25°C (Nielsen et al., 1989).

Specific volumes of O_2 (5, 2, 1, 0.5, and 0.0%) were mixed with 25, 28, 29, 29.5 and 30% CO_2 respectively and balanced with 70% N_2. Gas mixtures were selected in an attempt to imitate the MA (30% CO_2: 70% N_2) recommended for shredded cheese products (Farber, 1991; Ahvenainen, 1992). Specialty gas and pure N_2 was obtained from Brammer Welding (Roanoke, VA). Gas

composition was verified using a Fisher-Hamilton Model 29 (Fisher Scientific Co., Pittsburgh, PA) gas partitioner.

The atmospheric chamber was a modified version of the experimental incubation chamber described by Nielsen et al. (1989). A 1.3 cm (½ in) thick Lexan™ (polycarbonate) lid with hose connections and a septa seal was designed to fit a 13 x 23 cm (5 x 9 in) anaerobic nonvented jar. Gas mixtures were first humidified by passing through an airtight Erlenmeyer flask containing water. Nalgene™ tubing carried the gas to the bottom of the atmospheric chamber. Gas was vented out the top of the chamber and into an airtight flask with water. Gas flow was measured at the exit flask by a gas flow meter (Cole-Parmer, Niles, IL.).

Gas chromatographic analysis

Gas mixture inside the atmospheric chamber was monitored by pulling samples from the septa seal located on the top of each chamber. Gas samples were pulled 1 hr after the premixed gas was introduced into the chamber and once a day during the five-day incubation period. A 1.0 mL volume of gas was drawn using a 1.0 mL gas-tight syringe with a luer lock needle attachment. PrecisionGlide 23 gauge needles (Becton Dickinson & Co., Rutherford, NJ) were used.

Gas analysis was performed on a Fisher-Hamilton Model 29, (Fisher Scientific Co., Pittsburgh, PA) gas partitioner. A 30% Di(2-ethylhexyl) sebacate (60/80 mesh) on chromosorb PAW-DMCS column and a molecular sieve 13X (45/60 mesh) column were used in series. Helium served as the carrier gas at a flow rate of 40 mL min^{-1}. The column temperature was ambient and the cell temperature was 70°C. Peaks were recorded and analyzed using a Hewlett Packard integrator (Palo Alto, CA., Model 3396A). A premixed cylinder of Blood gas served as the standard (Brammer Welding, Roanoke, VA).

Growth measurement

Cultures were examined for colony formation visually on day 5. Initial response was "growth" vs. "no growth" for duplicate plates at the various modified atmospheres. Ten colony diameters were randomly measured on plates exhibiting growth and mean diameters were calculated (Ellis et al., 1993a; Nielsen et al., 1989; Nielsen et al., 1988).

Viability check of inoculum

Plates incubated under MA, which growth was not evident after 5 days, were incubated under normal atmospheric (20.9% O_2) conditions at 25°C. Growth under these conditions demonstrated that the plated spores were viable.

Experimental design

The experimental design was composed of five modified atmospheres, four mold types and three replicates per atmosphere. Specific volumes of O_2 (5, 2, 1, 0.5, and 0.0%) were mixed with a 25, 28, 29, 29.5 and 30% CO_2 respectively and balanced with 70 % N_2. Factors held constant include pH, a_w, and temperature. Inoculated plates (10^{-1} spores) were incubated at 25°C for 5 days under the modified atmospheres.

Data analysis

Data were analyzed using analysis of variance by the general linear model (GLM) procedure of the Statistical Analysis System (SAS® Institute, Inc., 1990). Significant differences among mean values were established using Tukey's studentized range (HSD) test. Significance was computed at $P < 0.05$.

RESULTS AND DISCUSSION

Neosartorya fischeri grew significantly ($P < 0.05$) slower than *A. niger, G. candidum* and *P. roqueforti* as indicated by the smaller colony diameter, 3.7 mm (Table 3.1). Growth rates of *A. niger* (11.7 mm) and *G. candidum* (11.9 mm), were not significantly ($P > 0.05$) different from each other. *Penicillium roqueforti* exhibited the fastest growth rate (13.0 mm), however it was not significantly ($P < 0.05$) different from *A. niger* and *G. candidum.*

Table 3.1 - Differences in growth rates (mm) of fungi cultured on potato dextrose agar with antibotics[1] at 25°C for 5 days under various oxygen concentrations. (N=3 reps.)

Fungi	Growth rate (mm) ± SD
Neosartorya fischeri	3.7 ± 3.0^a
Aspergillus niger	11.7 ± 8.4^b
Geotrichum candidum	11.9 ± 7.8^b
Penicillium roqueforti	13.0 ± 9.1^b

[1] Potato dextrose agar supplemented with 100ppm each of chloramphenicol and chlortetracycline-HCL (pH 5.6 ± 0.2).
[a-b] Tukey's studentized range (HSD) test. Means with the same letters are not significantly different ($P > 0.05$).

Large differences in growth rates resulted from decreasing the O_2 concentration (Table 3.2). The largest colony diameters (19.8 mm) were seen at 5% O_2. Growth rates under 1 and 2% O_2, 11.4 mm and 13.6 mm, respectively, were significantly lower than 5% but not significantly different from each other. As O_2 concentration dropped to 0.5%, fungal growth declined significantly ($P < 0.05$) to 5.7 mm. Germination of fungal spores was completely inhibited at 0.0% O_2.

Table 3.2- Effect of oxygen concentration on mean fungal growth rate (mm). Fungi were cultured on PDAA[1] at 25°C for 5 days. (N=3 reps.)

Oxygen Concentration[2]	Fungal Growth rate (mm) ± SD
0.0%	0.0 ± 0.0^a
0.5%	5.7 ± 2.6^b
1.0%	11.4 ± 4.8^c
2.0%	13.6 ± 5.3^c
5.0%	19.8 ± 7.2^d

[1] Potato dextrose agar supplemented with 100ppm each of chloramphenicol and chlortetracycline-HCL (pH 5.6 ± 0.2).

[2] Percent O_2 was balanced with CO_2 and 70% N_2.

[a-b] Tukey's studentized range (HSD) test. Means with the same letters are not significantly different ($P>0.05$).

Declining O_2 concentrations significantly ($P<0.05$) affected growth rates of within each organism. *Aspergillus niger* (Fig. 3.1) grew significantly ($P<0.05$) faster when incubated under 5% vs. 2% O_2. There was no significant growth difference ($P>0.05$) between 2% and 1% O_2. However 0.5% O_2 produced significantly less radial growth than 1%, 2% and 5% O_2. At 0% O_2, *A. niger* spores did not germinate.

Geotrichum candidum (Fig. 3.2) grew faster ($P<0.05$) under 5% O_2 than at 2% O_2. The growth rate was significantly ($P<0.05$) higher in 2% O_2 vs. 1%. This was also true when comparing growth rates between 1% to 0.5% O_2, with 1% O_2 exhibiting faster growth. Growth was significantly ($P<0.05$) inhibited when spores were incubated under 0% O_2.

Neosartorya fischeri (Fig 3.3) also grew significantly ($P<0.05$) faster at 5% vs. 2% O_2. There were no significant ($P>0.05$) differences in growth rates when spores were incubated under 2% vs. 1% O_2. Incubation at 0.5% O_2 produced significantly less growth than 1%, and 2% O_2. No growth was observed under 0% O_2 which was not significantly ($P>0.05$) different from the limited amount of growth observed at 0.5% O_2.

Penicillium roqueforti (Fig 3.4), like *A. niger*, *G. candidum*, and *N. fischeri* displayed significantly ($P<0.05$) higher growth rates when incubated under 5% vs. 2% O_2. There was no significant difference ($P>0.05$) in growth between 2% and 1% O_2. Spores incubated in 0.5% O_2 produced significantly less growth than spores incubated under 1% and 2% O_2. Growth of *P. roqueforti* was significantly ($P<0.05$) inhibited under 0% O_2.

It has been shown that CO_2 is not highly effective as a fungal inhibitor at non-refrigeration temperatures (Brody, 1989; Rosenthal et al., 1991). Therefore growth inhibition observed in this experiment is most likely due to the absence of O_2. All mold spores germinated and grew on PDAA when incubated under modified atmospheres containing as little as 0.5% O_2 (Fig. 3.1-3.4). No growth was observed at 0.0% O_2 during the 5 day incubation period. Exposure to air (20.9% O_2) after the 5 day incubation period resulted in spore germination with 24 hrs. These results indicate that 0.0% O_2 is fungistatic but not fungicidal which concur with the findings of Nielsen et al. (1989) and Rosenthal et al. (1991).
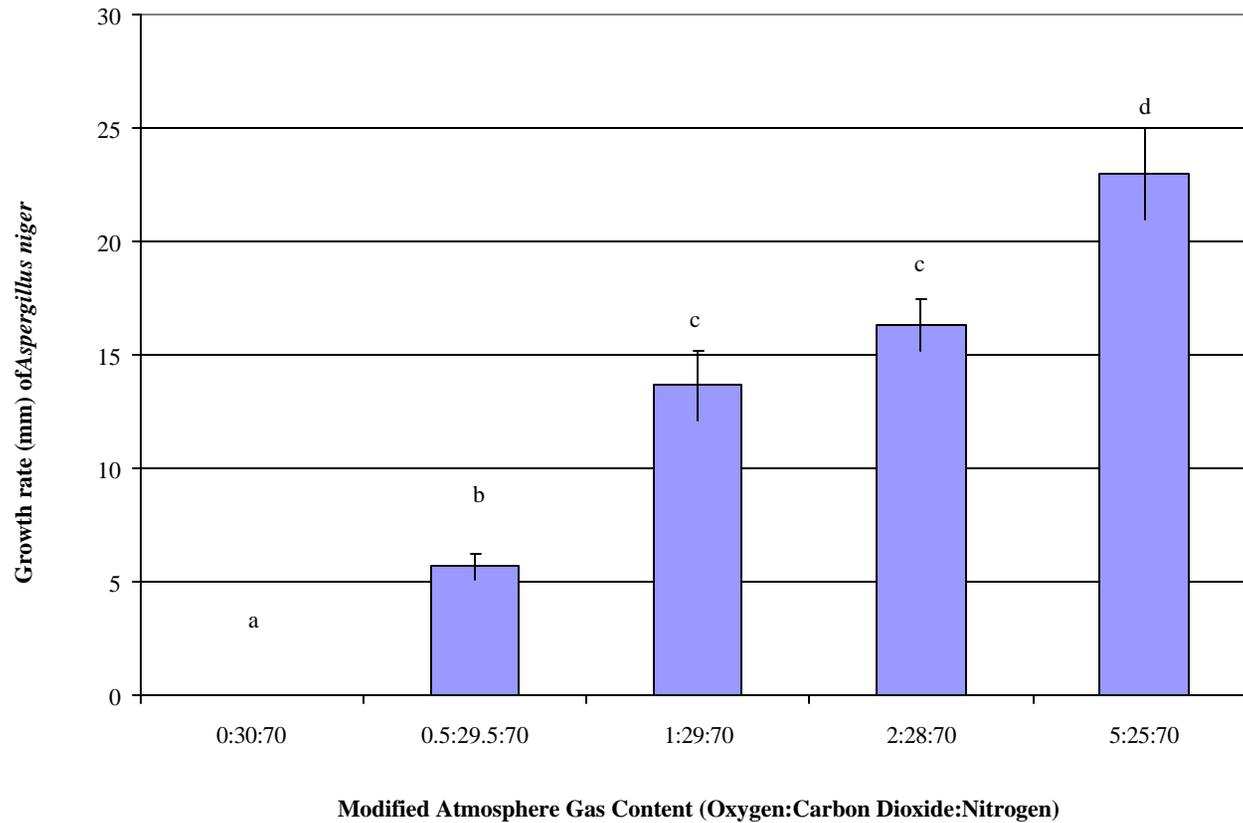
Fig. 3.1 –Influence of various modified atmospheres on the growth rate (mm) of *Aspergillus niger* **cultured on potato dextrose agar containing 100 ppm chlortetracycline-HCL and chloramphenicol (pH 5.6) at 25° C for 5 days**. Means (n=3 reps.) with a different letter are significantly different (P<0.05).
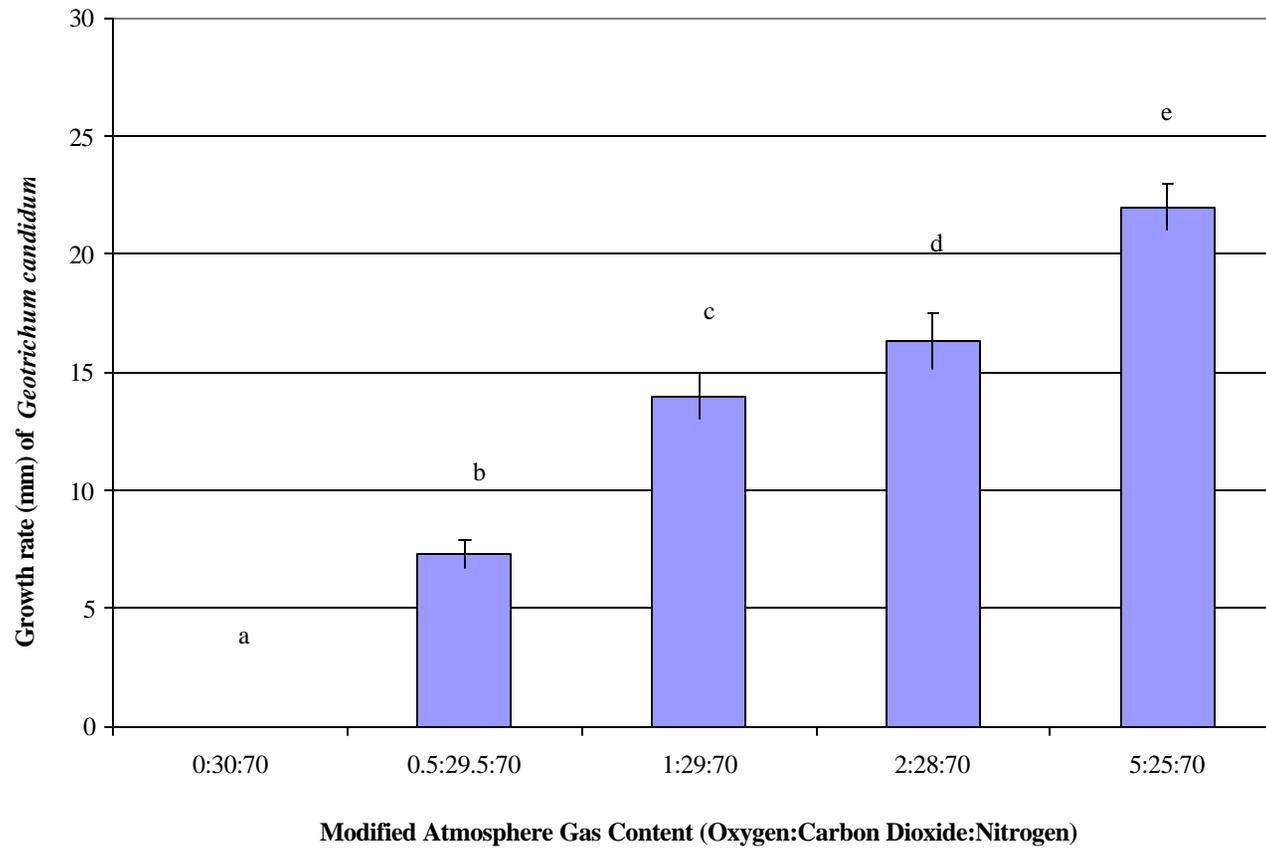
Fig. 3.2 –Influence of various modified atmospheres on the growth rate (mm) of *Geotrichum candidum* **cultured on potato dextrose agar containing 100 ppm chlortetracycline-HCL and chloramphenicol (pH 5.6) at 25° C for 5 days**. Means (n=3 reps.) with a different letter are significantly different (P<0.05).
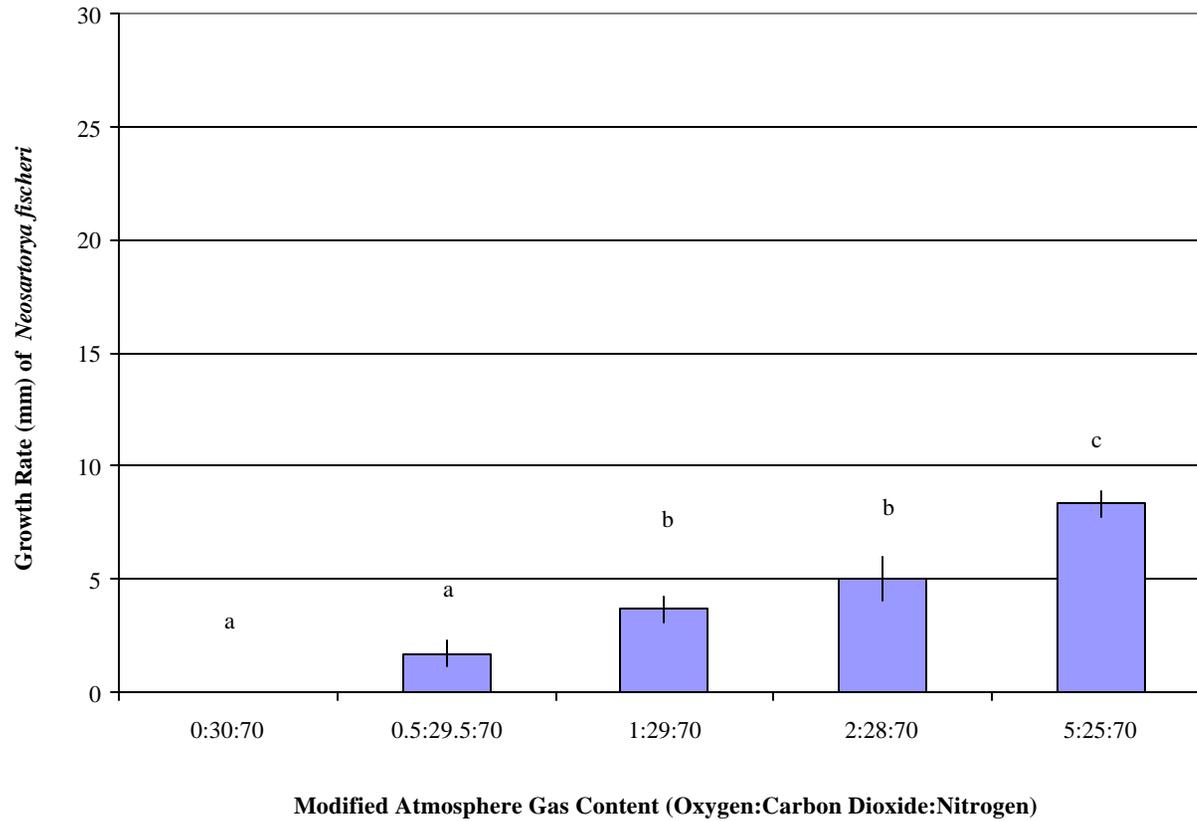
Fig. 3.3 –Influence of various modified atmospheres on the growth rate (mm)
Neosartorya fischeri **cultured on potato dextrose agar containing 100 ppm**
chlortetracycline-HCL and chloramphenicol (pH 5.6) at 25° C for 5 days. Means
(n=3 reps.) with a different letter are significantly different (P<0.05).

Fig. 3.4 –Influence of various modified atmospheres on the growth rate (mm) of *Penicillium roqueforti* **cultured on potato dextrose agar containing 100 ppm chlortetracycline-HCL and chloramphenicol (pH 5.6) at 25° C for 5 days**. Means (n=3 reps.) with a different letter are significantly different (P<0.05).
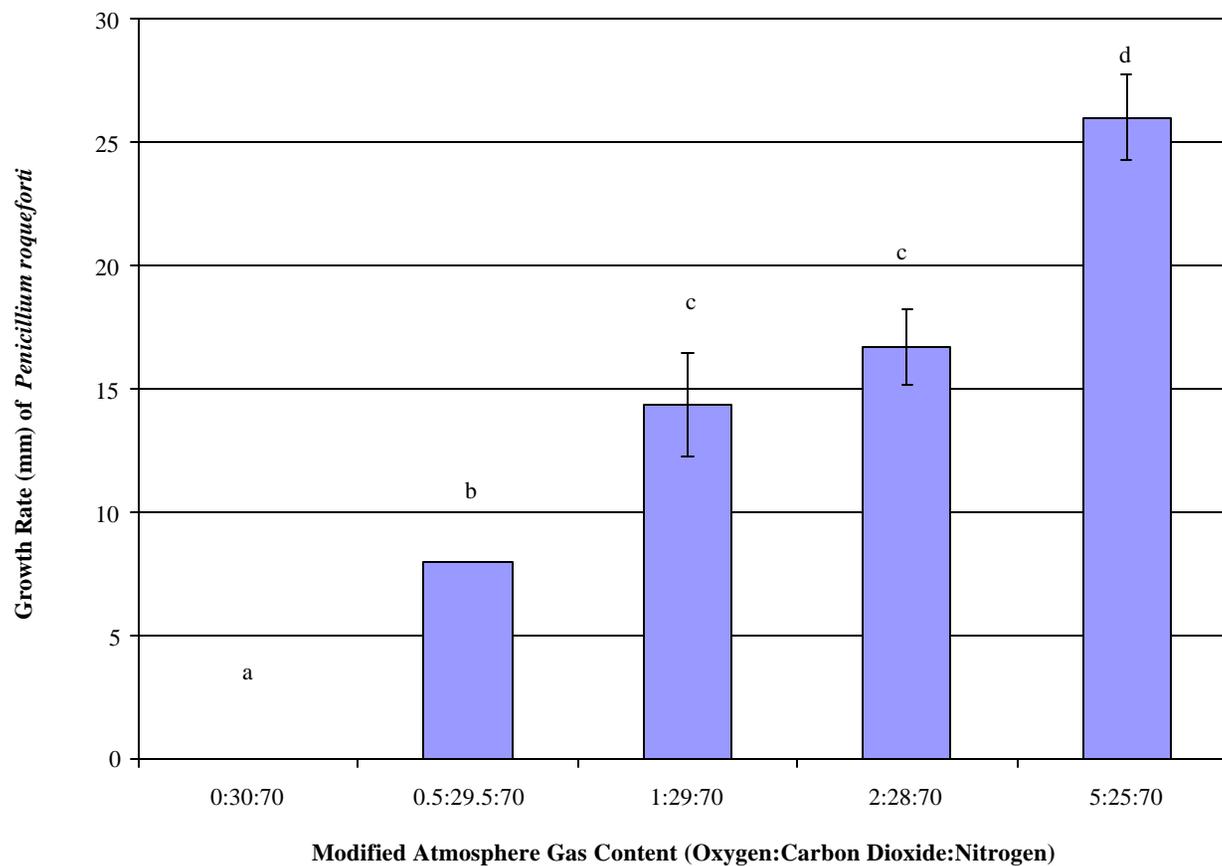
Gas composition within the chambers was essentially the same as the composition of the specialty gas mixture entering the chamber. When germination and growth occurred, there was no noticeable increase in CO_2 levels.

Viable mold count was significantly different between MA containing 0% and 0.5% O_2, for all four fungi (Table 3.3). No significant difference (P>0.05) was noted in viable count when MA containing 0.5% O_2 were compared with those containing 1%, 2%, or 5% O_2 for any of the molds except *A. niger*. However, highest mean viable count (49.7 x 10^1 per mL) resulted in largest growth rate (23 mm) for *A. niger* (Fig 3.1). These findings indicate that growth rates were not affected by the inoculum size used in this experiment, an issue described by Ellis et al. (1993b). Ellis and associates expected mycelial growth to be higher on plates inoculated with a higher inoculum level. However, results showed that mold growth was generally greater on plates with a low inoculum level (10^2 spores) vs. a higher level (10^4 spores), especially when spores were incubated in 5% O_2.

Other studies have shown that growth rate is affected more by lag phase than inoculum size. Gonzalez et al. (1988) determined that lag phases of some *Aspergillus* and *Penicillium* increased as the number of spores decrease, however, growth rates were unaffected. Nielsen et al. (1989) observed that germination of heat-activated ascospores was enhanced at pH 3.5 vs. pH 7.0, which may shorten the length of the lag phase. Their results indicated that growth rate was less affected by inoculum size than lag phase.

Table 3.3- Mean counts of molds incubated under various modified atmospheres on potato dextrose agar with antibiotics[1] at 25°C for 5 days. (N=3 reps.)

	10^1 Viable Spore mL^{-1} $\pm$ SD				
	Modified Atmosphere O_2:CO_2:N_2 (% Ratio)				
Fungi	0:30:70	0.5:29.5:70	1:29:70	2:28:70	5:25:70
A. niger	0^a	26.0 ± 5.0 [b]	38.0 ± 5.3 [c]	43.7 ± 5.7 [c]	49.7 ± 4.9 [c]
G. candidum	0^a	23.0 ± 5.3 [b]	25.7 ± 10.5 [b]	31.0 ± 11.4 [b]	35.3 ± 18.7 [b]
N. fischeri	0^a	22.7 ± 4.7 [b]	25.3 ± 7.8 [b]	32.7 ± 7.8 [b]	26.3 ± 7.5 [b]
P. roqueforti	0^a	24.7 ± 5.8 [b]	26.0 ± 8.7 [b]	37.3 ± 12.7 [b]	33.3 ± 19.9 [b]

[1] Potato dextrose agar supplemented with 100ppm each of chloramphenicol and chlortetracycline-HCL (pH 5.6 ± 0.2).

[a-c] Tukey's studentized range (HSD) test. Means with the same letters in each row are not significantly different (P>0.05).

CONCLUSION

Residual O_2 concentration in MA containing 30% CO_2, 70% N_2 affected the growth rate of common dairy spoilage molds when incubated under ideal environmental conditions. Reducing the residual O_2 concentration from 5.0 to 2.0% and 1.0 to 0.5% significantly decreased the radial growth over the 5 day incubation. Under ideal conditions, *Aspergillus niger, G. candidum, N. fischeri* and *P. roqueforti* are capable of growth at O_2 levels as low as 0.5%. Germination of spores was completely inhibited at 0.0% O_2. *Penicillium roqueforti* grew faster ($P>0.05$) than *A. niger* and *G. candidum* as indicated by the larger colony diameters (mm). The slowest growth rate ($P<0.05$) was seen in *Neosartorya fischeri*.

The lower atmospheric O_2 limit that will support growth of common dairy spoilage molds is less than 0.5% when incubated under ideal conditions. The most effective MA against mold spoilage is one that contains no residual O_2. Since MA are more effective against microbial growth as temperatures decrease, additional investigations are warranted to determine the critical level of residual O_2 in deterring fungal growth under refrigerated MA conditions.

ACKNOWLEDGEMENTS

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FUNGAL SPOILAGE IN COMMERCIALLY PROCESSED, EXTENDED SHELF-LIFE, ULTRA-PASTEURIZED FLUID MILK PRODUCTS

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ABSTRACT

Quality audits of commercial dairies showed a sporadic occurrence of yeast and mold in ultra-pasteurized (UP), extended shelf-life fluid milk products prior to the sixty-day shelf-life. Water, air, footbaths, paperboard, ingredients and raw milk were analyzed for yeast and mold content. Ten of each half-pint, pint, quart and half gallon filled cartons were randomly selected from all UP milk products available and stored at 7.2 °C. Samples, pulled at random on day 0, 15, 30, 45, and 60, were plated on Yeast and Mold Petrifilm™. Forty-seven percent of the UP products stored for 45 days tested positive for mold. Fungal growth was apparent down the side and along the bottom of the 5th panel. Contamination was traced to the presence of yeast and mold spores in paperboard cartons. Pinholes in the polyethylene coating and wicking at the unskived 5th panel allowed product seepage into the paperboard which provided favorable conditions for germination of mold spores. Fungi of similar origin and fatty acid profile were isolated from UP milk products, paperboard cartons that contained UP milk and blank cartons. Cartons and blanks contained less than 250 fungi g^{-1} of paperboard.

Key Words: extended shelf-life, ultra pasteurized, milk, fungi.

INTRODUCTION

Ultra-high temperature processing, 138°C for at least 2 seconds, of milk products in conjunction with clean packaging allows for a 60 day shelf-life, versus the typical 14 day shelf-life for high temperature short time pasteurization, 72 to 78°C for minimal 17 seconds (FDA, 1993). Extended shelf-life products enable dairies to expand their market segment, decrease distribution costs, and decrease financial losses due to the return of out-dated products. Cornell University estimated that 35% of total fluid milk losses were attributed to packaged milk returns (Stephenson et al., 1996).

Adequate refrigeration is an important factor in determining the shelf-life of ultra-pasteurized milk products. Schmidt et al. (1989) demonstrated that shelf-life decreased significantly as storage temperatures increased from 3 to 7 °C. Temperature abuse can occur at any time during distribution and the average temperature of retail and consumer refrigeration units is well above 4°C (Wyatt and Guy, 1980; Van Grade and Woodburn, 1987).

Commercially processed ultra-pasteurized (UP), extended shelf-life fluid milk products packaged in gable-top cartons (7.2°C) showed sporadic fungal contamination prior to the sixty-day shelf-life. Fungal contamination has been implicated in the spoilage of several processed dairy foods (Fedio, et al., 1994; Mannheim and Soffer 1996) and in citrus juice (Parish and Higgins, 1989; Parish and Higgins, 1990; Parish, 1991; Narciso and Parish, 1997).

Post-processing contamination is typically associated with unclean equipment, dirty packaging materials or poor air quality. *Cladosporium, Alternaria, Aspergillus, Penicillium, Acremonium and Fusarium* are among the more common airborne conidia that are associated with food spoilage (Alexopoulos et al., 1996). These same fungi have been isolated from wood, soil, (Alexopoulos, et al., 1996) and paperboard (Narciso and Parish, 1997). Many fungi are saprobes and attack dead matter as a food source. As agents of decay, fungi are the principle organisms associated with the decomposition of cellulose and lignin found in wood and wood products. Some Basidiomycetes are used by paper manufacturing facilities as biological pulping and bleaching agents in pulp and paper production (Alexopoulos et al., 1996).

During the paper manufacturing, wood fibers are subjected to a chlorination (Bendt, 1985). Free residual chlorine should be 0.2 to 0.5 ppm at the machine area in order to provide maximum protection. However, sulfite, sulfide, thiosulfate, coated broke, and urea formaldehyde that are used in the machine system will neutralize the effectiveness of chlorine which could lead to higher microbial counts in the paperboard. Microbial quality and safety guidelines set by FDA state that food-grade paperboard counts should not exceed 250cfu g^{-1} (Anonymous, 1991). These guidelines, commonly referred to as the "Dairyman's Standard", were established in 1948, when neither good sampling procedures, nor reliable plate count methods existed (May, 1994). Currently, no fungal standards exist for paper stock used in the manufacture of single service containers and closures for milk products.

Barrier films or laminates are applied to the food contact surface of paperboard to prevent the migration of bacteria and fungi into the food. Bleached board with a polyethylene (PE) coating is used to package milk and milk products. Imperfections in the PE coating can expose the food to the paperboard. The term "broken score line" describes a break in the PE coating along the score line. Breaks can be caused by low moisture content in the board or by impressing the score too deeply during the converting process. The board is stretched to a point where the board fibers break and the PE coating is ruptured. Broken score lines allow product to seep into the board which may result in a swollen appearance and possible leakage. Bottom sealing quality relates to the flow of polyethylene (PE) coating over the paperboard. This is measured by evaluating the number of channel penetrations and leaks. Bottom heat activation refers to the amount of pinholes in the PE coating due to bottom seal heating. High board moisture content or excessive heat upon sealing causes pin holing (Turner, 1996).

The objective was to isolate and identify the spoilage molds from UP, extended shelf-life milk and milk products and determine the origin of spoilage molds. A thorough examination of the dairy processing equipment, procedures, raw ingredients and finished products were necessary to accomplish these objectives.

MATERIALS AND METHODS

Plant sample collection

Line samples. Four ultra high temperature (UHT) fillers are used in the commercial processing plant from which all samples were collected. Filler 1, 2 and 3 fill half pints (HP), pints (PT), and quarts (QT); Filler 4 fills half gallons (HG). Each filler has two fill lines, designated as A or B. Eight samples (four from line A and five from B) of the particular product and size being filled were collected from each of the four fillers after sealing and dating. These samples never entered the cold storage facility.

Cold room samples. Ten samples, five from each line, of every UP product available were collected from the cold storage warehouse. Some products were already 10 to 30 days into code at time of collection.

Raw samples. Samples (50 ml) were collected from the raw milk and cream silos, cane sugar, fructose and corn syrup silos using sterile sample bags (Nasco, Fort Atkinson, WI). A cane sugar sample was collected as it entered the weigh tank to check for possible line contamination.

Dry storage samples. Bags of nonfat dry milk powder, UHT flavored base powder (cocoa), and half and half stabilizer were opened and sampled with the aid of sterile spoons and dispensed onto sterile 50g sample bags (Nasco, Fort Atkinson, WI).

Carton blanks. Eight flat carton blanks (unformed, unfilled cartons) were collected from each UHT filling machine in the fill room. In addition, 5 cartons of all sizes were requested for each of the UP products currently being processed at the dairy. These included the dairy's label as well as cartons used for subcontract packaging.

Erected, empty sealed cartons. Eight one-quart cartons (4 from line A and 4 from line B) were erected on Filler 2 without product in order to test the sterility of the magazine feed and the erectors within the machine. In the lab, cartons were injected with 200 mL of sterile malt extract broth using a sterile 50 mL syringe and needle. Cartons, periodically shaken once a week, were incubated at 25-28°C for the duration of the sixty-day code period. Two samples (one from each line, A and B) were randomly selected on day 14, 21, 45, and 60. They were opened and examined for mold growth and 1 mL malt extract broth was plated on Petrifilm™ Yeast and Mold (YM) Count Plates (3M Company, St. Paul, MN) (Beuchat et al., 1990; Beuchat et al., 1991).

Air samples. Air samples were collected throughout the plant using a Biotest Air Sampler RCS from Biotest Diagnostics Corp. (Denville, New Jersey) (Hickey et al., 1993). Air was sampled for two min using yeast and mold airstrips which were incubated at 25-28°C for 5 days and then counted.

Miscellaneous samples. Samples from the water supply, footbaths, and the cooling solution used to chill the mechanisms within the filling machines were collected.

Transportation and storage temperature

Samples were placed in coolers, covered with crushed ice and transported back to the university dairy laboratory for testing. Samples were stored in a walk-in cooler at 7.2°C for 0, 21, 45, or 60 days. This temperature was selected because previous audits showed that samples held at 7.2°C were more susceptible to mold growth than samples stored at 3°C.

Sample handling and enumeration

One g of each dry ingredient was suspended in a 9 mL and 99 mL phosphate/magnesium chloride dilution blank (Marshall, 1993), and mixed by shaking in a 45° arc for approximately 1 min. One mL duplicates were plated directly onto Yeast and Mold (YM) Petrifilm™ (3M Company, St. Paul, MN) and incubated at 25°C (Beuchat et al., 1990; Beuchat et al., 1991). Plates were counted after 5 days.

Two cartons per product and per container size were randomly selected from the cooler at day 0, 21,45, or 60 and shaken to insure a homogenous sample. Ethanol was used to sterilize a panel on the gable top lid. A flap measuring approximately 2.54 x 3.81cm was cut into the paperboard with a sterile knife, leaving the cartons top seal intact. One mL of milk was plated in duplicate on Petrifilm™ YM and incubated at 25°C for 5 days (Beuchat et al., 1991).

Raw samples were diluted by placing 1 mL of the sample into a sterile 99 mL phosphate/ magnesium chloride dilution blank (Marshall, 1993), and mixed using a 45° arc for approximately 1 min. In duplicate, 1 mL of the dilution was plated onto a Petrifilm™ YM and incubated at 25°C for 5 days.

The flap was closed by simply pushing the paperboard into the gabletop. Milk was held in the original carton until results were received from the initial screening with Petrifilm. Milk samples, positive for yeast and mold, were randomly selected and 0.1 mL of milk was plated on potato dextrose agar (Difco, Detroit, MI) with antibiotics (PDAA) as described by Beuchat (1979) and incubated at 25 °C for 5 days. The cartons were emptied and rinsed, then examined for mold as described below.

Carton evaluation

Carton blanks and cartons that contained moldy milk were evaluated using a modified version of the Disintegration method to determine the microbial content of paper container materials as described by Hickey et al. (1993). All procedures were conducted under a "bench type" airflow fume hood (Kewaunee Scientific Equipment Corp., Adrian, MI)

Four carton blanks were randomly selected from each of the nine types/sizes that were received from the plant. The side where the first and fifth panel join was sterilized on the surface and interior using ethanol and tissue wipes. Sterile scissors where used to cut a 5 g sample from the

blank which included the first and fifth panel. Sterile tweezers transported the sample to a 3% hydrogen peroxide solution that was used to mimic sterilization within the filling machine.

Surface sterility was determined by using a sterile cotton swab, pre-wetted with phosphate/magnesium chloride buffer. The swab was brushed over the surface of the paperboard sample, then placed on a PDAA plate and incubated at 25°C for 5 days.

The 5 g panel sample was cut into pieces (maximal size 1.3 cm), to aid in the grinding of the paperboard, and placed in a sterile Waring blender.   A 99 mL phosphate/magnesium chloride buffer was added to the blender and the blender covered with the lid. The mixture was blended at high speed for 5 min. After 2 min the blender was stopped and a sterile scrapper was used to remove paperboard pieces from the blender wall. High-speed degradation was resumed for an additional 3 min. The paper-blended liquid was decanted from the fibers and 1 mL of the liquid was plated in duplicate on Petrifilm™ YM. Plates were incubated for 5 days at 25°C. Twenty-four of the thirty-six carton blanks were also examined for aerobic bacteria. One mL of the paper-blended liquid was plated on 3M Petrifilm™ Aerobic Count (AC) plates and incubated at 37°C for 48 ± 3 hr. The lower limit of detection of the assay is 20 cfu g^{-1}.

Fungal mycelium was picked from Petrifilm™ YM with a sterile loop and streaked onto potato dextrose agar with antibiotics (PDAA) and incubated at 25°C for 5 days (Beuchat, 1979). The growth was then transferred, by a sterile loop, to sabouraud (SAB) dextrose broth or agar for growth, extraction and identification, as described below. Bacterial identification was not the objective of this study; therefore, no colonies were submitted for identification.

Mycology identification

 Microbial Identification System (MIS), distributed by Microbial ID, Inc. (Newark, DE), was used for computer identification of fungi. This system was previously used to identify bacterial contaminants in liquid paperboard (Pirttijarvi et al., 1996). The manufacturer's protocol, Fungal Library Culture Techniques (Appendix B), was followed for cellular fatty acid (CFA) extraction.

Erlenmeyer flasks (125 mL) containing 40 mL of sabouraud (SAB) dextrose broth (Difco Laboratories, Detroit, Mich.), were capped with reusable foam stoppers and autoclaved for 15 min (121°C @ 15 psi) then cooled. Broth was inoculated with the unknown mold mycelium. Broth cultures were incubated at 28°C in a shaking incubator at 150-RPM. Cultures were harvested, with the aid of a glass filtering system, once several mycelial balls appeared or when the broth turned cloudy. Flask contents were emptied into a funnel and a slight vacuum applied. Colonies, retained on the cellulose filter paper, were transferred to the extraction tube using a sterile spatula.

Fungal growth with yeast-like appearance was plated on 100-mm SAB dextrose agar (Difco Laboratories, Detroit, Mich.) plates. Cultures were incubated for 2 to 5 days at 28°C then colonies were transferred to fresh SAB dextrose agar and incubated for an additional 2 to 5 days at 28°C. Cells were transferred a total of three times before they were scraped from the plate.

Harvested cells were lysed and saponified with the addition of 1.0 mL sodium hydroxide-methanol solution (45g of NaOH, 150 mL methanol, 150 mL of deionized water). Tubes were

vortexed for 5 to 10 s, heated at 100°C for 5 min, vortexed for 5 to 10 s, heated for an additional 25 min at 100°C, then cooled. Fatty acids were methylated with 2 mL of a methanol-HCL solution (137.5 mL of methanol mixed with 162.5 mL of 6 N HCL), vortexed for 5 to 10 s, and heated at 80°C for 10 min. Tubes were rapidly cooled in an ice bath. Methylated compounds were extracted with 1.25 mL of hexane ether (200 mL hexane and 200 mL methyl-t-butyl ether) by rotating the tubes end over end for 10 min. Fungal colonies do not dissolve during the extraction procedure; thus, the top organic phase was transferred into a clean set of tubes and the bottom aqueous phase was discarded. Three mL of dilute sodium hydroxide (5.4 g of NaOH, 450 mL of deionized distilled water saturated with 125 g of NaCl) was added and tubes were tumbled end over end for 5 min. The upper solvent phase was transferred to a gas chromatograph sample vial, capped and analyzed according to the procedure described by Ghamen et al. (1991) and Moore et al. (1994).

The MIS (Microbial ID, Inc.) software was used to identify the peaks (by retention time) and to determine the area, the ratio of area to height, the equivalent chain length (ECL), the total area and the total area for the named compound. The profile of the unknown strain was compared to the library entries in order to find the reference that most closely resembled the unknown fungi. A "similarity unit" value of ≥0.500 is considered an acceptable identification, while a value less than 0.500 is a questionable identification (Ghamen et al., 1991).

Polyethylene coating test

Saffron red dye and Aerosol OT- Scarlet Moo stain was utilized to test the integrity of the bottom and top seals, as well as the interior polyethylene (PE) coating. Cartons were cut off approximately 7.5 cm from the bottom. Dye solution was poured into the bottom, allowed to stand for 15 sec, then poured out. Carton bottoms were rinsed under water, then corners were cut at the scores to achieve a flat bottom. Triangular gusset panels were lifted and the entire area was evaluated for dye marks according to diagrams supplied by Evergreen Packaging Division, Cedar Rapids, IA.

A similar procedure was used to evaluate "broken score lines", the term used to describe a break in the PE coating along the score line. Cartons were cut along the side seam, interior score lines were painted with dye and allowed to stand for 15 sec, then wiped off. Dye solution seeped into the paperboard at breaks in the PE coating.

Presence of pinholes, which occur on the interior PE coating, were also evaluated. Pinholes are due to the polyethylene coating being exceptionally thin or the use of excessive heat during the sealing process. Dye seeps into the board at the breaks in the PE coating, making a small dark red circle. Pinholes can develop along the score line or on the flat panel surfaces.

Experimental design

Two hundred and thirty (23 different types and/or sizes of UP milk, 10 cartons per type and/or size) samples were collected from the cold storage facility. Some of these samples were 26 days into their 45 or 60 day shelf-life at the time of collection. Forty-six samples (filling line A and B

from each of the 23 types) were evaluated for the presence of yeast and molds upon arrival at the laboratory. Future testing dates were selected over the remaining shelf-life and the balance of the 10 samples were tested at code date. Samples were plated in duplicate on Petrifilm™ YM.

Thirty-two (4 machines, 8 cartons per machine, 4 each from fill line A and B) line samples were taken directly off the filling machines. Two cartons were tested (one from line A and one from B) after storage of 0, 21, 45, and 60 days. Lab duplicates (1 mL) were plated on Petrifilm™ YM.

Fifteen additional samples including raw and dry storage samples were also tested for fungal contamination using Petrifilm™ YM. Dry samples were rehydrated in and raw samples were diluted in phosphate /magnesium chloride dilution blanks prior to the plating of lab duplicates.

Data analysis

Results were analyzed using a personal computer and a spreadsheet program capable of constructing graphs and tables (Microsoft® Excel 97, Microsoft® Corporation, USA, 1985-1996).

RESULTS AND DISCUSSION

Cold storage and line samples

Fifty-seven (25%) of the two hundred thirty samples analyzed from cold storage, were positive for mold growth by code date after being stored at 7.2°C. Two ultra-pasteurized milk products, a half-gallon and a quart carton of whole fat, vitamin D (HVD), were positive for mold within 10 days of processing. A non-fat quart, a 1% chocolate pint, a quart of 2% and a quart of strawberry, that were 28, 30, 31 and 37 days into code (expiration), respectively, also tested positive for mold growth. The remaining 51 samples were positive for mold at 45 and/or 60 days of code.

Line samples followed the same 45 day pattern. No samples were positive until 45 days into code, then 5 of the 8 (62.5%) tested positive. By the 60-day code date, 100% of the samples were positive for mold growth. Overall, 13 of the 32 (41%) line samples were positive for mold growth after being stored at 7.2°C.

Differences in the percent positive, 25% vs. 41%, is attributed to the fact that some samples collected from cold storage, 3°C (38°F), were 10 to 30 days into the code date (expiration) at the time of collection. These samples were held at 7.2°C (45°F) for the remainder of their shelf-life but the line samples were stored at the higher temperature (7.2°C) for their entire shelf-life. These results agree with Schmidt et al. (1989), who showed that shelf-life decreased as storage temperature increased from 3 to 7 °C.

Most positive cartons exhibited fungal growth along the unskived 5th panel and/or the raw edge of the bottom seal. Pirttijarvi et al. (1996) also noted bacterial growth along the unskived edge. The term "unskived" means it lacks a polyethylene coating, thus the product is exposed to the raw

paperboard. Some cartons contained a slimy film layer of fungi that was free floating or attached to the carton wall.

Product composition and package size

Products testing positive for YM occurred across all fat levels, in value-added products and in all container sizes (Table 4.1). Quarts of heavy cream, half gallons of 1% milk and half pints of table cream exhibited the highest percentage of positive samples, 60, 50, and 37.5% respectively. Pints of whole vitamin D, quarts of chocolate and half gallons of 1% chocolate milk were clear of fungal growth throughout the 60 day shelf life.

Fungal growth was evenly distributed among the four carton sizes; 21.4% of half-pints, 23.2% of the pints, 29.9% of the quarts, and 27.8% of the half gallons tested positive for mold growth. Positive samples were not limited to a single piece of filling equipment or an individual filling line (Fig. 4.1). Therefore, mold contamination could not be linked to unclean filling equipment, a particular container size or an added ingredient.

Miscellaneous Samples

Iodine solution collected from the filling room footbath averaged 194 cfu YM mL^{-1}. Dry ingredients showed no evidence of heavy mold contamination. Cane sugar, sampled at the weigh tank entrance, averaged 2 cfu YM mL^{-1}. However, freshly pasteurized products containing cane sugar were mold free, indicating that mold spores were eliminated by ultra pasteurization. Water supply and sanitation solution in the filling machine demonstrated no signs of fungal contamination.

Table 4.1- Percentage of ultra pasteurized (UP) milk products that tested positive for fungi while stored at 7.2°C and evaluated 0, 21, 45, and 60 days into code (expiration date).

		Percent (%) of Products Positive for Fungal Growth			
		Container Size			
UP Milk Product	N[1]	Half Pint	Pint	Quart	Half Gallon
Non Fat Milk	19	10.5	-[3]	21	-
1% Fat Milk	12	-	-	-	50
2% Fat Milk	38	-	15.8	15.8	10.5
Whole Vitamin D Milk	45	2.2	0	4.4	4.4
Table Cream	8	37.5	-	-	-
Half & Half	34	-	8.8	26	-
Heavy Cream	10	-	-	60	-
Chocolate Ice Cream Mix	9	-	-	-	33.3
Vanilla Lite Ice Cream Mix	9	-	-	-	11.1
Strawberry Milk	26	-	15.4	15.4	-
Chocolate Milk	29	-	3.4	0	3.4
1% Fat Chocolate Milk	23	-	8.7	-	0
N[2]	262	28	69	104	61
Percent of Positive Samples		21.4	23.2	29.9	27.8

[1] Number of samples collected per UP milk product.
[2] Number of samples collected per container size.
[3] No samples were collected.

Air samples

Cold storage air samples, which averaged 517 cfu YM m^{-3}, were well above the recommended level of <100 cfu m^{-3} (Biotest Diagnostics Corp., Denville, New Jersey). Filling room air counts were very low (12.5 cfu m^{-3}). The air sample taken at the filling head of filler 4 (1/2 gal) was clear of YM, indicating that the hepta filters were operating correctly. Comparison of cold storage and line sample results showed that a larger percentage (25% vs. 41%) of line samples tested positive for fungal growth. Line samples never entered the cold storage facility. Therefore, storage of dairy products in the cold room under elevated air counts had no apparent effect on the frequency of YM positive samples. Dry storage air samples were also high, averaging 458 cfu m^{-3}. Microbial evaluation of the dry ingredients in this storage area showed no evidence of mold contamination.
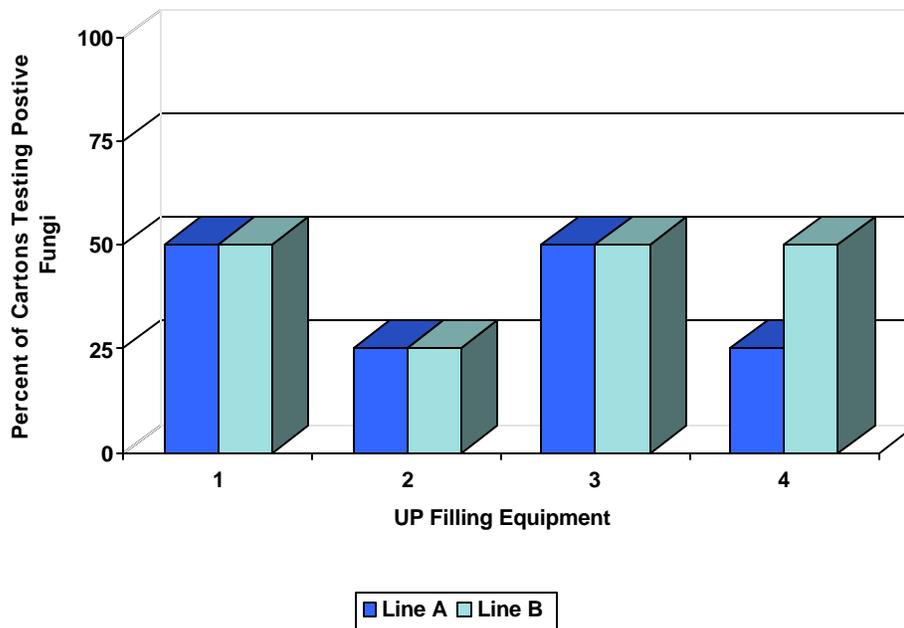
Fig. 4.1- Distribution of UP milk products testing positive for fungi over the various packaging machines.
Four paperboard cartons were collected per line per filler for a total of 32 samples. Samples were held at 7.2°C and analyzed on day 0,21,45,60. Fillers 1, 2 and 3 packages half pints (HP), pints (PT), and quarts (QT); Filler 4 packages half gallons (HG).

Bottom sealing and heat activation

Dye test performed on cartons that contained moldy milk indicated problems with the polyethylene coating and adhesives. Problems observed with bottom sealing quality included corner, side seam and/ or major flap channel leaks. Sealing quality is directly related to the quantity and smoothness of PE coating on the board.

Pinholes and broken score lines were noted on the bottom diagonal and bottom horizontal scores (Fig. 4.2). High board moisture content or excessive heat upon sealing is the typical cause of pin holing. Broken scores lines were also documented along the major vertical score. Breaks can be caused by low moisture content in the board or by impressing the score too deeply during the converting process. The board is stretched to the point where fibers break and the PE coating is ruptured. Broken score lines allow the product to seep into the board. This may result in a swollen appearance and/or a leaky package. The 5th panel leaked to the exterior of 4th panel in two cartons, and leaks were recorded at the #5 top diagonal score in most of the pints and quarts tested. These leaks are due to adhesive failure on the side panel (Anonymous, 1994).
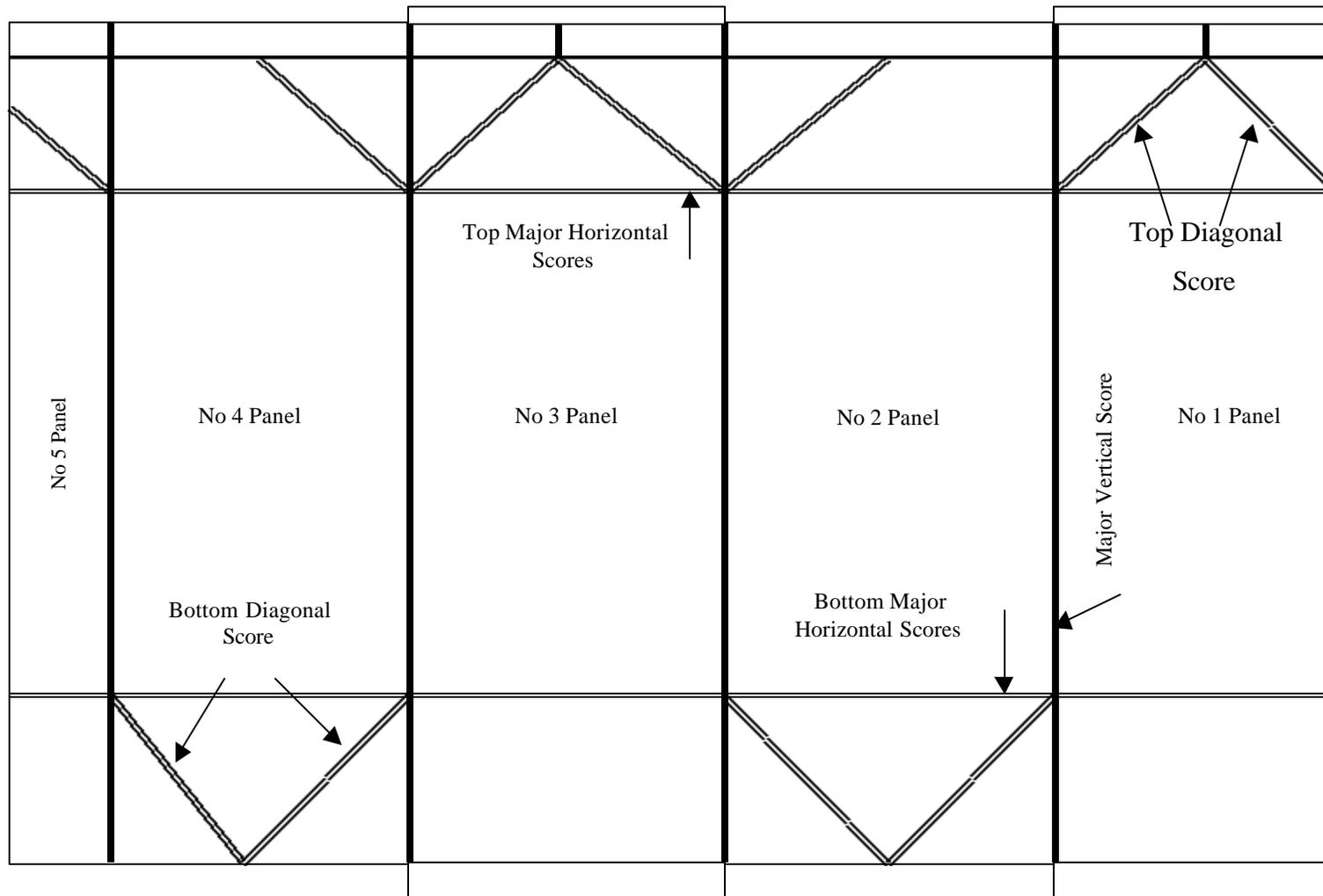
56

No 5 Panel

No 4 Panel

No 3 Panel

No 2 Panel

No 1 Panel

Top Major Horizontal Scores

Top Diagonal Score

Major Vertical Score

Bottom Diagonal Score

Bottom Major Horizontal Scores

Fig. 4.2 – Diagram of a paperboard carton "Blank"

Carton blanks (unformed, unfilled)

Six of the 36 carton "blanks" (17%) tested positive for fungal contamination, but total counts were less than 100 cfu g^{-1} (Table 4.2). Four of the 6 isolates were submitted for MIS identification. The system determined that the fatty acid profile of *Phoma* species best matched the profile of the unknowns. Second choices included *Acremonium, Aspergillus*, and *Geotrichum* species. Some cartons were subjected to irradiation by the manufacturer to reduce the microbial load, however they still tested positive for fungal contamination. Paperboard fibers from these cartons probably contained spores, which became viable under the proper growth conditions. Narciso and Parish (1997) isolated *Acremonium* and *Aspergillus* along with 12 additional genera of filamentous fungi from blank, gable-top paperboard fibers. These fungi are typically associated with environmental contamination of wood products (Leonard et al., 1990). Currently no fungal standard exists for paper stock used in the manufacture of single serve containers and closures for milk products.

Table 4.2 – Identification of fungi isolated from blank, gable-top paperboard cartons.

Carton Sample	Carton Size	Swab Test (Pos/Neg)	Y&M (CFU/g)	M.I.S.[a] Identification of Species	
				1st Choice (su)[b]	2st Choice (su)
1	Pint	Neg	79	Phoma (77)	Geotrichum (76)
"Irradiated"		Neg	41	Phoma (77)	Aspergillus (45)
		Pos	0		
		Neg	0		
2	Quart	Neg	0		
"Irradiated"		Neg	8	c	
		Neg	0		
		Neg	0		
3	Pint	Neg	21	Phoma (81)	Geotrichum (72)
		Neg	0		
		Neg	10	c	
		Neg	0		
4	Quart	Neg	0		
		Neg	63	Phoma (81)	Acremonium (71)
		Neg	0		
		Neg	0		
		Neg	0		

[a] Microbial Identification System (MIS) hardware and software used to identify fungi by comparing cellular fatty acid profiles with those of standard reference patterns.
[b] "similarity unit", a value of 0.500 or greater is considered an acceptable identification, < 0.500 is a questionable identification.
[c] Isolate not submitted for MIS Identification.

Twenty-four of the thirty-six "blanks" were also evaluated for aerobic spore count. Eleven paperboard cartons (42%) were positive for aerobic bacteria. All aerobic counts were less than 100 cfu g^{-1}, except for one QT container that contained 843 cfu g^{-1}. Bacterial contaminants were not identified by MIS. Pirttijarvi et al. (1996) isolated 218 strains of microbial contaminants from liquid packaging boards and blanks. Contaminants were aerobic spore forming bacteria, belonging mainly to the genera *Bacillus* or *Paenibacillus*. Isolation of yeast and molds was said to be rare. This may be because bacterial competition inhibits the growth of fungi (Beneke and Stevenson,

1987). Therefore, to achieve a true aerobic plate count, fungi need to be plated on selective agar and counted separately from bacteria.

"Sterile, formed" cartons

Erected, empty and sealed QT cartons, which were injected with sterile malt extract, followed the same pattern as cold storage and line samples. No growth was found in samples stored 15 or 28 days. However, cartons evaluated at 42, 49, and 58 days showed visual evidence of mold growth and counts were 10^5, 10^6, and 10^7 cfu mL^{-1}, respectively.   Results eliminated contaminated form, fill and seal equipment as a possible source of mold since cartons would have shown mold growth prior to the 42nd day of storage. Growth was concentrated at the unskived 5th panel and raw bottom edge. Fatty acid profile of the fungus isolated from the malt extract was identified by MIS as *Aspergillus* species. Fungi isolated from the "Sterile, formed" cartons' paperboard extract was identified as *Vlociadium* species.

Identification of mold isolated from UP milk and paperboard carton

Fungi isolated from the milk were identified by MIS as *Acremonium, Aspergillus, Candida, Cladosporium, Geotrichum, Penicillium, Phoma,* and *Vlocladium* species. Fungi isolated from the paperboard cartons, which contained this moldy milk, were classified as *Acremonium, Aspergillus, Botrytis, Candida, Chatomium, Geotrichum* and *Vlociadium* species. Many of these same species were isolated from cartons that had contained fruit juice (Narciso and Parish, 1997).

It is important to note that different species may have a similar fatty acid composition. Therefore it is critical that the library include as much fungal information as possible to prevent mislabeling of unknowns (Ghamen et al., 1991). However, a comprehensive fungal library does not exist at this time. Therefore, emphasis should not be placed on the genus assigned to the unknown because the genus may not be correct due to the limited database. However, the fact that the CFA profile of the fungi isolated from the milk is similar or identical to the profile of the paperboard isolate indicates that the contaminants are the same.

A good example of this is presented in Table 5.3. MIS determined that the fatty acid profile of a fungus isolated from a pint of contaminated milk was similar to *Acremonium* species (similarity unit = 0.519). The second choice was *Vlocladium* species, which had a similarity unit of 0.492. The fungus isolated from the paperboard fibers of that same pint of milk resembled *Vlocladium* species (0.499) more so than *Acremonium* species (0.491). The key is the CFA profile of the milk isolate is very similar to the paperboard isolate, thus the fungi that is in the paperboard is mostly likely responsible for contaminating the milk.

Table 4.3 – Methylated cellular fatty acids of fungi isolated from the Ultra Pasteurized (UP) milk and the paperboard cartons in which the milk was packaged.

FAME[a]		% (mean ± SD) of total chromatographic area in [b]:	
		UP Milk Isolate	Paperboard Isolate
16:0		15.54 ± 0.1	15.78 ± 0.2
18:0		3.73 ± 0.3	3.55 ± 0.1
18:1 cis 9 (w 9)		41.25 ± 0.0	43.64 ± 0.2
18:2 cis 9, 12/18:0a		35.87 ± 0.2	37.04 ± 0.1
MIS[c]	1st Choice similarity unit[d]	Acremonium 0.519	Vlocladium 0.499
	2nd Choice similarity unit[d]	Vlocladium 0.492	Acremonium 0.491

[a] FAME, fatty acid methyl ester.

[b] Values are the percentage of the total chromatographic area of products; -, minor peaks that were highly variable or undetected and not used in the MIS system's differential analysis.

[c] Microbial Identification System (MIS) hardware and software used to identify fungi by comparing cellular fatty acid profiles with those of standard reference patterns.

[d] "similarity unit", a value of 0.500 or greater is considered an acceptable identification, < 0.500 is a questionable identification.

Heat and steam in the paper making process normally kill vegetative cells of microorganisms. However a few species of bacteria, mainly *Bacillus* (Pirttijarvi et al., 1996) and many fungi, form spores that are very resistant to heat, chemicals, ultraviolet light, and ionizing radiation. The spores are dormant until the environmental conditions are favorable, then they germinate and vegetative cells develop (May, 1994). Immediate corrective action to prevent contamination would include: 1) limit exposure of UP product to the raw paperboard; 2) decrease paperboard spore loading; 3) improve the integrity of PE coating.

The citrus juice industry has limited exposure of the product to raw edges by switching to cartons with a skived fifth panel and a J bottom. A skived edge entails turning the raw edge of the 5th panel under, so the polyethylene coating is in contact with the product. Pirttijarvi et al. (1996) found few or no colonies on the inner, polyethylene-coated surfaces of skived cartons.

Eliminating fungal spores in the papermaking environment is the first step in reducing the spore loading. Raw material, water and additives used in the paper making process should be examined for fungal contamination (Bendt, 1985). Spore loading may also be reduced by treating the paperboard with an oxidizing agent, such as a hypochloride solution, prior to the polyethylene coating.

Paper manufacturers need to examine the integrity of the PE coating. Adhesive failure, pinholes and/or broken score lines were evident in all the cartons examined which contained moldy UP milk.

CONCLUSIONS

Fungi present in the paperboard cartons and blanks were of similar origin and fatty acid profile as those isolated directly from the UP milk products. Pinholes in the polyethylene coating and wicking at the unskived 5th panel allowed product seepage into the paperboard, which provided favorable growth conditions for mold spores. Under refrigeration temperatures (7.2°C), 45 days was adequate time for rehydration and proliferation of fungal mycelium. Growth was concentrated down the side and along the bottom of the unskived 5th panel. Results show the importance of proper refrigeration temperatures through out the 60 day shelf life.

Cartons contained less than 100 cfu $\bar{g}^1$ of yeast and mold spores. The Dairyman's Standard recommends a maximum level of 250 cfu g^{-1} for single-service food or milk containers. This guideline was established using general bacteriological growth media with no selective agents. Since fungi do not compete well with bacteria, a separate method and standard needs to be established to determine an acceptable level of yeast and mold spores that may be present in paperboard cartons. Many changes have occurred since the Dairyman's Standard was accepted as the basis for evaluating milk carton stock. It is time to reevaluate the microbiological quality and safety guidelines for paperboard. New specifications need to be established for paperboard that is being used to package extended shelf-life products.

ACKNOWLEDGEMENTS

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Glossary

Anamorph: the asexual stage in the life cycle of a pleomorphic fungus (cf. teleomorph, holomorph).

Asocmycetes: any of a class (Asocmycetes) of higher fungi (as yeast and molds) with septate hyphae and spores formed in asci.

Ascospore: one of the spores contained in an asci.

Ascus: the membranous oval or tubular spore case of an ascomycete.

Basidiomycetes: any of a large class (Basidiomycetes) of higher fungi having septate hyphae, bearing spores on a basidium, and including rusts, smuts, mushrooms, and puffballs.

Basidiospore : a spore produced by a basidium.

Basidium: a structure on the basidiomycete in which nuclear fusion occurs followed by meiosis and on which usually four basidiospores are borne.

Conidiogenous cell: a hyphal compartment or cell from which or in which a conidium is formed.

Conidiophore: a simple or branched hypha arising from a somatic hypha and bearing at its tip or side one or more conidiogenous cells.

Conidium: (pl. **conidia):** an asexually produced fungal spore formed on a conidiophore.

Deuteromycetes: fungi that only reproduce asexually.

Filamentous: an elongated thin series of cells attached to one another or a very long cylindrical single cell (as of some algae, fungi, or bacteria).

Hyphae: the threads that make up the mycelium of a fungus, increase the apical growth, and are coenocytic or transversely septate.

Mycelium: the mass of interwoven filamentous hyphae that forms the vegetative portion of the thallus of a fungus.

Obligate aerobes: oxygen is biologically essential for survival.

Phycomycetes: any of a large class (Phycomycetes) of highly variable lower fungi in many respects similar to algae.

Polyene : an organic compound containing many double bonds.

Septate: divided or having a septum.

Septum: a dividing wall or membrane especially between bodily spaces or masses of soft tissue.

Sterol: any of a group of predominantly unsaturated solid alcohols of the steroid group, such as cholesterol and ergosterol, present in the fatty tissues of plants and animals.

Classification of *Neosartorya fischeri* and its anamorph *Aspergillus fischeri* based on mode of spore production.

Classification	Asexual Spore Production	Sexual Spore Production
KINGDOM	*Fungi*	*Fungi*
DIVISION	*Eumycota*	*Eumycota*
SUBDIVISION	*Deuteromycotina*	*Ascomycotina*
CLASS	*Deuteromycetes*	*Ascomycetes*
SUBCLASS	*Hyphomycetidae*	*Plectomycetidae*
ORDER	*Moniliales*	*Eurotiales*
FAMILY	*Moniliaceae*	*Trichocomaceae*
GENUS	*Aspergillus*	*Neosartorya*
SPECIES	*fischeri*	*fischeri*
FRUITING BODY	Conidiophores	Cleistothecia, Conidiophores

APPENDIX C

Sensory Evaluation of Shredded Cheddar and Mozzarella Cheese

Experimental Design

Two bags of cheese per treatment were combined prior to sensory analysis in order to limit the number of samples evaluated by the sensory panel. The design resulted in a single response per treatment group, either *In* or *Out* of specifications for a saleable product. The dependent variable was dichotomous and coded as either zero (*Out*) or one (*In*).

Sensory results were analyzed using logistic regression. Logistic regression is used to model the effect of independent variables on a binary response variable (Glantz and Slinker, 1990; Hosmer and Lemeshow, 1989). The dependent variable represented two possible qualitative outcomes, *In* or *Out*. Logistic regression assumes that the probability of a success is a function of a set of regression variables. The objective was to find a subset of independent variables that could be combined to predict the value of the outcome variable.

Logistic regression could not be performed on treatments I-D-NS, I-D-S, I-I-NS and I-I-S due to quasicomplete separation in the data and the fact that the parameter estimates were not valid. Logistic regression was used to analyze results from the entire experiment (I-D-NS, I-D-S, I-I-NS, I-I-S, I-N-NS, NI-N-S, NI-N-NS, NI-D-NS, NI-D-S). The objective was to find a subset of independent variables that could be combined to predict the value of the outcome variable. The model attempted to predict the probability of the lower of the two values, which is not the desired result. However, the single bag per treatment response made it impossible to develop a model that would make valid predictions for sensory responses. Multiple responses per treatment should have been included in the original experimental design, or a different type of sensory analysis should have been utilized. To aid in the design of future experiments, it was decided that results would be interrupted with more leniency (P<0.25). Sensory research is considered exploratory and results must not be considered conclusive.

Results of sensory evaluation

The length of time that the package was open played a role (P < 0.25) in sensory evaluation of MAP shredded cheddar and mozzarella cheese. A 100% of the cheddar cheese samples were *"In"* specifications of a saleable and consumable product at day 0 (Fig. 1.0). By day 7, the proportion of bags that were "*In*" dropped to 82%. This decline in acceptability continued throughout the 21-day study with only 56% "*In*" specifications by day 14 and 3% "*In*" by day 21. On day 0, 70% of the mozzarella cheeses sampled were determined to be "*In*" specifications of a saleable product (Fig. 2.6). By day 7, 52% of the mozzarella samples were "*In*" and only 4% of the samples were "*In*" on day 14. Not a single bag of mozzarella cheese was "*In*" specifications after being open 21 days. Sensory scores of cheddar and mozzarella cheeses were not affected by storage period, *P. roqueforti*, oxygen scavenger and natamycin application and presence (P > 0.25).
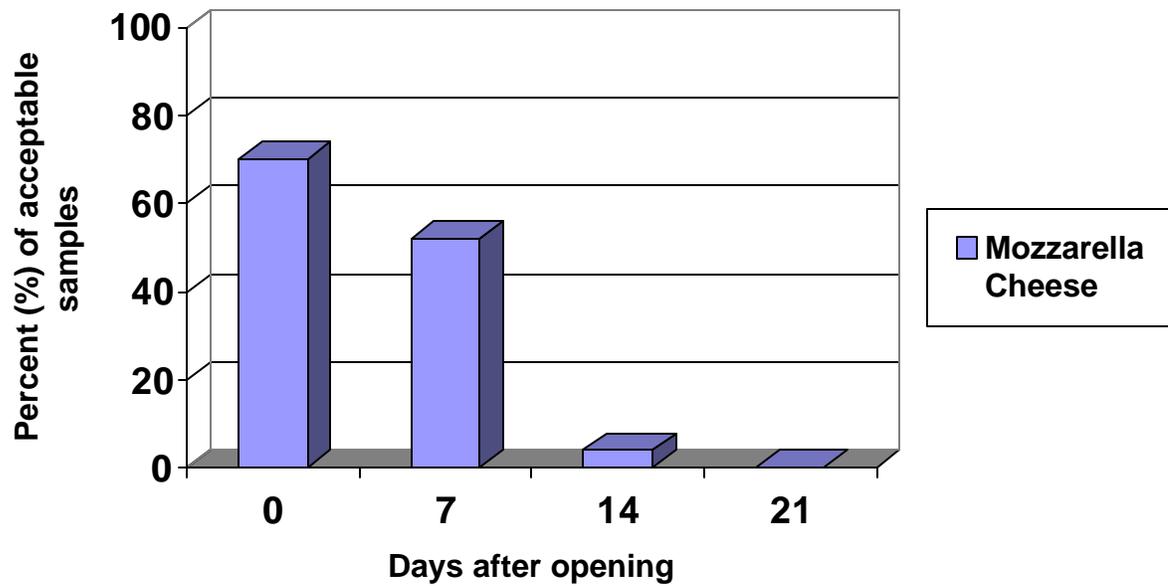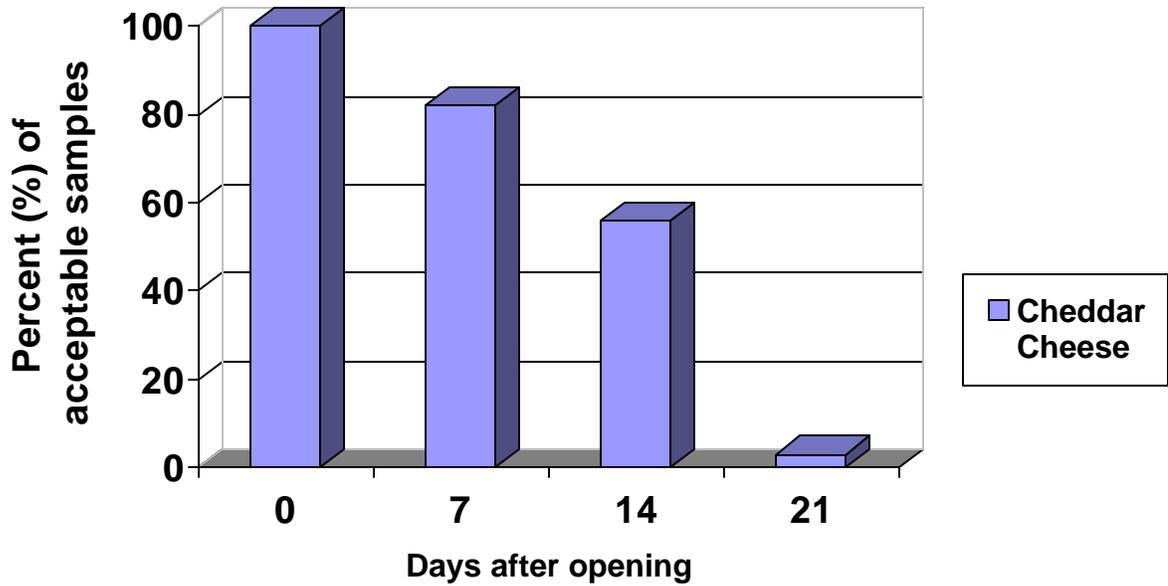
Fig. 1- Effect of length of time (days) a package has been open on the percent of MAP shredded cheddar and mozzarella cheese samples that are acceptable or "In" sensory specifications after 10°C storage. Cheddar (N=36) and mozzarella (N=27).

CURRICULUM VITA

Tina Moler Grove

Tina Marie Moler was born on October 18, 1965 in Winchester, Virginia to Charles Griffith and Juanita Demory Moler. She was the youngest of four children, John Jeffery Charles, Charmayne Danita, and Lisa Denise. She graduated from John Handley High School in 1984.

Tina received her BS degree in Dairy Science and a minor in Animal Science in May of 1988 from Virginia Tech, Blacksburg, VA. On November 19, 1988, Tina married Alan George Grove, son of George Milton and Ann Webb Grove of Waynesboro, Virginia. Alan is a 1985 graduate of the Virginia Tech Dairy Science department.

Tina completed her Master's degree in Dairy Science here at Virginia Tech in August of 1990, with an emphasis in physiology of lactation and management. That fall she entered the Ph.D. program in the Department of Food Science and Technology at Virginia Tech.   The next several years were a combination of working full–time and being a part-time student. In the spring of 2000, she received her Ph.D. in Food Science and Technology under the guidance of Dr. Joe Marcy and Dr. Cameron Hackney.

Honors:

       Alpha Zeta
       Gamma Sigma Delta
       Phi Sigma Society
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Grants:
       Virginia Tech Graduate Student Assembly Research Funding (96)

Public Service:

       Dairy Day at Valley View Mall, Roanoke, VA (91-95)
       IDFA's Capital Hill Ice Cream party (96)
       Mount Vernon's celebration of June is Dairy Month (94-95)
       Virginia Food Festival, Dairy exhibit, served ice cream   (90-94)
       Virginia State Fair, Dairy booth, served milk   (91-94)

Professional Organizations/Societies and University Service:

American Dairy Science Association. (84-97)
College of Agriculture & Life Sciences, Parking & Transportation Co-Chair (94-95)
College of Agriculture & Life Sciences, Strategic Planning Committee (93-94)
Institute of Food Technologists, National, CA/VA and Washington D.C. Sections (90-97)
Virginia Dairy Technology Society,   (94-97)
     Served as Corresponding Secretary /Treasurer (95-97)
     Second Vice-President (97-98)
     Virginia Tech Dairy Science Alumni Association: (88-97)
     Served as Treasurer, (92-94)
     Co-chaired the William M. Etgen Memorial Scholarship Fund (92-93)