Migration of *Penicillium spinulosum* from Paperboard Packaging to Extended Shelf Life Milk

by

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Abstract

Research was conducted to elucidate the cause of past fungal contamination experienced by local dairy processors in ultra-pasteurized, extended shelf life (ESL) fluid dairy products. Previous research at the Virginia Tech Department of Food Science and Technology indicated paperboard packaging as a potential source of fungi found spoiling the product. Preliminary research to isolate naturally occurring fungi from paperboard containers produced by various manufacturers found fungi to occur infrequently and sporadically.

Migration of *Penicillium spinulosum* from paperboard to milk during an extended shelf life, 60 days, was investigated through the inoculation of condia (spores) into paperboard test-squares created from gable-top carton flats designed for extended shelf life milk. These 57.2 by 57.2 mm (2 ¼ by 2 ¼ in.) test squares were sealed on three sides with melted paraffin wax, inserted with a steel pin at 3.2, 6.4, 9.5 and 12.7 mm from the uncoated edge (unskived edge) and sterilized by gamma-irradiation. A stock solution of *P. spinulosum* was injected at these four distances and the injection site sealed with melted paraffin wax. The squares were then incubated at 7°C in ultra-pasteurized skim milk for 60 days, allowing wicking of the milk to occur. An inverse relationship was seen between distance from the uncoated edge and presence of the organism in the surrounding milk. The percentage of milk samples containing *P. spinulosum* was 84% at 3.2 mm, 72% at 6.4 mm, 50% at 9.5 mm, and 28% at 12.7 mm from the uncoated edge. Survival of *P. spinulosum* in paperboard was investigated in two different studies. First, a study of survival over time used paperboard test-squares, which were inoculated, sealed on all four sides with paraffin wax and incubated as in the migration study in ultra-

pasteurized skim milk at 7°C. Five of the test-squares were separated to expose interior fibers and placed in malt extract broth with chloramphenicol to allow growth of any surviving fungi. Growth was transferred to potato dextrose agar and malt extract agar for identification. Survival was tested at 0, 10, 20, 30, 40, 50 and 60 days and it was found that *P. spinulosum* survived in the interior of paperboard for the entire incubation period. Secondly, survival was tested on all test-squares for which *P. spinulosum* was not detected in the surrounding milk in the migration study. Each test-square, excluding negative controls which were not inoculated with spores, was separated to expose interior fibers and placed in malt extract broth with chloramphenicol and identified as in the survival study. *Penicillium spinulosum* was detected in 94.4% of paperboard samples for which the milk tested negative.

The wicking characteristics of ultra-pasteurized skim and whole milk were investigated in four types of paperboard from gable-top carton flats designed for ultra-pasteurized fluid dairy products. Test squares were coated on 3 sides with melted paraffin wax and incubated in either ultra-pasteurized skim or whole milk at 7°C for 0, 10, 20, 30, 40, 50 and 60 days. A significant interaction was seen between the types of paperboard and milk. It is most likely that *P. spinulosum* at all inoculation distances had access to milk as a source of nutrition by day 40 in the migration study.

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Introduction

In the United States, all milk and milk products are subject to regulation by the Grade A Pasteurized Milk Ordinance (PMO). Under these guidelines all milk and milk products must be pasteurized except for a few specific exceptions. Conventionally pasteurized milk, at time and temperature combinations defined in the PMO, has been packaged with approximately a two-week code date. Two recent innovations, "ultra-pasteurized" and "ultra-high temperature" processed milk have provided additional options to consumers. Ultra-pasteurized milk is a refrigerated product with an extended shelf life when compared to the prevailing High Temperature, Short Time (HTST) pasteurization products. UHT milk is an aseptically packaged product requiring no refrigeration with a shelf life of several months. UHT milk is very popular internationally, because of the convenience of its long shelf life, but it has not found widespread acceptance in the United States.

The increased shelf life of ultra-pasteurized milk, while convenient for the consumer, has also increased the time available for growth of psychrotrophic spoilage microorganisms. Therefore, precautions must be taken to assure adequate thermal processing and to avoid post-pasteurization contamination. Contamination after pasteurization can occur from many sources including air, machinery, workers or packaging.

Most commercial packaging of milk is either in high density polyethylene (HDPE) jugs or gable-top paperboard cartons coated on both sides with polyethylene. In the United States, the Department of Health and Human Services sets the standards for milk packaging including bacterial quality of paperboard in "Fabrication of Single Service Containers for Milk and Milk Products". Later revisions of this publication include the use of reclaimed fiber for the production of paperboard milk cartons (FDA, 1993). While the use of recycled fiber is desirable for both environmental and economic reasons, paper products from recycled sources often contain more microorganisms than virgin paperboard.

Because of the extended shelf life of ultra-pasteurized milk, every effort must be taken to prevent contamination from paperboard packaging in order to ensure a high quality product. This research aims to examine the possibility of fungal contamination of milk by inoculating *Penicillium spinulosum* in paperboard packaging and investigating its ability to survive and grow in paperboard under refrigeration conditions.

Section I: Review of Literature

Ultra-Pasteurization

In order to increase the shelf life of milk, higher time-temperature combinations are used beyond that of normal pasteurization, but below that of ultra-high temperature (UHT) processing (Blake, et al. 1995). According to the Code of Federal Regulations 21, 131.3 (FDA, 1997b), and the Pasteurized Milk Ordinance (FDA, 1995) ultra-pasteurized milk is created by pasteurization at 138°C (280°F) or above for 2 or more seconds. This product must still be refrigerated and is referred to as extended shelf life milk (ESL). The extended shelf life allows longer shipping times and wider distribution of milk. The longer shelf life allows more time for growth of psychrotrophic microorganisms (Blake et, al. 1995).

ESL milk provides an intermediate shelf life product between HTST and UHT milk with greater consumer acceptability than aseptically packaged UHT milk. Blake et al. in 1995 evaluated the sensory characteristics of milk pasteurized by these different processes. They found that ESL milk was preferred over UHT processed milk, but conventional HTST milk was still preferred over both ESL and UHT milk.

Shelf Life of Milk

Because milk and other liquid dairy products are highly perishable, very stringent standards are enforced to ensure a safe product with a predictable shelf life. Many factors affect the shelf life of pasteurized milk including the quality of milk before processing, adequate heat processing, post-pasteurization contamination and storage temperature.

High quality milk prior to pasteurization is important in order to limit the number of thermoduric microorganisms capable of surviving pasteurization. Also, due to the ability of some organisms to produce enzymes that affect the character of the milk even after the organisms are eliminated, it is crucial to limit the levels of microorganisms as much as possible (Harding, 1995a; Nelson, 1981). Fecal contamination and mastitis infections seriously reduce the quality of milk and introduce not only potential spoilage organisms, but also pathogens (Harding, 1995a).

Pasteurization is intended to rid milk of possible pathogens, but not eliminate all spoilage organisms. Heat processing significantly reduces the number of psychrotrophic gram-negative bacteria in the milk, which are capable of rapidly causing spoilage, but leaves some of the more heat resistant gram-positive microorganisms that slowly grow to spoilage levels when milk is stored at proper temperatures (Harding, 1995b; Nelson, 1981). In addition to reducing microbial populations, pasteurization also inactivates the enzyme lipase naturally found in milk, which would otherwise result in rancidity of homogenized milk (Nelson, 1981).

Because pasteurization so significantly alters the microflora of milk, it becomes extremely important to prevent post-pasteurization contamination. Psychrotrophic spoilage organisms and pathogens capable of growth at low temperature can multiply with very little competition (Harding, 1995b; Nelson, 1981). *Listeria monocytogenes* is of great concern as an organism capable of contaminating milk after pasteurization because of its widespread occurrence and pathogenicity (Harding, 1995b).

In the United States the Pasteurized Milk Ordinance (FDA, 1997a) sets the highest allowable temperature for storage of pasteurized milk as 7°C or 45°F. The aim of this temperature is to significantly lengthen the generation time of most microorganisms to ensure a predictable shelf life for milk. Thermoduric organisms that may survive pasteurization are not usually well adapted to growth at low temperatures; therefore, proper storage can be a very effective means of inhibiting their growth (Harding, 1995b). Maintenance of temperature is important not only after pasteurization, but also before because of the ability of some bacteria to produce heat stable enterotoxins in raw milk and the general reduction of organisms in milk prior to processing (Nelson, 1981).

Contamination of Milk

A survey of spoilage microorganisms in dairy products by Walker in 1988 found that milk obtained directly from a healthy cow under hygienic conditions contains very few bacteria, yeasts or molds and generally is contaminated through handling and processing. This article states the four basic principles to prevent spoilage of dairy products as: (1) high quality raw materials, (2) consistent refrigeration at all stages, (3) monitoring critical processing points, and (4) good hygiene throughout. A study by Ren and Frank (1992) investigated aerosols at milk and ice cream plants as a source of contamination of milk. This study found a correlation between high levels of microbial aerosols and processing activity in the plant. To further examine microbial spoilage of milk, it is helpful to classify the contamination by type of organism: bacteria, yeast or mold.

Spoilage of Milk by Bacteria

Spoilage of milk by bacteria is particularly well documented. The bacterial contamination of pasteurized milk occurs by two methods when heat processing is properly performed, (1) by the ability to survive pasteurization or (2) by post-pasteurization contamination. Bacteria able to survive pasteurization and frequently isolated from pasteurized milk include the genera *Alacaligenes*, *Bacillus*, *Clostridium*, *Microbacterium*, *Micrococcus*, and *Streptococcus* (Stadhouders, 1975). In a spoilage incident of UHT chocolate milk in Trinidad, Antoine and Donawa (1990) attributed the cause to an *Enterobacter* and *Micrococcus* species, which were able to withstand high temperatures. It was concluded that these thermoduric organisms might have been able to withstand the UHT pasteurization process.

Of particular concern among heat resistant bacteria are those of the sporeforming, gram-positive *Bacillus* genus. Some of the *Bacillus* species pose a threat in pasteurized milk due to their ubiquitous nature, heat resistance, psychrotrophic growth and

enterotoxin production. Additionally, trends toward higher heat treatments and extended shelf life in pasteurized milk may tend to eliminate competition and select for *Bacillus sp*. (Meer et al., 1991). Several studies have shown that a low percentage of *B. cereus* isolates from pasteurized dairy products have the ability to both grow at refrigeration temperatures and produce toxins necessary for foodborne illness (Christiansson et al., 1989; Granum et al., 1993). Other *Bacillus* species of concern in pasteurized milk include *B. circulans*, *B. licheniformis*, *B. megaterium*, *B. pumilis* and *B. subtilis* (Meer et al., 1991; Stadhouders, 1975). Cromie et al. (1989) concluded that *B. circulans* posed a particular threat since it was isolated from milk pasteurized and stored at a variety of temperatures even when packaged aseptically.

Important gram-negative spoilage organisms of pasteurized milk include the genera *Achromobacter*, *Alcaligenes*, *Flavobacterium* and *Pseudomonas* due to the psychrotrophic nature of these organisms (Stadhouders, 1975). Other significant, but less frequently encountered gram-negative organisms in milk and dairy products include those of the genera *Acinetobacter*, *Aeromonas*, *Altermonas*, *Brucella*, *Campylobacter*, *Chromobacterium*, *Coxiella*, *Escherichia*, *Enterobacter*, *Hafnia*, *Klebsiella*, *Leptospira*, *Salmonella*, *Serratia*, *Xanthomonas* and *Yersenia* (Vasavada and Cousin, 1993). A study of Swedish and Norwegian pasteurized milk found the spoilage flora of pasteurized milk stored at 5°C for 3 weeks was 65% gram-negative bacteria (Ternström et al., 1993).

Spoilage of Milk by Yeasts

A variety of yeasts are frequently isolated from raw milk and raw milk products such as cheese, but are not considered to be a threat in pasteurized milk because of their susceptibility to heat processing (Cooke and Brazis, 1968). A study by Vadillo Machota et al. (1987) found that depending on the season, between 95 and 100 % of raw milk samples from a Spanish milk cooperative were positive for yeasts. Interestingly, a 1987 survey of pasteurized milk by Fleet and Mian found only a slightly reduced occurrence of yeasts with approximately 80% of samples positive for the presence of yeasts.

In pasteurized milk yeasts are often reported, but at low levels because the organisms usually are outcompeted by psychrotrophic bacteria at refrigeration temperatures (Fleet, 1990). The ability of fungi and yeasts to outgrow bacteria at lower temperatures was examined by Beuchat (1983). In circumstances where yeasts are not in competition with psychrotrophic bacteria for nutrients in pasteurized milk, yeasts are able to easily grow to large numbers and spoil the product (Fleet et al., 1987).

Spoilage of Milk by Fungi

Like yeasts, fungi do not compete well with bacteria in liquid products, such as pasteurized milk, unless the product contains high levels of sugar, salt or a high acidity which limits the growth of bacteria (Walker, 1988). However, the neutral pH of pasteurized milk provides an ideal environment for the rapid growth of psychrotrophic bacteria (Pitt et al., 1997). Mold spoilage of liquid dairy products in particular is not generally regarded as a critical concern; but is more predominant in solid and semi-solid dairy products such as cheese and cottage cheese (Walker, 1988). Molds and yeasts were implicated as a major factor in reducing the shelf life of pasteurized milk in Saudi Arabia in addition to psychrotrophic bacteria. In this study, molds, yeasts and psychrotrophic bacteria in combination were found to be the main cause of pasteurized milk reaching unacceptable sensory and microbiological health limits as set by the Saudi Arabian Standard Organization (SASO) (Salji, et al., 1988).

Isolation of fungi in raw milk has produced a variety of results. Vadillo Machota et al. (1987) isolated a variety of molds from raw milk with the most frequent genera in order of prevalence being *Geotrichum*, *Cladosporium*, *Penicillium*, *Aureobasidium* and *Aspergillus*. Comparatively, a similar study by Frevel et al. (1985) determined the most frequent genus of mold isolated from raw milk to be *Phoma*, followed by *Mucor*, *Fusarium*, *Penicillium* and *Aureobasidium*. In agreement with Frevel et al. (1985),

Cooke and Brazis (1968) found *Phoma herbarum*, a plant pathogen (Samson et al., 1996), to be the most frequently isolated species of fungi from raw milk.

Major fungal spoilers of dairy products in general include the genera *Geotrichum*, *Aspergillus, Penicillium, Mucor* and *Alternaria* according to a study by Walker in 1988. Due to storage conditions, the fungi that are found spoiling dairy products are generally psychrotrophic. Mold growth on dairy products results in spoilage because of off-flavors and aromas and/or visible growth on the products. These off-flavors and aromas are described by Walker (1988) as "ammoniacal, fruity or musty."

The occurrence of fungi in milk is reason for concern beyond loss of product due to spoilage; the potential for the production of mycotoxins must also be considered especially due to the high consumption rates of milk among youth. For pasteurized milk, most consideration is given to aflatoxin M_1 and M_2 , a metabolized product of aflatoxin B_1 and B_2 produced by *Aspergillus flavus* and *A. parasiticus*. This is a secondary form of mycotoxin contamination resulting from aflatoxin B_1 and B_2 in the feed of dairy cows, which is metabolized to M_1 and M_2 in the cow's body and then excreted in the milk (Lück and Wehner, 1979; van Egmond, 1989b). In a 1976 study by Paul et al., 6.2% of milk samples were contaminated with aflatoxin M, but no aflatoxin B or G was detected in any of the 81 samples. A study of 60 milk samples by Fritz and Engst (1981) found detectable levels of aflatoxin M_1 in milk produced during winter months when herds are fed stored grains as opposed to grazing. Ochratoxin A and sterigmatocystin are two mycotoxins that present potential carry-over effects in lactating cows from contaminated feeds, but are not considered a reason for serious concern (Lück and Wehner, 1979; van Egmond, 1989a).

Direct contamination of pasteurized milk by mycotoxins is not a frequent occurrence (Scott, 1989). Vadillo Machota et al. (1987) isolated 22 strains of *A. flavus* from unpasteurized milk, but only 1 was capable of producing aflatoxin even under ideal conditions. In 1986, Malik et al. found curdled milk was an adequate medium to support the growth of *Penicillium verrucosum* var. *cyclopium* and production of the mycotoxin

cyclopiazonic acid at 25°C. In light of the largely negative results for the detection of mycotoxins in pasteurized milk stored at proper temperatures, it is important to note that the number of fungi capable of producing toxic metabolites is very large and increasing with improving detection methods (Fritz et al., 1981).

It should be emphasized that low levels of fungi in unpasteurized milk do not pose a serious health threat unless the species isolated possesses heat resistant properties. This was the case in a study by Frevel et al. (1985) where fungi of the genus *Byssochlamys* were detected infrequently in raw milk samples. Several members of the genus *Byssochlamys* are known to produce such toxic metabolites as patulin and byssochlamic acid (Samson et al., 1996). Additionally, due to the high temperatures involved in UHT pasteurization, Pitt and Hocking (1997) concluded that most spoilage of UHT milk products due to fungi could be attributed to post-pasteurization contamination.

Milk Packaging

While paperboard production overall is increasing, production of paperboard milk cartons has been in decline since the early seventies when it reached a peak of 940.7 thousand tons for the year. In 1995, the figure dropped to 626.9 thousand tons according to *1996 Statistics Data through 1995* by the American Forest and Paper Association. However, production of sanitary paperboard food containers in general is slowly increasing with an estimated value of \$3.6 billion in 1998. Additionally, production is estimated to increase by 1% for 1999 (Huck, 1999).

A paper cap with a glass container was the packaging standard for milk for years. Paperboard milk containers coated with paraffin wax did not become widespread until the late 1930's, when the milk industry was among the first to use paperboard containers for a liquid product (May, 1994).

Guidelines for Paperboard Packaging of Milk

Guidelines for pasteurized milk containers in the United States are outlined by the Food and Drug Administration publication, "Fabrication of Single Service Containers and Closures for Milk and Milk Products" (1993). According to this document, containers should be made of paper stock containing less than 250 colonies per gram by disintegration method before lamination, 50 colonies per container by rinse method, or 10 colonies per container in those holding less than 100 ml. By swabbing, a container should contain less than or equal to 1 colony per square centimeter (FDA, 1993). This document does not contain specific guidelines for yeasts or molds on or in milk containers.

The 1966 publication of "Fabrication of Single Service Containers and Closures for Milk and Milk Products", defines acceptable paper stock as that which is derived from virgin pulp only:

"Clean sanitary paper stock shall mean any paper, paperboard, and laminated paper made from clean, sanitary virgin chemical or mechanical pulp or from broke, waste, or cuttings of such paper, paperboard, and laminated paper, provided they have been handled, treated, and stored in a clean, sanitary manner".

More current revisions of the FDA publication (1993) add the condition that paperstock could also be made of reclaimed fiber as governed by Title 21 Code of Federal Regulations, (CFR) 176.260.

Microbiology of Paperboard

Historically, bacteria have been isolated much more frequently from paperboard packaging than molds and yeasts and as a result have been the focus of considerably more research. This may be attributed to the belief that chemicals and heat used during paperboard production are more effective against fungi than bacterial spores (Pirttijärvi et al.,1996). Bendt (1985), Pirttijärvi et al. (1996), Suihko and Skyttä (1997), and Kneifel and Kaser (1994) concluded that aerobic, spore-forming bacteria are the most frequently encountered class of bacteria in food-grade paperboard because of their extreme resistance to heat and chemicals. According to May (1994), some of these spore-forming bacteria are capable of surviving the papermaking process as viable, but dormant cells remaining in the paper or paperboard, which can later germinate when conditions are favorable.

Bendt (1985) along with Väisänen et al. (1989) found *Bacillus*, a gram-positive, spore-forming rod, to be the genus encountered most often in finished paperboard. The species *B. cereus* elicits particular concern because of its frequency of occurrence in paperboard and its ability to cause foodborne illness. Pirttijärvi et al. (1999) detected numerous *B. cereus* isolates from food grade paperboard and paperboard machinery, many of which were capable of producing enterotoxin, which is believed to be responsible for the diarrheal form of the illness (Christiansson, et al., 1989; Granum et al., 1993; Meer et al., 1991). Pirttijärvi et al. (1996) found the most common isolates of microorganisms contaminating liquid paperboard packaging were spore-forming bacteria of the genus *Bacillus* and *Paenibacillus*.

In the 1994 study by Kneifel and Kaser, polyethylene coated paperboard milk containers were analyzed by disintegration with mean microbial counts as follows:

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|-----------------------------------|---|
| Type of Microorganism | CFU/g of |
| | carton material |
| Total aerobic mesophilic bacteria | 4.3×10^2 |
| Aerobic sporeformers | 3.1×10^2 |
| Anaerobic sporeformers | 1.5×10^{1} |
| Enterobacteria | less than10 |
| Yeasts and Molds | 1.4×10^{1} |

Table 1: Quantification of microorganisms in paperboard milk cartons

From Kneifel and Kaser, "Microbiological quality parameters of packaging material used in the dairy industry". 1994

A study by Väisänen et al. in 1991, found fungi at comparably low levels of not detectable to 10^2 CFU/g of carton material in polyethylene coated paperboard designed for foods. Suihko and Skyttä (1997) in a study of paperboards containing some recycled fiber also found low levels of fungi in finished board. This study additionally included pulp prior to chemical and heat treatment in the papermaking process where mold levels of 10^2 to 10^3 cfu/g were seen. However, Pirttijärvi et al. (1996) did not find any significant level of fungi or yeast in liquid paperboard packaging.

Suominen et al. (1997) in examining food grade paperboard, found bacterial counts varied significantly based upon the presence or absence of a mineral coating on the board just beneath the polyethylene barrier. Board without a mineral coating had bacterial levels of less than 10^3 CFU/g of paperboard, while board with a mineral coating had levels ranging from 8 x 10^4 to 2 x 10^6 CFU/g of paperboard. Even in paperboard without a mineral coating these researchers suggested that a possible origin of bacteria at the polyethylene-board interface might be starch used as a surface-sizing agent by board manufacturers.

May (1994) sites recycled pulp as a possible source of contamination of paperboard. He states that paperboard made from virgin wood usually contains a much lower level of bacterial contamination than recycled pulp does. Because of this, virgin pulp is usually within the standards set for milk containers. Vincent (1995) of James River Corp. supports the idea that food grade paperboard made from recovered materials likely poses no threat of microbial contamination, but that microorganism migration from raw edges of paperboard should be investigated before the widespread use of recycled fiber in food packaging.

Pirttijärvi et al. (1996) in an examination of the food-contact polyethylene surface of liquid packaging boards found very few microorganisms present. Because of these findings, the researchers concluded that microorganisms of paperboard origin would have to enter the product through "the raw edge, cutting dust from the processing of the blanks, or damaged polyethylene coating facing the board". Suominen et al. (1997)

exposed food grade polyethylene coated paperboard to water and nutrients under ideal conditions and found that over a 90-day exposure, bacterial numbers increased in the packaging when viewed with a laser scanning microscope. Their studies found that the bacteria in paperboard were generally at the interface of the paperboard and polyethylene coating facing the nutrient and water supply. Bacteria did not migrate into the paperboard matrix even after 3 months of incubation. They concluded that the bacteria that posed a threat of entering the product were those located at this polyethylene-paperboard interface.

Leonardi, Blakistone and Kyryk (1990) using electron microscopy on uninoculated samples determined that fungal and bacteria growth can extend through pits of paperboard and polyethylene coating in the case of Mead coated Natural Kraft. Their research showed that certain bacteria and fungi have the ability to degrade paperboard to some extent. Suominen et al. (1997) through analysis of disintegrated paperboard, found the packaging contained adequate nutrient levels and pH range for bacterial growth. These researchers concluded that water was the limiting factor for bacterial growth in paperboard.

Microbiology of Wood

Based on microscopy studies of microbial growth in paperboard, Leonardi et al. (1990) stated that: "It was concluded that manufactured wood-based products degrade similarly to wood in the native state". It is therefore appropriate to examine the degradation of wood to better understand the microbiology of paperboard. Fungi are the most common microorganisms to grow in and on wood and wood products, though bacteria do also play a limited role (Zabel and Morrell, 1992).

The requirements for growth of fungi in wood include free water, oxygen, appropriate temperature, pH, and a source of nutrients for metabolism (Zabel and Morrell, 1992). However, wood that is saturated with water is generally resistant to breakdown by fungi because of a lack of free oxygen, but is then more susceptible to the growth of bacteria (Zabel and Morrell, 1992). Most fungi are obligate aerobes, some of which are able to survive at low percentages of oxygen as long as the total amount of oxygen in solution is great enough (Pitt and Hocking, 1997). The ideal pH for most fungi is reported as 3 to 6 (Zabel and Morrell, 1992) or 4 to 6 (Rayner and Boddy, 1988) with an optimum of 5 to 6.5 for most plant pathogens (Zabel and Morrell, 1992). Wood decay fungi are usually mesophillic organisms, growing best between 20 and 30°C (Rayner and Boddy, 1988).

Fungi capable of wood decay must be able to obtain nutrients from the carbohydrates contained in the numerous associations of lignin, cellulose, hemicellulose and pectin found in wood. Lignin serves as a barrier to many microorganisms capable of digesting cellulose and hemicellulose. Cellulose, hemicellulose and pectin are broken down externally by oxidases and hydrolases requiring a thin layer of film for exchange of nutrients and contact with exocellular enzymes (Rayner and Boddy, 1988; Zabel and Morrell, 1992). Nitrogen is often a limiting factor in the ability of a species of fungus to be sustained by wood because of the low levels available and the high nitrogen needs of fungi (Zabel and Morrell, 1992).

Penicillium spinulosum

Penicillium spinulosum is a xerophile and psychroptroph not known to produce any mycotoxins (Pitt and Hocking, 1997). *Penicillium spinulosum* is highly similar to *P*. *glabrum* and is differentiated by the rougher exterior of the conidia of *P. spinulosum*. In addition, the phialades of *P. spinulosum* are narrower than those of *P. glabrum* (Pitt et al., 1990).

Penicillium spinulosum is a widely distributed organism that is frequently isolated from soil (Raper and Thom, 1984) and occasionally from grains (Basu and Mehrotra, 1976). In a 1997 study by Narciso and Parish, *P. spinulosum* was isolated from the paperboard orange juice cartons of all four manufacturers tested. In addition, this species

was found in juice contained in a carton of the type where *P. spinulosum* was isolated in this same study.

Contamination of Citrus Juices

Concerns similar to those of the dairy industry have been raised in recent years about fungal spoilage of citrus juices packaged in paperboard cartons. Wyatt and Parish (1995) concluded that the recent ability to extend the shelf life of citrus juices to 60 days or more through pasteurization provides any fungus in the product with the time needed to proliferate. This concept is repeated by Parish and Higgins in 1989 when they stated that molds and yeasts were not the greatest concern in short lifetime juices because of the superior ability of bacteria to grow in liquids.

In a study by Narciso and Parish (1997), a large variety of fungi were isolated from gable-top paperboard cartons for citrus juices, with the most common genus being *Penicillium* then *Aspergillus*. Several species of fungi isolated from paperboard cartons were also isolated from orange juice in the cartons by the same manufacturer. This research additionally showed the ability of several fungi to grow solely on sterilized paperboard carton material. Wyatt and Parish in 1995 confirmed that a number of orange juice spoiling fungi have shown the ability to grow at refrigeration temperatures with some able to germinate at temperatures as low as 0°C. Research by Sadler et al. (1992) found differences in yeast and mold levels of pasteurized orange juice packaged in paperboard cartons with and without an oxygen barrier. Paperboard cartons with the oxygen barrier layer had lower counts of yeast and mold in the orange juice than did cartons without the oxygen barrier.

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Section II: Migration of *Penicillium spinulosum* from Paperboard Packaging to Extended Shelf Life Milk

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Key words: P. spinulosum; extended shelf life milk; paperboard

Abstract

The growth and survival of the psychrotroph Penicillium spinulosum in paperboard was studied along with the wicking characteristics of ultra-pasteurized milk to understand sporadic fungal contamination of ultra-pasteurized, extended shelf life milk products. Previous research has indicated paperboard packaging as a potential source for the fungal contamination. Migration from paperboard to ultra-pasteurized skim milk during a 60-day shelf life, was investigated by inoculating condia (spores) into sterilized paperboard squares (57.2 by 57.2 mm) made from ultra-pasteurized milk cartons. Testsquares were sealed on three sides and inoculated at 3.2, 6.4, 9.5 and 12.7 mm from the uncoated (unskived) edge. The surrounding milk was tested for the presence of the fungus. Penicillium spinulosum was detected in 84% of samples at 3.2, 72% at 6.4, 50% at 9.5, and 28% at 12.7 mm from the uncoated edge. Survival in paperboard was investigated in sealed paperboard test-squares incubated in ultra-pasteurized skim milk at 7°C every 10 days up to 60 days. Penicillium spinulosum survived in the interior of paperboard for the entire incubation period. Survival was also measured on all testsquares for which *P. spinulosum* was not detected in the surrounding milk in the migration study. *Penicillium spinulosum* was detected in 94.4% of all negative samples. The wicking characteristics of ultra-pasteurized skim and whole milk were measured in four boards from gable-top cartons for ultra-pasteurized milk products. Test-squares were sealed on 3 sides and incubated in ultra-pasteurized skim or whole milk at 7°C. Wicking distances were measured every 10 days up to 60 days. A significant interaction was seen between the types of paperboard and milk. It is most likely that *P. spinulosum* at all inoculation distances had access to milk as a source of nutrition by day 40 in the migration study.

Introduction

Historically, bacteria have been isolated much more frequently from paperboard packaging than molds and yeasts and as a result have been the focus of considerably more research. This may be attributed to the belief that chemicals and heat used during paperboard production are more effective against fungi than bacterial spores (Pirttijärvi et al., 1996). Because of the lesser focus on fungi in paperboard, there are no specific guidelines for limits of fungi and yeast in paperboard as there are for bacteria (FDA, 1993)

Bendt (1985), Pirttijärvi et al. (1996), Suihko and Skyttä (1997), and Kneifel and Kaser (1994) concluded that aerobic, spore-forming bacteria are the most frequently encountered class of bacteria in food-grade paperboard because of their extreme resistance to heat and chemicals. According to May (1994), some of these spore-forming bacteria are capable of surviving the papermaking process as viable, but dormant cells remaining in the paper or paperboard, which can later germinate when conditions are favorable.

In the 1994 study by Kneifel and Kaser, polyethylene coated paperboard milk containers were analyzed by disintegration and found to have a mean fungal count of 1.4 x 10^1 CFU/g of carton material. A study by Väisänen et al. in 1991, found fungi at comparably low levels from not detectable to 10^2 CFU/g of carton material in polyethylene coated paperboard designed for foods. Suihko and Skyttä (1997) in a study of paperboards containing some recycled fiber also found low levels of fungi in finished board. This study also included pulp prior to chemical and heat treatment in the papermaking process where mold levels of 10^2 to 10^3 cfu/g were seen.

Subminent et al. (1997) in examining food grade paperboard found bacterial counts varied significantly based upon the presence or absence of a mineral coating on the board just beneath the polyethylene barrier. Board without a mineral coating had bacterial levels of less than 10^3 CFU/g of paperboard, while board with a mineral coating

had levels ranging from 8 x 10^4 to 2 x 10^6 CFU/g of paperboard. Even in paperboard without a mineral coating, these researchers suggested that a possible origin of bacteria at the polyethylene-board interface might be starch used as a surface-sizing agent by board manufacturers.

May (1994) sites recycled pulp as a possible source of contamination of paperboard. Paperboard made from virgin wood usually contain much lower levels of bacterial contamination than recycled pulp (May, 1994). Because of this, virgin pulp is usually within the standards set for milk containers. Vincent (1995) of James River Corp. supports the idea that food grade paperboard made from recovered materials likely poses no threat of microbial contamination, but noted that microorganism migration from raw edges of paperboard should be investigated before the widespread use of recycled fiber in food packaging.

Pirttijärvi et al. (1996) in an examination of the food-contact polyethylene surface of liquid packaging boards found very few microorganisms present. Because of these findings, the researchers concluded that microorganisms of paperboard origin would have to enter the product through "the raw edge, cutting dust from the processing of the blanks, or damaged polyethylene coating facing the board". Suominen et al. (1997) exposed food grade polyethylene coated paperboard to water and nutrients under ideal conditions and found that over a 90-day exposure, bacterial numbers increased in the packaging when viewed with a laser scanning microscope. Their studies found that the bacteria in paperboard were generally at the interface of the paperboard and polyethylene coating facing the nutrient and water supply. Bacteria did not migrate into the paperboard matrix even after 3 months of incubation. They concluded that the bacteria that posed a threat of entering the product were those located at this polyethylenepaperboard interface. Leonardi, Blakistone and Kyryk (1990) using electron microscopy on uninoculated samples determined that fungal and bacteria growth can extend through pits of paperboard and polyethylene coating in the case of Mead coated Natural Kraft.

Their research showed that certain bacteria and fungi have the ability to degrade paperboard to some extent.

More extensive research has been conducted in the citrus juice industry due to concerns similar to those of the dairy industry, which have been raised in recent years about fungal spoilage of citrus juices packaged in paperboard cartons. Wyatt and Parish (1995) concluded that the recent ability to extend the shelf life of citrus juices to 60 days or more through pasteurization provides any fungus in the product with the time needed to proliferate. This concept is repeated by Parish et al. in 1989 when he stated that molds and yeasts were not the greatest concern in short lifetime juices because of the superior abilities of bacteria to grow in liquids.

In a study by Narciso and Parish (1997), a large variety of fungi were isolated from gable-top paperboard cartons for citrus juices, with the most common genus being *Penicillium* then *Aspergillus*. Several species of fungi isolated from paperboard cartons were also isolated from orange juice in the cartons by the same manufacturer. Wyatt and Parish (1995) previously confirmed that a number of orange juice spoiling fungi have shown the ability to grow at refrigeration temperatures with some able to germinate at temperatures as low as 0° C.

Penicillium spinulosum is a xerophile and psychroptroph not known to produce any mycotoxins (Pitt and Hocking, 1997). *Penicillium spinulosum* is highly similar to *P*. *glabrum* and is differentiated by the rougher exterior of the conidia of *P. spinulosum*. In addition, the phialades of *P. spinulosum* are narrower than those of *P. glabrum* (Pitt et al., 1990). *Penicillium spinulosum* is a widely distributed organism that is frequently isolated from soil (Raper and Thom, 1984) and occasionally from grains (Basu and Mehrotra, 1976). In the1997 study by Narcisso and Parish, *P. spinulosum* was isolated from the paperboard orange juice cartons of all four manufacturers tested. In addition, this species was found in juice contained in a carton of the type where *P. spinulosum* was isolated in this same study.

The objectives of this study are (1) to evaluate the transmission of *P. spinulosum* through an uncoated edge of paperboard from milk containers into milk during an extended shelf life at refrigeration temperatures, (2) if transmission occurs, to determine a relationship between distance of fungal spores from the uncoated edge and the likelihood of milk spoilage at code date, (3) to assess the survival of *P. spinulosum* in the interior of paperboard and (4) to examine the effect of ultra-pasteurized skim and whole milk on the wicking characteristics of a variety of paperboard from gable-top cartons for extended shelf life liquid dairy products.

Materials and Methods

Organism

Penicillium spinulosum Thom, ATCC # 48226 (American Type Culture Collection, Manassas, VA), used in this study, is an isolate from rotting preserved wood in soil from Australia.

Spore Preparation

A supply of condia (spores) from *P. spinulosum* was harvested by flushing malt extract agar (MEA) (Difco, Detroit, MI) plates containing a 10-day growth of the selected organism with 10 ml of sterile 0.1 % peptone (Difco, Detroit, MI) dilution water three times then pipetted into sterile test tubes. These test tubes were vortexed for two min and the spore suspension was poured through sterile glass wool and collected in sterile centrifuge tubes. These tubes were centrifuged at low speed for 10 min without braking in order to prevent disturbance of spore pellets. The dilution water was pipetted off the spore pellet, which was resuspended in 5 ml of 0.1% peptone broth. With constant mixing, 1.0 ml of the spore suspension was pipetted into 2.0 ml cryogenic tubes, immersed in liquid nitrogen for 24hr and stored in a -80°C freezer. The overall spore

concentration was determined by spread plating 0.1 ml of the thawed spore suspension on MEA and incubated at 25°C for 4 days.

For each day of inoculation, a tube of spores was thawed and diluted in 0.1% peptone to a target of 100 cells per 0.025 ml. Depending on the overall concentration determined for the tubes of *P. spinulosum* spores, the spore suspension used for inoculation was diluted to range from $1.0 \ge 10^3$ spores/ml to $1.0 \ge 10^4$ spores/ml, equating 25 to 250 spores per 0.025 ml inoculation. For each day of inoculation, the spore supply was enumerated in duplicate on MEA.

Coating and Sample Preparation

Test-squares were cut (57.2 mm by 57.2 mm or 2 ¹/₄ in by 2 ¹/₄ in) from polyethylene-coated half-gallon flats intended for use with ultra-pasteurized milk in Virginia. The paperboard squares were coated with melted paraffin wax (Gulf Lite and Wizard Inc., Memphis, TN) on three edges, leaving the uncoated (raw) edge perpendicular to the machine direction, or coated on four sides for negative controls. The three edges were coated twice, allowing the wax to fully harden before the second coating. After cooling, inoculation points were marked off with a wax pencil at 3.2 mm (1/8 in), 6.4 mm (1/4 in), 9.5 mm (3/8 in) and 12.7 mm (1/2 in), measured from the uncoated edge. Preliminary tests with scarlet moo dye in 0.1% peptone broth indicated that the liquid migrates 2.2 mm from the point of injection (injection size 0.025 ml) using the type of polyethylene coated paperboard used in this study. In order to measure from the leading edge of the spore suspension, 2.2 mm was added to the inoculation points. A steel pin (44.5 mm) was inserted into the paperboard at the inoculation point, parallel to the uncoated edge to create a path for the injection. Samples were then placed into individual, sterile Whirl-pak[™] bags and gamma irradiated (SteriGenics International, Inc., Westerville, OH), receiving a dose of 27.2 to 33.5 kGy to achieve sterility. To assess sterility of irradiated test-squares, the interior fibers were exposed and then

incubated in a flask of 100 ml of sterilized malt extract broth (Difco, Detroit, MI) at 25°C. The flasks were monitored for growth up to 2 months.

Sample Inoculation

Test-squares were removed from Whirl-pak[™] bags in a biological safety cabinet (Nuaire, Plymouth, MN). A sterile 30 gauge needle and syringe (Becton Dickinson and Co., Franklin Lakes, NJ) was used to inject 0.025 ml of the diluted spore suspension between the layers of paperboard. The test-squares were allowed to sit with the injection site facing upward for 15 min to allow consistent absorption of the inoculum. The injection site was sealed with melted paraffin wax applied with a sterile swab. The paraffin wax was previously autoclaved at 121°C for 15 min and tempered to 55 to 60°C for optimal coating. Samples were given 2 min to cool before being placed uncoated side down in sterile Whirl-pak[™] bags with 200 ml of ultra-pasteurized skim milk and sealed. Ultra-pasteurized skim milk was obtained from a Virginia dairy and processed no more than 5 days prior to inoculation of each set of test-squares. Milk was transported on ice and kept in a 4°C refrigerator prior to use. All samples were randomly placed in a 7°C incubator in a vertical position to allow full coverage of the test-squares with the milk. This temperature, 7°C, represents the highest allowable storage temperature of milk in the United States (FDA, 1997).

Controls

For positive controls, test-squares were injected with 0.025 ml of the diluted spore suspension at 3.2 mm from the uncoated edge. The test-square were then cut into 4 pieces with sterilized scissors and placed in a sterilized Waring blender with 200 ml of ultra-pasteurized milk. The sample was disintegrated at high speed for 2 min. and poured into a sterile Whirl-pak[™] bag.

Negative 1 controls used test-squares which were injected with 0.025 ml of 0.1% peptone without spores at 3.2 mm from the uncoated edge. The test-square were then placed into a sterile Whirl-pak[™] bag with 200 ml of ultra-pasteurized milk.

For negative 2 controls, test-squares that were previously coated on all four sides were injected with 0.025 ml of spore suspension at 3.2 mm from the edge which was left uncoated on actual test-squares. The test-square were then placed into a sterile Whirlpak[™] bag with 200 ml of ultra-pasteurized milk. Controls were run on all media, diluents and milk used throughout this project.

After 60 days of incubation, 1.0 ml of milk from each sample after mixing was spread plated in duplicate on PDA with chloramphenicol (Fisher, Fair Lawn, NJ) plates and incubated at 25°C for up to 2 weeks. Any fungal growth was transferred aseptically to MEA plates and identified visually by microscopic comparison with a stock culture of *P. spinulosum* (examples in figures 1-3). Samples were recorded as positive or negative for the presence of *P. spinulosum* in the milk surrounding the test-square.

To better understand negative results, all samples for which *P. spinulosum* was not detected in the surrounding milk, the paperboard square was tested for the presence of fungus. Test-squares were removed from the milk and aseptically separated to expose the interior fibers and placed in 100 ml of malt extract broth with chloramphenicol. The samples were incubated at 25°C for 10 days. After thorough mixing of the sample, 1 ml from each dilution blank was spread plated onto PDA with chloramphenicol in duplicate and incubated for 7 days at 25°C. Any fungal growth was transferred aseptically to MEA plates and identified visually by microscopic comparison with a stock culture of *P. spinulosum*. Samples were recorded as positive or negative for the survival of *P. spinulosum* in the paperboard under incubation conditions.

Spore Survival Study

Survival of *P. spinulosum* spores in irradiated board was also tested over time at 0, 10, 20, 30, 40, 50 and 60 days. The paperboard squares used were coated on all 4 sides with melted paraffin wax and inserted with a steel pin perpendicular to the machine direction 3.2 mm from the edge. A sterile 30 gauge needle and syringe were used to inject 0.025 ml of the spore suspension between the layers of paperboard. The test-squares were allowed to sit with the injection site facing upward for 15 minutes to allow consistent absorption of the inoculum. The injection site was sealed with melted paraffin wax applied with a sterile swab. The paraffin wax was previously autoclaved at 121°C for 15 min and tempered to 55 to 60°C for optimal coating. Samples were given 2 min to cool before being placed in sterile Whirl-pak[™] bags with 200 ml of ultra-pasteurized skim milk and sealed. All samples were randomly placed in a 7°C incubator in a vertical position to allow full coverage of the test-squares with the milk.

After the incubation was complete 5 paperboard test-squares were removed and aseptically separated to expose the interior fibers and placed in 100 ml of malt extract broth with chloramphenicol. The samples were incubated at 25°C for 10 days. After thorough mixing of the sample, 1 ml from each dilution blank was spread plated onto PDA with chloramphenicol in duplicate and incubated for 7 days at 25°C. Any fungal growth was transferred aseptically to MEA plates and identified visually by microscopic comparison with a stock culture of *P. spinulosum*. Samples were recorded as positive or negative for the survival of *P. spinulosum* in the paperboard under incubation conditions.

Board Wicking

Absorption of ultra-pasteurized milk into paperboard was measured on 4 types of board from flats destined for use with ultra-pasteurized milk and milk products. Two containers were half-gallon and 2 were quart size. The basis weight ranged from 205 to 270 lbs/1000 ft². All board samples were manufactured with sizing agents to resist edge

wicking and coated on both sides with polyethylene. Test-squares of board (57.2 by 57.2 mm) were sealed on three sides with melted paraffin wax as in test squares. The exposed edge allowed milk to wick in the machine direction. The wicking distance of milk was then measured for the four types of paperboard used in fluid dairy packaging with ultra-pasteurized skim and ultra-pasteurized whole milk incubated at 7°C in Whirl-pak[™] bags. The wicking distance of 3 samples of each type of paperboard in each type of milk was measured at 10, 20, 30, 40, 50 and 60 days or until no substantial change was seen. The distance was measured for interior wicking of each sample by separating the layers of paperboard. The interior fibers were then immediately exposed to long wave ultra-violet light to aid in the visualization of wet areas on the paperboard because of the ability of milk to disperse UV light (Fox and McSweeney, 1998). A line was drawn through the apparent average wicking distance and each measured in mm.

Statistics

A sample size of 50 test-squares was used at each inoculation distance in the migration study to create a predictive model of fungal contamination under the conditions of this study through logistic and linear regression. Differences in mean wicking distance at 50 days were determined through analysis of variance using Tukey-Kramer HSD. Combined wicking data for both skim and whole milk was analyzed by split plot with paperboard and milk as whole plot factors and time and day squared as split plot factors. Statistical analysis was performed with JMP IN® version 3.2.1 for Windows (SAS Institute, Cary, N.C.)

Results and Discussion

Logistic regression of the proportion of contamination plotted by distance from the uncoated edge produced significant statistical evidence of a decline in the probability of contamination with distance (P less than 0.001) as seen in figure 4 (Agresti, 1990).

Simple linear regression of the proportion of contamination by distance from the uncoated edge was also highly significant over the distance range of 3.2 to12.7 mm from the uncoated edge ($R^2 = 0.98$, P = 0.004) as shown in figure 5. Over the range studied, the estimated rate of change of the probability is -15.0% per 2.5 mm with a standard error of 1.40. The significance is that this particular fungus is able to grow and extend from the interior of paperboard to spoil milk during its shelf life. Therefore, it is not only organisms located directly at the raw edge of paperboard containers that have the potential to spoil the product.

The ability of selected fungi to utilize paperboard as a source of nutrition has been shown by Narcisso and Parrish (1997). Suominen et al. (1997) through analysis of disintegrated paperboard, found the packaging contained adequate nutrient levels and pH range for bacterial growth. Suominen et al. concluded that water was the limiting factor for bacterial growth in paperboard. Based on these facts, it is likely that the P. spinulosum spores were able to germinate and grow to some extent on the diluent used during inoculation. This was seen by the fact that in 50% of the inoculated negative controls, the fungus was able to grow and extend past the coating to enter the surrounding milk as shown in Table 1. Therefore, the percentage of samples showing migration of the fungi into milk is likely higher than would have been seen if the spores were inoculated without a liquid. This emphasizes the importance of maintaining a low water activity in paperboard cartons through proper drying and storage prior to use. Also, to limit the ability of liquid, such as milk, to wick into paperboard, it would be helpful to eliminate the raw (uncoated) edge shown in figure 6. Because 50% of the fully coated controls (inoculated at 3.2 mm) and 84% of the test squares at 3.2 mm were positive for *P. spinulosum* in milk, it appears that coating the raw edge provided a measure of protection from contamination, but it was not as effective a barrier as distance in this study.

Survival of *P. spinulosum* in paperboard incubated in ultra-pasteurized milk at 7°C was investigated by two methods. In the study of survival over time, the fungus was

inoculated into paperboard that was sealed on all four sides. Survival was tested in five squares at 0, 10, 20, 30, 40, 50 and 60 days with all testing positive at every incubation time. In contrast, survival of all test squares showing negative results for the presence of *P. spinulosum* in the surrounding milk were tested in an attempt to determine the cause of the negative results. This second study found 94.4% of negative samples still retained viable *P. spinulosum* in the interior of the paperboard. While the two studies were not remarkably different, the reduced survival in the second study may be attributed to the increased incubation time prior to testing required to determine whether the surrounding milk was negative after 60 days. At least two weeks was required to confirm the presence of *P. spinulosum* in milk. Also, the first study used completely sealed test squares where competition for nutrients from psychrotrophic bacteria would have been eliminated, unlike the second study.

No fungi were detected in the pasteurized milk used prior to the migration study. Tests of the gamma-irradiated paperboard squares showed that sterility was achieved. The average number of *P. spinulosum* spores inoculated into the paperboard squares was 66 with a range of 61 to 70 spores/square, all within the target range of 25 to 250 spores/square.

Wicking studies showed that a substantial amount of milk was absorbed by the paperboard tested for both skim and whole milk as seen in figures 7 and 8. Type A represented the specific paperboard used in the migration and survival studies. *Penicillium spinulosum,* inoculated even at 12.7 mm from the raw edge, likely had access to skim milk as a source of nutrition by day 40 in the migration study (figure 7). Statistical analysis was performed on day 50 results because at day 60 both skim and whole milk had fully saturated all paperboard samples. Two-way analysis of variance at day 50 showed a significant interaction between type of paperboard and type of milk, p = 0.0001. Two-way analysis of variance also showed a significant interaction between type of paperboard and type of milk at day 10 and 40. Day 50 was further analyzed for statistical differences within the day as this data represents the farthest measurable

wicking distance in this study. Relatively few significant differences were seen in the wicking distance of samples at the 0.05 level at 50 days by type of paperboard and type of milk with Tukey-Kramer Honestly Significant Difference (HSD), shown in figure 9. Overall skim milk wicked farther than whole milk in extended shelf life paperboard, but the differences were slight. Graphs of skim and whole milk wicking over time, figures 7 and 8, both exhibit a bi-phasic characteristic separating between day 30 and 40. This may be attributed to the growth of microorganisms in the surrounding milk, affecting the properties of milk and separating components as time increases. These changes in the characteristics of milk may well be responsible for the increase in slope seen in the second half of the graphs. Split plot analysis of the combined data for whole and skim milk at all incubation days found that only 1.2% (by partial R^2) of the total variation in distance is accounted for by the interactions among paperboard type, milk type and day of incubation. While statistically significant interactions among paperboard type, milk type and day of incubation were seen, they were not practically important. Instead day of incubation and day squared accounted for 88.8% of the total variation in distance with p less than 0.001. Wicking distance by day for whole and skim milk combined is depicted in figure 10. Future research should examine the effect of different levels of pasteurization and also increase the sources of milk used to account for processing variations.

Based on the results of this study, to minimize migration of fungi from paperboard packaging and thereby reduce spoilage of the product, it is important to either limit the access of liquid to the microorganisms or limit the number of fungi in the packaging. This is most important for extended shelf life products, which allow a greatly increased opportunity for growth of psychrotrophic organisms. To protect the paperboard fibers from liquid, it would be helpful to coat the edge with a barrier or eliminate the raw edge during assembling by folding the edge into the interior of the package. The number of viable fungi and yeast in paperboard milk cartons could be controlled through specific regulations. Future research should focus on determining a relationship between the

concentration of fungi or yeast and the likelihood of contamination of milk in extended shelf life dairy products.

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| Inoculation | Ratio of milk | % | Ratio of negative milk | % |
|--------------------|----------------------|----------|------------------------|----------|
| distance from | samples positive for | Positive | samples which were | Positive |
| uncoated edge | P. spinulosum | | positive in paperboard | |
| (mm) | | | | |
| 3.2 (1/8 in.) | 42 / 50 | 84.0 | 7 / 8 | 87.5 |
| 6.4 (1/4 in.) | 36 / 50 | 72.0 | 14 / 14 | 100 |
| 9.5 (3/8 in.) | 25 / 50 | 50.0 | 23 / 25 | 92.0 |
| 12.7 (1/2 in.) | 14 / 50 | 28.0 | 35 / 36 | 97.2 |
| Negative 1 Control | 0 / 14 | 0.0 | N/A | N/A |
| 3.2 | | | | |
| Negative 2 Control | 7 / 14 | 50.0 | 6 / 7 | 85.7 |
| 3.2 | | | | |
| Positive Control | 8 / 8 | 100 | N/A | N/A |
| 3.2 | | | | |
| Total | 132 / 236 | 55.9 | 85 / 90 | 94.4 |

Table 1: Combined results of migration and spore survival study by inoculation distance and category.

- Figure 1: 40x magnification of a wet mount of *P. spinulosum* grown from frozen stock solution of conidia on malt extract agar showing condiaphores and condia (spores).

Figure 2: 40x magnification of a wet mount of *P. spinulosum* grown from frozen stock solution of conidia on malt extract agar showing elongated chains of conidia (spores) on conidiaphores typical of *P. spinulosum*.



Figure 3: 40x magnification of a wet mount of *P. spinulosum* grown from frozen stock solution of conidia on malt extract agar showing elongated chains of conidia (spores) on conidiaphores typical of *P. spinulosum*.



Figure 4: Logistic regression of the proportion of milk samples positive for *P*. *spinulosum* after 60 days by inoculation distance distance (3.2, 6.4, 9.5 and 12.7 mm) from the uncoated edge (P less than 0.001). Samples positive for the presence *of P*. *spinulosum* in milk are designated "1", while negative samples are designated "2".



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Figure 5: Simple linear regression of the proportion of milk samples positive for the presence of *P. spinulosum* after 60 days by inoculation distance (3.2, 6.4, 9.5 and 12.7 mm from the uncoated edge ($R^2 = 0.98$, P = 0.004).



Figure 6: Diagram of a $\frac{1}{2}$ gallon gable-top paperboard carton with fifth panel raw edge marked in red.







Figure 8: Plot of wicking distance of whole milk by time for four types of paperboard, A – D, intended for use with extended shelf life milk.



Figure 9: Wicking distance at 50 days for ultra-pasteurized skim and whole milk in four different types of paperboard, A – D, from extended shelf life milk cartons. The basis weights are 270, 265, 205 and 212 lbs/1000 ft² for A, B, C and D, respectively. Columns labeled with different letters indicate statistically different results (p less than 0.05).



Figure 10: Plot of overall wicking distance (mm) by incubation day at 10, 20, 30, 40 and 50 days for both skim and whole milk. Split plot analysis indicated that 88.8% of the total variance in distance is accounted for by day and day squared alone.



Appendix A

Future Research

Future research would benefit from a focus on microscopy to better understand the specifics of fungi migration from paperboard. Specifically, visualization of fungi in paperboard could best be accomplished by fluorescence or electron microscopy. A confocal scanning laser microscope, available in the Fralin Biotechnology building, offers the benefit of creating digital three-dimensional images of the interior of thin slices. Early attempts to apply this technology to fungi in paperboard were unsuccessful due to non-specific binding of the fluorescence labeled antibodies used to target chitin in the cell wall of fungi. This could be possibly overcome by transforming a fungus or yeast to express a fluorescence gene, which would eliminate the need for antibodies and the accompanying liquid needed.

The greatest challenge is to eliminate the use of liquid when inoculating paperboard with a microorganism. This is essential to create conditions that will mimic what actually occurs in paperboard in food applications. For this to happen, the microorganism would either have to be inoculated directly by hand, or as spores which are sprayed onto a surface or into the layers of paperboard. The hazard of this is that spores are difficult to contain outside of a liquid and accidental contamination becomes very likely.

This study of wicking of milk into paperboard showed that milk entered the paperboard more significantly than anticipated. The results are mostly useful for understanding the migration of fungi in this study specifically. The easiest and most interesting study on this subject would be accomplished by extensively testing the wicking distance of different types of milk in paperboard cut from cartons filled for

consumption. This would provide the most realistic and useful information for manufacturers and consumers.

Vita

Laura Sammons was born on August 22, 1974, the daughter of John and Deanna Sammons. In 1992, she graduated from Nansemond River High School in Suffolk, Virginia. She then attended Virginia Polytechnic Institute and State University where she received a bachelor's degree in Biology in 1996. After working at MedImmune, Inc. of Gaithersburg, MD for one year, she returned to Virginia Tech for her master's in Food Science and Technology, completed in 1999.