

Microbial Quality of Alfalfa Seeds and Sprouts after a Chlorine Treatment and Packaging Modifications

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ABSTRACT: Alfalfa seeds were treated with chlorine to determine the effect on microbial populations during soaking, sprouting, and refrigerated storage in three packaging environments. Chlorine was effective in reducing microbial populations on the seeds, but numbers increased during sprouting. Chlorine treatments had the most impact on yeast and molds during storage. Yeast and molds were significantly higher in sprouts that were stored in vacuum packages and in sprouts from non-chlorine treated seeds stored in MAP. Yeast and mold counts on all sprouts stored in perforated packaging did not significantly increase during storage. A combination of chlorine treatment of the seeds and prepackaged packaging of sprouts may increase the shelf-life.

Key words: Alfalfa sprouts, microbiological quality, chlorine, packaging

Introduction

THE TERM "SPOILAGE" IS DEFINED AS "ANY CHANGE THAT OCCURS IN A FOOD WHEREBY THE FOOD IS MADE UNACCEPTABLE FOR HUMAN CONSUMPTION" (Andrews and others 1979). Due to a short shelf life, alfalfa sprout producers report economic losses from product that becomes spoiled during distribution or in the supermarket. One of the primary causes of spoilage is visible mold growth and/or a musty smell from the sprouts. Preliminary data collected in our laboratory indicated that lactic acid bacteria and molds predominate the microflora of the spoiled sprouts. Producers report seasonal differences in spoilage, with peak losses occurring in the summer months. Spoilage also occurs sooner and more frequently when sprouts are not refrigerated in the supermarket and/or during distribution.

In addition to spoilage issues, pathogens could be introduced into sprouts from the seeds, in water used during production, and improper sanitation during production and marketing (Patterson and others 1980). Alfalfa sprouts have recently become a vehicle for many foodborne illness outbreaks. In 1995, an outbreak of *Salmonella stanley* and *Salmonella anatum* infections occurred in the United States because of consumption of contaminated alfalfa sprouts (Jaquette and others 1996). Outbreaks of food poisoning caused by *Escherichia coli* O157:H7 and infection by other *Salmonella* spp. have also been associated with the consumption of raw alfalfa sprouts (O'Mahoney and others 1990; Ponka and others 1995). Intervention methods that improve the shelf-life of alfalfa sprouts may also improve the safety of the sprouts by reducing pathogen loads.

Chlorine treatment of alfalfa seeds or sprouts at various stages of production has been suggested as an intervention method to reduce the total populations or to kill microorganisms such as *Salmonella* without adversely affecting the germination (Park and Sanders 1990; Brackett 1993; Jaquette and others 1996; Beuchat 1996, 1997). Reduction of total microorganisms using a chlorine treatment may possibly have the added benefit of extending the shelf-life of the sprouts. Soaking seeds in chlorinated water is an inexpensive, readily available intervention that can be utilized by traditional

sprout producers if effective in improving the microbial quality and extending shelf life.

Another intervention method that may improve the microbial quality of sprouts is alteration of the packaging conditions. Packaging alterations would be acceptable not only to the traditional sprout producer, but also to the organic producer who does not add chemicals to reduce microbial loads during production.

Alfalfa sprout producers use a variety of different packaging materials including heat-sealed or twist tie-sealed plastic bags, Ziploc® bags, heat-sealed cellophane bags, plastic boxes/cups with a film seal, clamshell packages, and produce boxes (Yam and Lee 1995). Many of these packaging systems include perforated packaging (PP) to allow for respiration, while others utilize nonpermeable films.

Modified atmosphere packaging (MAP), where the atmosphere surrounding the product contains a reduced O₂ concentration and elevated CO₂ concentration, has helped to maintain the fresh produce quality and to extend the shelf life of fresh produce (Kader 1986; Zagory 1988; Brackett 1993; Nguyen and Carlin 1994; Yam and Lee 1995). Carrots (Berrang and others, 1990), tomatoes (Beuchat and Brackett, 1991), and lettuce (Beuchat and Brackett, 1990) all benefited from an extended shelf life due to MAP, even though the total microorganisms were not impacted by MAP. As with other vegetables, MAP may have an impact on the microbial quality of alfalfa sprouts.

Another one of the common packaging methods in the food industry is vacuum and partial vacuum packaging. However, vacuum packaging (VP) may not be appropriate for fruit and vegetable products because it accelerates the deterioration of quality of some fruits and vegetables (Ponka and others, 1995). Additionally, the equipment needed for vacuum packaging as well as MAP may be too costly for a small sprout producer to purchase. Despite the added costs, mold growth appears to significantly contribute to microbial spoilage of sprouts, and reducing the amount of oxygen using either MAP or VP may reduce or eliminate the spoilage problems caused by molds and yeast. Additionally, PP may allow for more environmental contamination of the sprouts while nonpermeable

packaging used for VP and MAP may serve as a physical barrier to mold and bacterial contamination. On the other hand, altering packaging conditions are likely to alter the microbial profile on the sprouts. Altered profiles may have a beneficial or detrimental impact on spoilage microorganisms.

The objectives of this study were to determine the effect of seed soaking in 200 ppm chlorine on the microbiological quality of alfalfa seed and sprouts, and to evaluate the effect of different packaging modifications on the microbiological quality of alfalfa sprouts stored at refrigeration temperatures.

Materials and Methods

Sprouting Methods

Alfalfa sprouts were produced by the tray method (Sawyer and others 1985), which was modified using perforated trays with plastic cheesecloth on the bottom. Alfalfa seeds (*Alfalfa dakota*) were purchased from International Specialty Supply (ISS, Cookville, Tenn., U.S.A.). For each replication, 150 g of seeds were placed into each of six sterile (autoclaved) beakers. The seeds were washed with deionized water until the water became clear. Separate, duplicate 100 g aliquots of seeds were washed with deionized water and were retained for microbial analysis.

The washed seeds in beakers were divided into two groups. One was soaked for 6 h in 6 volumes of solution containing 200 mg of active chlorine per liter. The soaking solution was prepared with calcium hypochlorite (Ca (OCl)₂ (Fisher Chemical, Fair Lawn, N.J., U.S.A.). The active chlorine content of the solution was determined with Chlorine Test Paper (La Motte, Chestertown, Md., U.S.A.). The control group was similarly soaked, but solutions of sterile deionized water replaced the chlorine solution. All seeds were held in a dark environmental chamber at 23 °C (Percival, Boone, Iowa, U.S.A.) during the soaking period. Temperature and humidity were controlled by the chamber. The chamber was sanitized with a chloride solution and rinsed with distilled water to remove chlorine residue. The soaked seeds in both groups were rinsed with distilled water, and water was drained prior to sprouting. The soaked, drained seeds were placed on plastic cheesecloth in perforated trays. Perforated trays were sanitized by chlorinated distilled water before cheesecloth was placed on them. The trays with seeds were held in the dark chamber where the temperature and humidity were set to 23 °C and to 85% relative humidity to sprout for 4 d. After the 3rd day of the sprouting period, the sprouts were greened for 1 d under a fluorescent light. During the 4-d sprouting period, sprouts in both groups were rinsed with distilled water 3 times a day at 8-h intervals.

Packaging

At the termination of the sprouting period, all sprouts were rinsed with distilled water for 10 min to remove any remaining hulls and ungerminated seeds. The sprouts were drained on the perforated tray and placed onto absorbent paper for 10 min at room temperature.

Perforated packaging. Show Pack containers (ISS, Cookville, Tenn., U.S.A.) were used for perforated packaging. This packaging is a standard inexpensive commercial package for sprouts. The containers have vented bases and tight-fitting lids. Eighty g of alfalfa sprouts were put into containers for storage studies.

Vacuum packaging. Nylon EVOH 3 ml high-barrier co-extruded vacuum pouches containing 80 g of alfalfa sprouts

were sealed with a Multivac Chamber Machine, model AGV (Multivac, Kansas City, Mo., U.S.A.). A vacuum setting provided a package vacuum of 0.80 bar. In preliminary studies, higher vacuum levels resulted in crushing and mechanical damage to the sprouts.

Modified Atmosphere Packaging. Each pouch containing 80 g of alfalfa sprouts was evacuated, then backflushed with a gas mixture (5% O₂ and 10% CO₂, 85% N₂), and sealed using a Multivac Chamber Machine, model AG 500.

Storage

All packaged sprouts were stored in a walk-in cooler (Vollrath®, River Falls, Wis., U.S.A.) at 41 °F ± 1 for 8 d and removed as needed for each analysis. A storage period of 8 d was chosen because sprouts began physical deterioration after 4 to 8 d (depending on packaging method).

Microbiological Analysis

Twenty-five g of each sample were aseptically placed into a sterile stomacher bag with an appropriate amount of buffered peptone water (Difco, Detroit, Mich., U.S.A.) to achieve a 10⁻¹ dilution and pummeled for 2 min in a Tecmar 400 Stomacher (Tecmar® Co., Cincinnati, Ohio, U.S.A.). Serial dilutions were made in 0.1% buffered peptone water. Total aerobic bacteria, coliforms, generic *E. coli*, and yeast and molds were enumerated as they naturally occur on the alfalfa seeds and sprouts. Sprouts and seeds were also analyzed for the presence of *Salmonella*.

Aerobic Plate Count. Total aerobic plate counts (APC) were carried out for seeds, treated and untreated soaked seeds, and for alfalfa sprouts from each packaging modification during storage on day 0, 2, 4, 6, and 8. Appropriate serial dilutions with buffered peptone water were plated on plate count agar (PCA) (Difco) using the pour-plate method in duplicate. The plates were incubated for 48 h at 32 °C. After incubation, plates with 25 to 250 colony forming units (cfu) were enumerated with the assistance of a Quebec® Darkfield Colony Counter (Cambridge Instrument, Buffalo, N.Y., U.S.A.).

Enumeration of *Escherichia coli*/Coliforms. *Escherichia coli* and coliform enumeration was carried out on seeds prior to soaking, treated and untreated soaked seeds, and on alfalfa sprouts from each packaging modification on d 0, 2, 4, 6 and 8. Samples were plated onto 3M™ Petrifilm™ *E. coli* coliform count (EC) plates (3M, St. Paul, Minn., U.S.A.) in duplicate. The inoculated plates were incubated for 24 h at 37 °C. After incubation, plates were enumerated as coliform colonies which were bright red colonies associated with entrapped gas. The plates were incubated an additional 24 h at 37 °C to detect *E. coli*. After incubation, typical *E. coli* colonies (purple with gas production) were enumerated with the assistance of a Quebec Darkfield Colony Counter.

Enumeration of Mold and Yeast. Mold and yeast analysis for treated and untreated soaked seeds, and for alfalfa sprouts from each packaging modification during storage on day 0, 2, 4, 6, and 8, was performed (USFDA 1999). Serial dilutions were plated onto Potato Count Agar (PCA) which contained 100ug/mL chloramphenicol (Sigma Chemical, St. Louis, Mo., U.S.A.), and were incubated in the dark for 5 d at room temperature. After incubation, mold and yeast colonies were counted with the assistance of a Quebec Darkfield Colony Counter.

Statistical Analyses

A randomized complete block design was used to evalu-

ate the effects of seed-soaking conditions on the soaked seeds. A split-plot experimental design was used to evaluate the effects of seed-soaking conditions and the effect of different packaging modifications on the microbiological quality of alfalfa sprouts held at refrigerated temperatures. The sprouting process and the experiments were replicated three times. The data from three replications were statistically analyzed by analysis of variance using the mixed model of the SAS System® (SAS Institute Inc., Cary, N.C., U.S.A.). The Least Significant Difference test was used to determine differences between the treatment and packaging modifications. Significance of differences was set at $P < 0.05$.

Results and Discussion

Aerobic Plate Count (APC)

Prior to dividing the seeds into treatment groups, the APC of the seeds was determined to be $4.01 \log_{10}$ cfu/g (Figure 1). These results are similar to other reported values for APC of alfalfa seeds which range from $4.79 \log_{10}$ cfu/g (1) to $6.60 \log_{10}$ cfu/g (Prokopowich and Blank 1991). APC of seeds is variable due to differences in seed production, storage, and handling. Seeds can potentially become contaminated with microorganisms from animals housed near the production facility, manure fertilizer, contaminated water, and inadequate employee hygiene (USDA 1999).

After soaking, the APC of both treated and nontreated seeds was significantly higher ($P < 0.05$) than the APC of corresponding dry seeds (Figure 1). The APC of treated soaked seeds was 1 log cycle higher than that of dry seeds, while the APC of untreated soaked seeds was more than two log cycles higher than that of dry seeds. Results show the increase in APC on the seeds that did not receive a chlorine treatment was significantly higher ($P < 0.05$) than those that did receive a chlorine treatment. After the sprouting process, there were no significant ($P > 0.05$) differences in the APC of the sprouts regardless of seed treatment (Figure 1).

The APC of alfalfa sprouts from both chlorine-treated and nontreated seeds did not significantly increase ($P > 0.05$) during refrigerated storage, and there were no significant differences between treatments or packaging conditions. All APC's were greater than 10^8 cfu/g (data not illustrated). This population is not necessarily indicative of spoilage problems, be-

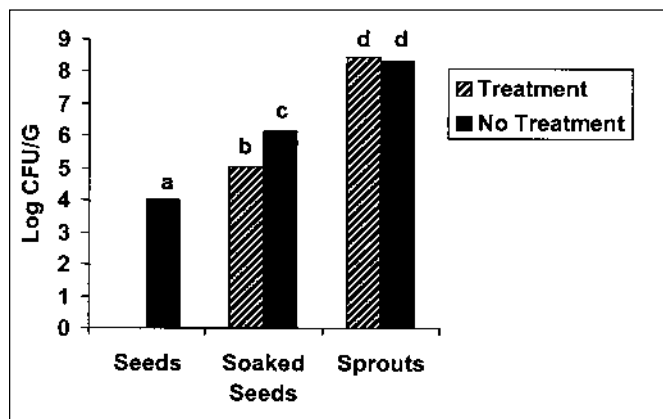


Figure 1—Aerobic plate count of chlorine treated (200 ppm) and nontreated alfalfa seeds and sprouts. Counts with different alphabetical notations are significantly different ($P < 0.05$)

Table 1—Total coliforms on packed alfalfa sprouts produced from treated and untreated seeds using different packaging systems during storage at 4 °C.

Days of Storage	Perforated packaging Log ₁₀ (cfu/g)		Modified atmosphere packaging Log ₁₀ (cfu/g)		Vacuum packaging Log ₁₀ (cfu/g)	
	T	NT	T	NT	T	NT
0	5.64 ^a	5.93 ^a	5.64 ^a	5.93 ^a	5.64 ^a	5.93 ^a
8	5.71 ^a	6.00 ^a	7.32 ^b	6.05 ^a	5.68 ^a	6.06 ^a

T = Sprouts produced from seeds soaked in 200 ppm chlorine. NT = Sprouts produced from seeds soaked in distilled water.
^aMeans values are duplicate from three replications; Values with different superscripts are significantly ($P < 0.05$) different.

cause 10^8 cells/g is not considered as a large population due to production conditions associated with the sprouts (Splits-toesser and others 1983).

Generic *Escherichia coli* and Coliforms

No *E. coli* was detected on seeds, soaked seeds, or sprouts; however, coliforms were detected. Total coliforms on the seeds used in this study prior to sprouting averaged $1.32 \log_{10}$ cfu/g (Figure 2). These numbers are similar to those previously reported in the literature (Andrews and others 1979; Prokopowich and Blank 1991).

In this study, total coliform counts on nonchlorine treated seeds increased significantly ($P < 0.05$) as a result of the soaking period (Figure 2). The total coliform counts of chlorine-treated soaked seeds were not significantly higher ($P > 0.05$) than that of dry seeds. While the chlorine treatment applied to seeds prevented an increase in coliforms during the soaking period, the treatment had no significant ($P < 0.05$) effect on total coliforms on the sprouts.

Total coliform counts did not significantly ($P > 0.05$) increase in sprouts produced from chlorine-treated or nontreated seeds packed in PP and in VP (Table 1). The coliforms increased in sprouts produced from chlorine-treated seeds and packaged in MAP. Because there were no significant changes in numbers of coliforms on day 2, 4, and 6, coliform data from day 0 and 8 only are illustrated.

Modified atmospheres inhibit the growth of certain mi-

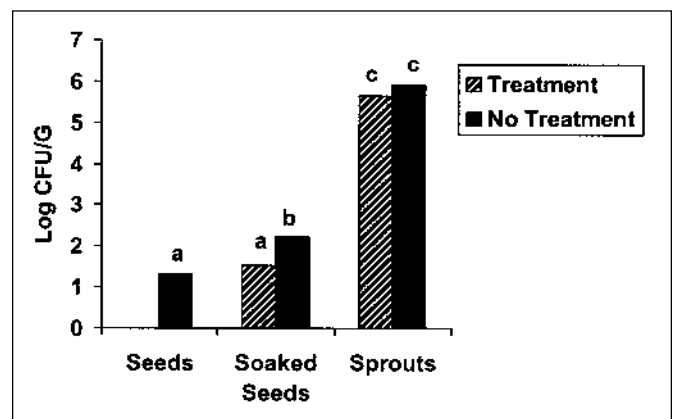


Figure 2—Coliform counts of chlorine treated (200 ppm) and nontreated alfalfa seeds and sprouts. Counts with different alphabetical notations are significantly different ($P < 0.05$)

croorganisms depending on O₂ and CO₂ concentration in the packaging (Kader 1986). MA conditions cause the respiration rates of vegetables to decrease and the metabolic activity of vegetables to become slower. Typically, this delays senescence and may reduce microbial spoilage (Kader 1986; Zagory 1988; Reyes 1996; Lopez and Chaves 1998). However, under certain circumstances, MA conditions accelerate senescence, physiological breakdown, and become more susceptible to postharvest microorganisms (El-Goorani and Sommer 1981). Also, diverse gases influence different microorganisms differently. Nitrogen has little effect on microorganisms, but CO₂ has an important role on the produce, displacing O₂, and on microorganisms by the reduction of pH in the cytoplasm of microbe's cells as well as the interference with normal cellular metabolism. Not all microorganisms are similarly limited by CO₂ (Daniels and others 1985). Additionally, MAP for long periods of time at refrigeration temperatures may select for psychotrophic organisms which could affect the microbial quality of the product. The combination of a modified atmosphere and chlorine treatments could have created an environment favorable to the growth of coliforms and unfavorable to the growth of naturally present competitive microflora that could inhibit the growth of coliform bacteria. Thus, we observed an increase in the number of coliforms in sprouts packaged in MAP and produced from seeds that were soaked in 200 ppm chlorine.

Mold and Yeast

Despite their significant contribution to spoilage, mold and yeast growth on alfalfa sprouts has not been investigated previously. Molds and yeast were not detected on treated soaked seeds in the present research, but mold and yeast growth was observed on sprouts produced from those treated soaked seeds (Figure 3). Molds and yeast may be introduced during sprouting or packaging from the environment or from handling the sprouts. On the untreated soaked seeds, the mold and yeast were detected at a level of 4.9 log₁₀ cfu/g, while there were no molds or yeast detected on chlorine-treated soaked seeds. Amounts on both treated and untreated soaked seeds increased to more than 4.0 log₁₀ cfu/g by the end of the sprouting period. Again, this increase indicates that the antimicrobial chlorine treatment reduced initial loads, but the effect was short-lived.

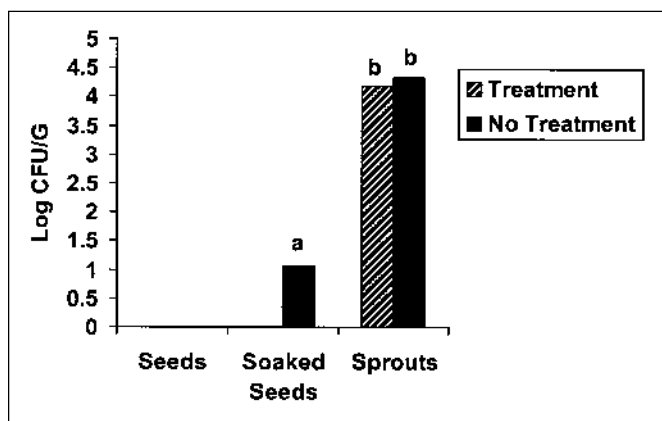


Figure 3—Yeast and mold counts of chlorine treated (200 ppm) and nontreated alfalfa seeds and sprouts. Counts with different alphabetical notations are significantly different ($P < 0.05$).

Table 2—Mold and yeast count on packed alfalfa sprouts produced from treated and untreated seeds using different packaging systems during storage at 4 °C.

Days of Storage	Perforated packaging ^a Log ₁₀ (cfu/g)		Modified atmosphere packaging ^a Log ₁₀ (cfu/g)		Vacuum packaging ^a Log ₁₀ (cfu/g)	
	T	NT	T	NT	T	NT
0	4.18 ^b	4.32 ^b	4.18 ^b	4.32 ^b	4.18 ^b	4.32 ^b
2	4.11 ^b	4.22 ^b	4.52 ^b	4.80 ^{bc}	5.25 ^c	3.57 ^{ab}
4	3.79 ^b	3.91 ^b	3.89 ^b	4.53 ^b	4.87 ^c	3.46 ^{ab}
6	5.17 ^b	3.50 ^{ab}	3.19 ^a	3.51 ^{ab}	4.94 ^c	3.75 ^{ab}
8	4.19 ^b	5.32 ^c	4.37 ^b	5.21 ^c	5.67 ^c	4.90 ^c

T = Sprouts produced from seeds soaked in 200 ppm chlorine. NT = Sprouts produced from seeds soaked in distilled water.
^{abc}Means values are duplicate from three replications; Values with different superscripts are significantly ($P < 0.05$) different.

Mold and yeast counts of sprouts were variable during storage in the different packaging systems. Sprouts produced from both chlorine-treated and nontreated seeds packaged in PP had no significant increases ($P > 0.05$) in yeast and mold counts after 8 d of storage (Table 2). All sprouts packaged in PP had no visible/physical signs of spoilage after 8 d of refrigerated storage (Table 3).

Sprouts produced from chlorine-treated seeds and packaged in MAP also had no significant increases in amounts of yeast and molds during storage (Table 2). Conversely, sprouts receiving no chlorine treatment and packaged in MAP had significantly more yeast and molds after the 8 d storage period. On day 8, sprouts produced from nontreated seeds had small amounts of visible mold growth and a musty odor (Table 3).

VP sprouts produced from chlorine-treated seeds had significant increases in molds and yeast after only 2 d of storage (Table 2), and had obvious physical signs of spoilage (Table 3). Sprouts receiving no chlorine treatment and VP increased significantly, but only after 8 d of storage. The yeast and mold counts on VP sprouts were significantly higher than the counts on sprouts packaged in PP and those produced from chlorine-treated seeds and packaged in MAP. Additionally, after 4 d of refrigerated storage, the chlorine-treated sprouts in VP had visible signs of mold growth and had a musty odor. Quality deteriorated on each subsequent d of analyses. Sprouts from nontreated seeds had visible mold growth and a musty odor after 8 d of refrigerated storage.

Sprouts produced from chlorine-treated seeds may have higher yeast and mold counts because the chlorine treatment may have reduced the presence of competitive microflora on the sprouts. While total APC did not increase or decrease, the numbers are not representative of the types of microorganisms present on treated and nontreated seeds.

We expected the numbers of molds and yeast to be less in vacuum-packaged sprouts than those in PP because of the reduced-oxygen environment. However, this study indicates that PP results in fewer molds and yeast than VP. In this study, the microflora of the sprouts was probably altered by the VP to select for molds and yeast and caused them to survive better than they did in PP. Additionally, we expected the APC of sprouts packaged in VP to decrease, compared to PP. The data indicated that the APC was the same for sprouts in both packaging environments.

The chlorine treatment with calcium hypochlorite (200

Table 3—Visible changes in alfalfa sprouts stored for 8 d at 41 °F in three packaging environments.

Days of Storage	Perforated packaging		Modified atmosphere packaging		Vacuum packaging	
	T	NT	T	NT	T	NT
0	None	None	None	None	None	None
2	None	None	None	None	Slight mold, musty odor	None
4	None	None	None	None	Slight mold, musty odor	None
6	None	None	None	None	Slight mold, musty odor	None
8	None	None	Slight mold, musty odor	Slight mold, musty odor	Slight mold, musty odor	Slight mold, musty odor

T = Sprouts produced from seeds soaked in 200 ppm chlorine. NT = Sprouts produced from seeds soaked in distilled water.

ppm active chlorine) applied to seeds for 6 h during the soaking period was effective in reducing total aerobic microorganisms, coliforms, and molds and yeast on the seeds, but it was not effective in reducing the same microbial loads on sprouts. However, the chlorine treatment had an impact on the predominant microflora because differences between treated and nontreated seeds were observed in MAP and VP sprouts.

Storage of the sprouts produced from treated and nontreated sprouts revealed that chlorine may result in the selection of coliforms and yeasts and molds in sprouts depending on the packaging method. Selection can occur because the yeast, molds, and coliforms survived the chlorine treatment, while microflora that may have competed with and inhibited the growth of these organisms may have been reduced by the chlorine treatment.

Microbial and visible results from this study indicate that perforated packaging, an economical, readily available package that can be used by both traditional and organic sprout growers, consistently resulted in the best microbial quality in the sprouts during refrigerated storage. Sprouts packaged in PP have a shelf life greater than 8 d, while those packaged in MAP and VP have a shorter shelf life.

Chlorine may be effective in reducing microbial populations on alfalfa seeds, but more than just an initial treatment to the seeds should be examined. Rinsing with chlorine solution could be used to reduce microbial populations on sprouts during the sprouting period. Additionally, inoculated studies using foodborne pathogens should be conducted to determine the effectiveness of chlorine on reducing the levels of pathogens in the final product.

Conclusion

THIS STUDY INDICATES THAT PACKAGING IN PERFORATED packaging materials will result in the longest shelf life of the final product. Sprout producers can achieve a product with less mold growth by utilizing economical, readily available, perforated packaging materials.

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