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Note

Microbial Quality of Chlorine Soaked Mung Bean Seeds and Sprouts

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The effect of 15 h, 300 ppm calcium hypochlorite soaking on microbial quality of mung seeds and sprouts was investigated. The extended chlorine soaking did not completely eliminate the microflora of the seeds but significantly reduced microbial proliferation rates during storage. Generally, microbial counts of the chlorine-soaked seeds were significantly lower than that of the control. Total plate count (TPC), lactic acid bacteria (LAB) and total coliform (TCC) populations of the seeds continuously increased during sprouting and sprout storage at 4.0℃, except for the yeast and mold counts (YMC) which increased during sprouting but decreased through the refrigerated storage. A significant decrease in the TPC of sprouts from chlorine-soaked seeds was observed after the 4th day of storage. *Escherichia coli* was not detected throughout the sprouting and storage periods. *Salmonella* was not isolated from the extended chlorine-soaked seeds, but was detected after the sprouting period.

Keywords: mung (*Vigna radiata* L. Wilczek) sprouts, chlorine treatment

Introduction

In many countries worldwide, including the Philippines, the consumption of seed sprouts has increased in recent decades with the advent of neutraceuticals, phytochemicals (Shetty *et al.,* 2003) and the shift of consumer preference toward health foods (Del Rosario, 2003; Pandrangi *et al.,* 2003). Mung bean (*Vigna radiata* L. Wilczek) sprouts are one of the most common vegetables consumed in the Philippines due to availability and nutritional value. The sprouts, locally known as *togue,* are available in markets all year round and contain high-quality proteins comparable to those obtained from expensive animal and marine sources (Del Rosario, 2003; FNRI, 1997). However, the nutritional quality and the sprouting methods employed by local sprouters make the commodity susceptible to microbial contamination and therefore compromises the safety and quality of the sprouts.

Unlike many developed countries where seed sprouts are produced in large industrial scales that allot enough capital for the procurement of appropriate sprouting equipment and facilities, sprouting in the Philippines is most commonly done on a micro scale as a part of the growing backyard industry. With capital limited for the procurement of raw materials and simple sprouting equipment, other necessities required in assuring sprout safety are often not available. Del Rosario (2003) reported that mung beans are sprouted by soaking the seeds overnight in water and then transferring the swollen seeds in jars or bamboo baskets covered with banana leaves. The seeds are then kept in the dark and watered several times for 3 d until they sprout. The seeds are finally de-hulled by soaking in water and allowing the seed coats to float to the surface. The Commonwealth Scientific and Industrial Research Organization and the Australian Food Industry Science Center (CSIRO-AFISC, 2000) have reported that the initial microbial flora of the seeds including pathogenic bacteria could increase by 1 $\log_{10}$ cfu g^{-1} after the soaking period alone and could reach up to 6 $\log_{10}$ cfu g^{-1} after sprouting, even under hygienic conditions. Consequently, the consumption of raw or partially cooked sprouts has thus become a major food safety concern (CIDRAP, 2002; OMAF, 2002; Sapers *et al.,* 2002). Taormina *et al.* (1999) cited that seed sprouts have been implicated in international outbreaks of *Salmonella* spp. and *Escherichia coli* O157: H7 with several reported cases in the United States, Canada and several European countries. Although pathogens like *Salmonella* spp. and *Listeria monocytogenes* have been isolated from mung bean sprouts in Asian countries like Malaysia and Thailand, only outbreaks of *E. coli* O157: H7 have been reported in Japan (Harris *et al.,* 2001; Taormina *et al.,* 1999). In the Philippines, since mung bean sprouts are traditionally eaten cooked in many home-style dishes (Del Rosario, 2003), few studies have looked into aspects related to microbial quality and safety. However, seed sprout safety is of concern in the Philippines because of the recent popularity of fast food establishments where raw mung bean sprouts are served as a side dish with soups and other meals. Furthermore, seed sprouts of alfalfa and broccoli are also becoming popular and an increasing number of supermarkets are selling these commodities.

Beuchat (1996) reported that alfalfa seeds and sprouts treated with chlorine have reduced microbial loads and

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that treatment kills pathogenic microorganisms including *Salmonella*. The U.S. Food and Drug Administration (FDA) currently recommends the use of 20,000 ppm chlorine to disinfect seeds prior to sprouting. Chlorine soaking was found to improve fresh produce microbial quality and shelf life without adversely affecting the viability of the seeds. Active chlorine exists as hypochlorous acid in solution and is responsible for the antimicrobial activity of the compound against microbial vegetative cells (Mossel *et al.,* 1995). However, even at recommended levels of 20,000 ppm for as long as 20 min, chlorine treatment can only partially eliminate pathogens (Pandrangi *et al.,* 2003). Although chlorine treatment is considered one of the cheapest sanitizing methods available, the additional cost it could entail might prevent some sprouters from practicing the seed sanitation step. Since seed sprout safety and quality cannot be compromised, studies on how to minimize costs while concurrently maximizing the use of this additional step should be done. Utilizing chlorine as a seed-sanitizing agent at lower concentrations but for extended period of time could potentially resolve this problem. This study was done to determine the effects of extended chlorine soaking (15 h; 300 ppm) on the microbial quality of mung bean seeds and sprouts.

Materials and Methods

Mung Seeds The study utilized the commonly known Philippine Centennial Mung variety with a Philippine Seed Board variety name of PSB Mg 6 and a line/hybrid name of Economic Garden Mung (EGM) 3995. The seeds were obtained from the Department of Agriculture, Bureau of Plant Industry, Los Baños National Crop Research and Development Center, (DA-BPI-LBNCRDC) University of the Philippines at Los Baños (UPLB), Laguna, Philippines. Developed by the Asian Vegetable Research and Development Center (AVDRC) in Taiwan and the DA-BPI-LBNCRDC under the Philippine Outreach Program (POP), this variety was the product of hybridization between *Vigna* Cross (VC) 2755A and VC 1482E. The mung seeds were planted and harvested in February and April 2003, respectively.

Chlorine Soaking and Sprouting of Mung Seeds The sprouting method used in this study was similar to those described by Del Rosario (2003) and the Philippine Council for Agriculture, Forestry and Natural Resources Research and Development (PCARRD, 1991). Mung seeds were placed in autoclaved 1000 ml beakers and washed twice with 6 volumes distilled water. The experiment was conducted in triplicate using 10 g of washed seeds that were pooled from the lot and subjected to microbial analyses.

A triplicate sample of 100 g washed mung seeds in sterile 1000 ml Erlenmeyer flasks was soaked in 600 ml 300 ppm calcium hypochlorite ($Ca(OCl)_2$) solution or distilled water (0 ppm $Ca(OCl)_2$) for 15 h. The seeds were then drained of the soaking media, rinsed once with 500 ml distilled water and transferred to sanitized polypropylene trays lined with autoclaved cheesecloth. Seed samples were obtained from each soaking lot for microbial and seed viability analyses. The seeds were sprouted in a dark place for 3 d at room temperature (29℃) following the 8-h distilled water-rinsing protocol described by PCARRD (1991). The mung bean sprouts were finally washed with distilled water twice to separate the testa.

Packaging and Storage Study The sprouts were drained and placed on absorbent paper for 10 min at 29℃ after the final rinsing. Ten grams of washed sprouts were obtained from the water- and chlorine-soaked lots for microbial and physicochemical analyses. Perforated polypropylene containers (PPC) with tight-fitting lids similar to those described by Soylemez *et al.* (2001) were used as packaging material for the sprouted mung seeds. Mung sprouts in PPC were stored at 4.0℃ and subjected to storage study where changes in the microbial and physicochemical qualities of the sprouts were monitored. Sprout quality analyses were conducted on storage days 0, 4, 6 and 8.

Mung Seed Viability Assay Seed viability assay similar to that described by Pandrangi *et al.* (2003) was followed in this study. A triplicate sample consisting of 100 unsoaked, water- and chlorine-soaked mung seeds were randomly pooled from each respective lot and placed in sterile Petri dishes containing sterile Whatmann No. 113 (qualitative, wet-strengthen) filter paper saturated with sterile distilled water. The seeds were incubated at 29℃ for 48 h and observed for signs of germination. Results were reported as% viability.

Physicochemical Analyses The mung sprout pH and hypocotyl texture were monitored during storage. Changes in the pH of the mung seeds and sprouts were measured using a Chemcadet pH meter (Model 5983–00, Cole-Parmer, USA). Ten grams sample was comminuted with 90 ml distilled water and filtered through Whatmann No. 113 filter paper prior to pH measurements. The texture of the sprouts was evaluated using a Humboldt Universal Penetrometer (Humboldt, USA) with fine needle attachment. The values for texture measurements were reported as the number of mm traveled or penetrated by the needle through the thickest portion of the sprout hypocotyls for 10 s (mm $10\,s^{-1}$).

Microbial Analyses Ten grams of the unsoaked, water- and chlorine-soaked seeds and sprout samples were homogenized with an appropriate amount of peptone water (PW) (Gelysate, BBL, USA) to make a 10^{-1} dilution. For Total Plate Count (TPC) analyses, 10-fold serial dilutions using PW diluents were pour-plated with Standard Methods Agar (SMA) (BBL, USA) and incubated at 37℃ for 24 h. Serial PW dilutions were also pour-plated with Potato Dextrose Agar (PDA) (Difco, USA) acidified with 10% tartaric acid for yeast and mold enumeration (YMC). Plates were incubated at 29℃ for 5 d. Lactic acid bacteria (LAB) were enumerated by pour-plating with Orange Serum Agar (OSA) (Merck, Germany) and incubating at 37℃ for 48 h.

Total Coliform Counts (TCC) and generic *Escherichia coli* enumerations were done by plating 1.0 ml of appropriate PW diluents onto 3M Petrifilm *E. coli*/coliform count (EC) plates (3M St. Paul, Minn., USA). The plates were

incubated at 37℃ for 24 h prior to total coliform enumeration that appeared as bright red colonies associated with gas formation. The plates were incubated for another 24 h before *E. coli* enumeration that appeared as dark blue colonies associated with gas formation.

Analysis for *Salmonella* spp. was done by first resuscitating sublethally injured cells by homogenizing 25 g seed or sprout samples with 225 ml buffered peptone water (BPW) and incubated for 16–20 h at 37℃. Duplicate 1 ml aliquots of the resuscitated cultures were inoculated into 10 ml Selenite Cystine Broth (SCB) (BBL, USA) and incubated for another 18–24 h at 37℃. Loopfuls of the incubated SCB cultures were streaked onto Bismuth Sulfite Agar (BSA) (Hi-Media, India) plates and incubated for 24–48 h at 37℃. Typical presumptive *Salmonella* colonies which appeared as gray or black colonies with or without metallic luster were subjected to confirmatory biochemical tests similar to those described in the Bacteriological Analytical Manual (BAM). The biochemical tests include inoculations into Lysine Iron Agar (LIA), Triple Sugar Iron Agar (TSI) and Urea Broth (UB) (Andrews *et al.,* 1992).

Statistical Analyses Data gathered from the experiments were subjected to single-factor Analyses of Variance (ANOVA) using the General Linear Model Procedure (PROC GLM) of the SAS statistical software version 8.0 (SAS Institute, Cary, N.C.) with Duncan Multiple Range Test (DMRT) for post-hoc determinations of significant differences. Treatment effects were considered significant when $p < 0.05$.

Results and Discussion

Seed Viability Results showed that the 15-h 300-ppm chlorine soaking did not have significant effects on the viability of mung seeds. Both the water- and chlorine-soaked batched retained 100% viability after the extended soaking period (data not shown). The results of the seed viability assay corroborated the findings of Beuchat (1996) who found that chlorine treatments do not adversely affect the viability of seeds intended for sprouting.

Physicochemical Characteristics The pH and texture changes of the sprouts during refrigerated storage were monitored. Generally it was observed that the pH of sprouts from both soaking lots decreased during storage, with the pH values significantly lower in the water-soaked seed sprouts (data not shown). Similar to the results obtained by Paglinawan (2001), the decreasing pH of refrigerated vegetables during storage was associated with increased microbial flora on the sprouts. The decrease in pH could have been due to organic acid production by the increased numbers of lactic acid bacteria and coliform populations in the sprouts from both soaking lots.

Sprout texture from both lots were measured using a Penetrometer with fine needle attachment and reported as mm penetrated by the needle for 10 s (mm $10\,s^{-1}$) through the thickest portions of the sprout hypocotyls. The values for the Penetrometer needle penetrations significantly decreased through the storage period in both lots, indicating increased in toughness of the sprout hypocotyls (data not shown). This could have been due to moisture loss and possible lignin formation as observed by Klaiber *et al.* (2004).

Microbial Quality The increased microbial counts (Table 1) obtained from seeds exposed to extended chlorine soaking were similar to previously reported values obtained from refrigerated chlorine-treated vegetables (Klaiber *et al.,* 2004; Paglinawan, 2001; Soylemez *et al.,* 2001). Total plate counts (TPC), lactic acid bacteria (LAB) and total coliform counts (TCC) significantly increased during the sprouting and storage period. Yeasts and mold populations on the other hand increased after sprouting but decreased during storage at 4.0℃. *Es-*

Table 1. Microbial quality of extended chlorine soaked mung seeds and refrigerated (4.0℃) sprouts in perforated packaging.

Microbial Quality Parameters	Changes in Microbial Populations per Soaking Medium										
	Unsoaked Seeds	0 ppm					300 ppm				
		15-h Soaked Seeds	Sprouts				15-h Soaked Seeds	Sprouts			
			Day 0	Day 4	Day 6	Day 8		Day 0	Day 4	Day 6	Day 8
Total Plate Count[1] (log_{10} cfu g^{-1})	4.44 ^{f}	5.87 ^{e}	7.18 ^{d}	7.77 ^{cb}	8.13 ^{cb}	9.07 ^{a}	5.39 ^{e}	6.80 ^{d}	8.27 ^{b}	7.98 ^{cb}	7.74 ^{c}
Lactic Acid Bacteria[1] (log_{10} cfu g^{-1})	<1.00 ^{d}	< 1.00 ^{d}	6.63 ^{b}	7.64 ^{a}	7.42 ^{a}	7.93 ^{a}	< 1.00 ^{d}	5.55 ^{c}	7.68 ^{a}	7.46 ^{a}	7.77 ^{a}
Yeast and Mold[1] (log_{10} cfu g^{-1})	<1.00 ^{e}	< 1.00 ^{e}	3.99 ^{a}	2.40 ^{cb}	2.72 ^{b}	1.94 ^{cd}	< 1.00 ^{e}	2.99 ^{b}	2.61 ^{cb}	3.08 ^{b}	1.57 ^{ed}
Total Coliforms[2] (log_{10} cfu g^{-1})	<1.00 ^{i}	3.77 ^{j}	6.40 ^{d}	6.20 ^{e}	6.95 ^{b}	7.95 ^{a}	<1.00 ^{i}	5.39 ^{h}	5.75 ^{g}	5.95 ^{f}	6.57 ^{c}
Escherichia coli[2] (log_{10} cfu g^{-1})	<1.00 ^{a}	<1.00 ^{a}	<1.00 ^{a}	<1.00 ^{a}	<1.00 ^{a}	<1.00 ^{a}	<1.00 ^{a}	<1.00 ^{a}	<1.00 ^{a}	<1.00 ^{a}	<1.00 ^{a}
Salmonella spp.[2, 3] ($\pm$ 25 g^{-1})	-	+	+	+	-	-	-	+	+	-	-

$^{a, b, c, \cdots}$ Values on the same row followed by the same letters are not significantly different from each other ($p>0.05$)
[1] Values are averages of three trials
[2] Values are averages of two trials
[3] *Salmonella spp.* was qualitatively analyzed and reported as ($\pm$)25 g^{-1} mung bean seed/sprout samples

cherichia coli were not isolated from any of the samples analyzed, while the extended chlorine soaking process retarded growth of Salmonella.

Total Plate Count Unsoaked seeds were found to have a TPC of 4.44 $\log_{10}$ cfu g^{-1}, similar to those reported by Soylemez *et al.* (2001) and Prokopowich and Blank (1991) for total aerobic plate count of alfalfa seeds. Possible sources of the microbial load of seeds include animals housed near the farming site, contaminated irrigation water, manure fertilizers and poor personnel hygiene (Pandrangi *et al.,* 2003; USDA, 1999). Both the water- and chlorine-soaked samples showed a significant increase in TPC after the 15-h soaking period. The sprouting procedure significantly increased the TPC of both the water- and chlorine-soaked seeds resulting in more than 1 $\log_{10}$ cfu g^{-1} rise in the total microbial load of the seeds. Increased sprout microflora were observed over the 8 d storage period. While the TPC of the sprouts from the water-soaked seeds showed a continuous and significant increase over the 8 d refrigerated storage period, sprouts from the chlorine-soaked seeds on the other hand exhibited a continuous and significant decrease after the 4th day of storage. Jinneman *et al.* (1995) reported that sprouts normally have TPCs of up to 7.99 $\log_{10}$ cfu g^{-1} and Soylemez *et al.* (2001) cited that, due to seed sprout production conditions, microbial populations of up to 8 $\log_{10}$ cfu g^{-1} are not indicative of microbial spoilage. The microbial flora of the sprouts instead reflect the sanitary quality of the processing steps as well as the microbial condition of the seeds prior to and during sprouting (Jay, 2000). Thus, by the 8th day of storage at 4.0℃, sprouts from chlorine-treated seeds can still be considered acceptable for consumption. Conversely, sprouts from the water-soaked mung seeds had counts greater than 9 $\log_{10}$ cfu g^{-1} by the end of the 8 d storage period. The extended chlorine soaking decreased the rate of multiplication of the total microflora of the treated seeds. It has been reported that aside from total inactivation, chlorine treatment can also cause sublethal cell damage (Pandrangi *et al.,* 2003). The microorganisms present on the chlorine-soaked seeds probably needed additional time for cell repair before cellular multiplications were reestablished.

Lactic Acid Bacteria Lactic acid bacteria were not isolated from unsoaked, water- and chlorine-soaked seeds ($<$ 1.00 $\log_{10}$ cfu g^{-1}). The sprouting process however significantly increased the LAB count in both lots, with the sprouts produced from water-soaked seeds having significantly higher LAB counts than the sprouts produced from chlorine-soaked seeds. The LAB counts for both lots increased significantly until the 4th d of storage. Increases in LAB counts in both treatment lots were insignificant after the 6th and 8th day of storage. These results were similar to those obtained by Klaiber *et al.* (2004) for chlorine-treated shredded carrots. Further, the results of LAB analyses agree with the initial findings made by Kakiomenou *et al.* (1996) and Soylemez *et al.* (2001) that LAB are amongst the dominating microbial flora of refrigerated vegetables, including seed sprouts.

Yeast and Molds Yeasts and molds were not isolated from the unsoaked and soaked seeds ($<$ 1.00 $\log_{10}$ cfu g^{-1}). Similar to the report of Soylemez *et al.* (2001), yeast and molds might have been introduced to the seeds during sprouting. The YMC of both water- and chlorine-soaked seeds significantly increased after the sprouting period with the YMC of the sprouts from water-soaked seeds being significantly higher than the chlorine-soaked lots. Decreased YMC was observed during the storage of the sprouts. A possible reason for the decrease in YMC of both sprout lots during storage could have been the continuously increasing population of competing bacterial microflora. In this study, results obtained for YMC were similar to those reported by Fabri *et al.* (1981) for refrigerated vegetables where the combined populations of LAB, mesophiles, psychrotrophs and other bacterial species were significantly higher than those of the combined yeast and mold populations.

Total Coliforms and Escherichia coli Coliforms and generic *E. coli* were not isolated from the unsoaked seeds ($<$ 1.00 $\log_{10}$ cfu g^{-1}), but Total Coliform Counts (TCC) of the water-soaked seeds increased significantly to approximately 4 $\log_{10}$ cfu g^{-1} after the 15-h soaking period. Conversely, the extended 300 ppm chlorine-soaking treatment maintained the TCC of seeds at undetectable levels. The TCC in the two soaking lots increased significantly during sprouting. By the end of the 3-d sprouting period, the water-soaked lots had TCC greater than 6 $\log_{10}$ cfu g^{-1} while that of the chlorine-soaked lots reached levels greater than 5 $\log_{10}$ cfu g^{-1}. The more rapid increase in TCC of the chlorine- rather than in water-soaked seeds during sprouting could possibly be ascribed to microbial competition for nutrients. Since the populations of TPC, LAB and YMC were initially relatively lower in the chlorine lot, the TCC increased faster than that in the water-soaked seed lot. During refrigerated storage of the sprouts, the TCC in the two soaking lots increased with values from water-soaked seeds always being significantly higher than that of the sprouts from the chlorine-soaked seeds. Generic *E. coli* was not isolated from any of the samples analyzed.

Salmonella spp. *Salmonella* spp. were not isolated from the unsoaked seeds or the extended chlorine-soaked mung seeds. *Salmonella* was however detected in the water-soaked seeds. Since the process for both treatments was performed under sterile conditions, the presence of *Salmonella* spp. can only be attributed to an inherent load of the mung seeds. *Salmonella* spp. present in the unsoaked mung seeds may have been in the viable but not culturable (VBNC) state. Jay (2000) reported that viable microbial cells like those of *Salmonella* spp. can remain undetected by ordinary plating techniques under certain conditions. The desiccated conditions on the unsoaked mung bean seeds could have induced the *Salmonella* spp. to go into the VBNC state. Soaking in water together with the many biochemical changes that accompany the germination of the mung seeds (Yaw-Huei and Wen-Hsiang, 1996) could have provided optimal conditions for microbial activation and growth. Conversely, the *Salmonella* spp. cells on mung

seeds soaked in 300 ppm chlorine remained undetected. The high-moisture sprouting condition at ambient could have similarly favored growth of *Salmonella* spp. resulting in the presence of the microorganism by the end of the 3 d sprouting period in both water- and chlorine-soaked lots. *Salmonella* spp. were detected in both treatment lots until the 4th d of storage. The increasing populations of other microflora could have prevented further multiplication of *Salmonella* and possibly also explain the negative results obtained after 6 and 8 days of storage in both treatments.

The results established in this study verified the findings of other studies that the conditions during mung bean sprouting are optimal for microbial growth. The chemical composition of the sprouts as well as the high-moisture conditions and sprouting temperature favor the multiplication of the microflora in the produce. This study demonstrated that, similar to other seed sanitizing procedures, the 15-h 300 ppm chlorine-soaking of mung seeds was not effective in totally eliminating the microbial flora of the seeds. A possible reason for the reduction in antimicrobial activity of chlorine during extended soaking is the formation of chloramines. Azanza *et al.* (2001) and Shapton and Shapton (1991) reported that chloramines are formed when hypochlorous acid reacts with organic substances thereby decreasing its antimicrobial activity. Yaw-Huei and Wen-Hsiang (1996) reported that mung seeds exhibit high proteolytic activities even before germination. Proteolytic enzyme activities in the germinating seeds could thus have contributed toward the organic species that reacted with the free chlorine during the period of soaking. However, the extended 300 ppm chlorine soaking process significantly reduced the rate of the microbial proliferation and was able to maintain microbial populations within acceptable limits (Jinneman *et al.,* 1995; Jay 2000; Soylemez *et al.,* 2001) until the 8th day of storage. Extended chlorine soaking of mung seeds can thus be considered a promising means with which to control or minimize the microbial decomposition of the resulting mung sprouts. However, since the study focused on the microbiological and physicochemical parameters for assessing the effects of extended chlorine treatment on mung seeds and sprouts, descriptive and affective sensory quality evaluations should also be done to better assess its efficacy as a pre-sprouting treatment for mung seeds. Inactivation of specific pathogenic microorganisms should also be studied.

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