

Survival and Growth of *Vibrio cholerae* O1, *Salmonella typhi*, and *Escherichia coli* O157:H7 in Alfalfa Sprouts

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ABSTRACT: The survival and growth of *Vibrio cholerae* O1, *Salmonella typhi*, and *Escherichia coli* O157:H7 during germination and sprouting of disinfected alfalfa seeds and alfalfa sprouts was determined. All pathogens showed ability to grow during germination and sprouting, reaching counts of approximately $6.0 \log_{10}$ CFU/g after 24 h. No growth was observed for any pathogen when the sprouts were inoculated after 24 h of seed germination. At this time, the background microflora was abundant. Numbers of pathogens inoculated on alfalfa sprouts decreased less than $1 \log_{10}$ CFU/g over 15 d of refrigeration. Alfalfa sprouts can be an important factor contributing to the endemicity for typhoid fever and cholera in México.

Key Words: alfalfa sprouts, food safety, *Salmonella typhi*, *Vibrio cholerae*, *E. coli* O157:H7

Introduction

SPROUTED SEEDS ARE TRADITIONAL FOODS IN SOME ORIENTAL countries. There is an increasing interest for these foods in many European countries and in the Americas, including México. Various bacterial pathogens have been isolated from sprouts of different seeds (alfalfa, beans, cress, mungbean, mustard, soybean) (Beuchat 1996). Recently, a number of human food-borne illness outbreaks have been reported due to consumption of soybean, radish, and alfalfa sprouts. Pathogens involved include *Salmonella* (O Mahony and others 1990; Ponka and others 1995), *Bacillus cereus* (Portnoy and others 1973), and *Escherichia coli* O157:H7 (CDC 1997). These bacteria may be present in the seeds or contaminate the produce during germination. Some studies show that sprouting seeds support the growth of bacterial pathogens, such as those mentioned (Hara Kudo and others 1997; Harmon and others 1987). Further, sprouts are commonly consumed raw or slightly cooked in soups, salads, or mixed with other vegetables. In México, sprouts are often consumed liquefied with water or mixed with other vegetables. This type of matrix increases the potential hazards associated with sprout consumption. *Vibrio cholerae* O1 appeared in 1991 in Latin America and is now endemic in many areas (CDC 1993). For instance, 3427 cases of cholera were reported in México between 1996 and 1997 (Sistema Nacional de Vigilancia Epidemiológica 1997 a,b). There is a paucity of research concerning the behavior of *V. cholerae* O1 in foods that represent a special hazard to people. *S. typhi* is also relevant to public health problems in México. For the period 1995 to 1996, 16,481 cases of typhoid fever were reported in México (Sistema Nacional de Vigilancia Epidemiológica 1997 a, b). A number of these cases may be associated with consumption of raw vegetables exposed to fecal contamination (Bryan 1997). Use of raw sewage to irrigate crops is an important mechanism that contributes to propagating conditions for cholera and typhoid fever. We have not found any information about the potential for survival and growth of *V. cholerae* O1 and *S. typhi* in sprout seeds. Finally, *E. coli* O157:H7 has been isolated from cabbage, cauliflower, celery, cilantro, and coriander obtained from markets in México City (Zepeda-López and others 1995); other vegetables, such as alfalfa sprouts, may be equally contaminated. The objective of this work was to determine the potential of *V. cholerae* O1, *S. typhi*, and *E. coli* O157:H7 to grow during germination

and sprouting using alfalfa seeds and alfalfa sprouts contaminated in the laboratory.

Results & Discussion

Although the initial concentrations of pathogens used in this study could be, and in fact are, unlikely to occur under real life conditions (Jaquette and others 1996), the possible hazards as shown by these results should be emphasized.

The aerobic plate count (APC) and coliform median of the 8 batches of alfalfa seeds examined were 4.3 (range, 3.9 to 6.0) and 1.3 (range, <1.0 to 3.9) \log_{10} CFU/g respectively. We selected the batch that contained $3.9 \log_{10}$ CFU/g of APC for further studies. Using this batch, APC were reduced almost 100 times, and coliforms lowered to undetectable levels (<10 CFU/g) after washing and disinfecting with 100 mg of chlorine per L (Table 1). The main objective of disinfection was to reduce interferences of native flora during monitoring of pathogen strains as seeds germinate and sprout. Use of thiosulfate citrate bile salt sucrose (TCBS) agar for *V. cholerae* O1 and bismuth sulfite agar for *S. typhi* had resulted in abundant spurious colonies on the plates. These experiences led us to make use of rifampycin- (Rif) resistant pathogen strains throughout this study. Native microorganisms in alfalfa sprouts ($8.8 \log_{10}$ CFU/g of commercial sprouts, and $9.0 \log_{10}$ CFU/g of sprouts prepared in the laboratory) were completely inhibited (<10 CFU) in plates of TSA containing 50 mg/L of Rif. Resistance of *S. typhi* and *E. coli* O157:H7 strains to Rif was not lost at least

Table 1—Effect of washing and disinfection of alfalfa seeds on APC and coliforms using sodium hypochlorite solutions containing 10 and 100 mg/L of free chlorine

APC		Coliforms					
		Washed-disinfected				Washed-disinfected	
Before washing	Washed	10 mg/L	100 mg/L	Before washing	Washed	10 mg/L	100 mg/L
3.9 ^{1a}	3.1 ^b	2.4 ^c	1.6 ^d	2.3 ^a	1.6 ^b	1.5 ^b	< 1 ^c
	(84.2) ²	(98.4)	(99.5)		(80)	(80)	(> 99)

¹ \log_{10} CFU/g

² (%) % reduction

Value within columns followed by the same letter are not significantly different ($p < 0.05$)

through 10 daily transfers on TSA free of Rif.

APC population increased rapidly in washed and disinfected seeds stored at 22 °C. After 24 h, sprouts from non-disinfected seeds showed 7.8 log₁₀ CFU/g (Fig. 1). Disinfected seeds behaved similarly, independent of the chlorine concentration (10 to 100 mg/L) applied after washing. It is interesting to note that coliforms grew actively, and on day 3, sprouts reached maximum colonization with 9 log₁₀ CFU of APC/g and more than 8 log₁₀ CFU of coliforms /g (Fig. 1).

V. cholerae O1 grew throughout the first 24-h period of seed germination (Fig. 2). *V. cholerae* O1 inoculum was close to that of APC in the seeds, approx. 2.5 log₁₀ CFU/g. Growth rate was also similar. After 24 h, *V. cholerae* O1 population reached 5.9 log₁₀ CFU/g while APC reached 6.4 log₁₀ CFU /g over the same period. *V. cholerae* O1 decreased over time, but surviving cells persisted for longer, at least 6 d. Interestingly, after 24 h the maximum con-

centration of *V. cholerae* O1 was only slightly different between seeds inoculated with a low (2.5 log₁₀) and a high dose (5.3 log₁₀) of the pathogen; final numbers were 5.9 and 6.6 log₁₀ CFU/g respectively. However, while APC maintained the same growth rate and reached 8.3 log₁₀ CFU/g at day 4, *V. cholerae* O1 progressively decreased its number to 3.7 log₁₀ CFU/g. *S. typhi* and *E. coli* O157:H7 did not follow the same pattern of growth shown by *V. cholerae* O1, although they behaved similarly to each other (Fig. 3). They grew freely after the first step when seeds began germination and reached 5.9 and 5.8 log₁₀ CFU/g respectively after 24 h. The numbers of both pathogens remained very close to each other until day 10. APC reached a maximum density (8.9 log₁₀ CFU/g) on day 3. Thus, it appears that *V. cholerae* O1 has less capacity than *S. typhi* and *E. coli* O157:H7 to survive or compete in the sprout environment, where an abundant background microflora is prevalent. It is important to consider that the pathogenic

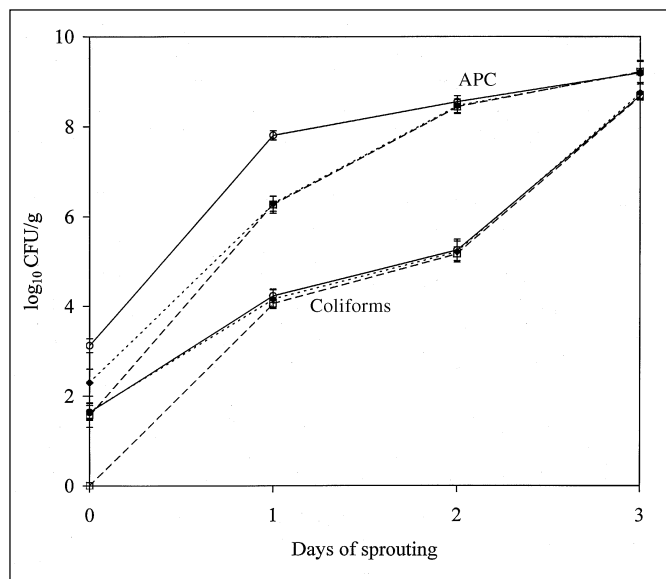


Fig. 1—Changes in aerobic plate count (APC) and coliform populations during sprouting from alfalfa seeds previously washed and disinfected with 0 (○—○), 10 (◆—◆) and 100 (□—□) mg/L of active free chlorine.

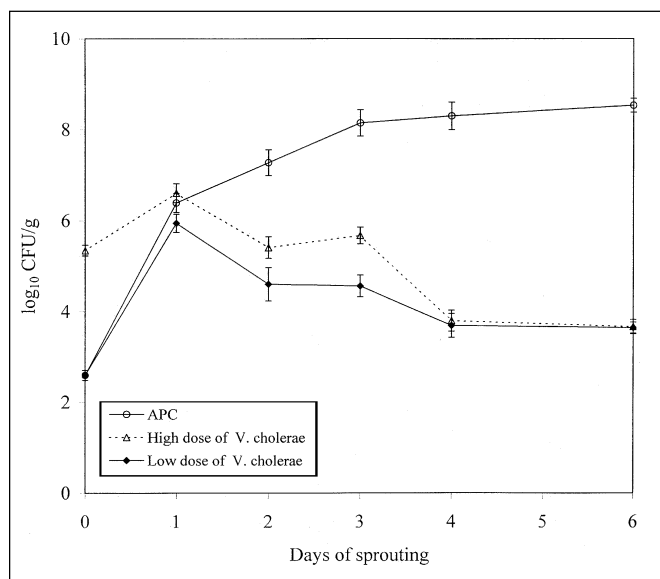


Fig. 2—Behavior of *V. cholerae* O1 and APC during sprouting of alfalfa seeds inoculated with 2 levels of the pathogen.

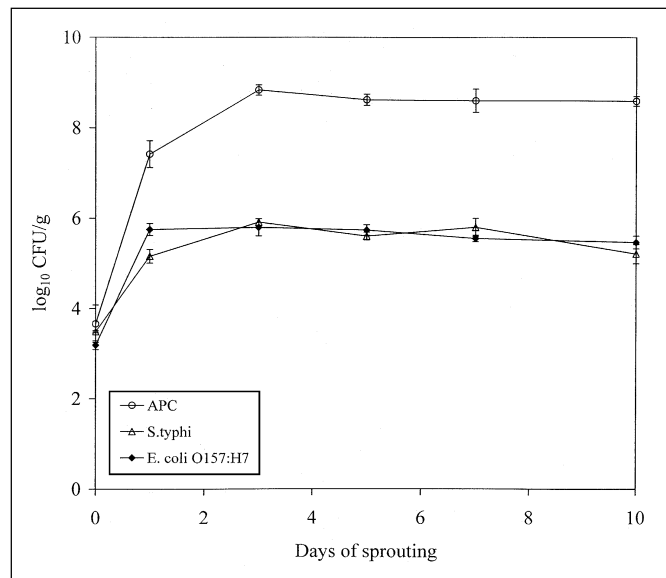


Fig. 3—Behavior of *S. typhi*, *E. coli* O157:H7, and APC during sprouting of seeds inoculated with each pathogen.

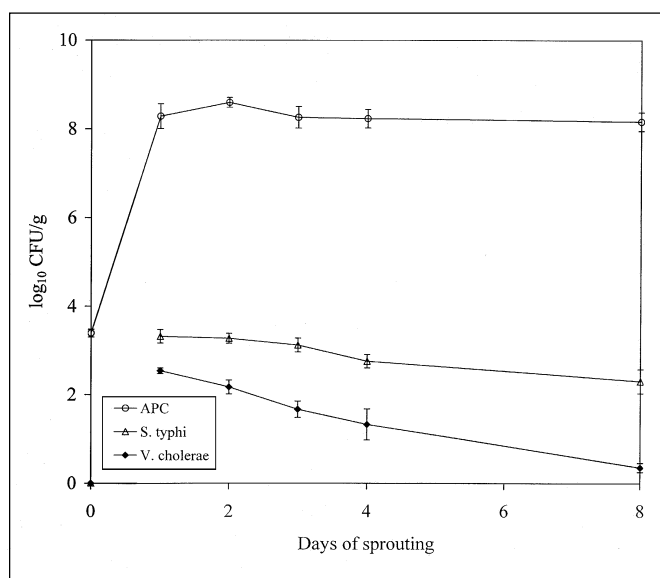


Fig. 4—APCs and counts of *V. cholerae* O1 and *S. typhi* on alfalfa seeds inoculated after germinating for 24 h.

bacterial growth we observed occurred under storage at 22 °C; eventually, room temperature may rise and, thus, activate the growth rate of these microorganisms.

When either *V. cholerae* O1 or *S. typhi* were inoculated on 24 h-germinating seeds, no growth was observed (Fig. 4). These experiments were done to simulate a situation in which water used to irrigate seeds could have been contaminated with either of these pathogens. There was a loss of viability of bacterial cells; and after 8 d of storage at 22 °C, *V. cholerae* O1 and *S. typhi* had decreased 2 and 1 log₁₀ respectively in relation to the initial number. APC followed the same patterns as before, and when the pathogens were inoculated at the end of day 1, its concentration had reached 7.5 log₁₀ CFU/g. The absence of growth of *V. cholerae* O1 and *S. typhi* on germinated seeds illustrates the principle of competitive exclusion, which is currently under investigation as an intervention to improve sprout safety.

Alfalfa sprouts are sometimes kept under refrigeration in stores or at home. Thus, it is also important to investigate if viability of pathogens is affected under such conditions. Refrigeration reduced the number of viable *S. typhi* from 4.8 log₁₀ to 4.5 log₁₀ CFU/g (50 % of reduction), and *V. cholerae* O1 from 3.1 log₁₀ to 2.1 log₁₀ CFU/g (90 % of reduction) after 15 d in storage. These limited decreases have no practical significance in the safety of

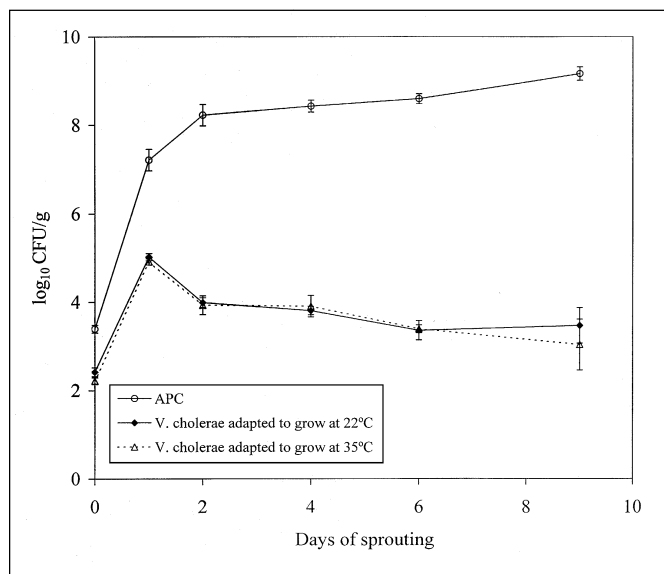


Fig. 5 Behavior of *V. cholerae* O1 adapted to grow at 22 or 35 °C during sprouting of contaminated alfalfa seeds.

the sprouts. It is again evident that the control of bacterial pathogens in sprouts is primarily preventive in nature. The well known content of nutrients of alfalfa sprouts combined with a nearly neutral pH and high moisture content (Hamilton and Vanderstep 1979), and the apparent absence of antimicrobial substances in the produce, make them an excellent substrate to support bacterial growth.

We did not observe any difference in the survival and growth pattern during germination and sprouting of *V. cholerae* O1 strain adapted to grow at 22 °C when compared to the strain that was always cultivated at 35 °C (Fig. 5). After 24 h of storage, both strains increased approx. 2.5 log₁₀, decreased 1 log₁₀ in the next 24 h, and then maintained a tendency to slowly decrease. However, after 9 d the concentration of vibrios remained above 3 log₁₀ CFU/g in both cases. Statistical analysis of data from Fig. 5 for both strains of *V. cholerae* O1 were compared by analysis of variance (Statistica, StatSoft Inc. 1993), and did not show any significant difference ($p < 0.01$). In contrast, APC was always increasing suggesting an antagonistic effect against vibrio populations.

Apparently, pathogenic bacteria are uncommon in vegetable sprouts and seeds (Prokopowich and Blank 1991). Data from developing countries (with high endemicity of infectious gastrointestinal illnesses) are lacking. Some common practices in México, and perhaps in other countries, make alfalfa sprouts particularly hazardous. The microbiological quality of water used to irrigate the seeds and sprouts by producers are dubious. Even if water available from the municipal supply is free of coliforms, inadequate storage conditions, the use of unclean utensils, and mishandling practices may introduce dangerous microorganisms. Alfalfa sprouts are sometimes distributed and offered unpacked to consumers, and in public markets (not supermarkets) the produce is not refrigerated. Further, sprouts are given indiscriminately and are sometimes given to susceptible groups of people, such as children, the elderly, and convalescent or weak individuals, to speed recovery or promote their nutritional condition. The critical control points are the sanitizing treatments applied to seeds prior to germination, sanitation of equipment used for germination, and assurance that the water supply is pathogen-free.

We concluded that due to the poor sanitary conditions under which foods are generally prepared and served, and to a high incidence of gastroenteritis among people in developing countries, it is expected that foods like sprouts are readily exposed to human contamination with enteropathogenic bacteria. It is a priority to regulate the production, shipping, and commercialization of alfalfa and other sprouts, which are becoming more accepted by consumers. It is also necessary to inform the public about adequate handling of products obtained from stores, and sanitary measures to obtain a safer sprouts from seeds at home.

Materials & Methods

Strains

Salmonella typhi LMS-4 isolated from a typhoid fever patient was obtained from the Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, México. *Escherichia coli* O157:H7 (E09) was kindly donated by Dr. M.P. Doyle, University of Georgia. *V. cholerae* O1 Inaba LMS-12 strain was isolated from a cholera patient in the State of Michoacan, México. Stock cultures were maintained at 4 to 7 °C with weekly transfer on tryptic soy agar (TSA). The organisms were cultured in tryptic soy broth (TSB) at 35 °C for 18 h; inocula were transferred to TSB at 3 consecutive 24-h intervals before use in experiments.

Seed samples and indicator bacteria counts

Eight batches of alfalfa seeds were purchased from food stores in 5 states. The seeds appeared generally clean and free of extraneous matter. Aerobic plate counts (35 °C, 24 h) were performed in triplicate using plate count agar, and total coliforms were determined using violet red bile agar (35 °C, 24 h) in 10-g seed portions (Vanderzant and Splittstoesser 1992). Samples showing the lowest bacterial content were selected for subsequent studies.

Preparation of inocula

Resistance to rifampycin (Ciba Geigy, Mexico City, Mexico) was induced in *Salmonella typhi*, *Vibrio cholerae* O1, and *Es-*

cherichia coli O157:H7 strains, according to Kaspar and Tamplin (1993). Briefly, a suspension of each pathogen was prepared by centrifuging 6-h cultures grown in TSB, discarding the supernatant and washing twice in peptone (0.1%) saline (NaCl 0.85%) water. Washed cells were finally resuspended in an appropriate volume of sterile 0.1% peptone to contain 1010 CFU/mL. For each pathogen, 1 mL of this suspension was spread over 5 TSA plates containing 100 mg/L of Rif. Inoculated Rif-TSA plates were incubated at 35 °C for 48 h, and 3 to 5 colonies were selected for streaking on TSA slants. A TSB culture of each pathogen was diluted in sterile 0.1% peptone to yield 103 to 105 CFU/mL dip suspensions.

Inoculation of seeds and germinated seeds

Alfalfa seeds (100 g) were aseptically weighed and transferred to a plastic strainer and rinsed thoroughly with tap water. They were then immersed just enough to cover the seeds with a sodium hypochlorite solution (pH 5.0, containing 10 or 100 mg/L free chlorine) contained in a 500 mL beaker in 2 separate trials. Chlorine solutions were prepared in deionized water, and the free available chlorine was determined by iodometric titration (Greenberg and others 1992). After 20 min contact with hypochlorite solution at room temperature, the seeds were drained and dipped in a sodium thiosulfate solution to inactivate any residual active chlorine. Seeds were soaked in sterile tap water at 21 to 22 °C for 4 h. Behavior of *S. typhi*, *V. cholerae* O1, and *E. coli* O157:H7 was studied both in seeds prior to germination; the behavior of *S. typhi* and *V. cholerae* O1 was studied 24 h germination began.

The strainer containing disinfected soaked seeds was separately dipped for 10 min in each bacterial dip suspension and allowed to drain with gentle periodic shaking for 10 min. Seeds

were then spread in a monolayer on a sterile stainless-steel tray and drained. Seeds that had been stored for 24 h germination were contaminated with suspensions of *S. typhi* or *V. cholerae* O1 in a similar manner as described for seeds. Trays were covered with plastic wrapping film to minimize moisture loss and microbial contamination. Seeds were incubated at 22 ± 1 °C and sprayed once per day with sterile tap water.

Monitoring *S. typhi*, *E. coli* O157:H7, and *V. cholerae* O1 during sprouting

On each storage day, inoculated samples were drawn and used to monitor the behavior of *S. typhi*, *E. coli* O157:H7, or *V. cholerae* O1 during sprouting. Numbers of CFU were counted on 10-g samples on TSA prepared with 60 mg/L of Rif. Aerobic plate count (APC) was determined (35 °C for 24 h) from each treatment in TSA free of Rif. In both cases, duplicate plated were counted after incubation and results were converted to log₁₀ CFU/g sample. Each trial was carried out by triplicate.

Statistical analysis was made for data presented in Table 1 (Duncan, 0.05). Limits of standard deviation are indicated for each experimental point in Figs. 1 to 5

Fate of *S. typhi* and *V. cholerae* O1 in alfalfa sprouts stored in refrigeration

In 2 experiments, alfalfa seeds were contaminated with suspensions of both *S. typhi* Rif R+ and *V. cholerae* O1 Rif R+ as mentioned. After sprouting for 7 d, sprouts were placed in a refrigerator at 4 to 7 °C for 15 d. Then, the populations of pathogens were determined in triplicate for 50-g portions as described.

All media used in this work were from BIOXON (Becton Dickinson, México City).

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