

COMUNICACIONES

Susceptibility of the bacterium *Vibrio cholerae* to acid pH in salad vegetables: An ultrastructural view

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Resumen: Se inoculó *Vibrio cholerae* (ca. 1×10^{12} UFC/ml) en ensaladas de vegetales y se evaluó su supervivencia adicionando jugo de limón ácido a la ensalada, lo que bajó el pH de 4.5 a 3.4. En estas condiciones no se recuperó la bacteria a los 30 minutos post adición del jugo de limón. Sin embargo, no hubo alteraciones morfológicas en las bacterias, detectables al microscopio electrónico de transmisión (tinción negativa) ni al microscopio electrónico de rastreo.

Key words: *Vibrio cholerae*, vibriocidal, acid foods, food microbiology, ultrastructure, electron microscopy.

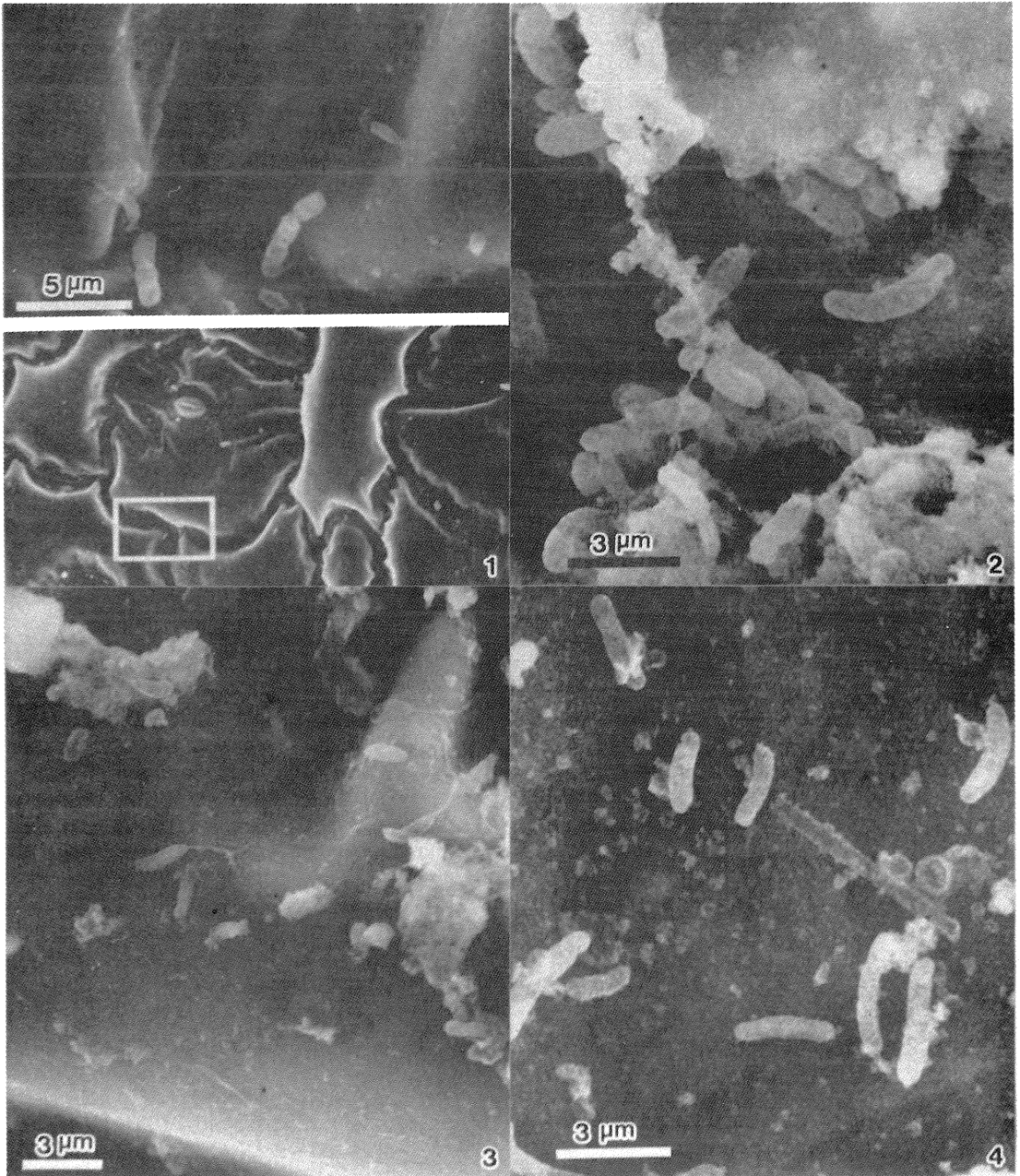
Morbidity and mortality associated with childhood diarrheal disease are a major public health problem in most non-industrialized American countries. But the reappearance of cholera in Latin America has extended the problem to virtually all age groups (Sagarnaga 1993). Diarrheal diseases are related to poor food-hygiene practice, sanitation, education, and scanty supply of drinking water, which also affect malnutrition (Blake 1993). These factors reflect the occurrence of more than 600,000 cases of cholera reported in Latin American countries in the second year of the epidemic (Sagarnaga 1993).

The main vehicles of *Vibrio cholera* are water and foods contaminated with feces from infected persons. But foods with pH lower than 4.5 do not permit the growth of this agent (WHO 1992); particularly, the vibriocidal effect of lemon juice is one of the most effective between other fruit juices, as previously was demonstrated by Mata (1992) and Gelli (1993). For this reason we evaluated the effect of lemon juice on the ultrastructure of *V. cholerae* and its inhibitory effect on salad vegetables.

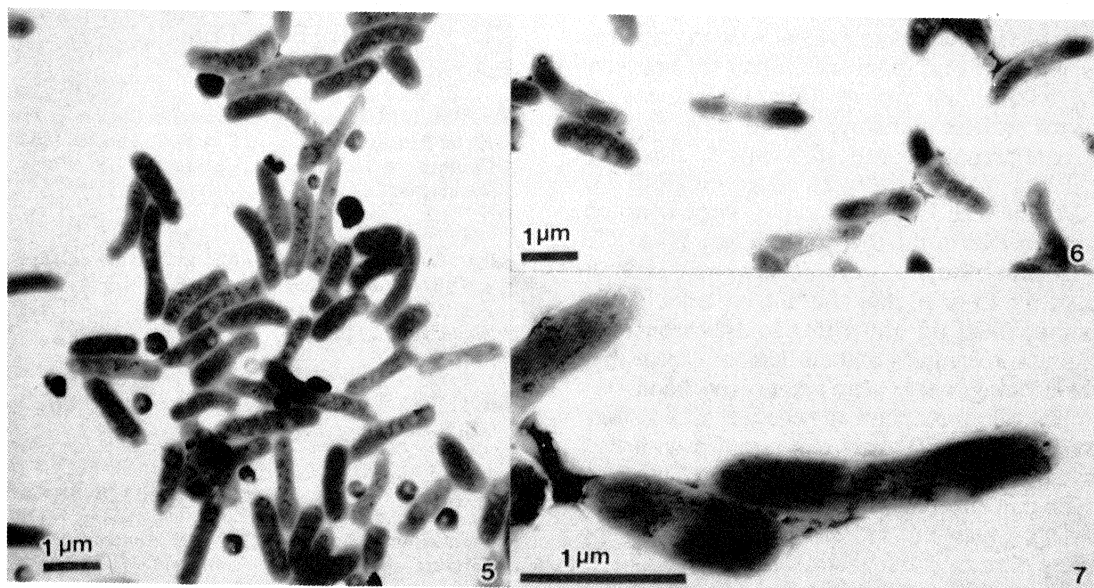
The recipe for salad vegetables was obtained from 130 housewives interviewed using a standardized questionnaire about ingredients, quantities, vegetable processing, time, and temperature of storage before consumption. The most common recipe was: one grated cabbage (350g), two grated carrots (100g), one sliced cucumber (190g), two diced tomatoes (340g), and the juice of one lemon. The pH was measured before and after the addition of lemon juice.

Four salads were prepared according to the above recipe. Each one was inoculated with ca. $1 \times 10^{10-12}$ CFU/ml of *Vibrio cholerae* O1 El Tor, serotype Inaba, strain 1800-82 CDC. Immediately after inoculation and again at 15 and 30 min later, 25g samples of the salads were taken, neutralized with NaOH to pH 7.0 - 7.4 and homogenized in 225 ml of alkaline peptone water (APW) and decimal dilutions were made in APW. Ten-fold dilutions were in APW. An aliquot of 0.1 ml of each dilution was inoculated in thiosulfate-citrate-bile salts-sucrose agar (TCBS) and incubated for 18 to 24 hours at 35°C. Also, the first dilution was incubated for 6 to 8 hours at room temperature and 0.1 ml were inoculated in TCBS. The same procedure was used with another four salads prepared without lemon juice.

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Figs. 1-4: Cabbage (Figs. 1 and 3) and carrot (Figs. 2 and 4) pieces inoculated with *V. cholerae*. The upper electron micrographs correspond to samples photographed at 30 min post inoculations and treatment with water. Figs. 3 and 4 were taken at 30 min after inoculation and treatment with lemon juice. S=stoma, bar=5 μm.



Figs. 5-7: *V. cholerae* on electron microscopy grids floated on distilled for 30 min. (Fig. 5) and floated on lemon juice for 30 min. (Figs. 6 and 7). Bar=5 μ m.

For scanning electron microscopy (SEM) analysis, small pieces (ca. 1 cm²) of cabbage and carrot were placed on a Petri dish, covered with a drop of the inoculum of *V. cholerae*, and kept at room temperature for 15 min. Six pieces of each vegetable were covered again with lemon juice, and another six were covered with distilled water. Immediately after adding lemon juice or water, and also at 15 and 30 min later, two pieces of each specimen were fixed in 2.5 % glutaraldehyde (GA), post fixed in 1 % osmium, and processed for SEM.

For transmission electron microscopy (TEM), the inoculum of *V. cholerae* was impregnated in formvar covered grids. Then the grids were floated on a drop of lemon juice or distilled water. Immediately after that, and also at 15 and 30 min later, two grids were fixed in GA, washed with distilled water, negative stained with phosphotungstic acid, and observed under a transmission electron microscope.

The pH of salads without lemon juice was 4.5 ± 0.2 , and after adding the juice was 3.4 ± 0.4 . The population of *V. cholerae* inoculated into salads without lemon juice decreased to $1 \times 10^5 - 10^6$ CFU/ml, depending to initial inoculum. Almost 1×10^6 CFU of the inoculated bacteria survived at least 15 min in the salad

(pH 4.5) and at 30 minutes the recovered population was ca. 1×10^5 . A dramatic reduction of the amount of bacteria was detected when lemon juice was added to the salad; out of 10^{12} CFU/ml added, the bacterial population at 30 minutes was only 1×10^3 CFU/ml or was not detected. Also, the bacteria were not recovered from the initial APW dilution taken after 30 min of inoculation; but twice were detected in the initial APW dilutions taken at 15 min after inoculation.

These experiments showed that, vegetable salads with pH around 4.5 reduced the inoculated *V. cholerae* in a proportion of about 10^4 CFU/ml. This effect was increased when lemon juice was added to the salads and their pH lowered to 3.4. Under those conditions an inoculum of 10^{10} to 10^{12} CFU/ml, that represent more than one infecting dose of *V. cholerae*, were reduced at 10^3 or less CFU/ml after 30 minutes of contact with lemon juice.

The SEM analysis of samples of cabbage and carrot inoculated with *V. cholerae* and then fixed without treatment with lemon juice showed the normal appearance of the bacteria (Fig. 1 and 2). On the cabbage surface there was less bacteria than on the surface of carrots (Figs. 1 and 2). In the former, small groups of 3

to 5 bacilli were more evident near the stomas, or individual bacillum were observed between the intercellular grooves (Fig. 1), whereas on carrot surface numerous bacilli were evident almost homogeneously distributed; often big clusters of bacteria were observed (Fig. 2). Also, on both kinds of samples were detected some bacilli in the division process. When the vegetable samples were covered with lemon juice for 15 or 30 min after the bacterial inoculation, they did not differ in appearance or amount in samples without lemon, except that the dividing shapes were scant (Figs. 3 and 4).

The ultrastructures of negative stained bacteria were similar between samples with and without lemon juice (Figs. 5 and 6). Thus, the inhibitory effect of low pH, in this case illustrated by the treatment with lemon juice, does not cause ultrastructural alterations.

These data suggest that an additional safety rule for the consumption of vegetable salads where *V. cholerae* is endemic, is to add lemon juice on the vegetable salads at least 30 min before consumption.

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