Antibacterial Activity of Hen Egg White Lysozyme against *Listeria monocytogenes* Scott A in Foods

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Egg white lysozyme killed or prevented growth of *Listeria monocytogenes* Scott A in several foods. Lysozyme was more active in vegetables than in animal-derived foods that we tested. For maximum activity in certain foods, EDTA was required in addition to lysozyme. Lysozyme with EDTA effectively killed inoculated populations of 10^4 *L. monocytogenes* per g in fresh corn, fresh green beans, shredded cabbage, shredded lettuce, and carrots during storage at 5°C. Control incubations without lysozyme supported growth of *L. monocytogenes* to 10^6 to 10^7/g. Lysozyme had less activity in animal-derived foods, including fresh pork sausage (bratwurst) and Camembert cheese. In bratwurst, lysozyme with EDTA prevented *L. monocytogenes* from growing for 2 to 3 weeks but did not kill significant numbers of cells and did not prevent eventual growth. The control sausages not containing lysozyme supported rapid and heavy growth, which indicated that lysozyme was bacteriostatic for 2 to 3 weeks in fresh pork sausage. We also prepared Camembert cheese containing 10^4 *L. monocytogenes* cells per g and investigated the changes during ripening in cheeses supplemented with lysozyme and EDTA. Cheeses with lysozyme by itself or together with EDTA reduced the *L. monocytogenes* population by ~10-fold over the first 3 to 4 weeks of ripening. In the same period, the control cheese wheels without added lysozyme with and without chelator slowly started to grow and eventually reached 10^6 to 10^7 CFU/g after 55 days of ripening. In the lysozyme-containing cheeses, however, *L. monocytogenes* also grew after 3 to 4 weeks of curing, which indicated that lysozyme was initially bacteriostatic but later allowed growth under the experimental conditions. Assay of lysozyme showed that lysozyme was still active in Camembert; therefore, changes during ripening in the cheese or in the bacterium probably overcame the inhibitory activity of lysozyme. In conclusion, results of this study suggest that egg white lysozyme could be useful as a preservative to protect against *L. monocytogenes* contamination in several foods, especially those prepared from fresh vegetables.

The four major outbreaks of listeriosis that have occurred since 1981 (2, 4, 10, 19) have proven *Listeria monocytogenes* to be an important pathogen. The incidents that occurred in Nova Scotia in 1981, Los Angeles in 1985, and Vaud, Switzerland, in 1988 confirmed by means of epidemiological and microbiological evidence that *L. monocytogenes* was transmitted by particular foods (28). In the Boston incident (4), epidemiological evidence suggested pasteurized whole milk as the vehicle, but microbiological confirmation was not obtained. These carefully investigated outbreaks have clearly demonstrated that *L. monocytogenes* can be transmitted in foods. In addition to these proven outbreaks, other listerioses have occurred in which epidemiological or microbiological evidence was unsatisfactory to prove food-borne transmittal (28). One of the first well-described food-related outbreaks of listeriosis occurred in 1979 in a Boston hospital, when 20 patients developed listeriosis probably by eating raw celery, tomatoes, and lettuce (8). Despite the lack of confirming evidence in many observed listerioses, the available data indicate that occurrence of food-borne listeriosis is increasing in several countries (28).

Food-borne transmission of listeriosis to humans probably occurs mainly through environmental contamination because of the uncommon ability of *L. monocytogenes* to colonize animals and food contact surfaces from environmental sources (16, 26, 28). *L. monocytogenes* is widespread in the environment and has been isolated by enrichment techniques from many sources, including sewage, vegetable matter, soil, straw, silage, dust, milk, and the feces of healthy animals and humans (3, 7, 13, 25, 27). Although listeriosis was once believed to be mainly a zoonosis, many experts now think that it contaminates food mostly from environmental sources (28).

Microbiological surveys of foods and food-processing plants have shown that the pathogen is thoroughly spread in the food supply and in food-processing plants (16, 26, 28). *L. monocytogenes* has been isolated from raw milk (1, 5, 16), cheeses (1, 2, 10, 16, 24, 28), eggs (17), vegetables (17, 28), prepared salads (17, 22), meat and poultry products (6, 7, 16, 19), and seafoods (16, 28).

In addition to its inherent contamination of many foods, *L. monocytogenes* seems to have an exceptional ability to colonize pasteurized or processed products and to be transmitted on-line by processing machinery, such as during slicing of meats or vegetables, filling of ice cream containers, or preparation of salads (14, 16, 28). In a survey conducted by the U.S. Food and Drug Administration beginning in 1986, 2.5% of 357 dairy processing plants were found to have products containing *L. monocytogenes* (5, 16). Postpasteurization contamination was thought to be mainly responsible for contamination. *L. monocytogenes* can grow at temperatures of 1 to 45°C (20), and refrigeration alone is not sufficient to prevent growth in foods. It can withstand 25% NaCl and acidic pHs for several months (21). The presence of *L. monocytogenes* in raw and certain processed foods may be currently unavoidable (28).

Safety of a food from *L. monocytogenes* depends on meeting three processing criteria (14): (i) eliminating the

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organism to the highest achievable extent by pasteurization or other processing methods, (ii) avoiding recontamination, and (iii) adding needed antimicrobial protection when intrinsic protection is inadequate. If recontamination of a food does occur, a secondary preservative is highly desired. Our laboratory showed earlier (9) that egg white lysozyme killed four pathogenic strains of *L. monocytogenes* in culture media and in buffer. We have extended our tests to foods and show in this study that lysozyme is effective in killing or preventing growth of *L. monocytogenes* Scott A in several foods.

MATERIALS AND METHODS

Materials. The sources of egg white lysozyme, *Micrococcus luteus*, and other materials were the same as previously described (9).

Organisms. *L. monocytogenes* Scott A was provided by E. H. Marth, University of Wisconsin, Madison. This strain is being used by the International Dairy Federation (group E64) for development of methods to detect and enumerate *L. monocytogenes* in foods (International Dairy Federation, group E64, Detection and Enumeration of *L. monocytogenes*).

Penicillium camemberti was used for surface ripening of cheese; it was originally subcultured from the Kenneth Raper culture collection in the Department of Bacteriology, University of Wisconsin, Madison. Spores were produced by 2- to 4-week growth at 25°C on potato dextrose agar.

Bacterial media, growth, and enumeration. *L. monocytogenes* was routinely grown in static culture in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) at 37°C. Growth in culture media was measured by optical density at 500 nm in a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). Optical density values of 0.1 and 1.0 represent viable cell counts of 2.7×10^8 and 2.0×10^9, respectively (Jean Schoeni, personal communication).

For growth studies in foods, *L. monocytogenes* Scott A was first cultured in brain heart infusion broth at 37°C and inoculated into the foods at a predetermined concentration of cells. To adequately monitor loss of viability, we usually used 10^4 cells per g of food. All food incubations were done in duplicate, and 25-g samples of foods were sampled and analyzed independently for *L. monocytogenes*.

L. monocytogenes was enumerated by direct plating on lithium chloride-phenylethanol-moxalactam agar as described previously (11, 12) except that glycine was used in place of glycine anhydride. Food samples (25 g) were placed in sterile plastic stomacher bags (model 400; Seward Medical Co., London, United Kingdom), and 225 ml of *Listeria* enrichment broth (see below for composition) was added. The samples were homogenized in a stomacher (Stomacher Lab Blender, model 400; Tekmar Co., Cincinnati, Ohio) for 2 min. Portions of 0.1 ml were plated on 10 lithium chloride-phenylethanol-moxalactam plates. When cells were present in high numbers ($\geq 10^4$/g), samples were serially diluted in 0.01 M phosphate buffer (pH 7.2), and 0.1 ml was plated on duplicate plates. The plates were incubated at 30°C for 5 to 7 days.

Enrichments were also performed to detect very low numbers of *L. monocytogenes*. We modified the enrichment procedure of McClain and Lee (12) by incubating cultures for 5 to 7 days in the stomacher bags at 30°C. *Listeria* enrichment broth (12) consisted of the following (per liter): proteose peptone (Difco), 5 g; tryptone (Difco), 5 g; Lab Lemco powder (Oxoid Ltd., Columbia, Md.), 5 g; yeast

extract (Difco), 5 g; NaCl, 20 g; Na_2HPO_4, 12 g; KH_2PO_4, 1.35 g; esculin (Sigma Chemical Co., St. Louis, Mo.), 1 g; and nalidixic acid (sodium salt; Sigma), 20 mg. Acriflavine hydrochloride (Sigma) (12 mg/liter) was filter sterilized and added to the medium after autoclaving. For primary enrichment, the food homogenates in the stomacher bags were incubated at 30°C for 5 to 7 days. Then 0.1-ml portions of the enrichments were inoculated into 10 ml of *Listeria* enrichment broth plus acriflavine hydrochloride (25 mg/liter) and incubated for 5 to 7 days as a secondary enrichment. These samples were streaked directly on lithium chloride-phenylethanol-moxalactam plates and also treated with KOH solution (12) to select for *L. monocytogenes*.

To confirm identification, five presumptive colonies per sample were examined for various phenotypic characteristics, including lack of pigmentation, morphology on tryptose agar, and positive catalase test. Three of the colonies were then tested for characteristic end-over-end tumbling motility in tryptose broth at ambient temperature and for umbrella motility just below the surface in motility test medium (Difco) at ambient temperature and at 37°C. One colony per food sample was further tested for a positive methyl red reaction in MRVP medium (Difco), for esculin hydrolysis in bile-esculin agar (Difco), and for reduction of litmus milk (Difco) after 2 days at 30°C. Serological analysis was also performed, using *Listeria* O antiserum type 4 (Difco). *Listeria* O antigen (type 4; Difco) was used as a positive control. We also tested ~15 isolates from the bratwurst experiment for acid production in purple broth (Difco) containing 0.5% (wt/vol) galactose, sorbitol, mannitol, dulcitol, maltose, rhamnose, xylose, glucose, lactose, and melezitose, and we found that the pattern of acid formation was the same as for Scott A.

Food studies. Several foods were used as substrates for *L. monocytogenes*. In all foods, we tested four conditions: (i) control (no additions); (ii) lysozyme, 100 mg/kg; (iii) EDTA, 5 mM (except for Camembert, which had 1 mM); and (iv) lysozyme plus EDTA. In each experiment, foods were prepared and individually placed in several containers for incubation. Two containers (duplicate samples) were tested at each time point of sampling. All food samples were incubated with and without experimentally inoculated *L. monocytogenes* to examine for natural contamination.

Foods were qualitatively examined at the conclusions of the experiments for lysozyme activity. *M. luteus* dried cells (0.25 mg/ml; Sigma) were suspended in 2.9-ml portions of 0.067 M phosphate buffer (pH 6.6). Food samples were diluted 10-fold with buffer and added to the *M. luteus* suspension. The decrease in A_{540} was measured after 2 h. A marked decrease in absorbance compared with that seen in the food samples without added lysozyme indicated that lysozyme activity was present. Also, fresh lysozyme (1 to 3 ppm [1 to 3 µg/ml]) was tested in buffer for comparison and in foods without added lysozyme to ascertain that inhibition was not occurring. The pH of each food sample was estimated after 10 g of food was homogenized in 90 ml of distilled water.

Preparations of foods. Fresh lettuce heads from a local market were coarsely shredded and divided into 1,800-g portions. Lysozyme was dissolved in 25 ml of 67 mM phosphate buffer (pH 6.6), and EDTA (tetrasodium salt; Sigma) was dissolved in 25 ml of distilled water. The solutions were sprinkled onto the 1,800-g portions of lettuce. The final concentrations of lysozyme and EDTA were 100 mg/kg and 5 mM, respectively. *L. monocytogenes* was diluted and placed in 25 ml of 67 mM phosphate buffer at a

concentration to give approximately 10^4 CFU/g of lettuce. The lettuce was then well mixed by hand. Portions of 100 g were placed in 8-oz (ca. 227-g) polystyrene containers (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.), and the containers were incubated at 5°C. Duplicate containers were sampled for *L. monocytogenes* after 0, 3, 7, and 12 days.

Fresh green cabbage obtained from a local market was processed as described above for lettuce. Duplicate containers were sampled after 0, 3, 7, 12, 20, 27, 34, 41, and 48 days.

Fresh ears of sweet corn were obtained at a local market, and kernels were removed. Procedures for lysozyme, EDTA, and *L. monocytogenes* additions and samplings were as described above for lettuce. Samples were taken after 0, 2, 4, 9, 11, and 15 days.

Fresh green beans were obtained from a local market and were snapped by hand into pieces 1 to 1.5 in. (ca. 2.5 to 3.8 cm) long. Other procedures were as described above for lettuce. Samples were taken after 0, 2, 5, 7, 9, and 15 days.

Fresh carrots were processed as described for fresh lettuce except that an inoculum of 10^3 CFU of *L. monocytogenes* per g was used. Samples were taken on days 0, 2, 5, 9, and 16.

Frozen corn and frozen green beans were treated as described above. Corn samples were taken on days 0, 2, 4, 7, and 10; green beans were sampled after 0, 4, 7, and 11 days.

For bratwurst samples, coarsely ground frozen pork trim (25 to 30% fat) was obtained from the Department of Muscle Biology, University of Wisconsin, Madison. Semithawed meat (15-lb [ca. 6.8-kg] samples) was placed in a Buffalo Mixer (John E. Smith's Sons Co., Buffalo, N.Y.); an ice slurry (750 ml) and 136 g of bratwurst spice (F. W. Witt and Co., Yorkville, Ill.) were also added. Then 100 ml each of distilled water with or without EDTA (5 mM, final concentration) and 67 mM phosphate buffer with or without lysozyme (100 ppm, final concentration) was added, and *L. monocytogenes* was inoculated to 10^4/g. The samples were mixed for 4 min with intermittent stops to shift the meat. The meat was then ground and stuffed into rinsed 32- to 35-mm natural hog casings (Vista International Packaging, Kenosha, Wis.) with a hand-cranked stuffing machine. Hand-twisted links (~6 in. [~15 cm]) were cut apart and vacuum packaged in 6.5-by-12-in. (ca. 16.5-by-30.5-cm) Curlon (grade 550) vacuum bags (Curwood, Inc., Chicago, Ill.) in portions of 0.75 to 1 lb (ca. 0.34 to 0.454 kg). The spice mix was formulated such that the prepared sausages contained approximately 20 oz (ca. 567 g) of NaCl per 100 lb (ca. 45.4 kg) of meat (F. W. Witt, personal communication). Throughout preparation of the sausages, reagents and equipment were kept cold and handling was minimized to keep the fat and muscle particulate. The packages of links were stored at 5°C for the duration of the experiment. Duplicate packages were examined for *L. monocytogenes* as described above after 0, 2, 6, 9, 15, 23, 33, 37, and 44 days.

Camembert cheese was prepared according to a standard protocol from the Cheese Research Institute (courtesy of Mark Johnson, Cheese Research Institute, University of Wisconsin, Madison). Safety precautions in preparation of the cheese with *L. monocytogenes* were followed as described by Ryser and Marth (18).

Fresh pasteurized whole milk (~60 lb [~27 kg]) was obtained from the University Dairy Plant. The milk was placed in a small cheese vat, and the temperature was adjusted to 33°C. A 10-ml amount of Superstart starter culture (Miles Laboratories, Inc., Madison, Wis.) and 6 ml of Marzyme single-strength microbial rennet (Miles) were

added. Lysozyme in 67 mM phosphate buffer and EDTA in distilled water were added to designated vats at 100 mg/kg and 1 mM, respectively (1 mM instead of 5 mM EDTA was used because the higher concentration inhibited curd formation). Finally, *L. monocytogenes* was suspended in 10 ml of sterile skim milk and added at 10^4 CFU/ml to designated batches. The contents of the vats were well mixed with a straining spoon, and curd was allowed to form for 50 min. The curd was cut into 0.75-in. (ca. 1.9-cm) cubes with cheese knives and allowed to heal for 15 to 20 min while being frequently stirred. The curds were poured into 12 cheese hoops (5 and 0.5 in. [ca. 12.7 and 1.3 cm]), which were placed on a draining board covered with cheesecloth. The hoops were covered with cheesecloth and turned after 1 and 5 h. The covered cheese wheels were left overnight at ambient temperature, and the next morning they were coated with cheese salt to achieve a salt content of ~2.5%. The wheels were left covered at 5°C on the second night. On the third morning, the wheels were dipped into 100 ml of sterile saline containing *P. camemberti* spores. The cheese wheels were placed on a cleaned draining board, covered with fresh cheesecloth, and incubated in the University of Wisconsin Biotron facility in an incubator held at 15°C and 90 to 95% relative humidity. The wheels were turned every 1 to 2 days for 14 days, at which time the wheels were individually wrapped in aluminum foil and stored at 5°C for the duration of the experiment. The curds were sampled on day 0, and duplicate slices were taken from single wheels of the ripening cheeses on days 2, 6, 13, 20, 27, 34, 48, 55, and 67. In addition, a cheese wheel was removed on day 2 just before mold inoculation and tested for salt and moisture contents. The salt concentrations were determined by using Quantab Chloride Titrators (no. 1176; Miles); individual determinations ranged from 1.5 to 2.7%. The moisture content was estimated by crumbling a slice and then mixing and placing 2- to 3-g portions into each of three tared aluminum boats, which were dried overnight at 94°C in a vacuum oven, allowed to cool in a desiccator, and weighed. Moisture contents ranged from 49.6 to 55.6%. Sampling and enumeration of *L. monocytogenes* in duplicate cheese slices were carried out as described above for the other foods.

RESULTS

Activity of lysozyme in vegetables. The antibacterial activity of lysozyme against *L. monocytogenes* Scott A was evaluated in several foods, especially those that have been shown or suspected to transmit listeriosis or to harbor the pathogen. Of the three confirmed food-borne outbreaks of listeriosis since March 1981 (28), two resulted from contamination of soft-ripened cheeses (2, 10) and one was attributed to coleslaw contaminated with infected cabbage (19). We first examined the activity of lysozyme in several vegetables, including cabbage.

L. monocytogenes grew well in shredded cabbage (Fig. 1); in the absence of inhibitory agents, the organism rapidly reached 3.5×10^6 CFU/g and then further increased to 10^8 CFU/g. (In all samplings, we examined duplicate containers at each time point. Duplicate enumerations of *L. monocytogenes* from individual containers varied $\leq$2-fold from the mean of the two determinations.) In incubations containing cabbage with 100 mg of lysozyme per kg or lysozyme with 5 mM EDTA, *L. monocytogenes* Scott A (CFU per gram) declined by approximately 10- or 100-fold, respectively, after 7 days of incubation at 5°C. *L. monocytogenes* in the cabbage samples with lysozyme grew marginally after 7 days
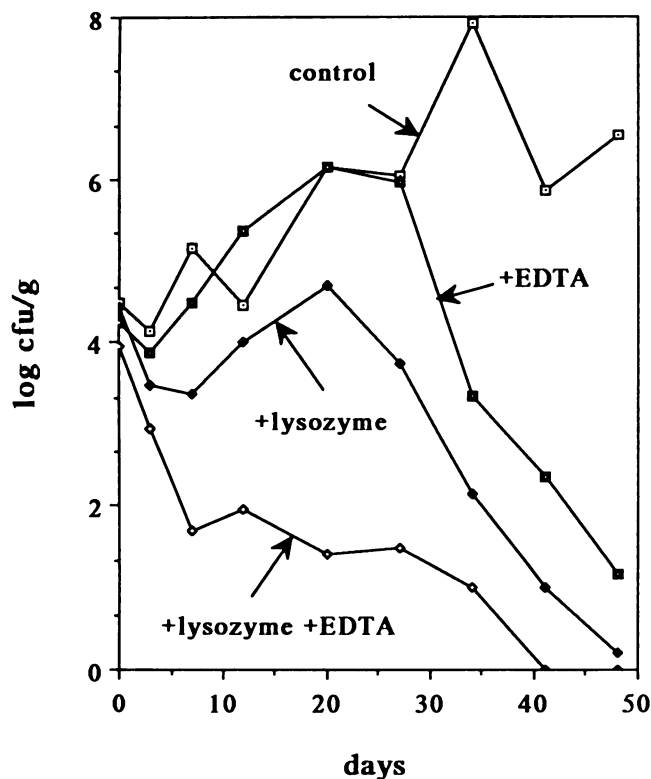
FIG. 1. Effect of lysozyme and EDTA on growth of *Listeria monocytogenes* in shredded cabbage. See Materials and Methods for experimental details.

FIG. 2. Effect of lysozyme and EDTA on growth of *L. monocytogenes* in shredded lettuce.

and later declined, whereas growth in the incubations with lysozyme and EDTA declined throughout the experiment. Bacteria in the treatments containing EDTA alone grew well and paralleled numbers in the control for 30 days, after which the population rapidly dropped. The unusual growth patterns of *L. monocytogenes* in cabbage containing EDTA were often seen in other foods as well (see below).

The loss in viability of *L. monocytogenes* in cabbage in the presence of lysozyme was not due to changes in pH, since all four incubation conditions gave approximately the same pH profiles. The initial pH of the cabbage medium was 4.8, which increased to 6.6 to 6.8 after 7 days and remained at this pH until 20 days of incubation. The pHs then dropped to 4.3 to 4.9 in the samples containing lysozyme, whereas the controls had final pHs of 5.8 to 5.85 after 40 days. The samples supplemented with EDTA alone had pHs ranging from 5.0 to 5.5 after 40 days. The unusual changes in pH may have been caused by changes mediated by *L. monocytogenes* and other bacterial flora in the refrigerated conditions, in which lactic acid fermentation may have been suppressed. Visible changes of the cabbage, including browning and softening, were noticed after 1 to 2 weeks.

The incubations with lettuce (Fig. 2) showed patterns of *L. monocytogenes* survival similar to those seen with cabbage. The combination of lysozyme and EDTA was actively listericidal, whereas lysozyme alone was listeristatic. The controls and the samples with EDTA alone grew to populations of 3.4×10^5 and 1.6×10^5. In contrast, samples with EDTA and lysozyme had no detectable *L. monocytogenes* after 12 days. The lysozyme samples had $\sim 2.5 \times 10^3$ organisms, a 2-fold decrease from the initial cell count. In the lettuce samples, there was little variation among the pH
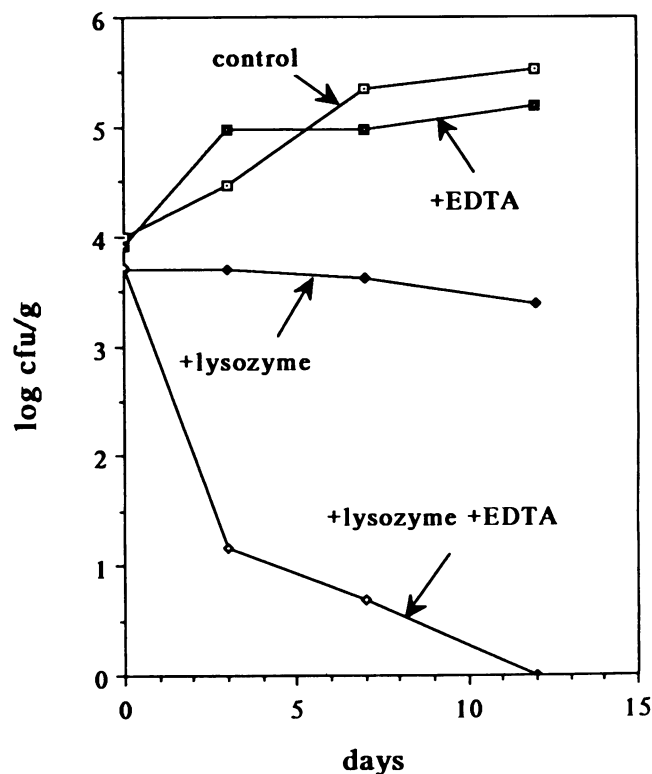
profiles except for the negative control, which had a lower final pH (4.8) than did the other incubations (pH range, 5.6 to 5.8). Therefore, lysozyme was actively listericidal in lettuce.

Lysozyme with EDTA was bactericidal in snapped green beans; after 1 week, a decrease of ~ 100-fold was observed (Fig. 3). In the other experimental variations growth occurred, although lysozyme slowed growth considerably compared with results for the control or EDTA incubations (Fig. 3). It appears that lysozyme with or without EDTA was less effective in green beans than in cabbage or lettuce. The initial pHs of the green beans varied from 5.94 to 6.07, and the final pHs of the different samples changed by less than 0.4 pH units. Possibly, the absence of pH stress during incubation supported survival of *L. monocytogenes*. Alternatively, since the green beans were snapped into fairly large pieces, it is possible that lysozyme did not rapidly penetrate to the interior and *L. monocytogenes* could colonize the center and grow in isolation.

Treatment of fresh corn kernels with lysozyme with and without EDTA resulted in a pattern of *L. monocytogenes* survival similar to that described above for green beans. The decline rates were much slower than on lettuce and cabbage, but it was still evident that lysozyme plus EDTA was listericidal whereas lysozyme alone was listeristatic (Fig. 4). In contrast, *L. monocytogenes* in the negative controls without any additions and the incubations containing EDTA alone grew to 1.0×10^7 and 1.8×10^5, respectively, after 9 days. Initial pHs of the fresh corn samples varied from 6.6 to 6.7 and did not change through day 9, but dropped to 4.5 to 5.0 after 15 days in the samples not containing EDTA. With EDTA present, the pHs did not drop below 6.0 after 15 days. EDTA seemed to affect subsequent bacterial spoilage but did not influence survival of *L. monocytogenes*. As with the
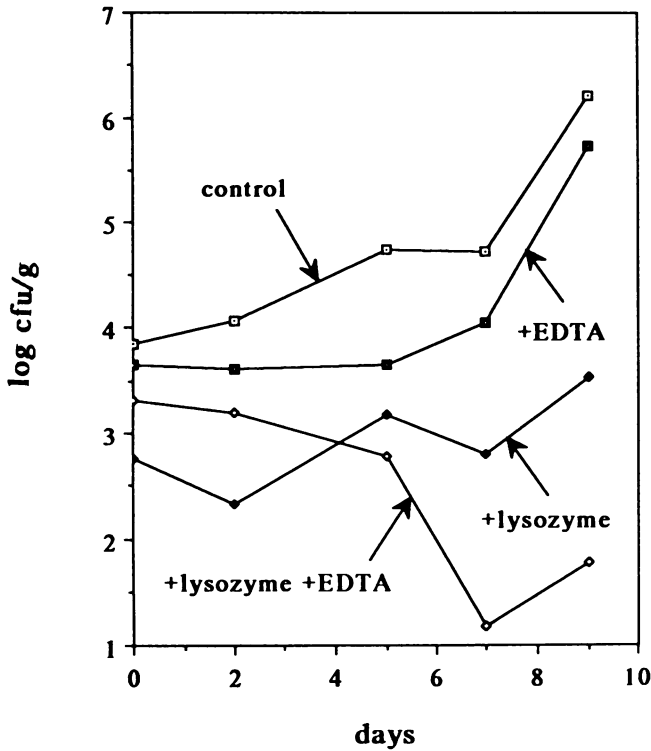
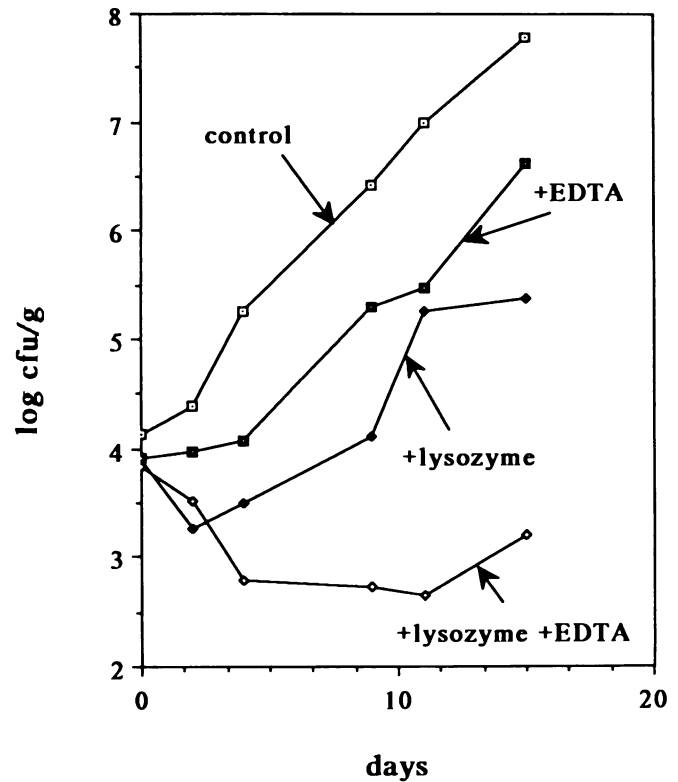FIG. 3. Effect of lysozyme and EDTA on growth of *L. monocytogenes* in fresh snapped green beans.

FIG. 4. Effect of lysozyme on growth of *L. monocytogenes* in fresh corn.

other fresh vegetables tested, lysozyme had a negative effect on *L. monocytogenes* growth in fresh corn. The slower rates of decline could have resulted from differences in food composition, such as the presence of high concentrations of carbohydrates, which could promote growth.

In fresh shredded carrots, incubation of lysozyme with and without EDTA and *L. monocytogenes* resulted in unusual responses of growth and killing (Fig. 5). In this experiment, *L. monocytogenes* was used at lower initial levels (~1,000 colonies per g). Lysozyme alone or with EDTA very rapidly killed *L. monocytogenes* in the carrot medium (Fig. 5). Bacteria in the control with no additions initially grew for approximately two doublings, and then detectable numbers also fell to zero after day 9. In contrast, EDTA initially supported a large increase in viable cell counts, which then rapidly declined after day 9. These results suggest that there may be factors in carrots, perhaps limiting metal ions or listericidal factors, that encourage the activity of lysozyme against *L. monocytogenes*. The pHs of the carrot samples were initially 6.2 to 6.4; these dropped steadily to 4.1 to 4.4 after 9 days. The change in pH did not appear to influence *L. monocytogenes* survival.

Overall, lysozyme was very effective in killing *L. monocytogenes* in most fresh vegetable products. During the winter, we also tested frozen commercial green beans and corn; in both cases, lysozyme did not kill the inoculated *L. monocytogenes*, although it did (in combination with EDTA) prevent growth (data not shown). We noticed that with the frozen green beans, but not with the corn, the pH of the frozen product dropped much faster than it did with fresh green beans, perhaps because of more rapid growth by acid formers. The reasons that lysozyme was not effective in thawed frozen products is not known, but they may be

related to inactivation of enhancing factors during processing of the fresh vegetables.

Activity of lysozyme in fresh sausages. We next examined the effectiveness of lysozyme against *L. monocytogenes* in bratwurst, which is a fresh meat sausage common in Wisconsin. Initial counts of *L. monocytogenes* in the sausages were 4×10^3 CFU/g. We found that the incubations containing lysozyme alone did not affect growth significantly compared with the negative control without lysozyme (Fig. 6). Both incubations grew to ~10^7 CFU/g in 1 to 2 weeks. The sausages containing EDTA alone were listeristatic for 2 weeks, after which *L. monocytogenes* grew to very high numbers. The most effective variation that inhibited *L. monocytogenes* was the combination of lysozyme and EDTA. When these two substances were included together, initial numbers fell from 3.9×10^3 to 1.4×10^3 after 9 days, and then *L. monocytogenes* slowly began to grow over the 44-day duration of the experiment. The pH remained at approximately 6 in all of the incubations.

In control sausages without inoculated *L. monocytogenes* Scott A, but not in the sausages containing EDTA, we detected and confirmed *Listeria* colonies in the absence or presence of lysozyme. Carbohydrate tests showed that a proportion of these colonies reacted differently than did *L. monocytogenes* Scott A, especially in assimilation of melezitose. We assume that the pork trim contained *Listeria* cells as inherent contaminants. These comprised <0.1% of the inocula we added and probably did not affect our results.

It is evident that in fresh pork sausages EDTA and lysozyme acted synergistically in preventing growth. Since the shelf life of fresh bratwurst is <2 weeks, it is likely that lysozyme together with a food-approved chelator could
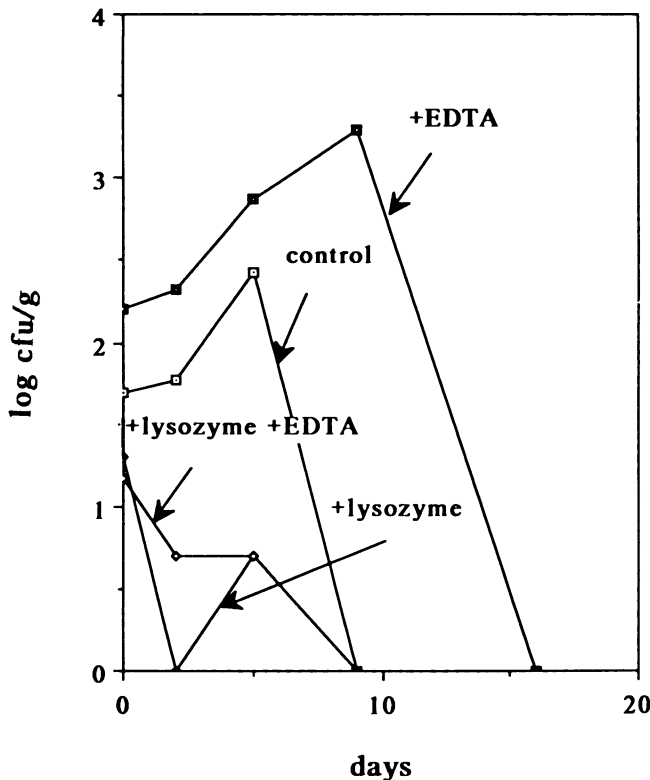
FIG. 5. Effect of lysozyme and EDTA and lysozyme on growth of *L. monocytogenes* in carrots.

pattern in which populations initially rose and then fell precipitously. These results have been seen with some other foods. Growth during the initial 41 days of cheese curing without inhibitory agents occurred steadily, and ~4.9 × 10^5 CFU/g was obtained. After this time, viable counts decreased to ~10^4 CFU/g. Unusual patterns of growth were also seen in the wheels containing *L. monocytogenes* with EDTA and lysozyme. During the initial 27 days of cheese ripening, counts dropped from 3.0 × 10^4 to 6.4 × 10^3 (range, 3.7 × 10^3 to 9.2 × 10^3). After this period, *L. monocytogenes* grew until 41 days and then dropped again to ~6 × 10^3. Eventually, relatively high counts were obtained (4.0 × 10^6). It is evident that numbers of *L. monocytogenes* varied considerably after 30 to 40 days of aging in the cheeses containing lysozyme with or without EDTA but not in the control. Soft-ripened cheese is a complex substrate, and more work is necessary to evaluate the effectiveness of lysozyme.

DISCUSSION

An important contributing factor for the control of *L. monocytogenes* in foods is the absence of technologies to thoroughly and permanently eliminate the pathogen from the processing plant environment (26, 28). The pathogen tenaciously contaminates processing plants despite extensive efforts to eliminate it through vigorous sanitation. Postprocessing contamination is an important factor in transmission of the pathogen to foods and is currently thought to be the major route of contamination (14, 16, 26, 28). Safe preservatives that can be added to foods to kill the organism if postprocessing contamination occurs are urgently needed.

provide needed protection. We are currently examining other meats, including fresh frankfurters. Various chelators, including natural substances such as conalbumin and lactoferrin, are also being tried in combination with lysozyme.

Effectiveness of lysozyme in mold-ripened Camembert cheese. Soft-ripened cheeses have been associated with transfer of listeriosis to humans. These products could benefit by inclusion of a safe preservative active against *L. monocytogenes*.

We prepared several wheels of Camembert cheese containing lysozyme, EDTA, and *L. monocytogenes*. Initial counts of *L. monocytogenes* from seven cheese wheels made in separate weeks ranged from 2.9 × 10^4 to 3.3 × 10^4. Figure 7 shows the results of including lysozyme with and without EDTA in the cheeses. The control cheeses harboring *L. monocytogenes* but not lysozyme or EDTA initially showed a slight decline in numbers during the first 3 weeks of curing, but thereafter *L. monocytogenes* grew steadily and reached 5 × 10^6 CFU/g after 55 days. After the 55-day sampling, detection in several of the experimental variations gave sporadic numbers from sample to sample, perhaps because of nonuniform spatial distribution of *L. monocytogenes* in the cheeses.

The cheeses containing *L. monocytogenes* and lysozyme also exhibited an initial decline that continued until the 31-day sampling. The initial numbers were 3.3 × 10^4 CFU/g, which dropped to 4.2 × 10^3 (range, 3.4 × 10^3 to 5.0 × 10^3) after 31 days of aging. *L. monocytogenes* then started to grow and reached 2.5 × 10^4 (range, 5.0 × 10^3 to 4.5 × 10^4) after 55 days (range, 4.5 × 10^4 to 5.0 × 10^4). Viable counts eventually declined to 2.5 × 10^4 (range, 5.0 × 10^3 to 4.5 × 10^4) after a climb in population to 2.3 × 10^5 to 3.5 × 10^5/g.

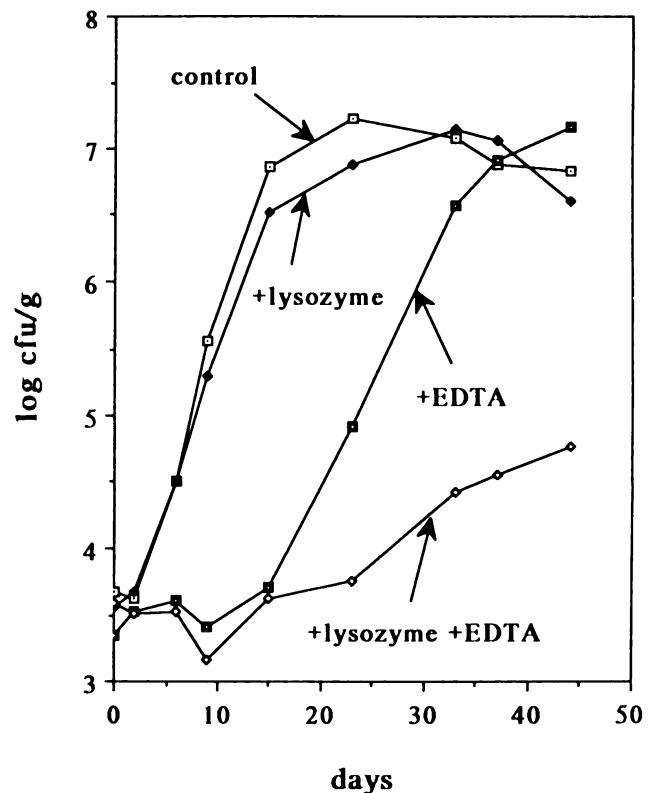The wheels containing EDTA showed an unusual growth

FIG. 6. Effect of lysozyme and EDTA on growth of *L. monocytogenes* in fresh sausages (bratwurst).
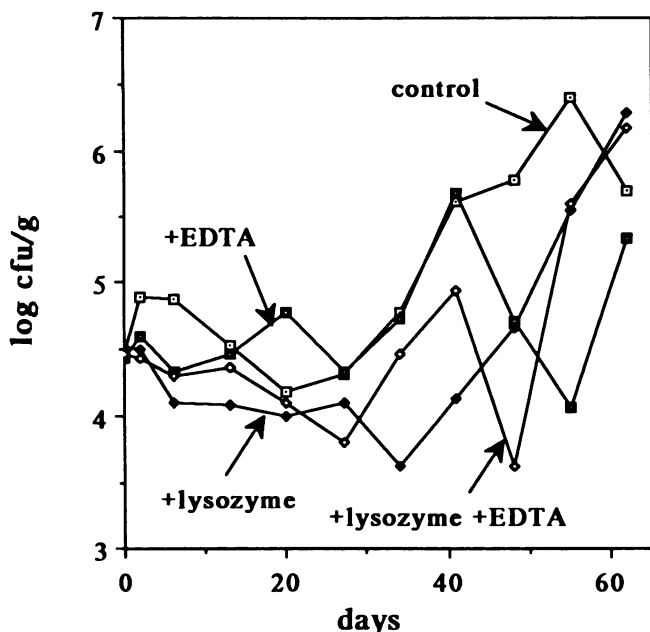
FIG. 7. Growth of *L. monocytogenes* in Camembert cheese containing lysozyme and EDTA.

The results of this study indicate that lysozyme is an effective preservative against *L. monocytogenes* Scott A in certain foods, especially fresh vegetables. In many of the vegetables we tested, lysozyme effectively killed high initial numbers of *L. monocytogenes*. In several vegetable foods, EDTA enhanced the activity of lysozyme and resulted in rapid and complete extermination of *L. monocytogenes*.

In the two foods of animal origin that we tested (fresh sausage and Camembert cheese), lysozyme with EDTA was bacteriostatic but did not rid the foods of viable *L. monocytogenes*, as occurred in many vegetables. Evidently, the growth substrates are critically important for bacterial growth and the effectiveness of lysozyme. Perhaps the presence of relatively high concentrations of certain nutrients such as heme, fat, or others are helpful to *L. monocytogenes* in avoiding killing by lysozyme.

The interaction of lysozyme with chelators needs more extensive study. The mechanism(s) by which they interact is not presently understood. There are at least two possibilities for the enhancing effect of a chelator: (i) it may slow growth by binding an important metal necessary for growth (e.g., iron [23]), thus allowing lysozyme to digest the cell wall faster than it can be synthesized, or (ii) it may disrupt the cell wall structure and enable better attack by lysozyme. Perraudin and Prieels (15) have shown that lactoferrin binds more readily to *M. luteus* cells to which lysozyme has previously bound. EDTA was used as a model chelator in our studies, and it is likely that other chelators, especially those that occur naturally with lysozyme in mammalian secretions, such as conalbumin, transferrin, ceruloplasmin, or lactoferrin, would work more effectively. Lactoferrin occurs naturally in bovine milk at lower concentrations than it occurs in milks of some other mammals. Preliminary experiments have shown that conalbumin enhances the activity of lysozyme against *L. monocytogenes* (V. L. Hughey, P. A. Wilger, and E. A. Johnson, experiments in progress). Conalbumin specifically binds iron, as opposed to EDTA, which

prefers divalent alkaline earth metals. Iron is quite important for growth and virulence of *L. monocytogenes* (23).

A second potential processing method for increasing the activity of lysozyme is to heat *L. monocytogenes* during or before exposure to lysozyme. Preliminary experiments have shown that this procedure dramatically increases the susceptibility of *L. monocytogenes* to lysis (A. DiNardo, V. L. Hughey, and E. A. Johnson, unpublished data).

Although lysozyme has been suggested to have many roles as a preservative (see reference 17 for review), it is currently used mainly in certain European cheeses to prevent blowing by sporeformers (17). Our previous findings that egg white lysozyme is highly effective against *L. monocytogenes* and some other pathogens (9), and the results presented here, showing that it is active in several foods, suggest that further applications can be found. Because lysozymes occur naturally in humans, and since egg white lysozyme is destroyed in the human stomach and during cooking of foods, it is likely that lysozyme could serve as a safe and widely used food preservative to protect against *L. monocytogenes* contamination.

LITERATURE CITED

1. Beckers, H. J., P. S. S. Soentoro, and E. H. M. Delfgou-van Asch. 1987. The occurrence of *Listeria monocytogenes* in soft cheeses and raw milk and its resistance to heat. Int. J. Food Microbiol. 4:249–256.
2. Bille, J., and M.-P. Glauser. 1988. Listeriose en Suisse. Bull. Bundesamtes Gesundheitswesen. 3:28–29.
3. Dijkstra, R. G. 1982. The occurrence of *Listeria monocytogenes* in surface water of canals and lakes, in ditches of one big polder and in the effluents and canals of a sewage treatment plant. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe B 176:202–205.
4. Fleming, D. W., S. L. Cochi, L. L. MacDonald, J. Brondum, P. S. Hayes, B. D. Plikaytis, M. B. Holmes, A. Audurier, C. V. Broome, and A. L. Reingold. 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. N. Engl. J. Med. 312:404–407.
5. Food and Drug Administration. 1986. FDA's dairy product safety initiatives. Preliminary status report. Food and Drug Administration, Washington, D.C.
6. Gitter, M. 1976. *Listeria monocytogenes* in oven ready poultry. Vet. Rec. 99:336.
7. Gray, M. L., and A. H. Killinger. 1966. *Listeria monocytogenes* and listeric infections. Bacteriol. Rev. 30:309–382.
8. Ho, J. L., K. N. Shands, G. Friedland, P. Eckind, and D. W. Fraser. 1986. An outbreak of type 4b *Listeria monocytogenes* infection involving patients from eight Boston hospitals. Arch. Intern. Med. 146:520–524.
9. Hughey, V. L., and E. A. Johnson. 1987. Antimicrobial activity of lysozyme against bacteria involved in food spoilage and food-borne disease. Appl. Environ. Microbiol. 53:2165–2170.
10. James, S. M., S. L. Fannin, B. A. Agee, B. Hall, E. Parker, J. Vogt, G. Run, J. Williams, L. Lieb, C. Salminen, T. Pendergast, S. B. Werner, and J. Chin. 1985. Listeriosis outbreak associated with Mexican-style cheese—California. Morbid. Mortal. Weekly Rep. 34:357–359.
11. Lee, W. H., and D. McClain. 1986. Improved *Listeria monocytogenes* selective agar. Appl. Environ. Microbiol. 52:1215–1217.
12. McClain, D., and W. H. Lee. 1988. Development of USDA-FSIS method for isolation of *Listeria monocytogenes* from raw meat and poultry. J. Assoc. Off. Anal. Chem. 71:660–664.
13. McLauchlin, J. 1987. *Listeria monocytogenes*, recent advances

in the taxonomy and epidemiology of listeriosis in humans. J. Appl. Bacteriol. **63:**1–11.

14. **Mossel, D. A. A.** 1987. Microbial recontamination of milk and dairy products. Mechanisms, health impact, management and monitoring. Microbiol. Aliment. Nutr. **5:**267–282.

15. **Perraudin, J.-P., and J.-P. Prieels.** 1982. Lactoferrin binding to lysozyme treated *Micrococcus luteus.* Biochim. Biophys. Acta **718:**42–48.

16. **Prentice, G. A., and P. Neaves.** 1988. *Listeria monocytogenes* in food: its significance and methods for its detection. Bull. Int. Dairy Fed. **223:**1–16.

17. **Proctor, V. A., and F. E. Cunningham.** 1988. The chemistry of lysozyme and its use as a food preservative and a pharmaceutical. Crit. Rev. Food Sci. Nutr. **26:**359–395.

18. **Ryser, E. T., and E. H. Marth.** 1987. Fate of *Listeria monocytogenes* during manufacture and ripening of Camembert cheese. J. Food Prot. **50:**372–378.

19. **Schlech, W. F., P. M. Lavigne, R. A. Bortolussi, A. C. Allen, E. V. Haldane, A. J. Wort, A. W. Hightower, S. E. Johnson, S. H. King, E. S. Nichols, and C. V. Broome.** 1983. Epidemic listeriosis—evidence for transmission by food. N. Engl. J. Med. **308:**203–206.

20. **Seeliger, H. P. R., and D. Jones.** 1986. Genus *Listeria* Pirie 1940, p. 1235–1245. *In* P. H. A. Sneath, N. S. Maine, M. E. Sharpe,

and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore.

21. **Shahamat, M., A. Seaman, and M. Woodbine.** 1980. Influence of sodium chloride, pH and temperature on the inhibitory activity of sodium nitrite on *Listeria monocytogenes.* Soc. Appl. Bacter. Tech. Ser. **15:**227–237.

22. **Sizmur, K., and C. W. Walker.** 1988. *Listeria* in prepacked salads. Lancet **i:**1167.

23. **Sword, C. P.** 1966. Mechanisms of pathogenesis in *Listeria monocytogenes* infection. I. Influence of iron. J. Bacteriol. **92:**536–542.

24. **Tham, W. A., and V. M.-L. Danielsson-Tham.** 1988. *Listeria monocytogenes* isolated from soft cheese. Vet. Rec. **122:**539–540.

25. **Watkins, J., and K. P. Sleath.** 1981. Isolated and enumeration of *Listeria monocytogenes* from sewage, sewage sludge and river water. J. Appl. Bacteriol. **50:**1–9.

26. **Wehr, H. M.** 1987. *Listeria monocytogenes*—a current dilemma. J. Assoc. Off. Anal. Chem. **70:**769–772.

27. **Weis, J., and H. P. R. Seeliger.** 1975. Incidence of *Listeria monocytogenes* in nature. J. Appl. Microbiol. **30:**29–32.

28. **World Health Organization.** 1988. Foodborne listeriosis. Report of a WHO informal working group. World Health Organization, Geneva.