Differences in survival of *Escherichia coli* O157:H7 under various conditions that re-enact the cooking of lunches implicated in an outbreak of haemorrhagic diarrhoea

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SUMMARY

Two elementary schools were served lunches that were cooked in the same kitchen. An outbreak of *Escherichia coli* O157:H7 occurred at one school where the dishes that were prepared for the school were lukewarm and kept for 33 min at an average temperature of 45 °C before serving. However, no outbreak occurred at the other school where dishes were hot and were kept for 60 min at an average temperature of 50 °C before serving. In a series of experiments on the survival of *E. coli* O157:H7 in the liquid portion of similarly prepared food, the population of *E. coli* O157:H7 was reduced by 10^{-3} by heating at 50 °C for 60 min and by only 10^{-1} by heating at 45 °C for 40 min. Further, *E. coli* O157:H7 survived at 45 °C for 40 min but not at 50 °C for 60 min at pH 4·0 with a 4·0% salt concentration that was similar to that of the liquid part of the food. These results indicate that pH and salt concentration of cooked food markedly affect the survival of *E. coli* O157:H7 and help to explain the occurrence of the disease outbreak at only one of the schools.

INTRODUCTION

A large outbreak of *Escherichia coli* O157:H7 occurred among schoolchildren in Sakai city, Osaka, Japan in July [1]. More than 6000 schoolchildren in 60 elementary schools in Sakai city were infected with *E. coli* O157:H7. Because school lunches were cooked at each school from common food materials and served through a centralized lunch programme, a large number of diarrhoea cases occurred at the same time. *E. coli* O157:H7 was not detected in the materials and food. By epidemiological analysis, white-radish sprouts supplied from a single farm were suspected as the source of the infectious agent in the outbreak [2–4]. However, because the kitchen of one

school (School A), was being repaired, the lunches of School A were prepared together with those of School B at the kitchen of School B. An outbreak of haemorrhagic *E. coli* O157:H7 diarrhoea occurred at School B but not School A. To determine why only one of these schools experienced an outbreak, a detailed analysis of the cooking procedure and time schedule of the foods at Schools A and B was made (Fig. 1).

Sweet and sour fried chicken with vegetables for both schools was separately cooked and materials such as chicken meat, leek, ginger, lettuce and white-radish sprouts were handled differently before the cooking. First, the chicken for School A was fried, and sweetened vinegar sauce was boiled in a pan. Half of the sauce for School B was transferred to another non-heated pan. The remainder for School A was added to cut lettuce and white-radish sprouts, then the fried chicken was added to the sauce for School A and mixed. Immediately the pan was covered with

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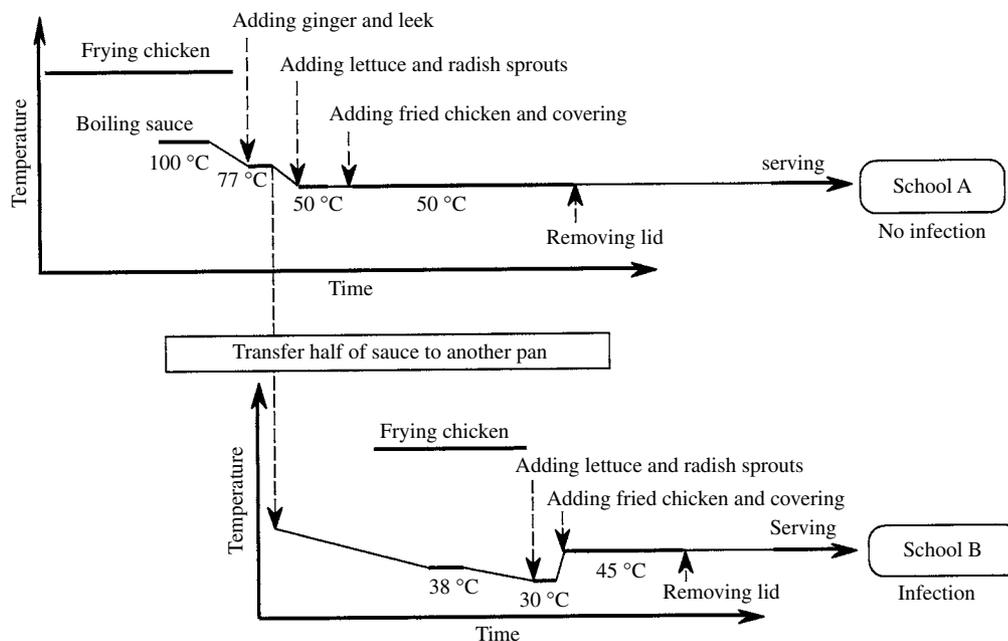(Email: ykudo@nihs.go.jp)

Fig. 1. The protocol for cooking the school lunch and the temperature of the food at each step.

a lid. Secondly, chicken for School B was fried. The sauce for School B was kept for 60 min at room temperature in a pan and was added to the cut lettuce and white-radish sprouts. Then fried chicken was also added and mixed. The pan was covered with a lid. The dish prepared for School A was served to the students after ~60 min. The temperature of the food in the pan averaged 50 °C (range 49·2–60·8 °C). On the other hand, the food for School B was served to students after ~33 min from a pan with a covered a lid. The temperature of food in this pan averaged 45 °C (range 42·5–45·3 °C).

Using these parameters, the aim of this study was to determine how *E. coli* O157:H7 survived under the cooking conditions for lunches of both schools. Furthermore, using model conditions in combination with salt concentration, pH and two kinds of acid were investigated to demonstrate important controlling factors that led to the *E. coli* O157:H7 infection.

MATERIALS AND METHODS

Cooked food and cooking procedures

Sweet and sour fried chicken with vegetables that were cooked for elementary students of the two schools was similarly prepared for this study. After the materials of the sauce (leek, ginger, soy sauce, apple vinegar, sugar and sesame oil) were boiled in a pan, cut vegetables (lettuce and white-radish sprouts) were added

and mixed. Then, fried chicken, immediately after frying, was added and mixed. After covering with a lid, the cooked food was similarly stored. The liquid part of the cooked food in the bottom of the pan was used for experiments for the survival of *E. coli* O157:H7.

Strain and culture

E. coli O157:H7 (No. 212) isolated from a patient of the 1996 outbreak was cultured in trypticase soy broth (TSB; Difco, Detroit, MI, USA) for 18 h at 37 °C. The culture was serially diluted to 10^{-6} with phosphate-buffered saline (PBS; Nissui, Tokyo, Japan). A total of 0·1 ml of the 10^{-3} dilution was inoculated into the liquid portion of the food or a model representation of the liquid portion of the cooked food. Trypticase soy agar (TSA) medium (Difco) was inoculated with a 10^{-6} dilution (0·1 ml) and was cultured for 18 h at 37 °C to estimate the inoculum size.

Chemical analysis of the liquid portion of the cooked food

The concentration of salt, pH and water activity (a_w) of the liquid portion of the cooked food were measured with a salt analyser (SAT-210, Toa Electronics, Tokyo Japan), a pH meter (IQ240, Toho, Tokyo, Japan) and a water activity meter (HydroPalm, Rotronic, Bassersdorf, Switzerland) respectively.

Experimental inoculation of the liquid portion of the cooked food

Aliquots (20 ml) of the liquid portion of the food were dispensed into two sterilized glass Erlenmeyer flasks (100 ml) and capped with a silicon stopper. Each flask was pre-incubated in a water bath (Personal-11, Taitec, Tokyo, Japan) for 15 min at 45 and 50 °C respectively. A total of 0·1 ml of the 10^{-2} dilution of *E. coli* O157:H7 was inoculated into each flask. Immediately after inoculation and at 20, 40 and 60 min after inoculation, 1·5 ml of the liquid portion of the cooked food was removed to perform a bacterial count. Each 0·2 ml of the liquid part of the inoculated cooked food was inoculated onto five plates of CHROMagar O157 (CHROMagar, Paris, France). Then 0·1 ml of 10^{-1} and 10^{-2} dilution of this liquid were inoculated onto CHROMagar O157 plates in duplicate. After incubation for 24 h at 37 °C, colonies of *E. coli* O157:H7 were counted. For confirmation of *E. coli* O157:H7, several colonies were tested for O157 antigenicity using a latex kit (Unikit, Oxoid, Basingstoke, Hampshire, UK). The experiment was performed in triplicate.

Experimental inoculation into a model solution of the liquid portion of the cooked food

Based on the chemical analysis of the liquid portion of the cooked food, we prepared a model solution that varied the salt concentration and pH. In the model solution, the concentration of salt was 3·0, 4·0 and 5·0% NaCl. The pH was adjusted to 3·0, 4·0, 5·0 and 6·0 with hydrochloric acid or acetic acid. Each concentration of salt was combined with each pH condition resulting in 24 kinds of sterilized solutions that were tested with inoculum at either 45 or 50 °C.

The experiment was performed in a similar fashion as the previous experiment using the liquid food portion. A total of 0·1 ml of the 10^{-3} dilution of *E. coli* O157:H7 culture was inoculated into each flask. Immediately after inoculation and at 5, 10, 20, 30, 40, 50 and 60 min after inoculation, 1·5 ml of the model test solution was removed and a bacterial count was performed. Briefly, each 0·2 ml of the model solution was inoculated onto five plates of TSA. Then 0·1 ml of the 10^{-1} and 10^{-2} dilution of the solution with PBS were inoculated onto TSA in duplicate. After incubation for 24 h at 37 °C, the colonies of *E. coli* O157:H7 were counted. Confirmation of representative colonies of *E. coli* O157:H7 was confirmed using the test kit as described previously. This experiment was performed in triplicate.
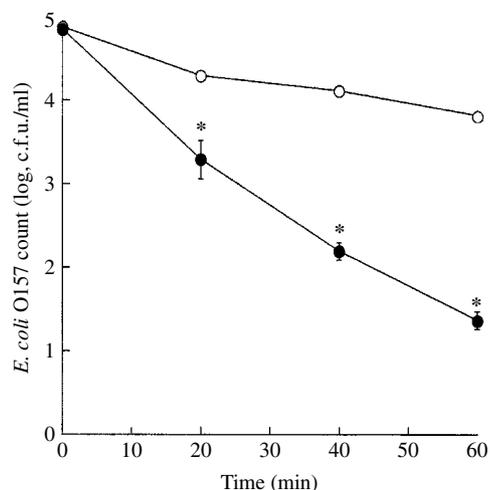

Fig. 2. Survival of *E. coli* O157:H7 at 45 °C (–○–) and 50 °C (–●–) in the liquid portion of sweet and sour fried chicken with vegetable. * Significant difference ($P < 0.05$) during the proportion of *E. coli* O157:H7 cells at 45 and 50 °C.

Statistical analysis

Data obtained for triplicate samples were analysed by Student's *t* test. Decimal reduction times (*D* values), in minutes, were determined from thermal inactivation curves ($D = -1/\text{slope}$) by linear regression analysis.

RESULTS

Chemical analysis of the liquid portion of the cooked food

The concentration of salt, pH and a_w of the liquid portion of cooked food were 4·2, 4·0 and 1·0% respectively.

Survival of *E. coli* O157:H7 in the liquid portion of the cooked food at 45 and 50 °C

When *E. coli* O157:H7 was added to the liquid portion of the cooked food, the initial count of the bacterium was estimated to be ~4·9 log c.f.u. (colony-forming units)/ml. The *E. coli* O157:H7 numbers decreased rapidly by heating at 50 °C while after 60 min the numbers were 1·4 c.f.u./ml (Fig. 2). On the other hand, by heating at 45 °C the numbers slowly reduced to 4·1 c.f.u./ml and by 40 min the cell number remained the same as at 60 min (Fig. 2). D_{45} and D_{50} were 56·0 and 17·0 respectively (Table).

Table. *D value for* Escherichia coli *O157:H7 under various conditions*

Acid	pH	NaCl (%)	D_{45} (min)	D_{50} (min)
Acetic acid	3·0	3·0	1·5	1·5
		4·0	1·5	1·5
		5·0	1·4	1·4
	4·0	3·0	18·3	7·9
		4·0	17·3	7·0
		5·0	10·1	5·0
Hydrochloric acid	3·0	3·0	2·8	1·5
		4·0	2·7	1·5
		5·0	2·8	1·5
	4·0	3·0	49·6	11·8
		4·0	47·1	11·7
		5·0	14·2	7·4
Liquid portion of cooked food	4·0	4·2	56·0	17·0

Survival of *E. coli* O157:H7 in a model solution of the liquid portion of the cooked food at 45 and 50 °C

When *E. coli* O157:H7 was added to the model solution, the initial count of the bacterium was estimated at ∼3·6 log c.f.u./ml. At pH 3·0 with either hydrochloric acid or acetic acid (Fig. 3), the population of *E. coli* O157:H7 quickly decreased to non-detectable levels in 3, 4 and 5% salt after incubation for <10 min at either 45 or 50 °C.

At pH 4·0 with acetic acid (Fig. 3*a*), incubation at 50 °C for 30 min reduced the cell numbers of *E. coli* O157:H7 to undetectable levels in 4 and 5% salt and almost undetectable levels in 3% salt. After incubation for 40 min at 45 °C, populations of *E. coli* O157:H7 decreased to an almost undetectable level in 5% salt and to around one tenth in 3 and 4% salt. The D_{45} value was more than twice as large as the D_{50} value at each concentration of NaCl (Table).

Hydrochloric acid at pH 4·0 reduced the population more slowly than acetic acid (Fig. 3*b*). Most of *E. coli* O157:H7 in 5% salt was inactivated after 50 min of incubation at 45 °C. With incubation at 50 °C, the population of *E. coli* O157:H7 decreased quickly. The D_{50} value of hydrochloric acid was larger than that of acetic acid (Table).

Acetic acid at pH 5·0 in 5% salt slightly reduced the population after incubation for 60 min at 50 °C (data not shown). But under other salt concentrations at pH 5·0, the initial population remained. No decrease in the cell numbers of *E. coli* O157:H7 at pH 6·0 was observed for 60 min (data not shown).

DISCUSSION

The lunch at School A, which did not have a diarrhoea outbreak, was served after storage for 60 min at 50 °C, while the lunch of School B, which did have a diarrhoea outbreak, was served after storage for 33 min at 45 °C. Our experimental data confirm the reasons why this occurred. In the experimental liquid portion of the food, the population (4·1 log c.f.u./ml) after incubation at 45 °C for 40 min was ∼500 times larger than that (1·4 log c.f.u./ml) for 60 min at 50 °C. Our data further suggest that heating the food at 50 °C (with 4% salt and pH 4·0) reduced the survival of *E. coli* O157:H7 but this was not the case at 45 °C where little reduction in numbers were seen. Heating for 40 min at 45 °C reduced the numbers by <1 log reduction whereas the numbers were reduced by >3 log reduction at 50 °C for 60 min. If the initial population of *E. coli* O157:H7 in the contaminated food is <10^3 c.f.u./ml, heating for 60 min at 50 °C would kill all the organisms. However heating for 40 min at 45 °C would not.

The initial contamination dose of *E. coli* O157:H7 in food associated with O157 diarrhoea outbreaks has been reported in previous outbreaks. In Kashiwa city, Japan in 1997, *E. coli* O157:H7 infections of 32 cases occurred when raw melon was served in a nursery school [5]. The melon was stored in a freezer as a test sample and afterward the melon was found to be contaminated with 43 c.f.u./g. The infectious dose was estimated to be 2000 c.f.u./patient. In an outbreak in the United States, raw hamburger patties were contaminated with *E. coli* O157:H7 at a concentration of <13·5–675 c.f.u./patty [6]. In an outbreak in Morioka city in which salad with boiled vegetable and boiled seafood sauce was implicated, the infectious dose was 9–57 c.f.u./patient [7]. These observations support the idea that low numbers of *E. coli* O157:H7 are able to induce infection, and that the cooking conditions for the lunch of School B would not have reduced populations of *E. coli* O157:H7 to non-detectable levels. Ironically, because the kitchen at School A was not available, the food was handled differently which prevented the occurrence of the outbreak.

The cooked food was acidic due to the use of apple vinegar (∼4·7 g of acetic acid/100 ml of apple vinegar). It is known that acetic acid has stronger effects on survival than other acids [8–10]. The experiment using the model solution in this study also demonstrates that acetic acid was more effective in reducing *E. coli* O157:H7 populations than
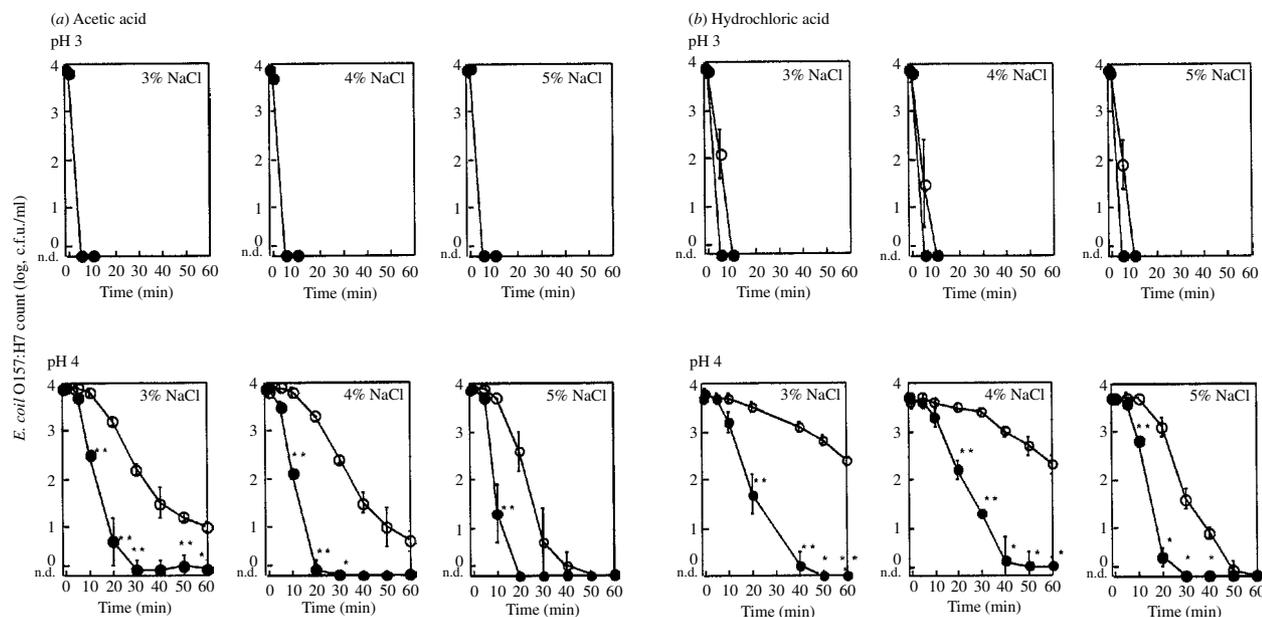
Fig. 3. Survival of *E. coli* O157:H7 at 45 °C (–○–) and 50 °C (–●–) under various conditions. n.d., Not detected (detection limit: 1 c.f.u./ml). Significant difference during the proportion of *E. coli* O157:H7 cells at 45 and 50 °C. *$P < 0.05$, **$P < 0.01$.

hydrochloric acid (Fig. 3). The undissociated form of organic acid can penetrate the cell membrane lipid bilayer easily and the organic acid subsequently dissociates. Protons generated from the intracellular dissociation of the organic acid reduce the intracellular pH. Because the relative proportion of undissociated forms of acetic acid is greater than that of hydrochloric acid, acetic acid has stronger effects than hydrochloric acid.

In the model solution, there was a marked difference in *E. coli* O157:H7 survival between 45 and 50 °C that was demonstrated only at pH 4·0. Therefore, it seems that a pH of 4·0 was the most important factor in the survival of *E. coli* O157:H7. In the solutions of pH 4·0 and 4% salt, the population of *E. coli* O157:H7 at 45 and 50 °C quickly decreased and reached a non-detectable level after incubation for 30 min at 50 °C respectively (Fig. 3). These results were not the same as the results in the liquid portion of the food (Fig. 2). However, these results are not inconsistent. The model solution did not contain organic substrate, the liquid food contained a lot of organic substrates such as lipid, protein and carbohydrate from chicken meat, vegetable, and oil. It is known that lipid induces heat resistance in bacteria [11, 12]. Therefore, the components of the food might also affect the survival.

Few reports describe the survival of *E. coli* O157:H7 with mild heating in acidic food. Aboul-Raouf et al. [8] show that a pH of 5·0 was more effective in

reducing the numbers of *E. coli* O157:H7 than a pH of 6·0 when heated to 54 °C in beef slurries. Benito et al. [13] described that heating for 60 min at 52 °C in TSB (pH 7·3) reduced the population of *E. coli* O157:H7 by 10^{-2}–10^{-3}. Cheng et al. [14] reported that from 10% down to 0·1% of *E. coli* O157:H7 in TSB survived heating for 60 min at 52 °C. However, there are no reports on the survival of *E. coli* O157:H7 using temperatures < 50 °C under acidic and salt conditions. Therefore, the effects of survival controlling factors such as pH, salt concentration and temperature on *E. coli* O157:H7 need to be further investigated. Ryu & Beuchat have studied the survival of *E. coli* O157:H7 in fruit juices (pH 3·56–3·98) after heating to 52 °C [15]. There were differences in the reduction of the *E. coli* O157:H7 population in each juice. The sensitivity to stress was different in each bacteria strain. From these findings, verification of food poisoning should be carried out using strains from patients from the outbreak and the most similar food to the cause in the suspected contaminated food.

The present study demonstrated that the different conditions of cooking between Schools A and B induced differences in survival. *E. coli* O157:H7 remained in the food stored at 45 °C for 40 min but not at 50 °C for 60 min. The results using the model solutions demonstrated that differences in salt concentration and pH in addition to the incubation time and temperature led to the occurrence of the outbreak

at only one of the schools, even though lunches for both were prepared in the same kitchen.

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