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Inactivation of *Escherichia coli* O157:H7 in Single-Strength Lemon and Lime Juices

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ABSTRACT

Survival of a five-strain mixture of stationary phase (nonadapted) and acid-adapted *Escherichia coli* O157:H7 in single-strength lemon and lime juices was evaluated at room temperature (22°C). The juices were reconstituted from concentrates that contained no preservatives and intrinsic pH values of 2.5 to 2.6 and titratable acidities of 4.51 to 4.53% (wt/vol, citric acid). A greater than 5-log reduction of stationary-phase cells was achieved in both lemon and lime juices after 72 h of incubation. Similar log reductions were obtained when the reconstituted juices were adjusted to pH 2.7, which is above the highest value normally observed in juice processing plants during the reconstitution of single-strength lemon or lime juice from concentrates. Lemon juice had a significantly higher inhibitory effect ($P < 0.05$) on *E. coli* O157:H7 than did lime juice. Validation tests with commercially produced shelf-stable lemon and lime juices confirmed that storage of the juices at room temperature (22°C) for 3 days may be an alternative to heat treatment to ensure the 5-log reduction of vegetative pathogens of concern required for the products under the U.S. Food and Drug Administration juice hazard analysis and critical control point regulations.

Several foodborne outbreaks associated with unpasteurized fruit juices occurred in the 1990s. Before these outbreaks, the common assumption was that juices were unlikely vehicles for foodborne illnesses because of their low pH and high levels of organic acids. However, major outbreaks have involved unpasteurized apple juice and cider contaminated with *Escherichia coli* O157:H7 *(1, 4, 6)* and unpasteurized orange juice contaminated with *Salmonella enterica (3, 5).* As a result, in 2001 the U.S. Food and Drug Administration (FDA) addressed the safety of juices by finalizing the juice hazard analysis and critical control point (HACCP) regulation that requires juice processors to develop and implement HACCP programs to control food safety hazards associated with juice products. The HACCP regulation includes a performance standard, which requires processors to achieve a 99.999% (5-log) reduction of the most resistant target microorganism of public health significance *(32).*

A number of studies have been published on the heat resistance of vegetative pathogens in single-strength juice *(17)* and juice concentrate *(12)* and on the survival of vegetative pathogens in juice concentrates *(11, 19, 20).* These studies have provided juice processors with the data necessary to identify control measures appropriate for attaining a 5-log pathogen reduction and thus comply with the HACCP performance standard for juice. Further studies on additional control measures will provide more options for juice processors to facilitate industry compliance with the HACCP regulation.

Single-strength juices, such as lemon and lime juices, are in some cases produced by using concentrates as an ingredient. Thermally processed juice concentrates generally are heated using time-temperature combinations that destroy vegetative pathogens, which can occur in raw juices. During storage, transportation, and reconstitution of the concentrates to single strength, there is a potential for recontamination of the juice by pathogenic bacteria. Although repasteurization of the single-strength juice is a straightforward means of inactivating pathogens acquired through recontamination, multiple thermal treatments may affect the quality of the product because of undesirable sensory changes such as browning and flavor loss *(23).* Undesirable sensory characteristics resulting from multiple heat treatments are a quality concern for reconstituted single-strength lemon and lime juices. Development of alternatives to heat treatment that still can deliver a 5-log pathogen reduction will help ensure product safety while maintaining optimum quality.

Previous work in our laboratory revealed that *E. coli* O157:H7, *Listeria monocytogenes,* and *Salmonella* were inactivated in lemon and lime juice concentrates *(19)* and in cranberry juice concentrates *(11, 19).* The bactericidal effect of some juices or juice concentrates may result from intrinsic antimicrobial properties such as very low pH, high titratable acidity, and possible presence of some antimicrobial compounds *(11, 19, 20, 24).* Derrickson-Tharrington et al. *(10)* reported that pretreatment of apple slices with household acidulants such as commercial lemon juice enhanced the destruction of *E. coli* O157:H7 during drying of the product. Beuchat et al. *(2)* found that *Salmonella, E. coli* O157:H7, and *L. monocytogenes* died in shelf-stable salad dressings. The types of salad dressing and the product

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pH (ranging from 2.8 to 3.5) affected pathogen inactivation when the products were stored at 25°C. Other researchers have explored antimicrobial activities of lime juice against *Vibrio cholerae* in food *(26)* and drinking water *(8)* and of lemon juice against *Salmonella* Typhimurium in mussels *(16),* vegetables *(27, 28),* and sliced fresh fruits *(13).* Tomotake et al. *(31)* found that juices of lemon (*Citrus limon* Burm. F.), lime (*Citrus aurantifolia* Swingle), and sudachi (*Citrus sudachi* Hort. ex Shirai) had antibacterial activity against several *Vibrio* species, including *V. cholerae, V. parahaemolyticus,* and *V. vulnificus.* Their results suggested that all citrus fruit juices tested had some bactericidal activity against *Vibrio* species because of the high concentration of citric acid but this effect only occurred at low pH values. Mossel and De Bruin (cited in a bulletin for the Australian food industry *(7)*), found that pathogenic *Enterobacteriaceae* at an inoculum of 10^6 CFU did not survive more than 1 day in lemon juice (pH 2.4 to 2.6) but survived for several days in products with pH greater than 3.0, such as apple, orange, or tomato juice, mayonnaise, and yogurt. The authors concluded that fruit juices with a pH greater than 3.0 must be considered a potential source of pathogenic bacteria. Low pH, storage conditions such as temperature, and the presence of antimicrobial compounds can be used as multiple hurdles to inactivate pathogens in products such as mayonnaise and mayonnaise-based dressings *(15),* eggplant salad *(29),* and other salad dressings and sauces *(30).*

The objective of this study was to determine the inactivation of a five-strain composite of *E. coli* O157:H7 in reconstituted single-strength lemon and lime juices and in commercially produced shelf-stable lemon and limes juices. The purpose of the study was to evaluate an alternative to pasteurization as a control measure to deliver a 5-log reduction of vegetative pathogens in single-strength lemon and lime juices.

MATERIALS AND METHODS

Juice products. Lemon and lime juice concentrates without preservatives at a °Brix level commonly produced by the industry were obtained from Grocery Manufacturers Association (GMA) member companies. Individual concentrates were diluted with sterile deionized water to reconstitute single-strength lemon or lime juices, which were defined by titratable acidity, not by °Brix. The reconstituted single-strength juices had 4.51 to 4.53% citric acid. The titratable acidity (percentage of citric acid, wt/vol) was determined by NaOH titration to a pH 8.1 endpoint. A 0.1 N NaOH solution standardized with potassium hydrogen phthalate was used in the titration. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Reconstituted juices were used to determine inactivation of *E. coli* O157:H7 over time to identify the conditions for 5-log reduction. Commercially produced shelf-stable single-strength lemon juice (with or without preservatives) and lime juice (with preservatives) were obtained from GMA members and used to conduct validation experiments. The preservatives in the shelf-stable products were sodium bisulfite and sodium benzoate.

Bacterial strains and culture conditions. Five strains of *E. coli* O157:H7 and five strains of *L. monocytogenes* were used in this study. All strains were from the GMA Culture Collection (Washington, DC). The *E. coli* O157:H7 strains used were N-4070 and N-4072 (isolated from apple juice implicated in a 1996 outbreak), N-4073 (isolated from a patient in a 1996 outbreak where apple cider was implicated), N-4064 (isolated from a patient in an outbreak where apple cider was implicated), and N-4087 (isolate from an outbreak involving salami). The *L. monocytogenes* strains used in the preliminary study were N-7003 (isolated from raw milk), N-7016 (isolated from meat), N-7175 (isolated from a meat plant environment), N-7031 (a human isolate, ATCC 7644), and N-7008 (a human isolate, ATCC 19113). Working cultures were made from freeze-dried stocks and maintained on tryptic soy agar (TSA; Difco, Becton Dickinson, Sparks, MD) slants at 4°C and transferred monthly for up to 3 months. Before inoculation, a loopful of each strain was transferred in 10 ml of tryptic soy broth (TSB; Difco, Becton Dickinson) and grown aerobically at 35°C overnight.

Both stationary-phase nonadapted and acid-adapted cells were prepared according to the procedures used in previously published studies from this laboratory *(11, 12, 17, 19).* For a stationary-phase inoculum (nonadapted cells), a second transfer was made to TSB for each strain, and the culture was incubated at 35°C overnight (approximately 18 h). From these individually grown cultures, a composite was made for nonadapted cells. Acid-adapted cells were obtained by transferring a loopful of an overnight pH 7.0 TSB culture to TSB adjusted to pH 5.0 with 1 N HCl and incubating the new culture at 35°C for approximately 18 h. The cells were rinsed and resuspended in cold 0.1 M citrate buffer (pH 4.0) and then refrigerated (4 ± 1°C) overnight before use.

Two types of cells were prepared in the study. A five-strain composite of either acid-adapted or nonadapted cells was made by mixing equal volumes of each of the five strains. The culture preparation of each strain was quantified on TSA before the composite was prepared to ensure approximately equal numbers of cells for each strain. The composite inocula also were quantified before inoculation.

Inoculation and monitoring survival. Reconstituted single-strength lemon or lime juice was dispensed in sterile 250-ml Erlenmeyer flasks (100 ml per flask) and individually inoculated with 2.0 ml of the composite of *E. coli* O157:H7 culture. The initial inoculum in the juice was 10^6 to 10^7 CFU/ml. Inoculated flasks were incubated at room temperature (22°C) for 1 week. Control flasks with uninoculated single-strength lemon or lime juice were incubated at the same temperature. Each experiment included two flasks for acid-adapted and nonadapted cell composites. Duplicate samples were taken from each flask for enumeration at predetermined time points for monitoring survival over time.

Immediately after inoculation and at a subsequent designated time, 10-ml samples were transferred into Erlenmeyer flasks containing 87 ml of universal preenrichment broth (UPB), and 2.7 ml of sterile 3 N NaOH was added to the sample to reach a final pH of 7.0 ± 0.1. The neutralization step prevented false-negative results during enrichment due to the low pH of the juice sample *(11, 19).* From those neutralized flasks, further decimal dilutions were made by transferring 1 ml of the sample into 9.0 ml of UPB (pH 7.0) and plating on TSA using a spiral plater (model AP4000, Spiral Biotech, Norwood, MA) to enumerate surviving cells. Colony counts were obtained using the Q count system (model 510, Spiral Biotech). Colonies on TSA were confirmed to be *E. coli* O157:H7 by streaking onto sorbitol MacConkey agar (Difco, Becton Dickinson). All plates were incubated at 35°C for 24 to 48 h before counting. The same procedure was repeated every 24 h for

TABLE 1. *Inactivation of a five-strain composite of stationary-phase (nonadapted)* E. coli *O157:H7 cells in reconstituted single-strength lemon and lime juices stored at 22°C*

Type of juice	Incubation time (h)	Log reduction (mean ± SD)[a]
Lemon, pH 2.5–2.6[b]	24	1.60 ± 0.56
	48	5.70 ± 1.15
	72	>6.75
Lemon, pH 2.7[c]	24	1.67 ± 0.99
	48	5.74 ± 1.04
	72	>6.75
Lime, pH 2.5–2.6[b]	24	0.81 ± 0.18
	48	3.94 ± 0.73
	72	>6.75
Lime, pH 2.7[c]	24	1.12 ± 0.56
	48	4.80 ± 1.30
	72	>6.75

[a] Log reduction for juices stored for 72 h were calculated based on the detection limits of the quantification method, i.e., 20 CFU/ml (based on plating of 50 μl of sample) or 4 CFU/ml (based on plating of 250 μl of sample). No *E. coli* O157:H7 cells were recovered from juices stored for 72 h. The log reductions in lemon and lime juices with adjusted pH (pH 2.7) were not significantly different ($P > 0.05$) from those observed for the respective products with a natural pH (pH 2.5 to 2.6) under the same experimental conditions.

[b] Juices with a natural pH of 2.5 to 2.6. Results were obtained from four different experiments.

[c] Juices with pH adjusted to pH 2.7. Results were obtained from two different experiments.

up to 5 days of storage of the inoculated lemon or lime juice samples. The initial UPB dilution (Erlenmeyer flask) also was incubated (35°C for 72 h) to detect pathogen survival when negative results were obtained with the direct plating.

Validation test. A validation test was conducted for the stationary-phase (nonadapted) *E. coli* O157:H7 cell composite after the conditions for a 5-log reduction had been identified from the experiments with the reconstituted juices. Commercially produced shelf-stable single-strength lemon juice without preservatives (in 1-liter bottles), single-strength lemon juice with preservatives (in 1-liter bottles), and single-strength lime juice with preservatives (in 450-ml bottles) were inoculated with a five-strain composite to give a final inoculum of approximately 10^6 CFU/ml in the juices. The inoculated samples were stored at 22°C for 72 h. Two experiments were conducted. In the first trial, after 72 h of storage, 10 ml of the juice sample was transferred into 90 ml of UPB or TSB, with the final pH adjusted to pH 7.0 ± 0.1. In the second trial, after 72 h of storage, 1.0 ml of the juice sample was transferred into 9.0 ml TSB (final adjusted pH 7.0 ± 0.1). All samples in UPB or TSB were incubated up to 72 h at 35°C and streaked onto sorbitol MacConkey agar to determine whether *E. coli* O157:H7 was present in the enrichment. All agar plates were incubated at 35°C for 24 to 48 h. No differences in survival were found when either UPB or TSB was used as the recovery and enrichment medium.

Experiments with nonadapted cells in reconstituted lemon and lime juices at natural pH (pH 2.6) were repeated four times. Experiments with acid-adapted cells in reconstituted lemon and lime juices with adjusted pH (pH 2.7) and those with nonadapted cells in commercially produced lemon and lime juice bottles were repeated twice. Statistical analysis was conducted for selected data with a single-factor analysis of variance from the add-in tool in Excel (Microsoft, Redmond, WA).

TABLE 2. *Inactivation of a five-strain composite of acid-adapted* E. coli *O157:H7 in reconstituted single-strength lemon and lime juices stored at 22°C*[a]

Type of juice	Incubation time (h)	Log reduction (mean ± SD)[b]
Lemon	24	2.92 ± 0.24
	48	6.31 ± 0.37
Lime	24	2.69 ± 0.35
	48	4.99 ± 1.50

[a] Juices with natural pH of 2.5 to 2.6. Results were from two different experiments.

[b] Log reductions in lemon and lime juice after 24 or 48 h were not significantly different ($P > 0.05$).

RESULTS

Preliminary experiments were conducted to determine the survival of stationary-phase and acid-adapted cells of *E. coli* O157:H7 and *L. monocytogenes* in single-strength lemon and lime juices stored at 0°C. A greater than 5-log reduction of *L. monocytogenes* was found after 48 h, but more than 1 week was needed for a 5-log reduction of *E. coli* O157:H7 (data not shown). In other preliminary experiments, a greater than 5-log reduction of both stationary-phase and acid-adapted *L. monocytogenes* cells in single-strength lemon and lime juices occurred after 72 h of storage at 22°C. For stationary-phase *L. monocytogenes,* reductions of 2.22 ± 0.43 and 2.09 ± 0.96 log CFU/ml were obtained after 24 h of incubation at 22°C in lemon and lime juices, respectively. The inactivation of nonadapted *L. monocytogenes* cells in both juices was >6.7 log CFU/ml after 72 h of incubation. At 22°C, acid-adapted *L. monocytogenes* cells were more sensitive (>5.3-log reduction in both juices after 24 h of incubation) than nonadapted *L. monocytogenes* cells and much more sensitive than acid-adapted *E. coli* O157:H7 cells (e.g., an average of 2.9-log reduction in lemon juice after 24 h of incubation). As a consequence we focused the present study on the inactivation of *E. coli* O157:H7 at room temperature (22°C).

Storage of single-strength lemon and lime juices at room temperature resulted in inactivation of *E. coli* O157:H7 in the products over time (Tables 1 and 2). After 24 and 48 h in reconstituted single-strength lemon juice with a natural pH of 2.5 to 2.6, average reductions of 1.60 and 5.70 log CFU/ml, respectively, were observed for stationary phase cells (Table 1), and average reductions of 2.92 and 6.31 log CFU/ml were observed for acid-adapted cells (Table 2). These results indicate that the stationary-phase (nonadapted) cells were more resistant to the antimicrobial conditions of the single-strength juices than were the acid-adapted cells under the same experimental conditions. We described a similar situation in a previous study, where we found that for all vegetative pathogens tested, the stationary-phase cells survived better than did the acid-adapted

TABLE 3. *Inactivation of a five-strain composite of* E. coli *O157:H7 in commercial shelf-stable single-strength lemon and lime juices stored at 22°C for 72 h*

Type of juice	°Brix	pH	Titratable acidity	Inoculum (log CFU/ml)	Cells recovered[a]	Log reduction
Lemon[b]	6.4	2.54	4.85	6.70	Yes	ND[c]
				5.95	No	>5.95
Lemon[d]	6.8	2.62	4.62	6.70	No	>7.70
				5.95	No	>5.95
Lime[d]	7.4	2.54	4.82	6.70	No	>7.70
				5.95	No	>5.95

[a] Cell recovery was attempted by streaking enrichment broth cultures onto sorbitol MacConkey agar. The detection limit for cultures from an inoculum of 6.70 log CFU/ml was –1 log CFU/ml (one cell in a 10-ml sample). The detection limit for cultures from an inoculum of 5.95 log CFU/ml was 0 log CFU/ml (one cell in a 1-ml sample).
[b] Without preservatives.
[c] ND, not determined.
[d] With preservatives (sodium bisulfite and sodium benzoate).

cells in the cranberry juice concentrates (pH 2.2 to 2.5) *(11).*

Under the same storage time and temperature conditions, inactivation of *E. coli* O157:H7 was greater in lemon juices than in lime juices for both nonadapted cells (Table 1) and acid-adapted cells (Table 2). For stationary-phase cells in reconstituted lemon and lime juices (pH 2.5 to 2.6), statistical analysis of four independent sets of data revealed that the log reduction of stationary-phase cells was significantly higher ($P < 0.05$) in lemon juice than in lime juice after storage for either 24 or 48 h (Table 1). Nevertheless, after 72 h storage, a greater than 5-log reduction was consistently achieved in both reconstituted lemon and lime juices with a natural pH 2.5 to 2.6 (Table 1).

Lemon and lime juices are standardized based on titratable acidity, and the single-strength juices produced under commercial conditions might reach as high as pH 2.7. Experiments with reconstituted lemon or lime juice with pH adjusted to 2.7 produced reductions similar to those observed for the respective products with a natural pH of 2.5 to 2.6 under the same experimental conditions (Table 1). After storage at room temperature for 72 h, a greater than 5-log reduction of *E. coli* O157:H7 stationary phase cells was consistently achieved in both lemon and lime juices with an adjusted pH of 2.7 (Table 1).

From the experiments with reconstituted juices, 72 h of storage time was selected for validation tests with commercially produced shelf-stable juices destined for the retail market. In the shelf-stable juices, a greater than 5-log reduction of *E. coli* O157:H7 in lemon juice (with or without preservatives) and lime juices (with preservatives) was consistently achieved after the products were stored at room temperature for 72 h (Table 3). In the first validation experiment, 10 ml of juice (after 24, 48, or 72 h storage) was transferred into 90 ml of recovery medium (TSB or UPB), and the enrichment broth culture was streaked on sorbitol MacConkey agar to recover surviving cells. *E. coli* O157:H7 was recovered from commercial juice samples inoculated with this pathogen and stored for 24 or 48 h (data not shown). After storage for 72 h, no *E. coli* O157:H7 was recovered from the flasks containing lemon or lime juices with preservatives; however, *E. coli* O157:H7 was detected in the enrichment broth from flasks containing 10-ml samples of lemon juice without preservatives (Table 3). The use of TSB or UPB made no difference in *E. coli* O157:H7 cell recovery. In the second validation test, 1.0 ml of sample was transferred into 9.0 ml of TSB and subjected to enrichment. The enrichment broth samples were streaked onto sorbitol MacConkey agar, and all samples were negative (Table 3), demonstrating that a greater than 5-log reduction of *E. coli* O157:H7 was achieved in the shelf-stable juices.

DISCUSSION

Conditions appropriate for inactivating *E. coli* O157:H7 in single-strength lemon and lime juices will likely result in inactivation of other potential pathogens of concern such as *Salmonella* and *L. monocytogenes.* The National Advisory Committee on Microbiological Criteria for Foods *(18)* recommended that *E. coli* O157:H7 or *L. monocytogenes* be considered appropriate target organisms for control because of outbreaks of *E. coli* O157:H7 infection caused by consumption of contaminated unpasteurized apple juice and the ubiquitous nature of *L. monocytogenes (33). S. enterica* Muenchen and Typhimurium serotypes have been associated with two major outbreaks associated with orange juice *(3, 5).* In previous studies from our laboratories, researchers investigated the destruction of *E. coli* O157:H7, *Salmonella,* and *L. monocytogenes* in juice concentrates (including lemon and lime concentrates) and found that *Salmonella* was inactivated faster than were the other pathogens *(19, 20).* Therefore, *Salmonella* was not included in the current study. Preliminary experiments with *L. monocytogenes* in this study revealed that although nonadapted stationary-phase cells had a sensitivity similar to that of *E. coli* O157:H7, acid-adapted *L. monocytogenes* was more sensitive to inactivation than was acid-adapted *E. coli* O157:H7 in single-strength lemon and lime juices. Therefore, the risk of *L. monocytogenes* contamination in lemon and lime juices is low because these products do not support growth of this organism and will eventually kill these cells. *E. coli* O157:H7 is more tolerant to acidic con-

ditions than is *Salmonella* and other foodborne pathogens and may have a lower infective dose. Inactivation of *E. coli* O157:H7 in the single-strength lemon and lime juices likely will result in inactivation of other pathogens of concern such as *Salmonella.* Therefore, *E. coli* O157:H7 is the most resistant vegetative pathogen of concern in the juice products under evaluation.

Results from this study are in agreement with data submitted to the FDA during the promulgation of the juice HACCP rule *(32),* which indicated that *E. coli* O157:H7 and *Listeria* do not survive in lime and lemon juice when stored at room temperature, but the actual log reduction in these organisms was not described. In the present study, a greater than 5-log reduction of *E. coli* O157:H7 was achieved after lemon and lime juices were stored at room temperature for 72 h, and *E. coli* O157:H7 survived better in single-strength lime juice than in lemon juice. Nogueira et al. *(19)* reported less effective inactivation of *E. coli* O157:H7 in lime than in lemon juice concentrates. In other studies, fresh squeezed lemon juice, lemon concentrates, and some lemon derivatives inactivated to various degrees pathogens such as *Salmonella (16, 25, 28)* and *V. cholerae (8, 9, 31).* Under some circumstances, lime juice consumption was encouraged to prevent foodborne transmission during cholera outbreaks *(26).* In the present study, the log reduction in lemon juice was significantly higher than that in lime juice after 24 or 48 h storage, and the pathogen was reduced to undetectable levels after both juices were stored for 72 h at room temperature.

The influence of temperature on pathogen survival under acidic conditions has been reported for products other than juice *(21, 22, 29, 33).* Survival of vegetative pathogens in some shelf-stable products such as mayonnaise, salad dressings, and sauces was reported to be much higher at refrigeration temperature than at room temperature. Lethality of bacterial pathogens in these products is most rapid under ambient storage conditions (20 to 30°C), whereas lower temperatures seem to protect organisms such as *E. coli* O157:H7, *Listeria,* and *Salmonella (14, 23, 30).* Chilling temperatures may inhibit the growth of the pathogens, but their survival can be enhanced under these conditions. *E. coli* O157:H7 and *Salmonella* survived longer in fruit juices under refrigeration than in those at room temperature *(23, 33).* Fisher and Golden *(14),* cited by the FDA *(32),* concluded that survival of *E. coli* O157:H7 in ground apples stored at various temperatures is enhanced when product is stored at refrigeration rather than stored at room temperature.

Because the required 5-log reduction of pathogen in single-strength lemon juice was not always obtained within 48 h, 72 h at 22°C is the appropriate condition to assure the required log reduction for lemon and lime juices. The validation tests in commercially produced shelf-stable products confirmed a greater than 5-log reduction of *E. coli* O157:H7 in single-strength lemon juice (with or without preservatives) and lime juice (with preservatives) stored at room temperature (22°C) for 72 h (3 days). The results from our study indicate that as an alternative to pasteurization, single-strength lemon and lime juices can be held for 72 h at 22°C to meet the FDA 5-log pathogen reduction performance standard.

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