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Phytic Acid and Sodium Chloride Show Marked Synergistic Bactericidal Effects against Nonadapted and Acid-Adapted *Escherichia coli* O157:H7 Strains

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The synergistic antimicrobial effects of phytic acid (PA), a natural extract from rice bran, plus sodium chloride against *Escherichia coli* O157:H7 were examined. Exposure to NaCl alone at concentrations up to 36% (wt/wt) for 5 min did not reduce bacterial populations. The bactericidal effects of PA alone were much greater than those of other organic acids (acetic, citric, lactic, and malic acids) under the same experimental conditions ($P < 0.05$). Combining PA and NaCl under conditions that yielded negligible effects when each was used alone led to marked synergistic effects. For example, whereas 0.4% PA or 3 or 4% NaCl alone had little or no effect on cell viability, combining the two completely inactivated both nonadapted and acid-adapted cells, reducing their numbers to unrecoverable levels (>7-log CFU/ml reduction). Flow cytometry confirmed that PA disrupted the cell membrane to a greater extent than did other organic acids, although the cells remained viable. The combination of PA and NaCl induced complete disintegration of the cell membrane. By comparison, none of the other organic acids acted synergistically with NaCl, and neither did NaCl-HCl solutions at the same pH values as the test solutions of PA plus NaCl. These results suggest that PA has great potential as an effective bacterial membrane-permeabilizing agent, and we show that the combination is a promising alternative to conventional chemical disinfectants. These findings provide new insight into the utility of natural compounds as novel antimicrobial agents and increase our understanding of the mechanisms underlying the antibacterial activity of PA.

Food safety has become an increasingly important aspect of public health. According to the World Health Organization, foodborne and waterborne diarrheal diseases kill an estimated 2 million people per year, many of whom are children (1). Although various chemicals are used to control the transmission of foodborne illnesses via the food and livestock industries (2, 3), several studies have suggested that synthetic sanitizers can have significant side effects (e.g., bleaching, formation of toxic compounds, and off odors) (4, 5). Because modern consumers tend to prefer the use of "natural" agents rather than "synthetic" ones (6), new antimicrobial agents are required; therefore, studies of natural compounds with antimicrobial actions are warranted.

Organic acids are a class of such natural antimicrobial agents. Several organic acids (particularly acetic, citric, and lactic acids) have long been used as preservatives or surface sanitizers for beef hides (7), carcasses (8), dairy products (9, 10), fresh-cut vegetables (11), and meat products (12). However, organic acids alone are not powerful enough to inactivate pathogenic bacteria after only a short exposure time (5, 13). Even if this problem could be overcome, applying adequate amounts of organic acids is impractical, because they generate strong acidic odors at working concentrations (5, 13). Thus, it is of vital importance to identify new alternatives or to develop alternative strategies to overcome barriers to the effective use of natural compounds.

Phytic acid (PA) (2,3,4,5,6-pentaphosphonooxycyclohexyl dihydrogen phosphate) is used by many grain and cereal plants as a source of stored phosphorous, where it represents 1 to 9% of dry weight (14). Several recent studies reported that PA has beneficial effects on human health through its antioxidant (15), anticancer (16), and antidiabetic (17) effects. It can also protect against the development of Parkinson's disease (18) and renal calculi (19). However, PA has not been widely studied as a natural antimicro-

bial agent. The mechanism by which organic acids exert their antimicrobial activity is generally explained by the weak acid theory, i.e., only undissociated forms of the acid can enter the cytoplasm, where they inactivate bacteria by gradually dissociating into charged ions that disrupt cytoplasmic pH homeostasis (20). The mechanism underlying the antimicrobial properties of PA is likely to be quite different from that of other organic acids, because PA has a unique structure (12 replaceable protons on six reactive phosphate groups bonded to a cyclic six-carbon ring, i.e., $C_6H_{18}O_{24}P_6$) (Fig. 1) and a wide acidity range (pK_a of 1.9 to 9.5) (21, 22). However, neither its bactericidal activity nor its mode of action has been examined in detail.

Sodium chloride is a typical condiment and food preservative that is widely used by the food industry and can be obtained from natural sources (it is present in the ocean at approximately 3 to 4% [wt/wt]) (23). The combination of organic acids and NaCl is a common example of hurdle technology in the food industry; many researchers have found that NaCl (at 3 to 5%) protects bacterial cells from the antimicrobial effects of organic acids (an-
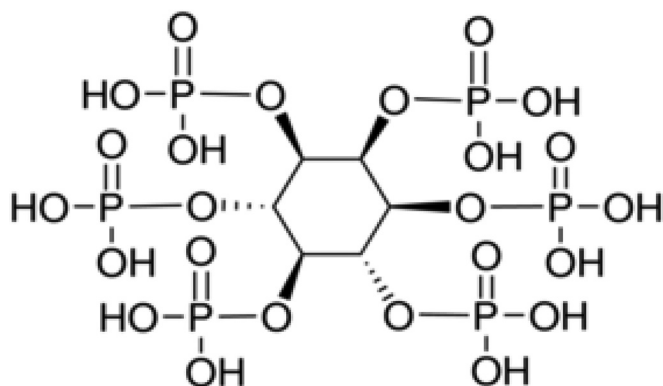
FIG 1 Chemical structure of phytic acid (2,3,4,5,6-pentaphosphonooxycyclohexyl dihydrogen phosphate).

tagonism) (24–30). However, it is unclear how PA interacts with NaCl.

Escherichia coli O157:H7 is a representative foodborne pathogen that produces Shiga-like toxins, ingestion of which causes watery and bloody diarrhea or, occasionally, a more serious condition called hemolytic-uremic syndrome (31). In the United States, the bacterium causes an estimated 73,000 cases of infection per year, with up to 61 deaths (32), and several large outbreaks have occurred worldwide (33, 34). *E. coli* O157:H7 is a highly virulent pathogen because it carries genes for Shiga toxin production and has an adaptive acid tolerance response system that results in strong acid resistance (35–37). Therefore, we selected *E. coli* O157:H7 as the target pathogen for study of the bactericidal effects of natural acidic compounds.

The aims of the study were 2-fold, namely, to examine the antimicrobial potential of PA and other organic acids generally used by the food and livestock industries against both nonadapted and acid-adapted *E. coli* O157:H7 and to examine the antimicrobial effects of PA and NaCl (alone and in combination) and the mechanism underlying the interactions between PA and NaCl.

MATERIALS AND METHODS

Bacterial strains. Three strains of Shiga-like-toxin-producing *E. coli* O157:H7, i.e., ATCC 35150 (harboring the stx_1 and stx_2 genes), ATCC 43889 (harboring stx_2), and ATCC 43895 (harboring stx_1 and stx_2), were obtained from the American Type Culture Collection (Manassas, VA, USA). All strains have inherent acid-adaptable (ATCC 43889) or acid-resistant (ATCC 35150 and ATCC 43895) characteristics (38). The strains were stored at −20°C in tryptic soy broth (TSB) (Difco, Becton Dickinson, Sparks, MD, USA) containing 20% glycerol and were subcultured on a monthly basis.

Bacterial cell suspensions. Each strain was cultivated for 24 h at 37°C in either TSB without glucose or TSB with glucose to obtain stationary-phase nonadapted cells or acid-adapted cells (cells adapted to pH 4.7 to 4.8), respectively (39). Cultures of individual cells of bacterial strains (either nonadapted or acid adapted) were mixed separately in a centrifuge tube and washed twice with 0.85% sterile saline by centrifugation at 3,000 × *g* for 15 min. The final cell pellet was resuspended in 0.85% sterile saline to yield a prepared cell suspension of 9 to 10 log CFU/ml.

Chemical agents. Purified acetic acid, citric acid, DL-lactic acid, DL-malic acid, and PA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions (50% [wt/wt]) were prepared by dissolving each compound in deionized water (DW). Stock solutions were then sterilized by microfiltration using a polytetrafluoroethylene syringe filter (DISMIC-25$_{HP}$; pore size, 0.45 μm; Toyo Roshi Kaisha Ltd., Tokyo, Japan) prior to

the experiments. An appropriate amount of NaCl (Junsei Chemical Co., Ltd., Tokyo, Japan) was dissolved in DW to yield a working concentration (2 to 36% [wt/wt]) and then was autoclaved. A hydrochloric acid solution (35% [wt/wt] in water; Matsunoen Chemical Ltd., Osaka, Japan) was prepared (5 M) in sterile DW and used to adjust pH levels. All working solutions were prepared on the day of the experiment, and each was mixed thoroughly using a vortex mixer (KMC-1300V; Vision Scientific Co. Ltd., Daejeon, South Korea) at high speed for 1 min immediately prior to use.

Treatments. An appropriate amount of each organic acid stock solution was added to sterile 0.85% saline (for single treatment, the final acid concentrations were 0.2%, 0.4%, 0.8%, and 1.6% [wt/wt]; for combined treatment, the final acid concentrations were 0.05%, 0.1%, 0.2%, and 0.4% [wt/wt] for PA and 0.4% [wt/wt] for other organic acids, in 2%, 3%, or 4% NaCl [wt/wt]). The effect of NaCl alone was assessed by exposing bacteria to solutions containing graded concentrations of NaCl (2%, 3%, 4%, 8%, 16%, and 32% [wt/wt]) approaching maximum solubility (36% [wt/wt] at 25°C). The net effect of pH was examined by using a sterile 0.85% saline solution, the pH of which was adjusted with 5 M HCl solution. Sterile glass culture tubes containing 9.9 ml of each solution were equilibrated to 22°C in a shaking water bath (VS-1205SW1; Vision Scientific Co. Ltd.) before the experiment. A prepared cell suspension (0.1 ml) was added to each solution (final concentration of target bacterial cells, 7 to 8 log CFU/ml). Both nonadapted and acid-adapted *E. coli* O157:H7 cells were exposed to each test solution for 5 min at 22°C, with shaking (100 rpm).

Viable cell counts and recovery of injured cells. Untreated and treated bacterial cells were diluted to 10^{-6} in sterile phosphate-buffered saline (PBS) (0.8% NaCl, 0.144% Na$_2$HPO$_4$, 0.024% KH$_2$PO$_4$, and 0.02% KCl [pH 7.4]). Both nondiluted samples (1 ml per five agar plates) and diluted samples (0.2 ml onto two agar plates) were spread plated onto tryptic soy agar (TSA) (Difco) plates and incubated at 37°C for 24 h (detection limit, 1 CFU/ml). Colonies growing on the plates were counted, and bacterial counts were expressed as a logarithmic value. To measure the residual antimicrobial effects of the treatment solution on target bacterial cells growing on the surface of the plates during incubation, 0.1 ml of cell suspension (7 to 8 log$_{10}$ CFU/ml) and 0.1 ml of working solution were plated onto TSA plates, and bacterial growth was monitored at 37°C for 24 h. All experiments were performed in triplicate. Test solutions of the combined treatment-bacterial cell combinations (1 ml) that resulted in complete inactivation of the inoculum were inoculated into 9 ml of TSB, and recovery of injured cells was examined after incubation at 37°C for 24 h.

pH measurements. The pH of each solution was measured three times using a pH meter (SevenCompact S220; Mettler-Toledo Inc., Columbus, OH, USA).

Statistical analysis. All data were evaluated by analysis of variance (ANOVA) using SAS software (version 9.4; SAS Institute Inc., Cary, NC, USA). Tukey's studentized range test was used to calculate the least significant differences between mean values ($P < 0.05$).

Cell staining. Propidium iodide (PI) Molecular Probes reagent (Invitrogen, Thermo Fisher Scientific Inc., Grand Island, NY, USA) was used for cell staining (40). A stock solution of PI (3 mM) was prepared by dissolving the compound in sterile DW. The stock solution was then stored at 4°C in the dark. Cell suspensions (approximately 6 to 7 log CFU/ml) of untreated cells (living cells), treated cells, or dead cells (after treatment with 70% ethyl alcohol at 22°C for 5 min) were prepared in sterile PBS. Immediately after treatment, cells were diluted 10-fold in sterile PBS to prevent further bactericidal activity. Residual chemicals were removed by two washes with centrifugation at 13,000 × *g* for 3 min at 4°C. The diluted bacterial cells were then reconcentrated (10-fold). All target cells were stained with PI (final concentration, 60 μM) at 37°C for 20 min in the dark. The stained cells were then washed twice with sterile PBS and used for flow cytometry.

Flow cytometric analysis. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA) with an excitation wavelength of 488 nm, generated by a

TABLE 1 Effects of various organic acids (at 0.2 to 1.6%) on nonadapted and acid-adapted *E. coli* O157:H7 cells treated at 22°C for 5 min

Organic acid and concn (% [wt/wt])	pH (mean ± SD)[a]	Effect of organic acid on indicated bacteria (log CFU/ml) (mean ± SD)[b]	
		Nonadapted cells	Acid-adapted cells
Acetic acid			
0.2	3.02 ± 0.01 A	−0.03 ± 0.06 Aa	−0.01 ± 0.05 Aa
0.4	2.86 ± 0.02 B	−0.02 ± 0.07 Aa	−0.01 ± 0.02 Aa
0.8	2.70 ± 0.01 C	−0.01 ± 0.08 Aa	−0.01 ± 0.03 Aa
1.6	2.53 ± 0.02 E	−0.04 ± 0.08 Aa	0.00 ± 0.09 Aa
Citric acid			
0.2	2.49 ± 0.02 E	0.01 ± 0.01 Aa	−0.01 ± 0.02 Aa
0.4	2.31 ± 0.01 F	0.03 ± 0.01 Aa	0.00 ± 0.02 Aa
0.8	2.13 ± 0.01 GH	0.01 ± 0.01 Aa	−0.03 ± 0.01Ab
1.6	1.97 ± 0.01 J	0.60 ± 0.08 Ba	0.01 ± 0.07 Ab
Lactic acid			
0.2	2.63 ± 0.01 D	0.01 ± 0.03 Aa	−0.02 ± 0.01 Aa
0.4	2.48 ± 0.02 E	0.01 ± 0.01 Aa	0.00 ± 0.02 Aa
0.8	2.29 ± 0.01 F	0.02 ± 0.01 Aa	0.00 ± 0.04 Aa
1.6	2.12 ± 0.01 H	0.64 ± 0.02 Ba	0.29 ± 0.12 ABb
Malic acid			
0.2	2.53 ± 0.01 E	0.02 ± 0.01 Aa	0.00 ± 0.04 Aa
0.4	2.35 ± 0.01 F	0.03 ± 0.01 Aa	0.01 ± 0.03 Aa
0.8	2.19 ± 0.01 G	0.03 ± 0.03 Aa	0.02 ± 0.03 Aa
1.6	2.04 ± 0.02 I	0.03 ± 0.03 Aa	0.04 ± 0.04 Aa
Phytic acid			
0.2	1.86 ± 0.01 K	0.46 ± 0.28 Ba	0.03 ± 0.20 Aa
0.4	1.64 ± 0.01 L	2.68 ± 0.39 Ca	0.41 ± 0.35 Bb
0.8	1.49 ± 0.04 M	5.77 ± 0.08 Da	4.01 ± 0.09 Cb
1.6	1.29 ± 0.08 N	>6.13 ± 0.02 Ea	4.54 ± 0.21 Db

[a] The initial pH value was 6.59 ± 0.02. Values in this column denoted by different capital letters (A through N) are significantly different ($P < 0.05$).

[b] The initial nonadapted and acid-adapted *E. coli* O157:H7 populations were 7.13 ± 0.02 log CFU/ml and 7.02 ± 0.06 log CFU/ml, respectively. Values in the same column (A to E) or row (a and b) but denoted by different letters are significantly different ($P < 0.05$).

blue argon laser (15 mW). Data were analyzed using BD CellQuest Pro software (Becton Dickinson Biosciences). An appropriate optical filter (FL3, ≤670 nm) was used to measure the red fluorescence emitted by PI. Light signals were detected as logarithmic signals by a photodiode detector with a forward scatter voltage setting of E02, and every 50,000 events were acquired at a flow rate of 12 μl/min. The acquired events were depicted as a histogram, and the M1 region was set using living and dead cells (both nonadapted and acid-adapted *E. coli* O157:H7 cells). The percentage of cells in the M1 region was calculated using the stat option in BD CellQuest Pro software.

RESULTS

Effects of treatment with phytic acid, other organic acids, or sodium chloride. Table 1 shows the pH and antimicrobial effects of PA and other organic acids against *E. coli* O157:H7 when used at the same concentrations (0.2%, 0.4%, 0.8%, and 1.6%). While the pH of acetic, citric, lactic, and malic acids at these concentrations ranged from 2.5 to 3.0, 2.0 to 2.5, 2.1 to 2.6, and 2.0 to 2.5, respectively, PA had a much lower pH in sterile 0.85% saline solution (pH 1.3 to 1.9; $P < 0.05$). Indeed, PA showed the strongest bactericidal activity and the lowest pH ($P < 0.05$). The other organic

acids had no effect on the bacterial population in the inoculum ($P > 0.05$), with the exception of citric acid (1.6%) or lactic acid (1.6%) treatment of nonadapted cells. Acid-adapted *E. coli* O157:H7 cells were significantly more tolerant to 0.4 to 1.6% PA than were nonadapted cells ($P < 0.05$). NaCl alone (up to its maximum solubility level of 36.0%) had no effect on the populations of nonadapted and acid-adapted *E. coli* O157:H7 cells after 5 min of treatment ($P > 0.05$) (data not shown).

Combination treatment with phytic acid or organic acids plus sodium chloride. To examine the effects of PA or other organic acids plus NaCl (either alone or in combination), each was used at a concentration that showed negligible or insignificant bactericidal activity, as follows: 0.05 to 0.4% for PA, 0.4% for organic acids other than PA, and 2 to 4% for NaCl. Figure 2 shows bactericidal activity against nonadapted (Fig. 2a) and acid-adapted (Fig. 2b) *E. coli* O157:H7 cells mediated by single or combined treatment with PA and NaCl at 22°C for 5 min, compared with that mediated by combinations of other organic acids with sodium chloride.

Figure 2a shows that acetic, citric, lactic, and malic acids had no effect on nonadapted cells when used in combination with 2 to 4% NaCl; the reductions in the bacterial population mediated by combined treatment were not significantly different from those mediated by individual treatment ($P > 0.05$). However, PA plus NaCl had significantly greater bactericidal effects than did treatment with either alone ($P < 0.05$) (Fig. 2a, gray panels). As the concentrations of PA and NaCl were increased, a clear synergistic interaction was observed. While treatment with PA alone (up to 0.2%) yielded a negligible reduction in the population of nonadapted *E. coli* O157:H7 cells (<0.6 log CFU/ml), treatment with 0.1 or 0.2% PA plus 3 or 4% NaCl had much greater effects (2.1 to 5.5 log CFU/ml) ($P < 0.05$). When 0.4% PA was tested in combination with 3 or 4% NaCl, all cells in the inoculum were completely inactivated (>7.3-log CFU/ml reduction) and no cells recovered after further culture in TSB at 37°C for 24 h (data not shown). Combination treatment resulted in an additional 4.5-log CFU/ml reduction in the bacterial population, compared with individual treatment (PA, 2.8 log CFU/ml; NaCl, <0.1 log CFU/ml).

Figure 2b shows the results from the same experiment using acid-adapted *E. coli* O157:H7 cells. In common with the experiment using nonadapted cells, the combination of acetic, citric, lactic, or malic acid (each at 0.4%) plus NaCl had no effect on the population of acid-adapted *E. coli* O157:H7 cells ($P > 0.05$). A negligible reduction (<0.5 log CFU/ml) was observed after treatment with PA or NaCl alone; however, treatment with 0.2% PA plus 3 or 4% NaCl or 0.4% PA plus 2 to 4% NaCl had significantly greater effects ($P < 0.05$) (Fig. 2b, gray panels). In particular, the combination of 0.4% PA plus 3 or 4% NaCl eradicated all cells in the inoculum (>7.0-log CFU/ml reduction), with no recoverable cells (data not shown). These results show that PA in combination with NaCl has clear synergistic bactericidal actions; indeed, treatment achieved an additional 6.6-log CFU/ml reduction, compared with that achieved with either PA (0.5-log CFU/ml reduction) or NaCl (<0.1-log CFU/ml reduction) alone.

The combination of 0.4% PA plus 3 or 4% NaCl (pH 1.4 to 1.6) was selected for the validation experiment because these conditions led to complete inactivation of the inoculum. When both nonadapted and acid-adapted *E. coli* O157:H7 cells were inoculated into a series of 0.85% saline solutions with the same pH
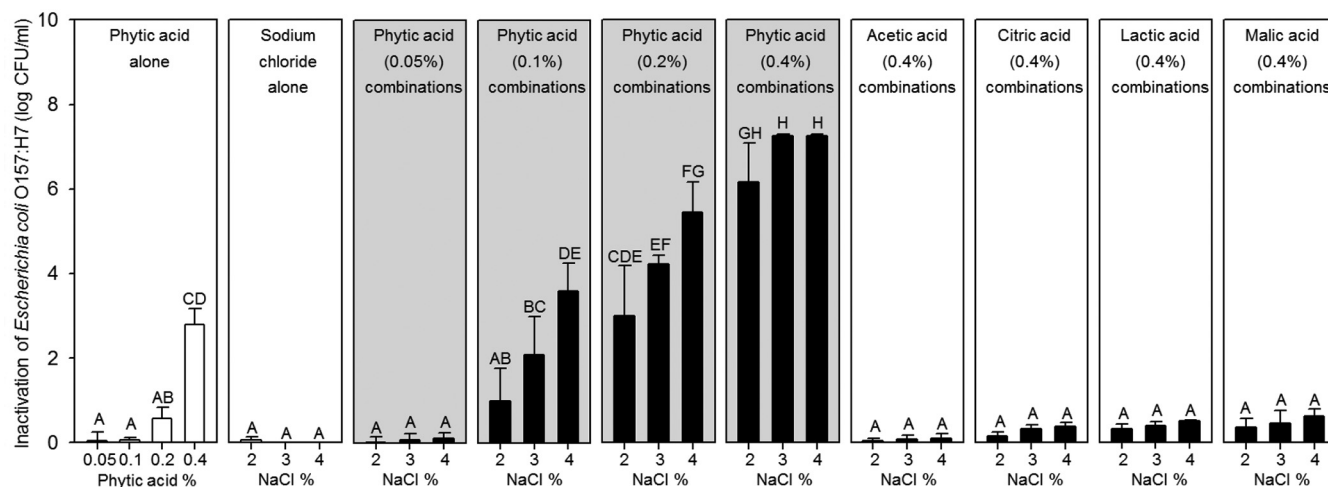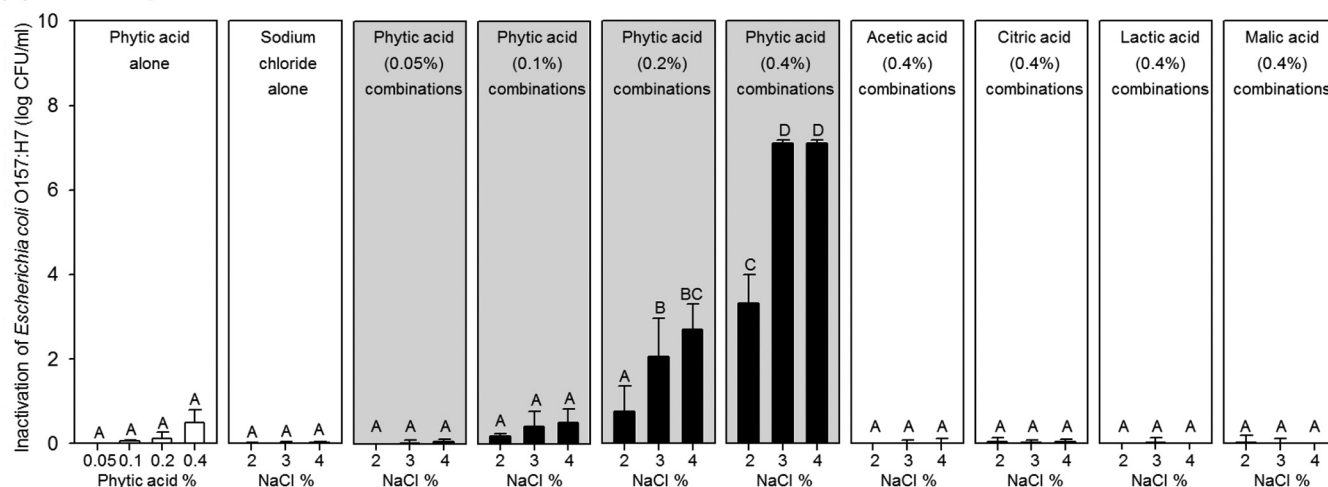
(a) Non-adapted *Escherichia coli* O157:H7

(b) Acid-adapted *Escherichia coli* O157:H7

FIG 2 Inactivation of nonadapted (a) and acid-adapted (b) *Escherichia coli* O157:H7 cells treated with 0.05 to 0.4% phytic acid alone, 2 to 4% sodium chloride alone, a combination of the two, or combinations of sodium chloride plus other organic acids (acetic, citric, lactic, and malic acid) at 0.4% (wt/wt). The gray panels represent combinations of sodium chloride and phytic acid. Initial nonadapted and acid-adapted *E. coli* O157:H7 populations were 7.13 ± 0.02 log CFU/ml and 7.02 ± 0.06 log CFU/ml, respectively. Values in the same panel but denoted by different capital letters (A to H) are significantly different ($P < 0.05$).

values as the PA-containing test solutions (pH 1.4 to 1.6), some cells in the inoculum survived after 5 min (nonadapted cells, 2.9 to 3.0 log CFU/ml; acid-adapted cells, 6.7 to 6.9 log CFU/ml) (data not shown). In addition, lowering the pH of 3 or 4% NaCl to the same level as that of the combined solutions (pH 1.4 to 1.6) did not eradicate all of the cells in the inoculum (survival of non-adapted cells, 2.3 to 2.5 log CFU/ml; survival of acid-adapted cells, 2.8 to 3.6 log CFU/ml) (data not shown).

Effects of phytic acid and other organic acids alone on membrane integrity. Figures 3 and 4 show how acetic acid, citric acid, lactic acid, malic acid, and PA affect the membrane integrity of nonadapted and acid-adapted *E. coli* O157:H7 cells, respectively. In addition, we measured the net effect of pH by treating cells with 0.85% saline solutions in which the pH had been adjusted to ensure it was the same as that in the PA solutions (pH adjusted with HCl). The percentage values for M1 indicate the proportions of damaged cells after each treatment. The results showed that the

membrane-permeabilizing effect of PA was clearly greater than that of the other organic acids. The number of damaged cells tended to be much higher in the PA-treated groups (Fig. 3e and 4e) than in the other acid-treated groups (Fig. 3a to d and 4a to d) or in groups treated with pH-equivalent 0.85% saline solutions (Fig. 3f and 4f).

For example, treatment with 0.4% PA led to the permeabilization of >50% of nonadapted (71.4%) and acid-adapted (53.9%) *E. coli* O157:H7 cells, whereas the other organic acids had little effect when tested at the same concentrations (cells in the M1 region, nonadapted, 5.6 to 9.3%; acid adapted, 3.8 to 7.0%). When the results for cells treated with 0.2 to 0.4% PA (pH 1.6 to 1.9) were compared with those for cells treated with pH-equivalent 0.85% saline solutions, we found that the percentages of permeabilized cells were much higher for the former (nonadapted, 31.8 to 71.4% [Fig. 3e]; acid adapted, 12.1 to 53.9% [Fig. 4e]) than for the latter (nonadapted, 16.9 to 32.5% [Fig. 3f]; acid adapted, 6.2 to 8.1%
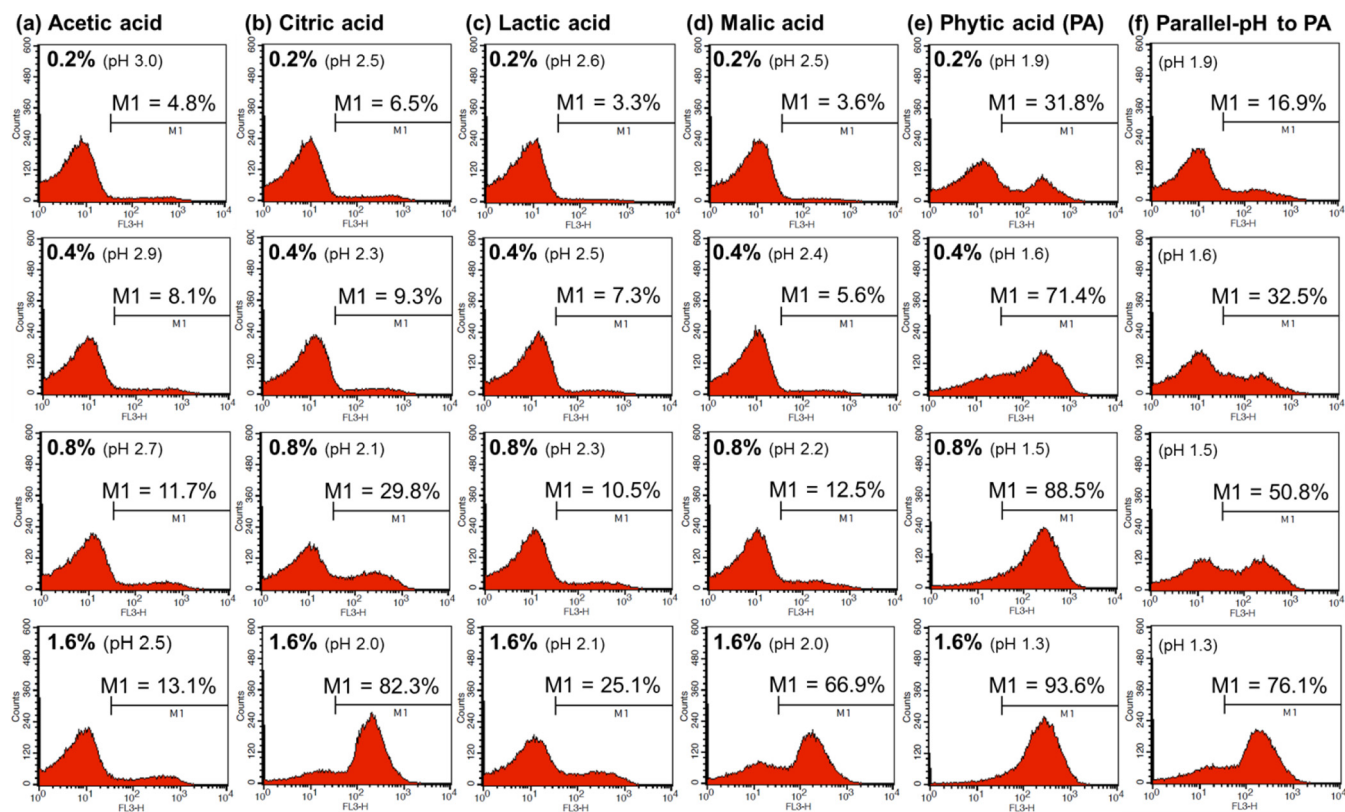
FIG 3 Flow cytometric analysis of nonadapted *Escherichia coli* O157:H7 cells treated with 0.2 to 1.6% acetic acid (a), citric acid (b), lactic acid (c), malic acid (d), or phytic acid (PA) (e) or with NaCl-HCl solutions at pH values equivalent to those of 0.2 to 1.6% PA solutions (f).

[Fig. 4f]).When 0.8% PA was applied, the percentages of damaged nonadapted and adapted cells reached 88.5% and 77.8%, respectively; when 1.6% PA was applied, the corresponding values were 93.6% and 92.8%, respectively. These percentages were much higher than those recorded after treatment with the other organic acids at the same working concentrations (cells in the M1 region, nonadapted, 10.5 to 82.3%; acid adapted, 4.7 to 12.5%). In addition, the membrane-permeabilizing effects of 0.85% saline solutions at pH values equivalent to those of the 0.8% and 1.6% PA solutions were much weaker than those of the PA solutions (nonadapted, 50.8 to 76.1% [Fig. 3f]; acid adapted, 25.2 to 45.5% [Fig. 4f]).

Changes in membrane integrity induced by treatment with phytic acid, sodium chloride, or a combination of the two. Finally, to investigate the mechanism underlying the synergistic activity of 0.2 to 0.4% PA plus 2 to 4% NaCl solutions, we examined the membrane integrity of *E. coli* O157:H7 cells before and after treatment. The histograms generated in flow cytometric analysis of nonadapted *E. coli* O157:H7 cells are shown in Fig. 5. While fewer cells (9.7 to 11.7%) were permeabilized by treatment with 2 to 4% NaCl alone (Fig. 5b to d), increasing numbers were damaged upon addition of 0.2% or 0.4% PA to the 2 to 4% NaCl solutions (cells in the M1 region after addition of 0.2% PA, 72.2 to 78.1%; after addition of 0.4% PA, 87.3 to 94.4%). In particular, the combination of 0.4% PA with 3 or 4% NaCl permeabilized almost all of the cells in the inoculum (cells in the M1 region, 93.2 to 94.4%). When 2 to 4% NaCl solutions with pH values equivalent to those of the 0.4% PA solutions were applied (Fig. 5p to r), the

proportions of damaged cells (51.7 to 71.6%) were significantly smaller than those induced by 0.4% PA plus 2 to 4% NaCl (87.3 to 94.4%).

The results for acid-adapted cells are presented in Fig. 6. As shown in Fig. 6b to d, treatment with NaCl alone had little effect on the membrane integrity of acid-adapted cells (cells in the M1 region, 12.7 to 15.0%). Addition of 0.2 or 0.4% PA to 2 to 4% NaCl increased the proportions of damaged cells (0.2% PA, 29.3 to 65.3%; 0.4% PA, 84.4 to 93.2%) (Fig. 6f to h or j to l, respectively); however, the increases were smaller than those observed for nonadapted cells (0.2% PA, 72.2 to 78.1%; 0.4% PA, 87.3 to 94.4%) (Fig. 5f to h or j to l, respectively). In agreement with the results obtained for nonadapted cells, the combination of 0.4% PA plus 3 or 4% NaCl damaged >90% of acid-adapted cells. The extent of the damage was much greater (cells in the M1 region, 90.8 to 93.2%) (Fig. 6k to l) than that caused by the net effect of pH and 3 or 4% NaCl (cells in the M1 region, 44.1 to 56.8%) (Fig. 6q to r).

DISCUSSION

The present study examined the antimicrobial potential of a "natural" compound, namely, PA, and its synergistic interaction with NaCl. The major findings were that PA exhibited significantly stronger bactericidal effects against acid-resistant *E. coli* O157:H7 than did other organic acids at equivalent working concentrations and that PA plus NaCl had marked synergistic antibacterial effects. A combination of PA (0.4%) plus NaCl (3 or 4%) resulted in an additional reduction of 4 to 6 log CFU/ml, compared with the
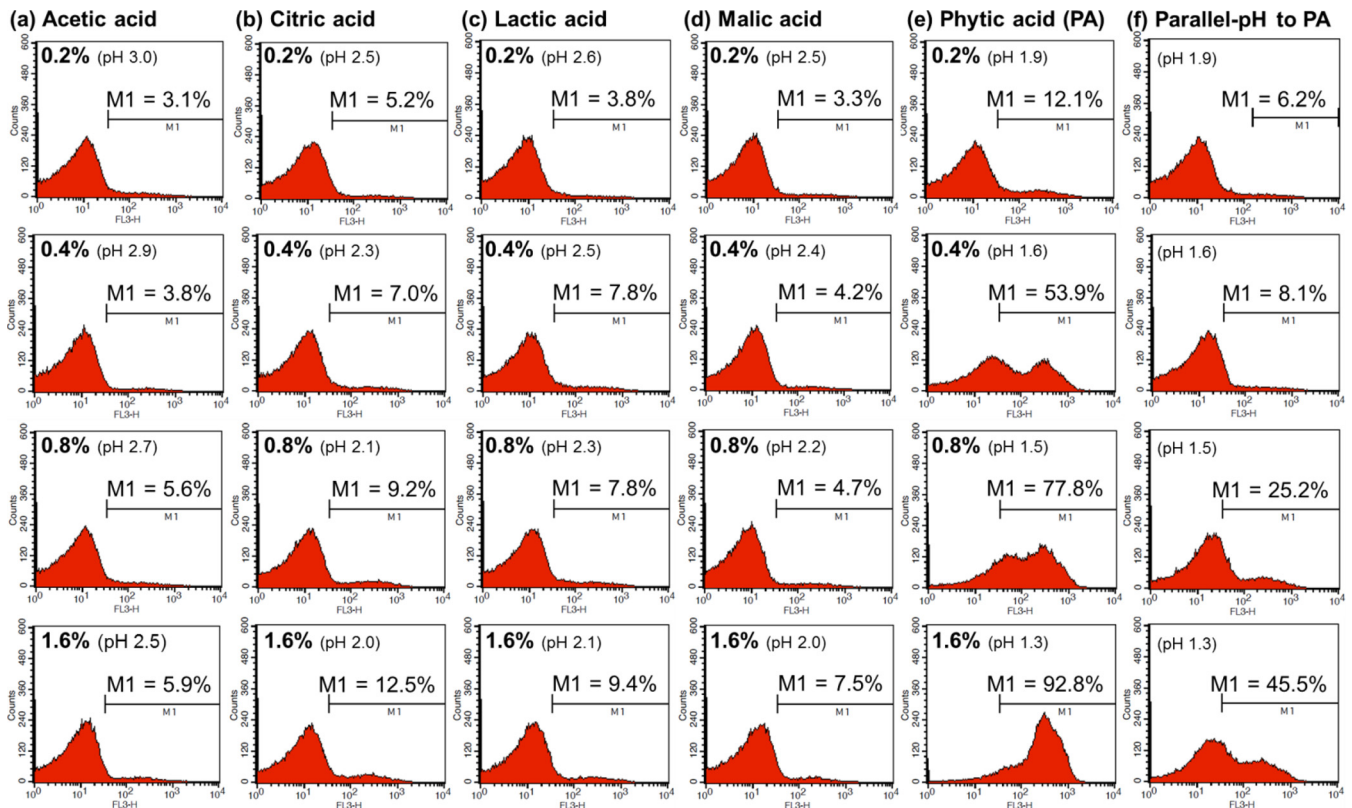
FIG 4 Flow cytometric analysis of acid-adapted *Escherichia coli* O157:H7 cells treated with 0.2 to 1.6% acetic acid (a), citric acid (b), lactic acid (c), malic acid (d), phytic acid (PA) (e), or NaCl-HCl solutions with pH values equivalent to those of 0.2 to 1.6% PA solutions (f).

sum of the effects of PA alone and NaCl alone after 5 min; this synergism was much greater for PA than for other organic acids.

A previous report showed that *E. coli* is much more able to survive under extremely acidic conditions than other *Enterobacteriaceae* (41). Lin et al. (42) found that *E. coli* O157:H7 could resist pH values of 2.0 to 2.5 for 3 h (survival rates, 16 to 98%). Here we found that treatment with 0.8 to 1.6% PA (pH 1.3 to 1.5) led to a >4-log CFU/ml reduction in *E. coli* O157:H7 numbers within 5 min. Compared with other organic acids at the same concentrations, PA showed significantly stronger effects and greater acidity (pH 1.3 to 1.9) than acetic, citric, lactic, or malic acid (pH 2.0 to 2.5). However, it is difficult to conclude that the low pH of PA was the main reason for its greater bactericidal effects, because acid solutions of equivalent pH did not generate the same bactericidal effects. Thus, the role of charged phytate ion merits attention as a factor that leads to significantly greater bactericidal activity against *E. coli* O157:H7.

Gram-negative bacteria such as *E. coli* possess an outer membrane (OM) that functions as a barrier to antimicrobial agents or antibiotics (43). The protective effect of the OM is due mainly to the presence of the lipopolysaccharide (LPS) molecules in the outer leaflet (44). Agents such as EDTA, citric acid, and lactic acid act on the OM (45–47) to disintegrate LPS by chelating OM-stabilizing divalent cations (e.g., Mg^{2+} and Ca^{2+}); this is an effective strategy against Gram-negative bacteria. PA contains six reactive phosphate groups, which are responsible for its strong chelating capacity (22). These groups gradually become negatively charged as the hydrogen ion dissociates under aqueous conditions. Previ-

ous studies have focused only on the food-spoiling qualities of PA and how to overcome them (14, 21, 48); however, this strong chelating agent may have potential as an effective surface sanitizer because it can disrupt the molecular interactions between LPS molecules expressed on the OM of pathogenic bacteria.

Here we showed that PA disrupts the membrane integrity of *E. coli* O157:H7 cells, which supports this hypothesis. To empirically measure the damage to the cell membrane, we stained the treated cells with PI, which cannot penetrate intact cell membranes (49, 50). The permeabilizing ability of PA was much greater than that of acid solutions of equivalent pH, as demonstrated by higher PI uptake and stronger red fluorescence signals (Fig. 3 and 4). The disruption of membrane integrity by PA (due to the presence of H^+ and phytate ions) was much greater (12.1 to 93.6% damaged cells) (Fig. 3e and 4e) than that by solutions containing HCl (which has the same number of H^+ ions but Cl^- rather than phytate) (16.9 to 76.1% damaged cells) (Fig. 3f and 4f). This may indicate that both H^+ and charged phytate ions play major roles in bacterial inactivation.

In general, we expected that PA would show greater antimicrobial efficacy than other organic acids because the acidity of the protons derived from PA varies from very strong to very weak (pK_a = 1.9 to 9.5) (22), whereas that of protons derived from the other organic acids is weak (pK_a = 3.1 to 6.4). This result has implications regarding the practical use of organic acids as surface-sanitizing agents (51, 52). At the same working concentrations, the bactericidal and membrane-permeabilizing activities of PA were much greater than those of the other organic acids. Also,
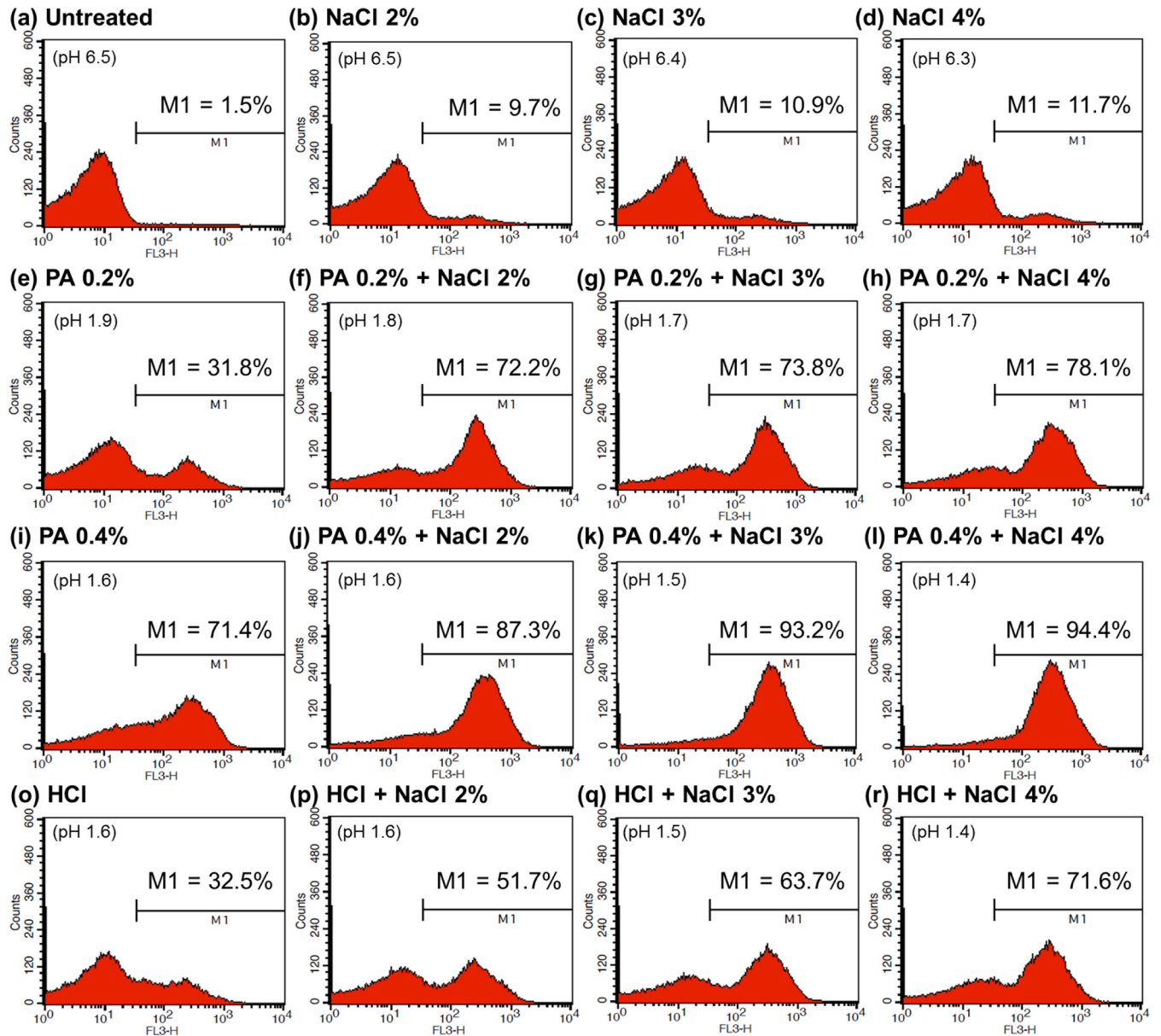
FIG 5 Flow cytometric analysis of nonadapted *Escherichia coli* O157:H7 cells that were untreated (a) or treated with 2 to 4% NaCl (b to d), 0.2% PA (e), 0.4% PA (i), combinations of 0.2% PA (f to h) or 0.4% PA (j to l) plus 2 to 4% NaCl, a solution with a pH equivalent to that of 0.4% PA (o), or 2 to 4% NaCl solutions with pH values equivalent to those of 0.4% PA plus 2 to 4% NaCl (p to r).

whereas the pungent odor of organic acids makes them unsuitable for use as surface sanitizers, PA is odorless. In addition, citric and malic acid are commonly used as natural chelating agents (53); therefore, the much stronger chelating ability of PA makes it a practical alternative agent for permeabilization of bacterial cells. Finally, the molarities of the 0.4% acetic, citric, lactic, and malic acid solutions were 66.9, 20.9, 44.6, and 30.0 mM, respectively, whereas that of PA was only 6.1 mM (9 to 29% that of other organic acids). Because PA showed much higher efficacy at lower molar concentrations, it might have potential as an alternative effective surface sanitizer.

We also found that the antibacterial action of PA was markedly enhanced in the presence of 3 or 4% NaCl. In contrast, the combination of other organic acids plus NaCl had no effect

under the same experimental conditions. Previous studies examining the antimicrobial relationship between organic acids and NaCl mainly used long exposure times (0.5 to 120 h) along with acetic or lactic acid (pH 3.0 to 4.2) and 2 to 8% NaCl in nutrient broth or TSB to model acidic foods (e.g., dressings, fermented foods, pickled vegetables, and sauces) (24–29). They all concluded that NaCl (3 to 5%) protects cells from the antimicrobial effects of organic acids. Casey and Codon (24) reported that NaCl protected bacteria by reducing the water activity, which decreases the cell volume and increases the cytoplasmic pH. Kitko et al. (30) also reported that osmolytes, including NaCl, contribute to cytoplasmic pH homeostasis by increasing microbial recovery from a rapid acid shift. However, we found that NaCl did not protect *E. coli* O157:H7 cells from
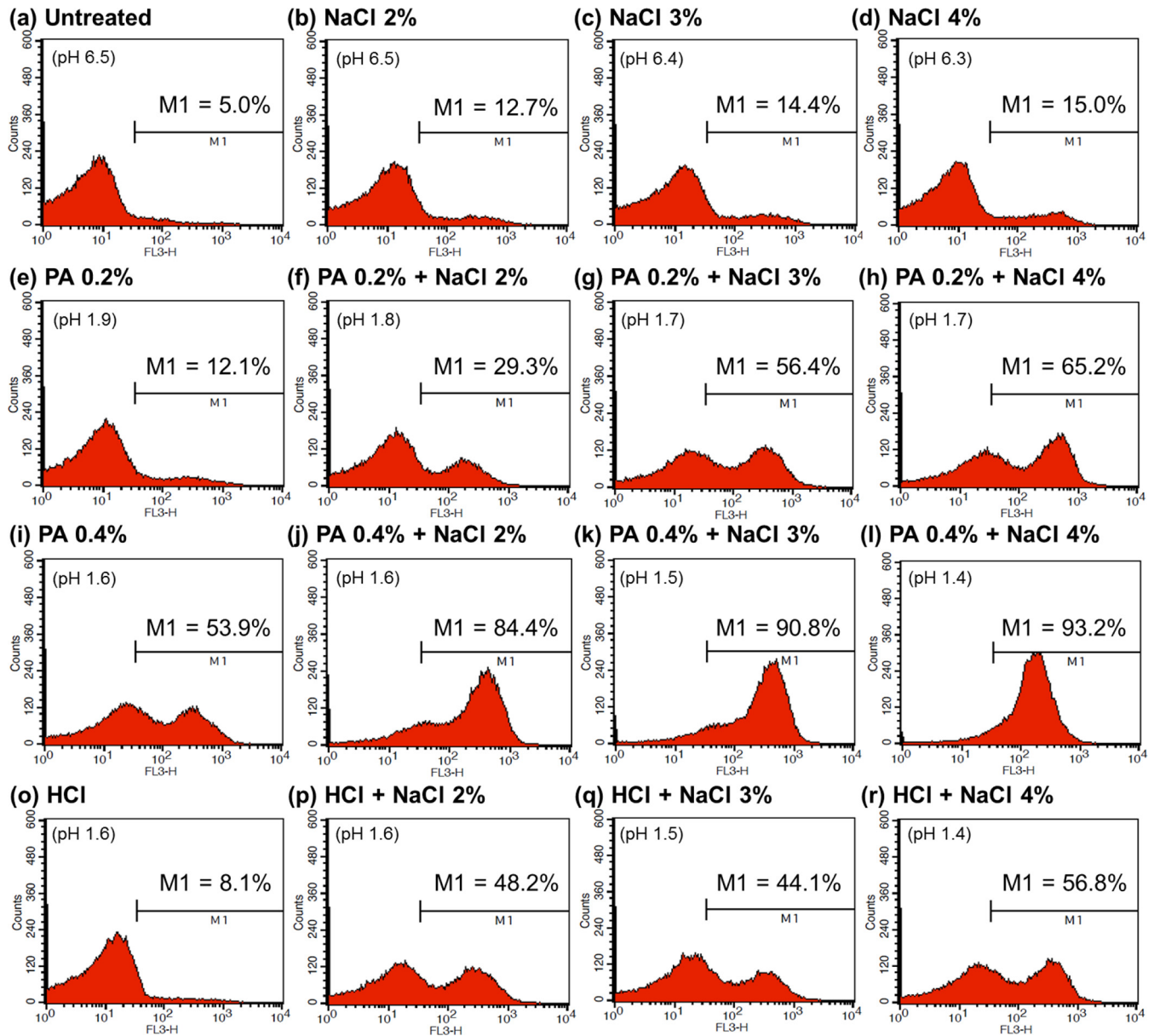
FIG 6 Flow cytometric analysis of acid-adapted *Escherichia coli* O157:H7 cells that were untreated (a) or treated with 2 to 4% NaCl (b to d), 0.2% PA (e), 0.4% PA (i), combinations of 0.2% PA (f to h) or 0.4% PA (j to l) plus 2 to 4% NaCl, a solution with a pH equivalent to that of 0.4% PA (o), or 2 to 4% NaCl solutions with pH values equivalent to those of 0.4% PA plus 2 to 4% NaCl (p to r).

the bactericidal and chelating activities of PA at low pH (pH 1.4 to 1.9), even after a short exposure (5 min).

Decad and Nikaido (54) found that cell wall damage was minimal at NaCl concentrations up to 0.3 M (approximately 1.7%); however, a large percentage of the cells underwent plasmolysis at NaCl concentrations greater than 0.5 M (2.9%). While NaCl (2 to 4% [0.35 to 0.71 M]) alone had no effect on viable *E. coli* O157:H7 counts, the results of the present study partially support those of Decad and Nikaido (54) because the membrane integrity of the cells was slightly affected by treatment with 2 to 4% NaCl (9.7 to 15.0% damaged cells). In addition, when cell membranes are permeabilized by the chelating action of 0.4% PA, a few Na^+ and Cl^- ions may significantly disrupt pH homeostasis inside the cells

(84.4 to 94.4% damaged cells). It can be presumed that both H^+ and phytate ions play leading roles in the marked synergistic effects of PA and NaCl, because addition of incremental amounts of PA to a NaCl solution had greater effects (90.8 to 94.4% damaged cells) than did NaCl-HCl solutions of equivalent pH (44.1 to 71.6% damaged cells).

Since acid adaptation significantly increases the acid tolerance of *E. coli* O157:H7 cells (36, 39, 42), we suggest that the combination of NaCl plus PA, rather than NaCl plus other organic acids, is an effective tool for controlling acid-resistant *E. coli* O157:H7. Based on the evidence presented above, we suggest the following mechanistic steps to explain the synergistic effects of PA and NaCl: (i) reactive phosphate groups on PA chelate divalent cations,

thereby releasing LPS from the OM; (ii) cells are permeabilized, but this damage is recoverable; (iii) Na^+, Cl^-, and H^+ ions more easily penetrate the cell membrane; and (iv) these charged ions attack the organelles, resulting in complete dysfunction of the cells.

PA is listed as a food additive in the Food Additive Code in Korea (55) and the Generally Recognized as Safe Notification (GRN) inventory published by the U.S. Food and Drug Administration (56). From a safety perspective, a number of repeated-dose studies have been conducted in rodents; no adverse effects were observed in a rat model with orally administered PA in the diet or drinking water at a concentration of approximately 1.0 to 2.5% for up to 12 weeks (17, 57–61). In addition, exposing the skin to PA may have more advantages than disadvantages. Some commercially available cosmetics contain PA, and several studies report that PA has the following positive effects on human skin: moisturization, lightening, increased elasticity, peeling, and improved acne vulgaris and melasma symptoms (62–64).

To conclude, the results of the present study suggest that PA is effective against *E. coli* O157:H7 and has a greater membrane-permeabilizing capacity than commonly used organic acids. It also shows outstanding synergistic effects when combined with NaCl. Both of these compounds are naturally occurring and cheap; PA is prepared from natural waste products from the rice-milling industry, and NaCl can be obtained from the ocean. The chelating characteristics of PA may enable other cell-impermeant antimicrobial agents (either electrically charged or hydrophobic molecules) to penetrate the cytoplasm, thereby increasing the susceptibility of microorganisms to hydrophobic antibiotics, detergents, lysozyme, or bacteriocins. These findings shed new light on the development of novel natural antimicrobial agents that have utility as disinfectants. Such agents may improve the microbiological safety of food and ensure hygiene in livestock industries and health care centers.

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