RESEARCH NOTE

MORPHOLOGICAL EFFECTS OF PULSED ULTRAVIOLET LIGHT AND ANOLYTE TREATMENT ON BACTERIAL CELL WALL

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ABSTRACT

Anolyte wash and pulse ultraviolet light (PUV) are approved for food use. Anolyte is a solution containing hypochlorous acid with a pH 6.0 - 6.5. PUV has a wide energy range. There is little information about the bacterial cell injury when the PUV treatment is combined with an anolyte wash. In this study an anolyte wash with a 300 ppm available chlorine for 3 min, PUV for 15 sec, and the PUV treatment followed by the anolyte wash was used to determine the morphological effect on *Salmonella*, *Listeria monocytogenes* (LM) and *Staphylococcus aureus* (SA) cells. Following each treatment, the cells were fixed for transmission electron microscopic (TEM) examination. The TEM images showed that *Salmonella* was more sensitive to the treatments. *Salmonella*'s cell membrane wrinkled after both the PUV and anolyte wash with increased cell wall damage and cytoplasm leakage after the PUV/anolyte treatment. The LM and SA showed less damage after the anolyte wash and PUV treatment, but increased cell damage did occur after the combined treatment of PUV followed by the anolyte wash. These results indicate that the gram negative cell wall is more sensitive to the anolyte treatments than the gram positives and PUV caused cytoplasmic disruption in both. The hurdle treatment (PUV followed by anolyte wash) is an effective way to inactivate bacterial pathogens.

INTRODUCTION

During food processing, cross contamination of pathogenic and spoilage bacteria can occur between the equipment and food surfaces. Sanitizers are used on the food contact surface to reduce this cross contamination. However, not all sanitizers are environmentally friendly due to their odor, low stability, toxic resides and reaction with metal equipment (25). The development of an effective, environmentally friendly sanitizer to reduce or eliminate bacteria in the commercial operation is crucial and hypochlorous acid is one such sanitizer.

Anolyte's main ingredient (92%) is electrolytically generated hypochlorous acid and is an approved sanitizer for food contact surfaces by the U. S. Food and Drug Administration (FDA) (4). Anolyte is produced by electrolysis of a saturated NaCl solution with a resulting pH of 6.0 – 6.5. Even though anolyte (i.e. hypochlorous acid) is classified as a weak acid (pKa ~ 7.5) and

dissociates slightly to H^+ and OCl^-, anolyte is a strong ozidizing agent and contains the most active form of residual chlorine (RCL) (8,17,18). Dychdala (8) reported that the biocidal activity of available chlorine (AC) in solution is pH dependent. At pH 6, it took 2.5 min for the AC in solution to inactivate bacterial spores by 99 % and increased contact time was required as the pH was increased. Anolyte, used as a bactericidal and fungicidal sanitizer for food contact surfaces, is non-toxic, environmentally friendly (non-corrosive), and leaves no residue (requires no rinsing) (1, 11).

Anolyte (up to 200 ppm AC) has the FDA (and U.S. Environmental Protection Agency approval for use on food contact surfaces (6, 7). There are reports on inactivation of microorganisms by electrically generated water from NaCl, but the resulting products were not called 'anolyte' by the authors (3, 11, 13, 14). Cao et al. (3), calling their product slightly acidic electrolyzed water (SAEW, pH 6.0 – 6.5), reported the effectiveness of SAEW to inactivate *Salmonella enteritidis in vitro*. They reported a 6.5 $\log_{10}$ cfu/g reduction of *S. enteritidis* after the SAEW wash (15 ppm AC – 3 min at all temperatures used) of contaminated shell eggs (3). Issa-Zacharia et al. (13) used SAEW (pH 5.6 – 20 ppm) for 5 min to reduce *Salmonella* spp. and *Escherichia coli* by 2 log on contaminated fresh strawberries. Guentzel et al. (11) produced ' neutral electrolyzed oxidizing water '(10 min, 120 ppm AC, pH 6.3-6.5) to wash contaminated spinach and lettuce. They reported that *E. coli* levels were reduced by >1 $\log_{10}$cfu/ml, whereas t *S. typhimurium*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Enterococcus faecalis* were reduced by 2.4 – 3.8 $\log_{10}$ cfu/ml (11). Similarly, Izumi (14) reported that after rinsing in running electrolyzed water (pH 6.8, 20 ppm AC) for 3 min, the background microflora on fresh vegetables was reduced by 1 log. Rajkowski and Sommers (21) used anolyte (300 ppm, pH 6.4, 3 min at 23 ^{o} C) to inactivate the background microflora, *Salmonella* and *L. monocytogenes* on catfish fillets. They reported a 1 $\log_{10}$ cfu/g reduction for the background microflora and *Salmonella* but no reduction of *L. monocytogenes* (21).

To determine the effects of AC on cell morphology, researchers examined bacterial cells after treatments by using transmission electron micrographs (TEM). The morphological effects of electrolyzed oxidizing water (EO water, pH 2.3 – 2.7) on *E. coli* and *Staph. aureus* and of electrolyzed acidic water (pH 2.7) on *Staph. saprophyticus* showed cell wall wrinkling and dissociation between the cytoplasm and cell wall (20, 24). The pH of the treatment waters in both these studies was acidic (pH 2.3 – 2.7). Rajkowski and Sommers (21) used anolyte (pH

6.4) to treat *Salmonella* and *L. monocytogenes* suspended in sterile distilled water. The resulting TEM micrographs of the treated bacteria showed similar morphological changes to the cell's membrane with increased damage as the contact time with the anolyte increased from 0.5 to 3 min (21).

In addition to liquid sanitizers, non-thermal interventions are also used to disinfect surfaces. Pulsed ultraviolet (PUV) light uses a Xenon bulb which is a powerful, non-mercury form of UV light. PUV delivers UV over a wide energy range (wide wave length) compared to a mercury generated UV energy, which is a single wave length. PUV light results in genetically damaged cells by the formation of lethal pyrimidine dimers (pyrimidone) on bacterial DNA (10). PUV light is used to decontaminate and sterilize smooth dry surfaces, such as glass, medical devices and packing materials (2). The FDA has approved the use of PUV light in the production, processing and handling of food contact surfaces (5). PUV light is an effective technology for inactivating food-borne pathogens on smooth surfaces (15,16, 22) and food powders (10). There is little documentation on the morphological effect of PUV light on bacteria cells. This research was conducted to observe morphological changes in the cell wall of bacteria after treatment with anolyte, PUV light and a combination of PUV light followed by an anolyte wash.

MATERIALS AND METHOD

MICROORGANISM: *Salmonella* Schwarzengrund 19535, *Listeria monocytogenes* HCC23 (serotype 4a) and *Staphylococcus aureus* 196E were obtained from the Eastern Regional Research Center's (Wyndmoor, PA) culture collection. The identity of each isolate was confirmed by both Gram stain and API identification strips (bioMerieux Vitek, Inc., Hazelwood, MO). Working-stock cultures of each strain were maintained in brain-heart infusion broth (Becton, Dickinson and Co., Franklin Lakes, NJ) and stored at 4^{o}C essentially as described (21).

In preparation for inactivation studies, each isolate was passed separately in tryptic soy broth (TSB, Becton, Dickinson and Co.) and incubated at 37^{o}C overnight. The 18 h cultures were centrifuged at 3600 x g for 10 min (Sorvall Legend ™ RT centrifuge, Kendro Laboratory Products, Newtown, CT) at 4^{o}C to remove the growth media and re-suspended in 1% buffered peptone water (BPW - Becton, Dickinson and Co.). All cell counts were determined by serial

dilution in 0.1 % peptone water (PW, Becton, Dickinson and Co.) and surface-plated on tryptic soy agar (Becton, Dickinson and Co.).

PREPARATION OF ANOLYTE: Anolyte, prepared daily by electrolyzing saturated NaCl solution, was obtained using the Mini-Lyte 50 System (Clarentis Technologies, LLC, Palm Beach Gardens, FL). The generator was preset to deliver anolyte with a residual chlorine level (RCL) of >700 ppm at a pH of 6.0 – 6.5. Before use, the anolyte was standardized by diluting with sterile deionized water to obtain a 300 ppm RCL at pH 6.2 – 6.5.

***In vitro* INACTIVATION:**

Before any inactivation treatment, samples were removed for cell count and transmission electron microscopic (TEM) analysis. One ml of each cell suspension was treated at 23^{o}C with 9 ml anolyte (300 ppm RCL) and mixed for 3 min. Ten ml of 2 X BPW was added to inactivate the anolyte. Cell samples were removed for TEM and for recovery counts. Twenty ml of the individual culture was added to a Petri dish. The top was removed before the dish containing the suspended cells was placed in the chamber on the middle shelf, which is 8 cm from the PUV source. The chamber was pre-chilled using dry ice and the chamber and cell suspension temperatures were monitored. The cell suspension was treated with pulsed ultraviolet light (PUV) for 15 sec. using the SteriPulse-XL® Sterilization System (Xenon Corp., Wilmington, MA). Temperature of the cell suspension after PUV was 30^{o}C. Samples were removed for TEM analysis, for recovery counts and for the anolyte treatment. One ml of the PUV cell suspension was added to 9 ml anolyte (300 ppm RCL) and mixed for 3 min at 23 ^{o} C before 10 ml of 2 X BPW was added to inactivate the anolyte. Cells samples were removed for TEM and for recovery counts. Each bacteria was treated and TEM samples prepared three times.

TRANSMISSION ELECTRON MICROGRAPH

Cells were suspended in a 2.5% gluteraldehyde solution (4^{o}C), (Electron Microscopy Sciences {EMS}, Hatfield PA, USA), to fix and allowed to sit for 30 min. The sample was then centrifuged for 30 min at room temperature to pellet cells and then re-suspended in a 0.1 M Imidazole solution (EMS) to wash out residual gluteraldehyde. Cells were centrifuged and re-suspended with 200 µL of a 1% Osmium Tetroxide, (EMS), solution under a fume hood for 1 h. The cells were re-suspended with a micropipette and the sample was allowed to stand for 1 h. Cells were centrifuged and re-suspended in 400 µL 0.1 M Imidazole for 30 minutes.

Dehydration used a graded ethanol solution of 50% ethanol gradually increased to 80% ethanol, (Warner-Graham Company, Cockeysville, MD), for 30 min. Each sample was centrifuged between series. The sample was then finally washed 3 X with 100% ethanol and allowed to stand for 30 min between each wash. Ethanol was then replaced with propylene oxide (EMS) twice for five minutes.

EMbed-812 (EMS) was mixed and used at a 50% to 100 % solution with propylene oxide starting with 500 µL 50% mixture.

Initial resin infiltration was done without catalyst in the mixture. The 100% resin without catalyst infiltration was incubated with mixing overnight. Final resin with catalyst was incubated overnight with mixing.

The 812 plastic was cured in a vacuum oven (Precision Scientific, Chicago, IL) at 90^{o}C and 25 in Hg overnight. Thin sections at approximately 70 nm were cut using a Reichert Ultracut S, (Leica Wien, Austria) with a Diatome (Fort Washington, PA) Ultra 45 degree diamond knife. Sections were collected on a copper 400 mesh grid (EMS) and stained with a saturated solution of Uranyl Acetate (EMS), for approximately 1 min, rinsed with distilled water and then counter stained with Reynolds Lead citrate for 1 min and then rinsed with distilled water.

Thin sections were observed using a Philips Transmission Electron Microscope CM 12, (Philips, Netherlands) with an accelerating voltage of 80KV and imaged with a DVC detector and processed with AMT software (Danvers, MA) and photographed.

RESULTS

Morphological changes following treatments: ***Salmonella.*** The 18 h *Salmonella* cell suspension in 1 % BPW had a 1-log reduction after treating with anolyte. This reduction is consistent with the 1-log reduction reported earlier on catfish fillets (21). Dychdala (8) reported that organic material (protein) inactivated AC. In both studies, the anolyte's bactericidal effect was inactivated by the protein in the BPW or on the surface of the fish. An additional reduction (4-log) was reported when *Salmonella* were suspended in sterile distilled water (21). The PUV treatment reduced *Salmonella* counts by 7-log; however, viable cells were still recovered by direct plating. Rowan et al. (22) reported a 4-log reduction for *Salmonella* after a 100-pulse PUV treatment (high UV light source – non restricted light output in the UV region), which did

not inactivate all cells. The PUV treatment followed by the anolyte wash inactivated the cells and none were recovered (≤ 1-log).

The morphological effects of the three treatments on *Salmonella* can be observed from the TEM photomicrographs. Figure 1A of the untreated cells shows cellular division and intact smooth outer cellular membranes. In Fig. 1B, the anolyte treated cells, the photomicrographs showed a thickening and wrinkling of the outer membrane. Nan et al. (19) reported similar morphological changes for *E. coli* after treatment with slightly acidic electrolyzed water (pH 6 (19). Their photomicrographs also showed cell wall thickening. The *Salmonella* showed disruption of the cytoplasm, wrinkling of the outer cellular membrane, and vascular formations after the PUV treatment (Fig. 1C). Following both the PUV and anolyte wash, *Salmonella* showed increased cytoplasm disruption, wrinkling of the outer wall with indications of cytoplasm leakage and vascular inclusion (Fig. 1D).

Morphological changes following treatments: *L. monocytogenes.* The 18 h *L. monocytogenes* (LM) cell suspension in 1% BPW had a 1-log reduction after treating with anolyte, whereas a 4-log reduction was reported for LM when suspended in sterile distilled water (21). When the growth media (organic material) was removed and the cell pellet used, Felicano et al. (9) treated *Listeria innocua* with electrolyzed water (pH 6.9). They also reported a 1-log reduction for *Listeria*. The PUV treatment reduced the LM to < 1 log cfu/ml from an initial count of 10 $\log_{10}$ cfu/ml, but viable cells were still recovered by plate count. MacGregor et al. (16) reported a 6-log reduction for LM on exposed agar after a pulsed zenon light source, which is consistent with our results. The PUV treatment followed by the anolyte wash inactivated the cells and no LM was recovered.

The morphological effects of the three treatments to inactivate LM were observed from the TEM photomicrographs. Figure 2A of the untreated cells shows cellular division and intact smooth outer cellular membranes. In Fig. 2B, the anolyte treated cells, the photomicrographs showed a thickening of the outer membrane, vacuoles and leakage of cytoplasm. Feliciano et al. (9) used electrolyzed water (pH 6.9) to inactivate *L. innocua* and reported that the morphological changes showed development of a thicker cell wall and vacuoles within the cytoplasm. The LM cells showed disruption of cytoplasm with vascular formations and thickening of the cell wall after the PUV treatment (Fig. 2C). Following both the PUV and anolyte wash, the LM cells showed

cytoplasm disruption, cytoplasm leakage and thickening of the cell wall (Fig. 2D). Zaika and Fanelli (23) reported that LM undergoes morphological changes when stressed by increased NaCl levels. In their report they showed a thickening of the cell envelope at the septum, which was also observed in this study (Fig. 2D).

Morphological changes following treatments: *Staph. aureus.* The 18 h *Staph. aureus* (SA) cell suspension in 1% BPW had a 1 log reduction after treating with anolyte. Nan et al. (19) reported no detectable SA (suspended in 0.1 % peptone water) after treatment with SAEW (pH6). The PUV treatment reduced the SA to < 1 log cfu/ml, but viable cells were still recovered. Krishnamurthy et al. (15) also reported an 8 log reduction of SA after PUV treatment. The PUV treatment followed by the anolyte wash inactivated the cells and no SA was recovered.

The morphological effects of the three treatments to inactivate SA were observed from the TEM photomicrographs. Figure 3A of the untreated cells shows intact smooth outer cellular membranes. In Fig. 3B, the anolyte treated cells, the photomicrographs shows a ruptured cell and vacuole inclusion within the cytoplasm of another. The outer membrane was not as affected as LM (Fig. 2B) but did show some outer cell wrinkling. This minimum morphological effect after the anolyte treatment was confirmed. Nan et al. (19) reported little damage to the SA cell after treating with SAEW (pH 6) and Hajmeer et al. (12) reported that SA was more tolerant of NaCl levels and had little morphological changes. However, Zeng et al. (24) reported that after a treatment of electrolyzed oxidizing water (pH 2.3 – 2.7) the SA outer cell membrane was wrinkled as observed in this study. The SA cells showed separation of the cell wall (thickening) after the PUV treatment (Fig 3C). Following both the PUV and anolyte wash, the SA cells showed increased cytoplasm disruption, and inclusion of vacuoles (Fig. 3D).

CONCLUSION

After examining the photomicrograph of the morphological changes by three separate TEM preparations, the results were the same as described. The cellular changes following the anolyte wash confirmed what previous authors reported for reactions to chlorine regardless of pH – disruption of the cell wall. Future studies are planned to determine the morphological changes using anolyte at lower AC concentrations with increased contact time. The results of the PUV treatment indicated that in addition to cytoplasm disruption, there were changes to the cell wall.

Future studies using PUV are planned to determine the extent of the cell wall damage. In this study, the combination of treatment (PUV + anolyte wash) using one set protocol was effective to inactivate both gram negative and gram positive bacterial pathogens. Future studies are planned to determine the effectiveness for an anolyte pretreatment followed by PUV.

The results of this study do indicate that both anolyte and PUV and the combination are effective in inactivating pathogens.

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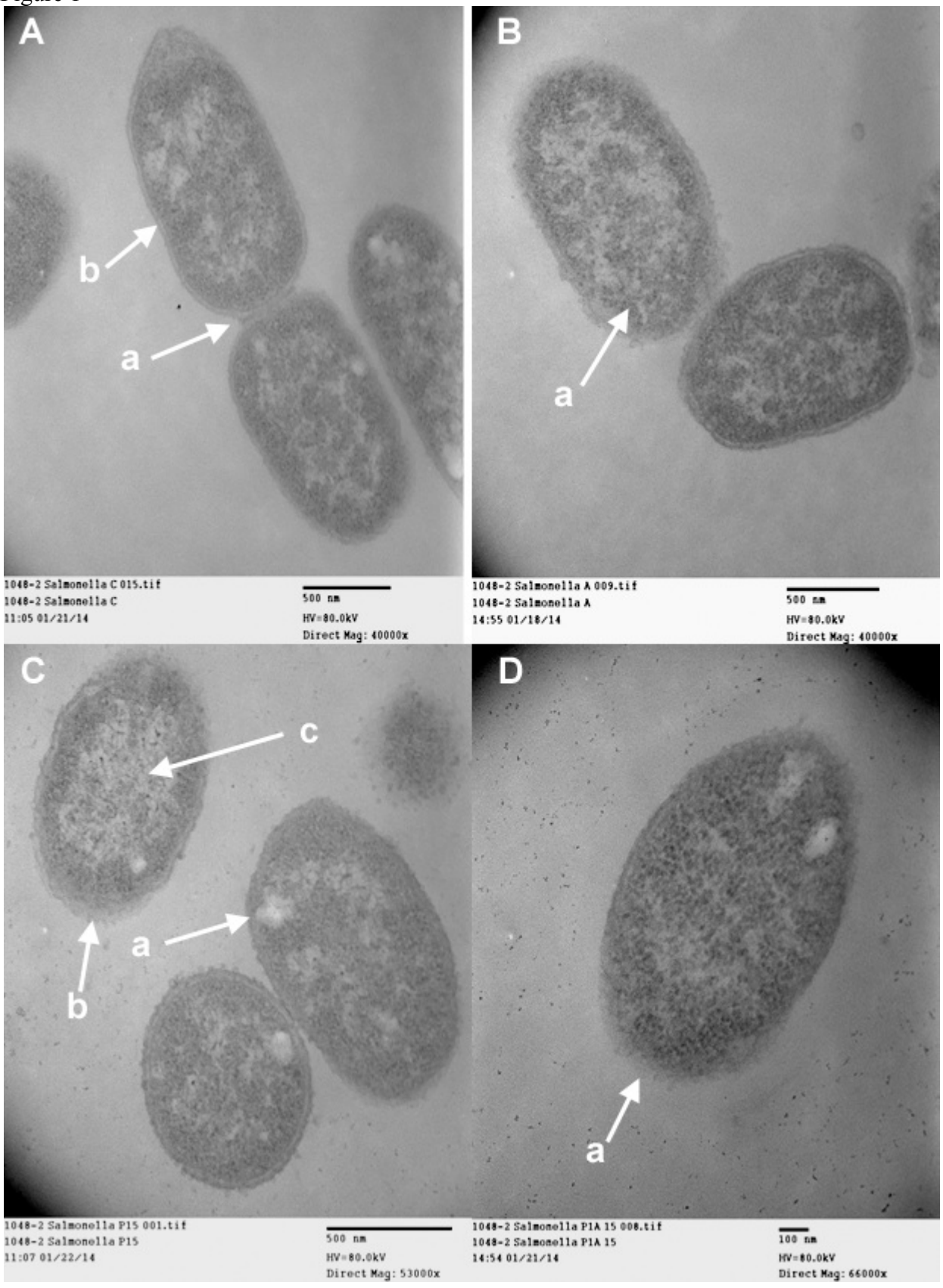
Figure 1. Transmission electron micrographs of untreated *Salmonella* (A), anolyte treated cells(B) , PUV treatment (C) and combined treatment (D.
A. a. completed cell division; b normal cell wall
B. a. wrinkled cell wall
C. a. vascular inclusion; b. wrinkled cell wall; c. cytoplasm disruption
D. a. cell wall disruption; b. vacuole

Figure 2. Transmission electron micrographs of untreated *L. monocytogenes* (A), anolyte treated cells (B) , PUV treatment (C) and combined treatment (D.
A. a. normal cell
B. a. vacuole; b. thickened cell wall; cell membrane disruption and cytoplasm leakage

C. a. thickened cell wall
D. a. vacuole formation; b. thicken septum; c. cytoplasm leakage

Figure 3. Transmission electron micrographs of untreated *Saph. aureus* (A), anolyte treated cells (B) , PUV treatment (C) and combined treatment (D.
A. a. normal cell wall
B. a. vacuole inclusion
C. a. cell wall disruption; b. cell wall thickening
D. a. wrinkling of cell wall; b. vacuole inclusion

406 Figure 1



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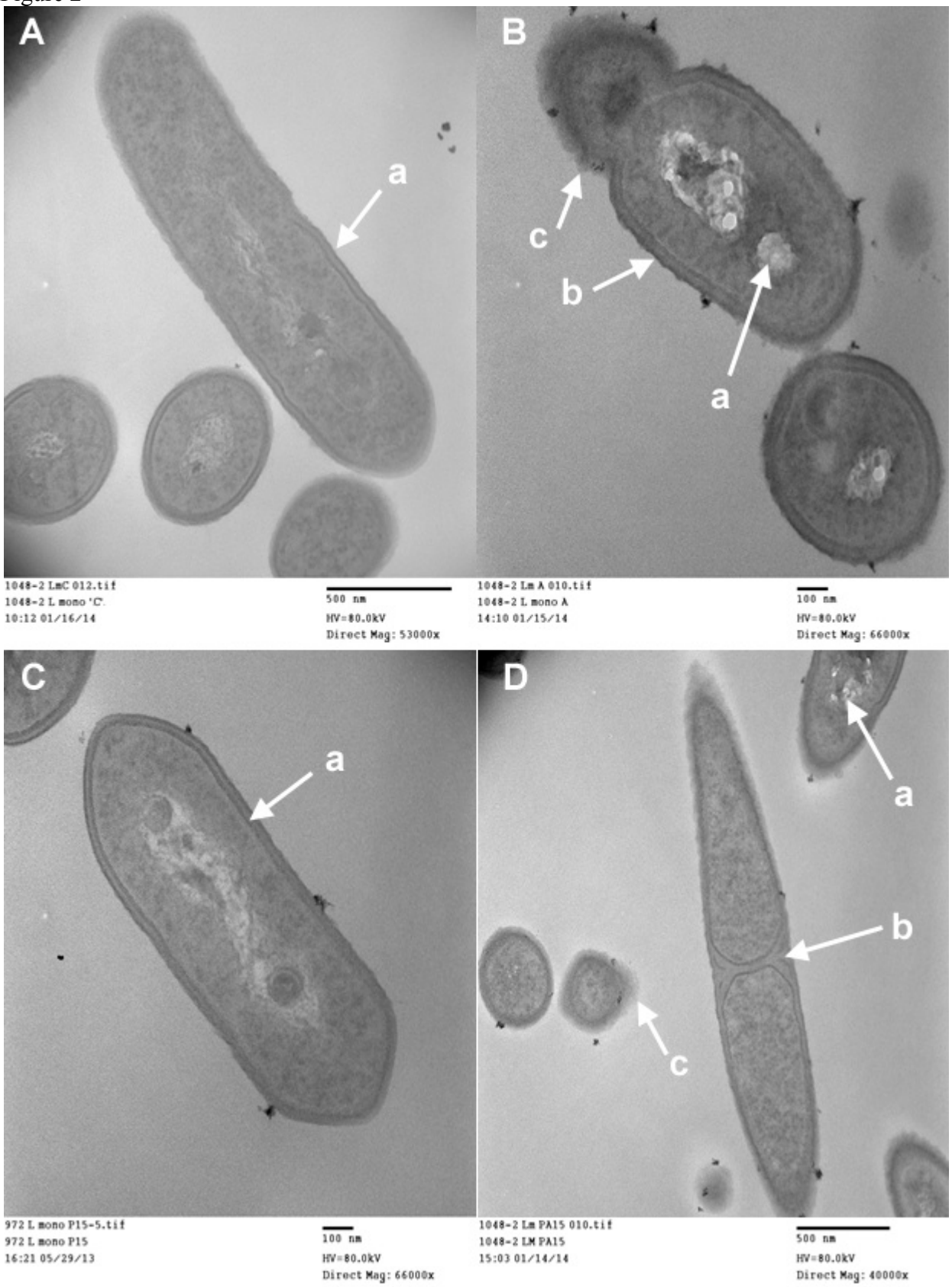

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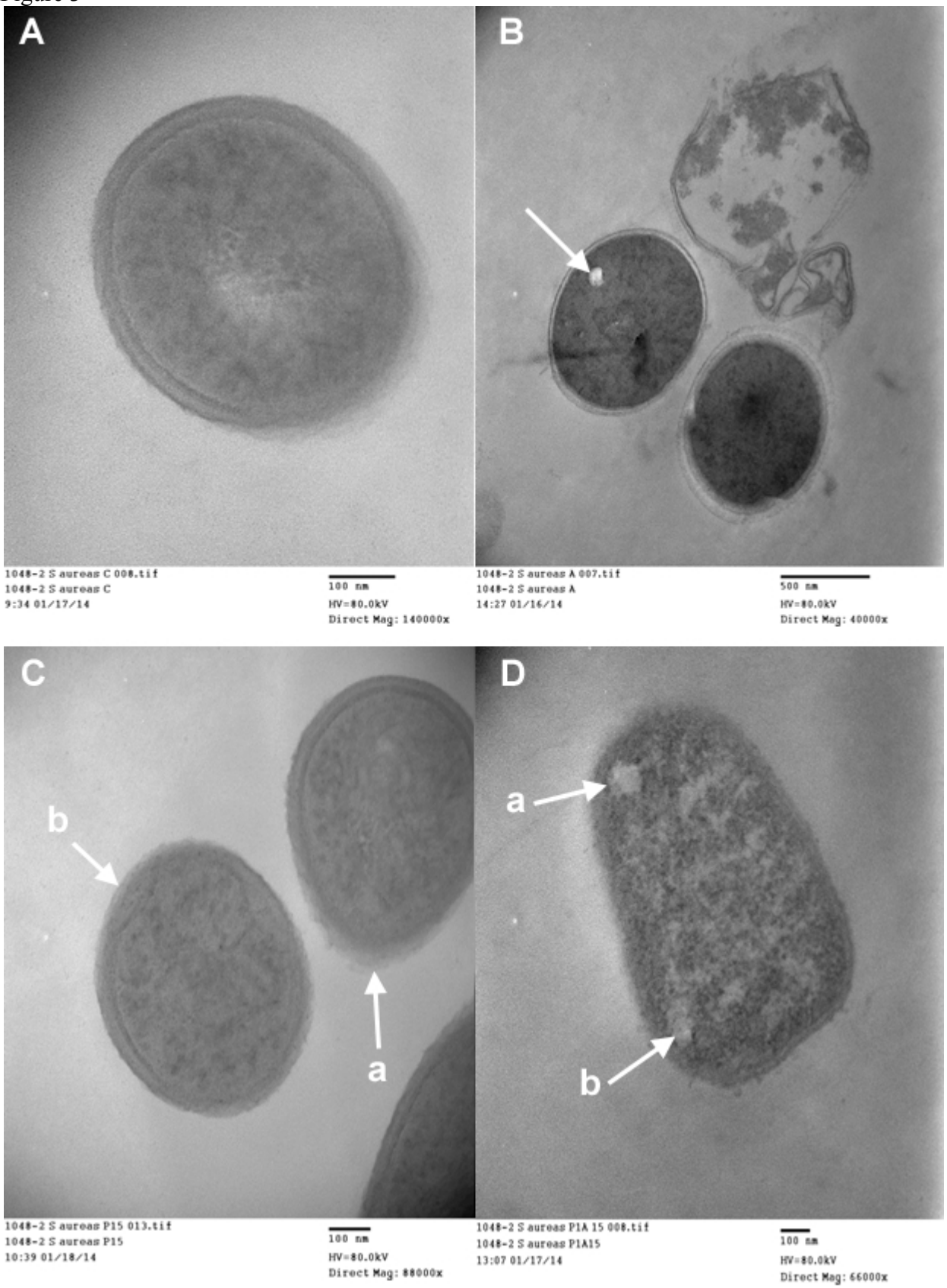

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