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ORIGINAL PAPER

# Combination of $TiO_2$-UV Photocatalysis and High Hydrostatic Pressure to Inactivate Bacterial Pathogens and Yeast in Commercial Apple Juice

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**Abstract** The purpose of this study was to investigate the effect of combined treatments using $TiO_2$-UV photocatalysis (TUVP) and high hydrostatic pressure (HHP) on inactivation of microorganisms in commercial apple juice as model liquid food. A synergistic effect was observed for combined treatments to inactivate microorganisms. Gram-positive bacteria, *Listeria monocytogenes* and *Staphylococcus aureus*, were completely inactivated from initial loads of 7.1 and 6.7 log CFU/mL, respectively, when treated with a combination of TUVP (8.45 J/cm$^2$) and HHP (500 MPa). In contrast, reductions of only 4.8 log CFU/mL (*L. monocytogenes*) and 2.4 log CFU/mL (*S. aureus*) were achieved with 500 MPa HHP alone. Gram-negative bacteria, *Escherichia coli* O157:H7 and *Salmonella* Typhimurium, were reduced by 7.1 and 7.2 log CFU/mL, respectively, after a combined treatment using 8.45 J/cm$^2$ TUVP and 600 MPa (*E. coli*) or 400 MPa (*S.* Typhimurium) HHP which were significantly higher than the effects of HHP alone. A 6.2 log CFU/mL reduction in *Saccharomyces cerevisiae* count was monitored after treatment with a combination of 8.45 J/cm$^2$ TUVP and 500 MPa HHP whereas even 600 MPa alone could not achieve complete *S. cerevisiae* inactivation. Combined treatments (TUVP + HHP) were more effective for microbial inactivation than alone treatments. Scanning electron microscopic images of microorganisms showed highly deformed morphologies after TUVP + HHP treatment. In conclusion, pretreatment of commercial apple juice using TUVP before HHP processing results in better disinfection and may assure complete disinfection.

**Keywords** $TiO_2$-UV photocatalysis · High hydrostatic pressure · Apple juice · Pathogenic bacteria · *Saccharomyces cerevisiae*

## Introduction

Fruit juices can be contaminated with pathogenic microorganisms that can grow and survive causing health problems for consumers (Guerrero-Beltrán and Barbosa-Cánovas 2005; Bayındırlı et al. 2006; Ukuku and Geveke 2010; Zhao et al. 2013; Ferrario et al. 2015). Apple juice products are consumed widely due to various health benefits. However, apple juice products can be contaminated with different kinds of pathogenic bacteria such as *Staphylococcus aureus*, *Salmonella* Typhimurium, and *Escherichia coli* that can cause serious health hazards if not inactivated using appropriate methodologies (Ukuku and Geveke 2010; Mihajlovic et al. 2013). In addition to bacterial pathogens, fruit juices including the apple juice are also susceptible to spoilage by yeast such as *Saccharomyces cerevisiae* or other foodborne yeasts because they can ferment acidic fruit products (Guerrero-Beltrán and Barbosa-Cánovas 2005).

Thermal processes have been commonly used to ensure the microbial safety of fruit juices; however, heat treatment can result in deterioration in the nutrient value (Song et al. 2007; Wang et al. 2010). Hence, the

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importance of minimally or non-thermally processed foods with an increased shelf life and better nutritional properties is increasing (Mohideen et al. 2015). HHP is a promising non-thermal technology that has gained an increasing application over the last 15 years due to its ability to preserve nutritional and sensory characteristics and to improve the overall quality of foods (San-Martín et al. 2002; Bayındırlı et al. 2006; Ghafoor et al. 2012; Patterson et al. 2012; Zhao et al. 2013; Serment-Moreno et al. 2015). Fruit juices are normally treated with HHP at 400 to 600 MPa for few minutes at low temperature to reduce the numbers of spoilage microorganisms and to prolong the product shelf life (Patterson 2005). Although many microorganisms can be destroyed by HHP treatment, some pathogens and enzymes are resistant to pressure (Jordan et al. 2001; Patterson 2005; Bayındırlı et al. 2006). Therefore, additional treatment is necessary to overcome this problem.

$TiO_2$-UV photocatalysis (TUVP) is a developing non-thermal technique that degrades pathogens under aqueous conditions by generation of strong oxidizing agents using UV light (Kim et al. 2013). A practical strategy for increasing the microbial inactivation efficiency during food processing is use of combined treatments to achieve a synergistic inactivation effect (Raso and Barbosa-Cánovas 2003; Ogihara et al. 2009; Wang et al. 2010; Yao et al. 2015). In a study carried out by Chai et al. (2014), no yeasts or molds, coliform bacteria, *Pseudomonas*, and *Bacillus cereus* were detected in *Angelica keiskei* juice after processing using TUVP + HHP treatment. Ferrario et al. (2015) found that combination of ultrasound and pulsed light was highly effective to inactivate spoilage microorganisms in commercial and natural apple juice. Sung et al. (2014) studied the combined effect of ozone and heat treatments in apple juice for microbial destruction. Therefore, it is important to find out effects of such combined treatments on inactivation of different kinds of pathogenic bacteria and spoilage-causing yeast in fruit juices.

This study investigated the inactivation effects of TUVP alone, HHP alone, and combination of both treatments (TUVP + HHP treatment) on Gram-positive bacteria (*Listeria monocytogenes* and *S. aureus*), Gram-negative bacteria (*E. coli* O157:H7 and *S.* Typhimurium), and yeast (*S. cerevisiae*) suspended in commercial apple juice. Morphological changes in these microbes were also studied using scanning electron microscopy (SEM). Microbial inactivation and morphological changes in bacterial and yeast cells were considered as indicators of synergistic effects of combined treatments and compared with those as results of TUVP-alone and HHP-alone treatment. The objectives of this study also included establishing an effective non-thermal process to ensure microbial safety of commercial apple juice as model liquid food.

## Materials and Methods

### Microbial Strains and Test Culture Preparations

A total of five microbial strains were used for the inactivation study. *E. coli* O157:H7 (NCTC 12079) were supplied by the National Collection of Type Cultures (London, England). *S. cerevisiae* (ATCC 38661) and *L. monocytogenes* (ATCC 15313) were supplied by the American Type Culture Collection (Rockville, MD, USA). *S.* Typhimurium (ATCC 14028) and *S. aureus* (ATCC 25923) were obtained from the Korean Culture Center of Microorganisms (Seoul, South Korea). Difco™ culture media were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA).

Stock cultures of *S.* Typhimurium, *S. aureus*, and *L. monocytogenes* were prepared in nutrient broth (NB) after incubation at 38 °C for 24 h with continuous agitation in a shaker. A 1.0 mL cell aliquot was transferred to 100 mL of fresh NB and incubated at 38 °C for 24 h with shaking. *E. coli* O157:H7 was inoculated into 100 mL of sterile mannitol broth at 38 °C for 24 h and gently agitated in a shaker. An amount of 1.0 mL of cell aliquot was transferred to 100 mL of fresh NB and incubated again for 24 h. *S. cerevisiae* was inoculated into 100 mL of sterile yeast/mold (YM) broth with shaking at 30 °C for 24 h. An amount of 1.0 mL of cell aliquot was transferred to 100 mL of fresh YM broth and incubated again for 24 h with shaking. Cell suspensions of each of the five microbial strains were centrifuged at 3000×*g* for 10 min. The cell pellet of each strain was suspended in an equal volume (30 mL) of a 0.85 % NaCl solution to be used as an inoculum.

### Sample Preparation

Commercially available apple juice (clarified) (Woongjin Foods Co., Seoul, South Korea) was purchased from a local store in Seoul and used as model liquid food. The purchased juice was thermally treated and contained no chemical additives. Juice bottles were kept at 4 °C and remained closed until their experimental use. The juice had a pH of 3.68±0.02 and 12.09 °Brix total soluble solids. Microorganisms ($10^3$–$10^7$ colony-forming unit (CFU)/mL) were added to the apple juice, mixed, and prepared for inactivation experiments.

### Sample Treatment

#### *TUVP Treatment*

A continuous TUVP reactor consisting of serially connected stainless steel chambers was used (Fig. 1). Each chamber was fitted with a $TiO_2$-coated quartz tube containing UV-C (wavelength=254 nm) lamps (16 W, Sankyo Denki Co. Ltd., Hiratsuka, Japan). The temperature in the chambers was kept ambient using ice water surrounding the chambers.

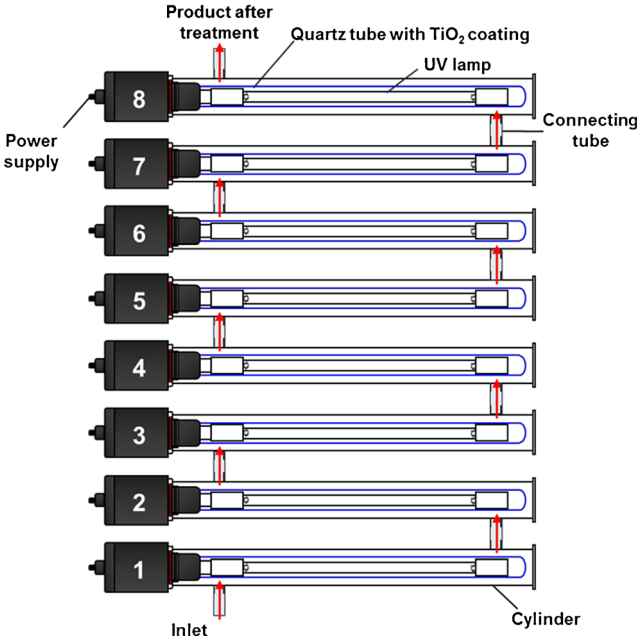


**Fig. 1** Schematic diagram of the continuous $TiO_2$-UV photocatalytic reactor

Commercial apple juice contaminated with microorganisms was injected into the bottom and recovered from the top chamber. The TUVP doses were 0.82 and 8.45 J/cm$^2$, calculated following the method described by Tran and Farid (2004) as

$$U = It/A$$

where $I$ represents the UV intensity of the lamp (16 W), $t$ is the UV exposure time (s) for juice and $A$ is the UV exposure surface area (cm$^2$) with $A=\pi DL$ cm$^2$ for which $D$ is the inner diameter of quartz tube ($D$=1.96 cm) and $L$ is the length of quartz tube ($L$=34.5 cm). The flow rate in the system was 2.5 mL/s and maintained using a peristaltic pump (RP-1000, Eyela, Tokyo, Japan). TUVP reactor was cleaned using tap water after each run with UV-C lamp turned on followed by autoclaving before the experiment. Experiments were performed in triplicate.

*High Hydrostatic Pressure Treatment*

HHP treatment was carried out at ambient temperatures using a hydrostatic pressurization unit (HHP-600, Baotou Kefa Co., Ltd., Inner Mongolia, China) having a capacity of 5.0 L. Distilled water was used as the pressure transmission fluid. Pressure was increased at a speed of 2 MPa/s, and the decompression time was 7 to 8 s. The pressure holding time in this study did not include the pressure increase and depressurization times. Juice samples previously TUVP-treated (or not-treated) were packed in flexible polyethylene terephthalate pouches (10×15 cm) and heat-sealed leaving no headspace. Packed samples were loaded into the pressure vessel and pressurized at 200–600 MPa for 1 min at 25 °C. HHP treatment resulted in an increase of product temperature through adiabatic heating approximately 3 °C per 100 MPa. Experiments were performed in triplicate.

## Microbiological Analysis

The total plate count was used to enumerate numbers of viable microbial cells in apple juice after inactivation treatments. Juice samples (before and after inactivation treatments) were serially diluted using a sterile 0.85 % NaCl solution, followed by inoculation of 1.0 mL of the solution onto duplicate plates containing an appropriate agar. Nutrient agar was used to detect viable *S.* Typhimurium, *E. coli* O157:H7, *S. aureus*, and *L. monocytogenes* cells after incubation at 37 °C for 24 h. Potato dextrose agar was used for enumeration of *S. cerevisiae* cells with incubation of plates at 30 °C for 36 h. Colonies were counted in each case at the end of the incubation period.

## Morphology of Microorganisms Using SEM

Morphological changes in microorganisms were observed by preparing samples after each treatment using the critical point drying technique. Samples were fixed in modified Karnovsky's fixative (2 % paraformaldehyde, 2 % glutaraldehyde, 0.5 % $CaCl_2$ in 0.1 M phosphate buffer) overnight, washed in 0.1 M cacodylate buffer, and postfixed using 1.0 % $OsO_4$ in 0.1 M cacodylate buffer. Dehydration was achieved by passing coverslips through 50, 60, 70, 80, 90, 95, and 100 % ethyl alcohol solutions (5 min in each), with final dehydration in 100 % ethyl alcohol for 10 min. Samples were dried using a critical point drying apparatus. Dried samples were mounted on SEM stubs, coated with approximately 300 Å of gold, and observed using field emission scanning electron microscopy (FE-SEM S-800, Hitachi Ltd., Tokyo, Japan). The resolution and tilt angle were adjusted prior to imaging.

## Statistical Analysis

Each experiment was independently repeated three times, and results of three replicate analyses ($n$=3) were analyzed using one-way analysis of variance (ANOVA) by Statistical Package for the Social Sciences (SPSS Version 21, IBM Corporation, NY, USA) and expressed as mean value±standard deviation. Statistical analysis was performed using Student-Newman-Keuls test, and confidence level for statistical significance was set at a probability value ($P$) of 0.05.

## Results and Discussion

### Inactivation Effects of TUVP-Alone, HHP-Alone, and TUVP + HHP Treatments on Gram-Positive Bacteria

*L. monocytogenes* is a facultative anaerobic, rod-shaped bacterium that is one of the most virulent foodborne pathogens. *S. aureus* is known as pressure-resistant facultative anaerobic bacterium. Results of inactivation of Gram-positive bacteria are shown in Table 1. The initial bacterial populations of *L. monocytogenes* and *S. aureus* in commercial apple juice were 7.1 and 6.7 log CFU/mL, respectively. The population of *L. monocytogenes* was reduced significantly ($P<0.05$) to 6.0 and 3.9 log CFU/mL using 0.82 and 8.45 J/cm$^2$ TUVP treatments alone, respectively. The counts for *S. aureus* also decreased significantly to 5.5 and 3.7 log CFU/mL using 0.82 and 8.45 J/cm$^2$ TUVP treatments alone, respectively. HHP treatment of 200 and 300 MPa alone had non-significant effects on Gram-positive bacteria inactivation, and reductions in their counts were lower than those obtained using two types of TUVP treatments. In case of juice treated using TUVP followed by higher pressures (400–600 MPa), the synergistic inactivation effect on *L. monocytogenes* was significant ($P<0.05$). *S. aureus* also demonstrated a synergistic inactivation effect at and above 300 MPa, and there was reduction of 6.7 log CFU/mL when a TUVP dose of 8.45 J/cm$^2$ was given before 500-MPa HHP treatment. For both *L. monocytogenes* and *S. aureus*, there was significant synergistic inactivation by HHP treatments (500–600 MPa), even with TUVP pretreatment at a lower dosage (0.82 J/cm$^2$). Combined use of TUVP at a dosage of 0.82 J/cm$^2$ and HHP at 500 MPa was sufficient to cause significant inactivation of *L. monocytogenes* and *S. aureus* in commercial apple juice whereas a complete inactivation of these bacteria was achieved using 8.45 J/cm$^2$ TUVP followed by 500-MPa HHP treatment. However, an additive effect was observed for 400-MPa HHP and 0.82 J/cm$^2$ TUVP pretreatment for inactivation of both bacteria. It is also noticeable that the inactivation of Gram-positive bacteria caused by HHP treatment alone was significantly lower than when it accompanied a TUVP pretreatment. HHP-alone treatment at 375 MPa for 15 min caused an approximately 2 log CFU/mL reduction in *L. monocytogenes* numbers in phosphate saline buffer at 25 °C (San-Martín et al. 2002). *S. aureus* was least affected by the HHP inactivation treatments used in this study, and an HHP-alone treatment of 600 MPa could reduce 5.0 log CFU/mL; however, this resistance was decreased when TUVP-pretreated juice was subjected to HHP treatment. Previously, it was reported that a 450-MPa HHP treatment caused an approximately 2 log CFU/mL reduction in the number of *S. aureus* (Wang et al. 2010). In brief, a combined treatment using TUVP (8.45 J/cm$^2$) and HHP (500 MPa) caused complete inactivation of *L. monocytogenes* (7.1 log CFU/mL) and *S. aureus* (6.7 log CFU/mL) in commercial apple juice.

### Inactivation Effects of TUVP-Alone, HHP-Alone, and TUVP + HHP Treatments on Gram-Negative Bacteria

*E. coli* is a facultative anaerobic, rod-shaped bacterium that is commonly found in spoiled food. *S.* Typhimurium is a rod-shaped, flagellated, aerobic bacterium. The outer membrane of these bacteria is largely of toxic lipopolysaccharide layer that is responsible for the pathogenicity. The results of inactivation of Gram-negative bacteria (*E. coli* and *S.* Typhimurium) using TUVP + HHP treatments are shown in Table 2. TUVP treatment alone caused significant ($P<0.05$) reductions of *E. coli* O157:H7 (3.2 log CFU/mL) and *S.* Typhimurium (4.0 log CFU/mL) at 8.45 J/cm$^2$ from an initial

**Table 1** Residual counts (log CFU/mL) of Gram-positive bacteria (*L. monocytogenes* and *S. aureus*) in commercial apple juice after treatment with $TiO_2$-UV photocatalysis (TUVP alone), high hydrostatic pressure (HHP alone), and combined (TUVP + HHP) treatments

| | TUVP dose | HHP (MPa) | | | | | |
|---|---|---|---|---|---|---|---|
| | (J/cm$^2$) | 0.1 | 200 | 300 | 400 | 500 | 600 |
| *L.monocytogenes* | 0 | 7.08±0.09 dC | 7.04±0.11 dC | 6.92±0.06 dC | 4.15±0.34 cB | 2.25±0.13 bC | 0.48±0.73 aA |
| | 0.82 | 6.02±0.13 dB | 6.02±0.13 dB | 4.66±0.08 cB | 4.01±0.11 bB | 0.65±0.50 aB | 0.36±0.54 aA |
| | 8.45 | 3.91±0.83 cA | 3.94±0.87 cA | 3.62±0.96 cA | 2.23±0.06 cA | ND | ND |
| *S. aureus* | 0 | 6.74±0.05 cC | 6.63±0.05 cC | 6.50±0.03 cC | 6.32±0.03 cC | 4.30±1.99 bC | 1.73±2.49 aB |
| | 0.82 | 5.53±0.22 cB | 5.31±0.04 cB | 5.28±0.03 cB | 5.23±0.01 cB | 1.37±1.15 bB | 0.05±0.16 aA |
| | 8.45 | 3.68±0.16 eA | 3.07±0.07 dA | 2.83±0.07 cA | 2.68±0.04 bA | ND | ND |

Values are expressed as mean±standard deviation ($n=3$). Different uppercase letters in the same column indicate a significant difference ($P<0.05$) for same bacterium. Different lowercase letters in the same row indicate a significant difference ($P<0.05$) for same bacterium

*ND* not detected, the levels of microbial populations were below 0.01 log CFU/mL

**Table 2** Residual counts (log CFU/mL) of Gram-negative bacteria (*E. coli* O157:H7 and *S.* Typhimurium) in commercial apple juice after treatment with $TiO_2$-UV photocatalysis (TUVP alone), high hydrostatic pressure (HHP alone), and combined (TUVP + HHP) treatments

| | TUVP dose | HHP (MPa) | | | | | |
|---|---|---|---|---|---|---|---|
| | ($J/cm^2$) | 0.1 | 200 | 300 | 400 | 500 | 600 |
| *E. coli* O157:H7 | 0 | 7.13±0.16 dC | 7.08±0.12 dC | 6.94±0.05 dC | 5.33±0.80 cB | 2.09±0.10 bB | 1.34±0.19 aB |
| | 0.82 | 5.83±0.28 cB | 5.66±0.16 cB | 5.60±0.05 cB | 1.53±1.87 bA | 0.46±0.69 aA | 0.15±0.35 aA |
| | 8.45 | 3.97±0.37 cA | 4.01±0.27 cA | 3.59±0.14 cA | 1.45±1.60 bA | 0.38±0.57 aA | ND |
| *S.* Typhimurium | 0 | 7.21±0.13 dC | 7.16±0.13 dC | 7.08±0.03 dC | 6.72±0.05 cC | 0.21±0.35 bA | ND |
| | 0.82 | 6.00±0.03 eB | 5.85±0.04 dB | 5.71±0.05 cB | 3.12±0.11 bB | 0.03±1.10 aA | ND |
| | 8.45 | 3.25±0.06 cA | 3.27±0.01 cA | 2.72±0.81 bA | ND | ND | ND |

Values are expressed as mean±standard deviation ($n=3$). Different uppercase letters in the same column indicate a significant difference ($P<0.05$) for same bacterium. Different lowercase letters in the same row indicate a significant difference ($P<0.05$) for same bacterium

*ND* not detected, the levels of microbial populations were below 0.01 log CFU/mL

load of 7.1 and 7.2 log CFU/mL, respectively. HHP treatment of 400 MPa alone caused 1.8 and 0.5 log CFU/mL reductions in initial load of *E. coli* and *S.* Typhimurium, respectively. When TUVP was applied before HHP, *E. coli* O157:H7 encountered significant ($P<0.05$) synergistic inactivation effects at 400–600 MPa HHP irrespective of TUVP dose. Complete inactivation (7.1 log CFU/mL reduction) of *E. coli* O157:H7 was, however, achieved by combined treatment of TUVP (8.45 $J/cm^2$) with HHP (600 MPa). In case of *S.* Typhimurium, the significant synergistic effects were obtained at 300 MPa and it was completely inactivated using 8.45 $J/cm^2$ and 400 MPa, although an increase in pressure resulted in a similar inactivation level in samples treated at a lower TUVP dosage (0.82 $J/cm^2$).

*S.* Typhimurium was found more sensitive to TUVP + HHP treatment than other bacteria inactivated in this study. *E. coli* showed resistance to HHP-alone and TUVP + HHP treatments, and more intense combination levels were required for its complete inactivation in apple juice samples. Jordan et al. (2001) reported that the numbers of *E. coli* in clarified apple juice treated at 500 MPa for 5 min were reduced by 5 log CFU/mL. However, an important food processing objective may be reduction in the process time to achieve desired results at lower pressures. It was observed in this study that the amount of *E. coli* O157:H7 inactivation was 5.0 log CFU/mL at 500 MPa in 1 min. Thus, the TUVP + HHP treatment resulted in a better inactivation rate at a lower pressure and in a shorter time. Patterson et al. (2012) showed that the numbers of total aerobes in carrot juice at a pressure of 500 MPa for 1 min were reduced by approximately 4.0 log CFU/mL. Yao et al. (2015) reported that combination treatment of nisin and mild HHP at 350 MPa created a synergistic effect for inactivation of *S. aureus* without affecting the sensory properties of meat slurry.

### Inactivation Effects of TUVP-Alone, HHP-Alone, and TUVP + HHP Treatments on Yeast

*S. cerevisiae* is a round-to-ovoid yeast, 5–10 μm in diameter, and known to reproduce by budding and can cause spoilage in foods including fruit juices. *S. cerevisiae* showed a higher inactivation rate with different combinations of TUVP + HHP treatment than the tested Gram-positive bacteria (Table 3). The initial count of *S. cerevisiae* in apple juice was 6.2 log CFU/mL. A significant reduction of 3.8 log CFU/mL was achieved using TUVP alone (8.45 $J/cm^2$). More than 5.9 log CFU/mL reduction of *S. cerevisiae* population in juice samples was achieved by combining the TUVP (8.45 $J/cm^2$) with HHP (300–400 MPa) whereas complete inactivation was achieved when 500 and 600 MPa HHP was followed by TUVP at 8.45 $J/cm^2$ through synergistic action. At 600 MPa, a TUVP pretreatment at 0.82 $J/cm^2$ also caused complete inactivation for *S. cerevisiae* in juice samples. At 200 MPa, the inactivation effect of combined treatment was additive at both TUVP pretreatment doses (Table 3). These results suggest that application of TUVP treatment results in significant inactivation of *S. cerevisiae* in juice, even at lower pressures.

Bacterial pathogens, yeast, and molds are known to have variable sensitivities to HHP treatment (Patterson 2005; Vega-Gálvez et al. 2012). Gram-positive and Gram-negative bacteria have different structural characteristics that result in different responses to HHP treatment. Gram-negative bacteria are likely to be more susceptible to pressure than Gram-positive bacteria (Patterson 2005), which have a thick superficial peptidoglycan layer. This layer may be thin and present inside of the outer cell membrane in Gram-negative bacteria. The thicker layer of Gram-positive bacteria may cause resistance to HHP treatment (Garcia-Gonzalez et al. 2009). Moreover, bacterial pathogens might be pressure resistant and even strong HHP or TUVP treatments alone may not be sufficient

**Table 3** Residual counts of *S. cerevisiae* (log CFU/mL) in commercial apple juice after treatment with $TiO_2$-UV photocatalysis (TUVP alone), high hydrostatic pressure (HHP alone), and combined (TUVP + HHP) treatments

| | TUVP dose | HHP (MPa) | | | | | |
|---|---|---|---|---|---|---|---|
| | ($J/cm^2$) | 0.1 | 200 | 300 | 400 | 500 | 600 |
| *S.cerevisiae* | 0 | 6.15±0.49 cC | 5.75±0.18 cC | 5.63±0.14 bcC | 5.10±0.14 bB | 1.30±1.42 aB | 0.39±0.59 aB |
| | 0.82 | 4.89±0.21 dB | 4.80±0.20 dB | 4.25±0.16 cB | 0.59±0.63 bA | 0.09±0.18 aA | ND |
| | 8.45 | 2.34±0.12 dA | 2.25±0.11 cdA | 0.22±0.08 cA | 0.22±0.33 bA | ND | ND |

Values are expressed as mean±standard deviation ($n$=3). Different uppercase letters in the same row indicate a significant difference ($P$<0.05). Different lowercase letters in the same column indicate a significant difference ($P$<0.05)

*ND* not detected, the levels of microbial populations were below 0.01 log CFU/mL

for complete inactivation. Zhao et al. (2013) reported that numbers of total aerobic bacteria in cucumber juice drink treated with HHP at 400 and 500 MPa were reduced only by 1.7 to 2.0 log CFU/mL, respectively. Therefore, strategies are being developed to combine pretreatments with HHP to obtain synergistic effects for microbial inactivation and food safety assurance. UV light possesses antimicrobial properties, hence frequently applied in different food disinfection studies (Ukuku and Geveke 2010; Tarek et al. 2015). However, limitations have been reported related to the low penetration of conventional UV irradiation into foods including the fruit juices with high suspended materials (Tran and Farid 2004; Keyser et al. 2008; Tarek et al. 2015). The use of UV light in association with $TiO_2$ is also being studied. Previous studies reported that illuminated $TiO_2$ can induce bactericidal activity (Maness et al. 1999; Hashimoto et al. 2005; Benabbou et al. 2007; Chai et al. 2014). Benabbou et al. (2007) studied the efficiency of photocatalytic oxidation reactions against *E. coli* under different domains of UV light and concentrations of $TiO_2$. Maness et al. (1999) suspended $TiO_2$ particles in a reaction vessel and continuously stirred the reaction medium to ensure maximal mixing and to prevent settling of $TiO_2$ particles. Lamps coated with $TiO_2$ particles to generate oxidizing radicals using UV light were used in this study. Use of $TiO_2$-coated UV lamps can save time, as $TiO_2$ particles are not necessary and reactions take place homogeneously with no concern for particle settling (Maness et al. 1999; Benabbou et al. 2007). Herein, the disinfection effect has been demonstrated to be positively correlated with the TUVP dosage. TUVP dosage of 8.45 $J/cm^2$ showed higher inactivation effects than a TUVP dosage of 0.82 $J/cm^2$ for all tested microbial strains. In this study, the order of sensitivity of different microbes to TUVP (8.45 $J/cm^2$) alone was *S.* Typhimurium > *S. cerevisiae* > *L. monocytogenes* > *E. coli* > *S. aureus*. The order of sensitivity of these microbes to HHP (600 MPa) alone was *S.* Typhimurium > *L. monocytogenes* > *E. coli* > *S. cerevisiae* > *S. aureus* (Tables 1, 2, and 3). Gram-negative *S.* Typhimurium was the most sensitive to both types of treatment alone, but similar type *E. coli* was less sensitive than Gram-positive *L. monocytogenes*. *S. aureus*, however, showed resistance to both types of individual inactivation treatments. Synergistic effects of these two techniques were sufficient for assuring complete inactivation of these microbes at variable combinations. *S.* Typhimurium was the most sensitive microbe to combined treatment followed by *S. cerevisiae* whereas *E. coli* seemed to be most resistant one in this case. *E. coli* and *S.* Typhimurium are both Gram-negative bacteria, but their sensitivities to different inactivation treatments varied in this study. Therefore, besides cell structure, there might have been various other factors that contributed to the resistance of a certain bacterium and yeast to the inactivation treatments. The type and composition of media during inactivation might also affect the inactivation of microbes. It has been demonstrated that the sensitivity of microorganisms is different in different medium types. For example, the ionic strength, type of ion, water activity, and pH values could all be contributing factors of the medium. Therefore, it is important to know the sensitivity of individual microorganisms to a selected medium (Patterson 2005; Mañas and Pagán 2005).

## Morphological Changes in Microorganisms After TUVP-Alone, HHP-Alone, and TUVP + HHP Treatments

SEM images have been used to demonstrate effects of inactivation treatments on the morphology and structure of microbial cells (Marx et al. 2011; Kim et al. 2013). SEM images of different bacterial cells and *S. cerevisiae* in commercial apple juice before and after different treatments are shown in Fig. 2. Control (no treatment), TUVP (8.45 $J/cm^2$)-treated, HHP (400, 500 MPa)-treated, and TUVP + HHP-treated microbial samples were examined. Untreated Gram-positive bacteria, *L. monocytogenes* and *S. aureus*, showed uniform and smooth configurations. *L. monocytogenes* treated with either TUVP (8.45 $J/cm^2$) or HHP at 400 MPa showed little morphological change. However, a combined TUVP (8.45 $J/cm^2$) and HHP treatment at both 400 and 500 MPa caused highly deformed cell morphology (Fig. 2a). This is in agreement with the inactivation effects of *L. monocytogenes* (Table 1) where the synergistic effect appeared at 400 MPa HHP when followed by a

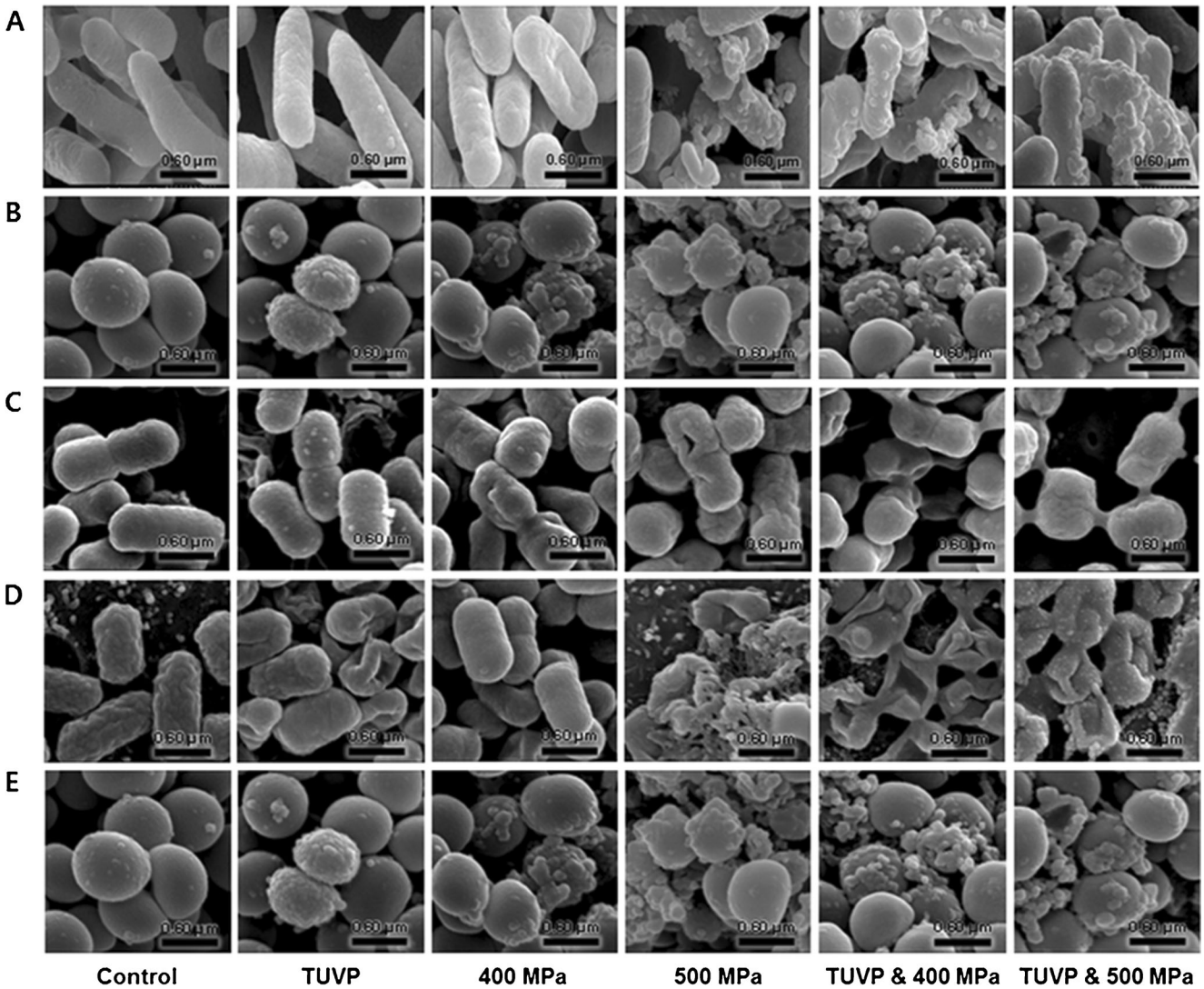


**Fig. 2** Scanning electron microscopic images of *L. monocytogenes* (**a**), *S. aureus* (**b**), *E. coli* O157:H7 (**c**), *S.* Typhimurium (**d**), and *S. cerevisiae* (**e**) cells. Cells were either untreated or treated using TUVP alone (8.45 J/cm$^2$), HHP alone (400, 500 MPa), and combined (TUVP + HHP) treatments

TUVP dose of 8.45 J/cm$^2$. SEM images (Fig. 2b) of *S. aureus* subjected to TUVP + HHP treatments showed highly deformed morphologies with pore formation and shrinkage.

*E. coli* O157:H7 and *S.* Typhimurium also showed similar invaginations, shrinkage, and cell fusion (Fig. 2c, d) after TUVP + HHP treatment. It is clear from the SEM images that the extent of physical damage in *S.* Typhimurium was much higher than that in *E. coli* which is consistent with results in Table 2. *S.* Typhimurium was completely inactivated in commercial apple juice after combined treatment using 8.45 J/cm$^2$ TUVP and 400 MPa HHP, and there was also a complete physical cell destruction as visible in SEM images (Fig. 2d) after this treatment.

Normal *S. cerevisiae* cells have a smooth surface with a continuous cell wall (Kopecká et al. 1974), but birth and bud scars have also been observed in their structure (Marx et al. 2011). SEM images of cells after TUVP + HHP treatment showed cell shrinkage and fusion (Fig. 2e) resulting in a cell volume reduction due to mass transfer between cells and surroundings (Pilavtepe-Çelik et al. 2008; Yoo et al. 2015).

TUVP induces destruction of the cytoplasmic membrane, DNA damage, and rupture of the internal organization of *E. coli*, *L. monocytogenes*, and *S.* Typhimurium cells in liquid culture leading to leakage of cytoplasmic contents and cell death (Kim et al. 2013). It was observed in another study that HHP caused destruction of the cell membrane, resulting in release of cell contents and death of living cells (Ghafoor et al. 2012). A TUVP pretreatment was important for ensuring maximum microbial inactivation effects before use of HHP. TUVP induces generation of reactive oxygen species (ROS), such as $OH^{\cdot}$, $O_2^{-}$, and $H_2O_2$. $TiO_2$ illuminated with UV light causes development of positive "holes" that are generated in the valance band of the $TiO_2$ molecules. $HO^{\cdot}$ radicals are formed when these holes react with adsorbed $H_2O$ or $OH^{-}$ at

$TiO_2$ surface. Electrons, generated in a conduction band, react with oxygen to form HO· radicals. These generated ROS can inactivate enzymes and cause acute oxidative stress in bacterial cellular components (Ireland et al. 1993). ROS also target cell DNA, RNA, proteins, and lipids and cause structural damage to the cell membrane, eventually leading to cell death (Ireland et al. 1993; Benabbou et al. 2007; Kim et al. 2013). HHP is also known to cause shrinkage, cell breakage, and cell rupture in living cells (Ghafoor et al. 2012). It has also been observed that HHP is capable of causing significant changes in the enzymatic activities and biochemical reactions of microbial cells, leading to disruption of cellular functions. HHP can also cause damage to chromosomes and the DNA of *Salmonella*, *E. coli*, *Shigella*, and *S. aureus*, resulting in failure of bacteria to grow and reproduce (Patterson 2005; Bayındırlı et al. 2006; Reineke et al. 2013). Complete inactivation of different pathogenic and spoilage-causing microorganisms by using HHP alone may require use of much higher pressures or longer processing time that may not be economical, and it can possibly deteriorate certain quality attributes of food products. TUVP is an emerging non-thermal microbial inactivation method for different food products, and TUVP + HHP treatment resulted in higher bacterial and yeast inactivation in commercial apple juice due to induction of multiple microbial inactivation mechanisms as discussed earlier. Hence, this synergistic approach is recommended for ensuring better microbial safety of fruit juices. The synergistic inactivation of microorganisms in sequential treatment might be due to a multiple damage mechanism caused by two different inactivation treatments resulting in different types of injuries to cell structures. Wang et al. (2010) assumed that combination of dissolved $CO_2$ and HHP promoted pressure-induced cell membrane permeabilization for higher inactivation of *S. aureus* and *E. coli* cells in a liquid culture via synergistic effect. Gayán et al. (2012) reported that the sequence of treatments seemed to be important for maximizing the lethal effect of combined treatments of UV light and heat treatment against *Salmonella* Typhimurium. Therefore, further work is required to get detailed insights to understand the mechanism of microbial inactivation of synergistic effect after combined treatments.

## Conclusions

Pretreatment with TUVP (8.45 J/cm$^2$) prior to HHP treatment (500 MPa) caused complete inactivation of *L. monocytogenes*, *S. aureus*, *S.* Typhimurium, and *S. cerevisiae* in commercial apple juice. Complete inactivation of *E. coli* O157:H7 was achieved using 8.45 J/cm$^2$ TUVP followed by 600-MPa HHP treatment. The resistance to various types of inactivation treatments varied with each microorganism. *S.* Typhimurium was the most sensitive, and *E. coli* was the least sensitive microbe with reference to TUVP + HHP treatment. SEM images of treated microbial cells showed significant cell deformation due to synergistic effects of disinfection methods, and these were mostly consistent with results about microbial inactivation in this study. This synergistic disinfection strategy is applicable in the liquid food industry provided that more studies are required to find out inactivation effects on other pathogenic and spoilage-causing microorganisms in various kinds of food matrix besides studying different quality attributes of end product.

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**Conflict of Interest** Authors declare that they have no conflict of interest.

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