Bacterial inactivation mechanism of SC-CD and TEO combinations in watermelon and melon juices

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Abstract

Inactivation mechanism of supercritical carbon dioxide (SC-CD) combination with *Thymbra spicata* essential oil (TEO) treatments on bacteria in tryptone soy broth (TSB) and juices was investigated. *Salmonella* Typhimurium was significantly inactivated by 3.45 and 5.55 logs after SC-CD+TEO treatment of TBS for 35 min at 7.6 and 10.13 MPa respectively. *Klebsiella pneumoniae* showed higher resistance to treatments than other two bacteria and *Escherichia coli* showed higher resistance to treatments than *S.* Typhimurium. Approximately 5.27, 4.68 and 3.82 logs inactivation of *S.* Typhimurium, *E. coli* and *K. pneumoniae* were obtained in the treatment of watermelon juice with SC-CD+TEO at 10.13 MPa after 35 min, while 6.89, 4.82 and 4.55 logs inactivated, respectively, were observed in melon juice. Research indicated that the inactivation mechanism involves inducing cell damage, increasing membrane permeability, increasing cell rupture, precipitation of cytoplasmic contents, extracting cellular substances and lysing cells. Different sensitivities of Gram-negative bacteria to the treatments may be attributed to the differences in the cell structure, compositions of membrane and presence of capsule, pressure level and exposure time. pH, total soluble solid and browning degree of juice did not significantly change after treatments and during 7 days of storage.

Keywords: supercritical carbon dioxide; *Thymbra spicata*; watermelon; melon; antimicrobial.

Practical Application: Inactivation mechanisms of Bacteria.

1 Introduction

Thermal heating methods are well-known and ancient techniques for reducing the microbial load of various foods. They have detrimental effects on the texture, color, flavor, aroma and nutrients of fruit products (Erkmen & Bozoglu, 2016c). Enteric bacteria (*Escherichia coli, Salmonella* strains and others) are Gram-negative rods, have great importance in public health and can associate with ready-made foods (such as fruits and fruit juice), dairy and meat products and seafood and vegetables (Erkmen & Bozoglu, 2016b). *K. pneumoniae* has been isolated from different foods, heated tomato juice (at 60 °C for 75 min), high sugar-containing acidic foods, wastewater and intestinal tract. In these senses, the increased consumer demand and awareness for fresh-like and natural food products with high and/or maximum organoleptic quality and extended shelf life, non-thermal food preservation methods have been proposed. Nonthermal pressurized carbon dioxide (CO_2) has several advantages in the inactivation of microorganisms in foods, such as low cost, ease of removal from the product after use, generally recognized as safe, non-toxic, inflammable and environmentally friendly (Moussé et al., 2016; Silva et al., 2020). Supercritical CO_2 (SC-CD) above its critical point (31 °C and 7.4 MPa) is characterized by a high density (close to liquids), a high diffusivity (close to gases), a low viscosity (as gases) and a very low surface tension. SC-CD were potentially used to inactivate microorganisms and enzymes in various foods such as watermelon, melon, milk, milk products, orange juice, apple juice, peach and carrot juice, apple juice and cheese, seeds, apple juice, peach and fresh-cut carrot, tomato juice and kimchi (Erkmen,

2000, 2012; Liu et al., 2012; Spilimbergo et al., 2013; Sikin et al., 2016; Marszałek et al., 2018; Pei et al., 2018; Wang et al., 2018).

Low limits of factors (such as pH, heat treatment, pressure, storage temperature, moisture content and concentration of antimicrobials) are used in the application of hurdles in the processing of foods. When only one hurdle is applied in food processing, it needs high severity in the application. This can cause significant damages on the sensory and nutritional quality of foods. The preference of consumer for fewer/no chemicals in food has led to research on the use of natural antimicrobials in the food process for controlling unwanted microorganisms. Essential oils (EOs) extracted from aromatic plants are mixtures of multiple compounds. Turkey is one of the most important medicinal and aromatic plant trader country of the World (Tutar, 2018). Spices have pleasant flavor, color and aroma, as well as antioxidant functions and action as natural preservatives (Souza et al., 2020). The genus *Thymbra spicata* var. *spicata* L., belonging to Lamiaceae family, vernacular "Zahter" in Turkey, is an economically significant plant and grows naturally in the Eastern Mediterranian countries. The major components of *T. spicata* being essential oil (TEO) obtained from Turkey are carvacrol, terpinen-4-ol, caryophyllene oxide, γ-terpinene, p-cymene, myrcene and thymol (Tutar, 2018). The antimicrobial effects of zahter on different microorganisms have been studied (Tutar, 2018).

Watermelon (*Citrullus lanatus*) and melon (*Cucumis melo*) are very popular and widely consumed fruits in the world. They are high in vitamins, some amino acids, minerals, lycopene,

antioxidants, and many other nutrients that protect the human body from free radicals (Ndiaye et al., 2018). They are mainly used in the raw state and their fleshes are directly consumed or transformed into juice. Juice obtained from fruit fleshes occasionally occur at the level of households or small businesses that do not very often meet the quality requirements for the international market (Sadji et al., 2018). They are suitable raw materials for the juice industry due to the high nutrient content and the pleasant aroma. The thermal processing of watermelon and melon juice results in off-flavor and undesirable color formation, and degradation of vitamins (Fonteles et al., 2012). Watermelon and melon caused 3602 illnesses, 322 hospitalizations, and 49 deaths in North America during 1973-2011 (Food and Agriculture Organization of the United Nations, 2011). The most common etiological agents reported from these juices were *Salmonella enterica* (47.1%) followed by Norovirus (22.4%), *E. coli* (5.9%), *Campylobacter jejuni* (3.5%) and *Shigella sonnei* (2.4%) (Muller, 2014). Cross-contamination of *E. coli* with watermelon caused an outbreak in a restaurant (Walsh et al., 2014).

Antimicrobial activity mechanisms of SC-CD+TEO combination have not yet been evaluated in broth, and juice of watermelon and melon. Besides, no research had been conducted about the hurdle approach of SC-CD and TEO. Therefore, the overall objectives of this study were as follows: (i) to detect minimum inhibitory and bactericidal concentrations of TEO for S. Typhimurium, *E. coli,* and *K. pneumoniae*, and use minimum inhibitory concentrations in the pressure treatment; (ii) to study bacterial susceptibility to SC-CD in TSB; (iii) to study the hurdle effects of SC-CD+TEO on these bacteria in TSB, describing the mechanism of hurdle in the inactivation of bacteria; and (iv) to evaluate the efficacy of hurdle method (SC-CD+TEO) at most effective pressure level and TEO in watermelon and melon processes.

2 Materials and methods

2.1 Preparation of bacterial cultures and materials

Essential oil extraction

Thymbra spicata var. *spicata* was obtained from June to August in 2017 from Gaziantep (Turkey). *T. spicata* was obtained as dry form and did not expose to sunlight to avoid loss of active components. Dry zahter (together with leaves, flowers and stems) were ground by a mechanical grinder. Ground plant materials were placed into a Clevenger vessel with double distilled water in 1:2 (material: water) ratio through opening and hydrodistilled for three hours (Oreopoulou et al., 2019). The collection of TEO was done through condensing of the extract in the cooling vapor and collected into a graduated column. The extracted TEO was dehydrated with anhydrous sodium sulfate (NaSO$_4$) and kept in a sterile dark bottle (10 mL) at 4 °C until use in experiments.

Preparation of watermelon and melon juice

Watermelon (*Citrullus lanatus*) and melon (*Cantaloupe melon*) were obtained from a local market in Gaziantep (Turkey) and chosen according to the maturity criteria which were basically the heaviness, the sound of the fruit after tapping and not affected by any diseases. Each of them was cleaned separately, dried and completely hollowed. Fleshes were cut into small pieces to facilitate grinding and at the same time seeds were removed. After one min grinding in a sterile blender (Waring blender 7011, Waring Commercial, Torrington, USA), the solution was filtered through cheesecloth in order to separate juice and puree. The pH of watermelon and melon juice was 5.38 and 6.40 respectively. Juice was stored in a cold place (at 4 °C) until use in the experiment. Fresh juice was prepared daily.

Preparation of bacterial culture

The following bacteria were used in this research: *Salmonella enterica* subsp. *enterica* var. Typhimurium KUEN 1357 (İstanbul University, İstanbul, Turkey), *Escherichia coli* KUEN 1504 (İstanbul University, İstanbul, Turkey) and *Klebsiella pneumoniae* ATCC 43816 (Manassas, VA, USA). Bacterial cultures were maintained on tryptone soy agar (TSA; Difco, Detroit) slants at 4 °C and subcultured monthly. Each bacterial was inoculated into tryptone soy broth (TSB; Difco, Detroit), incubated at 35 °C for 24 h and used as a stock culture. A working culture was prepared by diluting 24 h stock culture using a sterile physiological saline solution (PSS, 0.85% NaCl) at a volume ratio of 1:10 with the final bacterial number 10^7~10^8 colony-forming unit (cfu)/mL.

2.2 Treatments

MIC and MBC

The broth microdilution method (Seyyednejad et al., 2014) was performed to indicate antimicrobial activities (minimum inhibitory concentration, MIC and minimum bactericidal concentration, MBC) of TEO. Serial dilutions of TEO from 0.01 to 20.0 µg/mL were prepared in the sterile distilled water+0.15% Twin-80. The diluted TEO was added into the test tubes containing 1.5 mL of TSB to provide the required TEO concentration in 2 mL of TSB. After adding TEO, TSB containing TEO was completed to 1.9 mL by the required amount of TSB. From each of bacterial working cultures, 100 µL was inoculated into each of 1.9 mL TSB containing TEO. Therefore, the final volume of the TSB was 2.0 mL. Final TEO concentrations in the TBS were ranged from 0.1 to 2.5 µg/mL. Final bacterial numbers in tubes were 7.58x10^6 cfu/mL (6.89 logs) for *S*. Typhimurium, 5.62x10^6 cfu/mL (6.74 logs) for *E. coli* and 4.68x10^6 cfu/mL (6.67 logs) for *K. pneumonia*. The tubes were vortexed to mix suspension and incubated at 35 °C from 24 to 48 h in a water bath (ST-402; Nuve, İstanbul, Turkey). The water bath was shaken at 20 rpm. After incubation, the following antimicrobial activities on bacteria were predicted from tubes; (i) The lowest essential oil concentration in which microbial growth does not occur in the tube (without turbidity) was indicated as MIC (µg/mL); (ii) Subcultures were done starting from MIC tube to further three tubes without turbidity by streak plating onto TSA. The TSA plates were incubated at 37 °C for 24 h. The plates without growth (no colony formation) of bacterium are recorded as the respective TEO concentration completely killing bacterium and are indicated as MBC (µg/mL). Control broths with tween 80 and TEO in 2 mL TSB tubes without microbial culture inoculation were used to check the microbial contamination.

2.3 Supercritical carbon dioxide processing

Equipment

A cylindrical pressure vessel (maximum pressure tolerance level=100.76 MPa) with an internal volume of 266 mL was used for CO_2 pressurization. A schematic diagram of high-pressure CO_2 treatment is illustrated in Figure 1. The vessel closure has a gas-impermeable connection for sensors, and gas inlet and outlet. A pressure manometer (Klauser Fisher, 28124-1; Kardeşler Ticaret, Gaziantep, Turkey) and copper-constantan thermocouple (Heraeus, 24313651; Teknim, Ankara, Turkey) were installed to monitor pressure and temperature of the gas in the pressure vessel. After each run, the system was cleaned with 70% (v/v) ethanol followed by sterile distilled water to prevent possible contamination.

Supercritical carbon dioxide treatments

The following treatments were performed in this research.

- **(i) Treatment of TBS with SC-CD.** Five mL working bacterium culture was added into 35 mL of TSB, mixed and one mL sample was taken twice for initial cell count. Ten mL of TSB culture was added into sterile tubes (110×14 mm). Three loosely capped tubes were placed into the pressure vessel, the vessel was tightly closed and immersed in a thermostated water bath (ST-402; Nuve, Istanbul, Turkey) at 25 °C. When the temperature of the vessel was equilibrated, CO_2 with a purity of 99.99% (Kocerler, Gaziantep, Turkey) was introduced through the gas inlet valve from the gas cylinder into the vessel to reach the desired pressure (7.6 and 10.13 MPa). After the application of pressure for an exposure time (from 1 to 80 min), the pressure was decreased to the atmospheric level by opening the valve slowly in one min. Immediately after treatment, one mL of sample from each of three tubes in duplicate was removed. Samples were analyzed for surviving bacterium;

- **(ii) Treatment of TBS with the hurdle concept.** The effect of SC-CD in combination with TEO was evaluated with simultaneously combined treatments on three bacterial cultures separately. A sufficient amount of TEO was added into 40 mL of TSB (containing 5 mL of working bacterium culture) to provide MIC, mixed and one mL sample was taken twice for initial cell count. Ten mL of TSB culture was added into sterile tubes. SC-CD treatments were applied as explained above (i);

- **(iii) Treatment of watermelon and melon.** The treatments were carried out for each of watermelon and melon juice with three bacterial cultures separately by the hurdle approach of SC-CD+TEO at 10.13 MPa pressure and 25°C. A working bacterial culture (5 mL) was centrifuged at 4000 rpm (centrifuge 5810 R Eppendorf, Hamburg, Germany) for 15 min and the pellets were washed with PSS and re-centrifuged twice in PSS and then added into 40 mL juice in a tube. Then sufficient amount of TEO was added into 40 mL juice to provide MIC, mixed and one mL sample was taken twice for initial cell count. Ten mL juice containing bacterial cells and TEO were added into
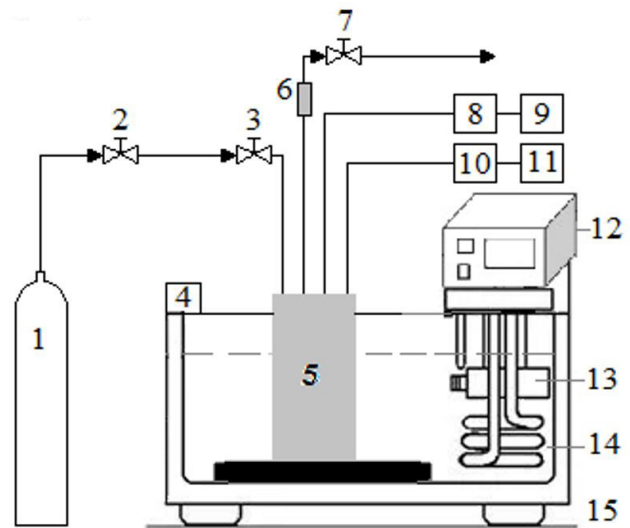
Figure 1. Schematic diagram of high-pressure CO_2 treatment. (1) = CO_2 cylinder; (2) = pressure regulator; (3) = needle valve for CO_2 inlet; (4) = thermostatic water bath; (5) = pressure vessel; (6) = line filter; (7) = needle valve for CO_2 outlet; (8) = thermocouple; (9) = temperature data logger; (10) = pressure transducer; (11) = pressure data logger; (12) controller; (13) water circulation pump; (14) Heater; (15) Immersion heater.

sterile tubes. SC-CD treatments were applied as explained above (i). Treated Watermelon and melon juice were stored in bottles at 4 °C until use in experiments.

2.4 Analysis

Enumeration of bacteria

Each sample obtained before and after treatment was diluted with PSS. The number of bacterial cells was counted by spread plating one mL of diluted (or non-diluted) samples on plates (in duplicate) of bismuth sulfide agar (BSA; Difco, Detroid) for *S.* Typhimurium, violet red bile agar (VRBA; Difco, Detroid) for *E. coli* and klebsiella chromoselect selective agar (KCSA; Merck, Interlab Laboratory Products, Adana, Turkey) supplemented with klebsiella selective supplement for *K. pneumoniae*. They were incubated at 35 °C for 24 h, after which all red colonies with a black zone on VRBA (Erkmen, 2015), all black colonies on BSA (Erkmen, 2015) and all purple-magenta colored colonies on KCSA (Moussé et al., 2016) were counted. Typical bacterial colonies (3 per plate) were streaked onto TSA slants for confirmation tests (Erkmen, 2015). The number of the bacterium was expressed as log colony-forming unit (cfu/mL). The inactivation curve was obtained by drawing Log (cfu/cfu_0) versus time. Initial bacterial numbers in tubes (TSB, watermelon and melon juice) were 7.58×10^6 cfu/mL (6.89 logs) for *S.* Typhimurium, 5.62×10^6 cfu/mL (6.74 logs) for *E. coli* and 4.68×10^6 cfu/mL (6.67 logs) for *K. pneumonia*.

2.5 Data analysis

Biological parameters, i.e., reaction rate constant (k) and decimal reduction in the surviving population (D), were evaluated with regression analysis of the first order rate inactivation models

using SigmaPlot 14.0 (Systat Software, Inc., Germany). Pressure inactivation of microorganisms was assumed to follow the first order rate inactivation models expressed as Equations 1 and 2:

$$\text{Log}\ (N\,/\,N_0)\ =\ -kt \tag{1}$$

$$\text{Log}\ (N\,/\,N_0)\ =\ -t\,/\,D \tag{2}$$

Where N = number of surviving microorganisms after a pressure treatment for time t (min), N_0 = initial number of microorganisms and k = reaction rate constant (min^{-1}), D = decimal reduction in the surviving population (min).

2.6 Determination of changes in juice

Determination of Browning Degree (BD)

The browning degree of watermelon and melon juice was evaluated using a spectrophotometric method described by Gui et al. (2006) Cloudy juice was centrifuged at 10000×g at 4 °C for 30 min, then passed through a 0.45 µm cellulose nitrate membrane (Product no: 10401131; Global Life Sciences Technologies, Tokyo, Japan). The browning degree was determined by measuring the A (absorbance at 420 nm) value using a Lamda-25 UV/VIS spectrometer (PerkinElmer, Shelton, CT, USA) at ambient temperature (25 ± 1 °C) with a 1 cm path length cell.

Determination of Total Soluble Solids (TSS)

TSS of watermelon and melon juice was determined by the refractometer (Index Instruments, PTR 46 X, Florida, U.S). The reading from the screen was recorded as percentage soluble dry matter and expressed as in terms of °Brix (Wang et al., 2018).

Determination of pH

The pH values of juices were measured at 25 °C with a Corning pH meter (EM78X, Fisher, Pittsburgh, USA) equipped with a glass electrode. A 20 mL sample was placed in a 50 mL beaker with a magnetic stir bar and measurement was recorded after pH was stable (Erkmen, 2015).

Determination of color

CIE LAB L*, a* and b* values of juices were determined by Hunter Lab Color Flex EZ spectrophotometer (HunterLab Color Meter, Color Flex, Virginia, USA). It was standardized by white and black disks. L*, a* and b* values of the disk were 93.41, -1.12 and 1.07 respectively. The sample was well mixed before filling it into a glass cup. The glass sample cup was filled with 25 mL juice and placed on the sample port. The calibration white tile was placed white side down on the sample cup. Each sample was evaluated on an L (lightness), a* (redness), b* (yellowness) scale at time 0 and after sitting for 10 min. Between samples, the glass sample cup was cleaned with sterile distilled water.

2.7 Statistical analysis

Two samples were removed at each sampling time and experiments were repeated three times. Data were evaluated using SPSS v.22 (IBM SPSS Corporation, Chicago, IL, USA) with a 99% confidence level. Error bars indicated in figures and ± correspond to the standard error of the mean. A "one-way analysis of variance" (ANOVA) and "paired-samples T-test" were performed to indicate the significant effects of treatments on each bacterium.

# 3 Results and discussion

3.1 MIC and MBC values of TEO

The MIC values of TEO on *S.* Typhimurium, *E. coli* and *K. pneumoniae* were 0.35, 0.45 and 1.00 µg/mL respectively. The MBC values of TEO on these bacteria were 0.60, 0.65 and 1.5 µg/mL in the order. The results showed that TEO was active against all tested Gram-negative bacteria while both MIC and MBC values were higher for *K. pneumoniae* than the others. Capsule formation and outer membrane of Gram-negative bacteria that contains polysaccharides and lipopolysaccharides, respectively, that could act as a barrier against essential oils (EOs) (Carvalho et al., 2018). Therefore, the higher amount of TEO was required in the inactivation of capsulated Gram-negative bacteria. Thymol and carvacrol of TEO are hydrophilic compounds that can cause damages to the membranes of microbial cells (Tutar, 2018).

## 3.2 Inactivation of bacteria by SC-CD and SC-CD+TEO in TSB

Inactivation of *S.* Typhimurium after treatments at 7.6 and 10.13 MPa (25 °C) up to 80 min in TSB are shown in Figure 2. SC-CD+TEO was showed a higher inactivation effect on *S.* Typhimurium than SC-CD at both pressures. About 4.36 and 6.10 logs *S.* Typhimurium inactivation were achieved with SC-CD+TEO at 7.6 and 10.13 MPa, respectively, in 35 min, while about 3.45 and 5.55 logs inactivation were achieved with SC-CD at the same conditions. All *S.* Typhimurium cells (6.89 logs) were completely inactivated after 45 and 40 min with SC-CD and SC-CD+TEO, respectively, at 10.13 MPa. The same inactivation occurred after 70 and 55 min with SC-CD and SC-CD+TEO, respectively, at 7.6 MPa. *S.* Typhimurium inactivation significantly (p<0.05) increased with pressure increase at both alone and combination treatments. Inactivation of *S.* Typhimurium is significantly (p<0.05) higher in the combination treatments than the alone.

The inactivation of *E. coli* after SC-CD and SC-CD+TEO treatments at 7.6 and 10.13 MPa (35 °C) in TSB are shown in Figure 3. *E. coli* inactivation by SC-CD was 3.05 and 4.07 logs after 35 min at 7.6 and 10.13 MPa respectively. The reduction of *E. coli* by SC-CD+TEO combination reached 3.48 and 4.91 logs after 35 min at 7.6 and 10.13 MPa, respectively, which were significantly (p<0.05) higher than SC-CD. All *E. coli* (6.74 logs) were inactivated in TSB after 55 and 45 min with SC-CD and SC-CD+TEO, respectively, at 10.13 MP. Inactivation of *E. coli* significantly (p<0.05) increased with pressure increase at both alone and combination treatments. Inactivation of *E. coli* is significantly (p<0.05) higher in the combination treatments than alone.
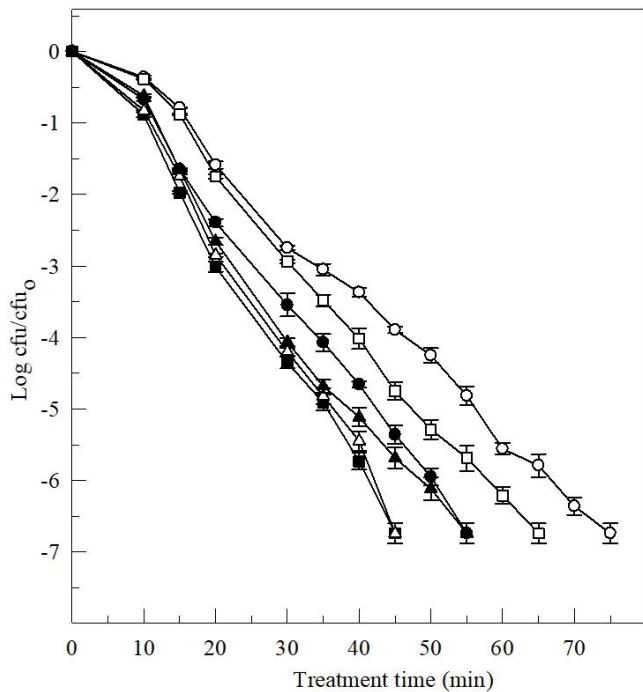
Inactivation of *K. pneumoniae* after SC-CD and SC-CD+TEO treatments at 7.6 and 10.13 MPa (25 °C) in TSB are shown in
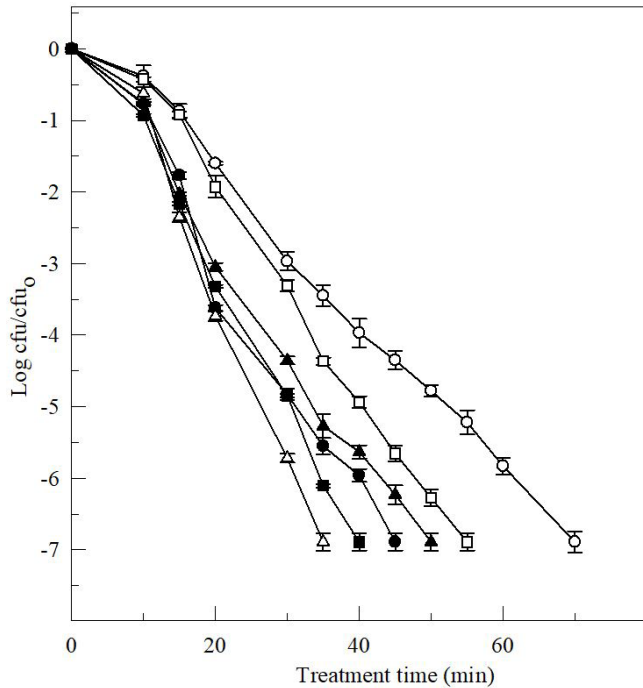
Figure 2. Antibacterial activity of SC-CO$_2$ and SC-CO$_2$+TEO on *S.* Typhimurium at 35 °C. Treatments are: 7.6 MPa (○) in TSB, 10.13 MPa (•) in TSB, 7.6 MPa+TEO (□) in TSB, 10.13 MPa+TEO (▪) in TSB, 10.13 MPa +TEO (Δ) in watermelon and 10.13 MPa+TEO (▲) in melon.
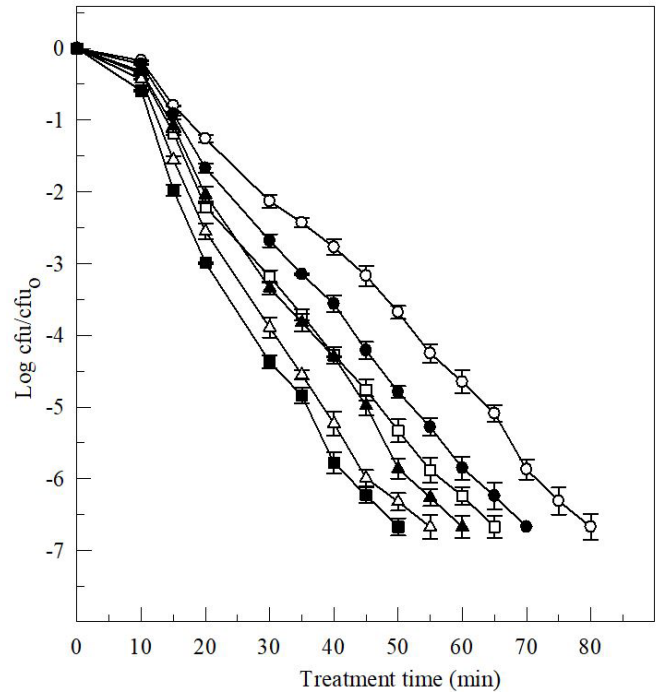
Figure 4. Antibacterial activity of SC-CO$_2$ and SC-CO$_2$+TEO on *K. pneumoniae* at 35 °C. Treatments are: 7.6 MPa (○) in TSB, 10.13 MPa (•) in TSB, 7.6 MPa+TEO (□) in TSB, 10.13 MPa+TEO (▪) in TSB, 10.13 MPa +TEO (Δ) in watermelon and 10.13 MPa+TEO (▲) in melon.

Figure 3. Antibacterial activity of SC-CO$_2$ and SC-CO$_2$+TEO on *E. coli* at 25 °C. Treatments are: 7.6 MPa (○) in TSB, 10.13 MPa (•) in TSB, 7.6 MPa+TEO (□) in TSB, 10.13 MPa+TEO (▪) in TSB, 10.13 MPa +TEO (Δ) in watermelon and 10.13 MPa+TEO (▲) in melon.

Figure 4. After 35 min of treatment with SC-CD at 7.6 and 10.13 MPa, 2.43 and 3.15 logs inactivation, respectively, were obtained, but a faster inactivation of *K. pneumoniae* with SC-CD+TEO

was observed with 3.71 and 4.84 logs at 7.6 and 10.13 MPa respectively. *K. pneumoniae* was a capsule forming bacterium, but *S.* Typhimurium and *E. coli* cannot form capsule. Antimicrobial effects of SC-CD and SC-CD+EO on *K. pneumoniae* cells were significantly lower ($p < 0.05$) than the other two bacteria. However, a large fraction of *K. pneumoniae* cells treated with SC-CD and SC-CD+TEO were significantly inactivated ($p < 0.05$). The bacterial capsule is an external protective layer to the outer membrane that acts as an additional barrier primarily with long-chain repeating polysaccharides (Band & Weiss, 2014). Moreover, SC-CD+TEO caused more antimicrobial effects ($p < 0.05$) on the *K. pneumoniae* than SC-CD which supported the synergistic effect of combination treatments in the inactivation of capsulated bacterial cells.

S. Typhimurium showed higher susceptibility ($p < 0.05$) to SC-CD or SC-CD+TEO than *E. coli* under the same conditions. Different sensitivity to the treatment between two Gram-negative pathogens may be attributed to the differences in the cell compositions especially in membranes. Four minor compounds have been detected in the structure of bacterial membranes (Ames, 1968): two partially characterized lipids, phosphatidic acid and phosphatidylserine. The phospholipid composition of the two bacteria is quite similar, the only difference is the absence of one of the minor components and a decreased level of other membrane components in *E. coli*. Growth conditions can also affect lipid composition (Ames, 1968). Therefore, differences in the membrane compositions may be the reasons to induce different pressure sensitivity among different bacterial strains. Due to the differences in the compositions of membranes, treatments would be shoved higher disorder effects on these

additional components of *S.* Typhimurium. Since these effects would be increased the disorder of phospholipid structure and that would be associated with a more permeable membrane to the antimicrobial agents (CO_2 and TEO). Treatments also showed more antimicrobial effects on young cells which would be included higher numbers among *S.* Typhimurium population (with short generation time) than *E. coli* population (with the long generation time).

To compare the effectiveness of SC-CO_2 and SC-CO_2+TEO treatments on three bacteria in TSB and to optimize process conditions, the k and D values can be used to compare the inactivation rate and resistance of microorganisms. D and k values obtained from regression analysis are presented in Tables 1-3. For model systems, the higher the pressure in the presence of TEO the smaller the D-value and the higher the k value. There were significant differences (p<0.01) among D and k values at constant pressure in TSB with and without TEO. The pressure resistance of microorganisms depends on the type of microorganism, level of pressure and presence of antimicrobial substance in the medium.

3.3 Inactivation mechanism of bacteria with the hurdle approach

The general mechanisms on the inactivation of microorganisms with CO_2 pressure process alone were indicated (Erkmen, 2012). The precise mechanism of bacterial inactivation with the SC-CD+EOs combination treatments is unclear. The possible antimicrobial mechanism of SC-CD+TEO combination on Gram-negative bacteria can be hypothesized as indicated in Figure 5. Under pressure, unhydrated CO_2 can diffuse through the cellular membrane and be accumulated in the lipophilic phospholipid bilayer. This can rupture lipid-protein interactions and order loss of the lipid chain. Additionally, solubilization of CO_2 in water in the environment of cells produces carbonic acid (HCO_3^-) and H^+ ion. These ions may alter the charged phospholipid groups and proteins on the surface area of the membrane, thereby altering the membrane function. At the same time, the synergistic aids can enhance the permeability of the membrane to TEO compounds and other external molecules due to the SC-CD-induced injury and damage on the cell membrane and cell wall (Figure 5a). The outer membrane and plasma membrane are the primary sites of damage for SC-CD resulting in altering the permeability and function of the membranes that can facilitate the transport of TEO through cell structures in a short time. Since, TEO compounds alone need a period time for their effect and penetration through membranes (Boskovic et al., 2017). The necessary time to inactivate cells with TEO was shortened by hurdle effects as compared with alone activity, which might cause a rapid synergistic antimicrobial effect of TEO on bacterial cells together with SC-CD. So on, TEO components easily diffused through membranes and SC-CD+TEO combination caused further disruption on the membranes with increasing permeability, acidification of cytoplasm, decreasing the pH gradient in the cell and altering osmoregulatory functions of the cytoplasmic membrane and cell wall. SC-CD+TEO leads to deformation on the cell structure, modifies membrane lipid, disordering the hydrocarbon lipid chains and rupturing

Table 1. Effect of SC-CO_2 and SC-CO_2+TEO on the inactivation rates (k) and decimal reduction times (D) of *S.* Typhimurium in TSB, watermelon and melon juice[a].

Medium	Pressure (MPa)	k (min⁻¹)		D (min)		r²
		$\overline{x}$ [b]	s_x	$\overline{x}$ [b]	s_x	
TSB	7.6	0.0962	0.0019	10.40	0.21	0.98
	10.1	0.1536	0.0053	6,5124	0.2259	0.97
ThymMIC-TSB	7.6	0.1211	0.0042	8,2587	0.2895	0.97
	10.1	0.1669	0.0055	5,9907	0.1964	0.98
ThymMIC+ Watermelon	10.1	0.1856	0.0115	5,3889	0.3349	0.95
ThymMIC+Melon	10.1	0.1409	0.0030	7,0995	0.1514	0.99

[a] $\overline{x}$ = means and s_x = standard deviations of three determinations, r² = regression coefficient; [b]There are significant differences (P < 0.01) between different treatments.

Table 2. Effect of SC-CO_2 and SC-CO_2+TEO on the inactivation rates (k) and decimal reduction times (D) of *E. coli* in TSB, watermelon and melon juice[a].

Medium	Pressure (MPa)	k (min⁻¹)		D (min)		r²
		$\overline{x}$ [b]	s_x	$\overline{x}$ [b]	s_x	
TSB	7.6	0.0885	0.0014	11.31	0.18	0.99
	10.1	0.1186	0.0018	8,4282	0.1272	0.99
ThymMIC-TSB	7.6	0.1021	0.0022	9,7948	0.2087	0.98
	10.1	0.1442	0.0031	6,9354	0.1506	0.99
ThymMIC+ Watermelon	10.1	0,1401	0.0039	7,1372	0.1975	0.98
ThymMIC+Melon	10.1	0.1255	0.0026	7,9650	0.1626	0.99

[a] $\overline{x}$ = means and s_x = standard deviations of three determinations, r² = regression coefficient; [b]There are significant differences (P < 0.01) between different treatments.

Table 3. Effect of SC-CO_2 and SC-CO_2+TEO on the inactivation rates (k) and decimal reduction times (D) of *K. pneumonia* in TSB, watermelon and melon juice[a].

| Medium	Pressure (MPa)	k (min⁻¹)		D (min)		r²
		$\overline{x}$ [b]	s_x	$\overline{x}$ [b]	s_x	
TSB	7.6	0.0785	0.0018	12,7310	0.2837	0.98
	10.1	0.0940	0.0018	10,6337	0.2056	0.98
ThymMIC-TSB	7.6	0.1046	0.0018	9,5593	0.1649	0.99
	10.1	0.1383	0.0035	7,2324	0.1809	0.98
ThymMIC+ Watermelon	10.1	0.1263	0.0031	7,9150	0.1946	0.98
ThymMIC+Melon	10.1	0.1107	0.0027	9,0293	0.2206	0.98

[a] $\overline{x}$ = means and s_x = standard deviations of three determinations, r² = regression coefficient; [b]There are significant differences (P < 0.01) between different treatments.

interactions of lipid-protein. This can cause order loss of the lipid chain (phase transition of phospholipid) in cell structures, the perforation of the cytoplasmic membrane and the formation
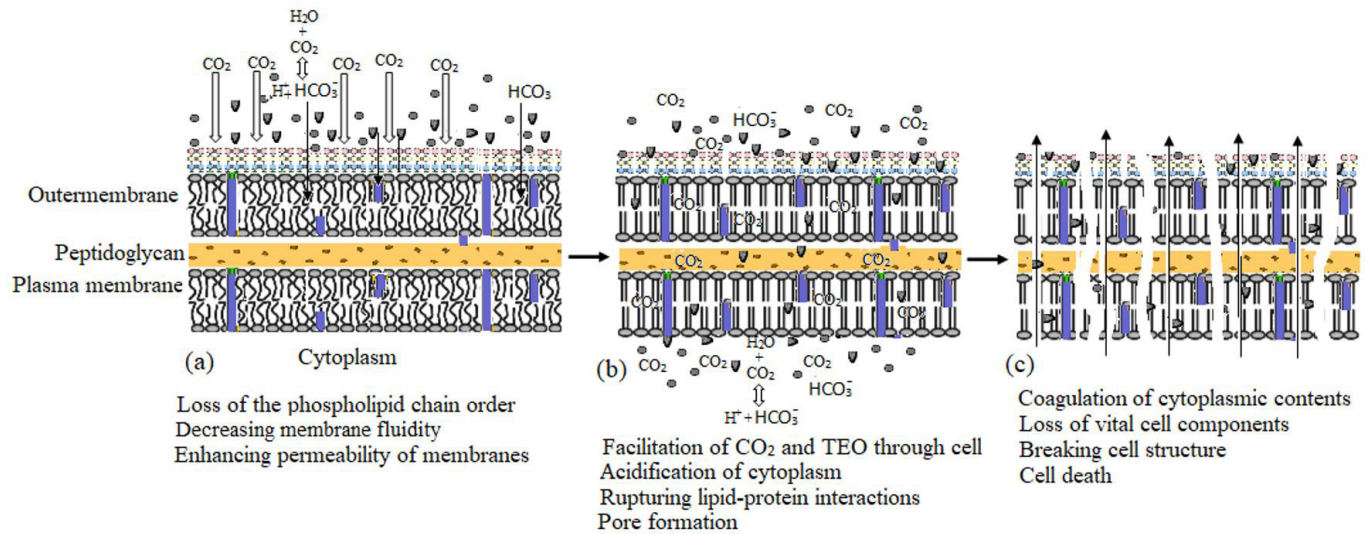
Figure 5. Mechanism of SC-CO$_2$ and TEO actions on cell structures and cytoplasm.

of pores on membranes (Figure 5b). Chung and Yousef (Chung & Yousef, 2008) indicated that pressure increases the lipid packing density in membranes and induces phase separations due to the differences in compressibility between lipids and proteins. Additionally, the presence of HCO$_3^-$ and H$^+$ ions in the cytoplasm may act on the cytoplasmic charged groups, thereby coagulation of cytoplasmic contents further altering the cellular structure. Different biochemical and physical effects are involved at multiple sites on the bacterial cell surface and within the cell. Hurdle effects may include chemical modifications of the cell membrane and cytoplasm, denaturation of enzymes and proteins, and misbalancing the biological characteristics of the microbial cell. SC-CO+TEO induced the membrane phase transition and decreases membrane fluidity. Breakage of cell structures resulted in leakage of cytoplasmic components concurrently with the loss of vital cellular material (loss of ions and metabolites) and finally caused cell death (Figure 5c).

A similar bacterial inactivation mechanism was also observed on the capsulated bacteria. Combination treatments showed that pressure disorders the polysaccharide structures in the capsule and this would aid the transport of CO$_2$ and TEO through the capsule. Due to this reason, combination treatments showed higher inactivation effects on capsulated bacteria than alone. EOs are active against *E. coli*, *S. aureus*, *B. cereus* and *Salmonella* spp. but are less effective against capsule forming bacteria that increase resistance to Eos (Martino et al., 2009).

Solubilization of CO$_2$ in water can acidify suspension and cytoplasm by the liberation of H$^+$ ions. The effects of low external pH (pH$_{ext}$) and internal pH (pH$_{int}$) on microbial cells are as follows: cells use too much energy that they may not able to regenerate and the low pH$_{ext}$ increase permeability of membrane and peptidoglycan layer to facilitate the penetration of antimicrobials into microbial cells (Erkmen & Bozoglu, 2016a). Low pH$_{int}$ denatures several enzymes and cellular proteins, and coagulate cellular components. Enhanced concentration of CO$_2$ and TEO in cells can easily interact with biomolecules and water, and destruct biological balance (Figure 5b). Many components

of TEO that are rich in terpenes and phenolics (such as carvon, thymol and carvacrol) can penetrate into the phospholipid bilayer of the outer membrane, where they bind to proteins and prevent them from their normal functions (Juven et al., 1994).

Previous results indicated that the pressure can disrupt the peptidoglycan layer and outer membrane in the cell wall (Nazzaro et al., 2013). Some of the *S.* Typhimurium cells showed swelling after treatment with thymol and the morphology of cells remarkably altered (Nazzaro et al., 2013). The hurdle effects would occur simultaneously and synergistically under pressure. These effects cause rapid and irreversible damages on the bacterial cells, preventing synthesis of new biomolecules that would be used in the repairing damaged side of the cell wall and membranes, prevent the cell division, compromise the microbial metabolism and prevent cell growth, breaking cell structure, and all the phenomena contribute bacterial inactivation and cell death (Figure 5).

3.4 Inactivation of bacteria in juices

The antimicrobial activities of SC-CD+TEO combination against bacteria in watermelon and melon juice at 10.13 MPa and 25 °C are also given in Figures 2, 3 and 4. All *S.* Typhimurium (6.89), *E. coli* (6.74 logs) and *K. pneumoniae* (6.67 logs) were inactivated after 50, 55 and 60 min, respectively, in melon juice. While higher lethality was observed in watermelon juice at the same conditions after 35, 45 and 55 min respectively. About 3.73, 2.85 and 2.55 logs of *S.* Typhimurium, *E. coli* and *K. pneumoniae*, respectively, were inactivated after 20 min in watermelon juice, while 3.05, 2.65 and 2.04 logs, respectively, were achieved in melon juice at the same conditions. Inactivation of bacteria in watermelon juice treated with SC-CD+TEO was significantly (p<0.05) higher than melon juice and TSB. This would be due to the additional synergistic effect (p<0.05) of the acidity of the watermelon juice (pH 5.38). The results indicated the synergistic combination effect of hurdle factors (SC-CD, TEO and acidity). The effect of the pressure process decreased in melon juice due to the presence of a higher amount

of organic components (such as carbohydrates) which immobilize and inactivate components in EOs and prevented easy diffusion of CO_2 in juice (Smid & Gorris, 2007).

The synergistic effect of EO and SC-CD observed in both TSB and juice was at least 5 min orders of magnitude decreased the inactivation time when compared to those observed with SC-CD alone. The number of cells was rapidly reduced with SC-CD and TEO combination compared with treated alone, indicating that bacteria injured by SC-CD processing might not be repaired in the presence of other hurdle factors. Previous studies suggested that the application of CO_2 pressure alone caused injury to the cells (Corradini & Peleg, 2012; Erkmen, 2012).

To compare the effectiveness of $SC-CO_2$ and $SC-CO_2$+TEO treatments on three bacteria in watermelon and melon juice and to optimize process conditions, the k and D values can be used to compare the inactivation rate and resistance of microorganisms. D and k values obtained from regression analysis are presented in Tables 1-3. For model systems, the higher the SC-CD pressure in the presence of TEO the smaller the D-value and the higher the k value. There were significant differences (p<0.01) among D and k values at constant pressure in watermelon and melon juice. The pressure resistance of microorganisms depends on the type of microorganism, level of pressure, and presence of antimicrobial substance and composition of the medium.

3.5 Effect of treatments on pH, total soluble solid, browning degree and color of juices

The influences of the combination of SC-CD+TEO treatment on total soluble solids (TSS) and pH of juices are given in Table 4. The initial TSS values of watermelon and melon juice were 11.29 and 9.47 °Brix, respectively. After SC-CD+TEO treatments, TSS showed fluctuations but did not change significantly (p>0.05)

after treatments and during storage compared with control. Hence, HPCD treatment did not affect carbohydrates (e.g. glucose, fructose and sucrose) which were the main components of TSS of watermelon juice (Liu et al., 2012). Similarly, HPCD treatment did not change TSS of carrot juice (Zhou et al., 2009) and a watermelon (Liu et al., 2012). Ultrafiltration of watermelon juice allowed an acceptable level of clarification in terms of sugar and ascorbic acid and also greatly reduced the suspended solids and the turbidity of the fresh juice (Bhattacharjee et al., 2018).

Table 4 shows the pH values for each juice after treatments during the 7 days storage. The initial pH values of watermelon and melon juices were 5.38 and 6.80. The pH values of the watermelon and melon juices were slightly decreased after treatments and during storage. Therefore, treatments had a significant (p<0.05) effect on the pH of juice compared with the control. There are two main reasons for the slight decrease in the pH value. First, the pressure may affect the ionization equilibrium of the aqueous solution. As the pressure rises, the ionization reaction of the carbonic acid in water will proceed in the direction of hydrogen ion generation, resulting in the reduction of the pH.

The influences of the SC-CD+TEO treatment on browning degree (BD) of watermelon and melon juices are given in Table 5. The initial BD of treated watermelon and melon juices were 1.12 and 1.09. BD values of the treated juices were not changed significantly (p>0.05) after treatment and during storage.

Tables 6 and 7 show the L*, a* and b* values watermelon and melon juices, respectively, after treatments and during the storage. Values of b* were unchanged after treatments (p>0.05) while a* value significantly (p<0.05) increased compared to control juices. Values of L* were slightly increased (p>0.05) after treatment and during the storage of juices. Through the comparison of total color difference values, SC-CD+TEO had little effect on the color

Table 4. Effect of SC-CO+EO treatment on total soluble solids and pH of watermelon and melon juices.

Day	Watermelone juice				Melon juice			
	Total soluble solid		pH		Total soluble solid		pH	
	Untreated	$SC-CO_2$+TEO	Untreated	$SC-CO_2$+TEO	Untreated	$SC-CO_2$+TEO	Untreated	$SC-CO_2$+TEO
0	11.29 ± 0.02*	11.32 ± 0.01	5.38 ± 0.01	5.32 ± 0.01	9.47 ± 0.21	9.47 ± 0.21	6.80 ± 0.15	6.69 ± 0.15
1	11.33 ± 0.03	11.36 ± 0.03	5.37 ± 0.02	5.30 ± 0.02	9.54 ± 0.18	9.58 ± 0.18	6.64 ± 0.20	6.62 ± 0.14
3	11.28 ± 0.03	11.30 ± 0.01	5.35 ± 0.01	5.28 ± 0.01	9.48 ± 0.19	9.55 ± 0.25	6.72 ± 0.13	6.58 ± 0.13
5	11.19 ± 0.02	11.28 ± 0.03	5.34 ± 0.02	5.27 ± 0.02	9.53 ± 0.13	9.51 ± 0.12	6.63 ± 0.09	6.59 ± 0.08
7	11.17 ± 0.03	11.30 ± 0.02	5.32 ± 0.03	5.27 ± 0.03	9.50 ± 0.09	9.54 ± 0.19	6.69 ± 0.12	6.62 ± 0.15

*Mean of three values ± Std Error.

Table 5. Effect of SC-CO+EO treatment on BD of watermelon and melon juices.

Day	Watermelon juice		Melon juice	
	Untreated[1]	$SC-CO_2$+TEO[1]	Untreated[1]	$SC-CO_2$+TEO[1]
0	1.12 ± 0.01	1.12 ± 0.02	1.09 ± 0.01	1.13 ± 0.01
1	1.12 ± 0.01	1.12 ± 0.01	1.08 ± 0.02	1.40 ± 0.01
3	1.11 ± 0.02	1.11 ± 0.02	1.08 ± 0.03	1.13 ± 0.02
5	1.09 ± 0.03	1.1 ± 0.03	1.07 ± 0.01	1.14 ± 0.03
7	1.07 ± 0.03	1.09 ± 0.03	1.08 ± 0.02	1.09 ± 0.01

Table 6. Effects of SC-CO+EO treatment on watermelon juice L*, a* and b* values after treatment (0) and during storage (7 days).

Day	Untreated[1]			SC-CO$_2$+TEO[1]		
	L*	a*	b*	L*	a*	b*
0	28.31 ± 1.23	19.34 ± 1.23	8.38 ± 0.23	30.87 ± 1.23	19.43 ± 1.23	8.58 ± 0.23
1	29.29 ± 1.10	17.56 ± 1.06	9.12 ± 0.12	31.23 ± 1.20	19.65 ± 1.14	8.78 ± 0.19
3	30.12 ± 2.05	16.61 ± 1.09	9.54 ± 0.08	31.17 ± 2.06	20.43 ± 2.12	8.82 ± 0.22
5	30.46 ± 2.14	17.36 ± 1.11	9.58 ± 0.11	31.02 ± 2.23	21.76 ± 2.08	8.54 ± 0.15
7	30.22 ± 2.07	17.44 ± 1.16	9.56 ± 0.12	31.68 ± 2.07	21.49 ± 1.12	8.23 ± 0.10

[1]Control, Without application of SC-CO$_2$+EO.

Table 7. Effects of SC-CO+EO treatment on melon juice L*, a* and b* values after treatment (0) and during storage (7 days).

Day	Untreated[1]			SC-CO$_2$+TEO[1]		
	L*	a*	b*	L*	a*	b*
0	24.25 ± 1.12	10.89 ± 0.12	19.75 ± 1.12	25.12 ± 1.12	10.45 ± 0.12	19.70 ± 1.14
1	26 ± 1.18	11.67 ± 0.15	19.65 ± 1.08	26.10 ± 2.18	10.10 ± 0.15	19.66 ± 2.08
3	25 ± 1.10	11.34 ± 0.16	18.98 ± 1.06	25.79 ± 1.10	9.62 ± 0.16	19.43 ± 1.06
5	26 ± 1.09	11.45 ± 0.09	19.45 ± 1.18	26.76 ± 1.09	9.30 ± 0.09	19.45 ± 1.18
7	25 ± 1.12	11.26 ± 0.013	19.24 ± 1.20	27.97 ± 2.12	9.08 ± 0.25	19.69 ± 2.20

of juice. The results of this study demonstrated the efficacy of SC-CD+TOE in inactivating microorganisms and maintaining the quality of juices. During the measurement, the juice color values (L*, a* and b*) were not uniform. The color settled out in a few hours if left undisturbed. Therefore, it was necessary to blend juice each day. Note that after 7 days, the SC-CD+TEO treated juice L* values stayed steady. Casas-Forero et al. (2020) indicated that centrifugal block freezing was increased the solid contents of the blueberry juice and the final color was darker than the fresh juice. The lightness of watermelon and melon juice significantly (p<0.05) increased between 8 and 12% after treatments with SC-CD+TEO, respectively, and slightly decreased during storage.

4 Conclusions

This study evaluated SC-CD+TEO non-thermal treatment mechanism to process watermelon and melon juice. SC-CD+TEO combination sufficiently inactivated pathogenic and spoilage bacteria. SC-CD+TEO combinations at 10.13 MPa were able to reduce all *S*. Typhimurium, *E. coli* and *K. pneumoniae* in TSB and juice. The mechanism of bacterial inactivation by SC-CD and TEO can be typically linked to the structure of bacteria, the type of microorganisms, the type of suspending medium, type of food, pressure level and exposure time. The combination of SC-CD with TEO as a hurdle approach can effectively enhance food safety, allow milder process conditions, increases product quality and reduces process costs. Using this technology, TSS and BD remained the same after the treatment storage of juices. The pH of juice slightly reduced after treatments and stable throughout the storage. Hurdle effects (SC-CD, TEO and acidity) represent an alternative to thermal processing in the shelf life of heat-labile watermelon and melon juices.

Acknowledgements

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