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Combined effect of high pressure and vinegar addition on the control of *Clostridium perfringens* and quality in nitrite-free emulsion-type sausage

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ABSTRACT

We evaluated the combined effect of high pressure (HP) and vinegar addition on control of *Clostridium perfringens* and quality in emulsion-type pork sausages. Sausages were manufactured with different levels of commercial white distilled vinegar made with jasmine tea extract (0, 1, and 2%) and sodium nitrite (0.02%, control). The sausages except for sodium nitrite treatment were subjected to HP at 500 MPa (four cycles and each for 3 min). After storage, the combined treatment of HP and vinegar showed the lowest number of *C. perfringens* vegetative cells (4.8-log CFU/g reduction) among the all treatments. The combined treatment also inhibited the growth of *C. perfringens* spores for five weeks (stored at 4 °C for two weeks followed by at 20 °C for three weeks). Among all treatment combinations, the best pH, water-holding capacity and chewiness, were found with the HP-vinegar sausage. In addition, vinegar treatment inhibited the lipid oxidation of sausage throughout the storage. Use of HP and vinegar addition inhibited growth of *C. perfringens* without observed adverse effects on quality of the emulsion-type sausage.

Industrial relevance: Currently, the use of natural additives and novel processing technologies for replacing sodium nitrite in meat products is gaining attention owing to increase in consumer demand for nitrite-free and safe meat products. The study showed that the addition of 1% (w/v) of vinegar and pressure treatment at 500 MPa (four cycles and each for 3 min) can replace sodium nitrite to inhibit growth of *C. perfringens* in emulsion-type sausages.

1. Introduction

For the past few years, *Clostridium perfringens* foodborne illness has been a major health hazard worldwide. *C. perfringens* is an anaerobic spore-forming bacterium, toxins of which cause severe illness and even death (Grass, Gould, & Mahon, 2013). The Center for Disease Control and Prevention (CDC) reported that approximately 965,958 cases of foodborne illnesses related to *C. perfringens* occur annually in the United States (Juneja, Baker, Thippareddi, Snyder Jr., & Mohr, 2013). In other countries, including Australia and Japan, *C. perfringens* is also one of the main reasons of bacterial foodborne outbreaks (Gould et al., 2004; Monma et al., 2015).

Meat and meat products are the major food items implicated in *C. perfringens* outbreaks. As *C. perfringens* requires > 12 different amino acids and an oxygen-free environment for growth, it thrives in vacuum-packaged meat products (Bauer, Carpenter, & Reagan, 1981; Park, Park, & Yoon, 2014). Fortunately, vegetative cells of *C. perfringens* are inactivated during cooking processes at temperatures over 75 °C. However, *C. perfringens* spores are difficult to inactivate, since those are extremely heat-resistant and can survive at 100 °C for ≤1 h (Labbé, 2000). Spores can easily germinate and cause foodborne illness when contaminated products are improperly cooled or temperature-abused after cooking (Evelyn & Silva, 2016). Temperature fluctuations are frequently observed in commercial and home refrigerators (Limbo, Torri, Sinelli, Franzetti, & Casiraghi, 2010). Therefore, methods for controlling *C. perfringens*, especially its spores, in packaged meat products post-cooking are necessary (Juneja et al., 2013; Labbé, 2000).

Nitrite addition is the most effective method for controlling the

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growth of vegetative cells and spores of *C. perfringens*. Nitrite addition also used for development of a characteristic red color and inhibition of lipid oxidation in meat products. Thus, synthetic nitrites, including sodium and potassium nitrite, are generally used in meat products for these purposes (Choi et al., 2017). However, owing to the growing concern over the use of synthetic food additives, the number of consumers avoiding nitrite-containing food products is also increasing (Lee et al., 2017). As meat products without nitrite are microbiologically hazardous, several studies have attempted to develop new methods of inactivating the vegetative cells and spores of *Clostridium* (Dutra et al., 2016; Valenzuela-Martinez et al., 2010).

High pressure (HP) treatment is a commercial non-thermal sterilization technology capable of inactivating bacterial vegetative cells (Kim, Ba, Dashdorj, & Hwang, 2018). However, since most microbial spores are extremely HP-resistant (withstanding 1000 MPa), inactivation of microbial spores has been a major challenge to HP process (Smelt, 1998). Paredes-Sabja, Gonzalez, Sarker, and Torres (2007) reported that HP at 650 MPa effectively inactivate *C. perfringens* vegetative cells but not for spores. However, when HP at 650 MPa was applied with high temperature or low pH, inactivation of *C. perfringens* spores was observed. Thus, HP should be combined with other treatments to inactivate both vegetative cells and spores of *C. perfringens*.

Traditionally, vinegar has been used in food processing to provide antimicrobial activity and functionality such as flavor enhancement or antioxidant activity (Choe & Kim, 2016; Valenzuela-Martinez et al., 2010). Recently, Smith, Dunn, Jefferies, Egget, and Steele (2018) reported that buffered vinegar inhibited outgrowth of *C. perfringens* spores in roast beef. In spite of these advantages of HP and vinegar, the application of them on meat products could lead to undesirable changes in the quality properties of the final meat products, such as texture, color and flavor. We hypothesized that combination treatment with HP and appropriate amounts of vinegar can inhibit *Clostridium* growth in meat products without adverse effects on quality. Therefore, the objective of the present study was to evaluate the combined effects of HP and vinegar treatment on inhibition of *C. perfringens* growth in nitrite-free cooked emulsion-type sausage under poor temperature control and to assess the changes in its physicochemical properties, including pH, water holding capacity (WHC), texture, and lipid oxidation, after 5 weeks of storage.

2. Materials and methods

2.1. Sample preparation and experimental design

2.1.1. Manufacture of emulsion-type sausage

Several pieces of pork hind leg meat and back fat were purchased from a commercial butcher (Seoul, Korea). Then, those were cut and ground through a grinder with a 5-mm plate (M-12S, Hankook Fujee Industries Co., Ltd., Hwaseong, Korea). White distilled vinegar made with jasmine tea extract as a natural flavoring agent (Verdad® Avanta™ F100, Corbin/Purac, Gorinchem, Netherlands) was purchased from a commercial market. This vinegar product contains lactic acid.

Three batches of typical emulsion-type sausage were manufactured with 0, 1, and 2% vinegar and one with 0.02% sodium nitrite as control. The ground meat was mixed with back fat, iced water, and additives in a silent cutter (Talsa K30, DSL Food Machinery Ltd., Valencia, Spain) depending on the formula of the four treatments. The formulation of the sausage is shown in Table 1, and the manufacturing process is Fig. 1. The temperature of the mixture was maintained below 10 °C and was monitored using a digital thermometer (TM-747DU, Tenmars Electronics Co., Ltd., Taipei, Taiwan). After emulsification, each meat batter was stuffed in collagen casing in (25-mm diameter; NDX, Viscofan, Ceske Budejovice, Czech Republic) and sausage was produced (approximately 10 g; 45-mm length). Then, the sausages were vacuum-packaged in linear low density polyethylene bags (LLDPE; 25 × 35-cm; oxygen permeability of 22.5 mL/m^2/24 h atm at 60% relative humidity/25 °C; water vapor permeability of 4.7 g/m^2/24 h at 100% relative humidity/25 °C). Approximately 10 sausages were included in one vacuum-package. Then, the one or two packages used were cooked together in a water bath (WB-22 Daihan, Wanju, Korea) at 80 ± 1 °C until the internal temperature of the sausages reached 75 °C. The internal temperature of the sausages was monitored using the extra

Table 1
Formulations (%) for manufacturing emulsion-type sausages with different levels of vinegar.

Ingredients	Sodium nitrite (PC)[a]	Addition level of vinegar		
		0%	1%	2%
Pork meat	66.34	66.34	66.34	66.34
Back fat	12.00	12.00	12.00	12.00
Iced water	17.70	17.70	17.70	17.70
Sodium chloride	1.20	1.20	1.20	1.20
Isolated soy protein	1.42	1.42	1.42	1.42
Spice mix	1.14	1.14	1.14	1.14
Transglutaminase	0.20	0.20	0.20	0.20
Total	100.00	100.00	100.00	100.00
Vinegar	–	–	1.00	2.00
Sodium nitrite	0.02	–	–	–

[a] PC, Positive control.

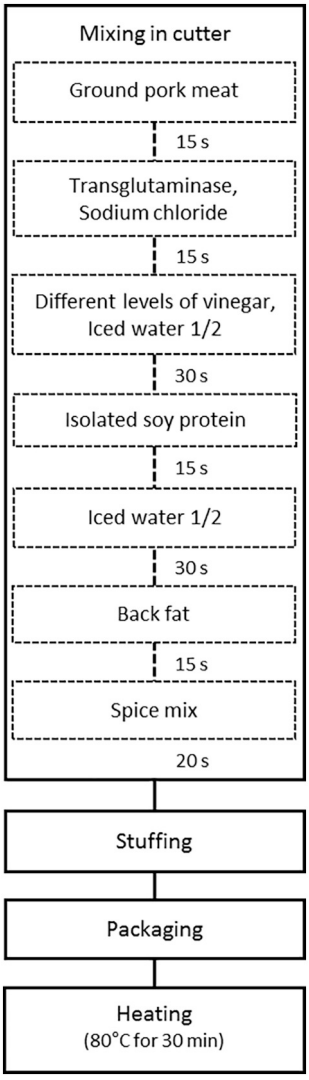

Fig. 1. Flow diagram for manufacturing process of emulsion-type sausages with different levels of vinegar.

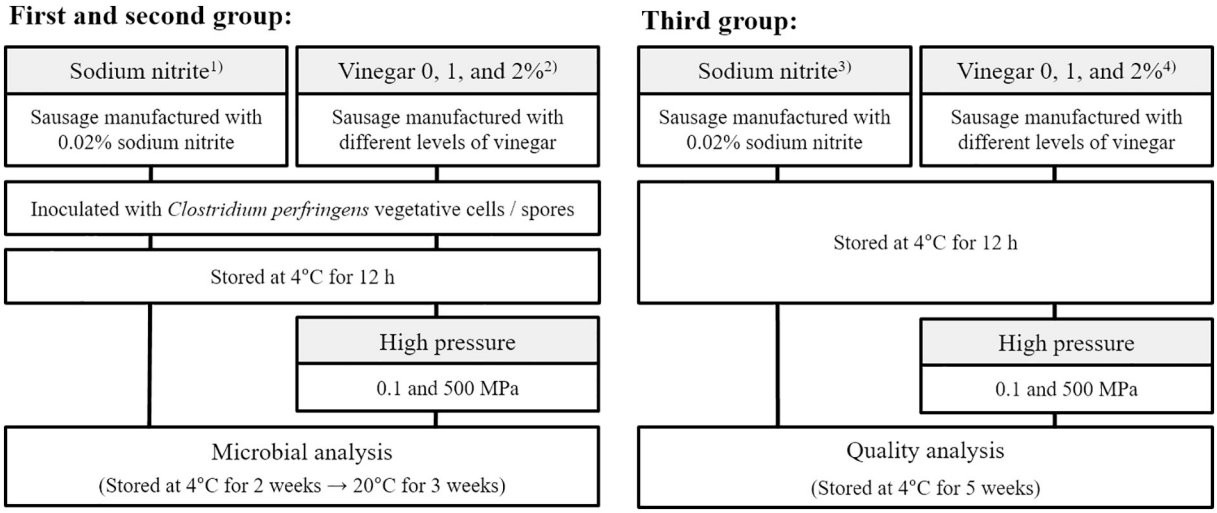

Fig. 2. Diagram illustrating the experimental procedure of the present study. Totally 112 sausages were actually used from one replication (batch). 1)Positive control for microbial analysis: type of microorganism (2, vegetative vs spore) × storage week (6, 0 ~ 5th week) = 12; 2)Type of microorganism (2, vegetative vs spore) × high pressure (2, 0 vs 500 MPa) vinegar addition (3, 0%, 1%, and 2%) × storage week (6, 0 ~ 5th week) = 72; 3)Positive control for quality analysis: storage week (4); 4)Quality analysis: high pressure (2, 0 vs 500 MPa) × vinegar addition (3, 0%, 1%, and 2%) × storage week (4, 0, 1, 3, and 5 week) = 24.

sausage pack by inserting the real time mode of thermometer with a probe type thermocouple (TM-747DU) into the center of the sausage. This cooking process ensured the absence of interference from endogenous bacteria prior to inoculation with *C. perfringens*. Next, the samples were cooled in iced water for 30 min.

Manufactured sausages were randomly divided into three groups. The first group was inoculated with *C. perfringens* vegetative cells, and the second group was inoculated with *C. perfringens* spores to confirm the antimicrobial effect of HP, vinegar, or the combined treatments. The third group was not inoculated and used to analyze the quality properties of the sausages affected by HP, vinegar, or combined treatments (Fig. 2). Eleven vacuum bags filled with sausages were prepared for first and second groups, respectively, while nine vacuum bags were prepared for third group. Subsequently, HP was applied to both groups except for sausages containing sodium nitrite.

2.1.2. HP treatment and storage

Vacuum-packaged sausages which were submerged in water in the treatment chamber (cylinder 200-mm internal diameter, 80-mm external diameter, 2250-mm length, and working volume of 50 L) were processed using the HP processing unit (VC-50, Innoway, Anyang, Korea). Then, the samples were pressurized with 4 cycles of HP of 500 MPa each for 3 min. This experimental condition was based on the results of other studies showing that multiple-cycle HP treatment was more effective than single-cycle treatments for inactivating pathogenic microorganisms in raw meat (Morales, Calzada, Ávila, & Nuñez, 2008; Morales, Calzada, Rodríguez, Paz, & Nuñez, 2009). The time required for pressure (500 MPa) to increase and release were 255 and 125 s, respectively. Temperature inside this equipment was not controlled, and hence only the initial and final temperature of the sausages could be monitored using an infrared thermometer (ST-101, Sinyoung Choukki Co., Ltd., Bucheon, Korea). The temperatures of the sausages were maintained below 5 °C. HP-untreated samples were maintained under atmospheric pressure (0.1 MPa) and stored at 4 °C. After HP treatment, sausage samples for microbial analysis were stored at 4 °C for 2 weeks, following which the temperature was increased to 20 °C and maintained at that temperature for 3 weeks. The two storage temperatures were selected to simulate proper refrigeration conditions (4 °C) and abused temperature (20 °C) which represented the typical "poor control" conditions encountered during distribution and storage of the product in the food chain that possibly favor spore germination and outgrowth. The sausages for quality analysis were stored at 4 °C for 5 weeks. A diagram illustrating the experimental procedure is shown in Fig. 2.

2.2. Microbial analysis

2.2.1. Preparation of C. perfringens

Four *C. perfringens* type F strains (KCCM12098, KCCM40946, KCCM40947, and KCTC5101) were cultivated independently in cooked meat medium (CMM; Oxoid, Hampshire, UK) at 37 °C for 24 h under anaerobic conditions. One milliliter of each starter culture was transferred to 9-mL brain heart infusion medium (BHI; Difco, Becton Dickinson, France), and anaerobically incubated in a jar using a AnaeroGEN™ Gas-Pack (Oxoid, Hampshire, UK) at 37 °C for 24 h. Then, the four strains were mixed and centrifuged at 1912 × *g* for 15 min at 4 °C (Continent 512R, Hanil Co., Ltd., Incheon, Korea). The vegetative cells of the strains were washed twice and suspended in sterile phosphate-buffered saline (PBS).

To obtain *C. perfringens* spores, four *C. perfringens* type F strains were also cultivated independently in CMM at 37 °C for 24 h under anaerobic conditions. The starter culture (100 µL) was transferred into 10-mL fluid thioglycollate (FTG; Difco) broth. The inoculated culture was heated at 75 °C for 20 min to inactivate the vegetative cells, and incubated at 37 °C for 18 h. Subsequently, the culture (100 µL) was transferred to freshly prepared FTG (10 mL) and incubated at 37 °C for 4 h. The incubated culture (500 µL) was then transferred to 49.5-mL modified Duncan-Strong medium (4.5-g proteose peptone, 1.2-g yeast extract, 0.3-g sodium thioglycolate, 3-g sodium phosphate, 1.2-g raffinose, 15-mL caffine, and 300-mL distilled water) and anaerobically incubated in a jar using a AnaeroGEN™ Gas-Pack (Oxoid) at 37 °C for 24 h. The culture of each strain was centrifuged at 1912 × *g* for 15 min at 4 °C (Continent 512R, Hanil Co., Ltd) and washed twice with PBS. Finally, the spores were suspended in PBS. The spore crops were mixed in equal quantities before making a mixture of strains for inoculation.

2.2.2. Inoculation

Before the HP treatment, sausage samples were inoculated with vegetative cells or spores of *C. perfringens*. Each sausage sample (10 g) was dipped in PBS (300 mL) containing the inoculum {which was approximately 8 log colony-forming units (CFU)/mL}and stirred for 2 min. After dipping, the samples were transferred to Petri dishes and air-dried for 10 min to allow attachment of *C. perfringens* to the dishes. Then, the sausages were vacuum-packaged in LLDPE bags. After

inoculation, the sausage samples were stored at 4 °C for 12 h to allow spore germination. The initial number of vegetative cells and spores in sausage were approximately 4.4 and 2.6 log CFU/g, respectively.

2.2.3. Enumeration

After HP treatment, the number of vegetative cells and spores of *C. perfringens* in sausage samples were analyzed during the storage period of 5 weeks. Sausage sample (10 g) was added to a sterile Whirl-Pak bags (19 × 30-cm^2; Nasco, Fort Atkinson, WI, USA) with 90-mL 0.1% (w/v) sterile buffered peptone water (BPW; Becton, Dickinson and Company, Sparks, MD, USA) as dilution fluid. And the contents were homogenized for 1 min at 8 strokes/s using a bag mixer (BagMixer; Interscience, St. Nom, France). Immediately after homogenization, the samples were serially ten-fold diluted with BPW. One-hundred microliters of appropriate dilutions were spread-plated in duplicate on tryptose-sulfite-cycloserine agar (TSC, Difco). After the plates were dried, they were overlaid with additional 10-mL TSC and anaerobically incubated at 37 °C for 18 h using the Gas Pak system (Oxoid Anaerogen 2.5-L Sachet, Thermo Fisher Scientific). Typical *C. perfringens* colonies were enumerated, and the counts were expressed as log CFU/g of sausage sample.

2.3. Quality analysis

2.3.1. pH

Each sausage sample (1 g) was homogenized with 9 mL distilled water (DW) using a homogenizer (T10 basic, Ika Works, Staufen, Germany) and centrifugation at 2265 × *g* for 10 min at 4 °C (Continent 512R, Hanil Co., Ltd). Then, the centrifuged homogenates were filtered through filter paper (Whatman No. 4, Whatman International Ltd., Kent, UK). The pH of the filtrate was measured using a pH meter (SevenGo, Mettler-Toledo International Inc., Schwerzenbach, Switzerland).

2.3.2. Water holding capacity

The sausage sample (5 g) was wrapped in a filter paper (Whatman No. 4) and placed in a centrifuge tube, followed by centrifuged (Continent 512R, Hanil Co., Ltd) at 252 × *g* for 10 min. The released water content was calculated as difference in sample weight before and after centrifugation and expressed as a percentage. The moisture content of sausages was determined according to Association of Official Analytical Chemists method (AOAC, 1995). The WHC percentage was calculated using the following formula:

$$\text{Released water content (\%)} = \frac{\text{Weight before centrifuging} - \text{Weight after centrifuging}}{\text{Weight before centrifuging}} \times 100$$

$$\text{Water holding capacity (\%)} = \frac{\text{Moisture content} - \text{Released water content}}{\text{Moisture content}} \times 100$$

2.3.3. Cooking loss

Meat batter (approximately 50 g) was vacuum-packaged with polyethylene bags for cooking. Samples were heated for 30 min at 80 °C in the water bath and weighed after removing the water on the surface and inside the casing using a paper towel. The weight changes of sausages before and after cooking were calculated as the percentage weight loss of a sample.

$$\text{Cooking loss (\%)} = \frac{\text{Weight before cooking} - \text{Weight after centrifuging}}{\text{Weight before cooking}} \times 100$$

2.3.4. Texture profile analysis

Sausage samples of 2.5-cm diameter and 2.0-cm length were compressed twice to 60% of their original height using a TA1 texture analyzer (AMETEK Lloyd instruments Ltd., Fareham, UK) attached with a 70-mm compression plate at a test speed of 2.0-mm/s and trigger force of 1 N. Texture profile analysis was performed using the NexygenPlus™ software (AMETEK Lloyd instruments Ltd.), and hardness (N), springiness, and chewiness (N) values were recorded.

2.3.5. Instrumental color analysis

The surface color of sausages was measured using a colorimeter (CM-5, Konica Minolta Co., Ltd., Osaka, Japan) with a 3-mm measuring port. The instrument was calibrated using a standard black and white plate. Then, the color values were expressed as L^* (+lightness, −darkness), a^* (+redness, −greenness), and b^* (+yellowness, −blueness) values.

2.3.6. Measurement of 2-thiobarbituric acid reactive substance level

Lipid oxidation was evaluated by calculating the concentration of malondialdehyde as a 2-thiobarbituric acid reactive substances (TBARS) value. The TBARS values of the sausage samples were measured according to method of Lee et al. (2018). Each sausage sample (5 g) was homogenized with 15-mL DW and 50-μL butylated hydroxytoluene (7.2% in ethanol) using a homogenizer (T10 basic, Ika Works). The supernatant (2 mL) was transferred to a test tube after centrifugation (Continent 512R, Hanil Co., Ltd) at 2265 × *g* for 10 min and mixed with 4-mL thiobarbituric acid (0.02 M)/trichloroacetic acid (15%) solution. Then, the test tubes were heated in a water bath at 90 °C for 30 min, cooled in an ice water for 30 min, and centrifuged (Continent 512R, Hanil Co., Ltd) at 2265 × *g* for 15 min. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer (X-ma 3100, Human Co. Ltd., Korea). The TBARS values were calculated using a standard curve of malondialdehyde (MDA) and expressed as mg MDA/kg sample.

2.4. Statistical analysis

Whole experimental procedures were conducted three individual trials (replication). In each trial, two measurements were performed. Then, statistical analysis was performed using one-way analysis of variance, and significant differences between mean values were identified using Student-Newman-Keuls multiple range test of the SAS statistical software (SAS, Release 9.4; SAS Institute Inc., Cary, NC, USA) with a significance level of $P < 0.05$. In addition, a multifactorial analysis of variance using the general linear model was applied to investigate the effect of HP and vinegar addition.

3. Results and discussion

3.1. Growth inhibition of vegetative cells and spores of C. perfringens

The HP and vinegar treatment of emulsion-type sausages differentially affected growth inhibition of the vegetative cells and spores of *C. perfringens* (Tables 2 and 3). HP treatment of sausages significantly reduced the number of vegetative cells of *C. perfringens*, whereas spore number was not significantly reduced. The initial amount of inoculated vegetative cells ranged from 4.0 to 4.6 log CFU/g (data not shown). Immediately after HP treatment, all treatments significantly reduced the vegetative cell number of *C. perfringens* in sausages. Irrespective of vinegar addition, HP treatment showed lower counts of vegetative cells by 2 weeks at 4 °C ($P < 0.05$). From 3 to 5 weeks at 20 °C, the group without HP and vinegar treatment showed rapid growth of vegetative cells, reaching 7.2 log CFU/g at the end of storage period, although HP treatment showed 3.8 log CFU/g. Therefore, HP can be used to reduce the initial number and retard the growth of *C. perfringens* vegetative cells compared to the non-pressurized group (0.1 MPa).

Table 2
Effect of high pressure (HP) and vinegar addition on *Clostridium perfringens* vegetative cell counts (log CFU/g) in emulsion-type sausages.

HP (MPa)	Treatments	Storage at 4 °C (weeks)			Storage at 20 °C (weeks)			SEM[1)]
		0	1	2	3	4	5	
0.1	Sodium nitrite	4.1^{Ba}	3.9^{Aa}	4.1^{Aa}	2.8^{Bb}	2.5^{Cb}	3.2^{BCab}	0.293
	0% vinegar	4.6^{Ac}	3.6^{ABd}	4.0^{Acd}	5.5^{Ab}	7.2^{Aa}	7.2^{Aa}	0.200
	1% vinegar	3.8^{BCa}	3.6^{ABa}	3.2^{Bb}	2.7^{Bc}	2.5^{Cc}	2.7^{BCc}	0.112
	2% vinegar	3.7^{Ca}	3.3^{Bab}	3.1^{Bb}	2.6^{Bc}	2.6^{Cc}	2.3^{Cc}	0.119
500	0% vinegar	2.7^{Dab}	2.6^{Cab}	2.3^{Cb}	4.0^{ABab}	4.4^{Ba}	3.8^{Bab}	0.412
	1% vinegar	2.5^{D}	2.4^{C}	2.4^{C}	2.5^{B}	2.3^{C}	2.6^{BC}	0.118
	2% vinegar	2.6^{D}	2.5^{C}	2.3^{C}	3.2^{B}	2.5^{C}	2.4^{C}	0.355
SEM[2)]		0.118	0.131	0.113	0.484	0.166	0.300	

$^{A–D}$Values with different letters within the same column differ significantly ($P < 0.05$).
$^{a–d}$Values with different letters within the same row differ significantly ($P < 0.05$)
1) Standard error of mean (n = 18).
2) (n = 21).

The addition of vinegar in sausages significantly reduced the growth of *C. perfringens* vegetative cells and spores irrespective of the amount added (Tables 2 and 3). The vegetative cell population was maintained during the storage period in samples with vinegar alone and in combination with HP treatment, and showed no significant difference with the sodium nitrite group during temperature abusive storage (Table 2). At the end of the storage period, the vegetative cell counts did not vary significantly between any groups with HP and/or vinegar treatment and those containing sodium nitrite ($P > 0.05$).

The number of *C. perfringens* spores in sausages did not vary significantly among the treatments for 2 weeks at 4 °C (Table 3). The population of *C. perfringens* spores in sausages was maintained for 5 weeks by the addition of vinegar (1 and 2%, respectively), even after temperature abuse at 20 °C, irrespective of HP treatment. However, the sausages without vinegar showed rapid increase in the number of *C. perfringens* spores, which was higher than that in the sodium nitrite group, irrespective of HP treatment ($P < 0.05$). These results suggest that vinegar inhibits the germination and outgrowth of *C. perfringens* spores over the entire shelf life of 5 weeks. These observations are in agreement with the results of Valenzuela-Martinez et al. (2010), who reported that vinegar is effective in controlling germination and outgrowth of *C. perfringens* spores in ground turkey roast without sodium nitrite. Similarly, Li et al. (2012) reported that the number of *C. perfringens* spores in sodium-roasted beef was controlled by vinegar treatment during abusive exponential cooling.

In our preliminary study, 0.5% vinegar was added to optimize its addition level in sausages following the manufacture process of Fig. 1 and Table 1. Vinegar treatment did not result in any significant differences in inactivation of *C. perfringens* vegetative cells and spores during storage (0, 1, 2, 4, 6, and 9 days) at 4 °C (data not shown). However, in the present study, vinegar treatment inhibited the growth of *C. perfringens* irrespective of the amount added (1 and 2%) during storage. Therefore, 1% vinegar may be sufficient for inactivating *C. perfringens* in sausages.

Overall, the HP treatment of sausages effectively inactivated the vegetative cells of *C. perfringens* but not its spores. Previous studies showed that HP treatment at 400–600 MPa for < 10 min is typically used for the inactivation of spoilage and pathogenic microorganisms in meat products and extension of shelf-life (Bajovic, Bolumar, & Heinz, 2012; Evelyn & Silva, 2016). However, spores are highly resistant to pressure, and are capable of surviving in up to 1200-MPa pressure (Larson, Hartzell, & Diehl, 1918). Furthermore, HP treatment of sausage samples without vinegar addition in this study showed earlier spore germination after 3 to 5 weeks at 20 °C (Table 3). This can be partially explained by the results of previous studies showing that HP treatment can lead to germination of spores of different microorganisms. This is probably because pressurization at 500–600 MPa opens channels for the release of dipicolinic acid (DPA) from the spore core, which induces spore germination (Paidhungat et al., 2002; Setlow, 2003). In addition, application of two cycles of pressure at 500 MPa slightly increased *Bacillus* spore germination compared to the use of single pressure cycle (Black et al., 2008). Therefore, treatment with a combination of HP and vinegar, but not HP alone, was required for inactivating *C. perfringens* spores. Sijtsema, Brookmeyer, and Yi (2017) observed that treatment with a combination of vinegar and jasmine tea extract controlled the growth of total aerobic bacteria and *Salmonella* and provided a clean label option for the meat industry (i.e., products that do not contain chemical ingredients). In this study, the addition of vinegar significantly inactivated the vegetative cells and spores of *C. perfringens* in sausages, showing values similar to that of the sodium

Table 3
Effect of high pressure (HP) and vinegar addition on *Clostridium perfringens* spore counts (log CFU/g) in emulsion-type sausages.

HP (MPa)	Treatments	Storage at 4 °C (weeks)			Storage at 20 °C (weeks)			SEM[1)]
		0	1	2	3	4	5	
0.1	Sodium nitrite	2.6	2.7	2.5	2.9^{CD}	3.9^{B}	2.5^{C}	0.387
	0% vinegar	2.6^{b}	2.1^{b}	2.6^{b}	5.4^{Ba}	5.2^{ABa}	5.1^{Ba}	0.506
	1% vinegar	2.7	2.7	2.7	2.6^{D}	2.4^{B}	2.8^{C}	0.116
	2% vinegar	2.5	2.7	2.6	2.3^{D}	2.2^{B}	2.3^{C}	0.213
500	0% vinegar	2.6^{b}	2.6^{b}	2.2^{b}	6.2^{Aa}	7.0^{Aa}	6.7^{Aa}	0.231
	1% vinegar	2.3	2.4	2.5	3.6^{C}	4.3^{B}	2.5^{C}	0.627
	2% vinegar	2.2	2.5	2.5	2.2^{D}	2.6^{B}	2.3^{C}	0.178
SEM[2)]		0.135	0.296	0.155	0.241	0.648	0.448	

$^{A–D}$Values with different letters within the same column differ significantly ($P < 0.05$).
^{a,b}Values with different letters within the same row differ significantly ($P < 0.05$).
1) Standard error of mean (n = 18).
2) (n = 21).

Table 4
Effect of high pressure (HP) and vinegar addition on the pH and water holding capacity (WHC) of emulsion-type sausages stored at 4 °C.

Traits	HP (MPa)	Treatments	Storage (weeks)				SEM[1)]
			0	1	3	5	
pH	0.1	Sodium nitrite	6.25^{Cb}	6.24^{Db}	6.26^{Dab}	6.28^{Da}	0.006
		0% vinegar	6.27^{Ba}	6.20^{Ec}	6.26^{Da}	6.24^{Eb}	0.004
		1% vinegar	6.28^{Bb}	6.26^{Cc}	6.31^{Ba}	6.31^{Ca}	0.003
		2% vinegar	6.35^{Ab}	6.33^{Ac}	6.34^{Ac}	6.36^{Aa}	0.003
	500	0% vinegar	6.24^{C}	6.24^{D}	6.23^{E}	6.24^{E}	0.002
		1% vinegar	6.29^{B}	6.28^{B}	6.29^{C}	6.29^{D}	0.004
		2% vinegar	6.35^{Aa}	6.33^{Ac}	6.34^{Ab}	6.34^{Bb}	0.002
	SEM[2)]		0.004	0.003	0.005	0.004	
WHC (%)	0.1	Sodium nitrite	74.50^{Eb}	73.28^{Cb}	87.71^{Da}	72.49^{Cb}	2.553
		0% vinegar	78.16^{Db}	77.77^{Bb}	89.31^{Ca}	74.10^{Cb}	1.126
		1% vinegar	80.84^{Bb}	77.26^{Bc}	89.82^{BCa}	79.37^{BCb}	0.557
		2% vinegar	80.53^{Bb}	78.90^{Bb}	91.53^{Aa}	78.85^{BCb}	0.475
	500	0% vinegar	79.75^{Cc}	78.02^{Bc}	89.23^{Ca}	82.21^{ABb}	0.629
		1% vinegar	84.20^{Ac}	83.00^{Ad}	90.92^{ABa}	87.68^{Ab}	0.232
		2% vinegar	84.74^{Ab}	83.00^{Ab}	91.78^{Aa}	83.85^{ABb}	0.495
	SEM[2)]		0.193	1.066	0.368	1.961	

$^{A–E}$Values with different letters within the same column differ significantly ($P < 0.05$).
$^{a–d}$Values with different letters within the same row differ significantly ($P < 0.05$).
[1)] Standard error of mean (n = 12).
[2)] (n = 21).

nitrite-treated group irrespective of HP treatment. Therefore, vinegar-treated products can meet the clean label criteria, as it is safe and provides antimicrobial activity.

3.2. pH and WHC

With the addition of increasing amounts of vinegar irrespective of HP treatment and storage period, the pH values of sausages increased (Table 4). The sausages treated with 2% vinegar showed the highest pH, which might be due to the inherently high pH of vinegar (7.50). The pH values were dependent on the amount of vinegar added. Similarly, Sijtsema et al. (2017) reported that addition of vinegar containing jasmine tea extract in chicken patties slightly increased the pH.

HP and/or vinegar treatment resulted in significantly higher WHC during 5 weeks of storage than sodium nitrite addition (Table 4). The highest WHC ($P < 0.05$) was observed in HP and vinegar-treated sausage samples irrespective of the amount of vinegar added. This result might be partially affected by the increase in pH values after vinegar addition ($P = 0.0092$). In addition, HP treatment of sausage samples significantly affected the increase in WHC ($P = 0.0009$). A previous study reported that HP treatment enhanced WHC of restructured pork meat (Hong, Park, Kim, & Min, 2006). In addition, Grossi, Søltoft-Jensen, Knudsen, Christensen, and Orlien (2012) observed that HP treatment of meat products increased myofibrillar protein solubility by disrupting electrostatic and hydrophobic interactions and hydrogen bonding, which increased WHC. In the present study, initial WHC was maintained until 1 week after all treatments, which peaked at week 3, and decreased thereafter until end of storage. However, the treatments did not differ significantly in terms of cooking loss (data not shown, $P > 0.05$).

3.3. Texture profile analysis

In this study, the hardness, springiness, and chewiness were affected by HP and/or vinegar treatment (Table 5). Regardless of HP treatment, the sausage samples without vinegar generally showed similar hardness and chewiness as sodium nitrite-treated samples ($P > 0.05$) during the entire storage period, except for hardness in the 3rd and 5th weeks.

Vinegar treatment significantly increased hardness of the sausages, which might be due to higher emulsification by the three-dimensional network formed after vinegar addition as suggested by Yang and Han (2002). The emulsion stability of mayonnaise dressing increased with vinegar concentration (Yang & Han, 2002). Kumar and Tanwar (2011) reported that chicken nuggets containing mustard showed higher sensory texture scores due to higher emulsification and WHC of mustard. In sausages at 5 weeks of storage, similar values ($P > 0.05$) in springiness were observed except for samples with 2% vinegar without HP treatment. Vinegar treatment of sausage samples decreased and increased chewiness at the initial and later storage time points, respectively. In general, HP treatment of sausage samples resulted in significantly higher chewiness. Fernandez, Cofrades, Solas, Carballo, and Colmenero (1998) reported that pressurization of chicken meat batters at 200 MPa and 70 °C increased hardness and chewiness compared to non-pressurized samples. A previous study reported that cooked sausages pressurized at 500 MPa were less firm than untreated ones (Mor-Mur & Yuste, 2003). In this study, the HP treatment did not critically affect changes in texture, and the cooking process possibly induced gelation before HP treatment. This may minimize the effect of HP treatment on changes in texture properties (Mor-Mur & Yuste, 2003).

3.4. Instrumental color analysis

Changes in color (L^*, a^*, and b^*) were dependent on HP, vinegar, and their combination treatment during 5 weeks of storage (Table 6). Independent HP and vinegar treatments decreased L^* values during the entire storage period except for samples treated with HP during initial storage. This indicates that cooked sausages tend to be darker. Similarly, Fernandez et al. (1998) reported decrease in L^*, a^*, and b^* values in chicken batters after HP treatment. This discoloration has been associated with pressure, which modifies hemoproteins, and sarcoplasmic and myofibrillar proteins of meat (Cheftel & Culioli, 1997). Furthermore, the reduction in L^* values was possibly due to the brownish tint of vinegar used in this study. This observation was different from that of Sikes, Tobin, and Tume (2009) who observed an increase in L^* value of pressurized beef sausage batters. However, when the product was cooked, myoglobin was converted into a nitrosyl-hemochromogen pigment, which is not affected by pressure (Carlez, Veciana-Nogues, & Cheftel, 1995; Mor-Mur & Yuste, 2003). This could explain why L^* value increased negligibly in this study.

As expected, sausage samples with sodium nitrite had the highest a^* values ($P < 0.05$) during the entire storage period. The a^* values were not generally affected ($P > 0.05$) by HP treatment during storage, but

Table 5
Effect of high pressure (HP) and vinegar addition on texture analysis of emulsion-type sausages stored at 4 °C.

Traits	HP (MPa)	Treatments	Storage (weeks)				SEM[1)]
			0	1	3	5	
Hardness (N)	0.1	Sodium nitrite	46.73^{CDb}	58.70^{Ca}	47.75^{Eb}	47.32^{Cb}	2.332
		0% vinegar	43.48^{Dc}	60.03^{BCa}	49.97^{DEb}	47.00^{Cbc}	1.107
		1% vinegar	63.62^{Bb}	71.30^{BCb}	81.31^{Ba}	69.14^{Ab}	2.998
		2% vinegar	70.94^{A}	75.57^{B}	70.26^{C}	73.51^{A}	1.788
	500	0% vinegar	51.37^{C}	64.40^{BC}	56.80^{D}	54.35^{B}	4.204
		1% vinegar	63.64^{Bd}	91.33^{Aa}	79.81^{Bb}	73.25^{Ac}	1.168
		2% vinegar	64.57^{Bc}	68.96^{BCbc}	92.23^{Aa}	74.76^{Ab}	2.421
	SEM[2)]		1.712	3.608	2.308	1.928	
Springiness	0.1	Sodium nitrite	0.89^{Aa}	0.84^{Aa}	0.75^{Aa}	0.43^{Bb}	0.073
		0% vinegar	0.72^{Ba}	0.84^{Aa}	0.68^{Aa}	0.52^{Ab}	0.046
		1% vinegar	0.60^{BC}	0.60^{AB}	0.40^{C}	0.39^{B}	0.056
		2% vinegar	0.60^{BCa}	0.59^{ABa}	0.49^{BCb}	0.41^{Bc}	0.022
	500	0% vinegar	0.68^{BCa}	0.59^{ABab}	0.61^{ABab}	0.40^{Bb}	0.057
		1% vinegar	0.57^{BCa}	0.45^{Bb}	0.46^{BCb}	0.35^{Bc}	0.016
		2% vinegar	0.52^{Ca}	0.54^{ABa}	0.50^{BCa}	0.36^{Bb}	0.016
	SEM[2)]		0.039	0.068	0.044	0.019	
Chewiness (N)	0.1	Sodium nitrite	8.05^{AB}	11.78^{B}	7.35^{C}	7.68^{D}	1.192
		0% vinegar	6.80^{B}	9.87^{B}	8.54^{C}	8.99^{CD}	0.818
		1% vinegar	9.04^{ABb}	11.43^{Bab}	15.42^{Ba}	16.23^{ABa}	1.287
		2% vinegar	10.78^{Ab}	12.01^{Bb}	8.12^{Cb}	18.97^{Aa}	1.446
	500	0% vinegar	9.85^{AB}	9.92^{B}	8.66^{C}	12.67^{BC}	1.884
		1% vinegar	9.20^{ABb}	16.40^{Aa}	11.80^{BCb}	17.99^{Aa}	0.915
		2% vinegar	9.20^{ABb}	8.10^{Bb}	20.53^{Aa}	20.18^{Aa}	0.535
	SEM[2)]		0.886	1.204	1.438	1.306	

[A–D]Values with different letters within the same column differ significantly ($P < 0.05$).
[a–d]Values with different letters within the same row differ significantly ($P < 0.05$).
[1)] Standard error of mean (n = 12).
[2)] (n = 21).

were affected ($P < 0.05$) by vinegar treatment at 3 and 5 weeks. In contrast, Yuste, Mor-Mur, Capellas, Guamis, and Pla (1999) reported that cooked sausages treated at 500 MPa had lower a^* and b^* values than non-treated samples. In general, the highest and lowest values (both $P < 0.05$) of a^* were observed for sausage samples treated with sodium nitrite and vinegar, respectively. Vinegar treatment only decreased b^* values, indicating that the color of the product turned blue rather than yellow.

Table 6
Effect of high pressure (HP) and vinegar addition on color analysis of emulsion-type sausages stored at 4 °C.

Traits	HP (MPa)	Treatments	Storage (weeks)				SEM[1)]
			0	1	3	5	
L^*	0.1	Sodium nitrite	67.49^{Aa}	66.80^{Aa}	67.10^{Ba}	64.83^{Bb}	0.234
		0% vinegar	67.39^{Aab}	66.71^{Ab}	68.51^{Aa}	67.06^{Aab}	0.436
		1% vinegar	64.68^{B}	64.16^{C}	65.38^{C}	64.71^{B}	0.389
		2% vinegar	64.83^{B}	63.75^{C}	63.97^{D}	63.70^{B}	0.364
	500	0% vinegar	67.38^{Aa}	65.67^{Bb}	67.35^{Ba}	64.70^{Bb}	0.394
		1% vinegar	66.23^{ABa}	63.76^{Cb}	64.86^{CDb}	62.34^{Cc}	0.400
		2% vinegar	64.83^{Ba}	63.01^{Cb}	62.93^{Eb}	62.05^{Cb}	0.348
		SEM[2)]	0.438	0.306	0.338	0.390	
a^*	0.1	Sodium nitrite	8.13^{Ac}	8.33^{Abc}	8.57^{Aab}	8.80^{Aa}	0.124
		0% vinegar	3.45^{BCb}	3.89^{Ca}	3.97^{Ba}	4.11^{Ba}	0.130
		1% vinegar	3.06^{C}	3.20^{C}	3.17^{C}	3.01^{D}	0.118
		2% vinegar	3.16^{C}	3.29^{C}	3.49^{C}	3.45^{C}	0.148
	500	0% vinegar	3.74^{B}	4.63^{B}	4.11^{B}	4.41^{B}	0.300
		1% vinegar	2.91^{Cb}	3.23^{Cab}	3.49^{Ca}	2.78^{Db}	0.136
		2% vinegar	3.39^{BC}	3.17^{C}	3.28^{C}	3.42^{C}	0.120
		SEM[2)]	0.140	0.244	0.122	0.122	
b^*	0.1	Sodium nitrite	12.02^{Cc}	11.83^{Cc}	13.30^{Ca}	13.93^{Cb}	0.134
		0% vinegar	14.77^{Ab}	14.65^{Ab}	16.16^{Aa}	15.18^{Bb}	0.250
		1% vinegar	13.05^{Bb}	13.27^{Bb}	14.37^{BCa}	13.67^{Cab}	0.261
		2% vinegar	13.01^{Bb}	13.03^{Bb}	14.09^{BCa}	13.52^{Cb}	0.156
	500	0% vinegar	15.37^{A}	14.87^{A}	15.61^{A}	15.79^{A}	0.242
		1% vinegar	13.32^{Bb}	13.50^{Bb}	14.71^{Ba}	13.57^{Cb}	0.221
		2% vinegar	13.67^{Bab}	13.07^{Bb}	13.74^{BCab}	14.01^{Ca}	0.236
		SEM[2)]	0.226	0.160	0.309	0.127	

[A–D]Values with different letters within the same column differ significantly ($P < 0.05$).
[a–d]Values with different letters within the same row differ significantly ($P < 0.05$).
[1)] Standard error of mean (n = 12).
[2)] (n = 21).

Table 7
Effect of high pressure (HP) and vinegar addition on the 2-thiobarbituric acid reactive substances (TBARS) values (mg malondialdehyde/kg sausage) of emulsion-type sausages stored at 4 °C.

HP (MPa)	Treatments	Storage (weeks)				SEM[1)]
		0	1	3	5	
0.1	Sodium nitrite	0.33^{B}	0.33^{D}	0.37^{C}	0.39^{C}	0.015
	0% vinegar	0.50^{Bc}	1.04^{Bb}	1.15^{Bb}	1.47^{Ba}	0.076
	1% vinegar	0.36^{B}	0.38^{C}	0.42^{C}	0.43^{C}	0.019
	2% vinegar	0.37^{B}	0.36^{CD}	0.39^{C}	0.39^{C}	0.011
500	0% vinegar	0.93^{Ad}	1.19^{ABc}	1.33^{Ab}	1.53^{Aa}	0.029
	1% vinegar	0.39^{Bb}	0.39^{Cb}	0.41^{Cab}	0.43^{Ca}	0.009
	2% vinegar	0.36^{Bb}	0.36^{CDb}	0.39^{Ca}	0.40^{Ca}	0.006
SEM[2)]		0.057	0.012	0.025	0.018	

[A–D]Values with different letters within the same column differ significantly ($P < 0.05$).
[a–d]Values with different letters within the same row differ significantly ($P < 0.05$).
[1)] Standard error of mean (n = 12).
[2)] (n = 21).

Overall, addition of vinegar changed the color attributes of the sausages by tarnishing the sample. These changes in L^*, a^*, and b^* values by vinegar treatment were probably due to its inherent color (L^*; 25.60, a^*; 6.12, b^*; 20.71). The color of the final products is mostly influenced by the color of the added ingredients. Color stability is critical for sausages as consumers use discoloration as an indicator of spoilage (Mancini & Ramanathan, 2008). In general, the difference between initial and final storage times was not significant for the color of sausages in this study. A number of studies reported that sausages added with natural ingredient shows lower L^* and a^* values compared to sausage added with synthetic nitrite, such as sodium nitrite (Deda, Bloukas, & Fista, 2007; Krause, Sebranek, Rust, & Mendonca, 2011). However, consumers' interest in “synthetic-nitrite-free” meats products is continuously increased in these days (Yong et al., 2018). Thus, even though sausage color was changed by addition of natural ingredient, using natural ingredients instead of synthetic additives, which are usually mentioned on the clean labels, may be more attractive for consumers (Bruhn, 2007).

3.5. Lipid oxidation

Generally, HP treatment induces lipid oxidation in food (Campus, Flores, Martinez, & Toldrá, 2008). The HP treatment of sausage samples without vinegar addition accelerated lipid oxidation by approximately 3 times (Table 7). Similarly, Cava, Ladero, González, Carrasco, and Ramírez (2009) reported that HP treatment of meat products significantly increased TBARS values even at lower pressure (200 MPa). However, in this study, addition of vinegar decreased the lipid oxidation of sausage samples treated with HP. In addition, vinegar treatment of sausage samples maintained ($P > 0.05$) initial TBARS values, showing 0.36–0.43 mg MDA/kg samples during the entire storage period, which was similar to the value obtained for the sodium nitrite-treated group. This is probably caused by the functional materials of vinegar with antioxidant activity, such as polyphenols and flavonoids, which is similar to that of sodium nitrite-containing meat products (Choe & Kim, 2016; Sakanaka & Ishihara, 2008). In addition, jasmine tea extract in vinegar contains catechins, which show strong antioxidant activity (Benzie & Szeto, 1999). However, the addition of different concentrations of vinegar did not inhibit TBARS values during the entire storage period ($P > 0.05$). These results suggest that sausages treated with 1% vinegar can substitute sodium nitrite-treated sausages in terms of inhibition of lipid oxidation.

4. Conclusion

Each HP and vinegar treatment of nitrite-free sausages inhibited the growth of vegetative cells and both vegetative cells and spores of *C. perfringens*, respectively, during the entire of storage period. In addition, vinegar treatment of sausages showed no significant difference from sodium nitrite treatment with respect to the populations of vegetative cells and spores of *C. perfringens*, even under abusive temperature storage at 20 °C. Therefore, sodium nitrite can be replaced by vinegar, and in combination with HP treatment, it can inhibit *C. perfringens* growth in emulsion-type sausages. In conclusion, a combination of HP and vinegar treatment extends the shelf-life and retains sausage quality albeit with discoloration of the final product. Hence, this method may be used for manufacturing safe products with a clean label, which may be perceived by consumers as natural meat products. Further studies are required to assess the effect of these changes on antioxidant and sensory characteristics of sausages.

Declaration of interest

None.

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