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Innovative Food Science and Emerging Technologies 8 (2007) 259 – 268
Innovative Food Science & Emerging Technologies
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Effects of high hydrostatic pressure on *Eimeria acervulina* pathogenicity, immunogenicity and structural integrity

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Received 10 November 2006; accepted 15 January 2007

Abstract

Eimeria acervulina is a protozoan parasite that can cause intestinal lesions and reduced weight gain in chickens. *E. acervulina* oocysts were treated by high hydrostatic pressure and evaluated for pathogenicity, immunogenicity, and structural integrity. Pressure treatment of *E. acervulina* oocysts at 550 MPa for 2 min at 4, 20 or 40 °C rendered the parasites nonpathogenic to chickens. Pressure treatment at 40 °C also prevented fecal shedding of oocysts. Upon challenge with non-pressurized *E. acervulina* oocysts, partial immunity was observed with a reduction in lesion severity in chickens that had been inoculated with pressure-treated oocysts. No changes to the fragility and permeability of the oocyst wall or excystation of sporocysts were observed as a result of pressure treatment. Light and scanning electron microscopy revealed no changes to the whole oocyst or sporocysts. Recovery and the morphology of excysted sporozoites were altered by pressure treatment. These results suggest that pressure affects sporozoite integrity.
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Keywords: High pressure processing; Inactivation; Microscopy; *Cyclospora* surrogate; Sporozoite; *Eimeria*; Immunogenicity; Vaccine

Industrial relevance: High-hydrostatic pressure processing has been shown to inactivate various microorganisms and is utilized commercially for enhanced food safety and quality. Some pathogenic microorganisms have been inactivated by HPP yet retain immunogenic properties suggesting potential application for vaccine development. *Eimeria acervulina* is a poultry pathogen for which new vaccines are sought. *E. acervulina* is also closely related to *Cyclospora cayetanensis*, a foodborne human pathogen. HPP was explored for effect on *E. acervulina* for potential vaccine development for chickens and for insight on HPP effects on parasites for enhanced safety of human foods.

1. Introduction

Eimeria acervulina is an avian protozoan coccidian parasite. It is one of seven *Eimeria* species known to infect chickens, and one of three species of particular commercial importance to the poultry industry. Annual losses of $450 million by the United States poultry broiler industry ($800 million worldwide) due to unrealized weight gain and inefficient feed utilization are attributed to coccidiosis (Allen & Fetterer, 2002). The infection initiates after the ingestion of sporulated oocysts of *Eimeria* species. Oocysts are mechanically broken open in the gizzard to release sporocysts; and in the small intestine, sporocysts excyst to release sporozoites in the presence of trypsin, bile salts and carbon dioxide (Allen & Fetterer, 2002). Sporozoites are the infectious stage that must invade the host for the parasite life cycle to continue through asexual (schizony) and sexual (gametogony) development, formation of unsporulated oocysts in the intestinal tract, and release of oocysts in feces (Reid, 1972). *Eimeria* infections cause intestinal tissue damage, disruption of normal feeding and nutrient absorption, dehydration, blood loss, and mortality in severe cases (McDougald & Reid, 1991). Poultry with coccidial infections are also more vulnerable to colonization by bacteria such as *Clostridium perfringens* and *Salmonella typhimurium*, which can further compromise poultry health as well as create a harborage for human pathogens (McDougald & Reid, 1991). The poultry industry manages coccidiosis primarily by medication of feed

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doi:10.1016/j.ifset.2007.01.004

with anti-coccidial drugs, but also by vaccination. Both approaches have limitations, one of the more notable being the emergence of resistance of *Eimeria* species to anti-coccidial drugs (Chapman, 1993).

Eimeria also bears morphological and genetic similarities to *Cyclospora cayetanensis* (Shields & Olson, 2003), a human pathogen responsible for foodborne outbreaks associated with raspberries, basil, and salad greens (Dawson, 2005). While traditional heat processing of foods can inactivate *C. cayetanensis*, organoleptic qualities of these foods can be dramatically changed by heat, and alternative processes are sought. However, *C. cayetanensis* is particularly difficult to study in the laboratory as it appears to be strictly limited to the human host. For this reason, *E. acervulina* has been utilized as a surrogate for *C. cayetanensis* (Lee & Lee, 2001).

The efficacy of high hydrostatic pressure processing (HPP) for inactivation of bacteria, fungi, and viruses has been well documented (Patterson, 2005), and thus, HPP has received considerable interest for foods. HPP technology has been selectively commercialized in food processing for the inactivation of microorganisms and has also been demonstrated to alter enzyme activity and modify rheological properties of foods (Hoover, 1993). HPP is believed to affect non-covalent interactions and disrupt large macromolecular assemblies, and therefore, may disrupt multiple biological structures and functions (Patterson, 2005). However, HPP has minimal effect on fresh-like attributes of foods, including flavors and nutrients (Patterson, 2005), as compared to changes observed with traditional heat processing. Potential for development of vaccines by HPP has also been demonstrated with success reported in inactivating disease agents while maintaining immunogenic properties (Silva, Luan, Glaser, Voss, & Weber 1992; Tian, Ruan, Qian, Shao, & Balny, 2000; Silva, Giongo, Simpson, da Silva Camargos, Silva, & Koury, 2001; Ishimaru, Sá-Carvalho, & Silva, 2004). However, while HPP has been commercialized for select food processes, to-date, no commercial vaccines are known to be developed utilizing HPP.

Limited studies on the effects of HPP on parasites have been reported. Kitching (1957) reported on the effect of pressure on various ciliates and flagellates. Larvae of the parasitic worm *Anasakis simplex* are reportedly sensitive to HPP treatment with potential application for processing affected fish (Molina-García & Sanz, 2002). Among Apicomplexan parasites, *Cryptosporidium parvum*, a protozoan predominantly affecting immune-compromised humans, appears to be sensitive to HPP at fairly high pressures, which may have potential application in the treatment of juices and water (Slifko, Raghubeer, & Rose, 2000). Likewise, *Toxoplasma gondii*, a protozoan associated with undercooked meat products, is sensitive to pressure in both media (Lindsay, Collins, Jordan, Flick, & Dubey, 2005) and ground pork (Lindsay, Collins, Holliman, Flick, & Dubey, 2006). Very limited information is available on the possible structures or functions of parasites that are disrupted by pressure.

In the present study, we explore the effect of HPP on *E. acervulina* pathogenicity, immunogenicity and structural integrity. The findings enhance the understanding of HPP efficacy and mode of action against protozoan parasites of interest for poultry and human health.

2. Materials and methods

2.1. *Eimeria acervulina*

E. acervulina, designated lab strain #12 (originally obtained from Auburn University, propagated at USDA/ARS for more than 30 years) was collected from the feces of Sex-Sal chickens (Moyer's Hatchery, Quakertown, Pennsylvania). Oocysts were suspended in 2.5% potassium chromate and sporulated by forced aeration for 48 to 72 h at 25.6 to 27.8 °C. Sporulated oocysts were further cleaned with bleach (30 min), followed by repeated water washes, and were stored in Dulbecco's Modification of Eagle's Medium with 4.5 g/l glucose and L-glutamine and sodium pyruvate (DMEM, Mediatech, Inc., Herndon, Virginia, USA) at 4 °C until used within 6 months of collection.

2.2. Treatment conditions

2.2.1. High hydrostatic pressure

Sporulated oocysts of *E. acervulina* in DMEM were transferred to sterile polypropylene pouches (VWR International, West Chester, Pennsylvania, USA), and pouches were heat-sealed. Pressure treatments were applied in an Avure PT-1 hydrostatic press (Avure Technologies, Kent, Washington, USA), a research-scale, batch-style processing unit 3 in.^{3} vessel capacity. Oocysts were treated under the following conditions: 550 MPa, 2-min hold time (exclusive of come-up time of approximately 30 s with instantaneous depressurization), with water as the pressure medium at 4 °C, 20 °C, or 40 °C.

2.2.2. Pressure versus temperature effects

To determine adiabatic heat changes, the temperature of the chamber bath in which the pouches were directly submerged during pressure treatment was measured using a type K stainless steel jacketed thermocouple fitted to the chamber cap. The temperature was recorded every 10 s. To differentiate the effects of temperature and pressure at 40 °C–pressure treatments, oocysts were also subjected to heat alone, and viability was determined using *in vivo* methods. The heating conditions determined during pressure were simulated using a thermocycler (Eppendorf Mastercycler® ep, Hamburg, Germany) according to the following program: 55.5 °C, 50.2 °C, 46.6 °C, 44.7 °C, and 43.3 °C consecutively for 24 s each then cooled to 4 °C. Each 0.2-ml PCR strip tube received 125 μl for heat treatment. After heating, the oocysts were collected with repeated washing of tubes with DMEM and resuspended to a final concentration of 4.4×10^5 oocysts per ml for the *in vivo* study.

2.3. Inactivation studies

2.3.1. In vivo infectivity study

Four-week old, Sex-Sal male chickens were used to determine infectivity of untreated, pressure- and heat-treated oocysts. *E. acervulina* oocysts were treated by pressure and heat as described above, refrigerated, and used within 48 h of treatment for the *in vivo* study. A total of eight birds were used per treatment group (four per independent trial) and maintained in separate cages

with fecal droppings disposed daily to minimize cross-contamination. Negative controls were administered sterile DMEM. Positive controls were administered untreated *E. acervulina* (average of 6.9×10^5 sporulated oocysts per ml DMEM) by oral gavage. The four treatment groups were administered *E. acervulina* oocysts pressure-treated at 4, 20, and 40 °C (average 7.5×10^5 oocysts/ml), and heat-treated oocysts (4.4×10^5 oocysts/ml) to simulate temperature increase realized during pressurization at 40 °C. Six days post-inoculation (p.i.), birds were evaluated for weight gain, feed conversion, and lesions (Johnson & Reid, 1970). Birds were sacrificed by cervical dislocation and the duodenal loop cut lengthwise to observe for color, lesions, and diarrhea. Handling of chickens and euthanasia was done according to the AVMA Panel on Euthanasia (2001). Fecal droppings were collected the previous 24 h and counted for oocyst shedding. Feces were suspended in an equal volume of water, mixed for 2 min, and overlayed with equal volume 2 M sucrose. The fecal sample was centrifuged at 3500 *g*, the top layer removed, washed well with water, and counted using a hemacytometer.

2.3.2. Intestinal oocysts

Intestines were collected from chickens infected with *E. acervulina* at 6 d p.i. The intestines were suspended in Hanks' Balanced Salt Solution (HBSS, Mediatech) and stored in a gaspak jar with anaerobic paks (BD BBL GasPak Anaerobic System Envelopes, 270304, Becton, Dickinson and Co., Sparks, MD, USA) and refrigerated to prevent sporulation of oocysts until use. Intestines were used within 2 days of collection, and were cut longitudinally, scraped with a glass slide, and washed of contents with HBSS. Unsporulated oocysts were suspended in 1 M sucrose, washed twice with deionized water and resuspended in DMEM. Centrifugation was done at 4 °C and tubes were kept in an ice bath during all other steps to prevent sporulation. Oocysts were left untreated to serve as a control or treated with pressure at 550 MPa for 2 min at 40 °C. Oocyst suspensions (5 ml) were dispensed into 20 ml glass beakers with a small stir bar (1/2 in × 5/16 in) to aerate, and covered with cheesecloth dipped in water to minimize drying. Ambient temperature was approximately 23 to 24 °C. Unsporulated and sporulated oocysts were counted prior to treatment, immediately after pressurization and approximately every 24 h for 4 d. Initial counts were approximately 10^5 unsporulated oocysts per ml. At time 0, some oocysts appeared to already be developing invaginations indicating the sporulation process had begun, although none were observed to have completed sporulation at the initial time point. Four replicate counts were determined with a hemacytometer at 400× magnification. At the final time point, oocysts were concentrated by centrifugation and recounted.

2.4. Immunogenicity study

In conjunction with one infectivity trial, four additional birds per treatment group were also evaluated for protective immunity against *E. acervulina* challenge. Birds were placed in suspended cages and fecal droppings collected in a pan to prevent cycling of oocysts. On day 14 post-primary inoculation (p.p.i.), feces were collected to verify that shedding of oocysts had ceased from the first inoculation with *E. acervulina*. Chickens from all groups except the negative control group were challenged at day 15 p.p.i. with untreated *E. acervulina* oocysts as described above. An additional group of four birds was added to this portion of the study to receive untreated *E. acervulina* to verify infectivity of the oocysts administered to the other birds during challenge. Feces were collected beginning 5 d post-challenge inoculation (p.c.i.). On day 6 p.c.i., the birds were sacrificed as described above and evaluated for weight gain, feed conversion, and intestinal lesions. Oocysts in feces were enumerated as described above. Handling of chickens and euthanasia was done according to the AVMA Panel on Euthanasia (2001).

2.5. Structural integrity

2.5.1. Fragility

Untreated and pressure-treated *E. acervulina* were examined for fragility of the oocyst inner wall as determined by ease of mechanically opening oocysts to release sporocysts. Oocysts (1.25 ml at 10^6 per ml) were added to 1.8 ml centrifuge tubes containing 0.18 g of 1-mm diameter glass beads. Oocysts and beads were vortexed at a speed setting of 2000 on a Deluxe Digital Vortex Mixer (Fisher Scientific) and samples withdrawn at 0, 7, 9, and 11 min of bead-beating. Intact oocysts were counted in duplicate at each time point using a hemacytometer (Bright-Line®, American Optical, Buffalo, New York, USA). Percentages of intact oocysts (relative to 0 min) were calculated for each time interval.

2.5.2. Excystation

After breaking the oocyst membrane, sporocysts were washed in cold saline A (0.14 M sodium chloride, 5 mM potassium chloride, 4 mM sodium bicarbonate, 6 mM dextrose in deionized water) then treated with excystation solution (0.25% trypsin, 0.014 M taurocholic acid in saline A) for 60 min at 41 °C to release sporozoites. Excystation fluid was removed by washing 3 times with cold saline A followed by centrifugation at 16,060 *g* for 10 min at 4 °C (AccuSpin Micro R, Fisher Scientific International Inc. Hampton, New Hampshire) and suspension in HBSS. Suspensions were examined microscopically (400×, Motic BA300, Motic Instruments Inc., Richmond, British Columbia, Canada) in 25 non-overlapping fields; counts were made in duplicate. Intact sporocysts and sporocyst ghosts were enumerated, and percent excystation was calculated according to: [# sporocyst ghosts / (sporocysts + ghosts)] × 100.

2.5.3. Permeability

The effect of pressure on oocyst membrane permeability was examined by uptake of methylene blue dye. *E. acervulina* in DMEM (4.4×10^6 oo/ml) was pressurized at 4, 20, or 40 °C as previously described. After pressure treatments, the 1-ml suspensions were centrifuged (16,060 *g*, 10 min, 4 °C) and the pellets resuspended in 1 ml methylene blue for 30 min at 4 °C. Untreated *E. acervulina* served as a negative control. For a positive control, *E. acervulina* in DMEM was exposed to 3 freeze–thaw cycles (−80 °C for 30 min and 41 °C water bath for 10 min) (Jensen, Nyberg, Burton, & Jolley, 1976; del Cacho et al., 2001), pelleted and resuspended in methylene blue (Poly Scientific, Bay Shore, New York, USA). The number of oocysts permeable to methylene blue was enumerated using a hemacytometer.

Table 1
Effect of HPP on *E. acervulina* pathogenicity and immunogenicity

Treatment	Primary inoculation				Challenge inoculation			
	Lesion score	%Weight gain*	Feed conversion	Oocysts (per g feces)	Lesion score	%Weight gain*	Feed conversion	Oocysts (per g feces)
Challenge control	ND	ND	ND	ND	3	ND	ND	4.42×10^9
Negative control (sterile DMEM)	0	100	2.36	0	0	100	1.79	0
Positive control (untreated *E. acervulina*)	4	69	2.85	7.83×10^8	0	104	1.7	0
HPP-4 °C	0	105	2.38	1.34×10^6	2	65	1.97	1.38×10^9
HPP-20 °C	0	105	2.35	1.11×10^5	0.75	53	2.18	8.76×10^8
HPP-40 °C	0	104	2.35	0	1	51	2.23	6.50×10^8
Heat treated	3	77	2.62	1.18×10^9	0	101	1.57	2.12×10^7

*Percent weight gain of negative control. ND, not determined.

2.5.4. Light microscopy

Sporulated oocysts of *E. acervulina* were pressure- (550 MPa, 2 min, 40 °C) and heat-treated (simulation of adiabatic heat at 550 MPa, 2 min, 40 °C pressure treatment), as described above. Oocysts were bead-beated and excysted to recover sporocysts and sporozoites as described above. Untreated oocysts were used as a control. Oocysts, sporocysts, and sporozoites were mounted on slides (Fisherbrand Plain Microscope slides, 25×75×1 mm, Fisher Scientific, Pittsburgh, PA USA, cat 12-550A), covered with coverslips (Fisherbrand, 22×22-1.5, 1 oz), and examined at each stage of preparation by light microscopy at 1000× magnification (Model BA300, Motic Instruments Inc., Richmond, B.C., Canada). Images were captured with a Moticam 2000 camera and Motic Images Advanced 3.2 Software.

2.5.5. Scanning electron microscopy

Heat- and pressure-treated sporulated oocysts (as described previously, either simulation of adiabatic heat at 550 MPa, 2 min, 40 °C pressure treatment or 550 MPa, 2 min, 40 °C) were observed by electron microscopy for visual damage. Oocysts were bead-beated and excysted to recover sporocysts and sporozoites as described above. Untreated oocysts were used as a control. SEM preparation procedures were adapted from methods by Bozzola and Russell (1999), Long and Strout (1984), Nyberg and Knapp (1970), and Zhu and McDougald (1992). Oocysts, sporocysts, and sporozoites were suspended in HBSS and suspended on coverslips precoated with poly-L-Lysine (P8920, Sigma-Aldrich, St. Louis, MO) for 60 min at 4 °C for adhesion to the coverslips. The suspension was drawn off and coverslips were flooded with freshly-prepared paraformaldehyde (2%)–glutaraldehyde (1%) (Electron Microscopy Sciences, Hatfield, PA, USA) in phosphate buffered saline pH 7.4 (PBS) for 1 h at ambient temperature to fix the various structures to the coverslips. The coverslips were rinsed three times with PBS and covered with 2% osmium tetroxide (19110, EM Sciences, Hatfield, PA) for 2 h at ambient temperature. The osmium tetroxide fixative was removed with three PBS washes. Adherent and fixed *E. acervulina* were then dehydrated in an ethanol series in ascending order of 25, 50, 75, and 95% for 10 min each. Coverslips were submerged in 95% ethanol and stored (no longer than 5 d) at 4 °C until further drying for SEM. Coverslips were submerged in 100% ethanol for 10 min, transferred to fresh 100% ethanol and dried in a Supercritical Autosamdri®–815B critical point dryer (Tousimis, Rockville, Maryland, USA). Samples were sputter coated with gold/palladium for 2 min with a Denton Bench Top Turbo III (Denton Vacuum, Moorsetown, NJ, USA). Samples were imaged on a Hitachi Field Emission Scanning Electron Microscope S-4700 (Hitachi High Technologies America, Inc., Pleasanton, California, USA).

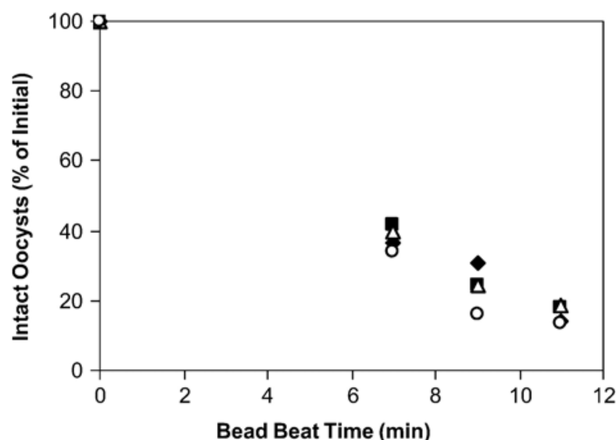

Fig. 1. Effect of HPP on fragility of *E. acervulina* oocysts. ◆, Control; ■, HHP 4 °C; △, HHP 20 °C; ○, HPP 40 °C.

2.6. Replicates and statistical analyses

All studies were conducted in duplicate unless otherwise stated. Means for each treatment among trials are presented; error bars represent the standard deviations. Differences in the means among treatments considering replications were determined according to a *t* test with a significance level of 5% (SAS Institute Inc., 1989).

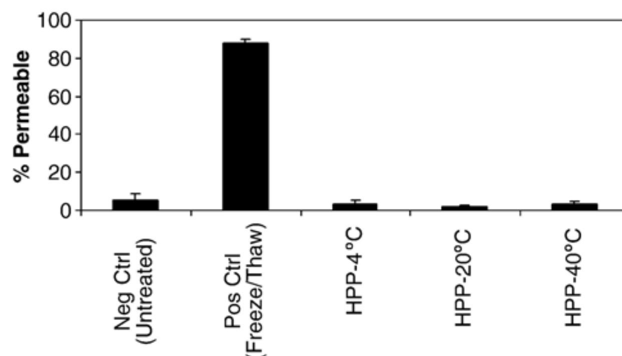

Fig. 2. Effect of HPP on permeability of *E. acervulina* oocysts. Y-error bars represent standard deviation.

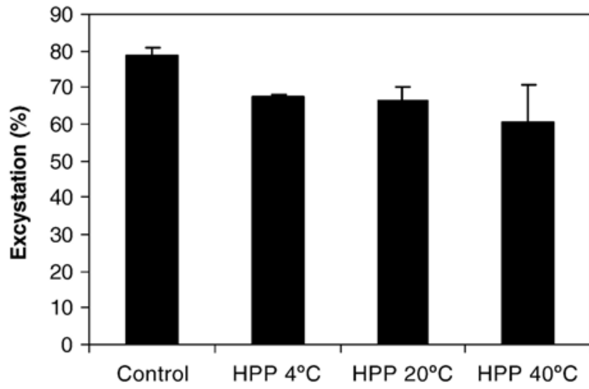

Fig. 3. Effect of HPP on excystation of *E. acervulina*. Y-error bars represent standard deviation.

3. Results

3.1. Inactivation

3.1.1. In vivo infectivity study

From the primary inoculation, the negative control birds exhibited no disease symptoms, had normal weight gain and feed conversion and did not shed oocysts in their feces (Table 1). The birds that received untreated *E. acervulina* showed heavy infection in the duodenal loop, decreased weight gain (69% of negative control group), a higher feed conversion ratio, and heavy shedding of oocysts in their feces. The birds receiving the pressure-treated *E. acervulina* oocysts did not exhibit disease symptoms in the form of lesions, suppressed weight gain or increased feed conversion. Birds that received oocysts pressure-treated at 40 °C did not shed oocysts. Pressure treatment at 4 and 20 °C reduced oocyst shedding by more than 2-and 3-$\log_{10}$, respectively, compared to birds that

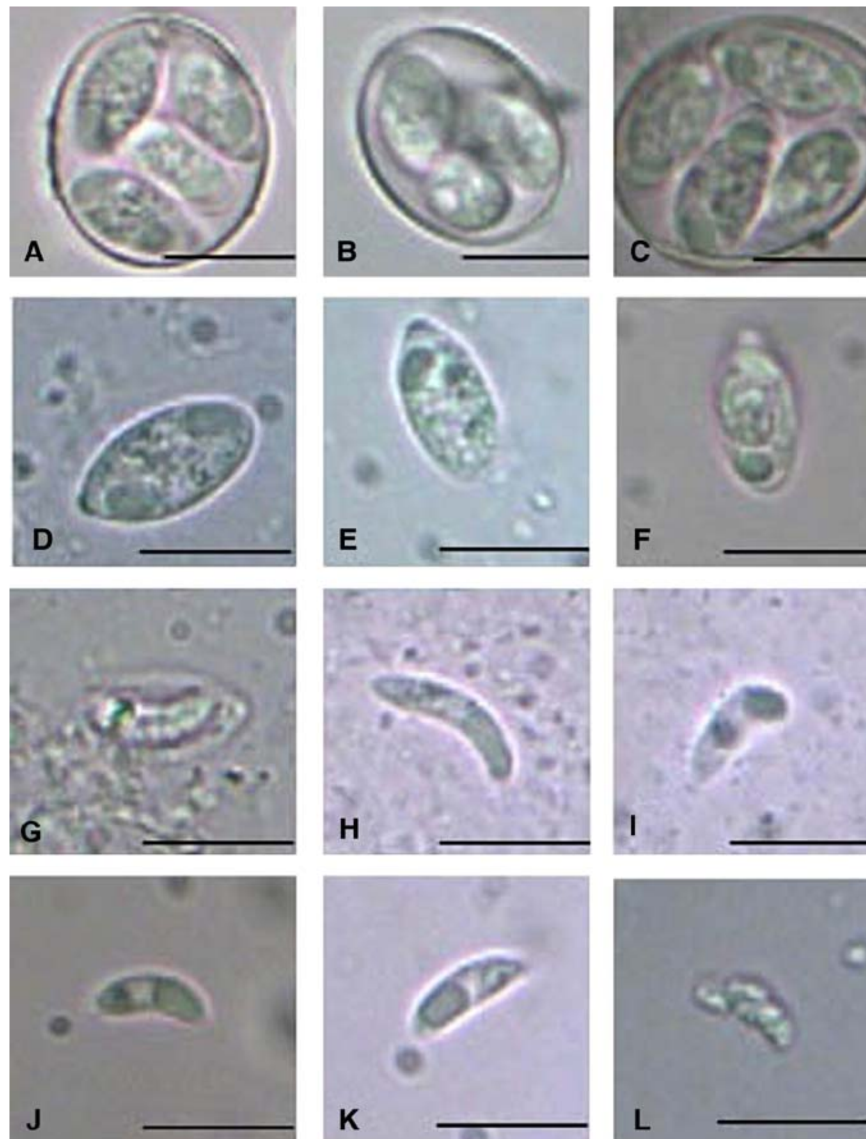

Fig. 4. Light microscopy of oocysts (A–C), sporocysts (D–F), and sporozoites (G–L) from untreated, heat-treated, and pressure-treated (550 MPa, 2 min, 40 °C) *E. acervulina* oocysts, respectively. Sporozoites (G–I) prior to exposure to excystation buffer, sporozoites (J–L) after exposure to excystation buffer from untreated, heat-treated, and pressure-treated oocysts, respectively. All bars represent 10 μm.

received untreated oocysts. The birds that received heat-treated oocysts exhibited disease symptoms in lesions, reduced weight gain (77% of negative control), increased feed conversion, and also shed oocysts in their feces comparable to the positive control group.

3.1.2. Intestinal oocysts

Untreated unsporulated oocysts (control group) were able to sporulate; and, although the percentage of sporulation was low for control samples, the comparison to treated samples was evident. For the untreated control sample, sporulation reached a plateau at 25 h to approximately 2×10^4 oo/ml from an initial unsporulated population of 2×10^5 oo/ml. No sporulated oocysts were detected from the pressure-treated sample at any time point even when the sample was concentrated and 25 fields were counted (data not shown).

3.2. Challenge study

From the challenge inoculation (Table 1), some disease symptoms were observed in birds previously inoculated with HHP-treated oocysts. The birds exhibited reduced weight gain and feed conversion and also shed oocysts. However, partial immunity was observed as lesions were less severe than those observed in birds with no prior oocyst exposure.

3.3. Structural integrity

3.3.1. Oocyst inner membrane

Oocyst membrane fragility was measured by the time necessary to release sporocysts from oocysts by bead-beating. There were no measured differences in oocyst membrane fragility as a result of HPP treatment (Fig. 1). HPP treatments did not increase permeability of oocysts to methylene blue as compared to the untreated *E. acervulina* (Fig. 2). Freeze/thaw treatments have been reported to increase permeability of *Eimeria* oocysts (Jensen et al., 1976), and uptake of the dye by these oocysts indicated the assay worked appropriately.

3.3.2. Excystation

Excystation of sporocysts to release sporozoites was not affected by HPP treatments and was comparable to the untreated control *E. acervulina* (Fig. 3). While the HHP 40 °C treatment yielded more variable results; there were no significant differences among treatment groups ($P>0.05$).

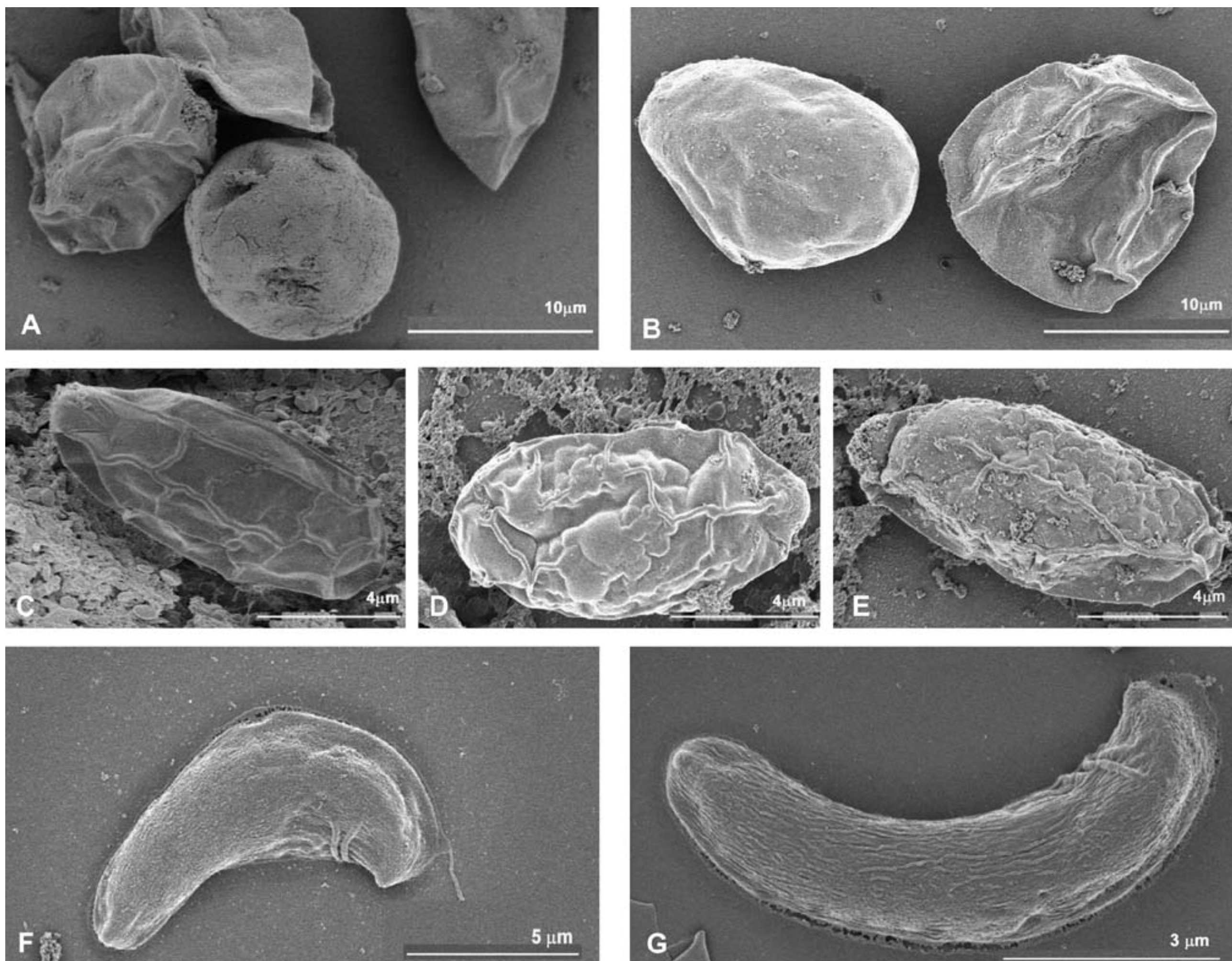

Fig. 5. SEM of oocysts (A, B) from untreated and pressure-treated *E. acervulina* oocysts, respectively. Sporocysts (C–E) from untreated, heated, and pressure-treated (550 MPa, 2 min, 40 °C) *E. acervulina* oocysts, respectively. Sporozoites (F, G) from untreated and heat-treated *E. acervulina* oocysts, respectively. Bars represent values as labeled.

3.3.3. Microscopy

No differences in appearance were observed among untreated, pressure-treated, and heat-treated oocysts or sporocysts by light microscopy (Fig. 4). Some sporozoites were released after bead-beating and these sporozoites appeared the same among treatment groups. However, after further treatment in excystation fluid, untreated and heated sporozoites looked the same while views of pressure-treated sporozoites were scarce and, when found, appeared distorted in edges and lacking internal smoothness (Fig. 4). Scanning electron microscopy revealed no differences in appearance among untreated, pressure-treated, and heat-treated oocysts or sporocysts (Fig. 5). Sporozoites of untreated and heat-treated oocysts were similar in appearance with no obvious surface damage. Unambiguous sporozoites from the pressure-treated oocysts were difficult to locate.

4. Discussion

The data presented herein provide the first evidence that high hydrostatic pressure can render a member of the Family Eimeriidae nonpathogenic to its natural host with apparent retention of some immunogenic properties. The data also provide some insight on the possible coccidial structures affected by pressure. Consideration is given to the potential use of HPP for inactivation of coccidia in human foods as well as vaccine development for chickens.

Sporulated oocysts treated with pressure (550 MPa, 2 min) at subambient (4 °C), ambient (20 °C), and mildly elevated (40 °C) temperatures did not cause lesions or compromise weight gain in chickens even when administered at a high dose of 10^5 oocysts. However, only chickens administered oocysts treated with pressure and mild heat did not shed oocysts in their feces. This suggests pressure without heat treatment may inactivate enough oocysts to reduce the dosage below the threshold to cause disease, while some oocysts are either unaffected or are reversibly injured and are able to complete their life cycle in the chicken. While sublethal injury by pressure has been detected in bacteria (Mañas & Pagán, 2005), this phenomenon has not been documented in parasites. The severity of coccidiosis symptoms can be correlated with dosage, and while this can vary with the isolate, ingestion of as few as 500 oocysts of *E. acervulina* has been demonstrated to cause weight depression and lesions (Swinkels et al., 2006). Therefore, if viable oocysts remained after pressure treatment in this study, the surviving population was likely to be fewer than 500 oocysts. The application of additional stresses such as heat has been reported to enhance the effectiveness of pressure for the inactivation of other microorganisms as well (Okazaki, Kakugawa, Yamauchi, Yoneda, & Suzuki, 1996; Patterson & Kilpatrick, 1998). Oocysts treated with heat alone caused disease symptoms; and therefore, adiabatic heating was not the sole source of oocyst inactivation. To simulate temperature effects of adiabatic heating that occurred at 550 MPa for 2 min with a surrounding bath temperature of 40 °C, the maximum treatment temperature was 55.5 °C for 24 s and declined to 4 °C within 2 min. Heat resistance data for *Eimeria* oocysts are limited; however, Reid (1972) reported that 65 °C for 15 min was usually sufficient to kill oocysts. McDougald and Reid (1991) reported that exposure to 55 °C kills oocysts very quickly. These data therefore provide more insight on the heat resistance of *Eimeria*.

Unsporulated oocysts may be able to sporulate under favorable conditions (aerobic, optimal temperature of 29 °C) (Graat, Henken, Ploeger, Noordhuizen, & Vertommen, 1994) and become infective; and therefore, the effect of pressure on sporulation is of interest. A population of predominantly unsporulated oocysts of *E. acervulina* collected from the chicken intestine did not sporulate after pressure–mild heat treatment as compared with untreated unsporulated oocysts. Therefore, in a mixed population of sporulated and unsporulated oocysts, pressure treatment would be expected to render the whole population nonpathogenic. The oocyst wall is a common structure between unsporulated and sporulated oocysts and does not appear to be damaged by pressure; therefore, pressure may have disrupted metabolic functions necessary to complete sporulation.

Additional studies with various combinations of pressures, exposure times, and temperatures are needed to fully characterize the inactivation kinetics of *E. acervulina* and how it compares to other microorganisms. Among microorganisms studied for pressure resistance, the resistance of *E. acervulina* appears to be less than that of bacterial spores (Patterson, 2005). The pressure resistance of *E. acervulina* is consistent with what has been reported for *C. parvum*, a protozoan infectious to humans and other animals. Pressure treatment of contaminated apple juice with 550 MPa for at least 1 min has been reported to render *C. parvum* (greater than 4 $\log_{10}$ reduction) nonviable and noninfectious (Slifko et al., 2000). *T. gondii* is reportedly inactivated by 340 MPa for 1 min while suspended in HBSS (Lindsay et al., 2005) and 300 MPa for 30 s in ground pork (Lindsay et al., 2006).

The pressure resistance of *C. cayetanensis*, another foodborne coccidial pathogen, has not been reported, and would be difficult to ascertain because it has no known hosts other than humans (Shields & Olson, 2003) thereby limiting resources of oocysts as well as presenting ethical issues for laboratory studies. Because *E. acervulina* and *C. cayetanensis* bear similarities in their morphologies, life cycles and genetics (Shields & Olson, 2003), the former has been utilized as a surrogate to evaluate the efficacy of processing treatments to render food safe if contaminated with the latter (Lee & Lee, 2001). *C. cayetanensis* is typically associated with fresh produce, in particular raspberries, salads, basil, and snow peas. Some studies have been conducted to determine the potential applicability of high hydrostatic pressure for fresh produce. High pressure does not appear to compromise several beneficial factors in various fruits, including anti-mutagenic and anti-oxidative factors (Butz et al., 2003). Also, the pressure demonstrated to inactivate *E. acervulina* in this study would likely be adequate to inactivate many spoilage and pathogenic microorganisms associated with fresh produce, with the probable exception of sporeforming bacteria on low-acid produce (Moerman, 2005; Arroyo, Sanz, & Préstamo, 1997).

The effect of such elevated pressures on organoleptic properties of produce/herbs varies depending on the food (Chauvin et al., 2005; Arroyo, Sanz, & Préstamo, 1999; Krebbers, Matser, Koets, Bartels, & Van Den Berg, 2002; Garcia-Palazon, Suthanthangjai, Kajda, & Zabetakis, 2004; Suthanthangjai, Kajda, & Zabetakis, 2005; Tangwongchai, Ledward, & Ames, 2000). More research is needed to determine the various HPP treatment conditions capable of inactivating coccidian parasites on produce and whether such conditions also inactivate other deleterious enzymes and microorganisms associated with produce while maintaining desirable fresh-like characteristics.

Several microbial pathogens that can be inactivated by high hydrostatic pressure have also been demonstrated to retain immunogenic properties, including *Leptospira interrogans* (Silva et al., 2001), Vesicular Stomatitis Virus (Silva et al., 1992), Infectious Bursal Disease Virus (Tian et al., 2000), Foot and Mouth Disease Virus (Ishimaru et al., 2004), and Coxsackievirus group B (Chen, Tian, & Ruan, 2001), and thus, development of a vaccine by high hydrostatic pressure is a theoretical possibility. Having established that high pressure can render *E. acervulina* non-pathogenic, we next explored whether immunogenic properties of the parasite were retained after pressure treatment and whether potential for vaccine development by high-hydrostatic pressure exists. Current commercial vaccines consist of live virulent or attenuated *Eimeria* species. Risks exist with any live vaccine including reversion of attenuated strains to more virulent states as well as mortality to the host. Attenuated *Eimeria* are characterized by reduced number of merogonic stages (Jenkins, 2001; Vermeulen, Schaap, & Schetters, 2001) and therefore cause less damage to the intestinal lining. Oocysts are generally administered in small doses and repeated exposure to oocysts in the litter (cycling) helps confer immunity (Vermeulen et al., 2001). The immunizing doses of oocysts vary among *Eimeria* species with *E. acervulina* generally requiring greater doses than other species. Previous studies reported the following doses of *E. acervulina* to provide partial or full immunity upon challenge: 80,000 and 160,000 oocysts at 2 and 4 weeks of age, respectively (Hein, 1968) or 2000 and 10,000 oocysts at 1 and 8 days of age, respectively (Long, Johnson et al., 1986). There are some data in the literature to suggest that complete immunity to *Eimeria* infections requires prior exposure to viable oocysts with sporozites capable of invading epithelial cells and carrying out some intracellular metabolism (Jenkins, Seferian, Augustine, & Danforth, 1993) and possibly schizony (Rose & Hesketh, 1976). However, potential vaccines based on *Eimeria* antigens have been demonstrated to provide partial protection to infection (Crane et al., 1991; Du & Wang, 2005). A vaccine derived from the whole oocyst and lacking pathogenicity upon primary infection without fecal shedding would be desirable.

In the present study, chickens previously administered pressure-treated oocysts then challenged with untreated oocysts exhibited disease symptoms, but generally to a lesser degree than chickens without prior exposure. Chickens with primary exposure to pressure-treated oocysts had reduced weight gain upon challenge as compared to those immune from primary inoculation with untreated oocysts. However, lesions in the duodenal loop were less severe than those observed in control chickens without any primary exposure. Chickens administered oocysts treated with pressure at 4 °C and 20 °C, and which shed oocysts in the feces upon primary infection, presumably did not receive a sufficient primary dose to confer immunity upon challenge. The nature of the partial immunity observed is curious in that weight gain was still compromised despite the reduction in lesion severity. Some possibilities for the partial response could be the sporozoites did not invade, surface antigens were altered, or the single primary dose (viable or nonviable) was insufficient to impart full immunity. Further studies of the bird immune response as well as investigating how much of the *Eimeria* life cycle is completed during primary inoculation would help elucidate why the pressure-treated oocysts conferred only partial immunity. Characterization of changes in *Eimeria* proteins after pressure treatment may also provide insight on antigens critical for induction of a full immune response. Future work in these areas should provide direction on what pressure treatment conditions are needed to improve the immune response.

Various mechanisms for the pressure-inactivation of microorganisms have been suggested. Covalent bonds are generally unaffected by pressure. Biochemical reactions can be affected by pressure. Pressure-induced volume changes in proteins can favor or disrupt certain functions such as the activity of enzymes. Nucleic acids and the DNA helix are reportedly stable to pressure; however, pressure can disrupt replication and transcription. Structural damage has been observed in bacterial cell membranes and walls (Patterson, 2005). One study with *C. parvum* points to possible pressure-induced structural damage to oocysts as suggested by a crumpled appearance (Slifko et al., 2000). In contrast, Lindsay et al. (2005) reported that the structures of *T. gondii* oocysts, sporocysts, and sporozoites appeared unaltered by pressure. No other data were found to suggest what structures or functions of coccidia are changed as a result of HPP.

In this study, we evaluated select properties of the three major *Eimeria* structures present, the oocyst inner wall, sporocysts, and the innermost sporozoites. The structural integrity of the oocyst wall did not appear to be compromised by pressure. No changes in resistance to mechanical breakage, permeability to a small dye, or appearance under light and scanning electron microscopies were detected in pressure-treated oocysts as compared to untreated controls. *Eimerian* oocyst wall composition has not been completely characterized, but is reportedly predominantly protein and lipid with less than 20% carbohydrate covalently bound to protein (Belli, Smith, & Ferguson, 2006). Dityrosine crosslinks are proposed to provide wall stability and resistance to environmental stress (Belli et al., 2006). If non-covalent structures are critical to the oocyst wall integrity, these particular structures do not appear to be affected by the HPP conditions employed. Despite the current classification as coccidia, Cryptosporidia differ from *Eimeria* in a number of ways (Barta & Thompson, 2006) including oocyst wall composition. The pressure-induced crumpling of *C. parvum* reported by Slifko et al. (2000) occurred with longer HPP exposure times; however, inactivation was seen with shorter treatment times suggesting such morphological changes

were not essential for inactivation. Some *Eimeria* oocysts appeared crumpled under SEM, but not to a greater degree than the untreated controls.

Two features of the sporocyst layer were evaluated for integrity after pressure treatment. The sporocyst layer was observed for structural changes by light and scanning electron microscopies. No changes were visible in sporocysts of pressure-treated oocysts compared to untreated samples. Additionally, the effectiveness of *in vitro* chemical excystation of sporocysts did not differ among treatments. It is not clear whether the ability of *Eimeria* species to excyst is an internal response to external stimuli and is therefore dependent on viability (Landers, 1960), or whether it is an externally-driven breakdown of proteins and is not a good indication of viability. It is generally accepted concerning the analysis of protozoa, that excystation overestimates viability (Kniel et al., 2003). Based on our data, excystation was not a good indicator of viability, or at least pathogenicity, of pressure-treated oocysts since excystation was unaffected by pressure but pathogenicity was. If the excystation of sporocysts is merely a chemical breakdown of sporocyst membranes, pressure did not alter the membranes such that digestive enzymes and acids could not excyst the sporocysts.

Microscopy studies of the sporozoite layer yielded interesting results. SEM studies yielded excellent and abundant views of sporozoites from untreated and heat-treated oocysts. However, indisputable sporozoites from pressure–heat-treated oocysts were not found. Replicate attempts yielded the same findings, and thus were unlikely to be a result of experimental error. Rather, we speculate pressure treatment altered the sporozoites such that either surface adhesive properties necessary for SEM, possibly surface charge or hydrophobicity, were compromised, or the sporozoites could not be recovered after standard *in vitro* excystation procedures. Difficulties were also encountered with attempts to view pressure-treated sporozoites by light microscopy. Sporozoites that were released from sporocysts after bead-beating and prior to exposure to excystation buffer appeared normal. However, after treatment with excystation fluid, difficulty in locating sporozoites was experienced with the few prospective sporozoites appearing distorted. The excystation step involves treatment with a digestive enzyme and acids at 41 °C and subsequent washing. Recovery is dependent on the appropriate physical and chemical properties of sporozoites including density and osmolality. Since the samples were observed in suspension rather than adhered to the slide, adhesion should not have been an issue for observing sporozoites by light microscopy. These studies suggest that pressure induced some change to sporozoites whether in vulnerability to excystation fluid or change in other properties that could affect subsequent recovery. Changes in such properties could also reasonably be expected to affect sporozoite activity in the poultry digestive system and may provide some explanation for the observed lack of pathogenicity and partial immunogenicity.

5. Conclusions

HPP treatment of 550 MPa for 2 min at 40 °C rendered *E. acervulina* nonpathogenic to chickens and prevented fecal shedding of oocysts. The same pressure conditions at 4 °C and 20 °C were sufficient to eliminate disease symptoms, but not fecal shedding of oocysts. HPP-treated *E. acervulina* retained some immunogenic properties and conferred partial protection to chickens challenged with untreated oocysts. The studies undertaken revealed no HPP-induced change to the structural integrities of the oocyst or sporocysts. Sporozoite integrity appeared to be compromised as evidenced by changes in recovery potential and morphology.

These data have two main implications. HPP may have potential use for development of a vaccine against *E. acervulina* derived from the inactivated, whole oocyst. This would be advantageous over current vaccines which rely on live attenuated oocysts and do not prevent fecal shedding. Considerable research is needed on the chicken immune response, mechanism of inactivation of *E. acervulina*, and optimal pressure treatment conditions to move toward this goal. These data also suggest HPP may have potential for inactivation of the closely-related human parasite, *C. cayetanensis*, which has been implicated in illness outbreaks due to contaminated fresh produce. Future studies with *Eimeria*, and ultimately *C. cayetanensis*, in these food systems are needed to assess the potential application of HPP for enhanced safety of these foods.

Acknowledgements

The authors gratefully acknowledge Deborah Powell for technical assistance with SEM studies and Andrea Laycock for assistance with chicken studies. This study was funded by the University of Delaware College of Agriculture and Natural Resources.

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