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Growth of *Clostridium perfringens* in *sous vide* cooked ground beef with added grape seed extract

Serap Cosansu[a,*], Vijay K. Juneja[b]

[a] Food Engineering Department, Engineering Faculty, Sakarya University, Esentepe Campus, Sakarya, Turkey
[b] Eastern Regional Research Center, Agricultural Research Service, United States Department of Agriculture, Wyndmoor, PA, USA

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ABSTRACT

The growth of *Clostridium perfringens* from spore inocula was studied in *sous vide* cooked ground beef with added 0 to 3% grape seed extract (GSE). *C. perfringens* did not grow at 4 °C with or without GSE present. Lag time (LT) was 95 h in control samples at 15 °C, whereas 1–3% GSE addition significantly ($p < .05$) extended LT to 244 h or longer. Generation time (GT) in 3% GSE added beef was similar to that of control (19 h, 3% GSE versus 18 h, control) at 15 °C. At 20 °C, GT was 1.5 h in samples without GSE; however, 1–3% GSE addition extended GT about 2–3 folds ($p < .05$). Lag time at 20 °C was 23 h in control samples, while LT was 40–59 h in samples containing GSE. Interestingly, GSE did not affect LT at 25 °C; however, significantly ($p < .05$) longer GT was observed in 3% GSE added samples than the other sample groups. Additionally, GSE from 1 to 3% in beef extended the period needed to reach 6 log cfu/g at 15 or 20 °C, while 3% GSE was required at 25 °C. The findings suggest that GSE exhibits concentration and temperature dependent inhibitory effect on growth of *C. perfringens* in *sous vide* cooked ground beef. Grape seed extract can be used to extend the shelf-life and ensure the microbiological safety of *sous vide* cooked meat products.

1. Introduction

Clostridium perfringens is an anaerobic, non-motile, sulfite reducing, endospore-forming, Gram-positive and rod-shaped bacterium that can be found ubiquitously in the environment (Juneja, Novak, Labbe, & Sofos, 2010; Talukdar, Udompijitkul, Hossain, & Sarker, 2017). The pathogen can be found in soil, dust, gastrointestinal systems of human and animals, surfaces of vegetables as well as in other raw or processed foods. Due to the high possibility of contamination during slaughtering, the isolation rates of this pathogen from meat and meat products can be as high as 47.4% (Juneja et al., 2010). The minimum and maximum temperatures for growth are 12 and 50 °C, respectively, while the optimum growth occurs between 43 and 47 °C (Schroder & Busta, 1971). However, some strains can grow at temperatures up to 52.3 °C under strict anaerobic conditions (Shoemaker & Pierson, 1976). At optimum growth temperature, generation time can be shorter than 10 min (Willardsen, Busta, Allen, & Smith, 1978). *C. perfringens* spores can survive during low-temperature long-time cooking of meat and slow cooling can result in germination of surviving spores (Doyle, 2002).

According to Bennett, Walsh, and Gould (2013), *C. perfringens* was determined to be the most common pathogen causing foodborne disease outbreaks (44%, $n = 536$ outbreaks), followed by *Staphylococcus aureus* and *Bacillus cereus*, out of 1229 foodborne outbreaks associated with these pathogens from 1998 through 2008. During sporulation of large number of ingested vegetative cells (generally 10^6 cells per gram of food or higher) in human intestine, an enterotoxin is released and this enterotoxin is the cause of illness with typical gastrointestinal symptoms including acute abdominal pain and diarrhea (Duncan, Strong, & Sebald, 1972; Hauschild & Thatcher, 1967). On the other hand, *C. perfringens* is considered as an indicator microorganism for safety of cooling processes since it grows faster than other spore-formers. While *C. perfringens* counts increased by 4–5 logs per gram of meat during 18-h cooling (Juneja, Bari, Inatsu, Kawamoto, & Friedman, 2007), other pathogenic spore-formers, i.e. *C. botulinum* and *Bacillus cereus*, cannot reach to this level in the same period as they grow slower than *C. perfringens* (Juneja, Snyder Jr, & Marmer, 1997). Therefore, heat-processed food must be cooled fast, and the temperature/time durations that correspond to the optimum growth temperature range of this pathogen must be as short as possible.

The cook-chill foods, including *sous vide* products, are considered as suitable niches for *C. perfringens* because of the rapid growth and ability to form a dormant stage, i.e. spores that enables survival post-thermal processing (Novak & Juneja, 2002). *Sous vide* is a cooking method used since the 1970s. In this method, foods such as meat, fish or vegetable

* Corresponding author.
E-mail address: scosansu@sakarya.edu.tr (S. Cosansu).

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are vacuum packaged, heat processed, cooled and then stored at low temperature (Baldwin, 2012). Thus, a cooked and ready-to-eat food product is produced. Microbial safety and shelf-life of this kind of product is highly dependent on the temperature and duration of the heat process as well as the temperature of storage after heat processing (Juneja & Snyder, 2007). Generally, mild heat treatments are applied to *sous vide* foods since cooking temperatures that inactivate *C. perfringens* spores will negatively affect the desirable sensory properties of the product. Therefore, *C. perfringens* spores are likely to survive in *sous vide* foods. Moreover, a mild heat treatment could function as an activation step for surviving spores. This situation puts *sous vide* cooked meat products at greater public health risk with regard to growth of *C. perfringens* from spores than other types of foods. A study by Barnes, Despaul, and Ingram (1963) revealed that the germination ratio was as low as 3% in spore population without prior heat shock, whereas heat shock resulted in germination of nearly all the spores. Under abusive temperatures, vegetative growth of *C. perfringens* is unavoidable following germination and outgrowth of spores (Juneja, 2003). In other words, *sous vide* cooked foods generally contain surviving spore-formers after thermal processing and thus, it is necessary to keep the foods at low temperatures (< 3.3 °C) during transportation and storage to prevent microbial growth (Baldwin, 2012). However, temperature abuse during various stages from manufacturing to consumer is a very common occurrence. Therefore, it is not realistic to rely on temperature control as the only prophylactic measure to ensure microbiological safety of *sous vide* processed products (Juneja, Fan, Peña-Ramos, Diaz-Cinco, & Pacheco-Aguilar, 2006). In this context, including additional hurdles other than relying on low storage temperature may help to prevent growth of heat activated spores in *sous vide* processed foods as recommended by National Food processors Association (NFPA, 1988).

Natural plant extracts have been proposed as an alternative to chemical and synthetic antimicrobials and antioxidants (Perumalla & Hettiarachchy, 2011). Because of the consumer concern over synthetic preservatives, the natural antimicrobials such as grape seed, grapefruit seed, green tea, etc. have attracted attention recently. Grape seed extract (GSE) is a by-product rich in phenolic compounds derived from grape seeds that are waste products of the winery and grape juice industry. Grape seeds are very rich in total extractable phenolics (60–70%) that is higher than in pulp (≤10%) and skin (28–35%) of this fruit (Shi, Yu, Pohorly, & Kakuda, 2003). GSE has GRAS (Generally Recognized As Safe) status approved by the Food and Drug Administration and has been proved to possess antimicrobial activity by means of its high content of phenolics that are effective on bacterial cell membranes (Perumalla & Hettiarachchy, 2011). In vitro antimicrobial effect of GSE on *C. perfringens* has been shown by Jakubcova et al. (2015). Molva and Baysal (2015) have reported that GSE inhibited growth of *Alicyclobacillus acidoterrestris* cells and spore germination/outgrowth in apple juice. Therefore, this study aimed to investigate the effect of static temperature abuse of refrigerated *sous vide* cooked ground beef on *C. perfringens* growth from spores and to assess the efficacy of GSE in controlling growth of this pathogen from spore inocula. While antioxidant properties of GSE are universally known, the findings in this study provide evidence to document the ability of this additive in controlling *C. perfringens* in meat.

2. Materials and methods

2.1. Preparation of Clostridium perfringens spore cocktail

Three different *C. perfringens* strains, including NCTC 82238, NCTC 8239 and ATCC 10288, were used. To prepare an active culture, 0.1 ml from the stock culture was transferred to freshly prepared Thioglycollate Broth (Difco). Spores of each strain were prepared separately in Duncan and Strong sporulation medium according to the procedure described by Juneja, Call, and Miller (1993). The spores were harvested by centrifugation (7212 ×*g* for 20 min at 4 °C), washed twice with sterile distilled water and then resuspended in distilled water. The spore suspensions were stored at 4 °C until use. The spore cocktail containing *C. perfringens* strains was prepared immediately prior to inoculation by mixing equal amounts from each spore suspension.

2.2. Preparation and inoculation of ground beef samples

Ground beef (96% lean) was obtained from a local grocery store, vacuum-packaged (200-g portions weighed into 8 × 12 vacuum pouches [BUNZL-Koch Supplies, Kansas City, MO]) and kept at −20 °C as frozen until use. A commercial grape seed extract (GSE) in liquid form (T.J. Clark; 6.25 mg/ml GSE containing 95% oligomeric proanthocyanidins, 0.125 mg/ml resveratrol, 11.25 mg/ml ascorbic acid and 0.1% potassium sorbate) was used. Ground beef was divided into four groups and each group was mixed with GSE in 0, 1, 2 or 3% (*v/w*) concentrations. Then, a 5-g portion from each group was weighed into sterile, full surface 20 × 25 cm micro-perforated filter blender bags (BagPage+, Interscience, Rockland, MA) and each bag was inoculated with 100 µl spore cocktail to a final 2–3 log spore/g in each ground beef sample. Spores were not heat-shocked prior to inoculation. The inoculated spores were distributed evenly in the samples by hand massaging followed by mixing in a lab blender (MiniMix 100, Interscience, Rockland, MA) for 2 min. Afterwards, ground beef samples in the bag were flattened to a uniform thickness of about 1 mm by pressing on a flat surface. The bags were evacuated to a negative pressure of 1000 mbars and vacuum-sealed with a Multivac gas-packaging machine (Model A300/16, Multivac Inc., Kansas City, MO). Inoculated and vacuum-packaged samples were stored at −20 °C < 45 days until cooking.

2.3. Cooking, cooling, storage and sampling

Before cooking, the frozen samples were left on a bench for 20 min for thawing. The vacuum packaged bags were immersed in a programmable water bath set at 23 C, simulating the conditions that occur in the retail food industry and institutional food service settings. Then samples were cooked for 1 h in a water bath where the temperature increased linearly from 23 °C to 75 °C in a period of 1 h. This low-temperature heat processing of ground beef for a long time was assumed to sufficiently heat shock *C. perfringens* spores. After cooling in an ice slurry, samples were stored in water baths adjusted to 4, 15, 20 and 25 °C. Sampling was done on days 0, 4, 7, 14, 21 and 28 for 4 °C; at hours 0, 24, 48, 72, 96, 120, 144, 192, 264, 336, 504, 672, and 840 for 15 °C; at hours 0, 24, 30, 48, 72, 96, 120, 144, 192 and 240 for 20 °C; and at hours 0, 2, 4, 6, 16, 21, 24, 27 and 30 for 25 °C.

2.4. Enumeration of C. perfringens

Samples were homogenized for 2 min using a lab blender after adding 5 ml sterile peptone water (0.1% peptone). Decimal dilutions were prepared using peptone water. From appropriate dilutions 0.1 ml-portions were spread on Tryptose Sulfite Cycloserine Agar (Difco) and plates were overlaid with approximately 10 ml of SFP Agar (Shahidi Ferguson Perfringens Agar, Difco) without supplement. The typical colonies were counted after anaerobic incubation at 37 °C for 24 h.

2.5. Data analysis

The experiments were conducted as two independent replicates. At each sampling point of each replicate, an average cfu/g of two plates was used to determine estimates of the growth kinetics. Bacterial growth curves were generated from the experimental data applying Gompertz equation (Gibson, Bratchell, & Roberts, 1987) and using DataFit software (DataFit for Windows version 9.1.32, Oakdale Engineering, Oakdale, PA, USA). Gompertz equation was as follows: