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Headspace oxygen as a hurdle to improve the safety of in-pack pasteurized chilled food during storage at different temperatures

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ABSTRACT

This study investigated the use of headspace oxygen in a model food system to prevent the growth of anaerobic pathogenic bacteria in in-pack pasteurized food at various storage temperatures. Three model food formulations prepared with tryptic soy broth and three agar concentrations (0.1, 0.4 and 1.0%) were sealed without removing the air from the package in high oxygen barrier pouches (OTR = 0.3 cm^3/m^2·day·atm). Important properties influencing bacterial growth, including pH and water activity (a_w) were determined. The oxygen sorption kinetics of each model food were obtained at three different storage temperatures (8, 12, and 20 °C) using an OxySense Gen III 300 system. An analytical solution of Fick's second law was used to determine the O_2 diffusion coefficient. Growth challenge studies at 12 and 20 °C were conducted at three selected locations (top, center and bottom layers) in model foods containing 1% agar. Model foods were inoculated with *Clostridium sporogenes* PA 3679 (300 spores/mL), and were classified as low-acid (pH > 4.5, a_w > 0.85). When the storage temperature decreased from 20 to 8 °C, the oxygen diffusion decreased from 0.82×10^{-9} m^2/s to 0.68×10^{-9} m^2/s. As the agar concentration was increased from 0.1 to 1.0%, the effective oxygen permeability decreased significantly ($p = 0.007$) from 0.88×10^{-9} m^2/s to 0.65×10^{-9} m^2/s. When the inoculated model foods were stored at 12 °C for 14 days, *C. sporogenes* PA 3679 was unable to grow. As the storage temperature was increased to 20 °C, significant bacterial growth was observed with storage time ($p < 0.0001$), and the *C. sporogenes* PA 3679 population increased by around 6 log CFU/g. However, the location of the food did not influence the growth distribution of *C. sporogenes* PA 3679. These results demonstrate that oxygen diffusion from the pouch headspace was primarily limited to the food surface. Findings suggest that the air/oxygen present in the package headspace may not be considered as a food safety hurdle in the production of pasteurized packaged food.

1. Introduction

Consumers today prefer food that requires minimal preparation time compared to conventional meals. They prefer high quality foods that are nutritious, low levels in preservatives, and minimally processed (Peck and Stringer, 2005; Rajkovic et al., 2010). Consumer preference has led to the development of in-package pasteurized foods. These foods are also known as refrigerated processed foods of extended durability (REPFEDs), cook-chill, ready-to-eat, and sous-vide foods (Choma et al., 2000; Daelman et al., 2013; Peck and Stringer, 2005). These types of products are gaining popularity due to the aforementioned consumer preferences (Brunner et al., 2010; Rodgers et al., 2003). For example, the total UK prepared chilled food market increased by 33% from November 2008 to January 2016 (Kantar WorldPanel, 2016).

REPFEDs are a heterogeneous group of food products typified by a variety of ingredients, processing conditions and packaging systems used in their production process. Based on production conditions, REPFED products can be categorized into three groups (Daelman et al., 2013):

1. Products pasteurized in-pack at 90 °C for at least 10 min or equivalent to achieve a 6D reduction of non-proteolytic psychotropic *Clostridium botulinum* spores.
2. Products pasteurized in-package at 70 °C for at least 2 min or equivalent to achieve a 6D reduction of *Listeria monocytogenes*.
3. Products pasteurized out of pack and then packed. These products are not defined by a specific P_{value}, and either the P_{90} or P_{70} pasteurization treatments are possible.

For REPFED products, there are a few microbiological safety

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concerns (Juneja and Snyder, 2008). First, these products are generally formulated with little or no preservatives and have low acid and high moisture content. Second, they undergo minimal thermal processing, are not commercially sterile, and must be refrigerated. Third, vacuum packaging provides a favorable environment for anaerobic and facultative pathogens such as *Clostridium botulinum* and *Bacillus cereus* to grow and produce toxins. Finally, there is a high probability of temperature abuse during distribution and storage.

The storage temperature of chilled foods may vary greatly during manufacturing, distribution, retail, and in-home storage. According to Bruckner et al. (2012), temperatures in trucks during poultry and milk distribution range from −3 °C to 15 °C and 3.6 °C to 10.9 °C, respectively. Additional temperature abuse may occur during retail display. Temperatures above 7 °C are often common in refrigerated display cabinets of convenience stores (Dodds, 1995; Koutsoumanis and Gougouli, 2015; Rybka-Rodgers, 2001; Walker, 1992). Before the stores and homes, there is little or no temperature control after products are purchased. Tamagnini et al. (2008) mentioned that Marklinder et al. (2004) found that 5–20% of foods in general were stored at temperatures above 10 °C in home refrigerators, with maximum temperatures from 11 °C to 18 °C. Koutsoumanis and Gougouli (2015) combined the results of nine surveys conducted in the UK, France, Ireland and Greece, finding that out of over 1000 consumer refrigerators, 64.1% were operating above 5 °C. Therefore, chilled foods may undergo temperature abuse conditions in the cold chain, and low-acid cook-chill foods are unprotected under these circumstances. Since we cannot rely exclusively on the maintenance of refrigerated conditions to assure safety, it is essential to address these challenges.

Non-proteolytic *C. botulinum* and *B. cereus* are spore-forming bacteria with the lowest minimum growth temperatures at 3.3 °C and 4 °C, respectively (ECFF, 2006). Based on this, as along with the ability of non-proteolytic *C. botulinum* and *B. cereus* spores to germinate and produce toxins, the recommended storage and distribution temperatures for cook-chill foods is under 5 °C (ECFF, 2006). If food products are subjected to temperatures above 10 °C for a prolonged time during the cold chain, proteolytic *C. botulinum* and *Clostridium perfringens* are also of concern. In addition, the absence of a competitive microbiota can increase proliferation of these pathogens.

However, the use of oxygen provides an alternative for in-package pasteurized food. Bacteria vary widely in their ability to use and tolerate oxygen (Prescott et al., 2002). *Clostridium botulinum* is an anaerobic bacteria that does not use oxygen for growth and eventually dies in the presence of oxygen (Prescott et al., 2002). Anaerobic bacteria do not have the elaborate system of defenses that aerobic bacteria have, since the system relies on a series of special enzymes in large quantities. These include super dismutase, catalase, and peroxidase, which can scavenge toxic compounds that form in an oxygen-rich atmosphere (Jasso-Chávez et al., 2015; Johnson, 2009). Anaerobic bacteria produce these enzymes in very small amounts, or not at all. Thus, the variability in oxygen tolerance of obligate anaerobes may be influenced by the amount of those enzymes that they can produce.

In order for oxygen to suppress the growth of *C. botulinum* in food, it should be able to dissolve in the food surface and diffuse throughout the product. In this study, we addressed these challenges by assessing the use of oxygen to improve the food safety design of cook-chill foods during temperature abuse. Oxygen diffusion in food model/packaging systems was observed at 8, 12 and 20 °C. Three food models with different matrices were compared. The growth of *Clostridium sporogenes* PA 3679 as a surrogate of *Clostridium botulinum* was monitored within the food (top, center and bottom layers).

2. Materials and methods

2.1. Food model preparation and properties measurements

Tryptic soy broth (TSB) culture medium was used as a model food.

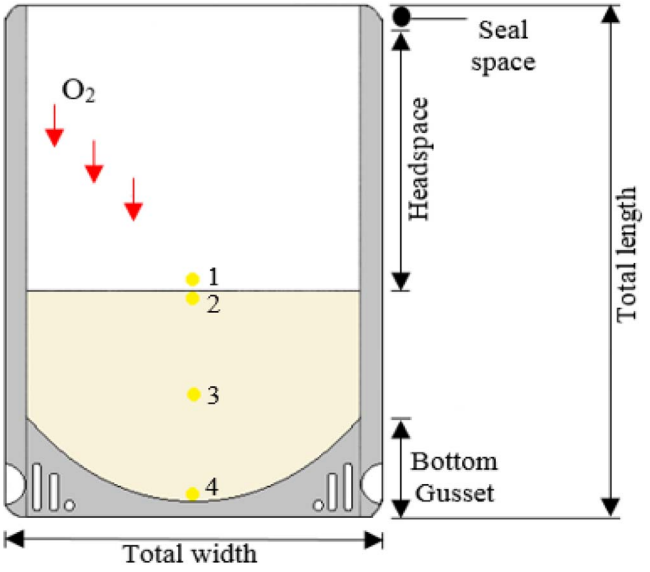

Fig. 1. Pouch dimension and set up. Pouch dimension: W × L × BG (13 cm × 18.5 cm × 3.5 cm). Oxydot 1 is located just above the food model surface, Oxydot 2 is located just below the food model surface, Oxydot 3 is located at the center of the food model column, Oxydot 4 is located at the food bottom layer. Distance between oxydots 2–3 and 3–4 is 3 cm. Sealed space was approximately 0.8 cm. Total height of food column is 7.5 cm.

The medium was prepared according to the manufacturer's instructions (Bacto, BD™) and was supplemented with different agar concentrations (0.1, 0.4, and 1.0% w/v). The resulting model foods were in liquid, semisolid and solid at temperatures below 45 °C. pH and water activity (a_w) were determined with a potentiometer (Mettler Toledo, EL 20) and a vapor sorption analyzer (Decagon Devices, Inc. VSA1042), respectively.

2.2. Food/packaging system set up

For oxygen sorption kinetics, light-sensitive oxygen sensors (OxyDot) were adhered at selected locations in the pouches to monitor O_2 concentration at the headspace, as well as within the food during storage time (Fig. 1). The adhesive used to glue the oxydots was RTV108-12C (Momentive, MIL-A-46106B Compliant) high performance silicone sealant. A small amount was applied to the inside of the pouch at selected locations. Using a vacuum pen, the oxydots were picked up with the coating side in contact with the pen, and then gently pressed to the adhesive inside the pouches. The adhesive was allowed to dry completely for 8 h, and then the model foods were dispensed for further analysis.

One batch of each formulation was dispensed in triplicate in 250 mL volumes in 13 cm × 18.5 cm × 3.5 cm stand up multilayered plastic pouches (Kurarister™ CF, Kuraray Company of America, Inc.). Next, the open edge of the pouches was sealed without a vacuum, using a manual impulse heat sealer (Hang bag sealer MP-12, Midwest Pacific®, Rocky Mount, MO, USA). This provided a water-tight and air-tight seal for an effective subsequent sterilization procedure by means of retorting. This thermal process allowed for preparation of the model foods without background microbiota, which could disturb the measurement of oxygen concentration in the different food/packaging systems. The oxygen transmission rate (OTR) of the film used in the pouch was 0.3 cm^3/m^2·day·atm. The pouches had a laminated structure with three layers of polyethylene terephthalate (12 μm thickness), biaxial oriented polyamide (15 μm) and cast polypropylene (50 μm) films.

2.3. Storage conditions and oxygen measurements

After sterilization, the pouches were stored at 8, 12, and

20 °C ± 0.5 °C for 2 days in a vertical position. The dissolved oxygen concentration at selected locations was monitored using the OxyDot-OxySense system (OxySense® 310, OxySense, Inc., Dallas, TX, USA). During the oxygen readings in pouches the incubator was opened several times and during these measurements only the incubator temperature fluctuated by ± 2 °C. This technique relied on a noninvasive, light sensitive sensor (OxyDot-O_2xyDot®) that was placed on the inside of the pouches before filling and sealing (Al-Qadiri et al., 2015). Next, the oxygen concentration was monitored with a fiber optic reader pen through the outside of the pouch. The pen reader device was connected to a computer that was equipped with OxySense's Gen-III software for data acquisition. The oxygen level was reported in parts per billion (ppb).

2.4. Oxygen sorption kinetics modeling and analytical solution

The transient oxygen transport process in a bulk medium was described using Higbie's (1935) penetration theory, which assumes that equilibrium exists at the gas-medium interface. The oxygen concentration at the gas-medium interface was determined by Henry's law (Eq. (1)) (Chaix et al., 2015).

$$C_{O_2,F} = S_{O_2} P_{O_2,HS} \tag{1}$$

where C_{O_2},F (mol/kg) is the oxygen solubility in the model food, P_{O_2},HS (Pa) is the partial pressure of O_2 in the package headspace, and S_{O_2} (mol/kg-Pa) is the solubility coefficient of O_2. Secondly, the transient oxygen diffusion within the medium was assumed to obey Fick's second law (Eq. (2))

$$\frac{\partial C_{O_2,F}}{\partial t} = D_{O_2} \frac{\partial^2 C_{O_2,F}}{\partial x^2} \tag{2}$$

where, $\partial C_{O_2},F$ (mol/kg) is the change in local oxygen concentration in food at any time t (s), and x is the distance from the gas-medium interface along the height of the medium (m). D_{O_2} is the diffusion coefficient of oxygen (m^2/s) and is assumed to be constant in the model food, which was considered to be homogenous and isotropic. The diffusion process was assumed to be one-dimensional along the height of the food sample. The medium was assumed to be semi-infinite, with the following initial and boundary conditions:

$$C_{O_2,F} = C_0,\ x \geq 0,\ t = 0 \tag{3}$$

$$C_{O_2,F} = C_s,\ x = 0,\ t \geq 0 \tag{4}$$

$$C_{O_2,F} = C_0,\ x \to \infty,\ t > 0 \tag{5}$$

where C_s is the oxygen concentration at gas-medium interface, in equilibrium with partial pressure of oxygen at the package headspace. During measurement, the oxygen ingress from the bottom and both sides of the medium can be neglected, as it was packaged with high oxygen-barrier pouches. Therefore, the system was considered to be insulated from both sides and bottom during the study.

The analytical solution of Fick's second law with aforementioned boundary conditions was given by Crank (1975):

$$\frac{C_{O_2,F} - C_0}{C_s - C_0} = erfc\left(\frac{x}{2\sqrt{D_{O_2} t}}\right) \tag{6}$$

The Eq. (6) was solved in Matlab 2013a (MathWorks Inc., Natick, MA, USA). The experimental data obtained from the oxydot located at 1.35 ± 0.15 cm below the surface was used for the computational process. The estimated D_{O_2} was used to predict the oxygen concentration at time t. The errors between experimental and predicted concentrations were minimized by using root mean square errors (RMSE) as follows:

$$RMSE = \sqrt{\frac{1}{p}\sum_{t=1}^{p} \left(C_{O_2,F_{et}} - C_{O_2,F_{pt}}\right)^2} \tag{7}$$

where p is the number of observations; C_{O_2},Fet and C_{O_2},Fpt are the experimental and predicted oxygen concentrations at time t, respectively.

2.5. Microbial growth study

The model food was microbiologically analyzed at two temperatures (12 and 20 °C) during a maximum period of 14 days to observe the behavior of *C. sporogenes* PA 3679 under temperature abuse. Since the minimum growth temperature for proteolytic *C. botulinum* is between 10 and 12 °C, the growth study was not carried out at 8 °C. The food/packaging system was prepared as described in Section 2.2. The model food containing 1.0% agar was selected for the study. This formulation allowed us to monitor microbial growth during storage time within the food due to its solid state, which facilitated slicing of the food to collect samples from the three selected locations (top, center and bottom layers) for microbial counts.

2.5.1. Microorganism and spores suspension preparation

The stock culture of *C. sporogenes* PA 3679 spores was obtained from the Center for Technical Assistance of the former National Food Processors Association (NFPA, Dublin, CA, USA). This strain is currently available from the American Type Culture Collection (ATCC 7955; ATCC, Manassas, VA, USA). The spore suspension was kept in a refrigerator at 4 °C until use (Mah et al., 2009). To prepare a working culture, a multi-step stage inoculation procedure was employed (Mah et al., 2009). Ten milliliters of tryptone-peptone-glucose-yeast extract broth (TPGY) was inoculated with 10μL stock spore suspension and incubated for 2 days at 32 °C in a GasPak™ 150 Anaerobic System anaerobic jar (BD Diagnostic Systems, Sparks, MD, USA). The anaerobic atmosphere inside the jar was generated using a BD GasPak EZ anaerobic container system sachet with indicator, Ref 260001 (BD). The TPGY broth medium consisted of 50 g of tryptone, 20 g of yeast extract, 5 g of peptone, 4 g dextrose, and 1 g sodium thioglycolate (all from Difco, Becton Dickinson, Sparks, Md., U.S.A) in 1 L of distilled water. Subsequently, 100 mL of TPGY broth was inoculated with 1 mL of the previous incubated culture, followed by 2 days incubation under the same conditions. Then the vegetative cell culture was transferred into 1 L of TPGY broth and the flask was incubated anaerobically for 2 days, as described previously. The culture was then washed three times by centrifugation with a Beckman J2-21 centrifuge (Beckman Instruments Inc., Palo Alto, CA, USA) with a Fiberlite F14B rotor (Fiberlite Centrifuge Inc., Santa Clara, CA, USA) at 15000*g* for 10 min at 4 °C. Washing was performed with sterile M/15 Sørensen's phosphate buffer, pH 7.0, consisting of 5.675 g sodium phosphate, dibasic (Na_2HPO_4); 3.63 g potassium phosphate, monobasic (KH_2PO_4); and 1 L deionized water; pH adjusted to 7.0 then sterilized by autoclaving.

To induce sporulation, the vegetative cell pellet was resuspended, first in 100 mL of a sporulation medium and then in 900 mL of the same medium, giving a total volume of 1 L. The sporulation medium consisted of 60 g tryptone, 1 g dextrose, 1 g sodium thioglycolate (all from Difco, Becton Dickinson, Sparks, Md., USA) in 1 L of distilled water. The medium pH was adjusted to 5.0 with 1 M HCl, and 5 g calcium carbonate (J.T. Baker Chemical Co., Phillipsburg, NJ, USA) was added, followed by sterilization by autoclaving. After 10 days of anaerobic incubation at 32 °C, the spore crop obtained was washed three times by centrifugation as aforementioned for vegetative cells and resuspended in M/15 Sørensen's phosphate buffer. The spore suspension prepared was found to contain around 9.3 ± 0.14 × 10^6 spores/mL, and was stored at 4 °C under aerobic conditions prior to use (Mah et al., 2009).

2.5.2. Inoculation of the model food in pouches

After sterilization, the model food in pouches was inoculated with a spore suspension of *C. sporogenes* PA 3679 previously heat shocked at 80 °C for 20 min. During inoculation, the temperature of the pouch

content was kept at 48 °C. To determine how much spore suspension (inoculum) was needed to get an inoculation dose of around 300 spores/mL of food in the pouches, the following calculations were conducted. First the desired spore dose value in the food (300 spores/mL) was multiplied by the total volume of the food in the pouches (250 mL). Then the product was divided by the spore crop concentration ($9.3 \pm 0.14 \times 10^6$ spores/mL). It was found that when using a spore crop with $9.3 \pm 0.14 \times 10^6$ spores/mL, the amount of inoculum needed for the desired spore dose was too small to dispense accurately. Therefore, the original spore suspension was diluted with peptone water to obtain a final concentration of around 10^4 spores/mL. The selected volume of spore inoculum was injected into the pouches under aseptic conditions using a manual syringe-septum technique (septum no. 940301, grey, 15 mm; Mocon, Inc., Brooklyn Park, MN) (Al-Qadiri et al., 2015). For this, septa were placed outside the pouches at the three selected locations. Then the total volume of the inoculum was injected in three portions, each corresponding to a location in the pouch (top, center or bottom). The pouches were gently shaken to evenly distribute the inoculum in the food, while avoiding aeration.

After inoculation, the pouches were stored at 12 °C and 20 °C for 14 days. The pouches were sampled in duplicate daily for those stored at 20 °C, and every 2 days for those stored at 12 °C. These sampling intervals were selected because bacteria were expected to grow faster at 20 °C. Food was allowed to equilibrate to the storage temperature for 1 h. Next, microbial counts were conducted and were taken as the 0 day condition. Four pouches, two inoculated and two non-inoculated, were stored at 12 °C and 20 °C. In these pouches, the oxygen concentration was monitored in the selected food locations and reported as a percentage. This growth experiment included two independent repetitions.

2.5.3. Microbial counts

The media used included Shahidi-Ferguson Perfringens (SFP) agar, phosphate-peptone water, and peptone water. The model foods were cut in half (Fig. 2), and then 10 g of each layer (top, center or bottom) was suspended in a stomacher bag, containing 90 mL of phosphate-peptone water. The food layer suspensions were homogenized for 45 s at 200 rpm in a Seward Circulator 400 stomacher (Seward, London, UK) and 10-fold serially diluted in sterile 0.1% peptone water. From each dilution, 1 mL by duplicate was pour-plated into Petri dishes using SFP agar. The SFP medium was autoclaved at 121 °C for 15 min and held in a 48 °C water bath prior to use. A negative control, consisting of 1 mL of 0.1% sterile peptone water pour-plated into Petri dishes using SFP agar, was also prepared in duplicate. Next, the plates were incubated anaerobically for 3 days at 32 °C. After incubation, dilutions yielding 25–250 colony forming units (CFU) were counted and CFU/g was calculated.

2.6. Statistical analysis

The oxygen mass transfer data were analyzed with the Statistical Analysis System (SAS 9.2, SAS Inst. Inc., Cary, NC, USA). The GLM procedure was used to conduct a two-way ANOVA test to observe the effects of agar content in the model food at different storage temperatures on oxygen diffusion and solubility coefficients. The same command was used to observe the effect of food location in *Clostridium sporogenes* (PA 3679) growth during storage time. Multiple comparisons were performed as needed by calculating the least square difference between the means (LSMeans). A significance level of 0.05 was applied in all tests. Bar charts for bacterial growth were generated with Microsoft Excel 2010 (Microsoft Corp, Redmond, WA).

3. Results and discussion

The pH values of the model foods ranged from 7.32 ± 0.01 to 7.34 ± 0.01, while a_w ranged from 0.994 ± 0.001 to 0.995 ± 0.001. These values allowed us to categorize the model foods as low-acid foods.

3.1. Oxygen solubility and diffusivity

The mean values of oxygen diffusion (D_{O_2}) and solubility (S_{O_2}) coefficients of 0.1, 0.4, and 1.0 agar percentage for 20, 12, and 8 °C are listed in Table 1. Statistical analysis showed that the interaction p-value was not significant, either for D_{O_2} ($p = 0.412$) or S_{O_2} ($p = 0.061$) coefficients. Therefore, how D_{O_2} and S_{O_2} change with temperature does not depend on the agar %, and vice versa. The ANOVA test was then re-run without the interaction (Ott and Longnecker, 2010), and the two main effects of storage temperature and agar % on D_{O_2} and S_{O_2} in the model foods were examined.

3.1.1. Main effects on oxygen solubility

Results show that each main effect p-value of temperature ($p < 0.0001$) and agar % ($p = 0.004$) was statistically significant in the oxygen solubility. The marginal means for both effects are plotted in Fig. 3. By increasing storage temperature from 8 to 20 °C, oxygen solubility decreased from 1.53×10^{-8} mol/kg/Pa to 0.95×10^{-8} mol/kg/Pa. When the agar content in the model food increased from 0.1 to 1.0%, the solubility decreased from 1.40×10^{-8} mol/kg/Pa to 1.28×10^{-8} mol/kg/Pa. The decrease in O_2 solubility due to temperature increase was 0.58×10^{-8} mol/kg/Pa, while that due to agar % increase was 0.12×10^{-8} mol/kg/Pa. Thus, it can be concluded that temperature had a higher impact in the O_2 solubility than agar %. In addition, the highest O_2 solubility in the model food was observed at 8 °C with 0.1% agar in the formulation.

The solubility of gases is not constant in all conditions, and the solubility of oxygen has been shown to be temperature-dependent. An increase in temperature results in a decrease of gas solubility in water and aqueous media. Chaix et al. (2014) compiled findings from several studies on oxygen solubility in water and some foods at different temperatures. They found that the oxygen solubility in water decreased from 2.3×10^{-8} mol/kg/Pa to 9.8×10^{-9} mol/kg/Pa as temperature

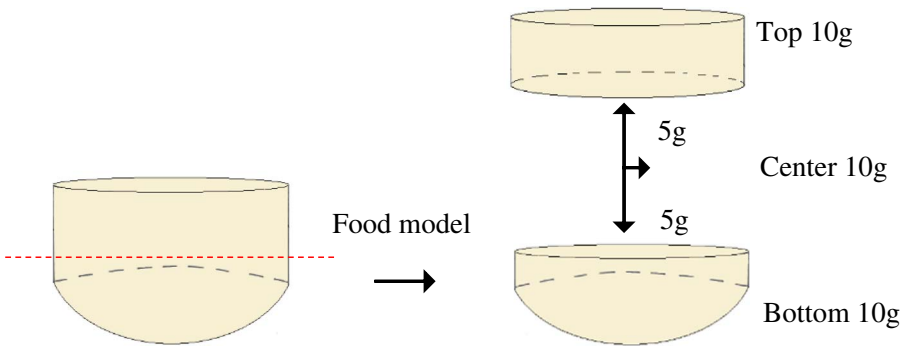

Fig. 2. Food model sample cutting and sub sample collection for *C. sporogenes* (PA 3679) was measured at three different locations (top, center and bottom).

Table 1
Oxygen diffusivity and solubility in model foods at different temperatures.

Agar (%)	Temperature (°C)	$D_{O2\ value}$ (10^{-9} m^2/s)	$S_{O2\ value}$ (10^{-8} mol/kg/Pa)
0.1	20	1.03 (0.11)	1.03 (0.03)
	12	0.87 (0.02)	1.56 (0.04)
	8	0.73 (0.22)	1.61 (0.03)
0.4	20	0.75 (0.05)	0.97 (0.05)
	12	0.78 (0.13)	1.60 (0.02)
	8	0.70 (0.05)	1.48 (0.05)
1.0	20	0.70 (0.05)	0.83 (0.13)
	12	0.74 (0.08)	1.53 (0.01)
	8	0.59 (0.09)	1.50 (0.01)

Data are presented as mean (standard deviation).

increased from 0 to 50 °C. An increase in agar has been found to increase the dry matter in model foods. The addition of dry matter has been reported to decrease oxygen solubility (Pénicaud et al., 2012).

3.1.2. Main effects on oxygen diffusivity

The main effect of agar percentage yielded an F ratio of F (2, 15) = 6.94, p = 0.007. As the agar percentage increased from 0.1 to 1.0% in the model food, oxygen diffusion decreased significantly from 0.88×10^{-9} m^2/s to 0.65×10^{-9} m^2/s (Fig. 4). Storage temperature did not affect significantly oxygen diffusion, at F (2, 15) = 3.25, p = 0.067. In general, when storage temperature decreased from 20 to 8 °C, oxygen diffusion decreased from 0.82×10^{-9} m^2/s to 0.68×10^{-9} m^2/s (Fig. 4). Overall, highest D_{O_2} were obtained at 20 °C and 0.1 agar %.

Oxygen diffusion in air and in water at 20 °C has been reported as 2.03×10^{-5} m^2/s and 2.10×10^{-9} m^2/s, respectively (Denny, 1993). In water, the diffusion coefficient of oxygen is 10,000 times smaller than in air. One reason is that the viscosity of water is 1.002 cP, while the viscosity of air is 0.018 cP at 20 °C (Abulencia and Theodore, 2009). Oxygen diffusion values obtained for the model foods used here are smaller than those reported for water in the literature.

In our study, water was used to prepare the model foods, and when the media culture powder and agar was added, the viscosity increased. This could reduce the mobility of oxygen throughout the food. It is also important to mention that the agar used here is a mixture of agarose and agaropectin molecules. The agarose molecules are responsible for gel structure formation (Labropoulos et al., 2001) while there are less agaropectin molecules which could form micro-aggregates that do not induce gelation. In our study, as the agar content increased, the model food showed a more solid-like behavior due to the development of a stable network structure. This structure traps or holds water in the food. It appears to be strong enough to increase the tortuosity and change the O_2 diffusion pathway, thus reducing oxygen mobility, since diffusion must go around the obstacles.

3.2. Microbial counts

In this study, we determined the growth behavior of *C. sporogenes* within the food at 12 and 20 °C during storage time (Fig. 5). When food was stored at 12 °C, bacteria did not grow. Slight changes were observed in bacteria counts and a logarithmic increment was not observed during storage. On the other hand, rapid growth was observed at 20 °C, and bacteria grew significantly ($p < 0.0001$) at around 6 log units from its initial point. Bacteria counts at 20 °C could not be recorded after four days of storage due to food spoilage. By day 5, bacterial growth caused significant changes in the food structure, and the food could not be cut into discrete layers. Therefore, local counts for the top, center, and bottom layers were not possible for the remainder of the storage time. The analysis of variance showed that the growth of *C. sporogenes* PA3679 was not significantly influenced by the food location (p = 0.296).

Growth of bacteria depends on external factors and the internal properties of food, such as pH and water activity. Food matrix properties were suitable for microbial growth in terms of pH, a_w, and dextrose in the food could be used as a carbon source. No growth occurred at 12 °C because minimum growth temperature for the bacteria to grow ranges between 12 and 15 °C (Hong et al., 2016). At this temperature, growth is delayed significantly, and it could not be detected during the storage time used in this study. The minimum growth temperature of proteolytic *C. botulinum* is often reported to range from 10 to 12 °C (Gunvig et al., 2013; Lindstrom et al., 2006; Peck, 2009; Peck et al., 2011). In some studies, a higher range is given, from 12.8 to 16.5 °C (Hinderink et al., 2009). In either case, our *C. sporogenes* PA 3679 minimum growth temperature results were in accordance with the minimum growth temperature ranges for proteolytic *C. botulinum*. However, comparing the growth kinetics of both bacteria is recommended before using *C. sporogenes* PA3679 as a surrogate to study the growth of proteolytic *C. botulinum* in food (Hong et al., 2016).

Although the inoculated model food stored at 20 °C, *C. sporogenes* PA 3679 grew well, the oxygen concentration gradient created within the food did not influence bacterial growth (Table 2). Headspace oxygen concentration during storage time did not dip below 21% (data not shown). At the beginning of storage time, oxygen concentration ranged from 6.58 to 9.37% in the top layer of the food. In the middle layer, it ranged from 1.16 to 1.65%, approximating zero at the bottom

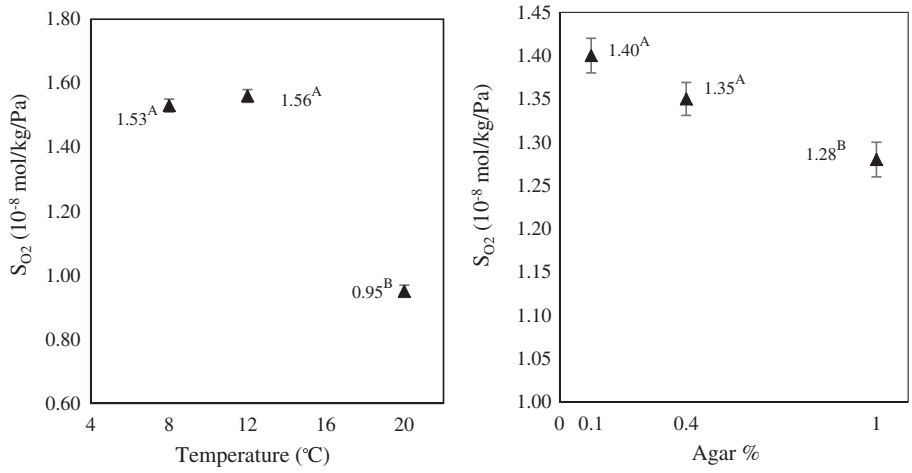

Fig. 3. Plot of oxygen solubility coefficient marginal means for temperature and agar % factors. Different letters for mean values represent significant differences. Standard error bars of the means.

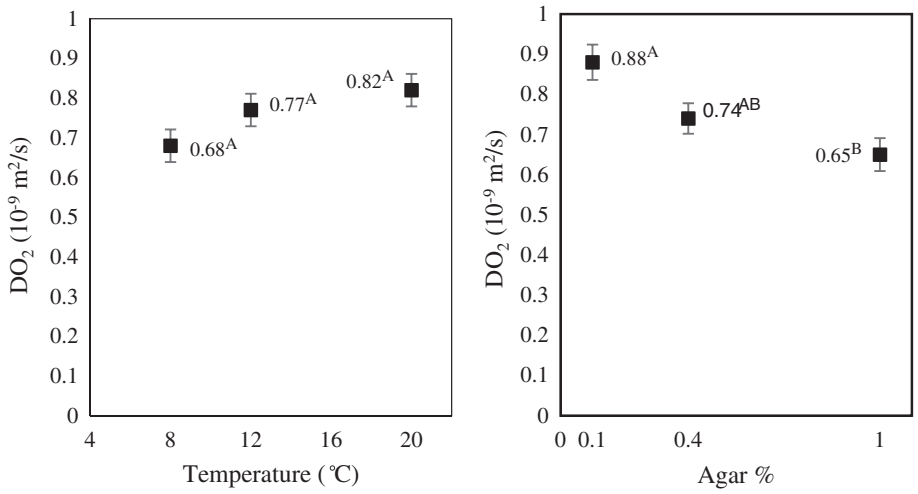

Fig. 4. Plot of oxygen diffusion coefficient marginal means for temperature and agar % factors. Different letters for mean values represent significant differences. Standard error bars of the means.

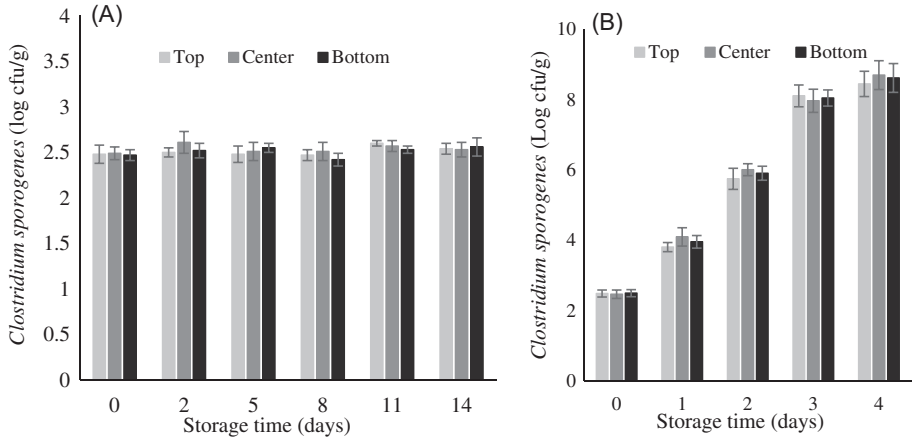

Fig. 5. *Clostridium sporogenes* (PA 3679) growth evolution within food (top, center and bottom layers) during storage time at 12 °C (A) and 20 °C (B).

layer. There was an increase in O_2 concentration at the top layer with storage time. However, there was no significant increase in subsequent layers. O_2 concentration at the top layer was higher at 12 °C than at 20 °C storage temperature. This was likely because oxygen is more soluble in food at lower temperatures. Results show that the O_2 amount present in the top layer did not prevent the growth of *C. sporogenes*. According to Meyer (1929), the oxygen threshold reported for *C. sporogenes* is 21%. Oxygen concentrations at the three locations were below 21%, supporting *C. sporogenes* PA 3679 growth. In addition, Meyer (1929) reported that there is a wide variability in oxygen tolerance among the *Clostridium* genus. The threshold of *C. botulinum* types A, B, and C is 6.75%, 8.4% and 2.7%, respectively. Thus, growth results within the food obtained in this study using *C. sporogenes* should not be used as a reference for *C. botulinum* growth behavior under air, since the threshold for both bacteria differs. However, our results indicate that oxygen diffusion within the model food is very slow. In addition, oxygen levels in the center and bottom layers did not increase at 12 °C or 20 °C during storage time. In these layers, O_2 levels ranged

Table 2
Oxygen concentration gradient within the model food at selected locations during storage time at two different temperatures. Oxygen concentration is expressed as percentage (%).

Temperature (°C)	Time (days)	Un-inoculated food			Inoculated food		
		Top	Center	Bottom	Top	Center	Bottom
12	0	8.00 (0.30)	1.16 (0.13)	0.63 (0.15)	9.37 (1.67)	1.18 (0.05)	0.71 (0.23)
	2	17.4 (3.64)	0.54 (0.05)	0.27 (0.02)	19.8 (0.61)	0.25 (0.14)	0 (0)
	5	11.6 (3.25)	0.56 (0.10)	0 (0)	19.0 (1.12)	0.16 (0.06)	0 (0)
	8	18.9 (1.34)	0.90 (0.27)	0 (0)	19.8 (0.12)	0.38 (0.16)	0.09 (0.01)
	11	17.2 (2.46)	0.33 (0.06)	0 (0)	17.1 (0.13)	0.57 (0.06)	0 (0)
	14	17.6 (1.33)	0.77 (0.14)	0 (0)	18.0 (1.47)	0.18 (0.03)	0 (0)
20	0	6.58 (1.12)	1.65 (0.10)	0.36 (0.15)	7.97 (1.67)	1.58 (0.11)	0.51 (0.23)
	1	9.63 (3.64)	0.73 (0.13)	0.09 (0.03)	10.1 (2.36)	0.65 (0.16)	0 (0)
	2	11.4 (2.3)	1.26 (0.10)	0 (0)	12.6 (2.10)	1.45 (0.12)	0 (0)
	3	12.1 (2.9)	1.21 (0.27)	0 (0)	14.0 (1.85)	1.38 (0.16)	0.10 (0.01)
	4	12.7 (2.3)	1.28 (0.13)	0 (0)	14.1 (1.32)	1.56 (0.11)	0.09 (0.03)

Data are presented as mean (standard deviation).

from 0 to 1.6%, conditions that could support the growth of *C. botulinum* at 20 °C, and for a longer term at 12 °C. Therefore, our findings suggest that air/oxygen in the package headspace, which results from packaging food without a vacuum, may not be considered as a hurdle in the food safety design of cook-chill products.

4. Conclusions

In this study, we investigated the effects of using oxygen in the package headspace to prevent the growth of *C. botulinum* in pasteurized chilled food during temperature abuse. *Clostridium sporogenes* PA 3679 was used as a model organism. Since this is a surrogate for proteolytic *C. botulinum*, the results are not necessarily 100% representative for *C. botulinum*. Therefore, the results should be considered as conceptual, with no firm conclusions for *C. botulinum*.

Storage temperature is an important factor in the margin of safety for pasteurized chilled food products. Storing food for 14 days at 12 °C has been found to prevent the growth of *C. sporogenes* PA 3679. During mild temperature abuse, food products may still be safe to consume. Bacterial growth increases rapidly when storage temperature increases to 20 °C. Therefore, exposing food products to severe temperature abuse may promote faster proliferation of proteolytic *C. botulinum*, compromising food safety. In this study, we found no beneficial effects of air/oxygen in the package headspace to prevent bacterial growth. Oxygen diffusion from the pouch headspace was primarily limited to the food surface layer. The structure and properties of the food significantly affected the mobility of the O_2 within it. Therefore, O_2 did not reach the center and bottom portions of the food during storage time, so that oxygen levels which favor *C. botulinum* growth were maintained. Our findings suggest that air/oxygen present in package headspace may not be considered as a hurdle in the food safety design of cook-chill products with a solid jelly-like structure and high water content.

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