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Using of infrared spectroscopy to study the survival and injury of *Escherichia coli* O157:H7, *Campylobacter jejuni* and *Pseudomonas aeruginosa* under cold stress in low nutrient media

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ABSTRACT

The inactivation and sublethal injury of *Escherichia coli* O157:H7, *Campylobacter jejuni* and *Pseudomonas aeruginosa* at three temperatures (22 °C, 4 °C and −18 °C) were studied using traditional microbiological tests and mid-infrared spectroscopy (4000–400 cm^{-1}). Bacteria were cultivated in diluted nutrient matrices with a high initial inoculation (~10^7 CFU/ml) levels. Both *E. coli* O157:H7 and *P. aeruginosa* survived and cell numbers increased at 22 °C for 5 days while *C. jejuni* numbers decreased one log_{10} CFU/ml. A two log CFU/ml decrease was observed for the three pathogens held at 4 °C for 12 days. *C. jejuni* survived poorly following incubation at −18 °C for 20 days while levels of *E. coli* O157:H7 and *P. aeruginosa* remained high (10^4 CFU/ml). Temperature stress response of microbes was observed by infrared spectroscopy in polysaccharide, protein, lipid, and nucleic acid regions and was strain specific. Level of cold injury could be predicted using cluster, discriminant function and class analog analysis models. Pathogens may produce oligosaccharides and potentially other components in response to stress as indicated by changes in spectral features at 1200–900 cm^{-1} following freezing.

1. Introduction

Escherichia coli O157:H7, *Campylobacter jejuni*, and *Pseudomonas aeruginosa* are food and waterborne pathogens that associated with several recent outbreaks. Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a major foodborne pathogen that causes hemorrhagic colitis and hemolytic uremic syndrome (HUS) (Wang and Doyle, 1998). The significant features of EHEC include transmission by a wide variety of food items and water, and a very low infectious dose is needed to cause disease, enabling high rates of attack and of person-to-person transmission (Nataro and Kaper, 1998). The major virulence factor and a defining characteristic of EHEC is Shiga toxin. This potent cytotoxin is the factor that leads serious symptoms and potentially death in patients infected with EHEC (Nataro and Kaper, 1998).

Campylobacter jejuni is a foodborne pathogenic bacterium, responsible for millions of cases of gastroenteritis annually, and is usually a relative mild and self limiting disease but still results substantial loss of productivity and lost work days particularly in developing countries and the strain is most commonly isolated from humans (Vandeplas et al., 2008). An estimated 250,000 cases of campylobacteriosis infections occur annually in the United States of America (USA) including several deaths (Levin, 2007).

Oligotrophic bacteria such as pseudomonads are capable of reproducing and forming biofilms under conditions that are usually considered to be nutrient restricted. Such organisms are found in low nutrient environments like drinking water, attaining population densities of 10^6 to 10^7 cells per ml in distilled water (McFeters et al., 1993). *P. aeruginosa* has been advocated as a means of assessing the hygiene quality of drinking water, and in certain circumstances, provides an indication of the general cleanliness of the water distribution system (Legnani et al., 1999).

Bacteria can survive under different storage and treatment conditions and harsh treatments may cause sublethal injuries to

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bacterial cells (Wu, 2008). Sublethally injured bacteria may lead to a significant health threat because they have the ability to repair themselves if conditions are favorable and then start to grow in food products presenting the potential for pathogenicity (Arpai, 1962; Rodrigues and Kroll, 1989; McCarthy, 1991; Williams and Golden, 2001). An important concern is that routine microbiological tests may give an underestimation of contamination level or false negative detections for sublethally injured cells, especially when using selective culture media. As a result, food could be presumed to be safe and free from pathogenic cells, but during storage may become unsafe due to the re-growth of injured cells (Williams and Golden, 2001; Bozoglu et al., 2004).

Fourier transform infrared (FT-IR) spectroscopy (4000–400 cm^{-1}) has been recently used to identify and differentiate microorganisms on the species and strains level based upon spectral features. The combination of attenuated total reflectance (ATR) of infrared spectrometer and advanced chemometrics (Workman et al., 1996) has been used in recent studies on: *E. coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica*, *C. jejuni*, *P. aeruginosa*, *Staphylococcus* spp., and *Cronobacter sakazakii* (Al-Qadiri et al., 2006a, Burgula et al., 2006; Lin et al., 2004; Mouwen et al., 2005, Al-Qadiri et al., 2006b; Goodacre et al., 1996; Lin et al., 2009). Our group was one of the first to pioneer the use of FT-IR to study bacterial injury caused by various factors, such as heating (Al-Qadiri et al., 2008b), sonication (Lin et al., 2004) and chemical treatment (Al-Qadiri et al., 2008a). In other studies, FT-IR was used to study heat-induced lethal and sublethal injury of *Lactococcus lactis* (Kilimann et al., 2006), osmotic and thermal induced injury of *E. coli* (Mille et al., 2002), pressure assisted thermal induced inactivation of *Clostridium tyrobutyricum* and *Bacillus sphaericus* (Subramanian et al., 2006; Subramanian et al., 2007) and radical induced damage of *Micrococcus luteus* (Lorin-Latxague and Melin, 2005).

The objective of this research work was to investigate the feasibility of FT-IR spectroscopy to monitor the degree and type of cell injury for *E. coli* O157:H7, *C. jejuni* and *P. aeruginosa* during storage at 22, 4, and −18 °C for up to 20 days.

2. Materials and methods

2.1. Bacterial strains and growth conditions

ATCC strains were obtained from Microbiologics® Inc. (St. Cloud, Minnesota, USA) for: *Escherichia coli* O157:H7ATCC 700728, *Campylobacter jejuni* ATCC 29428 and *Pseudomonas aeruginosa* ATCC 10145. To activate bacterial cells, *E. coli* O157:H7 and *P. aeruginosa* were inoculated into 50 ml of tryptic soy broth (TSB) (Bacto, Sparks, MD) and incubated at 37 °C for 24 h to yield a cell count of approximately 10^8–10^9 CFU/ml. *C. jejuni* was inoculated into 50 ml of campylobacter enrichment broth (Peyrat et al., 2008) consisting of campylobacter nutrient broth No. 2 (CM0067, Oxoid, England) and supplemented with campylobacter growth supplement (SR0232E, Oxoid, England). *C. jejuni* broth was then incubated in anaerobic jar at 42 °C for 24 h under microaerophilic conditions (10% CO_2, 5% O_2, and 85% N_2) using MGC-Pack-MicroAero™ (Mitsubishi Gas Chemical America, Inc. NY, NY).

2.2. Bacterial inoculation for survival studies

After the 24 h incubation, 1 ml broth of each strain was separately inoculated into 49 ml sterile saline solution (0.85% (w/v) NaCl) to yield a cell count of approximately 10^7 CFU/ml. After inoculation, bacterial viable counts were immediately measured in duplicate by serial decimal dilutions followed spread plating. Samples were then stored at 22, 4, and −18 °C for 5, 12, and 20 days. Control samples (1.0 ml broth without bacteria) were also prepared and stored as described above.

2.3. Recovery of bacteria and culture media

Bacterial viable counts were performed in duplicate by plating in parallel on selective and non-selective media using a spread plate technique. Non-selective R2A agar (Difco, Sparks, MD) was used to enumerate both *E. coli* O157:H7 and *P. aeruginosa*; agar plates were incubated at 37 °C for 24 and 48 h, respectively. Selective m-Endo agar LES (Difco, Sparks, MD) was used to enumerate *E. coli* O157:H7, whereas M-PA-C agar (BBL, Sparks, MD) was used to selectively enumerate *P. aeruginosa*; agar plates were incubated at 37 °C for 24 and 48 h, respectively. Preston campylobacter non-selective and selective culture media were used to enumerate survived bacteria (Peyrat et al., 2008). Preston campylobacter non-selective agar was prepared according to manufacturer's instructions, it consisting of campylobacter agar base (CM0689, Oxoid, England), campylobacter growth supplement (SR0232E, Oxoid, England) and lysed horse blood (Remel, Lenexa, KS). Preston campylobacter selective agar was prepared with addition of Preston campylobacter selective supplement (SR0117E, Oxoid, England). Non-selective and selective agar plates were incubated at 42 °C for 48 h under microaerophilic conditions (10% CO_2, 5% O_2, and 85% N_2).

2.4. FT-IR spectral measurements

At the end of each storage time, 50 ml of each sample was filtered through an aluminum oxide membrane filter (0.2 μm pore size, 25 mm OD) (Anodisc, Whatman Inc., Clifton, NJ) using a Whatman vacuum glass membrane filter holder (Whatman catalog no. 1960-032) to harvest bacterial cells. The anodisc membrane filter does not contribute spectral features between the wavenumbers of 4000 to 1000 cm^{-1}, providing a unique advantage compared with other types of substrates for spectral collection, such as cellulose ester filters (Burgula et al., 2006), polyethylene optical films (Mossoba et al., 2002) and hydrophobic grid membranes (Grasso et al., 2009). Aluminum oxide provides a smooth and flat surface onto which a bacterial film can form (Al-Qadiri et al., 2008a, Al-Qadiri et al., 2008b). The anodisc filters were then removed from the filtration apparatus and air-dried under laminar flow at room temperature for 5 min to allow a homogeneous dried film of bacterial cells to form. This method gives a more homogeneous distribution of cells and more reliable spectral results than recovering cells by centrifugation and then applying them to the membrane, thereby increasing the reproducibility of the spectroscopic method (Al-Qadiri et al., 2008a, Al-Qadiri et al., 2008b).

2.5. FT-IR spectroscopy and measurement

FT-IR spectra were collected using a Nicolet 380 FT-IR spectrometer (Thermo Electron Inc., San Jose, USA). The aluminum oxide membrane filter coated with a uniform and thin layer of bacterial cells was placed in direct contact with the diamond crystal cell (30,000–200 cm^{-1}) of attenuated total reflectance (ATR) detector. Infrared spectra were recorded from 4002 to 399 cm^{-1} at a resolution of 4 cm^{-1}. Each spectrum was acquired by adding together 32 interferograms. Four spectra were acquired at room temperature (22 °C) for each sample at different locations on the aluminum oxide filter for a total of 12 spectra for each treatment. Triplicate experiments ($N = 3$) were conducted and spectra from the first two times of experiments were used for establishment of chemometric models and the spectra from the third time of experiment were used for model validation.

2.6. Data processing and chemometrics

Infrared spectra were firstly pre-processed by EZ OMNIC 7.1a (Thermo Electron Inc.). Relevant background (control, aluminum oxide membrane filter coated with broth) was subtracted from each raw spectrum. Then, automatic baseline correction was employed to flatten baseline, following by a smooth of 5 (Gaussian function of 9.643 cm^{-1}). The pre-processed spectra were read by Matlab (2010a) (Math Works Inc., Natick, MA, USA) with xls format by Excel (Microsoft Inc., Redmond, WA, USA). The height and area of spectral bands were measured and calculated by OMNIC and Origin® 8.1 (OriginLab Co., Northampton, MA, USA). Second derivative transforms (with a gap value of 10 cm^{-1}) (Lu et al., 2010) and wavelet transforms (with a scale of 7) (Alsberg et al., 1997; Fu et al., 2005) were performed for spectral processing in Matlab. Chemometric models were established based on processed spectra, including cluster analysis (principal component analysis, PCA), dendrogram analysis (discriminant function analysis, DFA), class analog analysis (soft independent class of analog, SIMCA) and loading plot analysis (Lu and Rasco, In press). PCA is used to reduce the dimensionality of multivariate data (*i.e.* infrared spectra) while preserve most of the variances. Those selected unrelated principal components (PCs) are plotted and visualized in cluster forms (Wang et al., 2010). DFA can construct branched dendrogram structures with prior knowledge of sample's information (Lopez-Diez and Goodacre, 2004). Loading plots were derived from DF-PCA and used for explaining segregation of chemometric models based on molecular levels (Lu and Rasco, In press). SIMCA is a supervised classification method. The test samples are compared to study the analogy to the training set of samples (Al-Qadiri et al., 2008a). By using a combination of different chemometric models, it is possible to determine effects of various treatments on bacteria (Al-Qadiri et al., 2008b).

2.7. Statistical analysis

Three independent replicate trials were conducted and significant differences ($P < 0.05$) between microbial levels on selective and non-selective media, band area of spectra, and regression coefficient of loading plot (first 3 PCs) were determined by one-way analysis of variance (ANOVA) followed by *T*-test using Matlab.

3. Results and discussion

Table 1 shows the viable (log_{10} CFU/ml) of *E. coli* O157:H7, *C. jejuni* and *P. aeruginosa* after storage at 4, 22 and −18 °C held in a minimal media and then plated on selective and non-selective culture media at preset time intervals. *E. coli* O157:H7 cells were able to survive under these storage conditions. However, there was little increase in the number of viable counts on non-selective and selective agars (7.94 log_{10} and 7.32 log_{10} CFU/ml) during storage at 22 °C indicating little cells multiplication under these conditions. Viable *E. coli* on non-selective agar decreased by approximately 6.00 CFU/ml following storage at 4 °C. At −18 °C, there was approximately a one log difference in viable *E. coli* on selective and non-selective culture media, suggesting the presence of recovered sublethally injured cells.

In case of *P. aeruginosa*, cells were able to survive well, with 1.3 log_{10} and 3.1 log_{10} CFU/ml reductions observed on either selective or non-selective media for samples stored at 4 °C and −18 °C, respectively. *C. jejuni* cell numbers significantly declined to 5 log_{10} CFU/ml survived under −18 °C for 20 days, confirming the sensitivity of this pathogen to freezing (Jasson et al., 2007). Induced sublethally injured cells were also observed at this temperature due to the differences between selective and non-selective agars (Table 1).

Table 1
Viable counts (Log_{10} CFU/ml) of *E. coli* O157:H7, *C. jejuni* and *P. aeruginosa* after storage at 22, 4 and −18C for 5, 12 and 20 days, respectively.

Sample treatment	Viable counts (Log_{10} cfu/mL)[a]	
	Non-Selective	Selective
E. coli O157:H7		
Control	7.60 (0.11)	7.16 (0.28)
22 °C, 5 days	7.94 (0.32)	7.32 (0.09)
4 °C, 12 days	6.00 (0.25)	6.00 (0.14)
−18 °C, 20 days	5.92 (0.08)	4.98 (0.21)
P. aeruginosa		
Control	7.60 (0.25)	7.51 (0.30)
22 °C, 5 days	7.61 (0.27)	7.52 (0.22)
4 °C, 12 days	6.30 (0.09)	6.23 (0.13)
−18 °C, 20 days	4.51 (0.24)	4.19 (0.12)
C. jejuni		
Control	7.00 (0.12)	6.95 (0.21)
22 °C, 5 days	6.90 (0.12)	6.85 (0.09)
4 °C, 12 days	5.60 (0.25)	5.08 (0.15)
−18 °C, 20 days	2.00 (0.07)	1.60 (0.09)

[a] Samples were enumerated in duplicate using spread plate technique.

3.1. FT-IR spectral features of survived E. coli O157:H7, P. aeruginosa and C. jejuni

FT-IR spectral features of *E. coli* O157:H7, *P. aeruginosa* and *C. jejuni* were shown in Fig. 1, illustrating features including: cell membrane phospholipids, cell wall polysaccharides, cytoplasmic proteins and lipids and nucleic acids (Al-Qadiri et al., 2006b). The wavenumbers between 3000 and 2800 cm^{-1} represent lipid regions, with three bands (2966, 2929, and 2852 cm^{-1}) assigned to alkyl group of lipids (Table 2). The distinct band around 3300 cm^{-1} covers part of spectral features in lipid regions, resulting in a "shoulder" shape. The bands at 1637, 1545, 1458, 1395 cm^{-1} correspond to protein features (Table 2) with the band at 1637 cm^{-1} assigned to amide I of β-pleated sheet (Maquelin et al., 2002), the secondary protein structures, often the most prominent band in bacterial infrared spectra. This band has been extensively used for normalization of raw infrared spectra for compensation of variation of sample thickness (Al-Qadiri et al., 2008a; Lu and Rasco, In press). The band at 1545 cm^{-1} is assigned to amide II, the N—H banding of amide of proteins (Maquelin et al., 2002). The ratio of bands (both area and height) between amide I and amide II is close to 9.3:7 for microbial analytes (Naumann, 2001). The bands at 1458 and 1395 cm^{-1} are assigned to cell structure proteins (Lu and Rasco, In press). The band at 1235 cm^{-1} is assigned to P═O asymmetric stretching of PO_2^- phosphodiesters (Naumann, 2001), associated with phospholipid bilayers. The band at 1080 cm^{-1} is related to nucleic acids (Al-Qadiri et al., 2008a), however, it was worth noting that the wavenumbers between 1200 to 900 cm^{-1} (wide range) may reflect information about a variety of polysaccharides (Naumann, 2001). This may be the reason why the 1080 cm^{-1} band was wide with the appearance of smaller "shoulder" bands.

The variations of spectral patterns of *E. coli* O157:H7, *C. jejuni* and *P. aeruginosa* during storage at 22, 4 and −18 °C were apparent and reflected how cold injury affects these three bacterial strains differently. Fig. 1 shows the average of raw spectra of each treatment (22, 4, and −18 °C, respectively) after subtraction of media and filter backgrounds. Results from replicated experiments were consistent as reflected in the validated cluster analysis, dendrogram analysis and class analog models. In the case of *E. coli* O157:H7, an

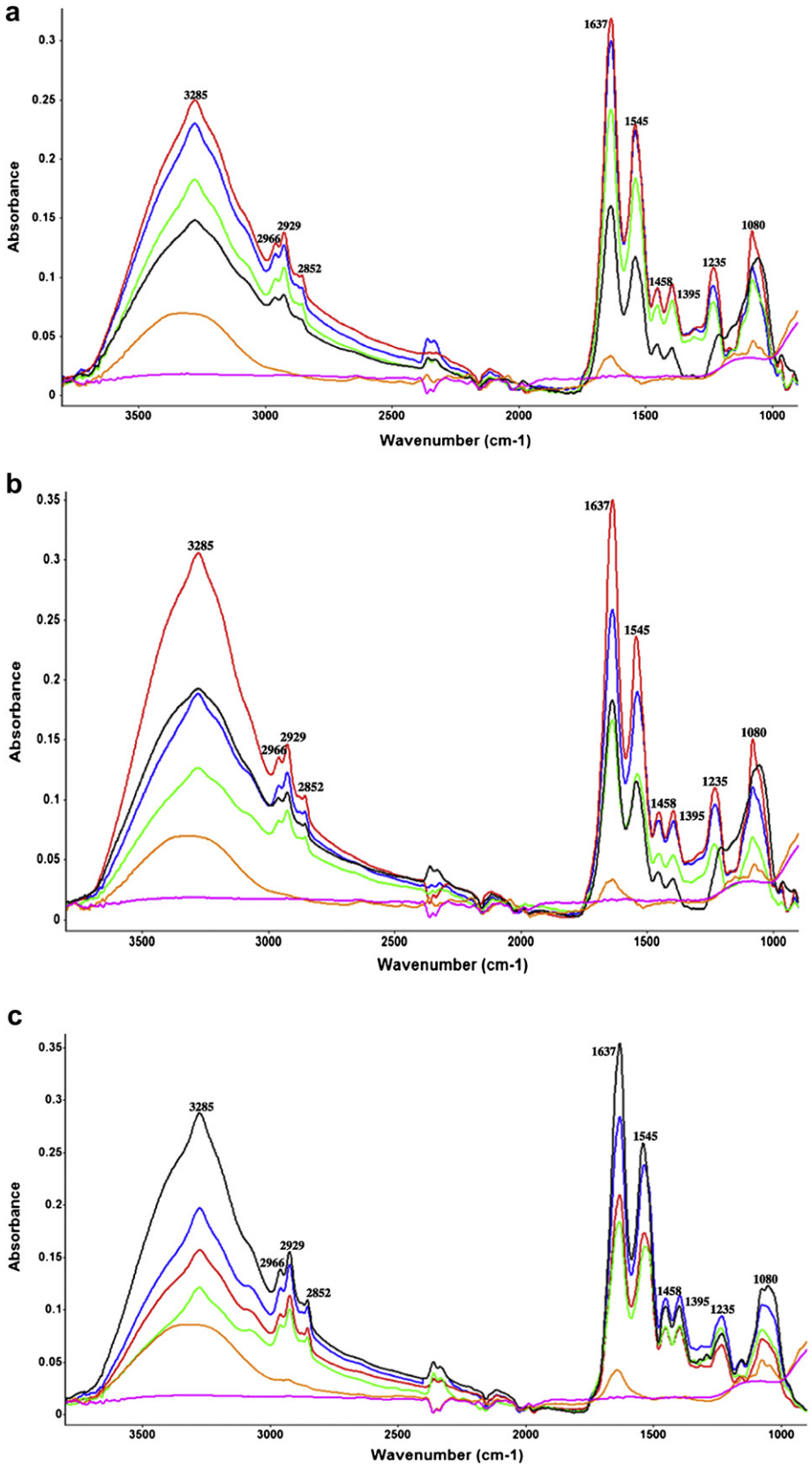

Fig. 1. (a). The variance of average spectral features of *E. coli* O157:H7 under selective treatments (blue: control; red: 22 °C for 5 days; green: 4 °C for 12 days; black: −20 °C for 20 days, orange: membrane coated with tryptic soy broth (1/50 diluted (v/v)); purple: aluminum anodisc membrane filter). The spectra were subtracted from background. (b). The variance of average spectral features of *P. aeruginosa* under selective treatments (blue: control; red: 22 °C for 5 days; green: 4 °C for 12 days; black: −20 °C for 20 days, orange: membrane coated with tryptic soy broth (1/50 diluted (v/v)); purple: aluminum anodisc membrane filter). The spectra were subtracted from background. (c). The variance of average spectral features of *C. jejuni* under selective treatments (blue: control; red: 22 °C for 5 days; green: 4 °C for 12 days; black: −20 °C for 20 days, orange: membrane coated with tryptic soy broth (1/5 diluted (v/v)); purple: aluminum anodisc membrane filter). The spectra were subtracted from background. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 2
Assignment of bands in FT-IR spectra of microorganisms (4000–400 cm^{-1}).

Wavenumber (cm^{-1})	Assignment
~3285	N–H str of proteins and O–H str of polysaccharides and water
~2966	C–H str (asym) of -CH_3 in fatty acids
~2929	C–H str (asym) of >CH_2 in fatty acids
~2852	C–H str (sym) of >CH_2 in fatty acids
~1740	>C=O str of esters
~1715	>C=O str of carbonic acid
~1695	amide I band component
~1685	>C=O in nucleic acid
~1655	amide I of α-helical structures
~1637	amide I of β-pleated sheet structures
~1620	str base carbonyl and ring breathing mode of nucleic acid
~1550–1515	amide II
~1469	CH_2 bending of the acyl chains (phospholipids)
~1458	C–H def of >CH_2 of proteins
~1395	C=O str (sym) of COO^- of proteins
~1235	P=O str (asym) of >PO_2^- phosphodiesters
~1161	str C–OH of serine, threonine, and tyrosine residues of cellular proteins
~1080	P=O str (sym) of >PO_2^- in nucleic acid
1200–900	C–O–C of polysaccharide and, str of phosphate
~1150	C–O str of carbohydrate
~1078	C–OH str of oligosaccharide
~1028	–CH_2OH and C–O str coupled with C–O bending of carbohydrate

increase in band area at 1080 cm^{-1} (nucleic acids) ($P < 0.05$) is indicative of microbial growth at 22 °C which was anticipated (Fig. 1 (a)) (Nataro and Kaper, 1998). During storage at 4 °C, areas of bands at 2966, 2929, 2852 (lipid), 1637, 1545 (secondary structure of protein) and 1235 cm^{-1} (phosphodiesters) decreased significantly ($P < 0.05$) compared to control ($t = 0$) samples. This might be associated with a decrease in viable counts (Table 1), cessation of cell growth, and cell death and appear to be concentration related. This microbe can produce cold shock proteins (Bollman et al., 2001) particularly following a sudden shift to a cold temperature environment playing an important role in maintaining cellular and physiological functions under stress, such as DNA modification, protein folding, sugar absorption and metabolic efficiency. Changes in spectral features in response to cold shock at 1458 and 1395 cm^{-1} may be associated with production of cold shock proteins. At −18 °C, the intensity of spectral features associated with lipid and structural protein dropped ($P < 0.05$) indicating cell death. Sublethally injured cells were present in significant numbers (Table 1). Phosphodiesters (1235 cm^{-1}) and structural proteins (1458 and 1395 cm^{-1}) features were lost and this provides an indication of that freezing damages phospholipid bilayers of cell membrane confirming findings of others who demonstrated that lipid bilayers may undergo both conformational alteration and structural damage in frozen cells in addition to the probable loss of phosphate (Kempler and Ray, 1978). However, the carbohydrate bands increased significantly ($P < 0.05$) offsetting changes in loss of nucleic acids (~1080 cm^{-1}) likely an indication of production of polysaccharides in response to stress.

For *P. aeruginosa*, significant spectral variations were observed in its response to different temperature treatments (Fig. 1(b)). The amide I (1637 cm^{-1}) and II (1545 cm^{-1}) bands increased significantly ($P < 0.05$) compared to control when samples stored at 22 °C, which might link to cell growth (Table 1). For cold stress (4 °C), band intensities decreased significantly ($P < 0.05$) compared to the control in what appears to be a concentration dependent effect. For freeze stress at −18 °C, loss in spectral intensity was significant ($P < 0.05$) compared to the control due to the reduction in viable cells and cessation of growth. The bands around 1080 cm^{-1} showed an increase in presumptive polysaccharides (Arpai, 1962; Buttner and Amy, 1989; EL-Kest and Marth, 1992; Restaino et al., 2001).

For *C. jejuni*, significant differences in the type of changes in spectra were observed compared to both *E. coli* O157:H7 and *P. aeruginosa*. Freezing inactivated *C. jejuni* (Jasson et al., 2007) rapidly, but the microbe survived at low temperature (4–6 °C). The temperature threshold at which survival becomes significantly affected is 16–22 °C (Hunter, 1993; Wang and Doyle, 1998). When *C. jejuni* strains were stored at 22 °C for five days, almost one $\log_{10}$ reduction occurred and this loss in cell numbers was reflected in the spectra where bands intensities decreased significantly ($P < 0.05$) from the control. *C. jejuni* is very sensitive to freeze stress as indicated by previous research (Sampers et al., 2010; Al-Qadiri et al., 2010; Doyle and Roman, 1981; Humphrey, 1986; Ray and Johnson, 1984). In general, the sensitivity of this bacterium to low temperatures varies widely and mainly depends upon population density, bacterial strain, procedure of recovery and cooling rate. Reduced osmolality (Reezal et al., 1998) and low nutrient media create a more stressful environment for fastidious microorganisms such as *C. jejuni* when compared to nutrient-rich broths or food (Höller et al., 1998). Freeze injury may result from continued exposure to concentrated solutes (low water activity) and physical damage caused by ice crystal formation (Archer, 2004). However, for the spectral features of amide I and II bands, these increased significantly compared to the control ($P < 0.05$), the same was observed among the nucleic acid and carbohydrate regions (~**1080** cm^{-1}). Moen et al. (2005) used FT-IR and DNA microarray to study the macromolecular changes of *C. jejuni* cell membrane under non-growth survival conditions and found that the amount of polysaccharides and oligosaccharides (1200–900 cm^{-1}) increased. The potential mechanisms for survival could be to down regulate most functions to save energy and to produce polysaccharides and oligosaccharides for protection against harsh environment. *C. jejuni* is also susceptible to another phenomenon that may be related to injury: the change in cellular morphology from curved, spiral rods to coccoid and donut forms (Buck et al., 1983; Ng et al., 1985).

3.2. Cluster, dendrogram and class analog analysis of *E. coli* O157:H7, *C. jejuni* and *P. aeruginosa*

The PC-DFA analysis was performed for *E. coli* O157:H7, *P. aeruginosa* and *C. jejuni* separately (Fig. 2, Fig. 3 and Fig. 4). Both cluster analytical model (principal component analysis) and dendrogram analytical model (discriminant function analysis) were established first using the spectra from the first two experiments. The spectra from the third experiment were used for model validation (red color in PCA model and star notation in DFA model). For all three pathogens, both PCA and DFA provided a clear segregation for the various treatments (control, 22, 4 and −18 °C). The *Mahalanobis distance* measurements were performed to compute between the centroids of classes (control, 22, 4 and −18 °C). The interclass distances ranged from 6.93 to 29.27, from 5.38 to 23.56 and from 9.41 to 37.83 for different classes of *E. coli* O157:H7, and *C. jejuni* and *P. aeruginosa* respectively. Clusters with interclass distance values higher than 3 are believed to be significantly different from each other (De Maesschalck et al., 1999). To classify each treatment for each pathogen, class analog analysis (SIMCA) was employed to compare spectral features of treatments (control, 22, 4 and −18 °C). Table 3 showed the SIMCA classification results of each treatment compared to the other test treatments. For both *E. coli* O157:H7 and *P. aeruginosa*, 90% correct rate of sorting was achieved, whereas 100% correct classification was achieved for *C. jejuni*. Accordingly, a high percentage of classification (>90%) can provide a good indication for the degree of similarity of the tested treatments (Al-Qadiri et al., 2006a).

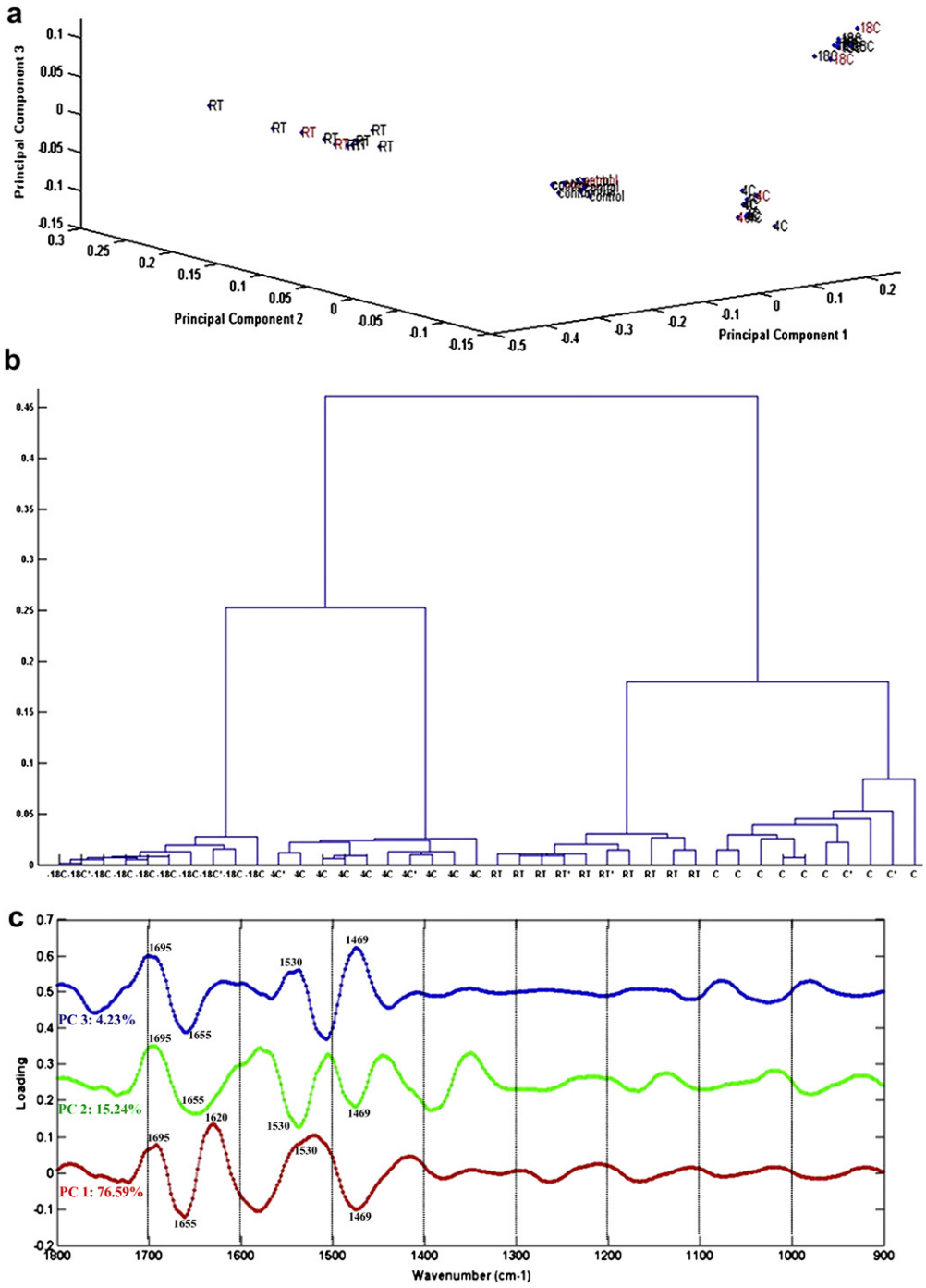

Fig. 2. (a). Principal component analysis of *E. coli* O157:H7 treated under room temperature for 5 days (RT), 4 °C for 12 days (4C), −18 °C for 20 days (−18C) and control (control). The cluster analysis model was validated by new treatments (red color in figure). (b). Hierarchical cluster analysis of *E. coli* O157:H7 treated under room temperature for 5 days (RT), 4 °C for 12 days (4C), −18 °C for 20 days (−18C) and control (C). The dendrogram function analysis model was validated by new treatments (* in figure). (c). Loading plots of the first (red), second (green), and third (blue) principal component obtained from PC-DFA of FT-IR spectra of *E. coli* O157:H7 treated under room temperature for 5 days, 4 °C for 12 days, −18 °C for 20 days and control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.3. Loading plots studies of *E. coli* O157:H7, *P. aeruginosa* and *C. jejuni*

Analysis of FT-IR loading plots showed that the chemical composition of bacterial cells was affected by temperature stress. The major bands on the first three PCs are most important to elucidate the physiological changes of bacterial cells during different treatments, such as cold and freeze stresses as determined by one-way ANOVA with *T*-test ($P < 0.05$) as applied to cluster analysis and dendrogram analysis. The first three PCs can explain approximately 90% segregation of cluster analysis (PCA) and dendrogram analysis (DFA) for *E. coli* O157:H7, *P. aeruginosa* and *C. jejuni* (Fig. 2(c), Fig. 3(c) and Fig. 4 (c)). Among of them, the first principal component can approximately contribute to 75%–80% of segregation.

For *E. coli* O157:H7, the three bands at 1695, 1655 and 1520 cm^{-1} are assigned to protein (Table 2). The variations of these protein

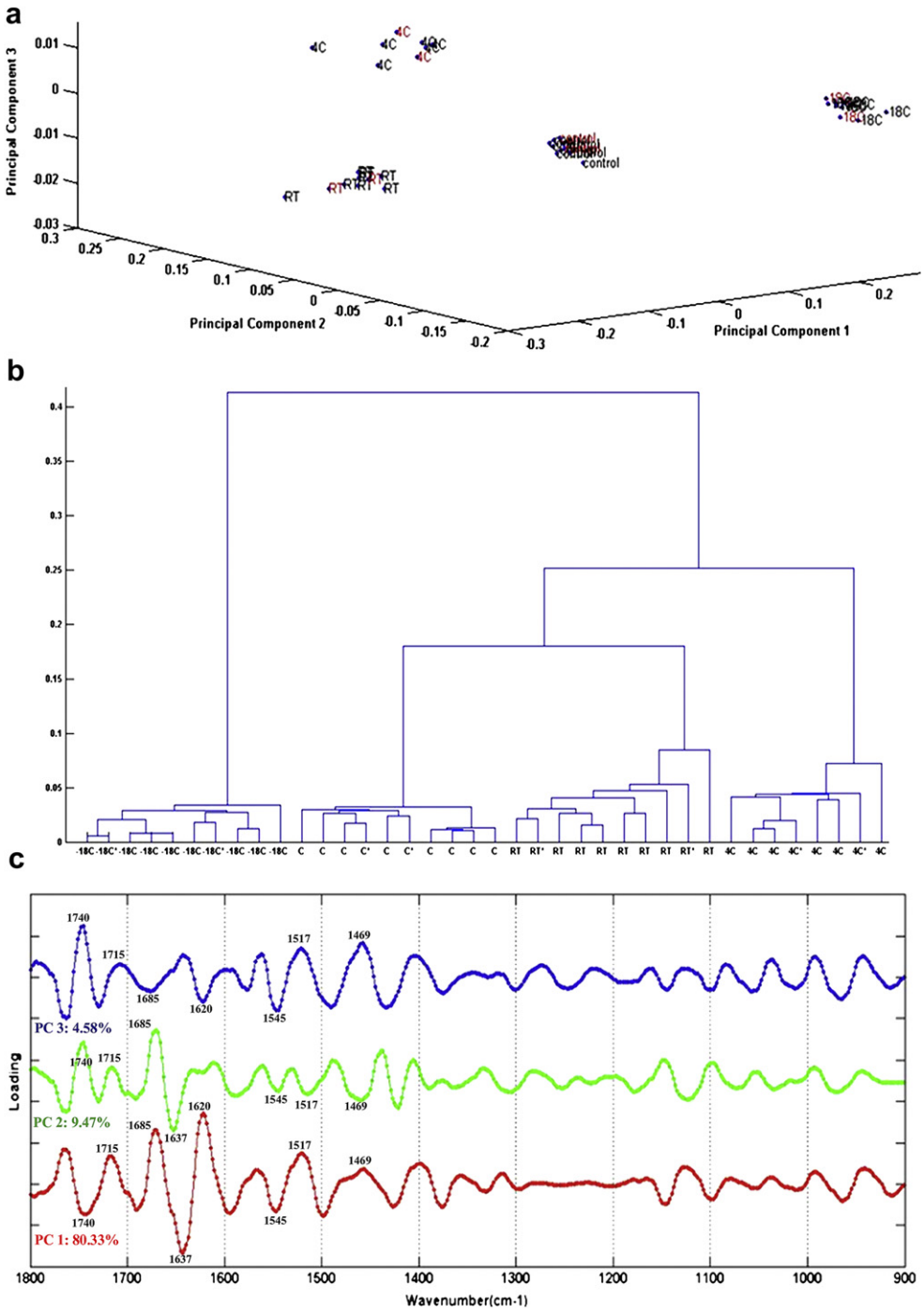

Fig. 3. (a). Principal component analysis of *P. aeruginosa* treated under room temperature for 5 days (RT), 4 °C for 12 days (4C), −18 °C for 20 days (−18C) and control (control). The cluster analysis model was validated by new treatments (red color in figure). (b). Hierarchical cluster analysis of *P. aeruginosa* treated under room temperature for 5 days (RT), 4 °C for 12 days (4C), −18 °C for 20 days (−18C) and control (C). The dendrogram function analysis model was validated by new treatments (* in figure). (c). Loading plots of the first (red), second (green), and third (blue) principal component obtained from PC-DFA of FT-IR spectra of *P. aeruginosa* treated under room temperature for 5 days, 4 °C for 12 days, −18 °C for 20 days and control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

bands can explain 86% of the total variation and this demonstrated that changes of protein secondary structure (amide I and II) are prominent during cold and freeze stress. The band at 1620 cm^{-1} is assigned to nucleic acid band (Naumann, 2001) while the band at 1469 cm^{-1} is assigned to phospholipid band (Movasaghi et al., 2008). Cell membrane damage was anticipated to happen during cold and freeze stress and the leakage of cell membrane also caused loss of nucleic acids inside of cells (Speck and Ray, 1977) and reduction in absorbance in these spectral regions for *E. coli* and *P. aeruginosa*. During freeze treatment, microorganisms are usually damaged by osmotic stress and elevated solute concentrations (low water activity) rather than being damaged mechanically by ice crystals (Gill, 2002).

For *P. aeruginosa*, the three bands at 1637, 1545 and 1517 cm^{-1} are assigned to protein (Table 2). The bands at 1685 and 1620 cm^{-1} are assigned to nucleic acids (Table 2) and the band at 1715 cm^{-1} to

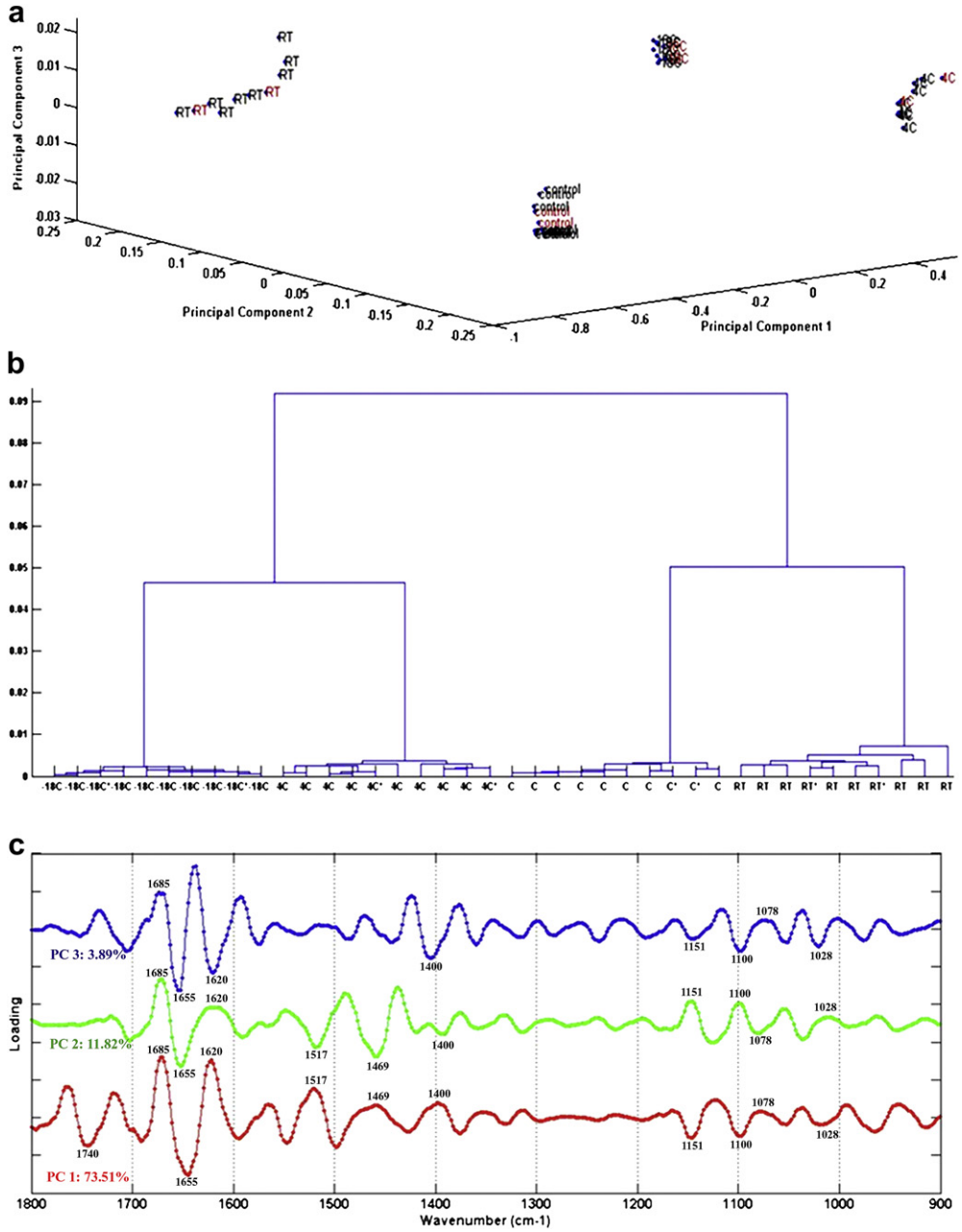

Fig. 4. (a). Principal component analysis of *C. jejuni* treated under room temperature for 5 days (RT), 4 °C for 12 days (4C), −18 °C for 20 days (−18C) and control (control). The cluster analysis model was validated by new treatments (red color in figure). (b). Hierarchical cluster analysis of *C. jejuni* treated under room temperature for 5 days (RT), 4 °C for 12 days (4C), −18 °C for 20 days (−18C) and control (C). The dendrogram function analysis model was validated by new treatments (* in figure). (c). Loading plots of the first (red), second (green), and third (blue) principal component obtained from PC-DFA of FT-IR spectra of *C. jejuni* treated under room temperature for 5 days, 4 °C for 12 days, −18 °C for 20 days and control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

carbonic acid (Naumann, 2001). The bands at 1740 (Al-Qadiri et al., 2006a) and 1469 cm^{-1} (Naumann, 2001) are related to lipids and phospholipids, respectively. For *C. jejuni*, the three bands at 1151, 1078, and 1028 cm^{-1} are assigned to carbohydrate bands (Oust et al., 2006). The variation of carbohydrate bands in *C. jejuni* were observed by others (Moen et al., 2005; Oust et al., 2006) using FT-IR to study bacterial cell response to stress (temperature, atmosphere, etc.). Oust et al. (2006) observed the increase of these three bands during cold stress and they postulated that the increase was due to production of osmoregulated periplasmic glucans, polysaccharide capsule, or glycoprotein in response to stress. The bands at 1740, 1469 and 1400 cm^{-1} correspond to lipid and phospholipid bands (Table 2). Since both lipids and phospholipids are abundant in the cell membrane and cell wall, changes of these macromolecules during stress response are reasonable. The effect of temperature on fatty acid composition in *Campylobacter* species was studied (Höller et al., 1998). The band at 1655 cm^{-1} is assigned to amide I of α-helical structures and 1637 cm^{-1} of amide I of β-pleated sheet structures. Thus, a change of protein secondary structure from β-pleated sheet to α-helix may occur during cold and freeze stress treatment to *C. jejuni*. The bands at 1685, 1620 and 1100 cm^{-1} associated with nucleic acid bands (Table 2) suggest that damage to

Table 3
SIMCA classification results for each treatment compared to the other test treatments.

Sample treatment	No. of correctly classified spectra	% of correctly classified spectra
E. coli O157:H7		
Control	10	100
22 °C for 5 days	9	90
4 °C for 12 days	10	100
−18 °C for 20 days	10	100
P. aeruginosa		
Control	9	90
22 °C for 5 days	9	90
4 °C for 12 days	10	100
−18 °C for 20 days	10	100
C. jejuni		
Control	10	100
22 °C for 5 days	10	100
4 °C for 12 days	10	100
−18 °C for 20 days	10	100

days, −18 °C for 20 days and control.

DNA was observed during freeze-thawed bacterial cells (Wesche et al., 2009).

4. Conclusion

FT-IR (4000–400 cm^{-1}) combined with various chemometric tools was used to study the survival and injury of three important Gram negative pathogens in bottled drinking water held in low nutrient media at different temperatures (22 °C for 5 days, 4 °C for 12 days and −18 °C for 20 days). The survival and injury of *E. coli* O157:H7, *P. aeruginosa* and *C. jejuni* was confirmed by both selective and non-selective media. The infrared spectral variation was distinct under each treatment and can reflect the changes of macromolecules on the surface of membrane, which may illustrate the mechanism of cell injury initiated from the morphology and physiological changes on the membrane. *C. jejuni* was most impacted by freezing but still survived for 20 days (~6 log reduction) and exhibited different signs of cell injury compared to *E. coli* O157:H7 and *P. aeruginosa* particularly in the carbohydrate region (1200–900 cm^{-1}). PCA, DFA and SIMCA correctly segregated >90% of bacteria by degree of injury. Loading plots indicated that various biochemical features are important for cold induced injury in bacteria. Further studies will focus on explorative multifactor approaches for investigating pathogen injury under various stresses, including DNA microarray, scanning electron microscopy, and vibrational spectroscopy.

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