Survival strategy of foodborne pathogenic bacteria under low water activity environment: Contribution of glass transition phenomenon of bacterial cells

（低水分活性環境における食品媒介食中毒細菌の生存戦略：
細菌細胞のガラス転移現象の寄与 ）

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Chapter 1

Introduction

1.1. Drying in the food industry

Drying is one of the oldest and most widely utilized methods for food preservation worldwide. Through various drying methods, moisture in the food is transferred to the surface, from where it evaporates into the air (Lewicki et al., 2006). Drying is important not only in the food industry but also in various other fields such as agriculture, manufacturing, chemistry, and the pharmaceutical industry. Removing water extends the shelf life of food and makes it easier to distribute, package, and operate by reducing the volume and mass of the product (Onwude et al., 2016). More than 200 different dryers and their respective methods have been reported, and 10–15% of the energy requirements of the food industry are used for drying in developed countries (Klemes et al., 2013).

Air and freeze-drying are the most commonly used methods for drying food. Air-drying is the process of exposing a product to air and evaporating the moisture of food into the air. It has been used since ancient times to preserve food. Air-drying is advantageous as it is simple to use and inexpensive. However, it is affected by several factors, such as temperature, weather, and water activity conditions, and is more time consuming than freeze-drying. In addition, air-dried products are usually of lower quality than the original foods, as the properties, such as color, appearance, taste, and flavor, are affected during air-drying procedures. (Hammami and Rene., 1997; Ratti., 2001). Freeze-drying is the most useful process for maintaining the initial

properties of the raw material of the original food after drying, and it has a high rehydration capacity compared to other drying methods (Hammami and Rene., 1997; Ratti., 2001). Freeze-drying uses the principle of sublimation of water in frozen foods with decompression, to remove the moisture content (Barbosa et al., 2015). Furthermore, it is easy to maintain the sensory quality of the food because freeze-drying is carried out under low-temperature conditions, the drying period is shorter, and less water content is retained in the food as compared to that in the air-dried food; therefore, the freeze-dried food can be stored for a longer period. Drying blueberries using various methods revealed that freeze-drying provided the highest nutrient retention compared with other methods, and it was reported that freeze-drying would be suitable for consumers because the rehydration ratio was high and bulk density was low (Yang and Atallah, 1985; Garcia-reverter et al., 1994; Hammami and Rene, 1997; Ratti, 2001). However, the processes and equipment used in freeze drying are more complex and expensive than those used in air-drying, and pose the risk of leakage of silicone oil (Hammami and Rene, 1997; Ratti, 2001; Meissner et al., 2011; Prosapio et al., 2017). Freeze-drying is more expensive than air-drying, as it costs approximately six times higher than the latter (Knorr, 1998).

1.2 Water activity and dried foods

As one of the constituents of food, water affects not only its physical properties but is also closely related to food quality and safety. The method of preserving food by adjusting the moisture content has been used since ancient times, and the index of moisture content has been used for measuring the water content present in food. This is the total amount of moisture present in food, expressed as a percentage of the total weight. Moisture content is not only useful for measuring dry weight but also for calculating the nutrient content in foods. However, it is difficult to determine the relationship between moisture content and deterioration of quality, such as food spoilage and microbial growth, in food (Labuza., 1980). An index called water activity (a_w), which measures the content of active moisture in food, was presented by Scott (Scott, 1957).

The a_w is calculated as the ratio of the vapor pressure of water in food to the vapor pressure of pure water as an indicator of the available water present in the material (Lewicki et al., 2004). This index not only provides information on food storage systems but is also an important factor in determining the shelf life of foods. Each microorganism has an optimal a_w value for growth. According to Labuza's study, most fresh foods exhibit >0.95 a_w (Labuza, 1980; Brewer, 1999). In contrast, foods, such as chocolate, honey, and powdered infant formula show <0.6 a_w. Most microorganisms show suppressed growth under <0.7 a_w, especially bacteria that can grow at >0.9 a_w (Brewer, 1999). The growth of microorganisms remains under control at <0.6 a_w, and foods exhibiting these conditions are called dried foods (Labuza, 1980).

1.3 Foodborne pathogenic bacteria in dried foods

Although the minimum a_w for the growth of most bacteria is reported to be 0.87, food poisoning accidents occur in low-moisture foods and dried-foods (Beuchat et al., 2013; Finn et al., 2013; Finn et al., 2013; Beuchat and Mann, 2015; Sanchez-Maldonado et al., 2018). A total of 714 cases of *Salmonella enterica* Typhimurium infection occurred in approximately 8 months in the United States, which were associated with peanut butter and other peanut products that have low moisture content (Cavallaro et al., 2011). A total of 439 *S. enterica* Oranienburg infections occurred in Germany in 2002, most of which were transmitted due the distribution of chocolates manufactured by a particular company (Werber et al., 2005). Several other *Salmonella* serotypes have been investigated in various low-moisture foods such as powdered infant formula (Cahill et al., 2011), sesame seed (Brockmann et al., 2003), and cereal (Russo et al., 2013). Although *Cronobacter sakazakii* has been investigated in various products, it is mainly found in powdered infant formula (Iversen and Forsythe, 2004; Friedemann, 2007; Henry and Fouladkha, 2019;). *C. sakazakii* can survive in powdered infant formula with low a_w, for more than 2 years (Edelson-mammel et al., 2005; Barron and Forsythe, 2007; Koseki et al., 2015). Moreover, it was found that *S. Typhimurium* and *C. sakazakii* have high tolerance to external environmental stresses such as drying time and thermal treatment under low water activity conditions (Lang et al., 2017; Lang et al., 2018). Since the reason these bacteria persist in low-moisture foods is not clear, it is necessary to investigate the mechanism by which they can have strong tolerance in extreme environments.

1.4. Glass transition phenomenon

As pathogenic bacteria persist even in low-moisture foods, the concept of a_w alone may be insufficient to define the microbial safety and stability of dried foods. Additionally, water activity only provides information about the amount of free and bound water, which does not explain changes in the physical properties of foods, the concept of glass transition phenomenon applies to foods as other alternatives (Rahman, 2009). In the case of monomers, as the temperature decreases, motility of the molecule also decreases, and it organizes itself into a highly ordered crystalline structure. However, in general, it is difficult to obtain a completely arranged form in polymers with long chains. Therefore, the molecule is organized into a non-crystalline structure in which the arrangement of the molecule is not constant, although it reduces the motility of the molecule. This form is called the glassy state (Fig. 1–1). Although the glassy state is solid, it has an amorphous form like a liquid; this property is displayed by various polymers (Noel et al., 1990). The phenomenon in which a rubbery or liquid state changes to a glassy state is called glass transition (Kauzmann, 1948; Dyre, 2006), and the temperature at which the glass transition occurs is called glass transition temperature (T_g). In the glassy state, the object is seemingly solid, but even if the molecules have a disordered shape, the mobility of molecules decreases sharply (Rahman, 2009). Typical examples from foods are pasta and sweets; in pasta, during the cooking process, the glassy state changes to the rubbery state through the glass transition phenomenon (Noel et al., 1990). Therefore, when heated above T_g, the molecule gains increased mobility and has a viscous flow similar to that of rubber. In contrast, when a substance is cooled below the T_g, it becomes brittle and glass-like, and the

molecules have low motility (Roos, 2010). T_g has been reported to be related to a_w (Sapru and Labuza, 1993). The studies investigating the T_g of each bacterial suspension (a mixture of *Lactobacillus bulgaricus* bacterial cells and fermented medium) and the fermentation medium (culture medium at the end of the fermentation), using differential scanning calorimetry (DSC), clarified that the glass transition temperature increases as the a_w decreases (Fonseca et al., 2001). It was also confirmed that organisms, such as African chironomid and tardigrade, can change to a glassy state called cryptobiosis and become viable in harsh environments (Sakurai et al., 2008; Horikawa et al., 2012). Therefore, it is hypothesized that the bacterial cells persisting in low a_w conditions are also associated with the glass transition phenomenon.

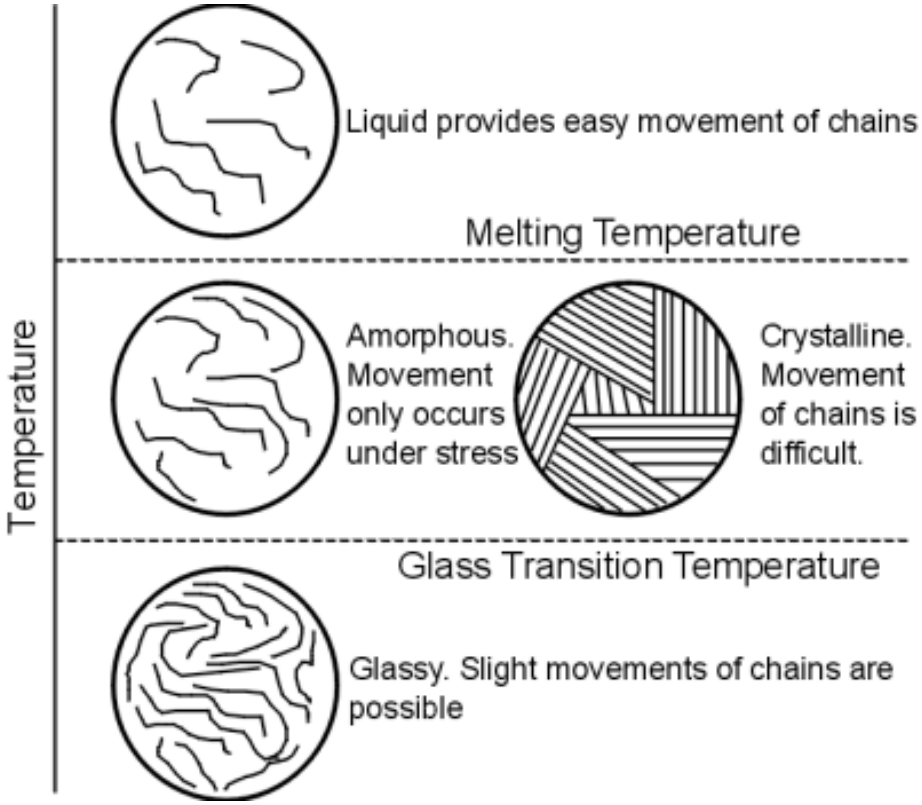


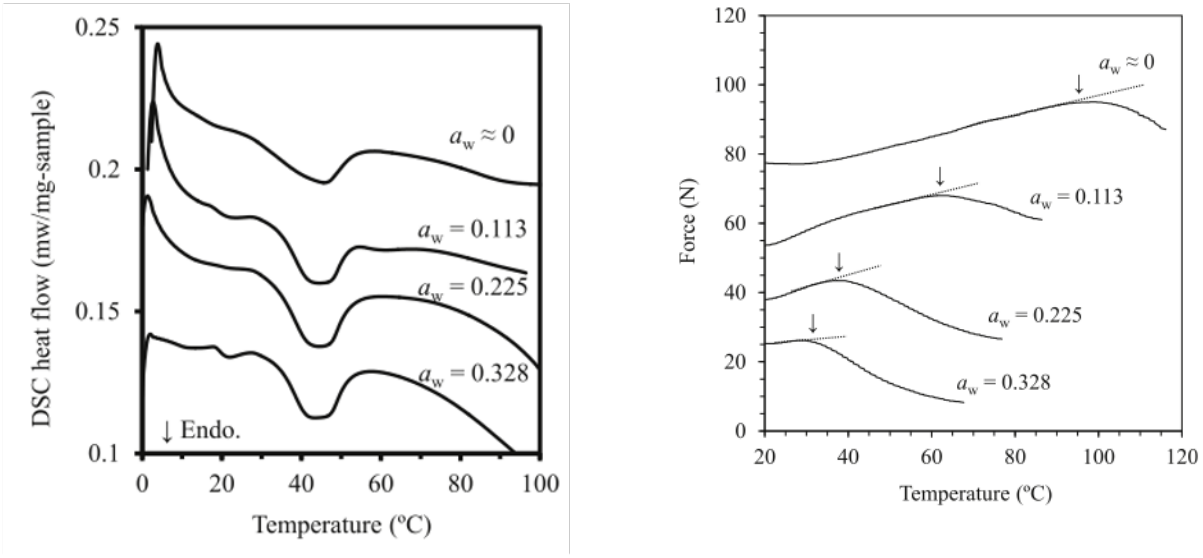
Fig 1-1. Arrangement of material depending on temperature (Askeland et al., 2003).

1.5. Thermal rheological analysis

Differential scanning calorimetry (DSC) is widely used as a method of measuring the T_g of material. DSC is a device that utilizes the principle of the changing heat flow of the sample and the reference sample at controlled temperature using a program (Hohne et al., 2003). However, measurement of the T_g of *Lactobacillus bulgaricus* cells using DSC makes it difficult to determine a specific numerical value of T_g because the value has a large range (-120–180 °C) (Fonseca et al., 2001; Santivarangkna et al., 2011). In another study, the T_g of *Bacillus subtilis* spores was investigated using DSC and found to be 90–115 °C, and the T_g value in a large range was also shown (Stecchini et al., 2006). A continuous thermal reaction causes changes such as melting fat, denaturation of protein, and gelatinization of starch. A wide temperature range is observed when measuring multiple components using DSC, and it is difficult to determine the T_g of the polymer material. (Kawai et al., 2014). Therefore, a mechanical approach that detects changes in mechanical properties, such as thermal rheological analysis (TRA), is more effective than DSC, which investigates the transition of heat capacity to determine the T_g of these substances. (Thuc et al., 2010; Sogabe et al., 2018; Jothi et al., 2018). The advantage of TRA analysis is that it is possible to measure the T_g that cannot be measured in DSC analysis because the T_g can be analyzed using the volume expansion of the sample. In a previous study, the T_g of the soup solid, which is a complex polymer sample, was measured with DSC and TRA, and DSC showed a broad peak of T_g (27–56 °C) (Fig 1-2a). On the other hand, TRA, which is a physical measurement method, revealed clear mechanical T_g (Fig 1-2b, Mochizuki et al., 2019). In addition, the mechanical

T_g of cookies (Kawai et al., 2014), hazelnuts (Ebara et al., 2018), and deep-fried foods (Jonthi et al., 2018) was measured using TRA. For measurement, the temperature of the sample is raised from below T_g to above T_g while compressing the sample at arbitrary stress. The temperature at which the pressure of the sample decreases is defined as the glass transition point, and the temperature at that point is the T_g (Mochizuki et al., 2019).

(a) (b)



DSC curve TRA curve

Fig. 1-2. DSC and TRA curves of soup powder sample (Mochizuki et al., 2019).

1.6. Research objectives

Despite the drying process for food preservation and the consequent production of dry foods with low a_w, several foodborne pathogenic bacteria may grow in these foods, and pose a potential threat to human health. In the present study, I investigated the cause of the survival of foodborne pathogenic bacteria under low a_w conditions, from the viewpoint of the glass transition phenomenon of bacterial cells, by assessing the physical properties of bacterial cells rather than using the conventional biochemical and molecular biological approaches.

Accordingly, the purpose of this study was to determine the glass transition phenomenon under various a_w conditions of dried *Salmonella enterica* and *C. sakazakii* that survive in low-moisture foods. The heat tolerance and viability of bacteria under various a_w conditions were investigated and the relationship between the glass transition phenomenon and the survival of bacteria in a low a_w environment was determined.

Briefly, I determined the T_g of different kinds of *Salmonella enterica* and demonstrated the relationship between a_w and thermal tolerance, as described in Chapter 2. I aimed to confirm that *S. enterica* exist in a glassy state in a low a_w environment and have high heat resistance. In Chapter 3, I describe the results from the measurement of the T_g of different strains of *C. sakazakii* and demonstrate the relationship between T_g and a_w among the different strains. Chapter 4 focuses on identifying differences in the viability of *C. sakazakii* processed using different drying methods, during storage. The differences in the T_g of *C. sakazakii* cells due to the drying method, the accompanying change in survival ratio during

storage, and sensitivity to mild heat treatment were also assessed. Finally, I aimed to understand the cause of bacterial survival under low a_w conditions according to change physical properties as a glass transition phenomenon.

Chapter 2

Relationship between the glass transition temperature and heat tolerance in *Salmonella enterica*

2.1. Introduction

Outbreaks of foodborne illnesses caused by dry foods, such as nuts (Kirk et al., 2004; Isaacs et al., 2005; Sheth et al., 2011), chocolate (Daoust et al., 1975; Gill et al., 1983; Hockin et al., 1989; Kapperud et al., 1990; Werber et al., 2005), cereals (Russo et al., 2013), and other foods, occur worldwide (Podolak et al., 2010; Beuchat et al., 2013; Santillana Farakos et al., 2014). Such foods have low water activity (a_w), and it is not possible to detect the growth of bacteria that cause foodborne illnesses. Therefore, microbiological hygiene control has not been regarded as important in dry food. However, cases of foodborne illnesses caused by dry food occur frequently, which means that pathogenic bacteria continue to survive even in a low-a_w environment. In particular, various *Salmonella* serotypes have been found in dry foods associated with disease outbreaks (Daoust et al.,1975; Gill et al., 1983; Hockin et al., 1989; Daoust et al., 1990; Kirk et al., 2004; Isaacs et al., 2005; Werber et al., 2005; Podolak et al., 2010; Sheth et al., 2011; Beuchat et al., 2013; Russo et al., 2013; Santillana Farakos et al., 2014; Kapperud et al., 2015). Indeed, several reports have shown that bacteria causing food poisonings, such as enterohemorrhagic *Escherichia coli* (EHEC) and *Salmonella*, continue to survive for long periods in a low-a_w environment (Burnett et al., 2000; Abushelaibi et al., 2003; Hiramatsu et al., 2005; Kimber et al., 2012; Nummer et al.,

2012; Keller et al., 2013; Santillana Farakos et al., 2013; Beuchat and Mann, 2015; Hokunan et al., 2016). In contrast, the rate of bacterial survival decreases under high a_w environments (Hiramatsu et al., 2005; Kataoka et al., 2014; Lian et al., 2015; Nakamura and Shiina, 2015), and it is inferred that a_w and the water content influence bacterial survival to some extent. From these previous studies, we consider that there are some associations between a_w and desiccation tolerance of bacterial cells. However, the mechanism of desiccation tolerance of bacterial cells under low-a_w conditions has not yet been clarified, and elucidation of the cause is required.

There is a clue to elucidate the mechanism of desiccation tolerance in other organisms. For example, microorganisms thriving in extreme environments, such as tardigrades and sleeping chironomids, which utilize cryptobiosis, are resistant to various external environments such as high temperature, high pressure, and dry environments (Sakurai et al., 2008; Horikawa et al., 2012; Hengher et al., 2015). In this study, I assume that bacterial cells would vitrify, and organisms in extreme environment are considered to acquire environmental stress tolerance. Vitrification of bacterial cells through the glass transition phenomenon might be one of the long-term survival factors in a low-a_w environment. The glass transition phenomenon refers to a change in state caused by the increase or decrease in molecular movement in a substance with change in the temperature and moisture content (Kawai et al., 2014; Jothi et al., 2018; Mochizuki et al., 2019). The state in which molecular movement is limited due to the decrease in temperature and a_w is called a glass state and the substance shows physical properties similar to a solid. Since molecular motion is almost

stopped in the glass state, the substance or organism shows high tolerance to various environmental stresses such as heat, desiccation, and pressure. In the present study, I hypothesized that bacterial cells are vitrified in low-a_w environments based on the physicochemical properties of solid particles. In other words, I assumed that bacterial cells enter a glass state due to a decrease in molecular movement accompanying a decrease in a_w, making it difficult for the bacteria to be affected by external factors. This, in turn, allows for long-term survival, even in dry environments.

While aiming to elucidate the survival mechanism of pathogenic bacteria, several studies reported a decline in the effect of bacterial thermal inactivation in dry foods (Jung and Beuchat, 1999; Mattick et al., 2001; Beuchat and Scouten, 2002; Shachar and Yaron, 2006; Ma et al., 2009; Ha et al., 2013; Li et al., 2014), and it is speculated that bacteria in dry foods may exhibit heat tolerance. I believe that the glass transition of bacteria is greatly involved in heat tolerance development, as is seen in other organisms living in extreme environments such as Tardigrada and sleeping chironomids (Sakurai et al., 2008; Horikawa et al., 2012; Hengherr et al., 2015).

I hypothesized that there would be a difference among a_w conditions in glass transition temperature (T_g) for bacterial cells. Differential scanning calorimetry (DSC) is widely used as a method for measuring the T_g (Abiad et al., 2009; Kawai et al., 2014; van Donkelaar et al., 2015). However, it is difficult to measure the T_g of a composite using DSC, because the thermogram shows intricate thermal responses (Kawai et al., 2014). Therefore, here, thermal rheological analysis (TRA) was used to measure T_g. TRA, which measures T_g through a

temperature control device attached to a rheometer, is based on the principle of thermal-mechanical analysis (Kawai et al., 2014; Jothi et al., 2018; Mochizuki et al., 2019). Previous studies have investigated the effect of water content on the T_g of cookies (Kawai et al., 2014; Sogabe et al., 2018), hazelnuts (Ebara et al., 2019), and deep-fried food (Jothi et al., 2018). To conduct the measurements, a sample is compressed at a temperature below T_g, and heated above T_g with compression. Then, the T_g of the sample can be determined as a force drop induced by the glass transition. This is a useful method for application to amorphous powders. By determining the T_g values, I could confirm the glass transition of bacterial cells. In addition, I sought to elucidate the influence of a_w on bacterial survival and its relationship with T_g. Finally, I aimed to elucidate the relationship between the state change of several *Salmonella* serotypes that are known to be present in low-water activity foods due to glass transition and the changes in thermal resistance in a desiccation environment. The results obtained here will help to understand bacterial survival in dry environments, which has not yet been clarified.

2.2. Materials and methods

2.2.1 Bacterial strains and culturing

Salmonella enterica Typhimurium (RMID 1985009 from the Research Institute for Microbial Diseases of Osaka University; isolated from patients in the sporadic case), *S. enterica* Chester, *S. enterica* Oranienburg (from the Aomori Prefectural Research Laboratory of Public Health; isolated from dried squid chips associated with an outbreak in 1999), *S.*

enterica Stanley (RIMD 1981001 from the Research Institute for Microbial Diseases of Osaka University; isolated from patients in sporadic case), and *S. enterica* Enteritidis (RIMD 1933001 from the Research Institute for Microbial Diseases of Osaka University, isolated from patients in sporadic cases) were used in this study.

These serovars were maintained at -80 °C in tryptic soy broth (TSB; Merck, Darmstadt, Germany) containing 10% glycerol. The strains were activated after incubating at 37 °C for 24 h on tryptic soy agar plates (TSA, Merck). An isolated colony of each bacterium was then transferred to 5 mL of TSB in a sterile centrifuge tube, incubated at 37 °C for 24 h, and then a 100 μL aliquot of cultured bacteria was added to 400 mL TSB and incubated at 37 °C for 48 h. The cultured cells were collected through centrifugation (3,000 × *g*, 10 min) and the pellets were resuspended in 5 mL of pure water. Bacterial cell pellets were obtained by pipetting off excess water and were collected on a plastic plate. The plates were frozen at -80 °C for 24 h before drying for 24 h using a freeze dryer (FDU-2200, EYELA, Tokyo, Japan). Dried bacterial cells were crushed, placed in an air-tight container at the desired relative humidity (% RH), which was produced using saturated salt aqueous solutions (43% RH: potassium carbonate, 57% RH: sodium bromide, 75% RH: sodium chloride, and 87% RH: potassium chloride), and stored at 4 °C for 48 h. The water activity and temperature in the air-tight container were continuously checked using a thermo recorder (TR-72wf, T and D, Nagano, Japan). The water activity of the bacteria was confirmed using a water activity meter (Aqualab 4TE, Decagon Devices, Washington, USA).

2.2.2. Determination of glass transition temperature (T_g)

Thermal rheological analysis (TRA) was used to measure T_g by attaching a temperature control device to a rheometer (EZ-SX, SHIMADZU, Kyoto, Japan) (illustrated in Fig. 2–1). The analysis is based on the principle of thermal-mechanical analysis (Kawai et al., 2014; Jothi et al., 2018; Mochizuki et al., 2019). A dried bacterial cell sample (ca. 100 mg) was placed in the forming die (φ = 3 mm) and compacted with a rheometer at ca. 10 MPa. Subsequently, the sample was compressed at ~ 5 MPa ca. for 1–3 min and then heated at a rate of approximately 3 °C/min until the temperature reached 120 °C. Pressure-time data were collected with the software provided with the rheometer. In parallel, a thermocouple was attached to the bottom of the forming die and time-temperature data were collected every second using a data logger. Subsequently, the relationship between the pressure and temperature data during heating was determined. Since pressure reduction begins at the point at which the bottom temperature of the sample reaches the mechanical T_g, the onset temperature of pressure reduction can be regarded as the T_g of the sample (Jothi et al., 2018).

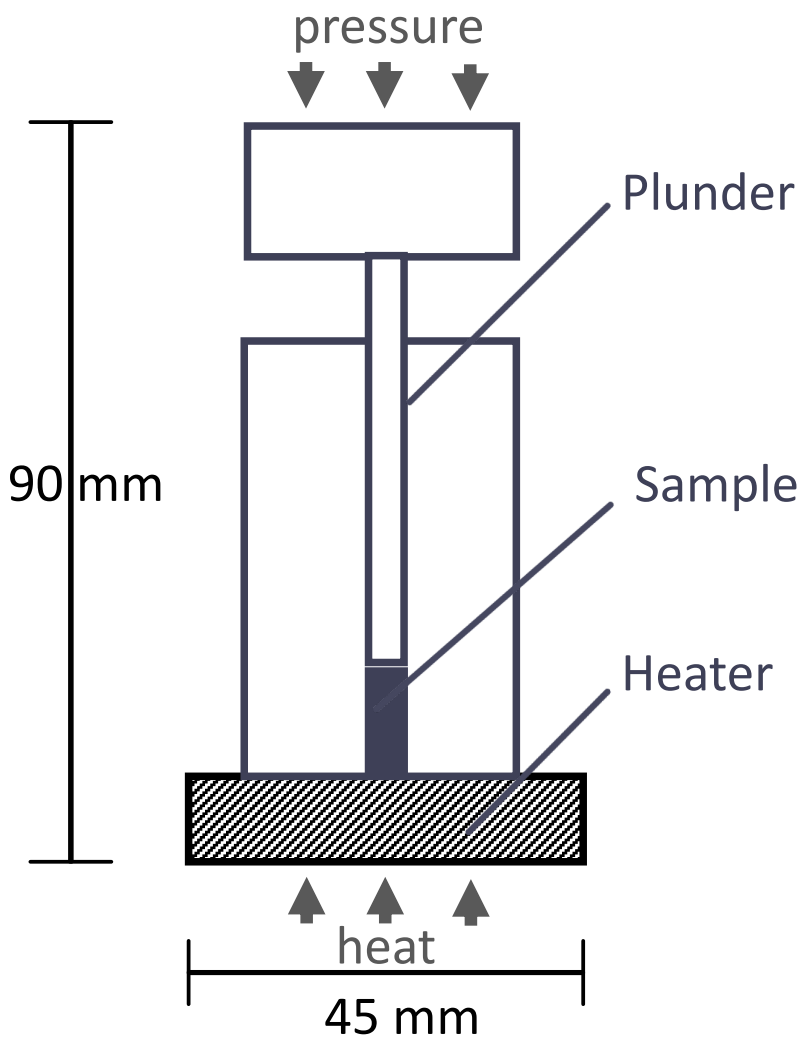


Fig 2-1. A schematic drawing of thermal rheological analysis (TRA).

2.2.3 Thermal inactivation under each water activity condition

Dried bacterial cells (ca. 50 mg), adjusted to each a_w condition, were placed into a small plastic bag (20 mm × 20 mm), making a thin layer, and the bag was vacuum-sealed before being submerged in a hot water bath at 60 °C for 10 min. In the same manner, dried bacterial cells (ca. 50 mg) were taken before heating and the viable cell number was determined as the initial condition. Following heating, the samples were combined with 500 μL of 0.1% peptone water, serially diluted in 0.1% peptone water, and surface-plated on TSA. The surviving populations were determined after incubating the plates at 37 °C for 24 h.

2.2.4. Statistical analysis

Triplicate trials for each experiment were performed. Data were expressed as mean ± standard deviation (SD) and subjected to the R statistical software (Version 3.4.1 for Mac OS X; http://www.r-project.org) for the Tukey-Kramer's multiple comparison test to determine the statistical significance ($P \leq 0.05$).

2.3 Results

2.3.1. Determination of glass transition temperature (T_g)

As a representative result, changes in the compressive stress over rising temperature were shown by TRA for *S.* Typhimurium (Fig. 2–2). The glass transition temperature (T_g) was determined as the intersection of the baseline and the tangent line of the inflection point of the obtained compressive stress vs. temperature curve. Clear softening behavior was observed during the temperature rising operation at a_w of 0.43, 0.57, 0.75, and 0.87. Similar results of the TRA analysis were observed in the other *S. enterica* serovars examined in this study. Accordingly, the results showed the possibility of determining the Tg of bacterial cells using TRA.

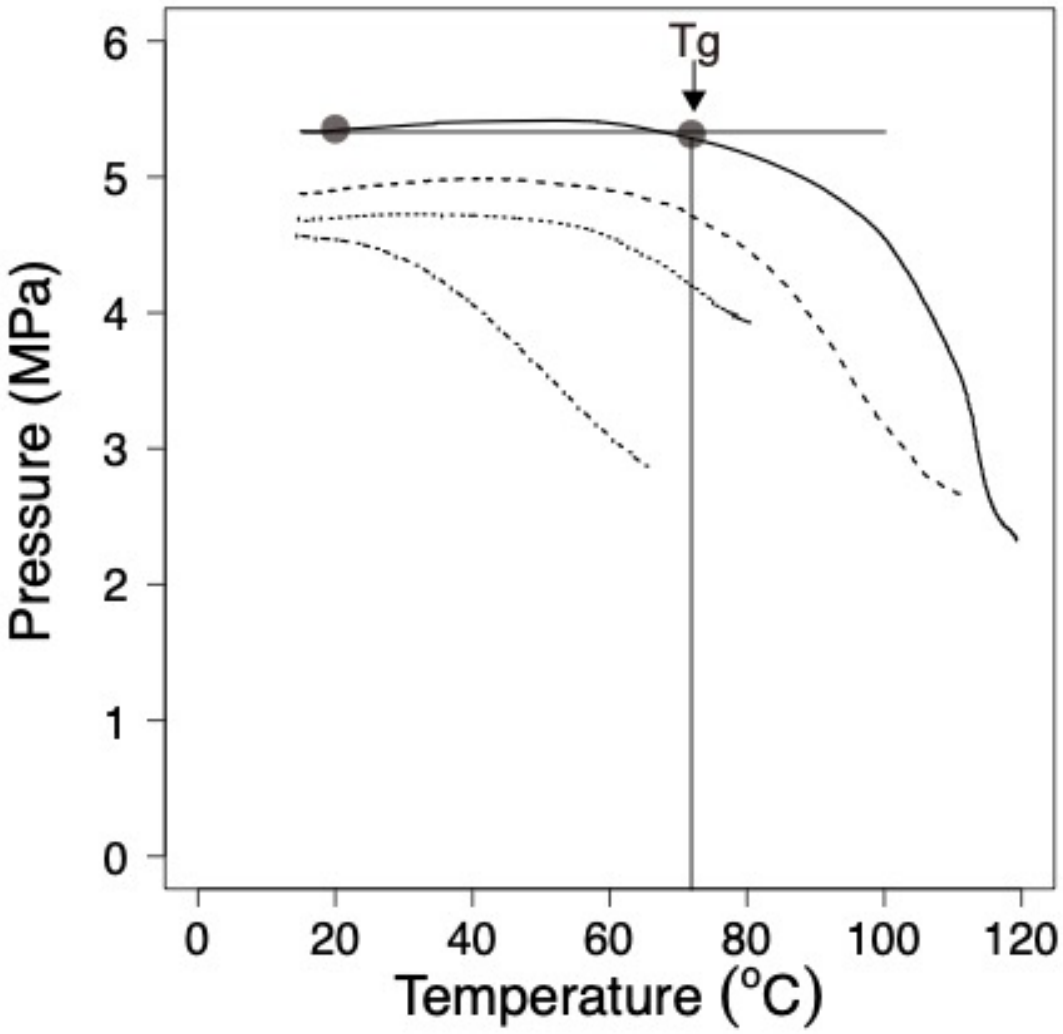


Fig 2-2. Measurement of the glass transition temperature of *S. enterica* ser.Typhimurium at a_w = 0.43 (solid line), 0.57 (dashed line), 0.75 (dotted line), and 0.85 (dot dashed line) by using thermal rheological analysis (TRA). The onset temperature of the pressure reduction was regarded as the T_g of the sample as shown in the figure.

2.3.2. Glass transition temperature (T_g) under each water activity condition

The T_g for each *Salmonella* serovar decreased with the increasing a_w of the bacterial cells (Table 2–1). For example, for *S. enterica* Typhimurium, the T_g was 73.7, 58.6, 48.4, and 23.2 °C at 0.43, 0.57, 0.75, and 0.87 a_w, respectively. Among the five strains, *S. enterica* Chester exhibited the highest glass transition temperature at a low a_w, whereas *S. enterica* Stanley exhibited the highest glass transition temperature at a high a_w. Compared to other *Salmonella* serovars, *S.* Stanley showed less extent of changes in glass transition temperature with increasing a_w.

Table 2-1. Relationship between water activity (a_w) and observed glass transition temperature (T_g) of *Salmonella enterica* serovar Typhimurium, *S.* Chester, *S.* Oranienburg, *S.* Stanley, and *S.* Enteritidis.

S. enterica serovars	Glass transition temperature (T_g, °C)			
	0.43 a_w	0.57 a_w	0.75 a_w	0.87 a_w
S. Typhimurium	$81.63 \pm 11.28^{aA\#}$	70.39 ± 3.26^{abA}	58.46 ± 2.23^{abB}	35.16 ± 2.92^{bC}
S. Chester	83.29 ± 2.04^{aA}	75.62 ± 2.64^{aA}	62.17 ± 1.37^{bB}	43.50 ± 2.35^{cC}
S. Oranienburg	81.26 ± 6.46^{aA}	73.14 ± 1.95^{abB}	63.39 ± 4.98^{bC}	43.00 ± 1.33^{cD}
S. Stanley	80.07 ± 2.96^{aA}	65.94 ± 3.64^{abB}	62.22 ± 3.31^{abB}	57.46 ± 8.06^{bB}
S. Enteritidis	77.10 ± 1.78^{aA}	51.25 ± 2.89^{bB}	45.37 ± 2.83^{cBC}	40.69 ± 2.76^{cC}

[#]Different lowercase letters in each column (a_w level) represent statistically significant differences ($P < 0.05$) among the five *S. enterica* serovars. Similarly, the different uppercase letters in each row (*S. enterica* serovar) represent statistical differences ($P < 0.05$) among the four different a_w levels.

2.3.3. Thermal inactivation under each water activity condition

The bacterial survival ratio increased with decreasing a_w levels at 60 °C for 10 min (Fig. 2–3), which reflected the rising T_g. The number of surviving *Salmonella* strains after thermal inactivation at 60 °C for 10 min decreased by ca. 1–5 log cycles at 0.87 a_w. There was an apparent difference in thermal tolerance among the five *S. enterica* serovars at 0.87 a_w. In contrast, under low-a_w conditions (e.g., 0.43 a_w), the decrease in bacterial numbers after inactivation was ca. 1–2 log cycles in all *S. enterica* serovars. *S. enterica* Stanley exhibited the smallest difference in survival ratio among a_w levels, and higher heat resistance than the other strains. Since *S. enterica* Stanley has a T_g >60 °C across all a_w, as shown in Table 1, the 60 °C treatment was not sufficient to affect bacterial inactivation. There were few differences in the bacterial inactivation effect between 0.43 a_w (Fig. 2-3a) and 0.75 a_w (Fig. 2-3b) for all *S. enterica* serovars except for *S.* Enteritidis. The apparent differences were observed in the bacterial inactivation effect between 0.87 a_w and the other two lower a_w, although there was a difference in the inactivation effect among the five serovars. This result is associated with the relationship between a_w and T_g, as shown in Table 2–1. The significant decrease in T_g from 0.75 a_w to 0.87 a_w (Table 2–1) increased the thermal inactivation effect (Fig. 2-3c). There is a possibility that the bactericidal effect of pathogenic bacteria decreases in a low-a_w environment.

The relationship between T_g and heat resistance (log reduction) of *S. enterica* serovars relationships is illustrated in Figs. 2–4. There seems to be *a* T_g dependency on heat resistance, which means that the bacterial inactivation effect increases with a decrease in T_g for the

tested *S. enterica* serovars except for *S*. Stanley. The correlation coefficients of *S.* Typhimurium, *S*. Chester, *S*. Oranienburg, *S*. Stanley, and *S*. Enteritidis were -0.54, -0.75, -0.80, 0.01, and -0.99, respectively. This result also confirms that T_g plays an important role in the heat resistance of *S. enterica* cells under dry conditions.

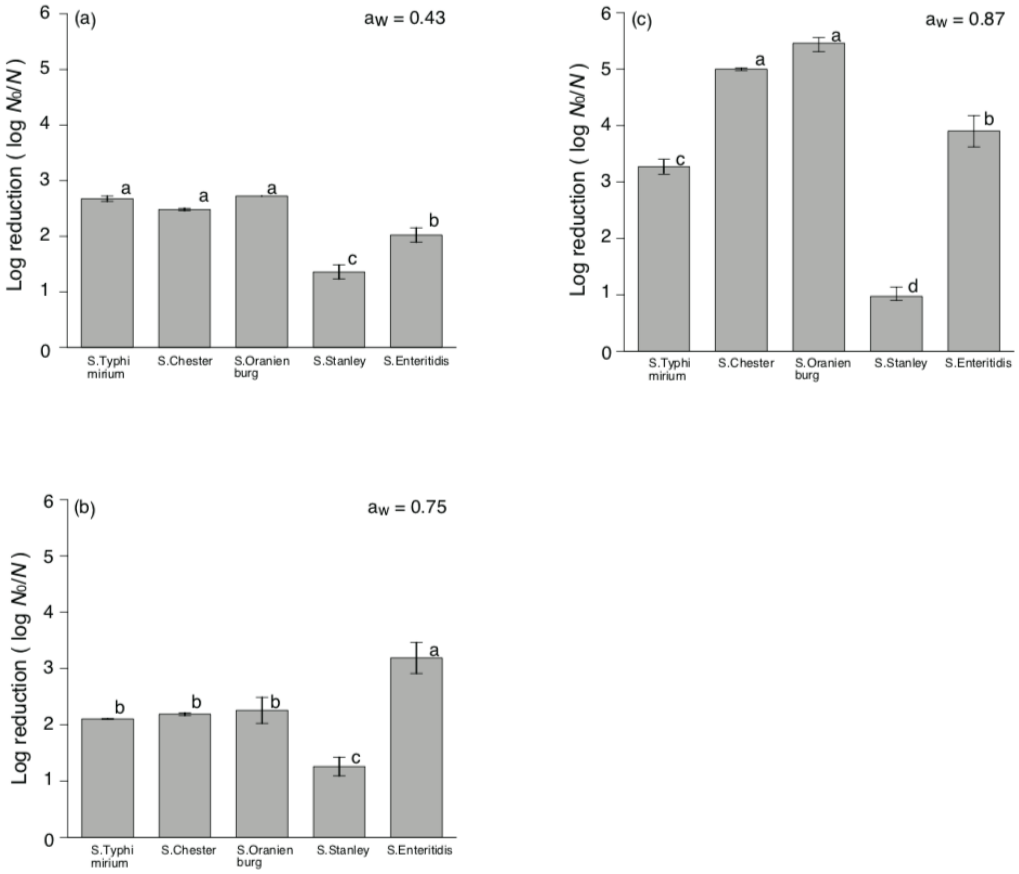


Fig 2-3. Comparison of the thermal inactivation effect (log reductions) of *Salmonella enterica* ser. Typhimurium, *S*. Chester, *S*. Oranienburg, *S*. Stanley, and *S*. Enteritidis heated at 60 ºC for 10 min under 0.43 (a), 0.75 (b), and 0.87 (c) a_w. Initial cell numbers (N_0) right before the heat treatment of dried bacterial cells ranged from 5–7 log CFU/mL depending on the serovar and a_w. The error bars represent the standard error of the mean (n = 3). Different lowercase letters among the five *S. enterica* serovars at the same a_w level represent statistically significant differences ($P < 0.05$). Similarly, different uppercase letters among different a_w levels for each *S. enterica* serovar represent statistically significant differences ($P < 0.05$).

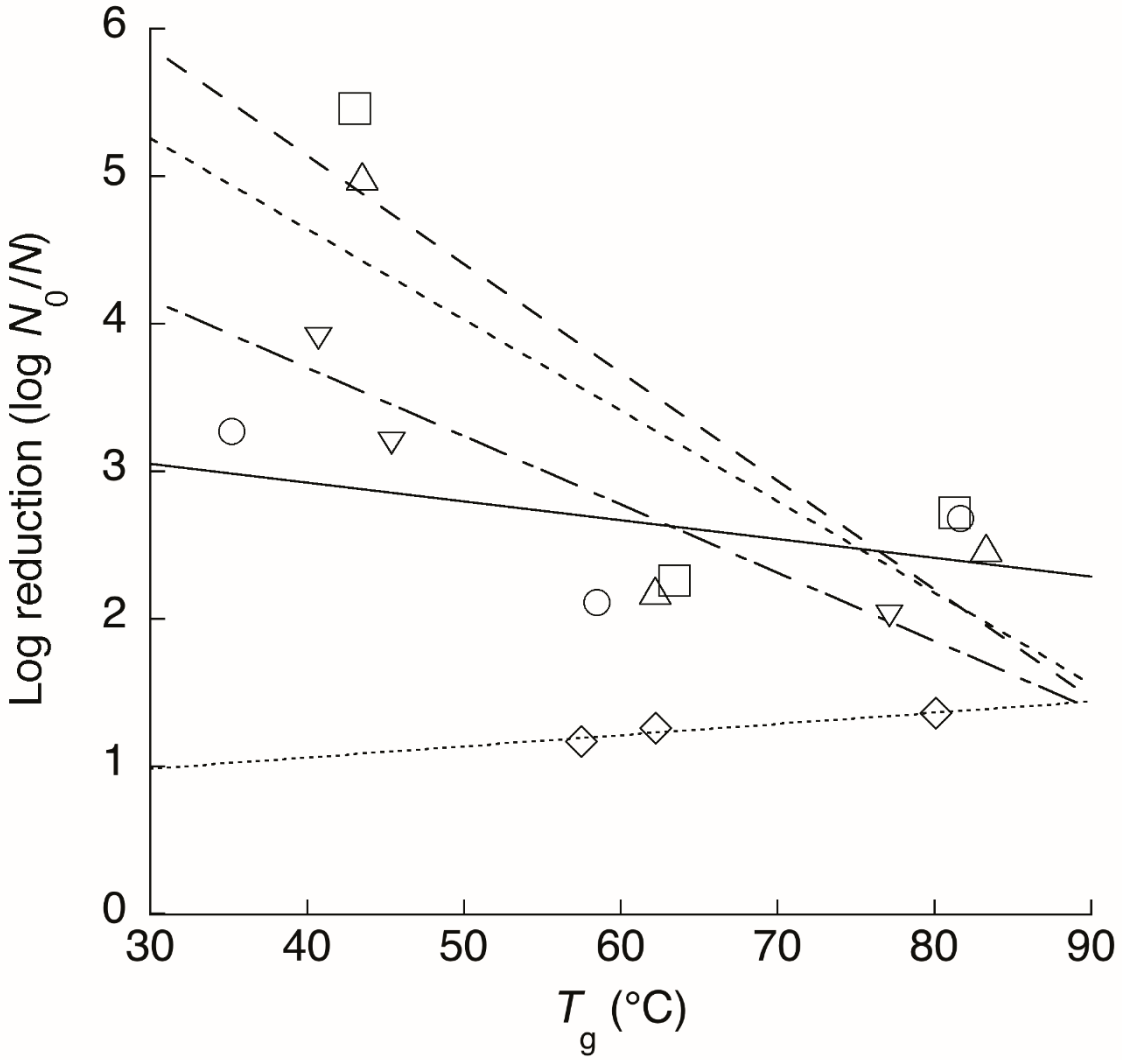


Fig. 2-4. Relationship between the glass transition temperature (T_g) and inactivation effect (log reductions) of *Salmonella enterica* ser. Typhimurium (○, solid line), *S.* Chester (△, dashed line), *S.* Oranienburg (□, dotted line), *S.* Stanley (◇, fine dotted line), and *S.* Enteritidis (▽, dot dashed line).

2.4. Discussion and conclusion

Previous studies have reported that a_w influences bacterial survival to a certain extent. Long-term survival of bacteria was observed under low-a_w conditions ($\leq$ 0.87 a_w), whereas bacterial death was found to be promoted under high a_w conditions (Lian et al., 2015; Nakamura and Shiina, 2015; Hokunan et al., 2016). The relationship between bacterial cell T_g and a_w is possibly a major factor influencing differences in survival across a range of a_w levels. The T_g of *Salmonella* tested at $a_w \leq 0.87$ was 30 ºC or higher in the present study. These cells would be in a glass state under room temperature conditions because room temperature (normally 20–22 °C) is lower than the T_g (30 ºC). In the glass state, the molecular movement in bacterial cells is almost completely stopped, and thus, is unlikely to be affected by external environments. It is inferred that bacteria acquire desiccation tolerance through the glass transition, accompanied with a decrease in a_w. Under high-a_w conditions, it is presumed that T_g would be considerably low, glass transition would not occur, and the rubber state would be maintained. Since molecular movement is not limited to the rubber state, bacterial cells would not acquire desiccation tolerance. I assumed that this state change is a key factor in the survival differences among bacteria. I preliminary examined the thermal inactivation effect on some *S. enterica* serovars, and confirmed an apparently higher inactivation effect of 6–7 log cycle reduction in a_w 0.99 than those of lower a_w levels on the same heat treatment (data not shown). Furthermore, since a negative correlation was observed between T_g and a_w in *Salmonella* cells (Fig. 2–4), the bacteria may exhibit stronger

desiccation tolerance as the a_w decreases. In addition, since T_g varies among bacterial species, the difference in desiccation tolerance will depend on T_g.

This study also showed that the thermal inactivation effect decreased under low-a_w conditions (Fig. 2–3). It has been reported the thermal inactivation effect in low-a_w food (Mattick et al., 2001; Shachar and Yaron, 2006; Ma et al., 2009; Li et al., 2014). The difference in thermal inactivation among different a_w levels is likely involved in the changing physical state properties of bacterial cells as well as in their survival differences under dry conditions. As described above, bacterial cells in a low-a_w environment will be in a glass state and exhibit a high tolerance to environmental stresses such as heat, pressure, and desiccation. For example, extreme environmental microorganisms, such as tardigrades and sleeping chironomids that utilize cryptobiosis, are also resistant to high temperature, high pressure, and dry environments (Sakurai et al., 2008; Horikawa et al., 2012; Hengherr et al., 2015). Bacterial cells would vitrify, similar to extreme environmental organisms that acquire environmental stress tolerance. Therefore, I attribute the reduced thermal bacterial inactivation in low-a_w conditions to changes in physical properties due to the glass transition of bacterial cells.

The differences in bacterial survival (Fig. 2–3) could be attributed to the difference in the T_g of each bacterium. *S. enterica* Stanley was found to have a higher T_g than the other *Salmonella* strains at high a_w (Table 2–1), which might mean differences in the ability to maintain the glass state. In other words, *S.* Stanley would have a stronger heat-tolerance than the other *Salmonella* strains. In a previous study, *S.* Stanley was reported to have a higher

long-term survival ratio under dry conditions, and *S.* Typhimurium showed the lowest T_g at high a_w, which was associated with a low survival rate (Hokunan et al., 2016). The difference in T_g among bacterial species/serovars could be attributed to innate (genetically) or acquired characteristics of each bacterial species or serovar. In particular, acquired characteristics might be due to habituation to various harsh conditions during the survival process. Based on these findings, I believe that bacterial acquisition of environmental tolerance and the glass transition phenomenon are closely related. Although the mechanism by which a_w exerts its influence on bacterial survival under desiccation and thermal conditions has not been elucidated, the present study demonstrates that the glass transition phenomenon of bacterial cells may play an important role in the survival of bacteria in stressful environments. Furthermore, I have successfully demonstrated that glass transition temperature influences the strength of desiccation and the thermal tolerance of bacteria. To elucidate the exact reason for the difference in T_g among bacterial species/serovars, further genetic and/or bacteriological investigations will be needed in the future.

In this study, I aimed to elucidate the role of glass transition phenomenon in pathogenic bacteria, in acquiring tolerance under low-a_w conditions. The experimental results not only confirmed the glass transition phenomenon of bacterial cells through thermal rheological analysis, but also showed a clear correlation between T_g and a_w. In addition, it was confirmed that the heat sterilization effect was reduced through the vitrification of bacterial cells. These results revealed that the glass transition phenomenon of bacterial cells is a major factor in the acquisition of bacterial stress tolerance.

Chapter 3

Strain variability according to the glass transition temperature of *Cronobacter sakazakii*

3.1. Introduction

Low moisture foods contain low water content naturally or by removal of water from foods with high water content, using artificial treatment (Young et al., 2015). Foods that contain less water can be stored for long periods and have a long shelf life (Finn et al., 2013). In addition, reduction in the moisture content of foods suppresses the growth of microorganisms. The minimum water activity (a_w) for bacteria to grow is 0.87 a_w, and other microorganisms also require at least 0.6 a_w (Beuchat et al., 2013). Therefore, low-moisture foods under 0.6 a_w, such as chocolate, cocoa, potato crisps, dried vegetables, and powdered infant formula, are less prone to foodborne pathogen growth and spoilage (Grant, 2004).

However, it has long been reported that *C. sakazakii* contaminates powdered infant formula, which is a low-moisture food (Biering et al., 1989; Acker et al., 2000; Restaino et al., 2006; Friedemann., 2007; Reich et al., 2010; Beuchat et al., 20013; Lang et al., 2017). *C. sakazakii* is a gram-negative, facultative anaerobic, motile, and nonspore-forming bacterium that can cause fatal diseases in infants (Nazarowec-white and Farber., 1997; Block et al., 2002; Beuchat et al., 2013; Lang et al., 2018). *C. sakazakii* causes diseases such as meningitis, necrosis, and bacteremia in infants, with infant fatality rates approaching 40–80% (Ha et al., 2014). *C. sakazakii* was not only confirmed to be preserved in infant milk formula

but was also found in various low-moisture foods such as grains, legumes, and teas (Tamura et al., 1995; Cottyn et al., 2000; No et al., 2002; Iversen et al., 2004; Richards et al., 2005; Restaino et al., 2006; Friedemann., 2007). However, the causes and mechanisms for the survival of this bacterium, despite being in an environment of low a_w, are still unknown. Survival under desiccation and low water activity conditions is associated with the bacterial osmotic phenomenon; therefore, upregulation of osmotic genes enhances their resistance to cell desiccation (Feeney et al., 2014). The osmotolerance gene of *C. sakazakii* was confirmed through a comparative genomic approach, which is related to the preservation of bacteria in low a_w environments. However, the collection of bacterial genome sequences provides only some indicators of protein structure and function and thus, additional experimental research is required (Feeney and Sleator., 2011). In addition, trehalose production, which is a component that induces phospholipid membrane and protein stabilization during the drying period, was confirmed in *C. sakazakii*. However, it is difficult to confirm whether the cause of desiccation tolerance is trehalose, since the synergistic effect of tolerance due to the addition of trehalose to the medium has not been investigated (Breeuwer et al., 2003). Therefore, I tried to approach the identification of this cause from the physical aspect, not from the aspect of a biochemical and microbiological viewpoint that were previously reported (Breeuwer et al., 2003; Feeney and Sleator., 2011; Feeney et al., 2014).

The mechanism of survival in low water activity environments can be inferred from the changing physical state of the molecule. For example, some organisms, such as tardigrades and sleeping chironomids, become resistant through the ability to transition to a physical state

such as glass under extreme conditions (Sakurai et al., 2008; Horikawa et al., 2012). The physical property that does not flow by changing from an aqueous and amorphous state is called a glassy state. The translational molecular motion is interrupted and the chemical reaction slowly progresses in the glassy state (Sapru and Labuza, 1993). Previous studies have reported that the central protoplast region of *Bacillus subtilis* spores exists in a glassy state containing low moisture and is maintained for a long time (Ablett et al., 1999). The temperature that causes a change from the rubbery state, which is viscous, to the glassy state is called the glass transition temperature (T_g). Materials with high T_g values become glassy at temperatures below T_g, and the atoms are fixed and the movement of molecules stops. Substances in which molecular movement has ceased have a strong tolerance to external stresses. Therefore, I considered that the resistance of bacteria to external stress changes according to the glass transition temperature.

Differential scanning calorimetry (DSC) is a glass transition temperature measurement method that is often used worldwide. However, it is difficult to investigate the T_g of bacteria because bacteria have the form of polymers displaying multiple thermal responses; therefore, the glass transition of bacteria is overlapped (Sogabe et al., 2018). In previous studies, DSC was also used to detect the T_g of bacteria, but the range was wide and it was difficult to determine the glass transition temperature at which multiple peaks were measured (Fonseca et al., 2001; Santivarangkna et al., 2011). In contrast, mechanical approaches, such as the thermal rheological analysis (TRA) method, are more effective in measuring the T_g of polymers and products (Jothi et al., 2018).

In this study, I aimed to evaluate the T_g of *Cronobacter sakazakii* strains under different conditions of water activity and could determine the relationship between glass transition temperature and water activity in bacteria according to this result. This study investigates the mechanisms by which *C. sakazakii* bacteria exist for a long period in powdered infant formula, which is a low water activity condition. In addition, the glass transition temperature results of *C. sakazakii* serve as the basis for research to control the T_g of foodborne pathogenic bacteria to enhance the safety of low-moisture foods.

3.2 Materials and methods

3.2.1 Bacterial strains and culturing

The *C. sakazakii* strains (JCM 1233, JCM 2127, JCM 24133, JCM 24135, and JCM 24136) used in this study were obtained from the Japan Collection of Microorganisms (Tsukuba, Ibaraki, Japan), and *C. sakazakii* NBRC 102416 was obtained from the NITE Biological Resource Center (Tokyo, Japan). All strains were maintained at -80 °C in tryptic soy broth (TSB, Merck, Darmstadt, Germany) containing 10% glycerol. They were recovered from a frozen stock and cultured on tryptic soy agar (TSA, Merck, Darmstadt, Germany) plates. The strains were precultured in 5 mL of TSB at 37 °C for 24 h. Then, 100 μL of *C. sakazakii* strains were subsequently added to 800 mL TSB and incubated at 37 °C for 24 h. The cultured bacteria were centrifuged (3,000 × *g*, Sorvall ST8, ThermoFisher Scientific, Jiangsu, China) for 10 min to collect cells. The collected pellets were washed twice with 30 mL of pure water. The cleaned *C. sakazakii* strains were stored on sterile Petri dishes and frozen at -30 °C for 24 h.

3.2.2 Freeze-drying and placed in desired water activity conditions

The frozen bacterial strains were freeze-dried using a freeze dryer (FDU-2200, EYELA, Tokyo, Japan) at -80 °C for 24 h. I pulverized the freeze-dried samples and stored them in air-tight containers set for different a_w that were adjusted using a saturated salt solution (0.43 a_w - potassium carbonate, 0.57 a_w - sodium bromide, sodium chloride - 0.75 a_w, and

potassium chloride - 0.87 a_w); the relative humidity in the container was continuously monitored using a thermo recorder (T&D Corporation, Nagano, Japan).

3.2.3 Determination of the glass transition temperature (T_g)

The thermal rheological analysis (TRA) was used to determine the T_g of *C. sakazakii*. As described in previous studies (Kawai et al., 2014; Sogabe et al., 2018; Jothi *et al*. 2018; Lee *et al*. 2020); this apparatus was designed using a rheometer (EZ-SX, SHIMADZU, Kyoto, Japan) and a temperature controller (TXN-700, AS ONE, Osaka, Japan). The temperature measurements on the surfaces of the samples were recorded separately using T-type thermocouples with a temperature data logger (midi logger GL240, GRAPHTEC, Kanagawa, Japan). A schematic diagram and a photograph of the TRA are shown in Fig. 3–1.

Approximately 100 mg of bacterial samples stored in the container were placed in the forming die having a diameter of 3 mm, and 7 MPa of pressure was applied using a rheometer. The samples were heated up to 120 °C at a rate of 1.5 °C /min using a temperature controller. The pressure change data were determined with the rheometer, and the temperature logger attached under the sample was simultaneously used to collect data on the temperature change on the sample surface. A scatter plot was used to determine the relationship between pressure and temperature. The point at which the properties of the samples changed and the pressure began to decrease as the temperature increased, was defined as the glass transition point, and the temperature was defined as T_g.

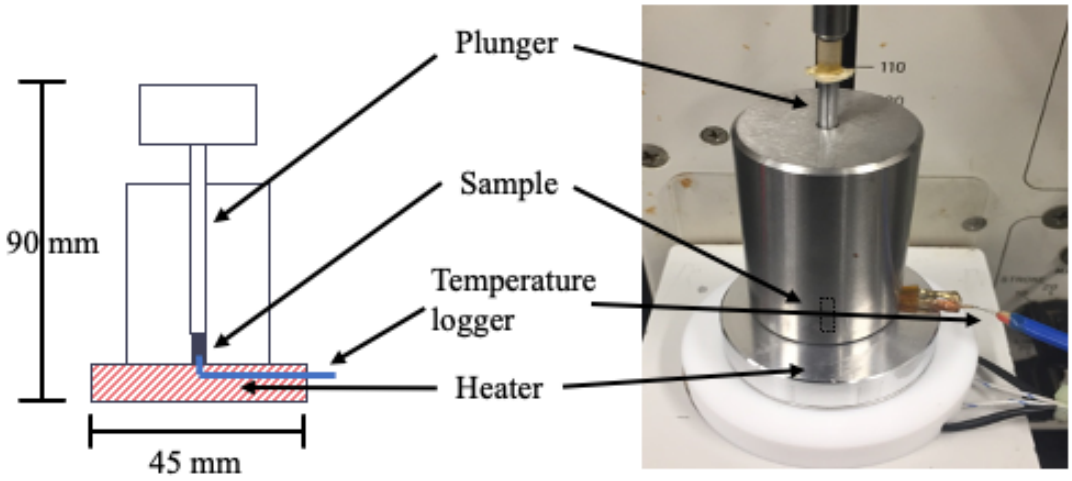


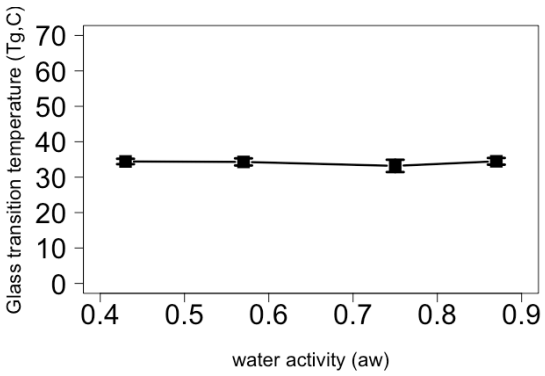
Fig. 3-1. A schematic diagram of the thermal rheological analysis (TRA).

3.2.4 Statistical analysis

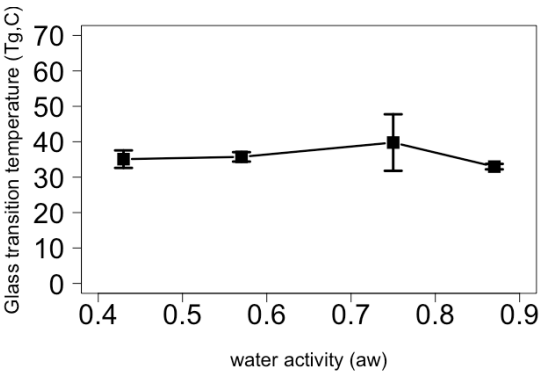
The T_g of each *C. sakazakii* strain was determined using triplicate samples. The triplicate observed T_g data were averaged and expressed as mean ± standard deviation (SD). To determine the statistical difference in T_g between strains and a_w levels, the data were analyzed using the Tukey-Kramer's multiple range test in the R statistical software (Version 3.4.1 for Mac OS).

3.3 Results

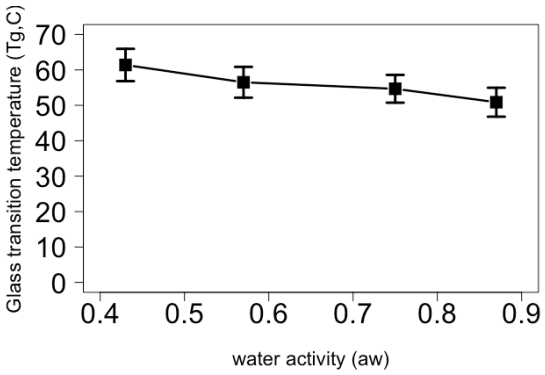
As shown in Fig. 3–2, the T_g of *C. sakazakii* strains was determined depending on the a_w. No significant difference in T_g due to water activity was observed in the *C. sakazakii* strains except 24135 and 24136. Strains 24135 and 24136 did not show any relationship between T_g and changed a_w (Table 3–1). *C. sakazakii* had T_g of 34.11–61.39 °C at 0.43 a_w, and *C. sakazakii* 24133 showed the highest T_g. Under the 0.57-a_w condition, the significantly higher T_g of *C. sakazakii* 24133 (57.51 °C) and 24136 (56.59 °C) was investigated compared with the other strains (33.01–39.71 °C). There were no significant differences in T_g among the strains at 0.75 a_w. A T_g of 32.96–50.87 °C was observed at 0.87 a_w, and *C. sakazakii* 24133 had the highest. The T_g of most *C. sakazakii* strains was <50 °C, and there were no significant differences between strains except for strains 24133 and 24136. Among the six strains, *C. sakazakii* strain 24133 exhibited the highest T_g (50.9-61.4 °C). For the difference between the highest and lowest T_g values for each strain, the *C. sakazakii* strain 1233 showed the smallest difference of 1.4 °C, while *C. sakazakii* 24135 showed a difference of 22 °C.



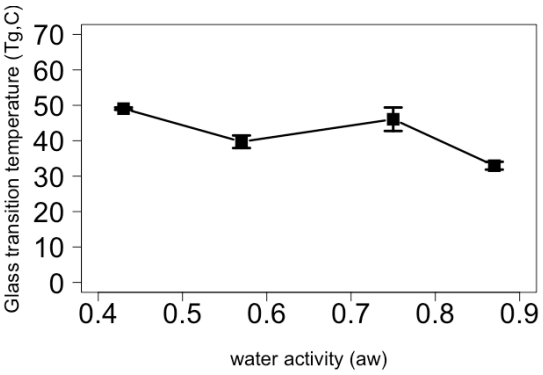
C. sakazakii 1233



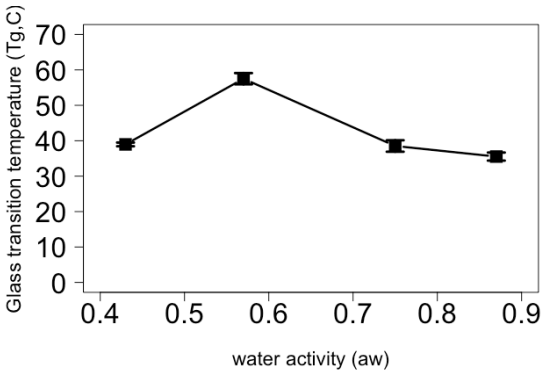
C. sakazakii 2127



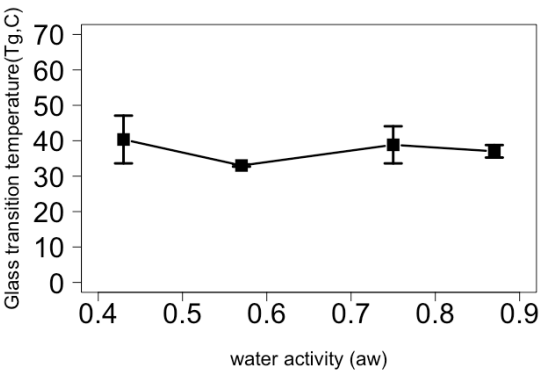
C. sakazakii 24133



C. sakazakii 24135



C. sakazakii 24136



C. sakazakii 102416

Fig. 3-2. Relationship between the glass transition temperature (T_g) and water activity (a_w) of *C. sakazakii* strains. Data are presented as mean values ± standard deviation of triplicate experiments.

Table 3-1. Relationship between the water activity (a_w) and observed glass transition temperature (T_g) of *Cronobacter sakakzakii* strains.

C. sakazakii	Glass transition temperature (T_g °C)			
strains	0.43 a_w	0.57 a_w	0.75 a_w	0.87 a_w
1233	$34.44 \pm 1.31^{bA\#}$	34.30 ± 3.26^{bA}	33.18 ± 2.23^{aA}	34.48 ± 2.92^{bA}
2127	35.08 ± 4.29^{bA}	35.73 ± 2.31^{bA}	39.77 ± 13.82^{aA}	32.98 ± 1.33^{bA}
24133	61.39 ± 7.90^{aA}	56.50 ± 7.53^{aA}	54.66 ± 7.84^{aA}	50.87 ± 7.07^{aA}
24135	49.06 ± 0.59^{abA}	39.71 ± 3.07^{bAB}	46.07 ± 5.75^{aA}	32.96 ± 1.91^{bB}
24136	38.97 ± 0.90^{bB}	57.51 ± 2.75^{aA}	38.52 ± 3.24^{aB}	35.53 ± 1.97^{bB}
102416	40.35 ± 11.67^{abA}	33.01 ± 0.55^{bA}	38.85 ± 9.08^{aA}	37.02 ± 2.50^{abA}

#Different lowercase letters in each column (a_w level) represent statistically significant differences ($P < 0.05$) among the six *C. sakazakii* strains. Similarly, the different uppercase letters in each row (*C. sakazakii* strain) represent significant differences ($P < 0.05$) among the four different a_w levels.

3.4 Discussion and conclusion

Low-moisture foods have low moisture content, and these foods can be stored for long periods, providing a sense of security against microorganisms to consumers and allowing them to be consumed without additional treatment such as heating (Young et al., 2015). However, outbreaks of foodborne illnesses due to various low moisture foods have increased in recent years (Tamura et al., 1995; Iversen et al., 2004; Restaino et al., 2006; Sanchez-Maldonado et al., 2018). Among foodborne pathogens, *C. sakazakii* is a concern in the dairy food industry because of its principal contamination of powdered infant formula for infants with low immunity. The present study evaluated the T_g of six strains of *C. sakazakii* under different a_w conditions, to identify the physical state of the bacteria under low a_w conditions, and to determine the relevance of the preservation mechanism of *C. sakazakii* in infant formula. Overall, it can be confirmed that all *C. sakazakii* strains exhibit high T_g more than room temperature at 0.43–0.87 a_w and the T_g was different depending on the strain, although it was the same bacterial species. In particular, *C. sakazakii* 24133 showed a significantly higher T_g of 61.39 °C at 0.43 a_w than that of the other strains. The lowest T_g was 34.44 °C for *C. sakazakii* 1233 under 0.43 a_w conditions. This suggests that *C. sakazakii* has a broad T_g range depending on the strain, and it is a point to be considered in the temperature setting of the process for sterilization of foodborne pathogenic bacteria. In the case of *Salmonella*, a high T_g was observed at low a_w, and the T_g decreased as the a_w increased (Lee et al., 2020). However, *Salmonella* had a high glass transition temperature of 77–83 °C under 0.43 a_w conditions. In contrast, the T_g of *C. sakazakii* was 37 °C at the same water activity condition

(0.43 a_w). Furthermore, the T_g of *C. sakazakii*, except strain 24133, was lower than that of *Salmonella enterica*, even in the high a_w environment. *Salmonella* generally may have a higher T_g than *C. sakazakii*. However, as the T_g of *C. sakazakii* is also higher than room temperature (25 °C), *C. sakazakii* is more likely to be in the glassy state in powdered infant formula. Although *Salmonella* showed a difference of 23–46 °C in T_g under 0.43–0.87 a_w levels, *C. sakazakii* exhibited a small difference in T_g (1–22 °C) under the same a_w conditions. The T_g of *C. sakazakii* would not be less affected by a_w levels than those of *S. enterica*. In other words, *C. sakazakii* might be in a glassy state, regardless of the a_w conditions. The presence of bacteria in a glassy state at room temperature means that the viscosity increases and the molecule movement becomes stopped (Santivarangkna et al., 2011). Some organisms have the characteristic of transitioning to a glassy state and overcoming unfavorable environments, which is called cryptobiosis (Jonsson, 2003). Since *C. sakazakii* has a higher T_g than room temperature under low a_w conditions, *C. sakazakii* might demonstrate similar characteristics to cryptobiosis. Such responses would lead to its survival for a long time in powdered infant formula.

In conclusion, the T_g of six strains of *C. sakazakii* was higher than room temperature, regardless of the a_w conditions ranging from 0.43–0.87 a_w. Based on the T_g of *C. sakazakii,* the *C. sakazakii* cells contaminating the commercial infant milk powder will be in a glassy state, and *C. sakazakii* might have a strong resistance to various external stresses such as heat. The characteristics of *C. sakazakii* on T_g and a_w may be the basis for the persistence of bacteria in low-moisture food, which has not been clarified so far. In addition, these data

suggest that high storage temperatures, such as >45 °C, might be useful in preventing the outbreak of foodborne illnesses due to the presence of *C. sakazakii* in low-moisture foods, because the T_g of most strains is approximately 40 °C.

Chapter 4

Effects of the drying methods of *Cronobacter sakazakii* on survival during storage and thermal tolerance

4.1 Introduction

Outbreaks of foodborne diseases have frequently been related to low-moisture foods in the recent years. *Salmonella* spp., *Bacillus cereus*, *Cronobacter sakazakii*, *Clostridium* spp., *Escherichia coli* O157:H7, and *Staphylococcus aureus* are the main foodborne pathogens reported to cause foodborne disease outbreaks related to low moisture foods (Cavallaro et al., 2011; Sheth et al., 2011; Beuchat and Mann, 2015; Hokunan et al., 2016; Syamaladevi et al., 2016; Sanchez-Maldonado et al., 2018).

Powdered infant formula, a low-moisture food, is used to provide many infants with the nutrients to grow. The product was formulated to mimic the nutritional constituents of human breast milk. As it is not a commercially sterile product, the safety of powdered infant formula is an issue to be addressed by manufacturers and food safety authorities.

C. sakazakii, formerly known as *Enterobacter sakazakii*, is associated with a severe form of life-threatening neonatal meningitis, resulting in mortality as high as 40–80%. *C. sakazakii* can also cause diseases in humans of all ages, which can be more serious in the elderly and immunocompromised people; the clinical symptoms of *C. sakazakii* infection include bacteremia (Iversen and Forsythe, 2003). It has been reported that *C. sakazakii* is present in various foods (Friedemann, 2007), including infant formula (Iversen and Forsythe, 2004). In

addition, *C. sakazakii* has persisted for a long time, more than 30 months, in powdered infant formula (Barron and Forsythe, 2007; Koseki et al., 2015). However, the route of contamination of powdered infant formula during processing, and the mechanism of survival of bacteria in dried foods are still unknown.

Dying is one of the oldest and most effective methods used to extend the storage period of food products (Bhandari and Howes, 1999; Ratti., 2001; Morgan et al., 2006; Lewicki, 2006; Finn et al., 2013a; Finn et al., 2013b, Onwude et al., 2016), as it controls the moisture content in foods. In general, most fresh foods, such as meat, fruit, vegetables, and dairy foods have an a_w of 0.95 or higher (Grant, 2004) and provide an environment for the growth of various types of microorganisms. Therefore, drying is used to reduce the a_w of food to create an environment in which microorganisms find it difficult to grow, to protect it against foodborne pathogens, and to extend its shelf life. The growth of foodborne pathogens that threaten human health is suppressed below 0.90 a_w (Brewer, 1999; Jung and Beuchat, 1999; Beuchat et al., 2013; Beuchat and Mann, 2015). Thus, drying as a food processing strategy is used to convert fresh foods to intermediate-moisture (a_w 0.6–0.84) or low-moisture ($a_w < 0.6$) foods to not only extend the storage time of food products but also preserve the organoleptic properties (Finn, et al 2013a).

In general, air-drying and freeze-drying are used as drying methods. Air-drying is a method that has been traditionally used to improve the preservability of foods and to remove moisture from foods using the principle of evaporation. Freeze-drying is a drying method that converts atmospheric pressure into a vacuum state under extremely low temperatures, such as

-80 °C, to cause sublimation of water. Freeze-drying is widely used to preserve the nutritional content of foods in the food industry. A recent study showed that fluidized bed drying, which is an air-drying method, exhibits strong stability of *Lactobacillus paracasei* than freeze-drying, and it was attributed to the lower porosity and the larger agglomerate size, which result in reduced absorption of water (Poddar et al., 2014). On the contrary, survival rates revealed that fluidized bed drying is more harmful to *Enterococcus faecium* and *Lactobacillus plantarum* than freeze-drying (Strasser et al., 2009). In addition, it has been reported that some bacteria were confirmed to be present even after freeze-drying, and the survival rates varied from 8.0–96.7% for 20 years (Miyamoto-Shinohara et al., 2000; Miyamoto-Shinohara et al., 2008). As such, the difference in bacterial survival, depending on the drying method, has not been clarified, and there is insufficient research on the viability of foodborne pathogens depending on the drying method.

In addition, the limitation of the concept of a_w in food has recently been confirmed. Water activity only provides information on the amount of free water and bound water, but does not explain properties such as mobility and reactivity. Thus, the concept of the glassy state and glass transition is applied as an alternative (Rahman, 2009). The protective effect of matrix material in a glass state is related to certain physical properties. Molecular translations in a glass state require an extremely long time and generate extremely high viscosity. Molecular movement is almost stopped and is unlikely to be affected by external stress in the glassy state. Both the time of molecular motion and the viscosity show very strong temperature dependence around the glass transition temperature (T_g) (Higl et al., 2007).

The aim of this study was to investigate the survival ratio of bacteria during storage under different water activity conditions, and the survival kinetics of *C. sakazakii* prepared under mild heat treatment by using different drying methods. Furthermore, the glass transition temperature of *C. sakazakii* under different drying methods was examined to determine the relationship between glass transition temperature and survival tolerance at different a_w.

4.2 Materials and methods

4.2.1 Bacterial strains

The two strains, *C. sakazakii* JCM 1233 and 2127 (NCBI, Tsukuba, Japan), which showed less change in T_g depending on a_w in Chapter 3, were compared according to the drying methods. The two strains were recovered from a frozen stock and cultured in tryptic soy agar (TSA, Merck, Darmstadt, Germany) plates. The cultures were incubated at 37 °C for 24 h, and an isolated colony of each bacterium was subsequently transferred to 5 mL of fresh TSB (Merck, Darmstadt, Germany) at 37 °C for 24 h; then, 100 μL of cultured *C. sakazakii* was added to 800 mL of TSB and incubated for 48 h. The cultured cells were centrifuged (3,000 × *g*, 10 min) and the pellets were washed with 30 mL of pure water. The washed bacteria were placed on a Petri dish and frozen at a low temperature of -30 °C.

4.2.2 Drying using different methods

The collected pellets of *C. sakazakii* were dried using two methods. One group of bacteria was dried by air-drying using the principle of diffusion. The bacterial cells were placed in a biological safety hood at room temperature for 48 h to completely dry the bacterial pellet. Another group of *C. sakazakii* was freeze-dried for 24 h using a freeze dryer (FDU-2200, EYELA, Tokyo, Japan) at – 80 °C and pressure of <10 Pa. Both groups of dried samples were powdered manually and stored in air-tight containers.

4.2.3 Survival of bacterial cells at different relative humidity environments (RH)

The powdered bacterial cells were stored in air-tight containers with the desired four levels of relative humidity (% RH). The relative humidity in the containers was adjusted using a saturated salt aqueous solution (43% RH – potassium carbonate, 57% RH – sodium bromide, 75% RH – sodium chloride, and 87% RH – potassium chloride). The bacterial cells for determining the T_g were stored in these air-tight containers with these four kinds of a_w and placed at 4 °C for 48 h to stabilize the a_w of the bacteria.

4.2.4 Survival kinetics of *Cronobacter sakazakii* in selected temperature and relative humidity conditions

The dried *C. sakazakii* cells stored in containers with different relative humidity (43% and 87%) at 4 °C, 25 °C, and 42 °C were investigated for survival kinetics of *C. sakazakii* during storage. An aliquot of 500 μL of 0.1 % peptone water was added to 50 mg of stored *C.*

sakazakii and was diluted serially. The diluted samples were cultured on TSA plates, and the numbers of colonies were confirmed after 24 h of incubation at 37 °C.

4.2.5 Effects of mild heat treatment and different drying methods on the survival of *C. sakazakii*

The dried *C. sakazakii* cells used for the investigation of heat tolerance were stored at 4 °C and 43% or 87% RH for 72 h. The bacterial cells (50 mg) stored under each condition were placed in a polyester film bag (20 mm × 20 mm) and vacuum-packed. Each sample was mildly treated with heat at 40 °C for 1, 3, 6, and 12 h in a water bath, and the heat-treated *C. sakazakii* cells were diluted with 0.1% peptone water. After dilution, *C. sakazakii* were cultured on TSA plates at 37 °C for 24 h to confirm the heat resistance of the dried bacteria depending on the drying method.

4.2.6 Determination of the glass transition temperature (T_g)

The T_g was investigated using thermal rheological analysis (TRA), which was devised in previous studies (Kawai et al., 2014; Sogabe et al., 2018; Jothi et al., 2018) and described in Chapter 2. TRA is an apparatus for determining the T_g of a polymer substance through pressure and heat regulation using a temperature control device attached to a rheometer (EZ-SX, Shimadzu, Kyoto, Japan). The dried samples (100 mg) were placed in the forming die and pressure was applied using a plunger (7 MPa). The compressed bacteria were treated with heat at a rate of 1.5 °C/min from 20–120 °C using a temperature controller. A data

logger was used to collect data between pressure and temperature, and information about the change in pressure was obtained as the temperature increased. The point at which the pressure changes and the physical properties begin to change is defined as the glass transition point, and the T_g can be confirmed through the temperature at this point.

4.2.7 Statistical analysis

The values of triplicate samples were averaged to represent the number of viable cells at each sampling time. Data were expressed as mean ± standard deviation (SD) and subjected to the R statistical software (Version 3.4.1 for Mac OS). One-way ANOVA was performed to compare the differential degrees between each treatment. The T_g and survival ratio were compared using the Tukey-Kramer's multiple comparison test. $P < 0.05$ was considered statistically significant.

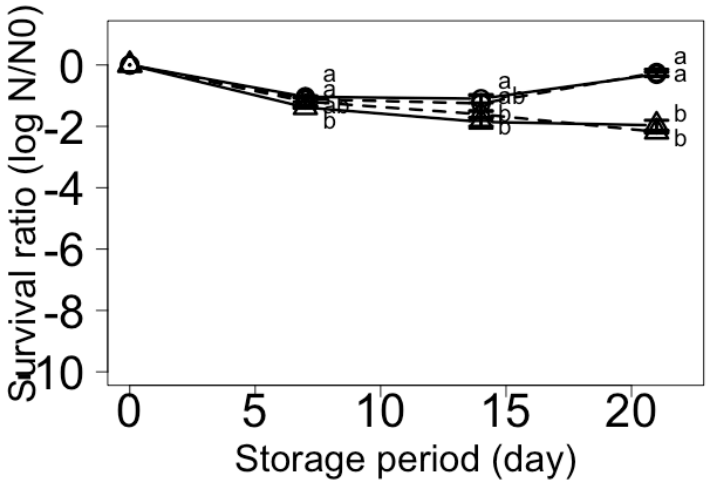
4.3 Results

4.3.1 Survival kinetics of *C. sakazakii* during storage

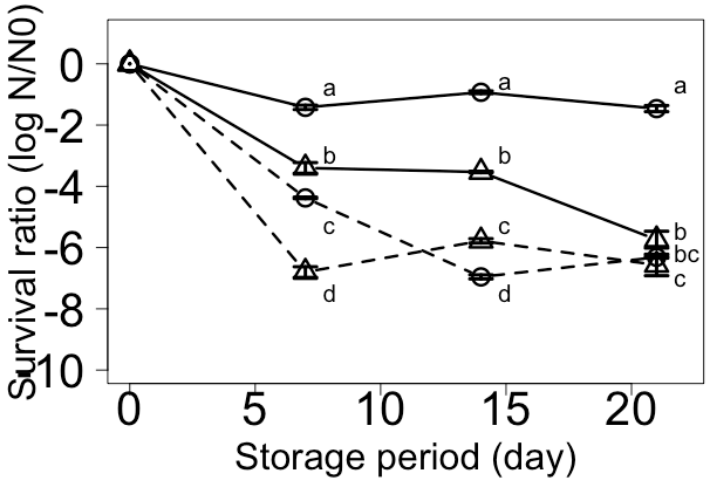
I investigated the survival kinetics of two *C. sakazakii* strains during storage at different temperatures and a_w conditions. The number of surviving air-dried *C. sakazakii* decreased by ca. 1 log cycles; in contrast, the number of viable of freeze-dried *C. sakazakii* declined by approximately 2–4 log cycles during storage at 4 °C. In contrast, under high temperature (42 °C), the decrease in the number of viable *C. sakazakii* was 6–10 log cycles. The changes in survival ratio under different a_w levels were not significantly different at 4 °C and 42 °C.

However, a large difference of survival ratio depending on a_w conditions was investigated at room temperature (25 °C) and *C. sakazakii* showed fast death at a high a_w of 0.87. When viability of the bacteria was confirmed using the drying method, it was found that the number of freeze-dried *C. Sakazakii* decreased faster than that of the air-dried *C. sakazakii*.

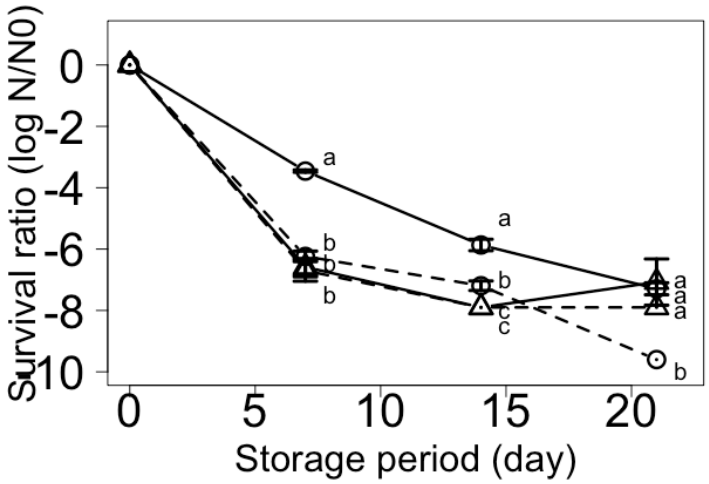
4 °C



25 °C

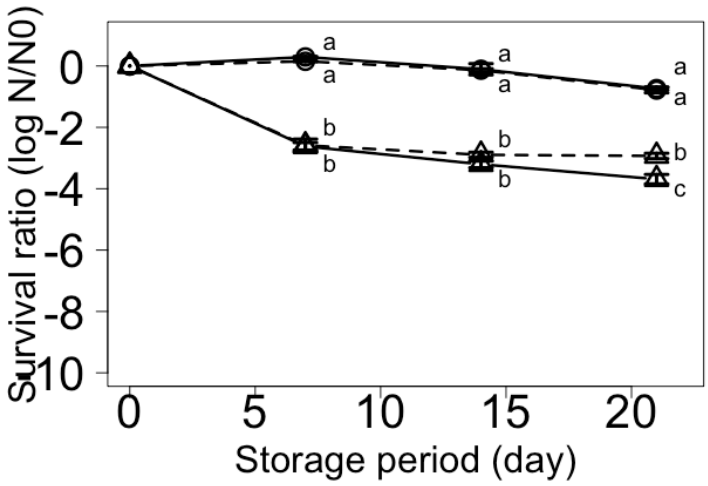


42 °C

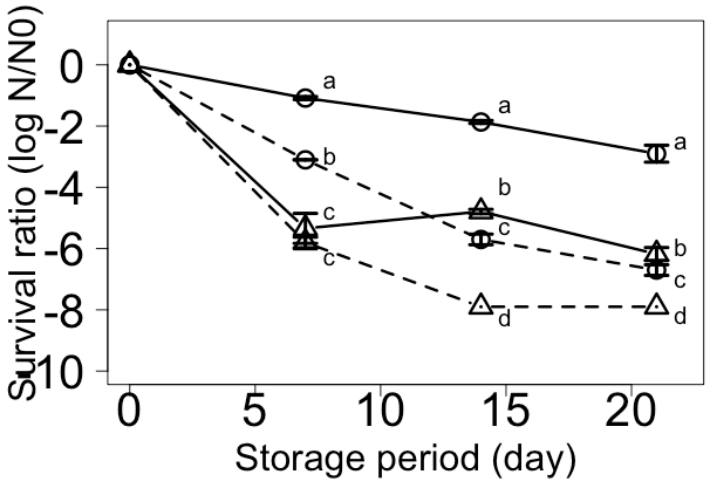


C. sakazakii 1233

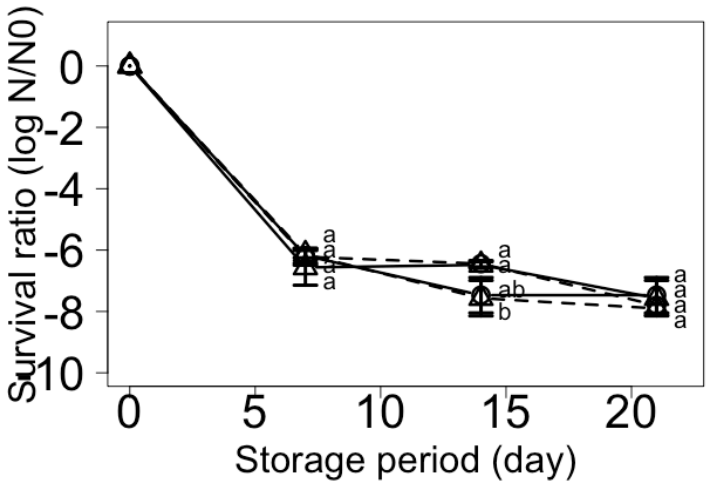
4 °C



25 °C



42 °C



C. sakazakii 2127

Fig. 4-1. Comparison of log reduction of air-dried (○) and freeze-dried (△) *Cronobacter Sakazakii* strains 1233 and 2127 stored under 0.43 a_w (solid line) and 0.87 a_w (dotted line) at different temperatures (4 °C, 25 °C, and 42 °C). The results are means ± standard deviations of three independent experiments. Values with different letters on the same sampling time between different conditions were significantly different ($P < 0.05$).

Table 4-1. Relationship between the survival ratio (log N/N0) and storage period (day) of *Cronobacter sakakzakii* 1233 depending on the drying methods and storage temperature (°C).

Temperature (°C)	Drying method	Water activity (a_w)	Storage period		
			7 days	14 days	21 days
4 °C	Air-dried	0.43 a_w	-1.04±0.03$^{aB\#}$	-1.10±0.14^{aB}	-0.30±0.08^{aA}
		0.87 a_w	-1.11±0.09^{aB}	-1.25±0.26^{abB}	-0.25±0.10^{aA}
	Freeze-dried	0.43 a_w	-1.38±0.03^{bA}	-1.85±0.14^{bB}	-1.97±0.16^{bB}
		0.87 a_w	-1.19±0.13^{abA}	-1.61±0.12^{bB}	-2.18±0.04^{bC}
25 °C	Air-dried	0.43 a_w	-1.42±0.07^{aB}	-0.93±0.05^{aA}	-1.46±0.10^{aB}
		0.87 a_w	-4.38±0.03^{cA}	-6.96±0.07^{dC}	-6.32±0.03^{bcB}
	Freeze-dried	0.43 a_w	-3.40±0.17^{bA}	-3.53±0.02^{bA}	-5.74±0.28^{bB}
		0.87 a_w	-6.80±0.17^{dB}	-5.78±0.07^{cA}	-6.57±0.35^{cB}
42 °C	Air-dried	0.43 a_w	-3.46±0.04^{aA}	-5.87±0.19^{aB}	-7.29±0.20^{aC}
		0.87 a_w	-6.24±0.17^{bA}	-7.19±0.16^{bB}	-9.60±0.00^{bC}
	Freeze-dried	0.43 a_w	-6.58±0.28^{bA}	-7.90±0.00^{cB}	-7.08±0.75^{aAB}
		0.87 a_w	-6.70±0.35^{bA}	-7.90±0.00^{cB}	-7.90±0.00^{aB}

$^{\#}$Different lowercase letters in each column (a_w level) represent statistically significant differences ($P < 0.05$) between the drying methods and water activity. Similarly, the different uppercase letters in each row (*C. sakakzakii* 1233) represent statistical differences ($P < 0.05$) among the four different storage periods.

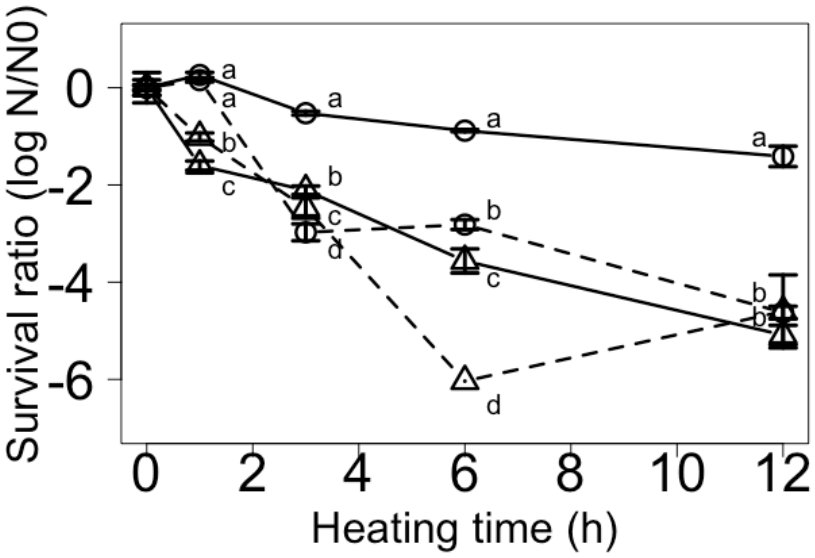
Table 4-2. Relationship between the survival ratio (log N/N0) and storage period (day) of *Cronobacter sakakzakii* 2127 depending on the drying methods and storage temperature (°C).

Temperature (°C)	Drying method	Water activity (a_w)	Storage period		
			7 days	14 days	21 days
4 °C	Air-dried	0.43 a_w	$0.29\pm0.02^{aA\#}$	-0.11 ± 0.18^{aB}	-0.74 ± 0.05^{aC}
		0.87 a_w	0.15 ± 0.02^{aA}	-0.14 ± 0.05^{aB}	-0.79 ± 0.09^{aC}
	Freeze-dried	0.43 a_w	-2.62 ± 0.12^{bA}	-3.20 ± 0.11^{bB}	-3.69 ± 0.15^{bB}
		0.87 a_w	-2.59 ± 0.21^{bA}	-2.89 ± 0.08^{bAB}	-2.93 ± 0.09^{cB}
25 °C	Air-dried	0.43 a_w	-1.09 ± 0.05^{aA}	-1.87 ± 0.05^{aB}	-2.90 ± 0.28^{aC}
		0.87 a_w	-3.10 ± 0.01^{bA}	-5.70 ± 0.17^{cB}	-6.70 ± 0.17^{cC}
	Freeze-dried	0.43 a_w	-5.34 ± 0.49^{cA}	-4.79 ± 0.07^{bA}	-6.18 ± 0.21^{bB}
		0.87 a_w	-5.80 ± 0.17^{cA}	-7.90 ± 0.00^{dB}	-7.90 ± 0.00^{dB}
42 °C	Air-dried	0.43 a_w	-6.18 ± 0.25^{aA}	-7.47 ± 0.58^{abB}	-7.47 ± 0.58^{aB}
		0.87 a_w	-6.22 ± 0.27^{aA}	-6.44 ± 0.10^{aA}	-7.80 ± 0.00^{aB}
	Freeze-dried	0.43 a_w	-6.57 ± 0.58^{aA}	-6.48 ± 0.10^{aA}	-7.57 ± 0.58^{aA}
		0.87 a_w	-6.13 ± 0.12^{aA}	-7.57 ± 0.58^{bAB}	-7.90 ± 0.00^{aB}

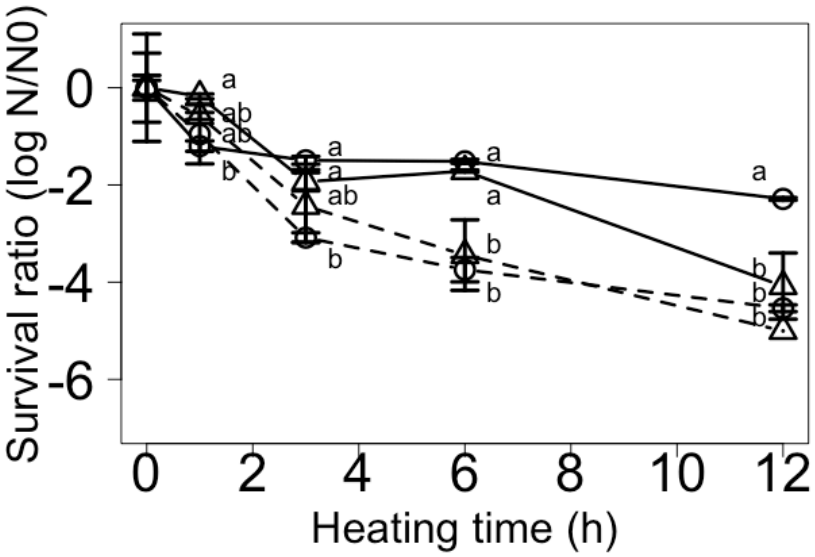
[#]Different lowercase letters in each column (a_w level) represent statistically significant differences ($P < 0.05$) between the drying methods and water activity. Similarly, the different uppercase letters in each row (*C. sakakzakii* 2127) represent statistical differences ($P < 0.05$) among the four different storage periods.

4.3.2 Heat sensitivity at different a_w of dried *C. sakazakii* cells prepared using different drying methods

A mild heat treatment was performed at 40 °C to examine the heat sensitivity of dried *C. sakazakii* to compare the effect of the drying methods. As shown in Fig. 4–3, the number of viable *C. sakazakii* cells decreased under all conditions over a mild heat treatment time. However, although the rate of death differed depending on the water activity environment and drying method, bacteria stored under low water activity conditions showed higher resistance to heat treatment. This indicated that the reduction in survival ratio was -1.41 ~ -5.09 (*C. sakazakii* 1233) and -2.29 ~ -5.00 (*C. sakazakii* 2127) log cycles during 12 h. However, there were no significant differences in survival rate reduction between the drying methods and a_w condition after 12 h of heating, except for air-dried bacteria stored at 0.43 water activity. Therefore, it was proven that the air-dried *C. sakazakii* bacteria stored at 0.43 a_w have a stronger resistance to heat treatment than the freeze-dried samples and air-dried *C. sakazakii* stored at 0.87 a_w.



C. sakazakii 1233



C. sakazakii 2127

Fig. 4-2 Comparison of the thermal inactivation effect (log reductions) of air-dried (○) and freeze-dried (△) *Cronobacter Sakazakii* heated at 40 °C under 0.43 a_w (solid line) and 0.87 a_w (dotted line). The results are means ± standard deviations of three independent experiments. Values with different letters on the same sampling time between different conditions were significantly different ($P < 0.05$).

Table 4-3. Relationship between the survival ratio (log N/N0) and heating time (h) of *Cronobacter sakakzakii* 1233 depending on the drying methods and water activity (a_w).

Drying method	Water activity (a_w)	Storage period			
		1 h	3 h	6 h	12 h
Air-dried	0.43 a_w	$0.26\pm0.05^{aA\#}$	-0.52 ± 0.04^{aB}	-0.88 ± 0.02^{aC}	-1.41 ± 0.21^{aD}
	0.87 a_w	0.16 ± 0.04^{abA}	-2.97 ± 0.17^{dB}	-2.82 ± 0.10^{bB}	-4.62 ± 0.13^{bC}
Freeze-dried	0.43 a_w	-1.60 ± 0.09^{cA}	-2.11 ± 0.09^{bB}	-3.56 ± 0.25^{cC}	-5.09 ± 0.20^{bD}
	0.87 a_w	-1.01 ± 0.08^{bA}	-2.47 ± 0.21^{cB}	-6.04 ± 0.00^{dC}	-4.60 ± 0.75^{bC}

[#]Different lowercase letters in each column (a_w level) represent statistically significant differences ($P < 0.05$) between the drying methods and water activity. Similarly, the different uppercase letters in each row (*C. sakakzakii* 1233) represent statistical differences ($P < 0.05$) among the four different heat treatment times.

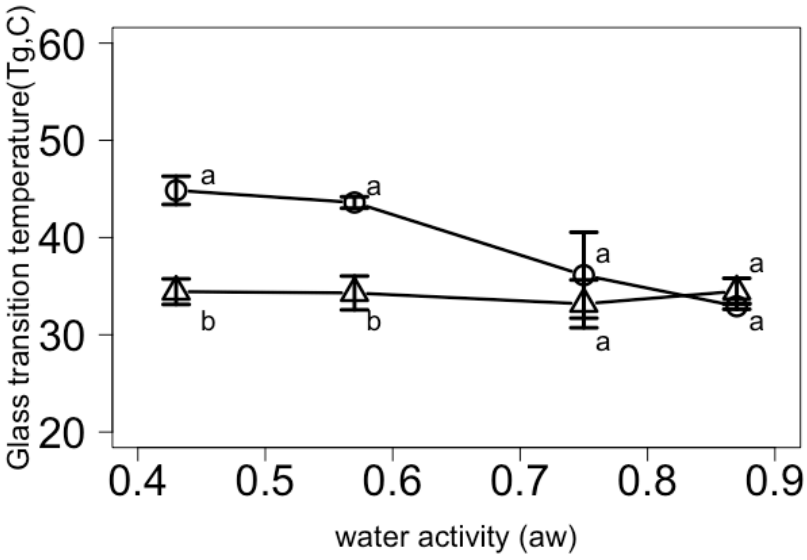
Table 4-4. Relationship between the survival ratio (log N/N0) and heating time (h) of *Cronobacter sakakzakii* 2127 depending on the drying methods and water activity (a_w).

Drying method	Water activity (a_w)	Storage period			
		1 h	3 h	6 h	12 h
Air-dried	0.43 a_w	$-1.20\pm0.11^{bA\#}$	-1.49 ± 0.08^{aB}	-1.51 ± 0.04^{aB}	-2.29 ± 0.03^{aC}
	0.87 a_w	-0.96 ± 0.61^{abA}	-3.08 ± 0.10^{bB}	-3.74 ± 0.25^{bB}	-4.52 ± 0.07^{bC}
Freeze-dried	0.43 a_w	-0.18 ± 0.05^{aA}	-1.93 ± 0.19^{aB}	-1.71 ± 0.02^{aB}	-4.08 ± 0.68^{bC}
	0.87 a_w	-0.58 ± 0.07^{abA}	-2.43 ± 0.75^{abAB}	-3.44 ± 0.73^{bB}	-5.00 ± 0.00^{bC}

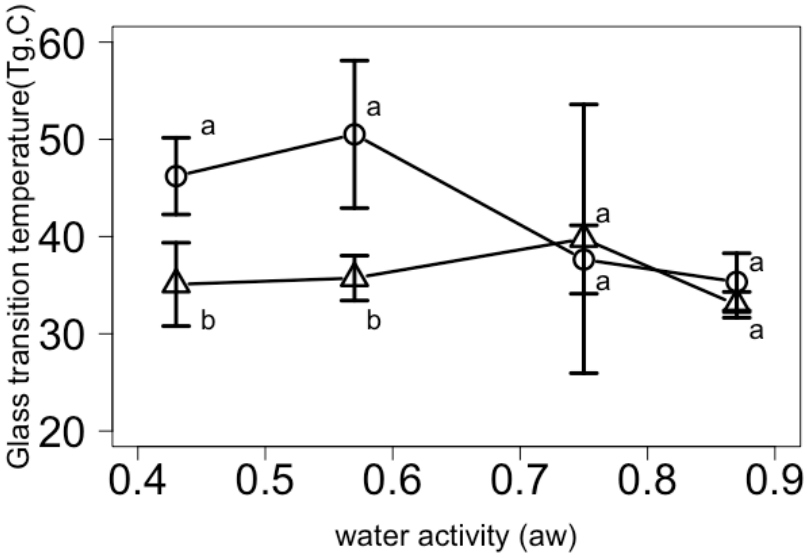
[#]Different lowercase letters in each column (a_w level) represent statistically significant differences ($P < 0.05$) between the drying methods and water activity. Similarly, the different uppercase letters in each row (*C. sakakzakii* 2127) represent statistical differences ($P < 0.05$) among the four different heat treatment times.

4.3.3 Glass transition temperature of *C. sakazakii*

To determine the relationship between T_g and the drying methods of bacteria, I examined the T_g of *C. sakazakii* 1233 *and C. sakazakii* 2127 stored under different a_w conditions (Fig. 4–1). *C. sakazakii* tended to show decreasing T_g as a_w increased. However, different T_g values at the same a_w were observed depending on the drying method. The air-dried *C. sakazakii* cells in both strains showed a higher T_g than that of the freeze-dried *C. sakazakii* cells under <0.57 a_w conditions. In particular, the largest difference in T_g between both drying methods was observed at 15 °C in the case of *C. sakazakii* 2127 stored at 0.57 a_w conditions. In contrast, there were no significant differences in the T_g between the two drying methods at a high a_w, such as >0.75. As a result, it was confirmed that both strains had a high T_g of air-drying under conditions of low a_w (0.43 and 0.57 a_w), and no significant difference was observed under conditions of high a_w (0.75 and 0.87 a_w).



C. sakazakii 1233



C. sakazakii 2127

Fig. 4-3. Relationship between the water activity (a_w) and observed glass transition temperature of air-dried (○) and freeze-dried (△) *Cronobacter sakazakii*. The results are means ± standard deviations of three independent experiments. Values with different letters on the same sampling time between different conditions were significantly different ($P < 0.05$).

Table 4-5. Relationship between the water activity (a_w) and observed glass transition temperature (T_g) of *Cronobacter sakakzakii* 1233 depending on the drying methods.

C. sakazakii	Glass transition temperature (T_g, °C)			
1233	0.43 a_w	0.57 a_w	0.75 a_w	0.87 a_w
Air-dried	$44.85 \pm 1.45^{aA\#}$	43.61 ± 0.59^{aA}	36.13 ± 4.42^{aB}	32.94 ± 0.31^{aB}
Freeze-dried	34.44 ± 1.31^{bA}	34.30 ± 1.74^{bA}	33.18 ± 2.46^{aA}	34.48 ± 1.34^{aA}

[#]Different lowercase letters in each column (a_w level) represent statistically significant differences ($P < 0.05$) between the drying methods. Similarly, the different uppercase letters in each row (*C. sakakzakii* 1233) represent statistical differences ($P < 0.05$) among the four different a_w levels.

Table 4-6. Relationship between the water activity (a_w) and observed glass transition temperature (T_g) of *Cronobacter sakakzakii* 2127 depending on the drying methods.

C. sakazakii	Glass transition temperature (T_g, °C)			
1233	0.43 a_w	0.57 a_w	0.75 a_w	0.87 a_w
Air-dried	$46.21 \pm 3.94^{aAB\#}$	50.51 ± 7.58^{aA}	37.64 ± 3.51^{aB}	35.34 ± 2.94^{aB}
Freeze-dried	35.08 ± 4.29^{bA}	35.73 ± 2.31^{bA}	39.77 ± 13.82^{aA}	32.98 ± 1.33^{aA}

[#]Different lowercase letters in each column (a_w level) represent statistically significant differences ($P < 0.05$) between the drying methods. Similarly, the different uppercase letters in each row (*C. sakakzakii* 2127) represent statistical differences ($P < 0.05$) among the four different a_w levels.

4.4 Discussion and conclusion

In the present study, I examined the survival rate, during the storage period and thermal tolerance, of *C. sakazakii* depending on the drying method. The effects of drying method on *C. sakazakii* stored at low (0.43 a_w) and high (0.87 a_w) water activity conditions during storage are shown in Fig 4–1. The difference in viability is thought to be due to changes in the physical properties of *C. sakazakii* due to differences in the water activity and drying methods. When the viability of the bacteria was confirmed according to the drying method, it was shown that the number of freeze-dried *C. sakazakii* decreased faster than that of the air-dried sample during storage. The freeze-dried *C. sakazakii* cells showed a lower T_g than that of the air-dried *C. sakazakii* cells, and the resistance to external stress might be low (Fig 4–3). In addition, since *C. sakazakii* showed a low T_g, such as 32–34 °C, under high a_w (0.87 a_w) conditions, *C. sakazakii* cells presented as a rubbery state, not a glassy state at 42 °C, because it is higher than T_g. *C. sakazakii* is estimated to exhibit a fast decreasing survival ratio at 42 °C than at the other temperature conditions, where it remains in a glassy state.

C. sakazakii has been shown to have stronger viability under low a_w conditions (0.43 a_w), showing a similar tendency that reduction in bacterial population tended to be greater at high a_w than at low a_w, as in previous studies (Gurtler and Beuchat, 2007; Lin and Beuchat, 2007). In addition, it is already known that *C. sakazakii* was shown low unculturable proportion and have decreased permeability after drying under low a_w conditions (Lang et al., 2018). In addition, it was confirmed that air-dried *C. sakazakii* at 20–25 °C survived for more than 2.5 years in powdered infant formula (Barron and Forsythe, 2007). The strong viability

characteristics in a low a_w environment of *C. sakazakii* is similar to cryptobiosis that is found in some organisms such as tardigrade and African chironomid. Cryptobiosis is a characteristic that confers a high survival rate through the transformation of organisms, similar to glass state, in harsh environments such as deserts (Sakurai et al., 2008; Horikawa et al., 2012). Therefore, I hypothesized that it is related to the glass transition phenomenon, which can explain the strong resistance of *C. sakazakii* under low a_w. As described above, *C. sakazakii* cells have a high T_g under conditions of low a_w; therefore, it is more likely to exist in the glass state at room temperature. The presence of bacteria in a glass state reduces the activity and fluidity of the substance through the cell membrane and makes it more resistant to external environmental stresses. Thus, *C. sakazakii* can be considered to exist for a long period with strong viability under low a_w conditions.

Fig 4–2 shows the effects of drying methods and a_w levels on the sensitivity of *C. sakazakii* to a mild heat treatment. *C. sakazakii* exhibited high thermal resistance when a_w was low (0.43), and was highly heat-tolerant when air-dried than freeze-dried. Based on Fig 4–2 and Fig 4–3, I believe that the acquisition of environmental tolerance of *C. sakazakii* cells is related to T_g. As shown in Fig. 4–3, the air-dried *C. sakazakii* cells (a_w = 0.43) had a higher T_g of 43 °C than that used for the mild heat treatment at 40 °C. Therefore, air-dried *C. sakazakii* cells stored at low a_w conditions, such as 0.43, remained in a glassy state during the mild heat treatment. On the contrary, air-dried *C. sakazakii* at 0.87 a_w and freeze-dried *C. sakazakii* existed in a rubbery state because the T_g values were lower than the heat treatment

temperature, as shown in Fig 4–3. This is considered to be the cause of the high death rate during heat treatment.

Nazarowec-white and Faber (1997) reported the *D*-value of *C. sakazakii* in a reconstituted dried-infant formula using varied temperature conditions. The *D*-values for the reconstituted dried-infant formula were $D_{52°C}$. = 54.8 min, $D_{54°C}$ = 23.7 min, $D_{56°C}$ = 10.3 min, $D_{58°C}$ = 4.2 min, and $D_{60°C}$ = 2.5 min. The relatively low D-values could be attributed to the examined temperatures that were higher than the T_g of *C. sakazakii* illustrated in the present study.

In this study, I aimed to elucidate the effect of *C. sakazakii* bacterial tolerance according to drying methods and to interpret it using the glass transition phenomenon. The results showed the effect of drying method on the survival rate of *C. sakazakii* bacteria during storage and heat treatment. It was confirmed that *C. sakazakii* bacteria exhibited different glass transition temperatures according to the drying method, and the change in bacterial resistance could be explained by the glass transition phenomenon. This approach serves as a valuable contribution to the selection of a drying method for inhibiting foodborne pathogens.

Chapter 5 Summary

5. Summary

Drying is one of the most effective techniques for food preservation and is widely used worldwide. Dry stress suppresses the microbial metabolic activity by reducing the free water in foods that is available to microorganisms. In particular, bacteria are not able to grow in low water activity (a_w) foods, although foodborne illnesses caused by dried foods are reported worldwide. The reason bacterial cells survive under low a_w conditions has been unclear. In order to clarify the cause of resistance of pathogenic bacterial cells to low a_w stress, this study focused on the glass transition phenomenon, which is a change in the physical properties of bacterial cells, instead of using conventional biochemical approaches.

1. Relationship between glass transition temperature and heat tolerance in *Salmonella enterica*

Salmonella enterica is a foodborne pathogenic bacterium that has been reported in various dried foods worldwide. To clarify the cause of the survival of *S. enterica* in dried foods under a low a_w environment, the glass transition phenomenon, which is a state change in substance, was the focus of this study. Five kinds of *S. enterica* serotype, under different water activity conditions, were examined for the glass transition temperature (T_g) using a newly developed thermal rheological analysis method. Under low a_w conditions ($a_w < 0.75$), all the five *S. enterica* serotypes showed a relatively high T_g, ranging from 45–62 °C. These results suggest

that *S. enterica* can be present in the glassy state at room temperature and/or below, in dried foods. Furthermore, the thermal tolerance of dried *S. enterica* at different a_w levels was examined at 60 °C for 10 min. The results demonstrated that the glassy state and the thermal resistance are related to each other, as *S. enterica* under low a_w conditions showed higher thermal resistance. These results would be the basis for understanding the survival of *S. enterica* in dried foods.

2. Strain variability according to the glass transition temperature of *Cronobacter sakazakii*

Cronobacter sakazakii is a widely known pathogenic bacterium that contaminates powdered infant formula worldwide. However, the reason for the contamination and survival of *C. sakazakii* in powdered infant formula with low a_w has been unclear for a long time. As the glass transition phenomenon of bacterial cells might be attributed to the survival of *C. sakazakii* under low a_w conditions, six strains of *C. sakazakii* were examined to determine their T_g. The results indicated that there were significant differences in the T_g of *C. sakazakii* among the six strains. However, the T_g of *C. sakazakii*, regardless of strain, ranged from 35–60 °C under low a_w conditions ($a_w < 0.57$), which suggests that *C. sakazakii* would exist in a glass state at room temperature and/or below. The survival of *C. sakazakii* in infant formula could be attributed to the glass transition of bacterial cells.

3. Effects of drying methods of *Cronobacter sakazakii* on survival during storage and thermal tolerance

Although there are some drying methods for producing dried foods, the effect of drying methods on the survival and thermal tolerance of dried bacterial cells has not been studied. A detailed investigation of the effects of drying methods on bacterial survival during storage under low a_w conditions would provide useful insights that may benefit the food industry. To clarify the survival behavior of *C. sakazakii* under low a_w conditions, the T_g of air-dried and freeze-dried bacterial cells, and the survival kinetics during storage were examined. The T_g of *C. sakazakii* depends on the drying method; in this study, the air-dried *C. sakazakii* exhibited a higher T_g than that of the freeze-dried cells. In addition, the air-dried *C. sakazakii* cells showed stronger viability for a long time during storage than the freeze-dried ones. Furthermore, the air-dried *C. sakazakii* cells demonstrated a higher survival rate during thermal treatment at 40 °C than that of freeze-dried ones, because the T_g of air-dried *C. sakazakii* was higher than 40 °C, in contrast to the lower T_g (ca. 33 °C) of freeze-dried *C. sakazakii*. These results suggest that the T_g of bacterial cells plays a key role in ensuring microbial food safety in dry foods.

References

Abiad M. G. *et al.* A Review on Methods and Theories to Describe the Glass Transition Phenomenon: Applications in Food and Pharmaceutical Products. Food Eng Rev. 1, 105–132 (2009).

Ablett S. *et al.* Glass formation and dormancy in bacterial spores. International Journal of Food Science and Technology. 34, 59-69. (1999)

Abushelaibi A. A. *et al.* Survival and growth of *Salmonella* in reconstituted infant cereal hydrated with water, milk or apple juice and stored at 4 °C, 15 °C and 25 °C. Food Microbiol. 20, 17–25 (2003).

Arku B. *et al. Enterobacter sakazakii* survives spray drying. International Journal of Dairy Technology. 61, 102-108 (2008).

Askerland D R. The science and engineering of materials. Chapman and Hall. 2nd edition.

Barbosa J. *et al* Comparison of spray drying, freeze drying and convective hot air drying of the production of a probiotic orange powder. Journal of Functional Foods. 17, 340-351 (2015).

Barron J.C. and Forsythe S.J. Dry stress and survival time of *Enterobacter sakazakii* and other Enterobacteriaceae in dehydrated powdered infant formula. J. Food Prot. 70, 2111-2117. (2007)

Belliveau B. H. *et al* Heat killing of bacterial spores analyzed by differential scanning calorimetry. 174, 4463-4474 (1992).

Beuchat, L.R. and Mann, D.A. Survival of *Salmonella* in cookie and cracker sandwiches containing inoculated, low–water activity fillings. J. Food Prot. 78, 1828–1834 (2015)

Beuchat L. R. and Scouten A. Combined effects of water activity, temperature and chemical treatments on the survival of *Salmonella* and *Escherichia coli* O157: H7 on alfalfa seeds. J Appl Microiol. 92, 382–395 (2002).

Beuchat L. R. *et al.* Low–Water Activity Foods: Increased Concern as Vehicles of Foodborne Pathogens. J Food Prot. 76, 150–172 (2013).

Bhandari B. R. and Howes T. Implication of glass transition for the drying and stability of dried foods. Journal of Food Engineering. 40, 71–79 (1999).

Biering G. *et al* Three cases of neonatal meningitis caused by *Enterobacter sakazakii* in powdered milk. Journal of Clinical Microbiology. 27, 2054–2056 (1989)

Block C. *et al* Cluster of neonatal infections in Jerusalem due to unusual biochemical variant of *Enterobacter sakazakii*. European Journal of Clinical Microbiology and Infectious Diseases. 21, 613–616 (2002).

Breeuwer P. *et al*. Desiccation and heat tolerance of *Enterobacter sakazakii*. Journal of Applied Microbiology. 95, 967-973. (2003)

Brewer M. S. Traditional preservatives – sodium chloride. Encyclopaedia of Food Microbiology. 3, 1723-1728 (1999).

Brockmann S. O. *et al Salmonella* in sesame seed products. Journal of Food Protection. 67, 178-180 (2004).

Burnett S. L. *et al.* Survival of *Salmonella* in peanut butter and peanut butter spread. J Appl Microbiol. 89, 472–477 (2000).

Cahill S. M. Powdered infant formula as a source of *Salmonella* infection in infants. Food Safety. 46, 268-273 (2008).

Cavallaro E. M. D *et al Salmonella Typhimurium* infections associated with peanut products. N. Engl. J. Med. 365, 601-610 (2011).

Cottyn B. *et al* Bacterial populations associated with rice seed in the tropical environment. Phytophathology. 91, 282-291 (2000).

Daoust J.Y. *et al. Salmonella eastbourne* outbreak associated with chocolate. Canadian Institute of Food Science and Technology Journal. 8, 181–184 (1975).

Dyre J. C. Colooquium: The glass transition and elastic models of glass-forming liquids. Rev. Mod. Phys. 78, 953-972 (2006).

Ebara T. *et al.* Effect of water content on the glass transition and textural properties of hazelnut. Journal of Thermal Analysis and Calorimetry. 135, 2629–2634 (2019).

Edelson-Mammel S. G. *et al*. Survival of *Enterobacter sakazakii* in a dehydrated powdered infant formula. J. Food. Prot. 68, 1900-1902 (2005).

Feeney A. and Sleator R. D. An in silico analysis of osmotolerance in the emerging gastrointestinal pathogen *Cronobacter sakazakii*. Bioengineered Bugs. 2, 260–270 (2011).

Feeney, A. *et al. Cronobacter sakazakii*: stress survival and virulence potential in an opportunistic foodborne pathogen. Gut Microbes 5, 711–718 (2015).

Finn S. *et al*. Mechanism of survival, responses, and sources of *Salmonella* in low-moisture environments. Frontiers in Microbiology.14, 1-15. (2013)

Finn S. *et al.* Phenotypic characterization of *Salmonella* isolated from food production environments associated with low-water activity foods. J.Food Prot 76, 1488-1499. (2013).

Fonseca F. *et al* State diagrams and sorption isotherms of bacterial suspensions and fermented medium. Thermochimica Acta. 366, 167-182 (2001).

Friedemann M. *Enterobacter sakazakii* in food and beverages (other than infant formula and milk powder). International Journal of Food Microbiology. 116, 1-10. (2007)

Garcia-reverter, J. *et al*. Low Temperature Blanching Affects Firmness and Rehydration of Dried Cauliflower Florets. Journal of Food Science 59, 1181–1183 (1994).

Gill O.N. *et al.* Outbreak of *Salmonella napoli* infection caused by contaminated chocolate bars. Lancet. 321, 574–577 (1983).

Grant W.D. Life at low water activity. Phil. Trans. R. Soc. Lond. B. 359, 1249-1267. (2004)

Gurtler J.B. and Beuchat L.R. Survival of *Enterobacter sakazakii* in powdered infant formula as affected by composition, water activity, and temperature. J. Food Prot. 70., 1579-1586 (2007).

Ha J.W. and Kang D.H. Synergistic bactericidal effect of simultaneous near-infrared radiant heating and UV radiation against *Cronobacter sakazakii* in powdered infant formula.

Applied and Environmental Microbiology. 80, 1858-1863. (2014)

Ha J W. *et al.* Inactivation of *Salmonella enterica* serovar Typhimurium and *Escherichia coli* O157:H7 in peanut butter cracker sandwiches by radio-frequency heating. Food Microbiol. 34, 145–150 (2013).

Hammami C. and Rene F. Determination of freeze-drying process variables for strawberries. Journal of Food Engineering. 32, 133–154 (1997).

Hengherr S. *et al.* High-Temperature Tolerance in Anhydrobiotic Tardigrades Is Limited by Glass Transition. Physiological and Biochemical Zoology. 82, 749–755 (2015).

Henry M. and Fouladkhah A. Outbreak history, biofilm formation, and preventive measures for control of *Cronobacter sakazakii* in infant formula and infant care setting. Microorganisums. 7, 1-22 (2019).

Higl B. *et al*. Impact of water Activity, temperature, and physical state on the storage stability of *Lactobacillus paracasei* ssp. *paracasei* freeze-dried in a lactose matrix. Biotechnol. Prog. 23, 794-800. (2007)

Hiramatsu R. *et al.* Ability of shiga toxin-producing *Escherichia coli* and *Salmonella spp*. to survive in a desiccation model system and in dry foods. Appl Environ Microbiol. 71, 6657–6663 (2005).

Hockin J. C. *et al.* An International Outbreak of *Salmonella Nima* from imported chocolate. J Food Prot. 52, 52: 51–54 (1989).

Hohne G *et al* Differential scanning calorimetry. Springer-Verlag, 1-8 (2013).

Hokunan, H. *et al*, S. Survival kinetics of *Salmonella enterica* and enterohemorrhagic *Escherichia coli* on a plastic surface at low relative humidity and on low-water activity foods. J. Food Prot. 79, 1680–1692 (2016).

Horikawa, D.D. *et al*. Tolerance of anhydrobiotic eggs of the tardigrade ramazzottius varieornatus to extreme environments. Astrobiology. 12, 283–289 (2012).

Isaacs S. *et al.* An international outbreak of salmonellosis associated with raw almonds contaminated with a rare phage type of *Salmonella enteritidis*. J Food Prot. 68, 191–198 (2005).

Iversen C. and Forsythe S. Isolation of *Enterobacter sakazakii* and other Enterobacteriaceae from powdered infant formula milk and related products. Food Microbiology. 21, 771-777. (2004)

Iversen C. and Forsythe S. Risk profile of *Enterobacter sakazakii*, an emergent pathogen associated with infant milk formula. Trends in Food Science & Technology. 14, 443-454. (2003)

Iversen C. *et al* A selective differential medium for *Enterobacter sakazakii*, a preliminary study. Int. J. Food Microbiol. 96, 133-139 (2004).

Iversen C. *et al* The growth profile, thermotolerance and biofilm formation of *Enterobacter sakazakii* grown in infant formula milk. Letters in Applied Microbiology. 38, 378–382 (2004).

Jiao R. *et al*. Effects of vacuum freeze-drying on inactivation of *Cronobacter sakazakii* ATCC29544 in liquid media with different initial inoculum levels. J. Dairy Sci. 100, 1674-1678. (2017)

Jonsson K. I. Causes and consequences of excess resistance in cryptobiotic metazoans. Physiological and Biochemical Zoology. 76, 429-435 (2003).

Jothi, J.S. *et al*. Effect of water sorption on the glass transition temperature and texture of deep-fried models. J. Food Eng. 237, 1–8 (2018).

Jung Y. S. and Beuchat L. R. Survival of multidrug-resistant *Salmonella* typhimurium DT104 in egg powders as affected by water activity and temperature. Int J Food Microbiol. 49, 1–8 (1999).

Kapperud G. *et al.* Outbreak of *Salmonella* typhimurium infection traced to contaminated chocolate and caused by a strain lacking the 60-megadalton virulence plasmid. Journal of Clinical Microbiology. 28, 2597–2601 (1990).

Kataoka A. I. *et al.* Survival of *Salmonella* Tennessee, *Salmonella* Typhimurium DT104, and *Enterococcus faecium* in Peanut Paste Formulations at Two Different Levels of Water Activity and Fat. J Food Prot. 77, 1252–1259 (2014).

Kauzmann W. The nature of the glassy state and the behavior of liquids at low temperatures. ACS Publications. 219-256 (1948).

Kawai, K. *et al*. Effect of sugar composition on the water sorption and softening properties of cookie. Food Chem. 145, 772–776 (2014).

Keller S. E. *et al.* Growth and survival of *Salmonella* in ground black pepper (*Piper nigrum*). Food Microbiol. 34, 182–188 (2013).

Kimber M. A. *et al.* Survival of *Salmonella*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* on inoculated almonds and pistachios stored at –19, 4, and 24° C. J Food Prot. 75, 1394–1403 (2012).

Kirk M. D. *et al.* An outbreak due to peanuts in their shell caused by *Salmonella enterica* serotypes Stanley and Newport – sharing molecular information to solve international outbreaks. Epidemiology and Infection. 132, 571–577 (2004).

Klemes J. *et al* Handbook of water and energy management in food processing. Boca Raton : Woodhead Publishing limited, 449-623 (2008).

Knorr D. Technology aspect related to microorganisms in functional foods. Trends in Food Science & Technology. 9, 295-306 (1998).

Koseki S. *et al*. Comparison of desiccation tolerance among *Listeria monocytogenes, Escherichia coli* O157:H7, *Salmonella enterica*, and *Cronobacter sakazakii* in powdered infant formula. J. Food Prot. 79, 104-110. (2015)

Labuza T. P. The effect of water activity on reaction kinetics of food deterioration. Food Technology, 36-41 (1980).

Lang E. *et al*. Cellular injuries in *Cronobacter sakazakii* CIP 103183T and *Salmonella enterica* exposed to drying and subsequent heat treatment in milk powder. Frontiers in Microbiology.13, 1-10. (2018)

Lang E. *et al*. Drying parameters greatly affect the destruction of *Cronobacter sakazakii* and *Salmonella* Typhimurium in standard buffer and milk. Food Microbiology. 62, 82-91. (2017)

Lee K. *et al.* Relationship between glass transition temperature, and desiccation and heat tolerance in Salmonella enterica. PLoS ONE. 15(5), 1-11. (2020)

Lewicki P. P. Design of hot air drying for better foods. Trends in Food Science & Technology. 17, 153-163 (2006).

Lewicki P. P. *et al* Effect of water activity on mechanical properties of dry cereal products. Acta agrophysica. 4, 381-391 (2004).

Lian F. *et al.* Survival of *Salmonella enterica* in skim milk powder with different water activity and water mobility. Food Control. 47, 1–6 (2015).

Lin L.C. and Beuchat L.R. Survival of *Enterobacter sakazakii* in infant cereal as affected by composition, water activity, and temperature. Food Microbiology. 24, 767-777 (2007).

Li C. *et al.* Comparative study of thermal inactivation kinetics of *Salmonella* spp. in peanut butter and peanut butter spread. Food Control. 45, 143–149 (2014).

Ma L. *et al.* Thermal Inactivation of *Salmonella* in Peanut Butter. J Food Prot. 72, 1596–1601 (2009).

Mattick K. L. *et al.* Effect of Challenge Temperature and Solute Type on Heat Tolerance of *Salmonella* Serovars at Low Water Activity. Appl Environ Microbiol. 67, 4128–4136 (2001).

Meissner U. *et al*. Detection of silicone oil leakages in freeze dryers. PDA Journal of Pharmaceutical Science and Technology. 65, 481–485 (2011).

Mochizuki T. *et al.* Effect of porous structure and water plasticization on the mechanical glass transition temperature and textural properties of freeze-dried trehalose solid and. J Food Eng. 247, 38–44. (2018)

Miyamoto-Shinohara Y. *et al*. Survival of freeze-dried bacteria. J. Gen. Appl. Microbiol. 54, 9-24 (2008).

Miyamoto-Shinohara Y. *et al*. Survival rate of microbes after freeze-drying and long term storage. Cryobiology. 41, 251-255 (2000).

Mochizuki, T. *et al*. Effect of glass transition on the hardness of a thermally compressed soup solid. J. Food Eng. 247, 38–44 (2019).

Morgan C.A. *et al*. Preservation of micro-organisms by drying; A review. Journal of Microbiological Methods. 66, 183-193 (2006).

Nakamura N. and Shiina T. Comparison of desiccation tolerance among *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella enterica*, and *Cronobacter sakazaki*i in powdered infant formula. J Food Prot. 78, 104–110 (2015).

Nazarowec-White. M. and Farber J. M. *Enterobacter sakazakii*: a review. International Journal of Food Microbiology. 34, 103–113 (1997).

Noel T. R *et al* Glass transitions in low-moisture foods. Trends in Food Science & Technology, 62-67 (1990).

No H. K. *et al* Antibacterial activities of chitosans and chitosan oligomers with different molecular weights on spoilage bacteria isolated from tofu. J. Food Sci. 67, 1511-1514 (2002).

Nummer B. A. *et al.* Survival of *Salmonella* in a high sugar, low water-activity, peanut butter flavored candy fondant. Food Control. 27, 184–187 (2012).

Olgenblum G.L. *et al*. Properties of aqueous trehalose mixture: glass transition and hydrogen bonding. J.Chem.Theory Comput. 16, 1249-1262. (2020)

Onwude D. I. *et al.* Modeling the thin-layer drying of fruits and vegetables: Review. Comprehensive Reviews in Food Science and Food Safety. 15, 599-618 (2016).

Patist A. and Zoerb H. Preservation mechanisms of trehalose in food and biosystems. Colloids and Surfaces B: Biointerfaces. 40, 107-113. (2005)

Poddar D. *et al.* Stability of probiotic *Lactobacillus paracasei* during storage as affected by the drying method. International Dairy Journal, 39, 1–7. (2014).

Podolak R. *et al.* Sources and Risk Factors for Contamination, Survival, Persistence, and Heat Resistance of *Salmonella* in Low-Moisture Foods. J Food Prot. 73, 1919–1936 (2010).

Prosapio V. *et al*. Optimization of freeze-drying using a Life Cycle Assessment approach: Strawberries' case study. Journal of Cleaner Production, 168, 1171–1179 (2017).

Rahman M. S. Food stability beyond water activity and glass transition: macro-micro region concept in the state diagram. International Journal of Food Properties. 12, 726-740 (2009).

Ratti C. Hot air and freeze-drying of high-value foods: a review. Journal of Food Engineering. 49, 311-319 (2001).

Reich F. *et al* Prevalence of *Cronobacter* spp. in a powdered infant formula processing environment. International Journal of Food Microbiology. 140, 214–217 (2010).

Restaino L. *et al* A chromogenic plating medium for the isolation and identification of *Enterobacter sakazakii* from foods, food ingredients, and environmental sources. J. Food Prot. 69, 315-322 (2006).

Roe K.D. and Labuza T.P. Glass transition and crystallization of amorphous trehalose-sucrose Mixtures. International Journal of Food Properties. 8, 559-574. (2005)

Roos Y. H. Glass transition temperature and its relevance in food processing. Ann. Rev. Food. Sci. Technol. 1, 469-491 (2010).

Richards G. M. *et al* Survival and growth of *Enterobacter sakazakii* in infant rice cereal reconstituted with water, milk, liquid infant formula, or apple juice. J. Appl. Microbiol. 99, 844-850 (2005).

Russo E. T. *et al* A recurrent, multistate outbreak of *Salmonella* serotype Agona infections associated with dry, unsweetened cereal consumption, United States. J. Food. Prot. 76, 227-230 (2013).

Sakurai, M., *et al.* Vitrification is essential for anhydrobiosis in an African chironomid, Polypedilum vanderplanki. Proceedings of the National Academy of Sciences. 105, 5093–5098 (2008).

Sanchez-Maldonado A. *et al*. Methods for the control of foodborne pathogens in low-moisture foods. Annu. Rev. Food Sci. Technol. 9, 177-208. (2018)

Santillana Farakos S. M. *et al*. Modeling the influence of temperature, water activity and water mobility on the persistence of *Salmonella* in low-moisture foods. Int J Food Microbiol. 166, 280–293 (2013).

Santillana Farakos S. M. S. *et al.* Predicting survival of *Salmonella* in low–water activity foods: An analysis of literature data. J Food Prot. 77, 1448–1461 (2014).

Santivarangkna C. *et al* Role of glassy state on stabilities of freeze-dried probiotics. Journal of Food Science. 76, R1-R5 (2011).

Sapru V. and Labuza T. P. Glassy state in bacterial spores predicted by polymer glass-transition theory. Journal of Food Science. 58, 445-448 (1993).

Scott W. J. Water relations of food spoilage microorganisms. Advances in Food research. 7, 83-127 (1957).

Shachar D. and Yaron S. Heat Tolerance of *Salmonella enterica* Serovars Agona, Enteritidis, and Typhimurium in Peanut Butter. J. Food Prot. 69, 2687–2691 (2006).

Sheth A.N. *et al.* A national outbreak of *Salmonella* serotype Tennessee infections from contaminated peanut butter: a new food vehicle for salmonellosis in the United States. Clin Infect Dis. 53, 356–362 (2011).

Sogabe T. *et al*. Effects of porous structure and water plasticization on the mechanical glass transition temperature and textural properties of freeze-dried trehalose solid and cookie. Journal of Food Engineering. 217, 101-107 (2018).

Stecchini M. L. *et al.* Glassy state in Bacillus subtilis spores analyzed by differential scanning calorimetry. International Journal of Food Microbiology. 106, 286-290 (2006).

Strasser S. *et al.* Influence of lyophilization, fluidized bed drying, addition of protectants, and storage on the viability of lactic acid bacteria. Journal of Applied Microbiology. 107, 167–177 (2009).

Syamaladevi R. M. *et al.* Influence of water activity on thermal resistance of microorganisms in low-moisture foods: a review. Comprehensive reviews in food science and food safety. 15, 353-370 (2016).

Tamura A. *et al* Flavor components and microorganisms isolated from suancha (sour tea, takezutsu-sancha in Japanese). J. Home Econ. Jpn. 46, 759-764 (1995).

Thuc T. *et al*. Measurement of Glass-Rubber Transition Temperature of Rice by Thermal Mechanical Compression Test (TMCT). International Journal of Food Properties. 13, 176–183 (2010).

Van Acker J. *et al* Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. Journal of Clinical Microbiology. 39, 293–297 (2001).

Van Donkelaar L. H. G. *et al.* Glass transitions of barley starch and protein in the endosperm and isolated from. Food Res Int. 72, 241–246 (2015).

Weber D. *et al* International outbreak of *Salmonella* Oranienburg due to German chocolate. BMC Infectious Diseases. 5, 1-10 (2005).

Yang C. S. T. and Atallah W. A. Effect of four drying methods on the quality of intermediate moisture lowbush blueberries. Journal of Food Science. 50, 1233-1237 (1985).

Young I. *et al*. Application of a rapid knowledge synthesis and transfer approach to assess the microbial safety of low-moisture foods. J. Food Prot. 78, 2264-2278. (2015)

Survival strategy of foodborne pathogenic bacteria under low water activity environment: Contribution of glass transition phenomenon of bacterial cells

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