Antibacterial activity of D-Tryptophan against food-borne pathogenic bacteria: Application to food processing and investigation of the mechanism

（D-トリプトファンによる食品有害細菌の増殖抑制：食品への応用と増殖抑制メカニズムの解明）

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Acknowledgements

I would like to express my special appreciation and deep gratitude to Professor Shigenobu Koseki, my research supervisors, for his patient guidance, enthusiastic encouragement and useful critiques of this research work. I would also like to thank Professor Shuso Kwamura, Professor Kazunori Iwamuchi, who help me with their valuable advice, support for completing my thesis.

I would also like to extend my thanks to my fellow: Hiroko Kudo, Kaito Kan or their feedback, cooperation. I am also grateful to the staffs of Center for Instrumental Analysis in Hokkaido University for the help and support.

I appreciate the fund support from China Scholarship Council (CSC), which gives me the opportunity to go abroad and study. Special appreciation goes to my master's supervisor Dr. Shiping Tian (Institute of Botany, The Chinese Academy of Sciences) for leading me into the field of food science and recommendation of doctoral study.

I would like to thank my friends for accepting nothing less than excellence from me. Last but not the least, I wish to thank my family, especially my mother, for their love, support and encouragement throughout my study.

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Chapter 1

General Introduction

1.1. Foodborne and environmental stress

Bacteria inhabit natural and artificial environments with diverse and fluctuating osmolalities, salinities, and temperatures (Wood et al., 2001). The general bacteriostatic or bactericidal stresses in these environments includes osmotic pressure, extremes of temperature, and pH, and starvation (Archer, 1996). In fact, various environmental conditions are encountered by bacteria during the infection process. Food is usually regarded as a friendly environment for bacteria. During the food preservation process, one or multiple environmental stresses are always utilized to slow or prevent bacterial growth. In particular, low temperature and osmotic pressure (low water activity) are two of the common methods to control bacterial growth during postharvest processing.

1.1.1 Osmotic treatment and low water activity in food preservation

During foods processing, osmotic treatments including lowering the water activity and salting, are commonly used to control the foodborne pathogens and preserve food stability. Osmotic treatment by lowering the water activity in foods, has been regarded as an effective method to reduce the growth of spoilage bacteria and limit the survival of both spoilage and pathogenic organisms (O'Byrne and Booth, 2002). As water activity-lowering agent, salts such as sodium chloride (NaCl), has long been used to preserve food products. Salting can also be

regarded as a simulated osmotic treatment process, which leads to a reduction in the amount of water available in foods (Bidlas and Lambert, 2008). Osmotic treatment is widely applied in the processing of vegetables, fruit, meat, and fish. During meat fermentation, 2–3% (w/w) NaCl is added to decrease the water activity as low as 0.96, to inhibit undesirable or pathogenic microorganisms and prolong the shelf life of the fermented product (Mellefont et al., 2003). During the process of cold-smoking salmon, a high NaCl concentration-mediated osmotic treatment is applied to the fish fillets to control spoilage bacteria, such as *Shewanella putrefaciens.* This growth inhibitory effect is dependent on the salt concentration (Leblanc et al., 2000). Osmotic treatment also affects the adaptation of foodborne bacteria to new environmental conditions. Sucrose-, KCl-, and NaCl-mediated shifts in water activity affect the duration of the lag phase in some foodborne bacteria (Mellefont et al., 2003). Increasing the external osmotic pressure by the addition of different amounts of NaCl increases the lag phase and decreases the growth rate of *Listeria monocytogenes* (Vasseur et al., 1999). Under low water activity or high osmolarity conditions, the growth of many non-halophilic bacteria is inhibited, while some bacteria, like *L. monocytogenes*, have the ability to accumulate compatible solutes, such as betaine and carnitine, to maintain homeostasis. Although lowering the water activity is widely exploited in food preservation (Albarracin et al., 2011), this common method may not be sufficient to control bacteria in foods with low water activity.

1.1.2 Chilling treatment and refrigeration in food preservation

Chilling treatment and refrigeration are some of the most common food preservation

processes. This is because the proper refrigeration temperature is expected to inhibit or reduce the growth of food-associated microorganisms. At low temperatures, the metabolic processes of microorganisms tend to slow down, increasing the time taken for microbial adaptation and growth initiation. Furthermore, exposure to low temperatures may induce cold injury in microbial cells. The extent of cold injury is determined by the temperature and the duration of the chilling stress. Generally, both rapid chilling and constant chilling impose additional injury to the microbial cell (Batt, 2014). Typical refrigeration temperatures normally range from 4–12°C (Russell, 2002). Nonetheless, cold-adapted psychrotrophic pathogens and spoilage microorganisms remain a major concern in refrigerated foods. These bacteria are commonly grouped as psychrophilic bacteria and include *Brochothrix thermosphacta*, *Pseudomonas* spp., *Micrococcus* spp., *Listeria monocytogenes*, and *Yersinia enterocolitica.* During long-term refrigerated storage, psychrophilic bacteria can survive and may grow to high levels, thus causing serious spoilage or food poisoning.

1.2 Compatible solute–based survival strategies of bacteria under stress

Unlike other life forms, bacterial species are perhaps the most versatile living organisms, as they can survive and grow in a wide range of environmental conditions, even under cold and osmotic stresses. When the external environment becomes a potentially lethal threat, bacteria can sense and respond to these environmental changes and eventually inhabit the environment (Wood et al., 2001). Bacteria are able to adapt in this way because they have evolved to cope with fluctuations in natural and artificial environments by the accumulation of a restricted

range of low molecular mass molecules that can balance the external pressure without affecting cellular functions (Sleator et al., 2002). These organic osmolytes are also called 'compatible solutes'. These organic osmolytes are also called 'compatible solutes'. The major compatible solutes in food-borne organisms include K^+, amino acids (e.g., glutamate, proline), amino acid derivatives (peptides, N-acetylated amino acids), quaternary amines (e.g., glycine betaine, carnitine), sugars (e.g., sucrose, trehalose), and tetrahydropyrimidines (ectoines) (Poolman and Glaasker, 1998).

Compatible solutes can accumulate at high levels by *de novo* synthesis or by transport, without interfering with cellular processes. Some compatible solutes are only available from the environment (e.g., choline, betaine, and ectoine), whereas others, like trehalose, are produced by *de novo* synthesis. Proline can be either synthesized or transported (O'Byrne and Booth, 2002). The complex organic nature of food means that it contains a rich source of compatible solutes or solute precursors and therefore, bacteria can easily and directly accumulate compatible solutes. Consequently, these low-molecular-weight and highly soluble compounds help foodborne bacteria overcome food preservation and safety barriers and thus, pose a potential risk to human health. The ability of foodborne bacteria to accumulate compatible solutes has been widely studied, particularly in relation to cold tolerance and osmotic tolerance. It has reported that compatible solutes, such as betaine, are involved in enhancing osmotolerance and psychrotolerance in *L. monocytogenes* (Ko et al., 1994). Since these compatible solutes play a crucial role in protecting bacteria from excessive stress, novel food preserving methods are required to inhibit foodborne bacteria by removing these

compounds from food products or by preventing their accumulation.

Consequently, these low-molecular-weight and highly soluble compounds play a crucial role in protecting bacteria from excessive stress and aid bacteria food borne bacteria overcome food preservation and safety barriers, and pose a potential risk to human health. Therefore, novel food preservative needs be required to inhibit food borne bacteria by removing the compatible solutes in food or preventing the accumulation of compatible solution.

1.3 D-amino acids and their antibacterial activity under conditions of stress

D-amino acids are now considered to be present in higher organisms, particularly in the mammalian brain (Hashimoto et al., 1992). In bacteria, D-amino acids, such as D-aspartic acid, D-glutamic acid, and D-alanine serve as constituents of the cell wall (Csapó et al., 2009). Synthesis of D-amino acids has been suggested as an adaptive strategy for bacteria in response to changing environmental conditions. Large quantities of free D-amino acids have also been found in the tissues of marine bivalves, where they may serve as important factors in regulating osmotic stress from seawater (Felbeck and Wiley, 2016). D-amino acids are widely present in harshly treated foods (e.g., roasted coffee) and fermentation products (e.g., cheese and yoghurt) (Palla et al., 1989). During food preparation and processing, D-amino acids can be generated from the corresponding L-stereoisomers. Heat and alkali treatments are the primary food technology methods to improve taste, texture, and shelf life. Exposure to alkali or high temperature conditions induce measurable quantities of D-amino acids in food proteins, such as soy protein. The presence of D-amino acids has also been investigated in milk and fermented

dairy products. Large amounts of free D-alanine, D-aspartic acid, and D-glutamic acid have been found in raw cow's milk. The free D-alanine content in raw milk gradually increases during refrigerated storage at 4°C and this is suggested as an indicator of milk contamination (Gandolfi et al., 1992). Besides bacterial activity, pasteurization and fermentation of raw milk also contribute to the occurrence of D-amino acids. With the increasing temperature or fermentation processing of raw milk and milk products, D-aspartate content can increase to over 3% (Man et al., n.d.). In addition, some D-amino acids have therapeutic properties. D-leucine and D-phenylalanine have been shown to relieve pain and may serve as potent, non-addictive analgesics for humans (Cheng and Pomeranz, 1979).

Recently, some D-amino acids, such as D-tryptophan (D-Trp), have been found to exhibit antibacterial activities against foodborne bacteria under conditions of osmotic stress. Some studies have subsequently confirmed that D-proline, D-alanine, and D-serine modestly inhibit bacterial growth under conditions of osmotic stress (Chin et al., 2010; Sasaki et al., 2007; Shahjee et al., 2002a). These incompatible solutes that are structurally similar to compatible solutes, have adverse effects on the growth of foodborne bacteria when under osmotic stress. Our previous study reported that D-Trp adversely affects the growth of foodborne bacteria under high-salt conditions (Koseki et al., 2015). One possible explanation for the antibacterial effect of D-Trp may be attributed to the D-amino acid-induced inhibition of biofilm formation, which plays a crucial role in multicellular bacterial communities and protects bacteria from environmental insults (Davies, 2003; Moran and Jarvik, 2010). For example, exposure to 10 mM D-Trp causes biofilm disassembly in *Cronobacter sakazakii* by reducing the initial

adhesion between cells and changing the properties of the extracellular matrix (Li et al., 2015). Therefore, D-Trp may be used as a novel preservative to control bacterial growth in foods.

1.4. Research objectives

Considering that some important foodborne bacteria, particularly those possessing strong resistance, are a potential threat to traditional food preservation barriers, such as salting and chilling, alternative or complementary control strategies are urgently needed. In the present study, we evaluated the use of an amino acid, D-Trp, as a novel natural food preservative in combination with traditional food preservation techniques, to provide safe and high-quality processed foods.

Accordingly, the objective of this study was to evaluate the inhibitory effects of D-Trp on salt-resistance in *Vibrio* spp. and cold-resistance in *Listeria monocytogenes* during osmotic treatment and chilling treatment, respectively. Subsequently, the addition of exogenous D-Trp as a food preservative in an actual food matrix was also investigated to increase the flexibility of food processing method design and to contribute to the improvement of food quality and extension of shelf life.

Briefly, Chapter 2, describes the confirmation of the working characteristics of D-Trp and clarification of the relationship between NaCl and D-Trp concentrations in inhibiting the growth of *Vibrio* spp. We aimed to determine the growth inhibitory effect of D-Trp on *Vibrio* spp. in live and shucked oysters in a seawater environment. In Chapter 3, we describe the evaluation of the effects of different concentrations of D-Trp on the psychrotrophic growth of

L. monocytogenes during long-term refrigerated storage and examination of the feasibility of utilizing D-Trp to control *L. monocytogenes* in pasteurized milk and all psychrotrophic bacteria in raw milk. The experiments reported in Chapter 4 focused on increasing our understanding of the mechanism of the antibacterial activity of D-Trp. We sought to clarify the role of intercellular and extracellular D-Trp in the inhibition of *Escherichia coli* via HPLC-based amino acid and microbiological analysis. Furthermore, we aimed to increase our knowledge of the impact of D-Trp treatment on the metabolism of *E.* coli cells.

Chapter 2

Growth Inhibitory Effect of D-Tryptophan under Osmotic Stress

2.1. Introduction

Vibrio parahaemolyticus and *Vibrio vulnificus* are two of the most notable pathogenic Vibrio species and they are known to contaminate ready-to-eat seafood, particularly raw oysters (*Crassostrea* spp.) (Fang et al., 2015; Hesselman et al., 1999). *V. parahaemolyticus* is implicated as the primary source of seafood-associated human gastroenteritis in the United States (Newton et al., 2012), whereas *V. vulnificus* is associated with a high fatality rate (approximately 50%) and is responsible for 95% of all the seafood-related deaths in the United States (Esteves et al., 2015; Murphy and Oliver, 1992).

Oysters are the most abundantly harvested shellfish worldwide. Raw oyster consumption is associated with a high risk of *Vibrio* infections, even after proper handling during harvest (Ellison et al., 2001). Recently, post-harvest treatments such as the use of electrolyzed water (Quan et al., 2010), heat shock (Wong et al., 2002), rapid chilling (Liu et al., 2009), irradiation (Mahmoud, 2009), and hydrostatic high pressure processing (Baker, 2016; Cook, 2003) have been studied to reduce the level of *Vibrio* spp. in oysters. However, these treatments have detrimental effects on oyster quality and residual bacterial populations may lead to *Vibrio* contamination during transport, storage, and retail. Therefore, a novel and economical post-harvest treatment with continuous effectiveness is required to control *Vibrio* spp. in oysters during the post-harvest period.

D-amino acids are now considered to be present in higher organisms, particularly in the

mammalian brain (Hashimoto et al., 1992). In bacteria, D-amino acid synthesis is a strategy for adapting to changing environmental conditions. Recently, some D-amino acids, such as D-tryptophan (D-Trp), were found to exhibit antibacterial activities against foodborne bacteria under conditions of osmotic stress. Our previous study reported that D-Trp adversely affected the growth of foodborne bacteria under high-salt conditions (Koseki et al., 2015). One possible explanation for the antibacterial effect of D-Trp may be attributed to D-amino acid-induced inhibition of biofilm formation, which plays a crucial role in multicellular bacterial communities and protects bacteria from environmental insults (Davies, 2003; Moran and Jarvik, 2010). For example, exposure to 10 mM D-Trp causes biofilm disassembly in *Cronobacter sakazakii* by reducing the initial adhesion between cells and changing the properties of the extracellular matrix (Li et al., 2015). Therefore, D-Trp may be used as a novel preservative for controlling bacterial growth in foods and in particular, in seafood, because of its high effectiveness in environments with high NaCl concentration (> 3%).

In this study, we aimed to evaluate the inhibitory effects of D-Trp on *V. parahaemolyticus* and *V. vulnificus* under conditions of varying NaCl and D-Trp concentrations in shucked and live oysters. This study serves as a foundation to highlight the potential application of D-Trp as a post-harvest technique to reduce *Vibrio* contamination in live oysters and to extend the shelf life of shucked oysters.

2.2. Materials and methods

2.2.1. Bacterial strains

*V. vulnificu*s (NBRC 103026) and *V. parahaemolyticus* (NBRC 12711) were obtained from the Biological Resource Center, NITE (NBRC) in Japan. Bacterial strains were stored at -80°C in brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) with 2.0% NaCl and 50% glycerol. For each experiment, bacterial cells were collected from the frozen stock and plated on trypticase soy agar (TSA, Merck) supplemented with 2% NaCl (TSA-2%). Individual colonies were selected and enriched in tryptic soy broth (TSB) with 2% NaCl (TSB-2%) at 25°C for 48 h to obtain a final concentration of approximately 10^9 CFU/mL.

2.2.2. Effects of D-Trp on the growth of *Vibrio* spp. in culture media

To evaluate the growth inhibitory effects of D-Trp (Wako Pure Chemical Industries, Ltd., Osaka, Japan) on *V. vulnificu*s and *V. parahaemolyticus* in TSB, bacterial cultures were prepared, as described above and were diluted in phosphate-buffered saline (PBS) to obtain a concentration of approximately 5×10^7 log CFU/mL. A 0.1-ml aliquot of this diluted bacterial culture was added to 0.9 ml of TSB supplemented with various concentrations of NaCl (3.5, 4.0, 4.5, and 5.0%; w/v) and D-Trp (0, 20, and 40 mM) and incubated at 25°C without shaking. Bacterial survival was determined via plate counts on TSA-2% after each 24 h incubation. Successive 10-fold serial dilutions were prepared using PBS (2% NaCl), and 100 μL aliquots of the diluted samples were then spread on TSA-2% plates and cultured at 25°C for 24 h.

2.2.3. Effects of D-Trp on survival of *Vibrio* spp. in shucked oysters immersed in peptone water.

This experiment was performed as described in a previous study (Fang et al., 2015), with

some modifications. Freshly shucked oysters were obtained from a local seafood market (Akkeshi, Hokkaido, Japan) and transported immediately in a styrene foam box with crushed ice. Each oyster was artificially contaminated by pipetting 300 μL (approximately 5.0×10^7 CFU/mL) of *Vibrio* spp. culture and air-dried at 25°C for 30 min to allow bacterial attachment. Subsequently, each *Vibrio*-contaminated oyster meat sample was individually transferred into a sterile plastic bag (85 × 60 × 0.04 mm), to which 30 mL of 0.1% peptone water containing 40 mM D-Trp was added. The NaCl concentration in the peptone water ranged from 3.5% to 5.0%. Samples were incubated at 25°C for 48 h. Treatment without D-Trp was considered as the control condition. To enumerate viable *Vibrio* spp., the culture and shucked oysters were aseptically placed in a sterile 400 mL Stomacher® filter bag and pummeled with a Stomacher® 400-T blender (Seward, UK). The stomached samples were diluted by 10-fold serial dilutions in PBS and plated onto *Vibrio* CHROMagar (CHROMagar, Paris, France), a selective media for *Vibrio* spp. *V. parahaemolyticus* appears as mauve colonies, whereas *V. vulnificus* appears as turquoise colonies (Shaw et al., 2014). Although the NaCl concentration of CHROMagar is high, at 5.14%, we previously confirmed that such salinity does not significantly impact the recovery of viable *Vibrio* cells, compared with TSA-2% (data not shown). CHROMagar only inhibits the recovery of natural microflora species other than *Vibrio* spp. Each condition was independently evaluated using three oysters and the number of viable bacteria in the homogenates was determined at 24-h intervals.

2.2.4. Effects of D-Trp on the growth of *Vibrio* spp. in artificial seawater of various salinities

The inhibitory effect of D-Trp on *Vibrio* spp. in artificial seawater containing salinities varying from 3.5 to 5.0% was also evaluated by the pour plating method as described above. Different salinities were achieved by altering the amount of NaCl added. The final salinity was measured by a salt meter (B-721; Horiba, Kyoto, Japan). All plates were incubated at 25°C for 48 h. Growth inhibition tests were performed three times. The colonies formed on the plates were counted and expressed as log CFU/mL. Results were recorded as the means ± standard deviation from three independent experiments.

2.2.5. Effects of D-Trp on survival of *Vibrio* spp. load on artificially inoculated live oysters.

Bacterial inoculum was prepared in broth culture, as described above. Stationary phase *Vibrio* cells were suspended at an initial concentration of approximately 5×10^4 CFU/mL in artificial seawater (2.7% salinity, Daigo's Artificial Seawater S; Nihon Pharmaceutical, Osaka, Japan) containing 40 mM D-Trp. Serial 10-fold dilutions in PBS were used to enumerate the *Vibrio* spp. by spread plating on *Vibrio* CHROMagar. The survival of *Vibrio* spp. was determined after incubation for 24 and 48 h. All experiments were independently performed three times.

The growth inhibitory effect of D-Trp on *V. parahaemolyticus* and *V. vulnificus* was further analyzed in experimental *Vibrio*-inoculated live oysters, as described in previously published oyster studies (1, 27), with some modifications. Live oysters (*Crassostrea gigas*) were harvested from Akkeshi (Hokkaido, Japan), transported in coolers on ice packs, delivered to the laboratories within 48 to 72 h, and then processed immediately. After 2 h of acclimation at

25°C, the oyster surface was washed with tap water to remove dirt and debris. Thirty oysters were placed in a sterile styrene foam box with 20 L of artificial seawater containing *V. vulnificus* or *V. parahaemolyticus* (approximately 10^5 CFU/mL) and incubated at 25°C for 24 h. Inoculated oysters were subsequently transferred to a new sterile styrene foam box with 5 L of artificial seawater, supplemented with or without 40 mM D-Trp and individually evaluated for the survival of *V. vulnificus* or *V. parahaemolyticus* after 0, 24, and 48 h. A laboratory water circulator (SM-05R; TAITEC, Koshigaya, Japan) was used to aid the accumulation of *Vibrio* in oysters. The water jet pump was set on the sterile styrene foam box (Fig. 2-1) and artificial seawater was circulated to keep dissolved oxygen levels favorable for oyster pumping and uptake of *Vibrio* spp.

To enumerate the *Vibrio* spp. in oysters, three oysters were randomly selected from the styrene foam box at each sampling time point and shucked aseptically with a sterile shucking knife. The oyster meat was collected aseptically in a sterile 400 mL Stomacher® filter bag. Samples were weighed and homogenized in an equal weight of PBS for 60 s at high speed in a Stomacher® blender (120 strokes per min). The stomached samples were then serially diluted 10-fold in PBS and plated on *Vibrio* CHROMagar to enumerate the *Vibrio* spp. Counts were recorded as log CFU/g.

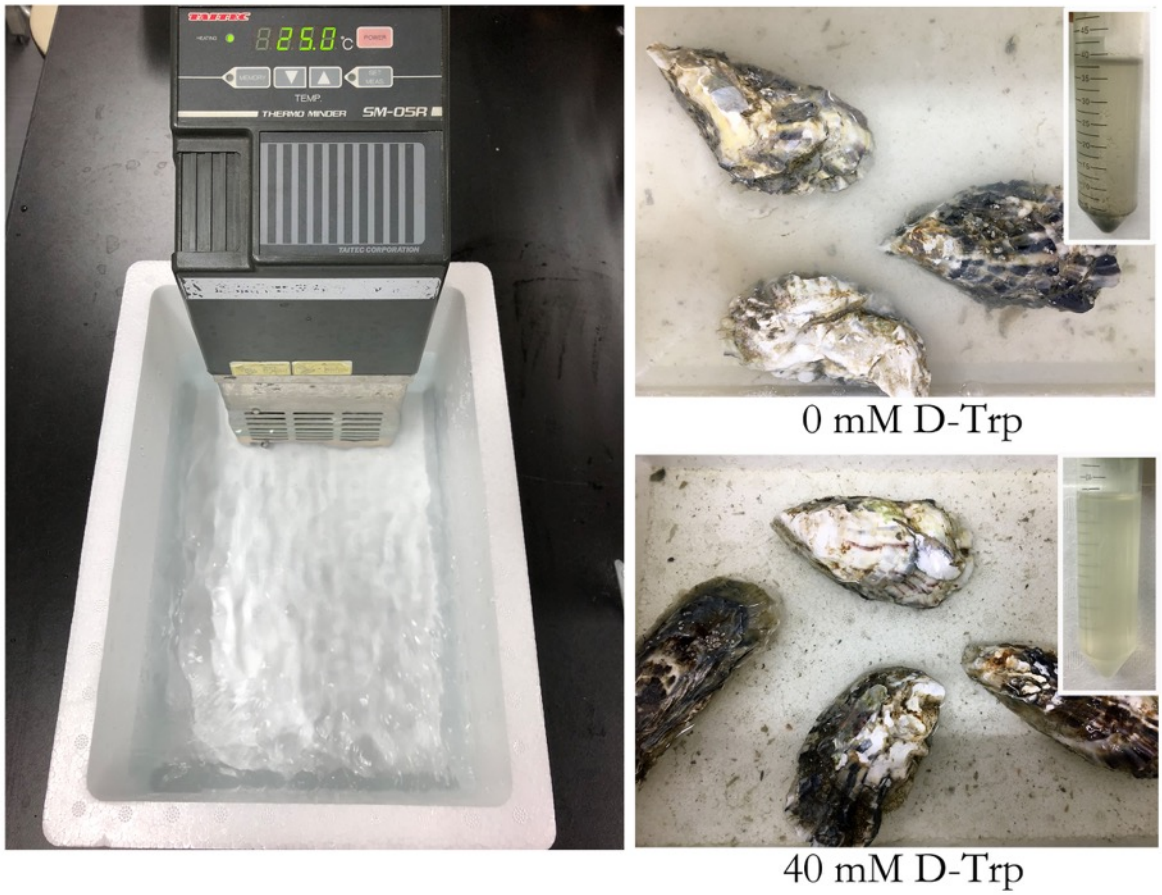


Fig. 2-1 The water jet pump was set on the sterile styrene foam box and artificial seawater was circulated to keep dissolved oxygen levels favorable for oyster pumping and uptake of *Vibrio* spp.

2.2.6. Combined efficacy of D-Trp and salinity on the total viable bacterial count in shucked oysters during refrigerated storage

This experiment was performed as described in a previous study (Fang et al., 2015), with some modifications. Freshly shucked oysters were obtained from a local seafood market (Akkeshi, Hokkaido, Japan) and transported immediately in a styrene foam box with crushed ice. Subsequently, each shucked oyster meat sample was individually transferred into a sterile

plastic bag (85 × 60 × 0.04 mm), followed by the addition of 30 mL of artificial seawater, with or without 40 mM D-Trp. The salinity levels of the artificial seawater were 3.5% or 5.0%. Samples were incubated at 25°C for 48 h. Treatment without D-Trp was considered as the control condition. To enumerate the total viable bacterial count, the culture and shucked oysters were aseptically placed in a sterile 400 mL Stomacher® filter bag and pummeled with a Stomacher® 400-T blender. The stomached samples were diluted in PBS with 10-fold serial dilutions and plated onto TSAYE (TSA supplemented with 0.6% yeast extract; Linton et al., 2003). The plates were then incubated at 25°C for 48 h. Each condition was independently evaluated using three oysters and the viable number of bacteria in the homogenates was determined after 1, 3, 5, and 7 days of storage.

2.2.7. Statistical analysis

Triplicate samples were collected at each sampling time. The colony-count data for the triplicate samples of each bacterium at each sampling interval were transformed to log CFU/mL or log CFU/g and the values of the triplicate samples were averaged to represent the number of viable cells at each sampling time. One-way ANOVA was performed to compare the differential degrees between each treatment. The viable cell counts in each condition were then compared by Tukey-Kramer's multiple comparison test. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using the commercially available software, KaleidaGraph 4.5 (Synergy Software, Reading, Pennsylvania, USA).

2.3 Results

2.3.1 Effects of D-Trp on the growth of *Vibrio* spp. in culture media

The growth inhibition of *Vibrio* spp. after D-Trp treatment was evaluated under various combinations of NaCl and D-Trp stress conditions (Figs. 2-2 and 2-3). Compared with the untreated control, samples treated with D-Trp showed significantly reduced ($P < 0.05$) populations of *V. parahaemolyticus* (Fig. 2-2) and *V. vulnificus* (Fig. 2-3) at 25°C. Exposure to 40 mM D-Trp resulted in more than a 4.0 log CFU/mL decrease in *V. vulnificus* and *V. parahaemolyticus* numbers at NaCl concentrations ≥ 4.5%, during incubation at 25°C. Under these conditions, an initial bactericidal effect was observed during the first 24 h and this was followed by a bacteriostatic effect. Although there was a slight increase in the number of *Vibrio* cells after 24 h of incubation at low NaCl concentrations, a gradual bacteriostatic effect was observed with increasing NaCl concentration. When the NaCl concentration was increased to 5.0%, D-Trp exerted the greatest overall bacteriostatic effect. Furthermore, *V. vulnificus* and *V. parahaemolyticus* showed different sensitivities to D-Trp. Under 20 mM D-Trp treatment, the population of *V. vulnificus* was significantly reduced, whereas a similar reduction was not observed in *V. parahaemolyticus*, regardless of NaCl concentration. Hence, higher concentrations of NaCl and D-Trp were required to achieve a bactericidal effect against *V. parahaemolyticus* to the same extent as the effect against *V. vulnificus*.

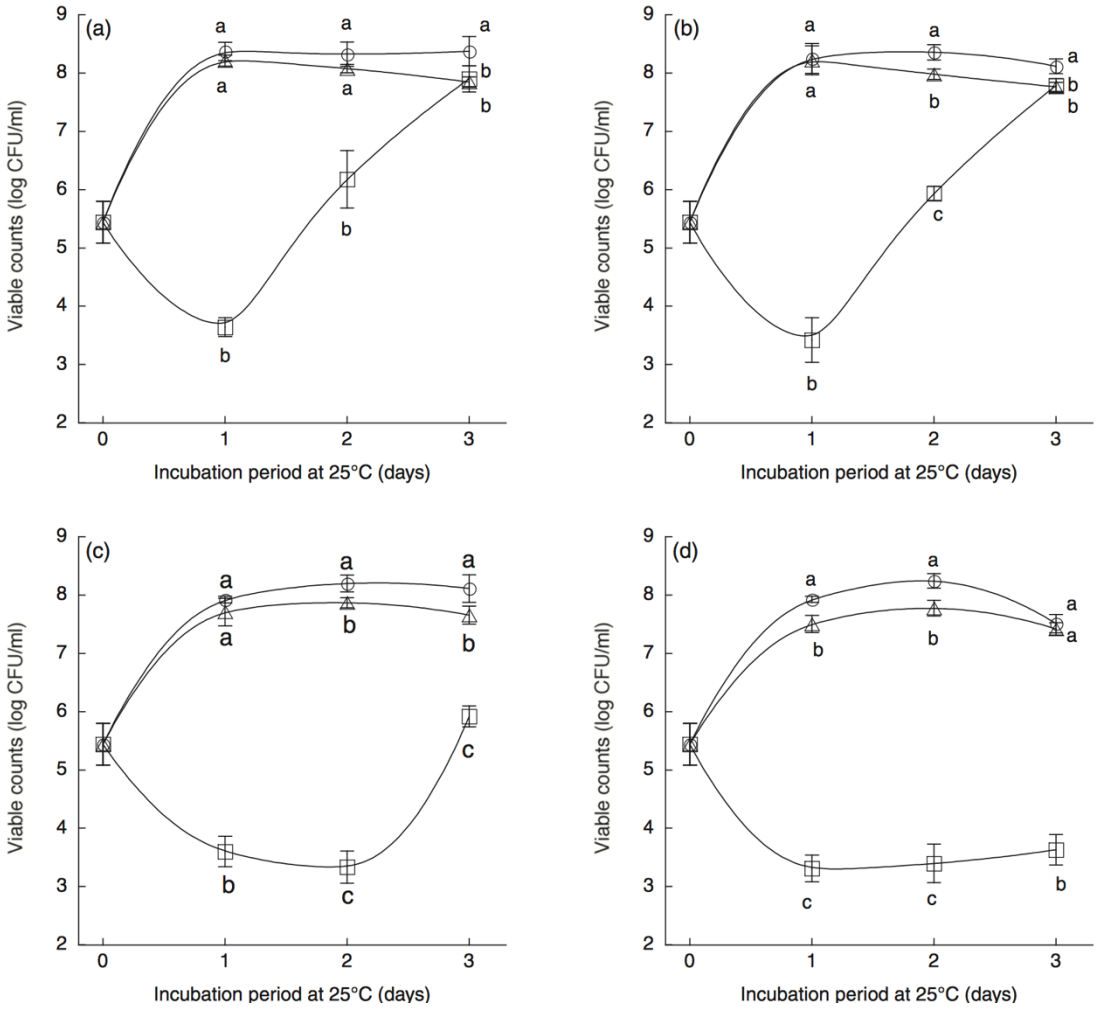


Fig. 2-2. Effects of D-tryptophan on viable numbers of *Vibrio parahaemolyticus* in tryptic soy broth containing different concentrations of D-tryptophan, (○) 0 mM, (△) 20 mM, and (□) 40 mM, under sodium chloride concentrations of (a) 3.5%, (b) 4.0%, (c) 4.5%, and (d) 5.0% at 25°C. Results are the means ± standard deviations of three independent experiments. Values with different letters on the same sampling time between different conditions were significantly different ($P < 0.05$).

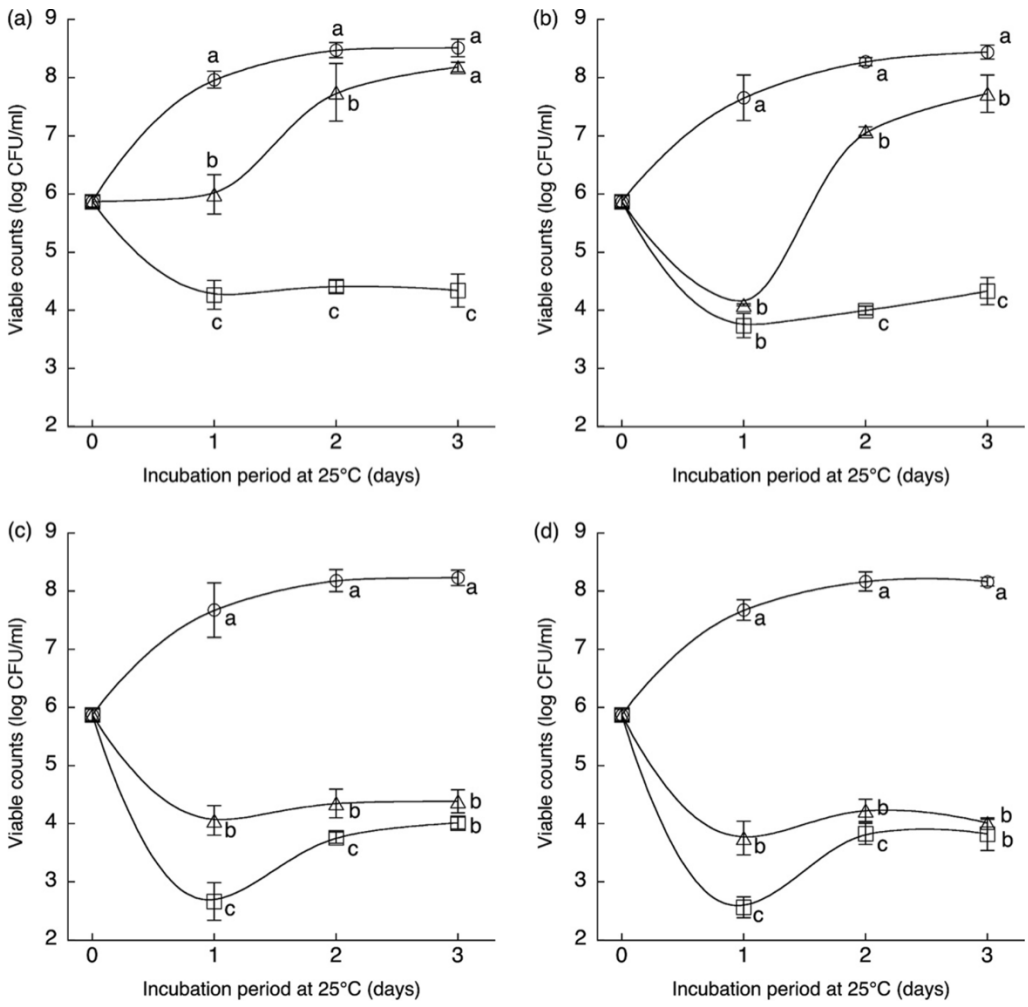


Fig. 2-3. Effects of D-tryptophan on number of viable *Vibrio vulnificus* in tryptic soy broth containing different concentrations of D-tryptophan (○) 0 mM, (△) 20 mM, and (□) 40 mM under sodium chloride concentrations of (a) 3.5%, (b) 4.0%, (c) 4.5%, and (d) 5.0% at 25°C. Results are the means ± standard deviations of three independent experiments. Values with different letters on the same sampling time between different conditions were significantly different ($P < 0.05$).

2.3.2 Effects of D-Trp on the survival of *Vibrio* spp. in shucked oysters immersed in peptone water

Considering the inhibitory effects of D-Trp on *Vibrio* spp. in culture media, we further examined the inhibitory effects of D-Trp on *V. vulnificus* and *V. parahaemolyticus* in sterile freshly shucked oysters immersed in peptone water, supplemented with various NaCl concentrations (3.5, 4.0, 4.5, and 5.0%, w/v). Treatment with 40 mM D-Trp significantly inhibited the growth of *V. parahaemolyticus* (Fig. 2-4) and *V. vulnificus* (Fig. 2-5) at 25°C, compared to their growth in untreated control samples (without D-Trp). Similar to the previous experiments, D-Trp exhibited a stronger growth inhibitory effect on *V. vulnificus* than on *V. parahaemolyticus* at 25°C. The mean number of *V. vulnificus* in D-Trp-treated samples was lower, at each NaCl concentration, than the number in respective control samples. In contrast, to achieve a comparable reduction in the number of *V. parahaemolyticus,* relatively higher concentrations of NaCl were required. Greater decreases in bacterial viability were observed with increasing concentrations of NaCl. The greatest inhibition was observed at 5.0% NaCl for both *V. vulnificus* and *V. parahaemolyticus*.

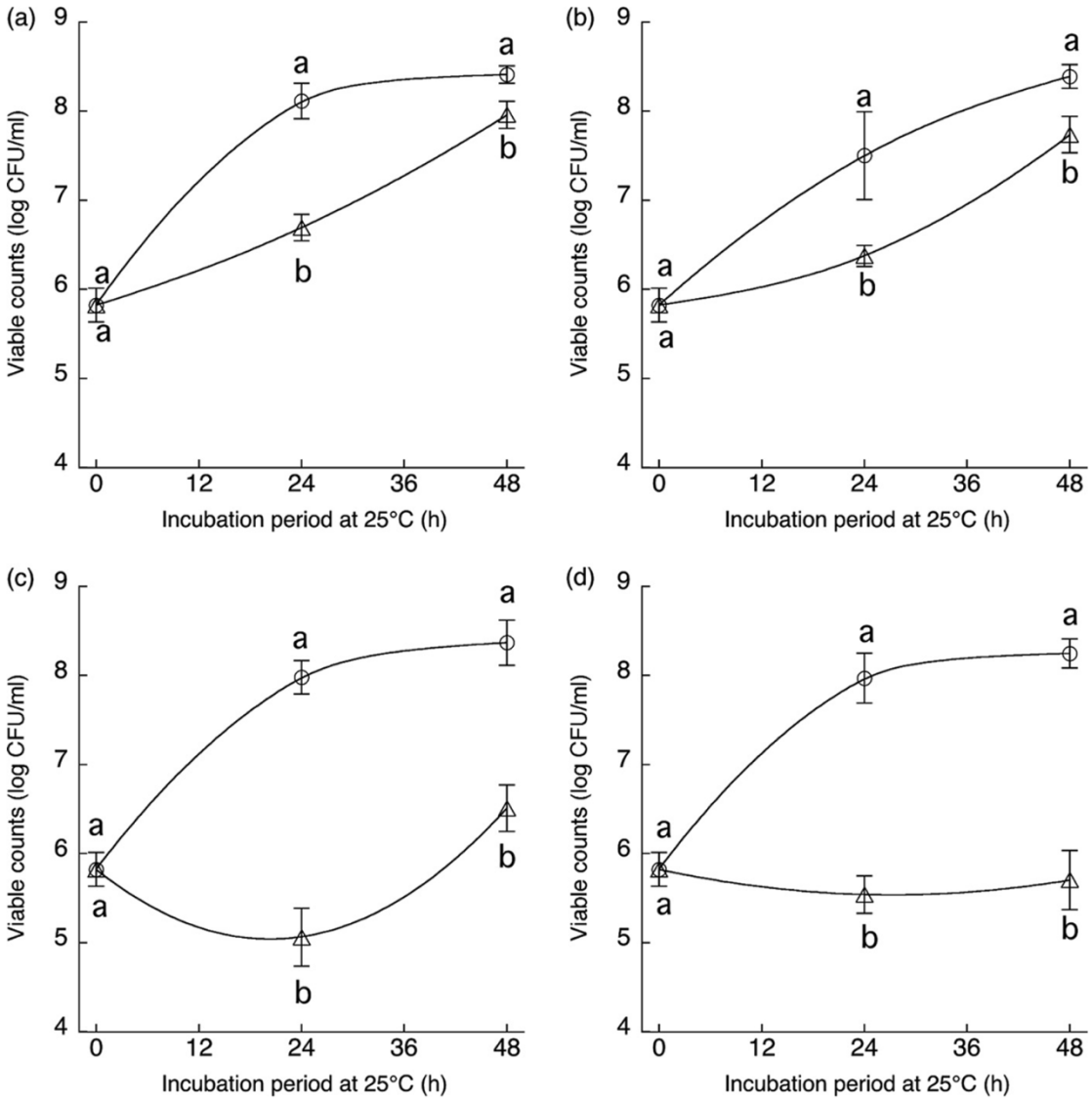


Fig. 2-4. Effects of D-tryptophan on the survival of *Vibrio parahaemolyticus* in experimentally inoculated shucked oysters stored in peptone water, under sodium chloride concentrations of (a) 3.5%, (b) 4.0%, (c) 4.5%, and (d) 5.0%, with (○) 0 mM or (△) 40 mM D-tryptophan, at 25°C. Results are the means ± standard deviations of three independent experiments. Values with different letters on the same sampling time between different conditions were significantly different ($P < 0.05$).

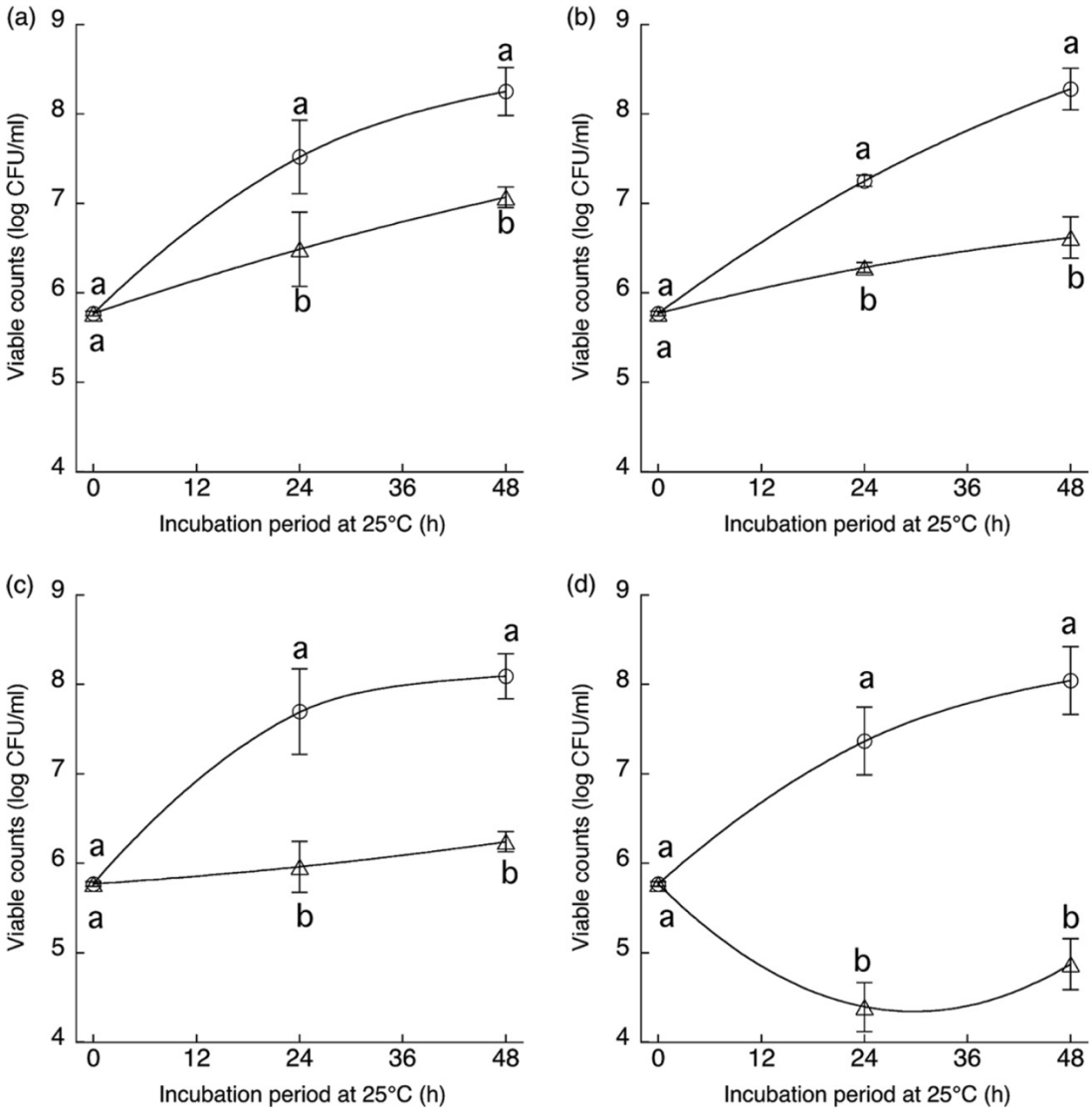


Fig. 2-5. Effects of D-tryptophan on the survival of *Vibrio vulnificus* in experimentally inoculated shucked oysters stored in peptone water under sodium chloride concentrations of (a) 3.5%, (b) 4.0%, (c) 4.5%, and (d) 5.0%, with (○) 0 mM or (△) 40 mM D-tryptophan, at 25°C. Results are the means ± standard deviations of three independent experiments. Values with different letters on the same sampling time between different conditions were significantly different ($P < 0.05$).

2.3.3 Effects of D-Trp on the growth of *Vibrio* spp. in artificial seawater

Due to the fact that seawater is the native environment for *Vibrio* growth, we further

examined the inhibitory effect of D-Trp on *Vibrio* survival in artificial seawater with salinities varying from 3.5 to 5.0% (Figs. 2-6 and 2-7). Interestingly, 40 mM D-Trp exhibited a significantly stronger inhibitive effect on the growth of both *V. vulnificus* and *V. parahaemolyticus* than TSB. As expected, salinity alone had little or no effect on suppressing the growth of either *V. vulnificus* or *V. parahaemolyticus*, with the exception of 4.5% and 5.0% NaCl concentrations. At these concentrations, bacterial growth of *V. vulnificus* was inhibited. However, after adding D-Trp into the artificial seawater, there were significant log reductions ($P < 0.05$) in the numbers of *V. vulnificus* and *V. parahaemolyticus*. This sensitivity of *Vibrio spp.* to D-Trp was consistent with the results seen in culture media. *V. vulnificus* tended to be more sensitive to D-Trp than *V. parahaemolyticus*. The growth of *V. parahaemolyticus* was significantly inhibited by 40 mM D-Trp and a gradual growth decrease was observed with increasing salinity. However, the population of *V. vulnificus* was reduced to less than 1.0 log CFU/mL (in TSB-2% for 24 h) during D-Trp treatment at each salinity level. Similar to previous results, the effectiveness of D-Trp tended to be enhanced at higher salinity levels. At the highest level of salinity tested (5%), even *V. parahaemolyticus,* which has been shown to be more resistant than *V. vulnificus* to D-Trp in TSB, was also significantly inhibited and declined continually from 5.8 to 3.1 log CFU/mL.

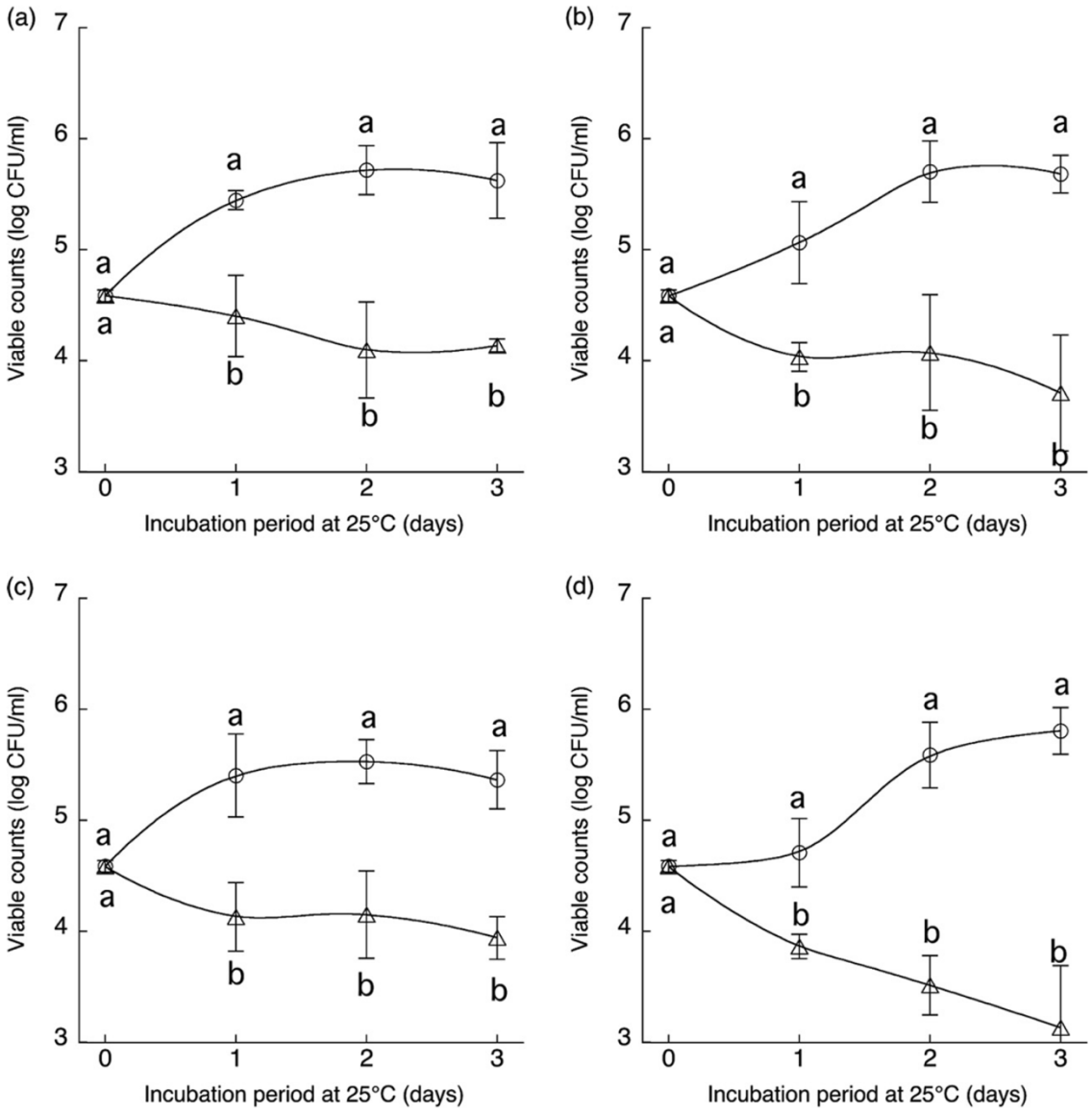


Fig. 2-6. Effects of D-tryptophan on the survival of *Vibrio parahaemolyticus* in artificial seawater under sodium chloride concentrations of (a) 3.5%, (b) 4.0%, (c) 4.5%, and (d) 5.0%, with (○) 0 mM or (△) 40 mM D-tryptophan, at 25°C. Results are the means ± standard deviations of three independent experiments. Values with different letters on the same sampling time between different conditions were significantly different ($P < 0.05$).

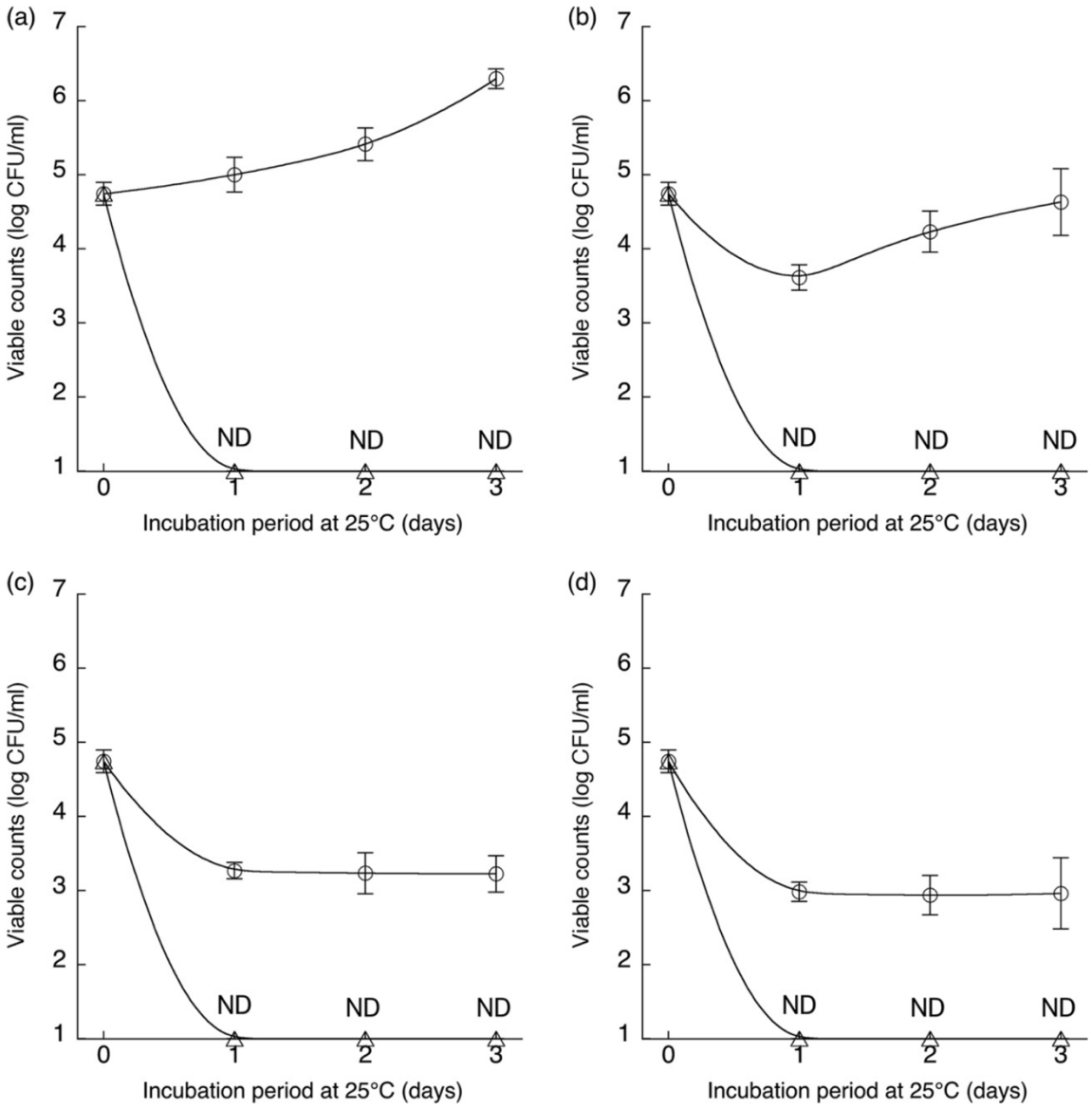


Fig. 2-7. Effects of D-tryptophan on the survival of *Vibrio vulnificus* in artificial seawater under sodium chloride concentrations of (a) 3.5%, (b) 4.0%, (c) 4.5%, and (d) 5.0% with (○) 0 mM or (△) 40 mM D-tryptophan, at 25°C. Results are the means ± standard deviations of three independent experiments. Values with different letters on the same sampling time between different conditions were significantly different ($P < 0.05$).

2.3.4 Effects of D-Trp on the survival of *Vibrio* spp. in experimentally inoculated live oysters

Since a high inhibitory efficiency of D-Trp was observed in artificial seawater, we further evaluated the effect of D-Trp on internal *Vibrio* growth in live oysters within their shells (Fig. 2-8). Thirty oysters were immersed in artificial seawater inoculated with *V. parahaemolyticus* or *V. vulnificus* (approximately 10^5 CFU/mL) and the oysters were allowed to internalize the bacteria via filter feeding. The bacterial numbers in individual oysters ($n = 3$) were determined via plate counts on *Vibrio* CHROMagar after 0, 24, and 48 h, with or without D-Trp exposure. As expected, after exposure to 40 mM D-Trp at 25°C, the growth of *V. vulnificus* and *V. parahaemolyticus* was significantly ($P < 0.05$) inhibited and yielded 2.9 log CFU/g and 2.2 log CFU/g reductions, respectively, after 48 h of treatment, compared to untreated controls.

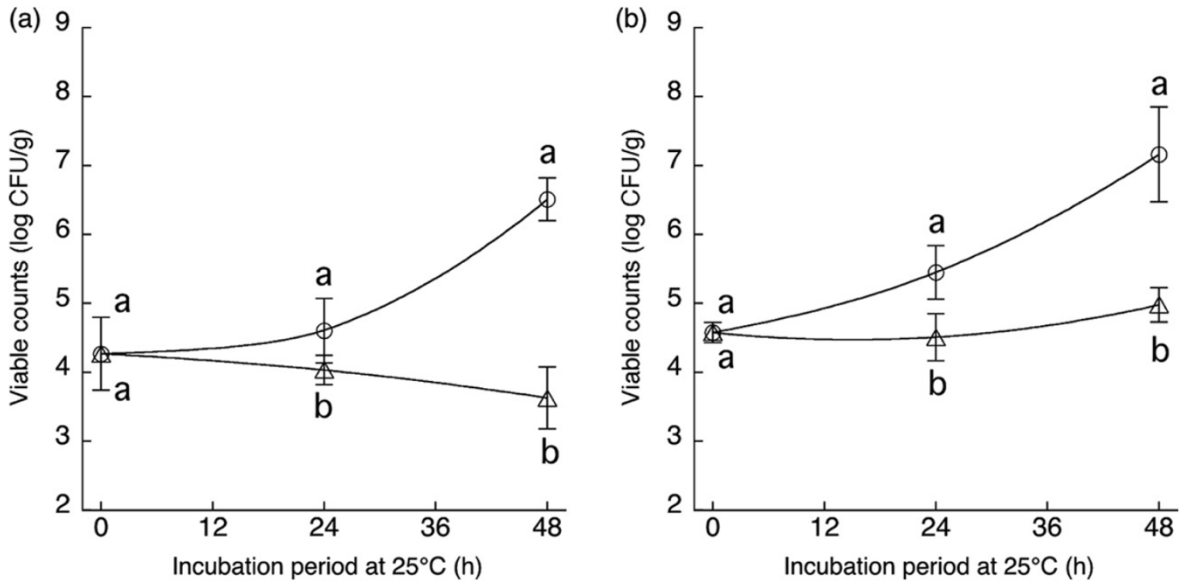


Fig. 2-8. Effects of D-tryptophan at concentrations of (○) 0 mM and (△) 40 mM on the survival of (a) *Vibrio vulnificus* and (b) *Vibrio parahaemolyticus* in experimentally inoculated live oysters. Results are the means ± standard deviations of three independent experiments. Values with different letters on the same sampling time between different conditions were significantly different ($P < 0.05$).

2.3.5 Combined efficacy of D-Trp and salinity on total viable bacterial count (TVC) in shucked oysters culture during refrigerated storage

Because shucked oysters are normally maintained under constant refrigeration to prevent spoilage, we subsequently examined the combined efficacy of D-Trp and salinity on total bacterial populations in sterile freshly shucked oysters immersed in artificial seawater at 4°C. The mean initial population of total viable bacteria in the shucked oyster culture was 3.3 log CFU/mL. In the untreated control (without D-Trp), the total bacterial count increased by approximately 2.6 log CFU/mL during five days of incubation, whereas the total bacterial population in artificial seawater with 3.5% salinity remained at its initial levels during the first 3 days of incubation and then slightly increased by approximately 1 log CFU/mL (Fig. 2-9). Consisted with previous experiments, a stronger inhibitory effect was observed at high salinity levels (5%). The total bacterial population in D-Trp-treated samples remained the same throughout the five-day incubation period.

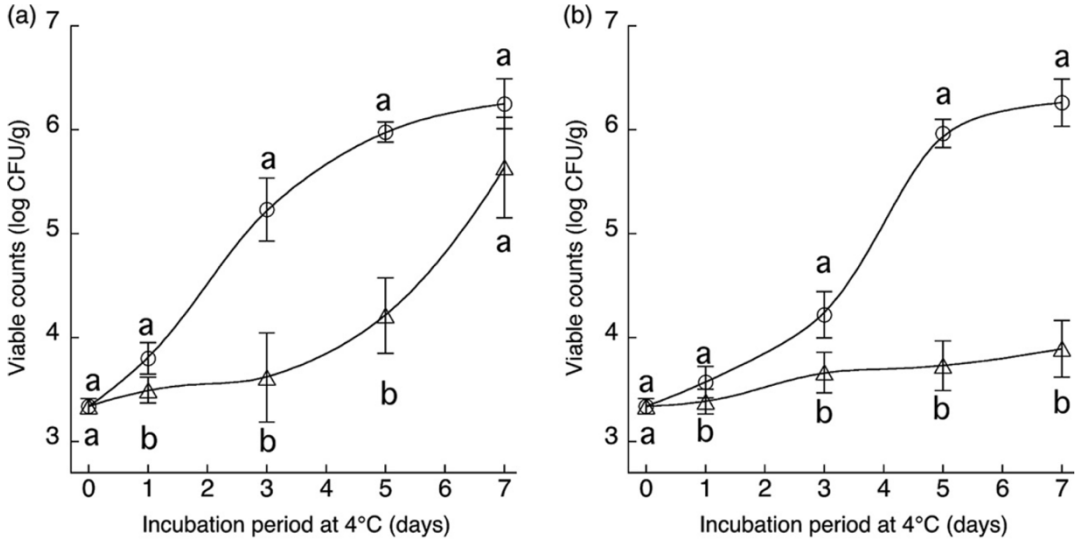


Fig. 2-9. Effects of D-tryptophan at concentrations of (○) 0 mM and (△) 40 mM, under salinities of (a) 3.5% and (b) 5.0%, on total bacterial population in freshly shucked oyster in artificial seawater at 4°C. Results are the means ± standard deviations of three independent experiments. Values with different letters on the same sampling time between different conditions were significantly different ($P < 0.05$).

2.4. Discussion

Recently, D-amino acids have been shown to exist in higher organisms, including humans, in a considerable quantity and have been shown to possess specific biological functions. For example, D-serine was detected in the mammalian brain, where it contributes to neurotransmission (Hashimoto et al., 1992; Stevens et al., 2003) and D-aspartate has been implicated in development and endocrine function (Ishio et al., 1998). Besides their therapeutic potential, D-amino acids, such as D-proline (Sasaki et al., n.d.), D-alanine (Manning et al., 1974), and D-serine (Shahjee et al., 2002b) have been shown to inhibit bacterial growth under conditions of osmotic stress. Moreover, in our previous study, we found that D-Trp significantly inhibited the growth of *Escherichia coli* O157:H7 and *Salmonella* in the presence of salt (Koseki et al., 2015). Therefore, utilizing exogenous D-amino acids, either free or incorporated in peptides or proteins, may be a novel strategy for food protection.

In this study, we found that the addition of exogenous D-Trp (20–40 mM) was effective at inhibiting *Vibrio* growth in TSB supplemented with ≥ 3.5% NaCl at 25°C. Compared with the negative control (without D-Trp), one day of treatment with 40 mM D-Trp significantly

reduced *V. parahaemolyticus* and *V. vulnificus* levels by 4.6 log CFU/mL and 3.7 log CFU/mL, respectively, at 3.5% NaCl. We further increased the salinity from 3.5% to 5.0% to examine the effect of D-Trp on bacterial inactivation under these conditions. Consistently, the populations of *V. parahaemolyticus* and *V. vulnificus* increased significantly even at the highest salinity level of 5%. However, when 40 mM D-Trp was added, increasing salinity resulted in an increased inhibition of bacterial growth. Exposure to 40 mM D-Trp inhibited the growth of *V. vulnificus* and delayed the growth of *V. parahaemolyticus* at NaCl concentrations ≥ 3.5%. When the NaCl concentration was increased to 5%, D-Trp strongly inhibited the growth of both of these bacteria. Thus, higher levels of NaCl are required for consistent and efficient growth inhibition of both *V. parahaemolyticus* and *V. vulnificus*.

In general, compared to *V. parahaemolyticus*, *V. vulnificus* is more sensitive to various stresses, such as gamma irradiation (Andrews et al., 2003), electrolyzed water (Ren et al., 2006), X-rays (Mahmoud, 2009), and high hydrostatic pressure (Cook, 2003). In our study, *V. vulnificus* was found to be more sensitive to D-Trp than *V. parahaemolyticus*. Although treatment with 20 mM D-Trp significantly ($P < 0.05$) reduced *V. vulnificus* levels by approximately 4 log CFU/mL after 24 h of exposure at NaCl concentrations > 3.5%, higher concentrations of D-Trp (i.e., 40 mM) were necessary for efficient growth inhibition of *V. parahaemolyticus*. These observed differences in the stress sensitivity of *Vibrio* spp. are consistent with previous reports.

Raw, freshly shucked oysters are one of the main products in the oyster processing industry (29, 30). After oyster shucking, the oyster meat is generally packed in flexible pouches

immersed in oyster culture solution. This closed environment poses a high risk of microbial contamination, because even if one piece of shucked oyster meat carried *Vibrio*, these residual populations would significantly multiply in oyster tissues during transport, storage, and retail and large numbers may be released into the surrounding culture solution, resulting in *Vibrio* cross-contamination. In this regard, we also investigated whether D-Trp could exhibit inhibitory effects in experimentally inoculated shucked oyster culture. Consistent with the culture media experiments presented here, after D-Trp was added into the shucked oyster culture, we observed a considerable inhibition in the growth of both *V. parahaemolyticus* and *V. vulnificus*. In addition, elevated levels of NaCl increased the inhibition efficiency, leading to a gradual reduction in *Vibrio* populations in the oyster culture. Therefore, D-Trp may be considered a novel preservative to control *Vibrio* contamination in freshly shucked oysters supplemented with seawater or sterile water with a high level of salinity, even when stored at room temperature.

Because seawater is the native environment for *Vibrio*, we further examined the inhibitory effects of D-Trp on the survival of *V. vulnificus* and *V. parahaemolyticus* in artificial seawater containing various levels of salinity (3.5, 4.0, 4.5, and 5.0%, w/v). Under all tested salinity conditions, D-Trp exhibited a stronger growth inhibitory effect on *V. vulnificus* and *V. parahaemolyticus* at 25°C than culture media (TSB) alone, indicating that the inhibitory effect of D-Trp can be achieved at even relative low levels of salinity. Moreover, a gradual growth decrease was observed with increasing levels of salinity. The reason why D-Trp had an unexpectedly stronger inhibitory effect on *Vibrio* in artificial seawater than in culture media is

not clear. However, one possible explanation is that some components of artificial seawater may increase bacterial sensitivity to D-Trp or the nutrient-poor environment of artificial seawater may contribute to bacterial growth inhibition. These results indicate that a lower concentration of NaCl and D-Trp would be needed to inhibit bacterial growth under low-nutrient conditions.

For live oysters, when post-harvest temperatures are not adequately controlled in supply chains, *Vibrio*. spp. can multiply and reach potentially hazardous levels. For example, *V. parahaemolyticus* is capable of growing rapidly by 1.7 or 2.9 log CFU/g in oysters after being exposed to 26°C for 10 or 24 h, respectively (Gooch et al., 2002). At room temperature, the population of *V. vulnificus* in oysters increases rapidly and reaches a peak during the first 12 h after harvest (Cook, 1994). In the present study, we conducted experiments at 25°C, which is generally considered as the optimal growth temperature for *Vibrio* spp. (Motes et al., 1998). Moreover, because D-Trp exhibited a strong inhibitory effect in artificial seawater and high levels of salinity may cause oyster death, artificial seawater (approximately 2.7% salinity), without extra NaCl added, was used in this experiment. As expected, D-Trp treatment reduced the *Vibrio* load in live oysters, which was consistent with the results of previous experiments. Additionally, *V. vulnificus* appeared to be more sensitive to D-Trp treatment than *V. parahaemolyticus*, exhibiting a greater reduction in growth.

Refrigeration is one of the most common methods to retard spoilage and extend the shelf life of fresh food (Mead et al., 1999). After shucking, oysters are normally maintained under constant refrigeration to effectively retard microbial growth and extend their shelf life during

postharvest processing. Refrigeration is a particularly common method for controlling *Vibrio* contamination (Andrews et al., 2003). For example, depuration at refrigeration temperatures (5 to 15°C) effectively reduces *V. parahaemolyticus* and *V. vulnificus* contamination in American oysters (Chae et al., 2009). In this study, as expected, the number of surviving bacteria in both *Vibrio* spp. gradually decreased at refrigeration temperatures, regardless of D-Trp treatment (data not shown).

Unlike *Vibrio* spp., the total bacterial load on shucked oysters increases gradually, even during refrigerated storage and this is expected to cause bacterial spoilage in oysters. Some studies have found that the TVC exceeded 10^6 CFU/g after 14 days and 9 days at 0.5°C and 4°C storage, respectively (Pace et al., 1988; He et al., 2002). Salinity, even at high levels, has little effect on controlling *Vibrio* spp. numbers in oysters (Motes et al., 1998). Based on this fact, we further evaluated the efficacy of combined D-Trp and salinity treatment for reducing the total bacterial population in shucked oysters in artificial seawater at 3.5% and 5.0% salinity. In controls (without D-Trp treatment), the TVC increased by 3 log units after storage at 4°C for one week and the average growth rate was approximately 0.4 log units each day, which is in good agreement with the results reported by Fernandez-Piquer (Fernandez-Piquer et al., 2011), who developed a predictive model for TVC in oysters stored at 3.6°C and 6.2°C. According to the U.S. Food and Drug Administration's microbiological standards, a TVC below 5×10^5 CFU/g in fresh bivalve mollusks is generally considered to be indicative of good quality (Cruz-Romero et al., 2008). Interestingly, we found that D-Trp could strongly inhibit the increase in TVC in shucked oysters in artificial seawater during storage at 4°C. Moreover, increasing the

salinity level to 5% further extended the shelf life of oysters by increasing the time taken for the TVC to exceed 5×10^5 CFU/g. These results demonstrate that D-Trp can reduce the TVC in oysters during refrigerated storage, indicating its potential use for shelf life extension of oysters during post-harvest storage and transport at refrigerated temperatures.

In conclusion, the contamination of raw oysters with *V. parahaemolyticus* and *V. vulnificus* may be reduced by holding oysters in artificial seawater containing D-Trp at a certain predetermined high NaCl concentration. However, further studies are warranted to investigate any detrimental effects that D-Trp or the combination of D-Trp and high salinity may have on oysters. Nevertheless, we suggest D-Trp as a potential novel alternative food preservative to control *Vibrio* contamination in oysters at room temperature and to extend the shelf life of raw oysters at refrigerated temperatures.

Chapter 3

Growth Inhibitory Effect of D-Tryptophan under Chilling Stres

3.1. Introduction

The gram-positive bacterium, *Listeria monocytogenes*, is an important foodborne pathogen and is the causative agent of listeriosis (Lianou and Sofos, 2007). This organism is problematic for food production and can be present in a wide range of processed foods, such as meat, dairy products, seafood, and vegetables. Besides its ubiquitous distribution, *L. monocytogenes* can grow and survive under adverse environment, including refrigeration temperatures, low pH, and high salt concentration (Tompkin et al., 1999). In particular, the low-temperature adaptation of *L. monocytogenes* is a crucial factor that makes the control of this foodborne pathogen difficult (Gandhi and Chikindas, 2007).

In the dairy industry, the psychrotrophic growth of *L. monocytogenes* at refrigeration temperatures is of particular concern in dairy foods that are usually consumed without any further processing (Greenwood et al., 1991). Dairy products, such as milk, are generally distributed and stored at refrigerated temperature to suppress bacterial proliferation (LeJeune and Rajala Schultz, 2009). However, this common method may not be sufficient to prevent *Listeria* growth in dairy products (Fleming et al., 1985). Once milk is contaminated with the bacterium, long-term refrigerated storage allows the psychrotrophic growth of *L. monocytogenes* by inhibiting other competing microorganisms, resulting in *L. monocytogenes* as the dominant contaminant in dairy products (Chan and Wiedmann, 2008; Gandhi and Chikindas, 2007). Although high-temperature, short-time (HTST) pasteurization has been

proven to destroy *Listeria* effectively, a minimal level of survival is still possible and a low level of contaminating *Listeria* is sufficient to cause human illness (Greenwood et al., 1991). In addition, excessive heat treatment and over-pasteurization have deleterious effects on the organoleptic and nutritional properties of milk. Nevertheless, by residual pasteurization survival or post-pasteurization contamination, this psychrotolerant organism, at a low contaminating level, can survive and multiply in milk during extended storage periods at low temperatures, leading to costly product recalls and a potential risk to human health (REINA et al., 1998). Therefore, a novel non-thermal processing technology with continuous effectiveness but no adverse effects on milk quality, is required to control the psychrotrophic growth of *L. monocytogenes* at refrigeration temperatures during extended post-harvest periods.

The adaptive ability of *L. monocytogenes* to low-temperature stress is mainly attributed to the fact that *L. monocytogenes* tends to uptake and accumulate organic osmolytes, such as glycine, betaine, and carnitine, under low temperature or osmotic stress conditions. These osmolytes, also known as 'compatible solutes', play a crucial role in the osmotolerance and psychrotolerance of *L. monocytogenes*. They usually provide a protective function, enabling *Listeria* survival, and deleting the transporters of these osmolytes significantly reduces *Listeria* growth (Wemekamp-Kamphuis et al., 2004). It has reported that compatible solutes, such as betaine, are involved in enhancing osmotolerance and psychrotolerance in *L. monocytogenes* (Ko et al., 1994).

Thus, compatible solutes allow *L. monocytogenes* to overcome food preservation and safety barriers, increasing the risk of listeriosis.

Unlike compatible solutes, some D-amino acids do not act as a compatible solutes, but exhibit antibacterial activities against foodborne bacteria under high conditions of osmotic stress (Chin et al., 2010; Sasaki et al., n.d.) (Shahjee et al., 2002a). Our previous study reported that D-Trp adversely affected the growth of foodborne bacteria under high-salt conditions. The synergistic effect of D-Trp and salinity has also been suggested as a novel and effective strategy for controlling *Vibrio* in oysters (Chen et al., 2018). Based on these results, the effect of D-Trp on *L. monocytogenes* and in particular, its psychrotrophic growth during long-term refrigerated storage, requires further investigation before its widespread application.

Refrigeration alone cannot stop the growth of psychrotrophic bacteria. Total psychrotrophic bacterial counts in raw milk increase remarkably quickly at refrigeration temperatures. The total psychrotrophic bacteria population is closely related to the quality of raw milk. During refrigerated storage, when the microflora of the milk exceeds 10^4 CFU/mL, it is dominated by psychrotrophic bacteria (Hantsis-Zacharov and Halpern, 2007). Moreover, psychrotrophic bacteria, even at low initial levels, can dominate the total bacterial population and usually account for more than 90% of the total microbial population in raw milk during storage at 4°C (Lafarge et al., 2004) (Magan et al., 2001). They are becoming increasingly problematic for the dairy industry, as the leading cause of spoilage and they can result in significant economic losses. Because of these psychrotrophic bacteria, the consumption of unpasteurized raw milk poses a potential risk to human health (Adamiak et al., 2015; Cousin, 1982). Therefore, refrigeration in combination with other methods, such as the addition of preservatives, is recommended.

The aim of the present study was to evaluate: (1) the effect of different concentrations of D-Trp on the psychrotrophic growth of *L. monocytogenes* during long-term refrigerated storage, (2) the combined effect of D-Trp and NaCl on the psychrotrophic growth *of L. monocytogenes* during long-term refrigerated storage, and (3) the feasibility of utilizing D-Trp to control *L. monocytogenes* in pasteurized milk and all psychrotrophic bacteria in raw milk.

3.2 Materials and methods

3.2.1 Preparation of bacterial strains

The *L. monocytogenges* strains (ATCC 13932, ATCC 15313, ATCC 19111, ATCC 19117, ATCC 19118, and ATCC 35152) used in this study were obtained from the American Type Culture Collection (Manassas, VA, USA). All strains were maintained at -85°C in BHI broth containing 10% glycerol. Strains were aerobically precultured in 10 mL of TSB at 37°C for 48 h. They were subsequently streaked onto individual plates of TSA and incubated at 37°C for 24 h. A single colony was selected and enriched in TSB at 37°C for 48 h (approximately 10^7–10^8 CFU/mL).

3.2.2 D-tryptophan, medium, and dairy production preparation

Considering the moderate solubility of D-Trp, in each treatment solution it was dissolved completely in an VS-100 ultrasonic cleaner (Iuchi Corp., Osaka, Japan) for 3 min at 45 kHz. Pasteurized milk was purchased from a local market. Raw milk samples were obtained from

bulk tanks in a local dairy farm and were kept refrigerated for a maximum of 3 h before use. PYG, TSA, and TSB media were prepared according to the manufacturer's instructions.

3.2.3 Growth curve of *L. monocytogenes* at registration temperature

To inhibitory effect of D-Trp on *L. monocytogenes* growth was determined at concentrations of D-Trp ranging from 0 to 40 mM, with increments of 10 mM. Survival of *L. monocytogenes* in PYG base medium was then assessed using optical density data. To generate a single sample of each pathogen comprising multiple strains, equal volumes of cell suspension from the four *L. monocytogenes* strains were combined to achieve approximately equal populations of each strain. The growth kinetics of *L. monocytogenes* were determined according to our previously published method (Kan et al., 2018; KOSEKI et al., 2015), with minor modifications. Bacterial cells were collected at the stationary phase after incubation at 37°C for 2 days and were diluted 100-fold in 0.1% peptone water. Then, 180 μL of peptone PYG, containing different concentrations of D-Trp (0 mM, 10 mM, 20 mM, 30 mM, and 40 mM) was added to a 96-well microplate and inoculated with 20 μL of bacterial cells to a final concentration of 10^4 CFU per well. The sample in each well was further overlaid with 20 μL of mineral oil to prevent evaporation. Bacterial growth was quantified continuously by measuring the absorbance at 595 nm using an iMark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Wells containing sterile PYG served as negative controls. The OD_{595} values for each condition were determined every 20 min for up to 450 h (approximately 20 days). The growth curves constructed from changes in OD_{595} values were fitted using the nls

package of R (version 3.4.1) statistical software, for a non-transformed four-parameter logistic model as follows:

$$OD_t = OD_{min} + \frac{OD_{max} - OD_{min}}{1 + \exp\left[-\mu_{OD}(t - t_{inf})\right]}$$

where t is time elapsed; OD_t is the optical density at time t; OD_{max} and OD_{min} are asymptotic maximum and minimum OD values, respectively; μ_{OD} is the maximum specific growth rate (μ_{max}) determined from the changes in the OD values; and t_{inf} is the time at the inflection point.

3.2.4 Survival of *L. monocytogenes* at registration temperature

We evaluated the inhibitory efficiency of D-Trp against *L. monocytogenes* alone and in combination with different salinity (NaCl) levels by plate enumeration. *L. monocytogenes* samples were collected at the stationary phase and diluted 100-fold in 0.1% buffered peptone water. A 1-ml aliquot was then directly added into 9 mL of PYG medium containing various NaCl concentrations (3.5, 4.0, 4.5, and 5.0%, w/v). Bacterial survival was determined by plate counts on TSA after each 10-days incubation period at 4°C. Successive 10-fold serial dilutions were prepared using 0.1% peptone water and 100 μL aliquots of the diluted samples were then spread on TSA plates and cultured at 37°C for 24 h.

3.2.5 Effect of D-Trp on the survival of *L. monocytogenes* in pasteurized milk

The antibacterial activity of D-Trp against *L. monocytogenes* in pasteurized milk was further evaluated. We evaluated the four strains (ATCC 15313, ATCC 19111, ATCC 19118, and ATCC 35152) separately, considering that *L. monocytogenes* may behave in a strain-

dependent manner in dairy products (Faleiro et al., 2003). *L. monocytogenes* samples were collected at the stationary phase and diluted 100-fold in 0.1% peptone water. A 1-mL aliquot was then directly added into 9 mL of milk, with or without 40 mM D-Trp. Bacterial growth was analyzed every 6 days during storage at 4°C. Ten-fold serial dilutions were prepared in buffered peptone water. An aliquot of 100 μL of the diluted sample was then spread on TSA and cultured at 37°C. The colonies were counted after 24 h and the percentage of viable bacteria was calculated.

3.2.6 Total psychrotrophic bacteria in raw milk

We determined the total psychrotrophic bacteria levels by counting the total bacterial population on TSAYE, according to the method of Patterson et al. (2010). Raw milk samples with initial microbial loads of approximately 5×10^3 CFU/mL were obtained from the university-affiliated farm at Hokkaido University (Sapporo, Japan). Milk samples were collected from a closed bulk milk container (4°C). D-Trp was mixed with raw milk samples firstly by vortexing and was then completely dissolved using a VS-100 ultrasonic cleaner for 3 min at 28 KHz. To enumerate the total psychrotrophic bacteria, each sample was diluted by 10-fold serial dilutions in 0.1% peptone water and plated onto TSA plates. The plates were then incubated at 4°C for 1 week. Each condition was independently evaluated using three milk samples and the viable number of bacteria in the homogenates was determined after 0, 3, 6, and 9 days of storage.

3.2.7 Statistical analysis

Triplicate samples were collected at each sampling time. The colony-count data for the triplicate samples of each bacterium at each sampling interval were transformed to log CFU/mL or log CFU/g and the values of the triplicate samples were averaged to represent the number of viable cells at each sampling time. One-way ANOVA was performed to compare the differential degrees between each treatment. The viable counts in each condition were then compared by Tukey-Kramer's multiple comparison test. $P < 0.05$ was considered statistically significantly. Statistical data were evaluated using the commercial software, KaleidaGraph 4.5.

3.3 Results

3.3.1 Effect of D-Trp on the growth curve of *L. monocytogenes* at 4°C

To evaluate the inhibitory effect of D-Trp, the growth curve of *L. monocytogenes* at 4°C in PYG containing different concentrations of D-Trp (0 mM, 10 mM, 20 mM, 30 mM, and 40 mM) was monitored by measuring the OD_{595} at different time intervals. As shown in Fig. 3-1, the psychrotrophic growth curves of a cocktail of *L. monocytogenes* strains was fitted to a log-logistic function. The *L. monocytogenes* sample without D-Trp (0 mM) showed a rapid increase in OD_{595}, as it was the first to leave the lag phase and reach the stationary phase. However, the addition of D-Trp decreased *L. monocytogenes* growth. As the concentration of D-Trp increased, the maximum OD_{595} value was reduced and the lag phase was prolonged. In addition, higher levels of D-Trp (>20 mM) also led to a decrease in the growth rate of *L. monocytogenes*.

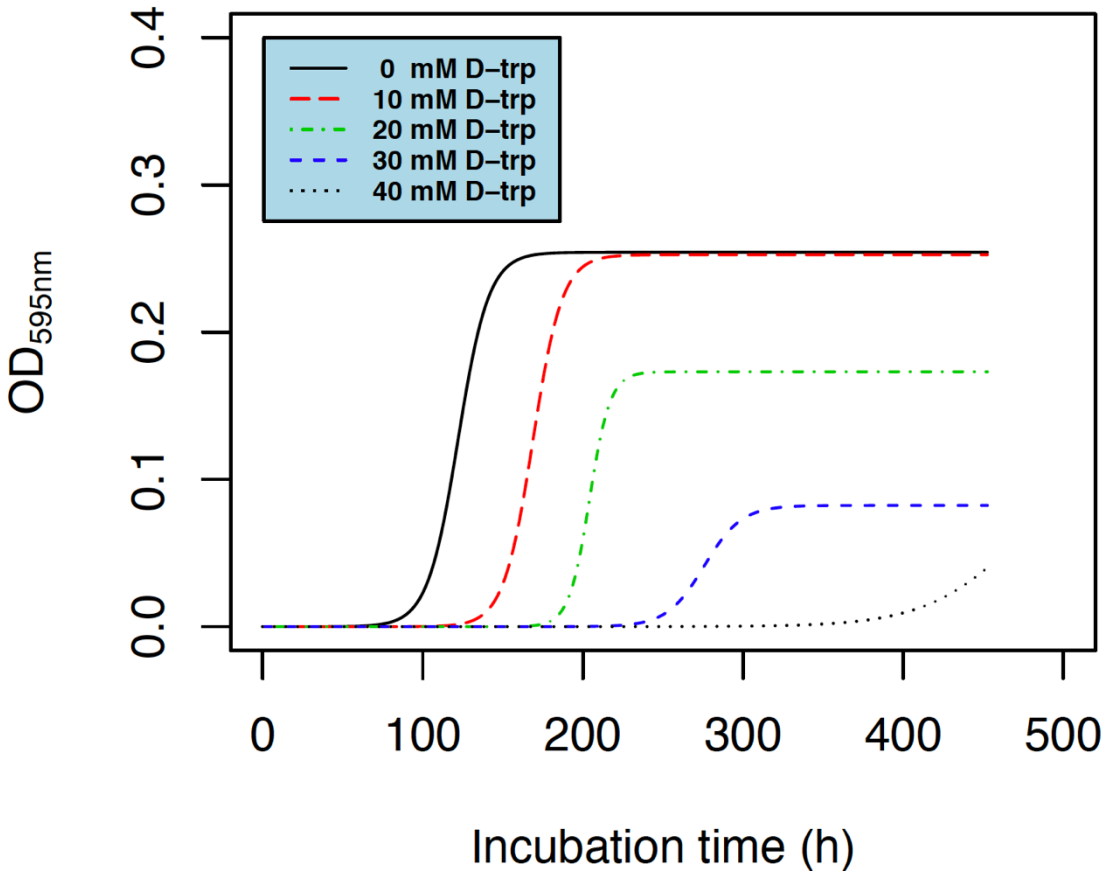
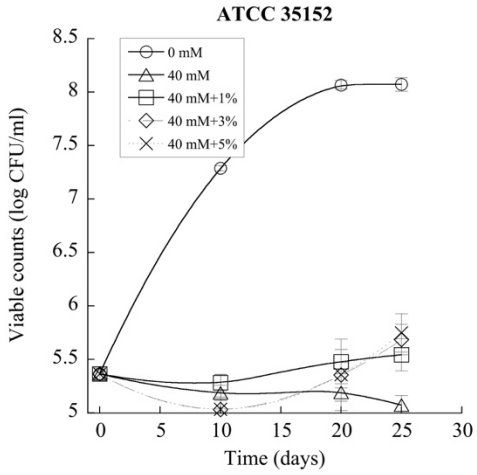


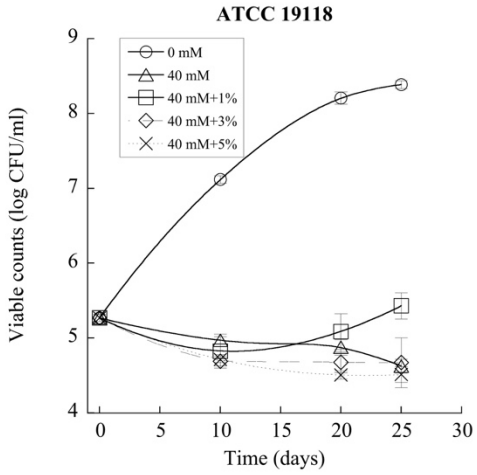
Fig. 3-1. Growth of *L. monocytogenes* strains determined by changes in optical density at 595 nm during incubation at 4°C in peptone-yeast-glucose medium supplemented with D-tryptophan at various concentrations: 0 mM (solid line), 10 mM (dashed line), 20 mM (dot-dashed line), 30 mM (short-dashed line), and 40 mM (dotted line). Presented curves are representative of triplicate experiments.

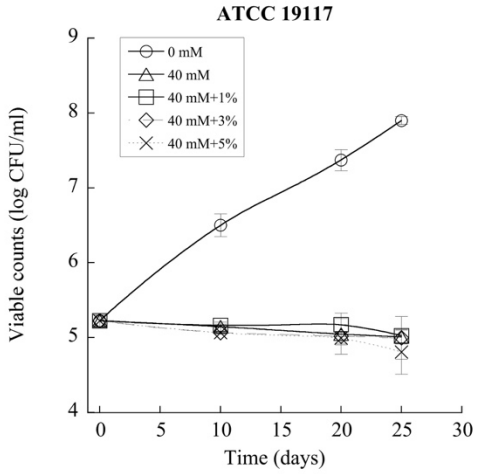
3.3.2 Effect of D-Trp on *L. monocytogenes* survival at 4°C

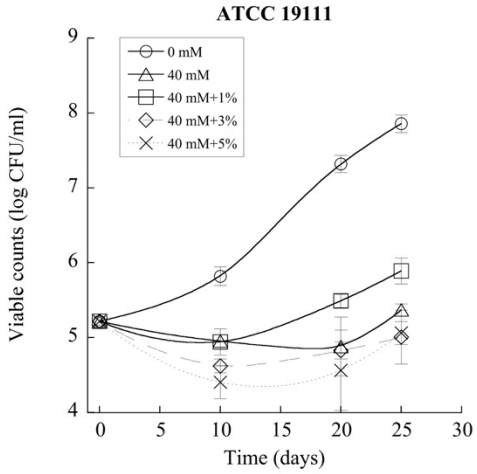
Considering that osmotolerance and psychrotolerance are two of the main factors that make *L. monocytogenes* a ubiquitous foodborne pathogen, we further examined the inhibitory efficacy of D-Trp combined with various NaCl concentrations on the psychrotrophic growth of *L. monocytogenes* in PYG during long-term storage at 4°C. The initial of *L. monocytogenes*

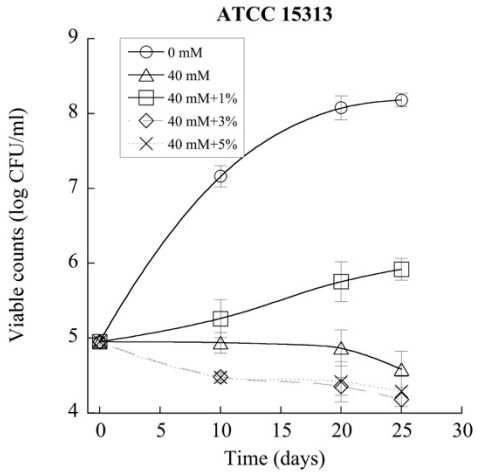
count was 5.2 log CFU/mL. In the controls (without D-Trp), the population of *L. monocytogenes* increased by over 3 log CFU/mL and exceeded 8.0 log CFU/mL during 30 days of incubation at 4°C in a preliminary investigation. However, when D-Trp was added, a reduction in the growth of *L. monocytogenes* was observed and this persisted throughout the incubation period at 4°C (Fig. 3-2). The growth of *L. monocytogenes* was retarded by 40 mM D-Trp, with a lag phase of 15 days. The combined addition of NaCl at 1%, 3%, or 5%, also showed a synergistic activity against *L. monocytogenes* growth. A gradual growth delay and decrease in *L. monocytogenes* counts was observed with increasing NaCl concentration. The combination treatment of D-Trp and high salinity levels had a partial bactericidal effect, reducing the initial microbial population in the first 15 days by 0.6 and 0.8 log cycles at 3% and 5% NaCl, respectively. D-Trp exerted the greatest overall bacteriostatic effect when used in combination with 5% NaCl. These results indicated that D-Trp exerted a greater bacteriostatic effect on *L. monocytogenes* during long-term refrigerated storage, when combined with high levels of salinity.


ATCC 35152
0 mM
40 mM
40 mM+1%
40 mM+3%
40 mM+5%
Viable counts (log CFU/ml)
Time (days)


ATCC 19118
0 mM
40 mM
40 mM+1%
40 mM+3%
40 mM+5%
Viable counts (log CFU/ml)
Time (days)


ATCC 19117
0 mM
40 mM
40 mM+1%
40 mM+3%
40 mM+5%
Viable counts (log CFU/ml)
Time (days)


ATCC 19111
0 mM
40 mM
40 mM+1%
40 mM+3%
40 mM+5%
Viable counts (log CFU/ml)
Time (days)


ATCC 15313
0 mM
40 mM
40 mM+1%
40 mM+3%
40 mM+5%
Viable counts (log CFU/ml)
Time (days)

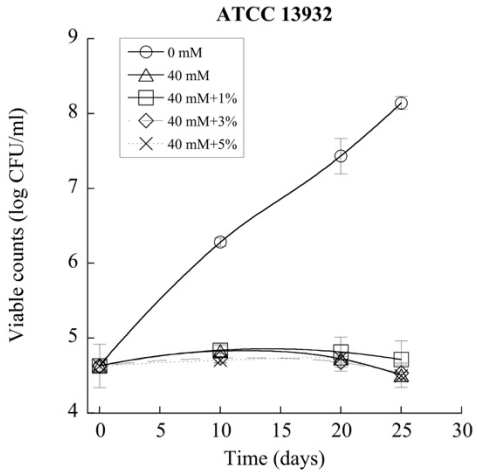
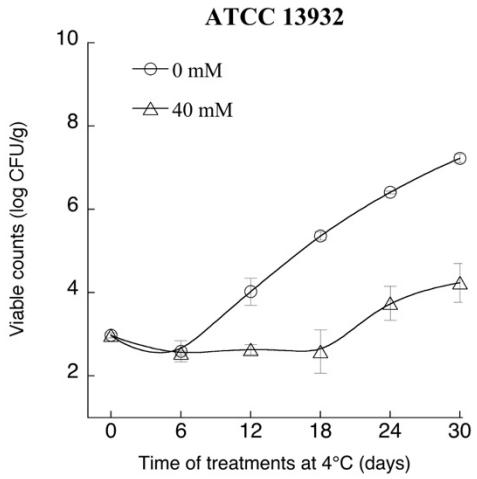

ATCC 13932
0 mM
40 mM
40 mM+1%
40 mM+3%
40 mM+5%
Viable counts (log CFU/ml)
Time (days)

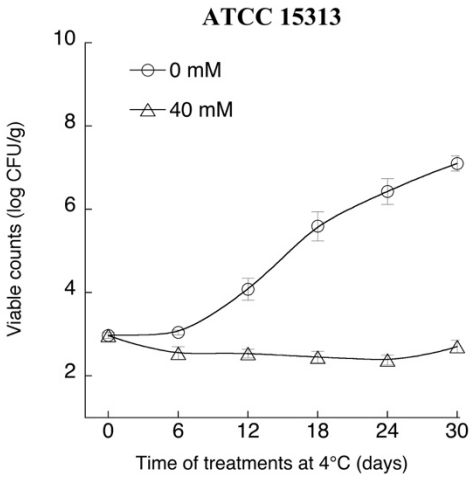
Fig. 3-2. Effects of (○) 0 mM and (△) 40 mM D-tryptophan, alone or in combination with different sodium chloride concentrations (1%, 3%, and 5%), on the survival of six strains of *L. monocytogenes* (ATCC 13932, ATCC 15313, ATCC 19111, ATCC 19117, ATCC 19118, and ATCC 35152) at 4°C. Results are the means ± standard deviations of three independent experiments. Values with different letters on the same sampling time between different conditions were significantly different ($P < 0.05$).

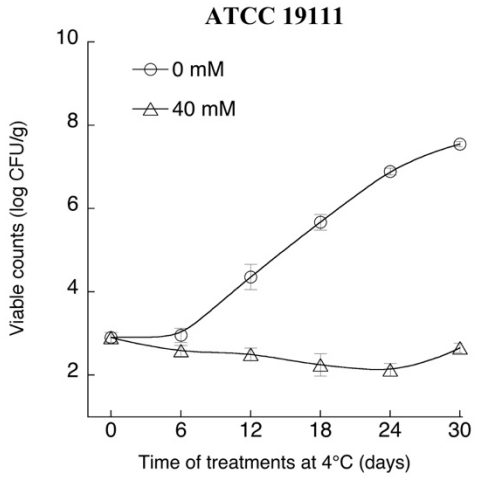
3.3.3 Effect of D-Trp on *L. monocytogenes* survival in pasteurized milk at 4°C

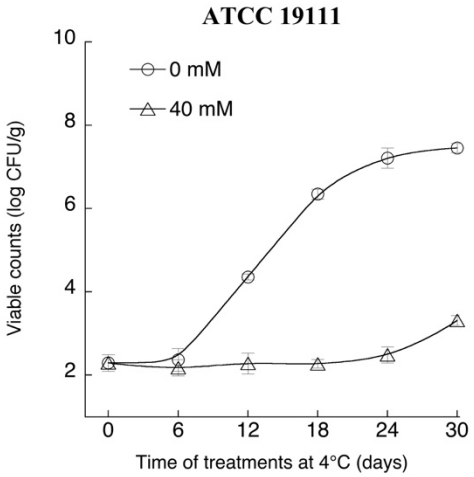
Considering the inhibitory effects of D-Trp on *L. monocytogenes* in culture media, we explored the feasibility of utilizing the D-Trp to control the psychrotrophic growth of *L. monocytogenes* in pasteurized milk during long-term refrigerated storage. Because *L. monocytogenes* may behave in a strain-dependent manner in dairy products, four strains of *L. monocytogenes* were tested separately in this experiment. With no D-Trp treatment, the numbers of each *L. monocytogenes* strain increased gradually at 4°C during 1 month of storage. The control samples of *L. monocytogenes* strains ATCC 15313, 19111, 19118, and 35152 were counted at 7.10, 7.55, 7.50, and 7.45 log CFU/mL after 30 days. Compared with the controls, the addition of 40 mM D-Trp caused a notable decrease in bacterial levels, with more than a 3.5 log CFU/mL reduction in each *L. monocytogenes* strain after 1 month of storage at 4°C (Fig. 3-3). In the case of *L. monocytogenes* strains ATCC 15313, 19111, and 35152, a notable bacteriostatic effect was observed during the whole storage period, when 40 mM D-Trp was

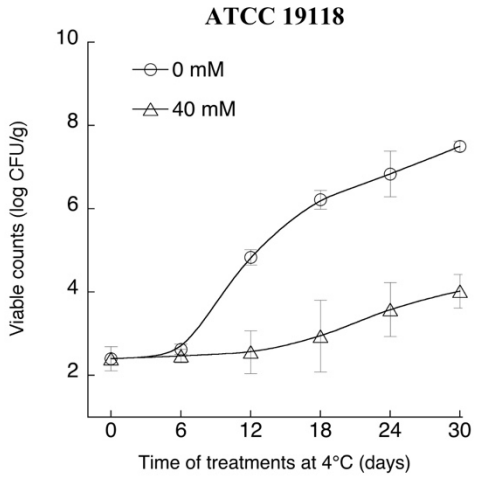
added and no cell growth was observed until 24 days. A slight increase in growth was observed for strain ATCC 19118 after 12 days of storage.


ATCC 13932
0 mM
40 mM
Viable counts (log CFU/g)
Time of treatments at 4°C (days)


ATCC 15313
0 mM
40 mM
Viable counts (log CFU/g)
Time of treatments at 4°C (days)


ATCC 19111
0 mM
40 mM
Viable counts (log CFU/g)
Time of treatments at 4°C (days)


ATCC 19111
0 mM
40 mM
Viable counts (log CFU/g)
Time of treatments at 4°C (days)


ATCC 19118
0 mM
40 mM
Viable counts (log CFU/g)
Time of treatments at 4°C (days)

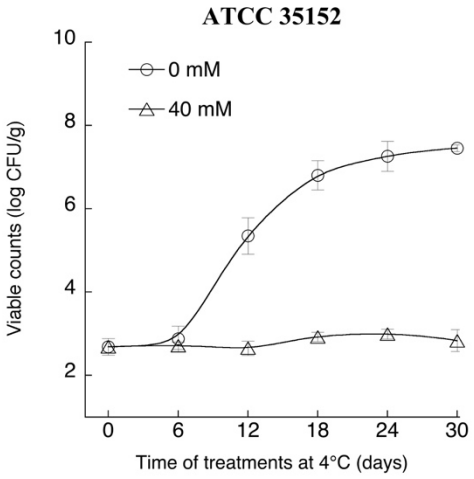

ATCC 35152
0 mM
40 mM
Viable counts (log CFU/g)
Time of treatments at 4°C (days)

Fig. 3-3. Effects of (○) 0 mM and (△) 40 mM D-tryptophan on the survival of four strains of *L. monocytogenes* (ATCC 15313, ATCC 19111, ATCC 19118, and ATCC 35152) in experimentally inoculated pasteurized milk at 4°C. Results are the means ± standard deviations of three independent experiments. Values with different letters on the same sampling time between different conditions were significantly different ($P < 0.05$).

3.3.4 Effect of D-Trp on total psychrotrophic bacteria counts in raw milk at 4°C

Because total psychrotrophic bacteria count is one of the major indicators of milk quality (Cempírková, 2012), we subsequently examined the efficacy of D-Trp on total psychrotrophic bacterial levels in raw milk on TSAYE, according to the method of Patterson et al. (2010). The initial mean population of total viable bacteria in raw milk was approximately 5×10^3 CFU/mL. In the untreated control (without D-Trp addition), the total count increased markedly by approximately 3 log CFU/mL during 9 days of incubation. However, a strong inhibitory effect of D-Trp on total psychrotrophic bacteria in raw milk was observed during storage at 4°C. The total count in milk with D-Trp added remained at the same level during first 3 days of incubation and this was followed by delayed growth, with an increase of approximately 2 log CFU/mL over the remaining 6 days (Fig. 3-4).

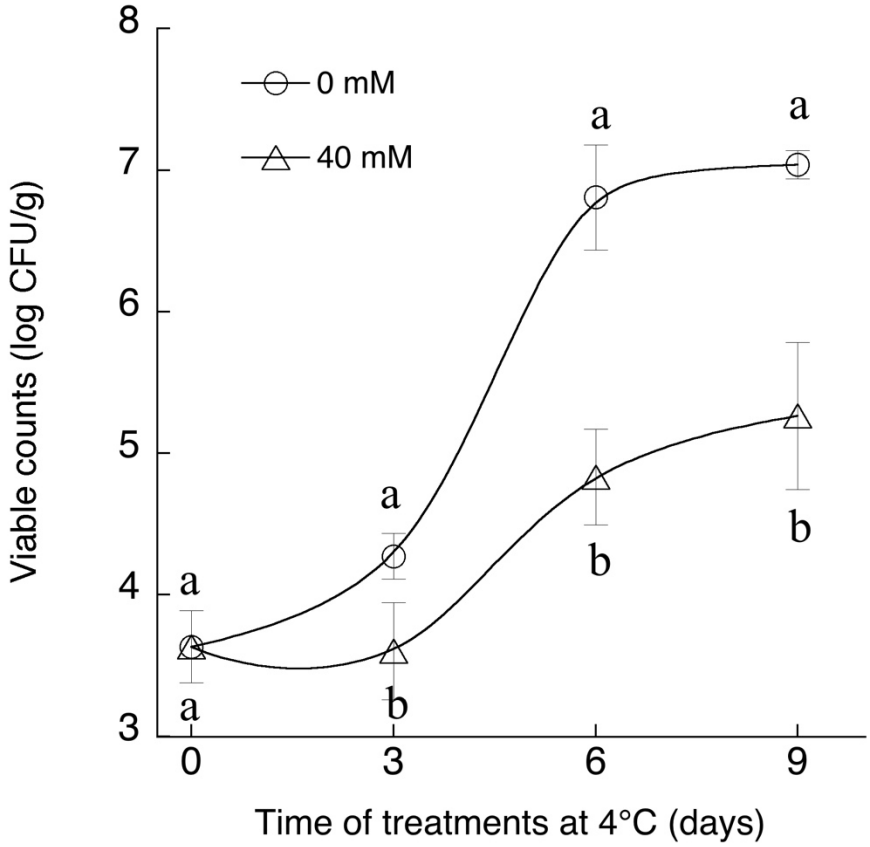


Fig. 3-4. Effects of (◯) 0 mM and (△) 40 mM D-tryptophan on total psychrotrophic bacteria in fresh raw milk at 4°C. Results are the means ± standard deviations of three independent experiments. Values with different letters on the same sampling time between different conditions were significantly different ($P < 0.05$).

3.4 Discussion

Refrigeration is one of the most common methods to retard spoilage and extend the shelf life of food (Mead et al., 1999). Nevertheless, long-term refrigerated storage may not be sufficient to prevent bacterial growth, especially for psychrophilic bacteria. The term

psychrotroph refers to a microorganism that is able to grow at low temperatures. Among these microorganisms, *L. monocytogenes* is a major concern in the food industry due to its adaptation to low temperatures. In the present study, we evaluated the effect of D-Trp on *L. monocytogenes* under low temperature stress. Interestingly, we found that D-Trp treatment inhibited the growth of *L. monocytogenes* in culture media and led to bacteriostatic effects during long-term storage at refrigerated temperatures (4°C). Furthermore, the effect of D-Trp was dependent on its concentration. As the concentration of D-Trp increased, *L. monocytogenes* growth rate decreased and the time in lag phase increased. Similar experiments were conducted at 37°C, which is regarded as the optimum growth temperature for *L. monocytogenes*. However, treatment with D-Trp, even high concentrations, had little or no effect on *L. monocytogenes* growth at 37°C (data no shown). Based on these results, we hypothesize that D-Trp does not directly interfere with cell metabolism in *L. monocytogenes* under optimal growth conditions, but may decrease its resistance to adverse environments.

The effectiveness of D-Trp may be enhanced with increased NaCl levels. Our previous study showed that D-Trp can cause bacterial growth inhibition in the presence of NaCl and this application of D-Trp has shown promise in controlling the foodborne pathogenic bacteria, *Vibrio*, in oysters. In artificial seawater with high levels of salinity (5% NaCl), D-Trp completely inhibited the growth of *Vibrio vulnificus* and *Vibrio parahaemolyticus* (Chen et al., 2018). However, this combined effect was strain-dependent and considerable variation has been demonstrated between gram-positive and gram-negative microorganisms. A combination of 40 mM D-Trp and high levels of salinity (NaCl > 3%) has been shown to significantly inhibit

the growth of *Escherichia coli* O157:H7 and *Salmonella enterica*, but not the gram-positive bacterium, *L. monocytogenes* (Koseki et al., 2015). A novel combination of D-Trp and NaCl should be developed for widespread application of this preservation method in the future. Because adaptation to low temperatures and osmotic stress are the main factors that make *L. monocytogenes* a ubiquitous foodborne pathogen (Ko et al., 1994), we further evaluated the synergistic inhibition of *L. monocytogenes* by D-Trp and NaCl at 4°C for 1 month. A combination of 40 mM D-Trp and elevated osmolarity caused notable growth inhibition in *L. monocytogenes* under low temperature stress. Moreover, higher levels of NaCl led to a gradual reduction in *L. monocytogenes* growth. Although adding NaCl, even up to 5%, did not cause the complete inhibition of *L. monocytogenes* growth, an overall bacteriostatic effect with a partial bactericidal effect was observed. With D-Trp treatment, *L. monocytogenes* appeared to lose its resistance to cold temperatures and become more sensitive to osmotic stress.

Milk and milk products are generally considered as vehicles for *L. monocytogenes*. The consumption of pasteurized milk can also lead to outbreaks of *listeriosis*, because *L. monocytogenes* can grow during refrigerated storage (Fleming et al., 1985). At 4°C, *L. monocytogenes* generally survives and grows in milk until it reaches the stationary phrase, at levels above 10^7 CFU/mL. This maximum population of *L. monocytogenes* can persist for an extended time, even longer than 2 months (Rosenow and Marth, 1987). Therefore, the psychrotrophic growth of *Listeria* in pasteurized milk should be taken into consideration. Based on this fact, we separately examined the inhibitory effects of D-Trp on the survival of four *L. monocytogenes* strains in artificially contaminated pasteurized milk during storage at

4°C. Consistent with previous reports, the number of each *L. monocytogenes* strain in the control samples (no D-Trp addition) increased gradually and reach 10^7 CFU/mL after 30 days of incubation at 4°C. However, when D-Trp was added, *L. monocytogenes* growth was significantly inhibited in three of the four strains, maintaining an initial low level of approximately 10^3 CFU/mL throughout the 1-month refrigeration period. The exception was *L. monocytogenes* strain ATCC 19118, which slightly increased after 12 days storage. Therefore, D-Trp may be considered a novel preservative to control *L. monocytogenes* contamination in long-term packaged milk at refrigeration temperatures. However, the strain-dependent sensitivity of *Listeria* to D-Trp should also be taken into consideration when applying this method for the preservation of pasteurized milk.

The total psychrotrophic bacterial population is closely related to the quality of raw milk. In general, psychrotrophic bacteria, even at low initial levels, can dominate the total bacterial population and usually account for more than 90% of the total microbial population in raw milk during storage at 4°C (Lafarge et al., 2004) (Magan et al., 2001). In this study, we also found that the addition of 40 mM D-Trp inhibited the increase in total viable bacterial count in raw milk during refrigerated storage. We hypothesize that D-Trp inhibited the growth of certain psychrotrophic bacterial species that dominated the microorganism population in raw milk. Therefore, D-Trp may serve as a novel temporary preservative for raw milk and may extend the shelf life of refrigerated milk.

In conclusion, D-Trp is a promising food preservative for controlling the psychrotrophic bacterium, *L. monocytogenes*, at refrigerated temperatures. The inhibitory effect of D-Trp alone

and the combined effect of D-Trp and salinity may be appealing to the dairy food industry. Further understanding of the mechanism whereby D-Trp affects the low-temperature adaptation of *Listeria* is required. Nevertheless, these data suggest that D-Trp serves as an additional safety barrier to prevent bacterial growth at refrigerated temperatures.

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Chapter 4

The Mechanism of the Antibacterial Activity of D-Tryptophan

4.1 Introduction

Osmotolerance is an important determinant of bacterial survival at high osmotic stress in their natural environment. When exposed to osmotic stress, bacteria are capable of accumulating a restricted range of low-molecular-weight organic compounds, termed osmolytes or compatible solutes, such as potassium, proline, and beanie. These compatible solutes can accumulate at high levels by *de novo* synthesis or by transport, without interfering with cellular processes. It has been reported that some halophilic bacteria are capable of *de novo* biosynthesis of compatible solutes, such as glycine betaine, to maintain osmotic equilibrium in response to a high-salt external environment (Shivanand et al., n.d.).

Escherichia coli has been studied extensively with respect to the accumulation of compatible solutes (Ishida et al., 1994). The addition of compatible solutes, such as betaine or proline, to growth medium increases the osmotic tolerance and growth rate of *E. coli* cells subjected to osmotic stress (Weymarn et al., 2001). In minimal medium, potassium is the primary osmolyte accumulated by *E. coli* cells under osmotic stress (Ishida et al., 1994). When betaine or proline is present in the growth medium, the uptake of proline and glycine betaine has priority over the synthesis of trehalose, probably because more energy is required for biosynthesis than for uptake from the growth medium (Cayley et al., 1992). Moreover, glycine betaine has been shown to have significantly greater osmoprotective capacity than proline in

E. coli K12 (Milner et al., 1987).

D-amino acids are now considered to be present in higher organisms, particularly in the mammalian brain (Hashimoto et al., 1992). In bacteria, D-amino acids such as D-aspartic acid, D-glutamic acid, and D-alanine are constituents of bacterial cell walls (Csapo et al., n.d.). Synthesis of D-amino acids has been suggested as an adaptive strategy for bacteria in response to changing environmental conditions. Large quantities of free D-amino acids have also been found in the tissues of marine bivalves, where they may serve as important regulatory factors in response to osmotic stress from seawater (Felbeck and Wiley, 2016). In food, D-amino acids are widely present in harshly treated foods (e.g., roasted coffee) and fermentation products (e.g., cheese and yoghurt; Palla et al., 1989). During food preparation and processing, D-amino acids can be generated from their L-stereoisomers. Heat and alkali treatments are the primary food technology techniques to improve taste, texture, and shelf life. Exposure to alkali or high temperature conditions induces measurable quantities of D-amino acids in food proteins, such as soy protein. The presence of D-amino acids has also been investigated in milk and fermented dairy products. Large amounts of free D-alanine, D-aspartic acid, and D-glutamic acid have been found in raw cow's milk. The free D-alanine content in raw milk gradually increases during storage at 4°C and it is suggested as an indicator of milk contamination (Gandolfi et al., 1992). Besides bacterial activity, pasteurization and fermentation processes also contribute the occurrence of D-amino acids in raw milk. With the increasing extent of heat or fermentation processing of raw milk and milk products, D-aspartate content can increase to over 3% (Man

et al., n.d.). In addition, some D-amino acids have therapeutic properties. D-Leucine and D-phenylalanine have been shown to cause pain relief and may serve as potent, non-addictive analgesics in humans (Cheng and Pomeranz, 1979).

Recently, some D-amino acids, such as D-tryptophan (D-Trp), have been found to exhibit antibacterial activities against foodborne bacteria under conditions of osmotic stress. Some studies have subsequently confirmed that D-proline, D-alanine, and D-serine modestly inhibit bacterial growth under conditions of osmotic stress (Chin et al., 2010; Sasaki et al., n.d.) (Shahjee et al., 2002a). These incompatible solutes, which are structurally similar to compatible solutes, have adverse effects on the growth of foodborne bacteria under conditions of osmotic stress. Our previous study reported that D-Trp adversely affects the growth of foodborne bacteria under high-salt conditions (Koseki et al., 2015). Therefore, D-Trp appears to be a novel preservative for controlling bacterial growth in foods. However, there are several unanswered questions regarding the mechanism of the antibacterial activity of D-Trp. For example, is D-Trp taken up into bacterial cells as a compatible solute by mistake, but actually acts as an incompatible solute that contributes to growth inhibition? Is D-Trp itself toxic, with direct detrimental effects on the metabolism of bacterial cells? Is it D-Trp or osmotic stress that contributes to bacterial growth inhibition? Finally, what is the relationship between D-Trp and osmoprotectants, such as proline?

The objectives of this study were to examine the role of intercellular and extracellular D-Trp in bacterial inhibition and to increase our knowledge of the impact of D-Trp treatment on *E. coli* cell metabolism. We used capillary electrophoresis time-of-flight mass spectrometry

(CE-TOF/MS) to perform metabolome analyses of *E. coli* cells inhibited by D-Trp under osmotic stress conditions and compared the principal component differences in metabolites between D-Trp-treated cells and untreated cells. Furthermore, we tested the effect of D-Trp on proline uptake, to clarify the relationship between D-Trp and compatible solutes.

4.2 Materials and methods

4.2.1 Bacterial strains

In this study, we chose *Escherichia coli* as the representative bacterial strain, because its osmoregulation has been extensively investigated. *E. coli* ATCC 25922 (obtained from the American Type Culture Collection) was recovered from a frozen stock and cultured in TSB. The cultures were incubated at 37°C for 24 h without agitation and 100 μL of culture was subsequently transferred to 5 ml of fresh TSB at three successive 24-h intervals to obtain a more homogeneous and stable inoculum.

4.2.2 Sample preparation and D-Trp treatment

Osmotic stress was induced by dissolved NaCl, which is widely used in food processing. Bacterial samples were prepared as described previously (Kan et al., 2018). In brief, broth containing peptone (5.0 g/L; Difco, Becton Dickinson, Sparks, MD, USA), yeast extract (2.0 g/L, Difco), and glucose (1.0 g/L, Wako) was used as the base medium for bacterial growth experiments.

Because our previous study showed that 40 mM D-Trp in PYG broth, supplemented with 3% NaCl, could completely inhibit *E. coli* growth at 25°C, combined treatment with 40 mM D-

Trp and 3% NaCl was defined as the growth inhibition condition. For this condition, 40 mM D-Trp was added to the PYG base medium, supplemented with 3% (wt/vol) NaCl. Considering the moderate solubility of D-Trp, each treatment solution was dissolved completely in a VS-100 ultrasonic cleaner for 3 min at 45 kHz. The D-Trp solution was then filtered through a membrane filter (0.2-mm pore size; Millipore, Billerica, MA, USA).

4.2.3 Microbiological analysis

Cell growth was monitored by measuring the OD_{595} and by plate counting. For the optical density method, an aliquot (20 μL) of the stationary-phase bacterial culture (10^7 CFU/mL) was added to each treatment solution (180 μL) on a plate, and mineral oil (20 μL) was added to prevent evaporation of the samples. The inoculated microplate was incubated in an iMark microplate reader at 25°C for 5 days and the changes in OD_{595} during this incubation period were continuously recorded.

For plate counting, bacterial survival was determined via plate counts on TSA after each treatment. Successive 10-fold serial dilutions were prepared using 0.1% peptone water and 100 μL aliquots of the diluted samples were then spread on TSA plates and cultured at 25°C for 24 h.

4.2.4 Intracellular amino acid analysis

Free amino acid analysis was performed according to a previously published method (Moghaddam et al., 2016). In brief, 500 μL of a methanol/chloroform/demineralized H_2O

solution (10/5/4, by volume) was added to 30 mg of dry material. After shaking for 5 min, 130 μL of chloroform and 130 μL of demineralized H_2O were added and the mixture was shaken again for 5 min. Finally, the mixture was centrifuged at 8000 g for 3 min and the hydrophilic top layer, containing the free amino acids, was separated for subsequent analyses. The aqueous phase was analyzed by HPLC, using an amino acid analyzer (L-8900, Hitachi, Tokyo, Japan) at the Center for Instrumental Analysis, Hokkaido University.

4.2.5 Metabolome analysis

CE-TOF/MS metabolome measurements were performed using an Agilent CE-TOF/MS system (Agilent Technologies, Santa Clara, CA, USA) and an in-house library created by running standard compounds from Human Metabolome Technologies (HMT, Tsuruoka, Japan), as described previously (Kimura et al., 2018; Ohashi et al., 2008). Samples were prepared as described above for intracellular amino acid analysis. An internal standard solution (HMT) was added to the cell suspension and the mixture was filtered through a regenerated cellulose membrane (UltrafreeMC-PLHCC for metabolome analysis, HMT). The filtered samples were stored at -80°C until used. Metabolome analyses were repeated in biological duplicates for each treatment.

4.2.6 Statistical analysis

Colony-count data for triplicate samples of each bacterium at each sampling interval were transformed to log CFU/mL or log CFU/g and the values of the triplicate samples were

averaged to represent the number of viable cells at each sampling time. One-way ANOVA was performed to compare the differential degrees between each treatment. The viable counts in each condition were then compared by Tukey-Kramer's multiple comparison test. $P < 0.05$ was considered statistically significant.

CE-TOF/MS peaks were assigned for the 727 target molecules listed for *E. coli* (Ohashi et al., 2008). The KEGG (http://www.genome.jp/kegg/), Ecocyc, and Visualization and Analysis of Networks Containing Experimental Data databases were used for pathway analyses and for mapping metabolites to their metabolic pathway. Principal component analysis (PCA) was performed using SampleStat ver. 3.14 software (HMT).

4.3 Results

4.3.1 Microbiological analysis in PYG medium (compatible slute rich)

To determine the inhibition and growth condition of D-Trp, we examined the growth of *E. coli* ATCC 25922 in PYG media. Bacteria grew reasonably well in PYG medium supplemented with 40 mM D-Trp alone, whereas *E. coli* growth was inhibited in PYG medium containing 40 mM D-Trp and 3% NaCl (Fig. 4-1).

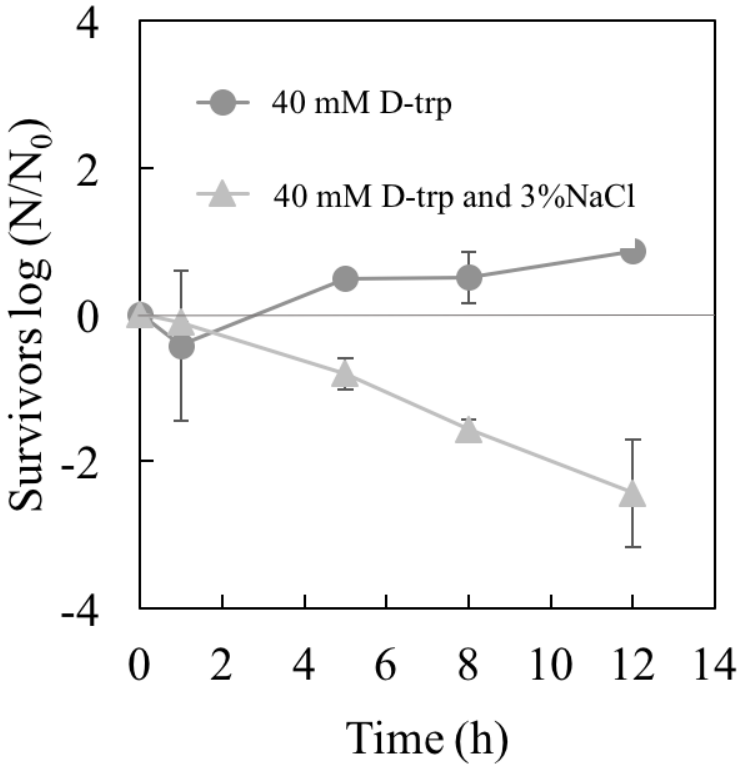

Fig. 4-1. Effects of 40 mM D-tryptophan on the survival of *Escherichia coli* ATCC 25922 in peptone-yeast-glucose medium supplemented with (○) 0% NaCl or (△) 3% NaCl at 37°C. Results are the means ± standard deviations of three independent experiments. Values with different letters on the same sampling time between different conditions were significantly different ($P < 0.05$).

4.3.2 Intracellular changes of D-Trp in *Escherichia coli*

To confirm whether D-Trp was actually taken up into bacterial cells and consequently, caused a metabolic disorder and growth inhibition under osmotic stress, we examined changes in intracellular D-Trp levels by HPLC.

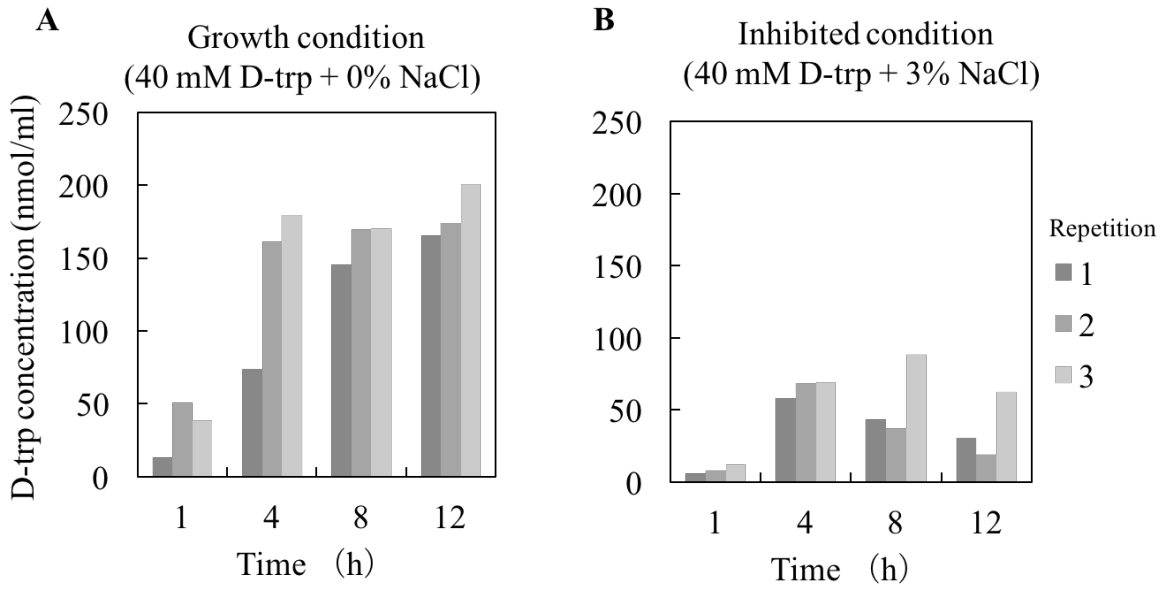

Fig.4-2. The effect of osmotic stress on D-Trp uptake in *Escherichia coli* ATCC25922 cells in peptone-yeast-glucose medium supplemented with 40 mM D-Trp at (A) 0 % NaCl or (B) 3% NaCl.

4.3.3 Extracellular presence of D-Trp and its inhibitory effectiveness

To investigate the role extracellular D-Trp in bacterial growth inhibition udner osmotic stress, we measured the growth curve of *E. coli* in YGP containing 3% NaCl supplemented with or without D-tryptophan at 25°C. As shown in Fig. 4-3, the growth of *E.coli* was not inhibited in the high-salinity culture media when D-trp was absence as a gradual increase in optical density at 595 nm since the initial 5 h. Whereas, adding exogenous D-trp into YGP containing 3% NaCl led to a complete inhibition in *E.coli* growth. Interestingly, after replaced the inhibited cells wth 8 h of D-trp treatment to a fresh high-salinity PYG media in the absence of D-trp, a rescued growth was observed and subsequently exhibited a similar trend to the one

in D-trp absence.

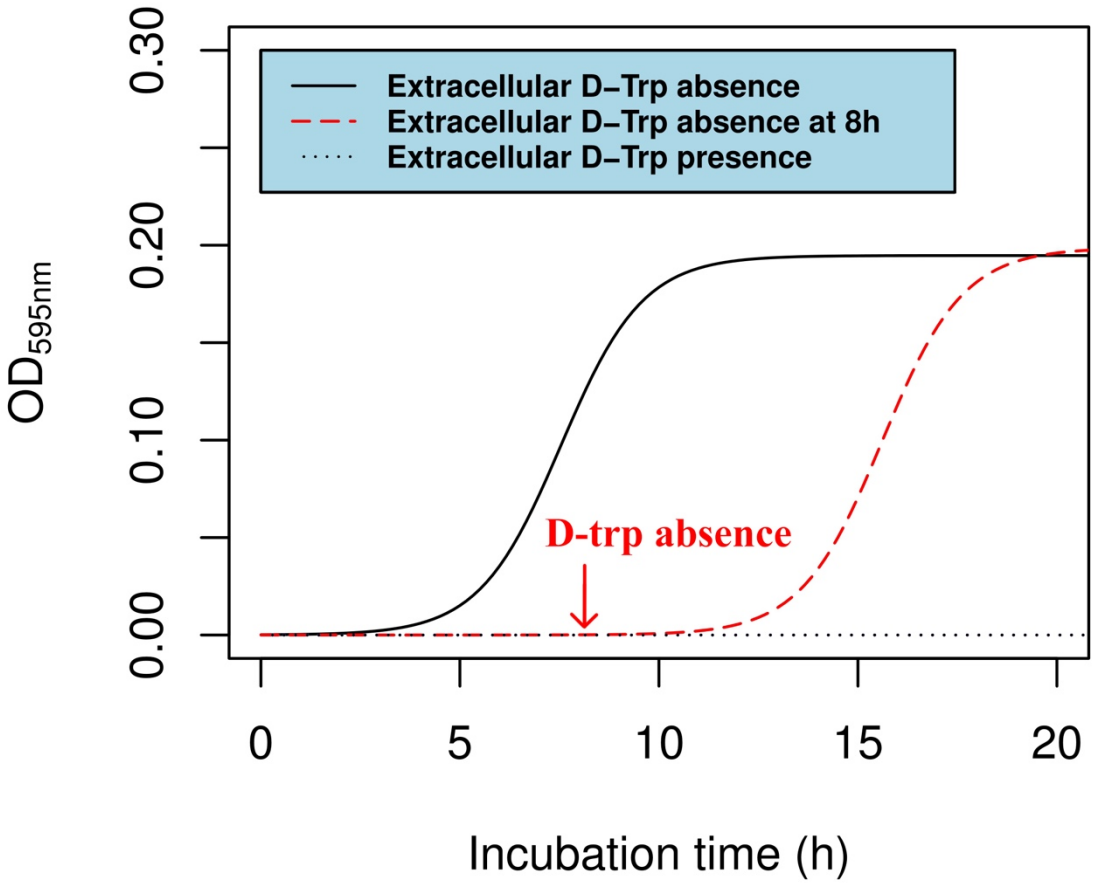

Fig.4-3 The effect of extracellular D-Trp (40 mM) on the changes of optical density at 595 nm in *E. coli* ATCC25922 cells in 3%-NaCl-contained PGY media. The solid black lines indicates extracellular D-trp was absent during the treatment; The solid dotted line indicates extracellular D-trp was present during the treatment;The dashed line indicates D-trp was present during the first 8 h and was absent after 8h.

4.3.4 Metabolome analysis

To assess the impact of D-Trp treatment on *E. coli* cell metabolism, we used CE-TOF/MS to compare low-molecular-weight metabolites (including sugars, nucleotides, amino acids, and

lipids) extracted from inhibited cells (sample treated with 40 mM D-Trp and 3% NaCl) and growing cells (samples in 3% NaCl, but without D-Trp treatment), with those extracted from living control cells. A total of 176 metabolites were annotated and 70 of these were mapped to metabolic pathways (Fig. 4-5). The first and second principal components (PC1 and PC2) derived from the metabolome data explained 59.7% and 16.6% of the total variance (Fig. 4-4). Although the PCA score plot showed that both inhibited cells (40 mM D-Trp and 3% NaCl) and growing cells (3% NaCl) clearly differed from the untreated control, there was no notable separation between the inhibited cells (40 mM D-Trp and 3% NaCl) and growing cells (3% NaCl). Further detailed metabolic pathway analysis showed that both inhibited cells and growth cells had a similar alterations in terms of the main metabolic pathways (lipid, fatty acid metabolism, sugar metabolism, TCA cycle and amino acid metabolism) (Fig. 4-6). Under osmotic stress, 40 mM D-Trp treatment did not lead to a D-Trp-dependent changes in metabolic pathways with the exception of some metabolites such as adipic acid, quinic acid, and guanosine 3', 5'-bispyrophosphate (ppGpp), which showed D-Trp-dependent alterations. The levels of these metabolites were significantly elevated after D-Trp treatment.

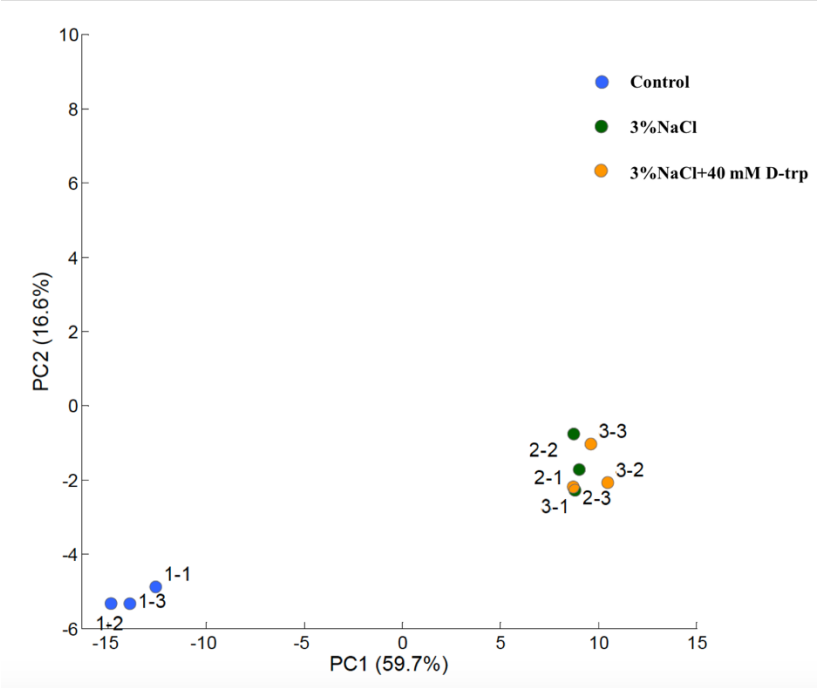

Fig. 4-4. A biplot of principal component analysis of CE-TOF/MS peaks of metabolites derived from cell lysates. Dots indicate scores from duplicate results. Blue dots, untreated control cells; green dots, growing cells with high osmolarity (3% NaCl); yellow dots, inhibited cells with high osmolarity (3% NaCl) and D-Trp treatment at 40 mM. Principal component analysis was performed using SampleStat ver. 3.14 software.

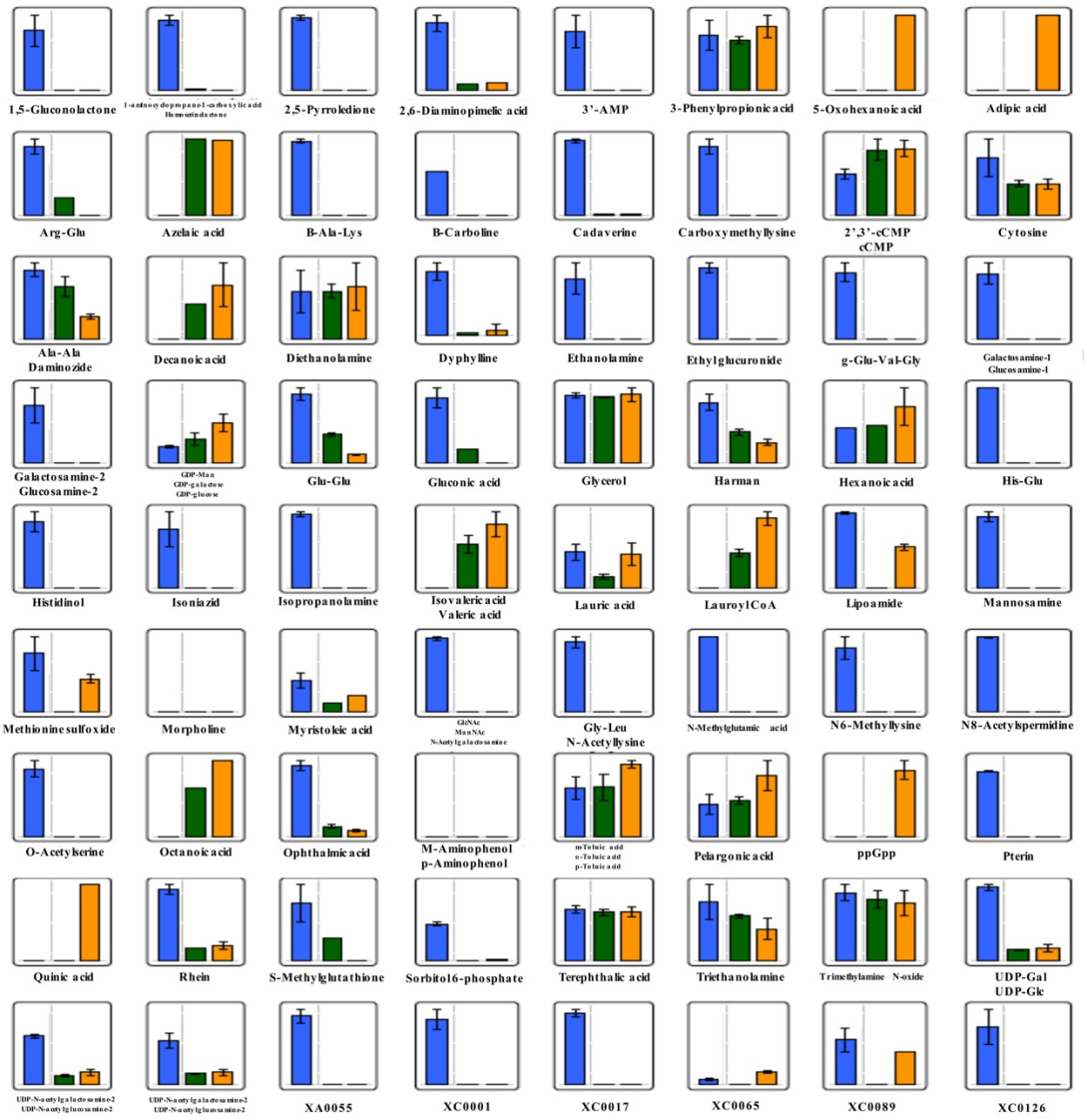

Fig.4-5 Concentration of metabolites detected in each cell samples. Blue bar indicates untreated control cells; Green bar indicates growth cells with high osmolarity (3% NaCl)-treated; Yellow bar indicates inhibited cells with high osmolarity (3% NaCl) and D-Trp treated at 40 mM.

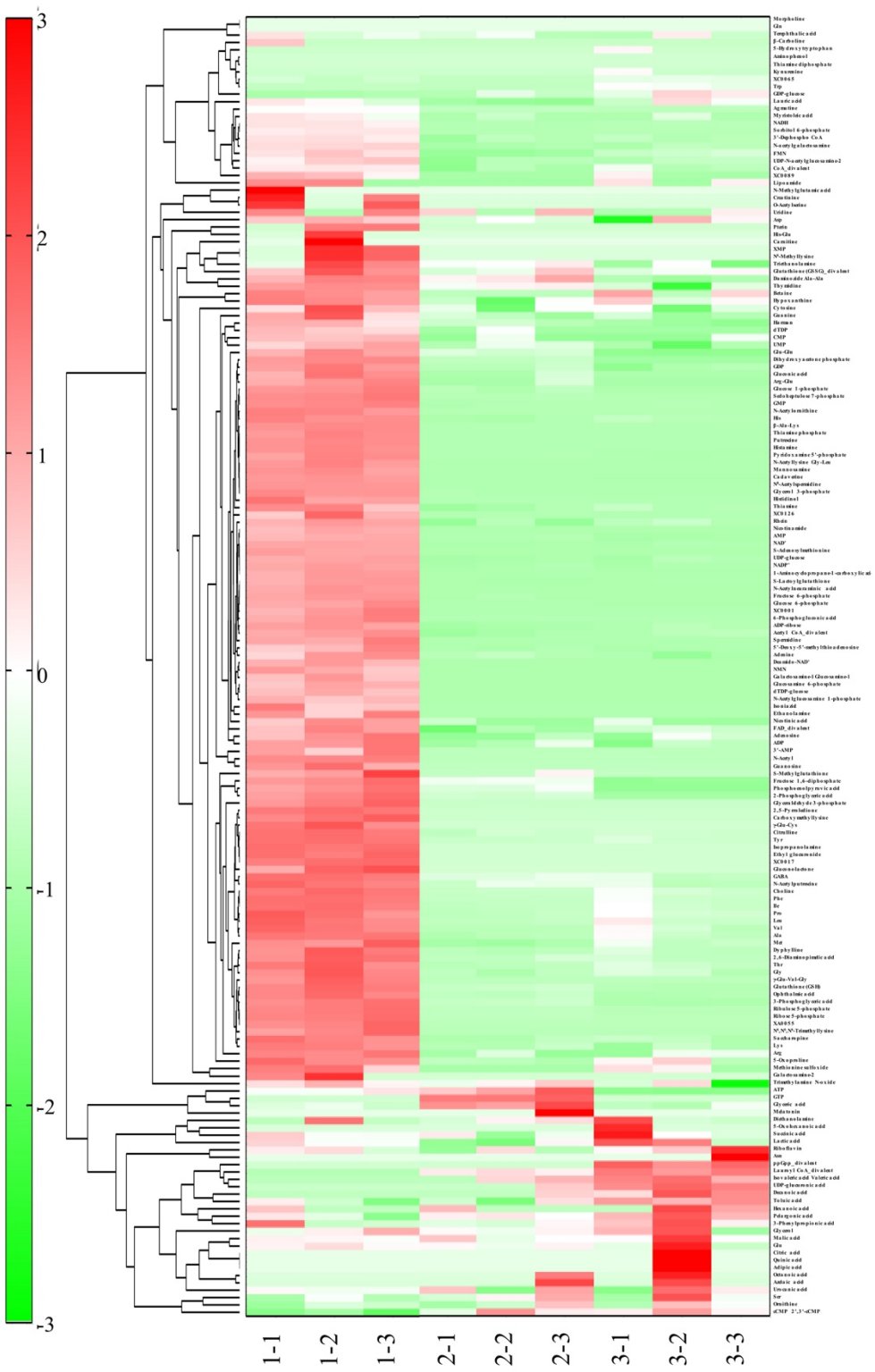

Fig.4-6 Heatmap of the main metabolites in untreated control cells (1-1,1-2,1-3), cells under osmotic stress of 3% NaCl (2-1,2-2,2-3), and cells treated with 40 mM D-Trp while under osmotic stress of 3% NaCl (3-1,3-2,3-3).

4.4 Discussion

The inhibitory effect of D-Trp is favored by the presence of high osmolarity. (KOSEKI et al., 2015) reported that D-Trp could only inhibit *E. coli* growth under high osmolarity (3% NaCl) conditions. Consistent with this result, we also found that 40 mM D-Trp strongly deceased *E. coli* survival at 3% NaCl, but did not exhibit equivalent inhibitory effects at 0% NaCl. This suggests that *E. coli* growth will not be affected, even at high levels of exogenous D-Trp, without high osmolarity. Therefore, high osmolarity is indispensable for consistent growth inhibition by D-Trp. Therefore, in this study, treatment of *E. coli* with 40 mM D-Trp and 3% NaCl was regarded as the inhibitory condition, whereas treatment with either 40 mM D-Trp or 3% NaCl alone was defined as the growth condition.

Although D-amino acids have been shown to be present in organisms and foods, some D-amino acids can exert an isomer-specific toxic effect under certain conditions (Masters et al., n.d.). It has been reported that D-Trp accumulation inhibits beta-galactosidase synthesis in *E. coli* (Schlammadinger and Szabó, 1973) and induces hemolysin production in *Vibrio parahaemolyticus* (Cherwonogrodzky et al., 1984). Thus, we hypothesized that D-Trp is taken up into bacterial cells as a compatible solute by mistake, but actually acts as an incompatible solute that contributes to growth inhibition. During this process, high osmolarity, such as with high NaCl concentrations, may support the accumulation of intracellular D-Trp, as it does for other compatible solutes. (Cherwonogrodzky et al., 1984) found that exogenous D-Trp accumulate in *V. parahaemolyticus* cells during initial growth inhibition and subsequently disappeared when growth recommenced, accompanied by hemolysin production. They

concluded that there may be a correlation between the presence of D-Trp in the cell and the inhibition of culture growth and substrate utilization. Consistent with this study, our amino acid analysis of cell lysates also showed that the intracellular levels of D-Trp gradually increased and then tended to decrease slightly during the inhibition period. Nevertheless, it appears that the intracellular levels of D-Trp do not correlate with bacterial growth inhibition. This was supported by the observation that, under both growth and inhibition conditions, the intracellular level of D-Trp increased during 12 h of exposure to 40 mM D-Trp. Moreover, when exposed to D-Trp, *E. coli* cells in the growth condition actually maintained a relatively higher intracellular D-Trp content than those in the inhibition condition, indicating that the uptake and accumulation of intracellular D-Trp, even at high levels, did not directly contribute to the growth inhibition of *E. coli*. D-Trp was found to accumulate at considerably high levels, regardless of the presence of high osmolarity. Based on this fact, it unlikely that growth inhibition occurred due to D-Trp toxicity.

We further suspected that intracellular D-Trp may be transformed into toxic compounds, which then cause a metabolic disorder and bacterial growth inhibition during conditions of osmotic stress. If this hypothesis was true, growing cells treated with a high level of D-Trp would tend to be inhibited when transferred to a high osmolarity environment. Since exposure to D-Trp alone could result in a considerably high level of D-Trp in cells, we first precultured cells in YPG containing 40 mM D-Trp and then replaced the media with fresh YPG, supplemented with 3% NaCl. According to the microbiological analysis results, similar growth inhibition was not observed in cells precultured with D-Trp. Therefore, it is unlikely that

intracellular D-Trp or its derivatives, contribute to bacterial cell inhibition during osmotic stress.

In general, osmotic stress induces changes at the metabolite level, but does not lead to the growth inhibition of *E. coli.* In our study, we also confirmed that *E. coli* is able to grow in PYG liquid culture media containing 3% NaCl (data not shown). In light of these observations, we decided to investigate whether the addition of D-Trp in high osmolarity media would cause a specific metabolite response that leads to growth inhibition. Metabolites extracted from cells grown under inhibition and growth conditions, were analyzed by metabolome measurements using CE-TOF/MS. PCA showed that there was a notable difference between bacteria under high osmolarity stress and those under normal conditions. However, this difference in metabolites was largely attributed to the high osmolarity stress rather than the addition of D-Trp, since there was no specific metabolite difference between inhibited cells (40 mM D-Trp and 3% NaCl) and growing cells (3% NaCl) during osmotic stress. Under osmotic stress, adding exogenous D-Trp had negligible influence on intracellular metabolic changes. Therefore, these data indicated that, that under high osmolarity stress, D-Trp treatment had no specific influence on the main intracellular metabolic changes. An exception was observed for some minor metabolites, such as ppGpp. ppGpp is an interesting candidate, because this polyphosphate has a significant role in controlling the survival and growth rate of bacteria under environmental stresses, such as osmotic shock or nutrient limitation (Greenway and England, 1999). The ppGpp synthetase, RelA, is involved in the adaptation to various environmental conditions (Burgess et al., 2016). As a stringent response to nutrient starvation, *E. coli* synthesizes ppGpp to increase the intracellular ppGpp levels. Moreover, an inverse

linear correlation exists between growth rate and intracellular ppGpp concentration, suggesting that the more ppGpp produced, the more severe the amino acid or carbon source starvation and the greater the inhibition of rRNA transcription (Condon et al., 1995). Therefore, in this study, the increased ppGpp level seen in inhibited cells may be regarded as a response to D-Trp-induced nutrient starvation. Under such conditions, ppGpp would serve as an effector to control growth rate. Nonetheless, it does not seem possible that D-Trp affected the majority of metabolic pathways that contribute to subsequent growth inhibition during osmotic stress.

The extracellular matrix and its components are essential for bacterial survival and resistance to stress. For example, biofilm growth is a protective mode of bacterial growth that allows cells to survive in hostile environments and to colonize new niches by dispersal of microorganisms from the microbial clusters (Hall-Stoodley et al., 2004). Recent studies have reported that exposure to 10 mM D-Trp causes biofilm disassembly in *Cronobacter sakazakii*, by reducing the initial adhesion between cells and changing the properties of the extracellular matrix (Li et al., 2015). Therefore, one possible explanation for the antibacterial effect of D-Trp may be attributed to the presence of D-Trp in the extracellular matrix, which plays a crucial role in bacterial multicellular communities and protects bacteria from environmental insults (Davies, 2003; Moran and Jarvik, 2010). If the presence of extracellular D-Trp is a prerequisite for bacterial growth inhibition, the efficacy of D-Trp uptake-induced growth inhibition would not be continuous when culture conditions change. Consistent with our hypothesis, a recommencement of growth was observed when we replaced the inhibiting cell medium with fresh (D-Trp-free, normal osmolarity) liquid PYG medium, indicating that bacterial inhibition,

under conditions of osmotic stress, required the presence of extracellular D-Trp. Taken together, we attribute the observed growth inhibition to extracellular D-Trp, which exerted its antibacterial effects at the extracellular matrix.

In conclusion, the uptake and accumulation of D-Trp at high levels did not directly contribute to bacterial growth inhibition. It does not seem possible that the uptake of D-Trp affected the majority of metabolic pathways that contribute to subsequent growth inhibition during osmotic stress. The majority of metabolite responses in cells treated with D-Trp were nearly identical to the responses due to osmotic stress, indicating that uptake of D-Trp did not cause a remarkable metabolic disorder in bacterial cells. The elevation of intracellular ppGpp levels reveals that bacterial cells may have suffered some severe stress that may be linked to D-Trp treatment. In addition, the antibacterial mechanism of D-Trp treatment most likely took place outside the bacterial cell, instead of the intracellular matrix.

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Chapter 5 Summary

5. Summary

In the present study, we investigated the antibacterial effect of D-Trp and added this novel antibacterial molecule into actual food matrices to control important foodborne bacteria.

In Chapter 2, we report the investigation of the antibacterial effects of D-Trp on *V. parahaemolyticus* and *V. vulnificus*, which are high osmolarity-resistant bacteria that grow and survive in high-salt environments (e.g., seawater). We demonstrated that the addition of a small amount of D-Trp inhibited the growth of *V. parahaemolyticus* and *V. vulnificus* under a high-salt environment, even at ambient temperatures. We further investigated different D-Trp treatment conditions and investigated the relationship between salt and D-Trp concentration for optimal growth inhibitory effects in *Vibrio* spp. These results provide a useful method for enhancing the effectiveness of D-Trp, by increasing salinity levels. Furthermore, in a nutrient-free environment (artificial seawater), a stronger inhibitory effect was observed at relatively lower salinity levels, indicating that D-Trp may be regarded as an effective food preservation technique to enable reduced salinity. Therefore, we suggest the use of exogenous D-Trp as a novel and effective strategy for controlling *Vibrio* in live oysters in a seawater environment, even at ambient temperatures and also for effectively retarding the growth of spoilage bacteria and extending the shelf life of shucked oysters at refrigerated temperatures.

In Chapter 3, we discuss the applicability of D-Trp, combined with refrigeration treatment, for controlling the psychrotrophic growth of *L. monocytogenes*. We demonstrated that adding exogenous D-Trp could reduce and delay the psychrotrophic growth of *L. monocytogenes* at

4 °C. Moreover, either increasing the level of D-Trp or adding NaCl further decreased the growth rate and extended the duration of the lag phase of *L. monocytogenes*. Furthermore, in artificially contaminated pasteurized milk, the addition of 40 mM D-Trp also significantly ($P < 0.05$) inhibited the growth of *L. monocytogenes* during extended refrigerated storage. The *L. monocytogenes*-contaminated milk samples maintained a low listerial load of approximately 10^3 to 10^4 CFU/mL throughout the 1-month refrigeration period. In addition to the effect on *L. monocytogenes*, 40 mM D-Trp significantly reduced the total bacterial count in raw milk and extended its shelf life for more than 6 days with a total psychrotrophic bacterial count < 10^5 CFU/g at 4°C. These results indicated that D-Trp could significantly retard the psychrotrophic growth of *L. monocytogenes* by decreasing its chill tolerance at refrigeration temperatures and therefore, it may serve as a novel food preservative to extend the shelf life of milk products.

In Chapter 4, we present studies aimed at elucidating the mechanism of the antibacterial effect of D-Trp. The view now generally held is that, under osmotic stress, bacteria can survive and grow due to their ability to uptake compatible solutes. Recently, our lab found that this adaptive ability appears to be limited when D-Trp is present, resulting in D-Trp-dependent growth inhibition. At first, we attributed this phenomenon to D-Trp uptake by cells, which subsequently served as an incompatible solute affecting cell metabolism and consequently, leading to growth inhibition. However, amino acid analysis of cell lysates showed that the uptake and accumulation of D-Trp, even at high levels, did not directly contribute to bacterial growth inhibition. Further microbiological analysis showed that D-Trp, even after uptake, cannot provide continual inhibitory effects on *E. coli* cells once the culture conditions change.

Therefore, it is unlikely that D-Trp itself is toxic to bacterial cells. Metabolome analysis further confirmed that the inhibited cells do indeed experience metabolic alterations. However, the majority of metabolite responses were nearly identical to the responses to osmotic stress, indicating that D-Trp uptake did not cause a remarkable metabolic disorder in bacterial cells. The metabolic influence of D-Trp seems to be negligible, with the exception of its effect on ppGpp. The intracellular levels of ppGpp were clearly elevated in bacteria under osmotic stress, after treatment with D-Trp, suggesting that bacterial cells may have suffered a severe stress during D-Trp treatment. Nonetheless, it does not seem possible that the uptake of D-Trp affected the majority of metabolic pathways that contribute to growth inhibition during osmotic stress. Subsequent microbiological analysis indicated that extracellular D-Trp is a prerequisite for bacterial growth inhibition. This was supported by the observation that growth inhibition does not continue to occur in the absence of extracellular D-Trp in bacterial cells under osmotic stress. In conclusion, we propose that D-Trp itself is not toxic to bacterial cells and its antibacterial mechanism most likely takes place outside the bacterial cell, rather than in the intracellular matrix.

References

Adamiak, A., Górska, A., Mróz, B., 2015. Psychrotrophic bacteria in raw milk and dairy productsS. Żywność. Nauka. Technologia. Jakość, (101),36-48.

Albarracin, W., Sanchez, I.C., Grau, R., Barat, J.M., 2011. Salt in food processing; usage and reduction: a review. Int. J. Food Sci. Tech. 46, 1329-1336.

Andrews, L., Jahncke, M., Mallikarjunan, K., 2003. Low dose gamma irradiation to reduce pathogenic *Vibrios* in live oysters (*Crassostrea virginica*). J. Aquat. Food Prod. 12, 71-82.

Angelidis, A.S., Smith, G.M., 2003. Role of the Glycine Betaine and Carnitine Transporters in adaptation of *Listeria monocytogenes* to chill stress in defined medium. Appl. Environ. Microbiol. 69, 7492-7498.

Archer, D.L., 1996. Preservation microbiology and safety: Evidence that stress enhances virulence and triggers adaptive mutations. Trends Food Sci. Tech. 7, 91-95.

Sleator, R. D. and Hill, C., 2001 Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. FEMS Microbiol. Rev. 26, 49-71.

Baker, G., 2016. Food safety impacts from post-harvest processing procedures of molluscan shellfish. Foods 5, 29-12.

Batt, C.A., 2014. Encyclopedia of Food Microbiology. Academic Press.

Bidlas, E., Lambert, R.J.W., 2008. Comparing the antimicrobial effectiveness of NaCl and KCl with a view to salt/sodium replacement. Int. J. Food Microbiol. 124, 98-102.

Burgess, C.M., Gianotti, A., Gruzdev, N., Holah, J., Knøchel, S., Lehner, A., Margas, E., Esser, S.S., Sela Saldinger, S., Tresse, O., 2016. The response of foodborne pathogens to osmotic and desiccation stresses in the food chain. I Int. J. Food Microbiol. 221, 37-3.

Cayley, S., Lewis, B.A., Record, M.T., 1992. Origins of the osmoprotective properties of betaine and proline in *Escherichia coli* K-12. J. Bacteriol. 174, 1586-1595.

Cempírková, R., 2012. Psychrotrophic vs. total bacterial counts in bulk milk samples. Vet. Med. Czech. 47, 227-233.

Chae, M.J., Cheney, D., Su, Y.C., 2009. Temperature Effects on the Depuration of *Vibrio parahaemolyticus* and *Vibrio vulnificusfrom* the American Oyster (*Crassostrea virginica*). J. Food Sci. 74, M62-M66.

Chan, Y.C., Wiedmann, M., 2008. Physiology and genetics of *Listeria Monocytogenes* survival and growth at cold temperatures. Crit. Rev. Food Sci. 49, 237–253.

Chen, J., Kudo, H., Kan, K., Kawamura, S., Koseki, S., 2018. Growth inhibitory effect of D-tryptophan on *Vibrio* spp. in shucked and live oysters. Appl. Environ. Microbiol. 84, e01543-18.

Cheng, R.S., Pomeranz, B., 1979. Correlation of genetic differences in endorphin systems with analgesic effects of D-amino acids in mice. Brain Res. 177, 583-587.

Cherwonogrodzky, J.W., Skinner, M.A., Clark, A.G., 1984. Effect of D-tryptophan on hemolysin production in V*ibrio parahaemolyticus*. J. Clin. Microbiol. 20, 909-911.

Chin, J.P., Megaw, J., Magill, C.L., Nowotarski, K., Williams, J.P., Bhaganna, P., Linton, M., Patterson, M.F., Underwood, G.J.C., Mswaka, A.Y., Hallsworth, J.E., 2010. Solutes determine the temperature windows for microbial survival and growth. P. Natl. Acad. Sci. USA 107, 7835-7840.

Condon, C., Squires, C., Squires, C.L., 1995. Control of rRNA transcription in *Escherichia coli*. Microbiol. Mol. Biol. Rev. 59, 623-645.

Cook, D.W., 2003. Sensitivity of Vibrio species in phosphate-buffered saline and in oysters to high-pressure processing. J. Food Protect. 66, 2276-2282.

Cook, D.W., 1994. Effect of time and temperature on multiplication of *Vibrio vulnificus* in postharvest Gulf Coast shellstock oysters. Appl. Environ. Microbiol. 60, 3483–3484.

Cousin, M.A., 1982. Presence and activity of psychrotrophic microorganisms in milk and dairy products: A Review. J. Food Protect. 45, 172-207.

Cruz-Romero, M., Kelly, A.L., Kerry, J.P., 2008. Effects of high-pressure treatment on the microflora of oysters (*Crassostrea gigas*) during chilled storage. Innov. Food Sci. Emerg. 9, 441-447.

Csapó, J., Albert, Cs., Csapóo-Kiss, Zs., 2009. The D-amino acid content of foodstuffs (a review). Acta Univ. Sapientiae Acta Univ Sapientiae Alimentaria 2, 5-30.

Davies, D., 2003. Understanding biofilm resistance to antibacterial agents. Nat. Rev. Drug Discov. 2, 114-122.

Ellison, R.K., Malnati, E., DePaola, D., Bowers, J.C., Rodrick, G.E. 2001. Populations of *Vibrio parahaemolyticus* in retail oysters from Florida using two methods. J. Food Protect. 64, 682-686.

Esteves, K., Hervio-Heath, D., Mosser, T., Rodier, C., Tournoud, M.-G., Jumas-Bilak, E., Colwell, R.R., Monfort, P., 2015. Rapid Proliferation of *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Vibrio cholerae* during Freshwater Flash Floods in French Mediterranean Coastal Lagoons. Appl. Environ. Microbiol. 81, 7600-7609.

Faleiro, M.L., Andrew, P.W., Power, D., 2003. Stress response of *Listeria monocytogenes* isolated from cheese and other foods. Int. J. Food Microbiol. 84, 207-216.

Fang, L., Wolmarans, B., Kang, M., Jeong, K.C., Wright, A.C., 2015. Application of chitosan microparticles for reduction of *Vibrio* species in seawater and live oysters (*Crassostrea virginica*). Appl. Environ. Microbiol. 81, 640-647.

Felbeck, H., Wiley, S., 2016. Free D-amino acids in the tissues of marine bivalves. Biol. bull-US 173, 252-259.

Fernandez-Piquer, J., Bowman, J.P., Ross, T., Tamplin, M.L., 2011. Predictive models for the effect of storage temperature on *Vibrio parahaemolyticus* viability and counts of total viable bacteria in pacific oysters (*Crassostrea gigas*). Appl. Environ. Microbiol. 77, 8687-8695.

Fleming, D.W., Cochi, S.L., MacDonald, K.L., 1985. Pasteurized milk as a vehicle of infection in an outbreak of *listeriosis*. Am. J. Infect. Control 13, 284-285.

Gandhi, M., Chikindas, M.L., 2007. *Listeria*: A foodborne pathogen that knows how to survive. Int. J. Food Microbiol. 113, 1-15.

Gandolfi, I., Palla, G., Delprato, L., Nisco, F.D., Marchelli, R., Salvadori, C., 1992. D-amino acids in milk as related to heat treatments and bacterial activity. J. Food Sci. 57, 377-379.

Gooch, J.A., DePaola, A., Bowers, J., 2002. Growth and survival of *Vibrio parahaemolyticus* in postharvest American oysters. J. Food Protect. 65, 970-974.

Greenway, D.L.A., England, R.R., 1999. The intrinsic resistance of *Escherichia coli* to various antimicrobial agents requires ppGpp and sigmas. Lett. Appl. Microbiol. 29, 323-326.

Greenwood, M.H., Roberts, D., Burden, P., 1991. The occurrence of *Listeria* species in milk and dairy products: a national survey in England and Wales. Int. J. Food Microbiol. 12, 197-206.

Hall-Stoodley, L., Costerton, J.W., Stoodley, P., 2004. Bacterial biofilms: from the Natural environment to infectious diseases. Nat. Rev. Microbiol. 2, 95-108.

Hantsis-Zacharov, E., Halpern, M., 2007. Culturable psychrotrophic bacterial communities in raw milk and their proteolytic and lipolytic traits. Appl. Environ. Microbiol. 73, 7162-7168.

Hashimoto, A., Nishikawa, T., Hayashi, T., Fujii, N., Harada, K., Oka, T., Takahashi, K., 1992. The presence of free D-serine in rat brain. FEBS Lett. 296, 33-36.

He, H., Adams, R.M., Farkas, D.F., Morrissey, M.T., 2002. Use of high-pressure processing for oyster shucking and shelf-life extension. J. Food Sci. 67, 640-645.

Hesselman, D.M., Motes, M.L., Lewis, J.P., 1999. Effects of a commercial heat-shock process on *Vibrio vulnificus* in the American oyster, *Crassostrea Virginica*, harvested from the Gulf Coast. J. Food Protect. 62, 1266-1269.

Ishida, A., Kawatake, Y., Ono, N., 1994. Osmotic stress conditioning for induction of acquired osmotolerance in *Escherichia coli*. J. Gen. Appl. Microbiol. 40, 35-42.

Ishio, S., Yamada, H., Hayashi, M., Yatsushiro, S., Noumi, T., Yamaguchi, A., Moriyama, Y., 1998. D-Aspartate modulates melatonin synthesis in rat pinealocytes. Neurosci. Lett. 249, 143-146.

Kan, K., Chen, J., Kawamura, S., Koseki, S., 2018. Characteristics of D-Tryptophan as an antibacterial agent: Effect of sodium chloride concentration and temperature on *Escherichia coli* growth inhibition. J. Food Protect. 81, 25-30.

Kimura, K., Inaoka, T., Yamamoto, K., 2018. Metabolome analysis of *Escherichia coli* ATCC 25922 cells treated with high hydrostatic pressure at 400 and 600 MPa. J. Biosci. Bioeng. 126, 611-616.

Ko, R., Smith, L.T., Smith, G.M., 1994. Glycine betaine confers enhanced osmotolerance and cryotolerance on *Listeria monocytogenes*. J. Bacteriol. 176, 426-431.

Koseki, S., Nakamura, N., Nakamura, N., Shiina, T., Shiina, T., 2015. Growth inhibition of *Listeria monocytogenes*, *Salmonella enterica*, and *Escherichia coli* O157:H7 by D-Tryptophan as an incompatible solute. J. Food Protect. 78, 819-824.

Lafarge, V., Ogier, J.C., Girard, V., Maladen, V., Leveau, J.Y., Gruss, A., Delacroix-Buchet, A., 2004. Raw cow milk bacterial population shifts attributable to refrigeration. Appl. Environ. Microbiol. 70, 5644-5650.

Leblanc, L., Leroi, F., Hartke, A., Auffray, Y., 2000. Do stresses encountered during the smoked salmon process influence the survival of the spoiling bacterium *Shewanella putrefaciens*? Lett. Appl. Microbiol. 30, 437-442.

LeJeune, J.T., Rajala Schultz, P.J., 2009. Unpasteurized milk: A continued public health threat. Clin Infect Dis 48, 93-100.

Li, H., Ye, Y., Ling, N., Wu, Q., Zhang, J., 2015. Inhibitory effects of D-tryptophan on biofilm development by the foodborne *Cronobacter sakazakii*. Int Dairy J 49, 125-129.

Lianou, A., Sofos, J.N., 2007. A review of the incidence and transmission of *Listeria monocytogenes* in ready-to-eat products in retail and food service environments. J. Food Protect. 70, 2172-2198.

Linton, M., Mc Clements, J.M.J., Patterson, M.F., 2003. Changes in the microbiological quality of shellfish, brought about by treatment with high hydrostatic pressure. Int. J Food Sci. Tech. 38, 713-727.

Liu, C., Lu, J., Su, Y.-C., 2009. Effects of flash freezing, followed by frozen storage, on reducing *Vibrio parahaemolyticus* in pacific raw oysters (*Crassostrea gigas*). J. Food Protect. 72, 174-177.

Magan, N., Pavlou, A., Chrysanthakis, I., 2001. Milk-sense: a volatile sensing system recognises spoilage bacteria and yeasts in milk. Sensor Actuat. B-Chem. 72, 28-34.

Mahmoud, B.S.M., 2009. Reduction of *Vibrio vulnificus* in pure culture, half shell and whole shell oysters (*Crassostrea virginic*a) by X-ray. Int. J. Food Microbiol. 130, 135-139.

Man, E.H., nutrition, Bada, J.L., 1987. Dietary D-amino acids. Annu. Rev. Nurr. 7, 209-225.

Manning, J.M., Merrifield, N.E., Jones, W.M., Gotschlich, E.C., 1974. Inhibition of bacterial growth by beta-chloro-D-alanine. Proc. Natl. Acad. Sci. U. S. A. 71, 417-421.

Maria-Rosario, A., Davidson, I., Debra, M., Verheul, A., Abee, T., Booth, I.R., 1995. The role of peptide metabolism in the growth of *Listeria monocytogenes* ATCC 23074 at high osmolarity. Microbiology 141, 41-49.

Masters, P.M., Friedman, M., 1980. Amino acid racemization in alkali treated food proteins chemistry, toxicology, and nutritional consequences. *In Chemical Deterioration of Proteins* (ACS Symposium Series, No. 123) (J.R. Whitaker and M. Fujimaki, eds.) pp. 165-194, American Chemical Society, Washington, DC.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., Tauxe, R.V., 1999. Food-related illness and death in the United States. Emerging Infect. Dis. 5, 607-625.

Mellefont, L.A., McMeekin, T.A., Ross, T., 2003. The effect of abrupt osmotic shifts on the lag phase duration of foodborne bacteria. Int. J. Food Microbiol. 83, 281-293.

Milner, J.L., McClellan, D.J., Wood, J.M., 1987. Factors reducing and promoting the effectiveness of proline as an osmoprotectant in *Escherichia coli* K12. Microbiology 133, 1851-1860.

Moghaddam, J.A., Boehringer, N., Burdziak, A., Kunte, H.-J., Galinski, E.A., Schäberle, T.F., 2016. Different strategies of osmoadaptation in the closely related marine myxobacteria *Enhygromyxa salina* SWB007 and *Plesiocystis pacifica* SIR-1. Microbiology 162, 651-661.

Moran, N.A., Jarvik, T., 2010. Lateral transfer of genes from fungi underlies carotenoid production in aphids. Science 328, 624-627.

Motes, M.L., DePaola, A., Cook, D.W., Veazey, J.E., Hunsucker, J.C., Garthright, W.E., Blodgett, R.J., Chirtel, S.J., 1998. Influence of water temperature and salinity on *Vibrio vulnificus* in Northern Gulf and Atlantic Coast oysters (*Crassostrea virginica*). Appl. Environ. Microbiol. 64, 1459-1465.

Murphy, S.K., Oliver, J.D., 1992. Effects of temperature abuse on survival of *Vibrio vulnificus* in oysters. Appl. Environ. Microbiol. 58, 2771-2775.

Newton, A., Kendall, M., Vugia, D.J., Henao, O.L., Mahon, B.E., 2012. Increasing rates of *Vibriosis* in the United States, 1996-2010: Review of surveillance data from 2 systems. Clin. Infect. Dis. 54(Suppl. 5): S391-S395.

O'Byrne, C.P., Booth, I.R., 2002. Osmoregulation and its importance to food-borne microorganisms. Int. J. Food Microbiol. 74, 203-216.

Ohashi, Y., Hirayama, A., Ishikawa, T., Nakamura, S., Shimizu, K., Ueno, Y., Tomita, M., Soga, T., 2008. Depiction of metabolome changes in histidine -starved *Escherichia coli* by CE-TOFMS. Mol. Biosyst. 4, 135-147.

Pace, J., Wu, C.Y., Chai, T., 1988. Bacterial flora in pasteurized oysters after refrigerated storage. J. Food Sci. 53, 325-327.

Palla, G., Marchelli, R., Dossena, A., Casnati, G., 1989. Occurrence of D-amino acids in food. J. Chromatogr. A 475, 45-53.

Patterson, M.F., McKay, A.M., Connolly, M., Linton, M., 2010. Effect of high pressure on the microbiological quality of cooked chicken during storage at normal and abuse refrigeration temperatures. Food microbiol. 27, 266-273.

Poolman, B., Glaasker, E., 1998. Regulation of compatible solute accumulation in bacteria. Mol. Microbiol. 29, 397-407.

Quan, Y., Quan, Y., Choi, K.-D., Choi, K.D., Chung, D., Chung, D., Shin, I.-S., Shin, I.S., 2010. Evaluation of bactericidal activity of weakly acidic electrolyzed water (WAEW) against *Vibrio vulnificus* and *Vibrio parahaemolyticus*. Int. J. Food Microbiol. 136, 255-260.

Reina, L.D., Jin, Z.T., Zhang, Q.H., Yousef, A.E., 1998. Inactivation of *Listeria monocytogenes* in milk by pulsed electric field. J. Food Protect. 61, 1203-1206.

Ren, T., Su, Y.C. 2006. Effects of electrolyzed oxidizing water treatment on reducing *Vibrio parahaemolyticus* and *Vibrio vulnificus* in raw oysters. J Food Protect. 69, 1829-1834.

Rosenow, E.M., Marth, E.H., 1987. Growth of *Listeria monocytogenes* in skim, whole and chocolate milk, and in whipping cream during incubation at 4, 8, 13, 21 and 35°C. J. Food Protect. 50, 452-459.

Russell, N.J., 2002. Bacterial membranes: the effects of chill storage and food processing. An overview. Int. J. Food Microbiol. 79, 27-34.

Sasaki, H., Takaki, A., Oshima, A., Ishida, A., Nagata, S., 2007. Comparison of the function of L- and D-proline as compatible solute in *Escherichia coli* K-12 under high osmolarity. Annal. Microbiol. 57: 265-268.

Schlammadinger, J., Szabó, G., 1973. The effect of D-tryptophan upon induced beta-galactosidase synthesis in *Escherichia coli*. Acta Biochim. Biophys. Acad. Sci. Hung, 8, 257-259.

Shahjee, H.M., Banerjee, K., Ahmad, F., 2002a. Comparative analysis of naturally occurring L-amino acid osmolytes and their D-isomers on protection of *Escherichia coli* against environmental stresses. J. Biosciences 27, 515-520.

Shaw, K.S., Rosenberg Goldstein, R.E., He, X., Jacobs, J.M., Crump, B.C., Sapkota, A.R., 2014. Antimicrobial susceptibility of *Vibrio vulnificus* and *Vibrio parahaemolyticus* recovered from recreational and commercial areas of Chesapeake Bay and Maryland Coastal Bays. PLoS One 9, e89616.

Shivanand, P., Mugeraya, G., 2011. Halophilic bacteria and their compatible solutes–osmoregulation and potential applications. Curr. Sci. 100, 1516-1521.

Stevens, E.R., Esguerra, M., Kim, P.M., Newman, E.A., Snyder, S.H., Zahs, K.R., Miller, R.F., 2003. D-serine and serine racemase are present in the vertebrate retina and contribute to the physiological activation of NMDA receptors. Proc. Natl. Acad. Sci. U. S. A. 100, 6789-6794.

Tompkin, R.B., Scott, V.N., Bernard, D.T., Sveum, W.H., Gombas, K.S., 1999. Guidelines to prevent post-processing contamination from *Listeria monocytogenes*. Dairy Food Environ. Sanit. 19: 551-562.

Vasseur, C., Baverel, L., Hebraud, M., Labadie, J., 1999. Effect of osmotic, alkaline, acid or thermal stresses on the growth and inhibition of *Listeria monocytogenes*. J. Appl. Microbiol. 86, 469-476.

Wemekamp-Kamphuis, H.H., Sleator, R.D., Wouters, J.A., Hill, C., Abee, T., 2004. Molecular and physiological analysis of the role of osmolyte transporters BetL, Gbu, and OpuC in growth of *Listeria monocytogenes* at low temperatures. Appl. Environ. Microbiol. 70, 2912-2918.

Weymarn, Von, N., Nyyssölä, A., Reinikainen, T., Leisola, M., Ojamo, H., 2001. Improved osmotolerance of recombinant *Escherichia coli* by *de novo* glycine betaine biosynthesis. Appl. Microbiol. Biotechnol. 55, 214-218.

Wong, H.C., Peng, P.Y., Lan, S.L., Chen, Y.C., Lu, K.H., Shen, C.T., Lan, S.F., 2002. Effects of heat shock on the thermotolerance, protein composition, and toxin production of *Vibrio parahaemolyticus*. J. Food Protect. 65, 499-507.

Wood, J.M., Bremer, E., Csonka, L.N., Kraemer, R., Poolman, B., van der Heide, T., Smith, L.T., 2001. Osmosensing and osmoregulatory compatible solute accumulation by bacteria. Comp. Biochem. Physiol. A 130, 437-460.

Antibacterial activity of D-tryptophan against foodborne pathogenic bacteria: application to food processing and investigation of the mechanism

March, 2018

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