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Growth Properties and Sensitivities to Various Bactericidal Methods of Cold-Tolerant Microorganisms Isolated from Packed Tofu

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Abstract: Disinfection and sterilization against cold-tolerant microorganisms are very important for enhancing food safety and hygiene management under refrigeration conditions. We isolated *Exiguobacterium* sp. and *Pantoea* sp. from an immersion solution of packed tofu and evaluated their growth properties and sensitivities to various bactericidal methods. These microorganisms were classified as psychrotrophic bacteria, according to their ability to grow at 4 °C, with an optimum growth temperature of 30 °C. *Exiguobacterium* sp. and *Pantoea* sp. were more sensitive to heat and sodium hypochlorite than *Escherichia coli* (*E. coli*). *Exiguobacterium* sp. was sensitive to acetic acid and citric acid, while *Pantoea* sp. was relatively insensitive and *E. coli* was insensitive to these organic acids. All microorganisms used in this study were resistant to sodium hydroxide solution (pH 8.00). *Exiguobacterium* sp. showed resistance to ozonated water and UV; therefore, particular attention should be paid to the contamination of this microorganism in food factories. Both *Exiguobacterium* sp. and *Pantoea* sp. were inactivated by pulsed electric field (PEF) treatment that did not cause lethal damage to *E. coli*. The results obtained in this study suggest that the bactericidal methods targeting the fragile cell membrane with high permeability are effective for the inactivation of cold-tolerant microorganisms.

Keywords: sterilization; tofu; cold tolerance; psychrotrophic bacteria; pulsed electric field

Citation: Matsui, M.; Tanino, T.; Ito, M.; Nomura, C.; Guionet, A.; Takahashi, K.; Takaki, K.; Ohshima, T. Growth Properties and Sensitivities to Various Bactericidal Methods of Cold-Tolerant Microorganisms Isolated from Packed Tofu. *Agronomy* **2022**, *12*, 233. <https://doi.org/10.3390/agronomy12020233>

Academic Editor: Sung-Cheol Koh

Received: 26 December 2021

Accepted: 17 January 2022

Published: 18 January 2022

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1. Introduction

From the standpoint of food safety and hygiene management, the methods of microbial control, for example, bacteriostatic treatment, decontamination, disinfection, and sterilization are very important. Since the most common food spoilage microorganisms are mesophiles [1], refrigeration and freezing are commonly employed as effective food preservation methods. However, the deterioration and spoilage of chilled food occurs frequently by cold-tolerant microorganisms, such as psychrophilic and psychrotrophic bacteria [2]. With the growth of the global cold chain market size, there is a great demand for enhancing the microbiological safety and quality of various foods under refrigeration conditions, especially perishable ones. Hence, the isolation, identification, and inactivation of cold-tolerant food spoilage microorganisms have been recognized as significant research issues.

Tofu is a gel-like food, prepared by coagulating soy milk. Owing to the health benefits of tofu as an excellent protein source with high quality nutrients, it has become very popular

all over the world. Tofu is particularly susceptible to microbial contamination because of its high moisture (>80%), high protein content [3,4] (6–8.4%) [5], and near-neutral pH, in the range of 5.8 to 6.2 [4]. Thus, the shelf life of tofu is very short, even under preservation conditions like refrigeration. For example, the shelf lives of fresh and packed tofu in Japan are 1 to 5 days and about 1 week, respectively [6]. The isolation and identification of cold-tolerant food spoilage microorganisms from tofu have been performed from the standpoint of microbiological quality management [3,7,8], estimation of shelf life [9], and quantitative microbial risk assessment [10]. In order to improve the microbiological safety and quality of tofu, it is necessary to determine the effective bactericidal methods for these microorganisms. However, the sensitivities of these microorganisms toward various bactericidal methods have not been sufficiently investigated.

In this study, we aimed to isolate cold-tolerant food spoilage microorganisms from packed tofu and to evaluate their growth properties and sensitivities to various bactericidal methods. To obtain ozone-resistant microorganisms, the filling water of the packed tofu was substituted with 5 ppm ozonated water. *Exiguobacterium* sp. and *Pantoea* sp. were isolated from an immersion solution of packed tofu stored at 4 °C for 6 days. The growth properties of each microorganism in soy milk were evaluated from growth curves obtained at different temperatures, ranging from 4 to 37 °C. Furthermore, sensitivities of these microorganisms toward various bactericidal methods, such as heat, sodium hypochlorite (NaOCl), organic acids, alkaline, ozone, ultraviolet (UV), and pulsed electric field (PEF) were investigated. The results were compared with those of *Escherichia coli* (*E. coli*), which is recognized as a hygiene indicator organism in foods [11]. The factors affecting the sensitivity of each microorganism to various bactericidal methods were also discussed, based on the difference in the structure of cells and functions of intracellular components.

2. Materials and Methods

2.1. Isolation and Identification of Microorganisms

The packed tofu used in this study was purchased at a grocery store in Japan. Ozonated water was made from tap water using an ozonizer (Masuda Research Inc., Tokyo, Japan). To remove chlorine by evaporation, tap water was left to stand at room temperature overnight. The concentration of ozonated water was determined by the measurement of the absorbance at 260 nm by a spectrophotometer (UV-mini 1240: Shimadzu Corp., Kyoto, Japan). The filling water of the packed tofu was substituted with 5 ppm ozonated water, and the pack was sealed again, with the sample then stored at 4 °C. An aliquot of the filling solution collected after 6 days was serially diluted with distilled water and 0.1 mL of each dilution was plated on a nutrient agar plate (Bacto peptone 10 g/L, Bacto beef extract 5 g/L, NaCl 5 g/L, agar 15 g/L). After incubation at 37 °C for 24 h, isolated orange and yellow colonies were obtained. Samples of isolated microorganisms were sent to BEX Co., Ltd., Tokyo, Japan and identified by 16S rRNA gene sequence analysis. Polymerase chain reaction (PCR) amplification of the 16S rRNA genes of isolates was carried out using the primers 10F (5'-GTTTGATCCTGGCTCA-3') and 800R (5'-TACCAGGGTATCTAATCC-3'). The PCR products were sequenced using an automated DNA sequencer. Taxonomic identification was performed using Basic Local Alignment Search Tool (BLAST) database [12].

2.2. Culture of Microorganisms at Different Temperatures

The isolated microorganisms (*Exiguobacterium* sp. and *Pantoea* sp.) were cultivated in 10 mL of nutrient broth (Bacto peptone 10 g/L, Bact beef extract 5 g/L, NaCl 5 g/L) at 37 °C for 18 h with shaking. After cultivation, 0.1 mL of each cell culture was inoculated into 10 mL of soy milk (Kikkoman Co., Ltd., Chiba, Japan) and then cultivated at different temperatures ranging from 4 to 37 °C for various periods up to 6 days with shaking. A pharmaceutical refrigerator with freezer (SANYO Medicoool MPR-411F, SANYO Electric Co., Ltd., Osaka, Japan) and a laboratory incubator (SANYO Incubator MIR-151, SANYO Electric Co., Ltd., Osaka, Japan) were used to maintain culture temperatures at 4 °C and at 10–37 °C, respectively. Cell cultures of 0.4 mL were collected at constant time intervals and

serially diluted with distilled water. Next, 0.1 mL of the dilutions were plated on nutrient agar plates. The plates were incubated at the same temperature at which the collected cell culture was cultivated. After incubation for various periods, colonies were counted to determine the numbers of colony forming units (CFU). Growth curves were obtained by plotting CFU/mL against the cultivation time. The doubling time and specific growth rate were calculated to evaluate the growth characteristics of each microorganism.

2.3. Preparation of Cell Suspensions

Exiguobacterium sp. and *Pantoea* sp. were cultivated in 10 mL of nutrient broth at 4 °C for 5 days with shaking. After cultivation, the cells of each microorganism were collected using a centrifuge (10,000 rpm, 5 min, 4 °C) and washed twice with distilled water. The cells suspended in distilled water were collected using a centrifuge (10,000 rpm, 5 min, 4 °C) and the pelleted cells were resuspended in distilled water. The initial cell concentration of each microorganism was about 10^7 – 10^9 CFU/mL.

E. coli K-12 strain was cultivated in 10 mL of Luria–Bertani (LB) medium (Bacto peptone 10 g/L, Bacto yeast extract 5 g/L, NaCl 5 g/L) at 37 °C for 18 h with shaking. After cultivation, *E. coli* cells were collected using a centrifuge (10,000 rpm, 5 min, 4 °C) and washed twice with distilled water. The cells suspended in distilled water were collected using a centrifuge (10,000 rpm, 5 min, 4 °C) and the pelleted cells were resuspended in distilled water. The initial cell concentration was about 10^7 – 10^9 CFU/mL.

2.4. Bactericidal Methods

2.4.1. Heat Treatment

Cell suspensions of 5 mL were dispensed into test tubes and held in a thermostatic water bath (Shimadzu Water Bath BW-4: Shimadzu Corp., Kyoto, Japan) at 45, 50, 55, and 60 °C for 20 min. Then, the suspensions of 0.4 mL were collected at 5 min intervals and immediately quenched in an ice bath. The collected samples were serially diluted with distilled water and 0.1 mL of the dilutions of *Exiguobacterium* sp. and *Pantoea* sp. cells, and *E. coli* cells were plated on nutrient agar plates and LB agar plates (Bacto peptone 10 g/L, Bacto yeast extract 5 g/L, NaCl 5 g/L, agar 15 g/L), respectively. After incubation at 37 °C for 24 h, colonies were counted to determine the number of CFUs. Each experiment was performed three times. The data in the figures are means of the experimental results and error bars indicate maximum and minimum values.

2.4.2. Ozonated Water Treatment

Ozonated water was made from tap water using the ozonizer. To remove chlorine by evaporation, tap water was left to stand at room temperature overnight. The concentration of ozonated water was determined by the measurement of the absorbance at 260 nm by the spectrophotometer. Cell suspensions of 3 mL were dispensed into test tubes and equal volumes of ozonated water were added to obtain final ozone concentrations of 0.5, 1, and 1.5 ppm. The mixtures were left to stand at 20 °C for 9 min, and sample solutions of 0.4 mL were collected at 3 min intervals. The collected samples were serially diluted with distilled water, and 0.1 mL of the dilutions of *Exiguobacterium* sp. and *Pantoea* sp. cells and *E. coli* cells were plated on nutrient agar plates and LB agar plates, respectively. After incubation at 37 °C for 24 h, colonies were counted to determine the number of CFUs. Each experiment was performed three times. The data in the figures are means of the experimental results and error bars indicate maximum and minimum values.

2.4.3. UV Irradiation

Cell suspensions of 30 mL were dispensed into glass Petri dishes (90 mm in diameter) and placed below the germicidal lamp (Mitsubishi GL 15: Mitsubishi Electric Corp., Tokyo, Japan) on a clean bench. The distance between the germicidal lamp and the liquid surface was 507 mm, and the thickness of cell suspension was about 6 mm. Cell suspensions were stirred at 900 rpm using a magnetic stirrer (CT-1AN Pasolina mini stirrer: AS ONE

Corp., Osaka, Japan) during the UV irradiation at room temperature and cell suspensions of 0.4 mL were collected at 1 min intervals for up to 3 min. The collected samples were serially diluted with distilled water and 0.1 mL of the dilutions of *Exiguobacterium* sp. and *Pantoea* sp. cells and *E. coli* cells were plated on nutrient agar plates and LB agar plates, respectively. After incubation at 37 °C for 24 h, colonies were counted to determine the number of CFUs. Each experiment was performed three times. The data in the figures are means of the experimental results and error bars indicate maximum and minimum values.

2.4.4. Sodium Hypochlorite Treatment

Sodium hypochlorite solution (197-02206, FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) and sodium thiosulfate pentahydrate (197-03585, FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) were used as received. Cell suspensions of 1 mL were dispensed into test tubes, and equal volumes of diluted sodium hypochlorite solutions were added to obtain final sodium hypochlorite concentrations of 0.25, 0.5, 1, 2, and 5 ppm, respectively. The mixtures were allowed to stand for 5 min at room temperature, and 0.1 mL of sample solutions were collected at 30 s intervals for the first 1 min and at 2 min intervals for the next 4 min. The collected samples were immediately neutralized with sodium thiosulfate solutions and serially diluted with distilled water. Then, 0.1 mL of the dilutions of *Exiguobacterium* sp. and *Pantoea* sp. cells and *E. coli* cells were plated on nutrient agar plates and LB agar plates, respectively. After incubation at 37 °C for 24 h, colonies were counted to determine the number of CFUs. Each experiment was performed three times. The data in the figures are means of the experimental results and error bars indicate maximum and minimum values.

2.4.5. Organic Acids and Alkaline Treatments

Acetic acid (017-00251, FUJIFILM Wako Pure Chemical Corp., Osaka, Japan), citric acid (030-05525, FUJIFILM Wako Pure Chemical Corp., Osaka, Japan), hydrochloric acid (080-01066, FUJIFILM Wako Pure Chemical Corp., Osaka, Japan), and sodium hydroxide (198-13765, Wako Pure Chemical Corp., Osaka, Japan) were used as received. Cell suspensions of 1 mL were dispensed into test tubes, and equal volumes of acetic acid solutions (pH 2.68), citric acid solutions (pH 2.00), and sodium hydroxide solutions (pH 8.00) were added. The mixtures were left to stand for 5 min at room temperature. Sample solutions of 0.1 mL were collected at 30 s intervals for the first 1 min and at 2 min intervals for the next 4 min. The collected acidic sample solutions and basic sample solutions were immediately neutralized with sodium hydroxide solutions and hydrochloric acid solutions, respectively, and serially diluted with distilled water. Then, 0.1 mL of the dilutions of *Exiguobacterium* sp. and *Pantoea* sp. cells and *E. coli* cells were plated on nutrient agar plates and LB agar plates, respectively. After incubation at 37 °C for 24 h, colonies were counted to determine the number of CFUs. Each experiment was performed three times. The data in the figures are means of the experimental results and error bars indicate maximum and minimum values.

2.4.6. PEF Treatment Chamber and PEF Treatment Procedure

The PEF treatment chamber was composed of stainless steel plates, acrylic plates, silicone sheets, resin bolts and nuts. Stainless steel plates (1 mm thickness) used as the high voltage electrode and the earth electrode were placed on both sides of an acrylic plate (100 mm × 100 mm, 10 mm thickness) with a hole (30 mm in diameter) and an inlet at the top. These components, silicone sheets (1 mm thickness), and acrylic plate (3 mm thickness) were fixed by resin bolts and nuts, and the resulting gap between the electrodes was 10 mm.

A pulsed high voltage of 15 kV was generated with a gap-type pulse generator (Masuda Research Inc., Tokyo, Japan) with a frequency of 50 Hz. The cell suspension of 8 mL was poured into the chamber, then pulsed high voltages were applied for 1 min. The suspensions of 0.4 mL were collected at 30 s intervals. The collected samples were serially diluted with distilled water and 0.1 mL of the dilutions of *Exiguobacterium* sp. and *Pantoea* sp. cells and *E. coli* cells were plated on nutrient agar plates and LB agar plates,

respectively. After incubation at 37 °C for 24 h, colonies were counted to determine the number of CFUs. Each experiment was performed three times. The data in the figures are means of the experimental results and error bars indicate maximum and minimum values.

3. Results and Discussion

3.1. Isolation, Identification, and Growth Properties of Microorganisms

Two types of microorganisms, forming orange and yellow colonies on nutrient agar plates, were isolated from an ozonated immersion solution of packed tofu, stored at 4 °C for 6 days (Figure 1). This time period corresponds to 4 days after the shelf-life date of the selected tofu sample. Thus, these microorganisms may be involved in the microbial deterioration and spoilage of tofu. Since the tofu sample was preserved under refrigerated conditions in a grocery store and in our laboratory, they could be considered to be cold-tolerant microorganisms. The microbial isolates, with orange and yellow colonies, were identified as *Exiguobacterium* sp. and *Pantoea* sp., respectively, based on the 16S rRNA gene sequence analysis.

Figure 1. The microbial isolates with orange (a) and yellow colonies (b) from an ozonated immersion solution of packed tofu stored at 4 °C for 6 days.

Bacteria of the genus *Exiguobacterium* are Gram-positive, non-sporing, facultative anaerobes [13] with varying morphologies, depending on the strain and growth conditions [14]. *Exiguobacterium* spp. have been isolated or molecularly detected not only from natural habitats, including a pond [15], a lake [16], and a tidal flat [17], but also extreme environments, such as permafrost [14,18–20], a hot spring [21], the alkaline effluent of a potato-processing factory [13,22], the drain of a fish processing plant with high hydrogen peroxide (H₂O₂) concentrations [23], high altitude lakes located at 4400 m [24], a radioactive region [25], and the deep sea at 3044 m depth [26]. Strains from these extreme environments exhibit resistance to low temperature [14,18–20], high temperature [21], high pH [13,22], high concentrations of H₂O₂ [23], high salinity and UV [24], radiation [25], and high pressure [26]. The adaptability of these strains to various extreme environments is attracting great biological interest, though these unique properties are also very troublesome from the standpoint of microbial control in food safety and hygiene management. For example, when fresh and processed foods are contaminated with cold-tolerant strains that can grow at −6 °C [14], −3 °C [20], −2.5 °C [18,19], 4 °C [18,20,23], and 5 °C [27], it is difficult to prevent the microbial deterioration and spoilage of the foods, even under refrigeration or freezing conditions. The effect of bacteria of the genus *Exiguobacterium* on the quality of tofu has not been clearly elucidated, but the contribution of a bacterium belonging to the same genus as the spoilage of water-boiled salted duck at 4 °C has been reported [28]. Since tofu is a protein-rich food like meat, the risk of its deterioration or spoilage by bacteria of the genus *Exiguobacterium* should be considered concerning. To the best of our knowledge, this study is the first report of a bacterium of the genus *Exiguobacterium* being isolated from tofu. Although no *Exiguobacterium* strains have been isolated or molecularly detected from soybeans, *Exiguobacterium indicum* (*E. indicum*) has been isolated from the various utensils and tools used during the tofu manufacturing process [29]. Therefore, the equipment used is considered to be a potential source and risk factor for the microbial contamination of tofu.

Bacteria of the genus *Pantoea* are Gram-negative, non-sporing, facultative anaerobes, shaped like a straight rod [30]. *Pantoea* spp. have been isolated or molecularly detected from a wide variety of sources, such as river water [31], spring water [32], soil [33–35], various plants [30,36–45], humans [46,47], and other animals [48,49]. Some strains possess beneficial properties, such as endophytes with plant growth-promoting abilities [41,42,45] and biocontrol agents, which can counter bacterial and fungal plant diseases [39,44]. In contrast, others exhibit deleterious characteristics, such as plant pathogens [40,43] and ice nucleation-active bacteria, that cause frost damage to plants [50,51]. Several cold-tolerant strains that are capable of growth at 3 °C [39], 5 °C [33–35], 8 °C [52], and 10 °C [51] have also been reported. When fresh and processed foods are contaminated with these strains, it is difficult to prevent the microbial deterioration and spoilage of the foods, even under refrigeration conditions. In fact, *Pantoea agglomerans* (*P. agglomerans*, synonym: *Enterobacter agglomerans*) was isolated from spoiled tofu at 2–3 °C [7] and at 7 °C [8]. Fouad et al. reported that this strain caused a putrid odor in spoiled tofu [7]. Since several strains of the genus *Pantoea* have been isolated or molecularly detected from leaves [36–38,40] and the rhizosphere [33] of soybeans and humans [46,47], it is assumed that the ingredients and workers are potential sources and risk factors for the microbial contamination of tofu.

Growth properties of *Exiguobacterium* sp. and *Pantoea* sp. were evaluated from growth curves obtained at different temperatures, ranging from 4 to 37 °C (Figure 2a). Assuming the contamination of these microorganisms during the process of tofu manufacturing, soy milk was selected as the culture medium. Since the proliferation of *Exiguobacterium* sp. and *Pantoea* sp. was observed at all cultivation temperatures, it was confirmed that these microorganisms can utilize soy milk as a nutrient source. Interestingly, these microorganisms were able to grow at refrigeration temperatures (4 and 10 °C). Although the growth of each microorganism was slow, at 10 °C or below, the maximum cell concentration in the stationary phase reached about 10⁹ CFU/mL, this value being almost the same as those obtained at room temperature (15–25 °C) or above. When these microorganisms contaminate packed tofu, they continue to grow, even under refrigeration conditions, and it is estimated that after 4 to 5 days, the cell concentration will reach the same level as that of packed tofu left at room temperature for half a day. These results suggest that these microorganisms may be involved in the microbial deterioration and spoilage of tofu under refrigerated conditions. To understand the growth properties of *Exiguobacterium* sp. and *Pantoea* sp. in more detail, the specific growth rate and doubling time of each microorganism was calculated using following equations:

$$\mu t = \ln \frac{n}{n_0} \quad (1)$$

where μ is the specific growth rate (1/time), t is the cultivation time, n_0 is the initial number of cells, n is the number of cells after time t .

$$t_d = \frac{\ln 2}{\mu} \quad (2)$$

where t_d is the doubling time, μ is the specific growth rate (1/time). The optimal growth temperature for each microorganism was estimated to be around 30 °C, at which the doubling time was at its minimum and the specific growth rate was at its maximum (Figure 2b,c). The value was close to the optimal temperatures of other strains, for example, 34 °C for *E. oxidotolerans* T-2-2^T [23] and 30 °C for *P. agglomerans* CPA-2 [39]. According to Morita's definition [53], *Exiguobacterium* sp. and *Pantoea* sp. were classified as psychrotrophic bacteria because they can grow at low temperatures (0–5 °C) with an optimum growth temperature above 20 °C.

Figure 2. Growth curves of *Exiguobacterium* sp. and *Pantoea* sp. at different temperatures (a). Doubling time and specific growth rate of *Exiguobacterium* sp. (b) and *Pantoea* sp. (c). Symbols indicate doubling time (closed circles) and specific growth rate (open circles).

3.2. Sensitivities of Each Microorganism to Various Bactericidal Methods

3.2.1. Heat Treatment

Heat treatment has been widely used in the food industry because of its capacity to inactivate vegetative cells, bacterial spores, yeasts, and molds [54]. In most cases, especially under severe treatment conditions, such as sterilization above 100 °C, the organoleptic and nutritional properties of foods are severely modified [54]. To minimize the required heat treatment, determining the heat resistance of microorganisms is very important. In this study, heat treatment of microorganisms was performed at different temperatures ranging from 45 to 60 °C (Figure 3). The viable cell density of *Exiguobacterium* sp. maintained almost the same level as the initial value, at 45 °C for 20 min. At higher temperatures, it was reduced slowly over time and was not detected at 50 °C for 15 min, and at 55 °C or higher for 5 min, respectively (Figure 3a). Similar behavior was observed for *Pantoea* sp., although the time to reach almost undetectable levels at 50 °C was shortened to 10 min (Figure 3b). *E. coli* showed the highest heat resistance among these microorganisms. At 50 °C, only 1 log reduction in CFU/mL was observed. No viable cells were detected at 55 °C for 20 min (Figure 3c). The activation energies for thermal inactivation of each microorganism were calculated from the slopes of the Arrhenius plots. The activation energy values were 826, 605, and 480 kJ/mol for *Exiguobacterium* sp., *Pantoea* sp., and *E. coli*, respectively. The values for *Exiguobacterium* sp. and *Pantoea* sp. are higher than for *E. coli*, suggesting that their heat tolerance properties are theoretically lower than that of *E. coli*. In fact, these microorganisms are inactivated at lower temperatures, with shorter treatment times than *E. coli* (Figure 3). The experimental data shows good agreement with the theoretical result. We predicted that

soybeans could be the potential source of contamination of several strains belonging to the genus *Pantoea* for tofu. If the lethal temperatures for these strains are comparable to that for *Pantoea* sp., our prediction is not acceptable because ground soybeans are cooked by steam above 100 °C in the tofu production process [7].

Figure 3. Effect of heat treatment on *Exiguobacterium* sp. (a), *Pantoea* sp. (b), and *E. coli* (c). Symbols indicate temperatures of 45 °C (closed circles), 50 °C (closed triangles), 55 °C (closed squares), and 60 °C (open circles). N.D. means not detected.

3.2.2. Sodium Hypochlorite Treatment

Sodium hypochlorite is the most common disinfectant in the food industry. Typically, fruits, vegetables, and cooking utensils are immersed in 50–200 ppm sodium hypochlorite solution [55,56] for 5 min [56]. When cell suspensions of *Exiguobacterium* sp., *Pantoea* sp., and *E. coli* were treated with sodium hypochlorite solution under the above-mentioned conditions, no viable cells were detected within 30 s (data not shown). Thus, the bactericidal effect of sodium hypochlorite was evaluated at one tenth of the minimum concentration of the normal operating condition (50 ppm) or lower (Figure 4). The viable cell density of *Exiguobacterium* sp. decreased by approximately 1 log, 1 log, and 4 log CFU/mL, at 0.25, 0.5, and 1 ppm for 5 min, respectively (Figure 4a). At 2 ppm or higher, it was not detected within 30 s. Although no bactericidal effect against *Pantoea* sp. was observed at 0.25 ppm, around a 3 log and 5 log reduction in CFU/mL was achieved at 0.5 and 1 ppm for 5 min, respectively (Figure 4b). No viable cells were detected at 2 ppm for 3 min and 5 ppm for 30 s. For *E. coli*, no significant reduction in viable cell density was observed at 0.25 to 2 ppm (Figure 4c). No viable cells were detected at 5 ppm for 3 min. The sensitivities of *Exiguobacterium* sp. and *Pantoea* sp. to sodium hypochlorite were higher than that of *E. coli*. In an aqueous solution, chlorine exists in chlorine gas (Cl_2), hypochlorous acid (HOCl), and hypochlorite ion (OCl^-), and Cl_2 readily escapes from the solution. HOCl exhibits a greater germicidal property than OCl^- because it can penetrate the cell membranes [57]. Since their relative proportions are dependent on the pH of the solution, the pH level and the available chlorine content should be considered to evaluate the bactericidal effect of the sodium hypochlorite solution. The pH values of 0.25, 0.5, 1, 2, and 5 ppm sodium hypochlorite solutions were 6.11, 6.29, 6.89, 7.68, and 8.19, respectively. At 0.25, 0.5, and 1 ppm, HOCl is the major component because the percentages of this molecule are calculated at about 96, 94, and 80%, respectively. Under these conditions, the permeability of the cell membrane of each microorganism would be related to their sensitivity to sodium hypochlorite. In general, microorganisms which grow at low temperatures contain more unsaturated fatty acids than those which grow at high temperatures [58,59]. This phenomenon was explained as part of a mechanism to maintain the fluidity and permeability of the cell membrane [60]. Since *Exiguobacterium* sp. and *Pantoea* sp. were cultured at 4 °C, the proportion of unsaturated

fatty acids in their cell membranes may be increased. As a result, the fragility of the cell membrane was enhanced due to the decrease in the gel to liquid-crystalline phase transition temperatures [61]. The bacterial cells of these should become more permeable to HOCl than that of *E. coli* cultured at 37 °C; therefore, they are more sensitive to sodium hypochlorite than *E. coli*. At 2 and 5 ppm, ^-OCl becomes the predominant chlorine species because the percentages of HOCl are calculated at about 30 and 7.8%, respectively. Although the germicidal activity of ^-OCl is weak, the concentration of this species might be enough to kill all of the microorganisms used in this study, through an oxidizing action from outside the cell.

Figure 4. Effect of sodium hypochlorite treatment on *Exiguobacterium* sp. (a), *Pantoea* sp. (b), and *E. coli* (c). Symbols indicate concentrations of 0.25 ppm (closed circles), 0.5 ppm (closed triangles), 1 ppm (closed squares), 2 ppm (open circles), and 5 ppm (open triangles). N.D. means not detected.

3.2.3. Organic Acids Treatments

Acetic acid is the main component of vinegar and is used as a potential disinfectant agent, with a broad spectrum of antibacterial activity [62,63]. Citric acid is naturally found in citrus fruits and is applied for the inhibition of the growth of various microorganisms, including food poisoning bacteria [64,65]. In order to evaluate the bactericidal effect of these organic acids at the actual pH levels of sour foods, the pH values of the acetic acid solution (pH 2.68) and the citric acid solution (pH 2.00) were adjusted within the pH ranges of vinegar (pH 2.40–3.40) [66] and lemon juice (pH 2.00–2.60) [66], respectively. When the cell suspension of *Exiguobacterium* sp. was treated with the acetic acid solution, about an 8 log reduction in CFU/mL was observed at 30 s and thereafter (Figure 5a). The viable cell density of *Pantoea* sp. decreased by about 1 log CFU/mL for 5 min (Figure 5b). No bactericidal effect against *E. coli* was observed because the viable cell density of this microorganism maintained almost the same level as the initial value for 5 min (Figure 5c). The bactericidal effect of citric acid against these microorganisms showed a similar tendency to that of acetic acid (Figure 6). These results suggest that *Exiguobacterium* sp. is sensitive, *Pantoea* sp. is less sensitive and *E. coli* is insensitive to acetic acid and citric acid. The bactericidal effect of organic acids has been assumed to be due to the penetration of their undissociated forms into the membrane of the bacterial cell, and the subsequent dissociation and release of protons (H^+) in the cytoplasm [67,68]. In this study, acetic acid and citric acid would be present largely in the undissociated forms because the pH of each acidic solution was lower than their acid dissociation constant (pKa) values (acetic acid: 4.76 [69], citric acid: 3.13, 4.76, and 6.41 [70]). Thus, the sensitivities of these microorganisms toward these acids are considered to be dependent on the difference in membrane permeability. Since the *Exiguobacterium* sp. and *Pantoea* sp. were cultured at 4 °C, their cell membranes became fragile due to the relatively high content of unsaturated fatty acid in phospholipid molecules. The bacterial cells of these should become more permeable to the undissociated

forms of organic acids than that of *E. coli*. The experimental result of *Exiguobacterium* sp. can be explained by this assumption. The contradictory result of *Pantoea* sp. suggests that this microorganism may have the natural acid resistance and/or acid tolerance response (ATR) system similar to that of *P. agglomerans* CPA-2, a microbial antagonist against the major postharvest diseases of citrus and pome fruits [71].

Figure 5. Effect of acetic acid treatment on *Exiguobacterium* sp. (a), *Pantoea* sp. (b), and *E. coli* (c). N.D. means not detected.

Figure 6. Effect of citric acid treatment on *Exiguobacterium* sp. (a), *Pantoea* sp. (b), and *E. coli* (c). N.D. means not detected.

3.2.4. Alkaline Treatment

In order to investigate the sensitivities of *Exiguobacterium* sp., *Pantoea* sp., and *E. coli* to alkaline conditions, these microorganisms were treated with a sodium hydroxide solution (pH 8.00). As shown in Figure 7, no bactericidal effect against each microorganism was observed, even after 5 min of treatment time. This result suggests the potential risk that alkaline sanitizers, such as sodium bicarbonate solution [72] and neutral electrolyzed water (NEW) [73], are ineffective to these microorganisms. However, if the alkaline sanitizers contain other biocidal reagents, the bactericidal effect should be expected because these microorganisms were inactivated by 2 and 5 ppm sodium hypochlorite solutions, with pH values of 7.68 and 8.19, respectively (Figure 4). NEW seems to be an effective sanitizer against these microorganisms because it contains HOCl and ^-OCl [73].

Figure 7. Effect of alkaline treatment on *Exiguobacterium* sp. (a), *Pantoea* sp. (b), and *E. coli* (c).

3.2.5. Ozonated Water Treatment

Ozone is a strong oxidant and favorable disinfectant. Because it is easier to handle than ozone gas, ozonated water is applied as the effective sanitizer for various foods [74]. In this study, the bactericidal effect of ozonated water was investigated within the common concentration ranges (0.5–2.0 ppm) used in vegetables and fruits industries [74]. As shown in Figure 8a, no bactericidal effect against *Exiguobacterium* sp. was observed, even at the highest concentration of 1.5 ppm, with the longest treatment time of 9 min. In contrast, *Pantoea* sp. and *E. coli* were completely inactivated at the lowest concentration of 0.5 ppm, with the shortest treatment time of 3 min (Figure 8b,c). It was confirmed that *Exiguobacterium* sp. is resistant to ozone, and particular attention should be paid to the contamination of this microorganism in food factories, where cleaning and sanitizing are performed using ozonated water, with concentrations of 1.5 ppm or below. Although these microorganisms were isolated from an ozonated immersion solution of packed tofu, *Pantoea* sp. was not resistant to ozone. There are two possibilities to explain this result. One possibility is a rapid decrease in ozone concentration due to the spontaneous process, and oxidation with tofu and its water-soluble organic compounds, such as proteins, peptides, and polysaccharides. Another possibility is that the planktonic cells of *Pantoea* sp. were detached from the biofilm, on the surface or the relatively central portion of the tofu, after the decomposition of ozone was completed. The bactericidal effect of ozone is based on the destruction of bacterial cells, which occurs due to the interaction of ozone molecules, reactive oxygen species (ROS) [75,76] and reactive nitrogen species (NOS) [75], with the cell wall [76]. In addition, intracellular components, such as enzymes and nucleic acids are also targeted [76]. Therefore, the sensitivity of each microorganism to ozonated water might be related to the difference in the structure of the cell wall and the cell membrane, as well as the activities of antioxidants and antioxidant enzymes. Considering the selectivity of ozone towards double bonds of unsaturated lipids in the cell membranes [77], *Exiguobacterium* sp. and *Pantoea* sp. should become vulnerable to ozone, because the proportion of unsaturated fatty acids in the cell membranes of these microorganisms may be increased at a low culture temperature of 4 °C. However, the experimental result of the former did not obey this assumption (Figure 8a). The involvement of the factors that contribute to ozone resistance should be considered concerning. Since *Exiguobacterium* sp. is a Gram-positive bacterium, a thick peptidoglycan layer (20–80 nm) [78] may protect bacterial cells from ozone and other reactive species. It is also necessary to think about the defense systems of antioxidants and antioxidant enzymes, with reference to the examples of other strains of the same genus. *Exiguobacterium* sp. may produce carotenoids because the characteristic of this microorganism to form orange colonies (Figure 1a) was consistent with several carotenoid-producing strains [24,25]. Other strains can produce highly active antioxidant

enzymes. For example, the catalase (CAT) activity in the cell extract of *E. oxidotolerans* T-2-2^T is 567 times higher than that of *E. coli* [23]. The activity of superoxide dismutase (SOD) purified from *Exiguobacterium* sp. OS-77 is 1.4 and 7.5 times higher than those of *E. coli* and *Thermus thermophilus*, respectively [27]. The involvement of carotenoids and highly active antioxidant enzymes in the protection of *Exiguobacterium* sp. from ROS and NOS can also be expected. *Pantoea* sp. and *E. coli* are Gram-negative bacteria, and the peptidoglycan layer of these cells is thin (1–10 nm) [78], which makes them susceptible to damage by ozone and other reactive species. Similar to several carotenoid-producing strains [79,80], *Pantoea* sp. formed yellow colonies (Figure 1b). Other strains also exhibit the enzymatic activities of glutathione-S-transferase (GST) [81], CAT [81,82], and SOD [81,83]. Although the ability of *Pantoea* sp. to produce carotenoids and antioxidant enzymes is expected, the activities of these molecules seem to be insufficient to detoxify ROS and NOS. Normally, *E. coli* does not synthesize carotenoids [84], as there is no defense system by this antioxidant. Although the microorganism can produce CAT [85], SOD [85], glutathione reductase (GR) [86], GST [87], and glutathione peroxidase (GPX) [88], the activity of these antioxidant enzymes may not be sufficient to quench ROS and NOS.

Figure 8. Effect of ozonated water treatment on *Exiguobacterium* sp. (a), *Pantoea* sp. (b), and *E. coli* (c). Symbols indicate concentrations of 0.5 ppm (closed circles), 1 ppm (closed triangles), and 1.5 ppm (closed squares). N.D. means not detected.

3.2.6. UV Irradiation

UV disinfection is based on DNA damage that leads to an inhibition of DNA replication [89,90]. It can be utilized as a potential non-thermal technique for inactivating the pathogenic and spoilage microorganisms in the food industry [90]. UV is classified into UVA (320–400 nm), UVB (280–320 nm), and UVC (190–280 nm) [91]. Among them, UVC is commonly used due to its excellent germicidal properties [90]. In this study, the bactericidal effect of UV irradiation was evaluated by using a germicidal lamp that emits UVC at around 254 nm. No bactericidal effect against *Exiguobacterium* sp. was observed, even after irradiation for 3 min (Figure 9a). In contrast, the viable cell densities of *Pantoea* sp. and *E. coli* were reduced over time and were not detected at 2 min or thereafter (Figure 9b,c). *Exiguobacterium* sp. was found to be UV-resistant; therefore, particular attention should be paid to the contamination of this microorganism in food factories, where microbial disinfection has been performed by UV irradiation. The sensitivity of *Pantoea* sp. to UV irradiation was similar to that of *E. coli*. The mechanisms underlying the bactericidal effect of UVC are explained as follows: UVC irradiation leads to the formation of cyclobutane-type pyrimidine dimers, the (6,4) photoproduct (and corresponding Dewar isomer), and thymine glycols [92]. In addition, singlet oxygen generated by UVC causes 8-oxoguanine (8-oxoG) [93]. Such damage to DNA can block replication and can result in cell inactivation. In prokaryotes, these

lesions are repaired by various pathways, such as nucleotide excision repair (NER) [94–98], base excision repair (BER) [99–101], methyl-directed mismatch repair (MMR) [96,102–104], UV damage endonuclease-dependent excision repair (UVER) [105], SOS response [97], and photoreactivation [106]. Numerous DNA repair enzymes are involved in these pathways. In addition, singlet oxygen-induced DNA damage can be reduced by carotenoids [107]. Therefore, the sensitivities of these microorganisms to UV irradiation might be related to the activities of DNA repair enzymes and antioxidants. Although DNA repair enzymes of bacteria belonging to the genus *Exiguobacterium* have not yet been fully elucidated, genes encoding UvrABC [108,109], MutM [109], MutS–MutL [108,109], SSB [109], LexA [109], RuvAB [109], and photolyase [108,109] have been identified in a few strains of the same genus. As noted above, the characteristic of *Exiguobacterium* sp. to form orange colonies (Figure 1a) was consistent with several carotenoid-producing strains [24,25]. It is presumed that *Exiguobacterium* sp. also has DNA repair enzymes and carotenoids, and their superior activities, may be responsible for the resistance to UV irradiation. In the case of bacteria belonging to the genus *Pantoea*, only one gene encoding photolyase has been identified in *P. agglomerans* [110]. It is believed that some of the DNA repair pathways (NER, BER, and MMR) are highly conserved from bacteria to human [98,101,104]. Thus, *Pantoea* sp. may have a number of DNA repair enzymes. This microorganism may also have carotenoids because they form yellow colonies (Figure 1b), similar to other carotenoid-producing strains [79,80]. However, the activities of these molecules might not be sufficient to protect against UV and singlet oxygen-induced DNA damage. *E. coli* has a wide variety of DNA repair enzymes [94–97,99–104,106], but does not have carotenoids [84]. This microorganism is thought to be inactivated due to the insufficient activity of these enzymes and the absence of the defense system by carotenoids.

Figure 9. Effect of UV irradiation on *Exiguobacterium* sp. (a), *Pantoea* sp. (b), and *E. coli* (c). N.D. means not detected.

3.2.7. PEF Treatment

PEF treatment is a non-thermal bactericidal method that can minimize the loss of taste, aroma, color, and nutrients in food [111,112]. The inactivation of the microorganism is achieved by the electrical breakdown of the cell membranes during exposure to electric fields. The lethal effect of PEF treatment is dependent on the electric field strength and the treatment time [113]. When the cell suspensions of each microorganism were treated with PEF at the electric field strength of 15 kV/cm for 1 min, the viable cell densities of *Exiguobacterium* sp. and *Pantoea* sp. decreased by about 5 log and 4 log CFU/mL, respectively (Figure 10a,b). In contrast, no bactericidal effect against *E. coli* was observed (Figure 10c). Since the temperatures of these cell suspensions reached the lethal level of the respective microorganisms (approximately 50 °C), it is necessary to think about the effect

of heat on the bactericidal effect. In the heat treatment experiment, about 1 log and 2 log reductions in CFU/mL for *Exiguobacterium* sp. and *Pantoea* sp., respectively, were observed at 50 °C for 5 min (Figure 3a,b). If the treatment is completed within 1 min, the decrease in the number of viable cells is estimated to be more suppressed. Therefore, the bactericidal effect against each microorganism observed here is not considered to be due only to the generated heat. The possibility of the combined effects of heat and PEF treatment on the bactericidal effect [111,114] should be considered. *Exiguobacterium* sp. and *Pantoea* sp. were inactivated by PEF treatment that did not cause lethal damage to *E. coli*. As we discussed in the previous work [111], the sensitivity of microorganisms to PEF treatment is considered to be dependent on the difference in the stability of cell membranes. Since *Exiguobacterium* sp. and *Pantoea* sp. were cultured at 4 °C, their cell membranes should become fragile due to the relatively high content of unsaturated fatty acid in phospholipid molecules. Thus, they become more sensitive to PEF treatment. The results obtained here show good agreement with our previous work and support our hypothesis that the bactericidal effect of PEF treatment depends on the culture temperature of the objective microorganism [111]. Because PEF would primarily attack the cell membrane, this method may also be applicable to the inactivation of other cold-tolerant microorganisms.

Figure 10. Effect of PEF treatment on *Exiguobacterium* sp. (a), *Pantoea* sp. (b), and *E. coli* (c). Symbols indicate cell concentration of PEF treated sample (closed circles), temperature of PEF treated sample (closed triangles), cell concentration of sample without PEF treatment (open circles), and temperature of sample without PEF treatment (open triangles).

3.2.8. Sensitivities of Each Microorganism to Various Bactericidal Methods

Table 1 shows the condition of each bactericidal method in which the highest bactericidal effect was obtained. *Exiguobacterium* sp. was sensitive to heat, sodium hypochlorite, acetic acid, citric acid, and PEF, but resistant to sodium hydroxide, ozonated water, and UV. The sensitivities of *Pantoea* sp. to various bactericidal methods were almost similar to those of *E. coli*, except for the responses to PEF. *Pantoea* sp. was inactivated at a lower temperature and a lower concentration of sodium hypochlorite than *E. coli*. The former was sensitive and the latter was insensitive to PEF.

Table 1. Comparison of the effects of various bactericidal methods.

Treatment	<i>Exiguobacterium</i> sp.	<i>Pantoea</i> sp.	<i>E. coli</i>
Heat	50 °C, 15 min	50 °C, 10 min	55 °C, 20 min
Sodium hypochlorite	2 ppm, 30 s	2 ppm, 3 min	5 ppm, 3 min
Acetic acid	30 s	No bactericidal effect	No bactericidal effect
Citric acid	30 s	No bactericidal effect	No bactericidal effect
Sodium hydroxide	No bactericidal effect	No bactericidal effect	No bactericidal effect
Ozonated water	No bactericidal effect	0.5 ppm, 3 min	0.5 ppm, 3 min
UV	No bactericidal effect	2 min	2 min
PEF	>1 min	>1 min	No bactericidal effect

4. Conclusions

Exiguobacterium sp. and *Pantoea* sp. were isolated from an ozonated immersion solution of packed tofu, stored at 4 °C for 6 days. These microorganisms were classified as psychrotrophic bacteria, according to their ability to grow at 4 °C and optimum growth temperature of 30 °C. Equipment and workers are considered to be the potential sources and risk factors for the microbial contamination of tofu.

Exiguobacterium sp. was sensitive to heat, sodium hypochlorite, acetic acid, citric acid, and PEF, but resistant to sodium hydroxide, ozonated water, and UV. Therefore, particular attention should be paid to the contamination of this microorganism in food factories where cleaning, sanitizing, and microbial disinfection are performed by ozonated water and UV irradiation. The resistance of *Exiguobacterium* sp. to ozonated water was probably due to the presence of a thick peptidoglycan layer and the defense systems of carotenoids and highly active antioxidant enzymes. The superior activities of DNA repair enzymes and carotenoids may be responsible for the resistance of this microorganism to UV irradiation. *Pantoea* sp. was sensitive to heat, sodium hypochlorite, ozonated water, UV, and PEF, but resistant to acetic acid, citric acid, and sodium hydroxide. The resistance of this microorganism to organic acids may be derived from natural acid resistance and/or the ATR system. *Exiguobacterium* sp. and *Pantoea* sp. were resistant to sodium hydroxide. This result suggests the risk that alkaline sanitizers are ineffective to these microorganisms. However, if the alkaline sanitizers contain other biocidal reagents, such as HOCl and ^-OCl , the bactericidal effect should be expected. Both *Exiguobacterium* sp. and *Pantoea* sp. were inactivated by PEF treatment that did not cause lethal damage to *E. coli*. Since these microorganisms were cultured at 4 °C, their cell membranes should become fragile due to the relatively high content of unsaturated fatty acid in phospholipid molecules. Thus, they become more sensitive to PEF treatment.

From this research, we have provided practical recommendations for the improvement of the microbial safety and quality of tofu in the manufacturing process. Equipment should be cleaned, sanitized, and disinfected by heat treatment and sodium hypochlorite. The workers are required to sanitize their hands with organic acids and ozonated water. PEF treatment of the water for cooling, washing, and packing of the tofu blocks could also be effective to prevent or minimize microbial contamination.

The results obtained in this study suggest that bactericidal methods targeting the fragile cell membrane with high permeability are effective for the inactivation of cold-tolerant microorganisms. In particular, PEF treatment can be applied as a powerful tool for

the inactivation of cold-tolerant microorganisms. This method may contribute to enhancing the microbiological safety and quality of various foods under refrigeration conditions.

Author Contributions: Conceptualization, T.T. and T.O.; methodology, M.M., T.T. and T.O.; software, A.G., K.T. (Katsuyuki Takahashi) and K.T. (Koichi Takaki); validation, A.G., K.T. (Katsuyuki Takahashi) and K.T. (Koichi Takaki); formal analysis, A.G., K.T. (Katsuyuki Takahashi) and K.T. (Koichi Takaki); investigation, M.M., T.T., M.I., C.N. and T.O.; resources, M.M., T.T. and T.O.; data curation, M.M., T.T., M.I., C.N. and T.O.; writing—original draft preparation, M.M., T.T. and T.O.; writing—review and editing, M.M., T.T., A.G., K.T. (Katsuyuki Takahashi), K.T. (Koichi Takaki) and T.O.; visualization, M.M., T.T., M.I., C.N. and T.O.; supervision, K.T. (Koichi Takaki) and T.O.; project administration, K.T. (Koichi Takaki) and T.O.; funding acquisition, K.T. (Katsuyuki Takahashi), K.T. (Koichi Takaki) and T.O. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by a Grant-in-Aid for Scientific Research (S) from the Japan Society for the Promotion of Science, Grant Number 19H05611.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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