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Influence of pH on bioactivity of cinnamon oil against *Legionella pneumophila* and its disinfection efficacy in hot springs

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ABSTRACT

Cinnamon oil extracted from leaves of *Cinnamomum osmophloeum* has recently been proved as a promising antibacterial agent against *Legionella pneumophila*, an etiological agent of human pneumonia known as Legionnaires' disease. However, the pH effects on the efficacy of cinnamon oil against *L. pneumophila* and its applicability to recreational spring water remain unknown. We therefore determined the bactericidal activity of cinnamon oil at pH 3–10 in phosphate-buffered saline (PBS) and in four kinds of springs with various conductivity (259–5595 $\mu\text{S cm}^{-1}$) and pH (2.1–7.7) levels. Results show *L. pneumophila* cells were more susceptible to cinnamon oil at pH 8–10 than at pH 4–6 in PBS, which became more evident as increasing contact time from 10 to 60 min. An increase in concentration of cinnamon oil and contact time significantly increased the anti-*L. pneumophila* activity ($P \leq 0.001$), indicating a consistent biocidal effect regardless of pH. Interestingly, this dose-response biocidal effect was also observed in spring waters. Moreover, *L. pneumophila* of 4 log CFU ml^{-1} in spring waters was completely inactivated within 60 min by cinnamon oil at 300–750 $\mu\text{g ml}^{-1}$, with the highest inactivation in alkaline hydrogen carbonate spring. The great bioactivity of cinnamon oil demonstrates its potential to be used to control *Legionella* growth in recreational spring water and possibly other niches generally at basic pH, e.g., cooling towers.

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1. Introduction

Legionella pneumophila, the major causative agent of Legionnaires' disease (LD) and Pontiac fever, has been isolated from spring waters with pH between 6.2 and 8.9 (Furuhata et al.,

2004; Verissimo et al., 1991). Investigations conducted in spring sources, runoff channels, water-retaining tanks, hot spring spa and baths demonstrate the presence of *Legionella* at concentrations up to 3–6 log CFU l^{-1} (Bornstein et al., 1989; Furuhata et al., 2004; Verissimo et al., 1991) with

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L. pneumophila as the predominant species. Exposure to *Legionella*-contaminated spring water during bathing activities is associated with sporadic and lethal outbreaks of LD (Bornstein et al., 1989; Ito et al., 2002; Miyamoto et al., 1997; NIID, 2003). Therefore, it is imperative to maintain good quality of spring water.

For spa pools that are commonly installed in hot spring resorts, European guidelines suggest continuously filtering and treating water with chlorine (EWGLINET and EWGLI, 2005). While appropriate in general, the disinfection activity of chlorine is known to be significantly reduced at the alkaline pH (EWGLINET and EWGLI, 2005), which is commonly found in hot springs (Furuhata et al., 2004; Hsu et al., 2006; Verissimo et al., 1991). Moreover, formation of trihalomethanes, the most common disinfection byproducts of chlorination process, increases with higher water temperature (Chu and Nieuwenhuijsen, 2002). Adverse health effects such as spontaneous abortion and urinary tract defects have been associated with exposure to trihalomethanes (Nieuwenhuijsen et al., 2000), implying the potential health risk for humans bathing in chlorinated hot springs. Finally, the design of water supply and drainage system in certain hot spring facilities makes it difficult to maintain adequate chlorine residuals needed for continuous disinfection of the water (Hsu et al., 2006). It is therefore desirable to explore other disinfection methods to control *Legionella* in recreational hot spring sites.

Essential oils, which are the natural mixtures of the secondary metabolites from plants, have been used historically in warm baths for aromatherapy. Many essential oils have antibacterial properties against waterborne bacteria (Kalemba and Kunicka, 2003). Recently we have successfully demonstrated that cinnamon oil extracted from the leaves of *Cinnamomum osmophloeum* Kaneh. (Lauraceae), an endemic tree in the hardwood forests of Taiwan, possesses strong bioactivity against *L. pneumophila* at 42 °C, and has the great potential to be used as an antibacterial agent to control LD associated with recreational hot water (Chang et al., 2008). In addition to *L. pneumophila*, this particular cinnamon oil also inhibits the growth of *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, methicillin-resistant *S. aureus*, *Klebsiella pneumoniae*, *Salmonella* sp., and *Vibrio parahaemolyticus* (Chang et al., 2001). However, the bioactivity of cinnamon oil against *L. pneumophila* was exhibited at pH 7 in phosphate-buffered saline (PBS) (Chang et al., 2008). Its anti-*Legionella* activity in hot springs has not been investigated, nor has the effects of pH on the antibacterial activity of cinnamon oil.

In light of the presence of *Legionella* in springs and of the antibacterial activity of cinnamon oil, we conducted this study to determine the bioactivity of cinnamon oil against *L. pneumophila* at various pH and in hot spring waters typical in Taiwan. The culturability of *L. pneumophila* at pH 2–10 and in hot springs was also assessed in order to characterize the risk of *Legionella* exposure during bathing activities. Moreover, the effects of dilution with tap water on cell culturability and on inactivation efficacy of cinnamon oil in spring waters were also investigated as this practice of blending is commonly conducted in spring bathing to lower water temperature.

2. Materials and methods

2.1. Test organism

L. pneumophila serogroup 1 ATCC 33152 was incubated on BCYE α agar (Sigma Chemical Co., MO, USA) at 37 °C for 2 days in an atmosphere of 5% CO₂ (Chang et al., 2007). Colonies formed were removed and serial dilutions were performed to obtain a concentration of 6 log CFU ml⁻¹ in PBS.

2.2. Cinnamon oil

Mature leaves of *C. osmophloeum* were collected from the Da-Pin-Ting of Taiwan Sugar Farm. The species was identified by Mr. Yen-Ray Hsui of the Taiwan Forestry Research Institute and the voucher specimens were deposited at the Laboratory of Wood Chemistry, School of Forestry and Resource Conservation, National Taiwan University. Cinnamon oil was extracted from the collected leaves by water distillation for 6 h, and stored in airtight containers. The extracted cinnamon oil, classified as cinnamaldehyde type based on its major component (accounting for 91.32% of cinnamon oil) (Chang et al., 2008), was used in the present study.

2.3. Collection of spring water

Hot spring water was taken from the pipes of natural springs in four hyperthermal locations (A–D) of Taiwan. Hot springs of A, B, and C are within the Yangmingshan's national park, an area renowned for its volcanic features and topography. Hot spring of D is within a historical resort south of Taipei city. According to the chemical composition, these four hot springs were classified as: acid sulfate (A), acid hydrogen carbonate (B), acid sulfate chlorine (C), and alkaline hydrogen carbonate spring (D) (Song and Liu, 2003). Approximately 300 ml of spring water was collected in sterile screw-capped bottle. Four water samples were taken on two different sampling days from each spring location.

Collected spring water was first filtered through a 0.22 μ m mixed cellulose esters filter (Millipore, MA, USA) within 24 h after sampling. One half of the filtered spring water was then diluted at a ratio of 1:1 with sterile tap water.

2.4. Characteristics of spring water

Temperature and pH of the springs were determined by a portable pH meter (Thermo Orion, MA, USA) at the spring sources. The conductivity was also measured using a conductivity meter (YSI, Ohio, USA), which was calibrated by a standard solution (YSI catalog # 3167) of 1000 μ S cm⁻¹ provided by the manufacturer. Because the pH and conductivity of the collected samples might change due to the shift in the carbonate equilibrium between the water and air over time and due to the dilution treatment with tap water, the pH and conductivity of both diluted and non-diluted spring samples were determined again at the beginning and end of 60-min heating experiment at 42 °C (described below). Results are presented as the average $\pm$ standard deviation (SD) over 60 min.

2.5. Culturability of *L. pneumophila*

The culturability of *L. pneumophila* in PBS at pH 2–10 and in hot springs was determined at 42 °C, a typical bathing temperature. The pH of sterile PBS was adjusted by adding 0.2 N HCl (Aldrich, MO, USA) and 2 N NaOH (Aldrich). The vials containing 500 µl of PBS at various pH were placed in a water bath (Sheldon Manufacturing, Cornelius, OR, USA) preheated at 42 °C, to which 25 µl of *L. pneumophila* culture was added to give a final inoculum of 4 log CFU ml⁻¹. After 10- and 60-min exposure at 42 °C in vials with 50 rpm shaking, 0.1 ml of cell suspension was plated on BCYEα agar and incubated at 37 °C for 5 days in an atmosphere of 5% CO₂. The forming colonies were counted to determine the culturability. The culturability of the unheated samples was also measured. All the experiments were repeated three times.

As for the tests in springs, *L. pneumophila* of 4 log CFU ml⁻¹ was subject to 42 °C-preheated diluted or non-diluted spring water for 10 and 60 min. The culturability of cells was determined as described previously with the unheated controls.

2.6. Antibacterial activity of cinnamon oil

To determine the pH effects on bioactivity of cinnamon oil against *L. pneumophila*, the extracted oil was first dissolved in 95% ethanol and diluted with PBS at various pH, resulting in five to eight concentrations at 10–1000 µg ml⁻¹. As for the antibacterial tests of cinnamon oil in springs, five to nine concentrations of cinnamon oil were prepared at 50–1200 µg ml⁻¹ in filtered spring water previously diluted or non-diluted with sterile tap water. *L. pneumophila* cells of 4 log CFU ml⁻¹ were then subjected to cinnamon oil for 10 and 60 min at 42 °C. The negative controls were also prepared with PBS and spring water to which only *L. pneumophila* was added in absence of cinnamon oil.

Cinnamon oil-treated samples and negative controls (0.1 ml) were plated on BCYEα agar and colonies were counted. The percent reduction in CFU, defined as the inactivation rate of *L. pneumophila* with exposure to cinnamon oil, was determined at each pH (or spring) for a specific period of contact time (10 and 60 min) as follows: [(Ca – Cb)/Ca]100, where Ca: CFU of negative control at 42 °C, and Cb: CFU of cinnamon oil-treated specimen at 42 °C. The minimal bactericidal concentration 100 (MBC₁₀₀), defined as the concentration of cinnamon oil that inactivated at least 99.9% *L. pneumophila* and resulted in no growth of *L. pneumophila* on BCYEα agar, was also determined. All the experiments were performed in triplicate.

For positive controls, the method suggested by British Society for Antimicrobial Chemotherapy (BSAC, 2004) with modification of use of BCYEα agar was followed to test the susceptibility of *L. pneumophila* to erythromycin (potency ≥ 850 mg g⁻¹, Sigma-Aldrich, MO, USA) and rifampicin (95% purity, Sigma-Aldrich). It was observed the minimum inhibitory concentrations of erythromycin and rifampicin were always within the ranges of 0.06–0.5 mg l⁻¹ and 0.004–0.06 mg l⁻¹, respectively, recommended for *L. pneumophila* (BSAC, 2004), demonstrating the present test strain appropriately responded to antibacterial agents.

2.7. Statistical analysis

The inactivation rates of *L. pneumophila* against concentrations of cinnamon oil were plotted, and the best-fit model was created by Sigmaplot software (SPSS Inc., IL, USA) at $R^2 \geq 0.98$. The values of MBC₅₀ and MBC₈₀, which represented the minimum concentration of cinnamon oil that inactivated 50% and 80% of *L. pneumophila*, respectively, were determined from the best-fit model.

In addition, the univariate analysis of variance was undertaken using SPSS software (SPSS Inc.) to examine the effects of contact time, pH, spring type and dilution treatment on culturability of *L. pneumophila*. With addition of two more factors, i.e., concentration of cinnamon oil and cell culturability at specified pH, the univariate analysis of variance was also applied to anti-*L. pneumophila* activity of cinnamon oil. Pairwise comparisons between any of two types of springs were further conducted to examine whether the mean differences of inactivation rate and cell culturability were statistically significant. Stepwise regression analysis was also performed for each spring to explore the factors significantly affecting cell culturability and inactivation activity of cinnamon oil. All results were considered statistically significance at $P < 0.01$.

3. Results

3.1. Characteristics of spring water

Among the four tested springs, Table 1 showed Spring C was the most acid spring (pH = 2.1) with the highest conductivity at 6460 µS cm⁻¹. The lowest conductivity was found in Spring A (acid sulfate spring) with a pH of 5.6. Springs B and D were both carbonate springs exhibiting moderate conductivity.

Storage of spring water at 4 °C and dilution of the springs with tap water resulted in a decrease in conductivity and an increase in pH. Consequently, the means of pH and conductivity of the tested spring waters ranged at 2.1–7.7 and 259–5595 µS cm⁻¹, respectively. For each of tested springs, the pH of spring samples measured at the beginning and the end of experiments remained the same and the variation of conductivity was limited during the 60-min heating tests. Stable pH and conductivity values indicated that heating at 42 °C did not noticeably change the characteristics of springs.

3.2. Culturability of *L. pneumophila* in PBS

At room temperature, Fig. 1 showed *L. pneumophila* maintained the culturability in PBS at pH 2–10. However, cells completely lost their culturability at pH 2 within 10 min at 42 °C. An increase in contact time from 10 to 60 min at 42 °C resulted in a further consistent decline of cell culturability at all pH tested, whereas *L. pneumophila* maintained their culturability better at pH 4–6 than at pH 8–10. Univariate analysis of variance revealed that pH and contact time were both significant parameters affecting the culturability of *L. pneumophila* in PBS (all $P < 0.01$).

3.3. Culturability of *L. pneumophila* in springs

Fig. 2(a) showed that the culturability of *L. pneumophila* was comparable in non-diluted Springs A, B, and D at either room

Table 1 – Characteristics of hot springs (A–D)

Types of hot spring	A	B	C	D
	Acid sulfate	Acid hydrogen carbonate	Acid sulfate chlorine	Alkaline hydrogen carbonate
<i>At the sampling site</i>				
Temperature (°C)	50.1	53.0	45.1	61.5
pH	5.6	6.2	2.1	7.1
Conductivity ($\mu\text{S cm}^{-1}$)	557 $\pm$ 197 ^a	2188 $\pm$ 619	6460 $\pm$ 537	2073 $\pm$ 762
<i>Within 1 day after sample collection</i>				
(a) No dilution with sterile tap water				
pH	6.3	6.6	2.1	7.5
Conductivity ($\mu\text{S cm}^{-1}$)	382 $\pm$ 48	1546 $\pm$ 98	5595 $\pm$ 526	1331 $\pm$ 31
(b) 1:1 dilution with sterile tap water				
pH	6.5	6.8	2.1	7.7
Conductivity ($\mu\text{S cm}^{-1}$)	259 $\pm$ 36	915 $\pm$ 20	2881 $\pm$ 99	787 $\pm$ 17

a Mean $\pm$ standard deviation.

temperature or 42 °C. In contrast, only half of *L. pneumophila* remained culturable in Spring C at room temperature. Exposure to 42 °C caused further declines in culturability, resulting in only 1281 CFU ml⁻¹ of *L. pneumophila* detected at the end of 60-min contact.

Comparison of cell culturability between Fig. 2(a) and (b) revealed that no obvious difference in Springs A, B, and D, indicating the culturability of *L. pneumophila* in these three Springs was not affected by the dilution treatment with tap water. However, an evident change was found in Spring C at exposure for 60 min at 42 °C: The mean of 8688 CFU ml⁻¹ was detected in diluted spring, which was 7 times that measured in non-diluted spring water.

Results of univariate analysis of variance showed both the spring type and contact time had statistically significant

effects on cell culturability in springs ($P < 0.001$ and $=0.009$), whereas dilution treatment had not ($P = 0.72$). Pairwise comparison revealed cell culturability was significantly lower in Spring C than in any other three springs (all of $P \leq 0.001$), while no statistical difference was detected among Springs A, B, and D.

3.4. Anti-*L. pneumophila* activity of cinnamon oil in PBS

The bioactivity of cinnamon oil against *L. pneumophila* was evaluated at pH 3–10 in PBS where culturable cells were present. Table 2 showed, at 10- and 60-min contact with cinnamon oil, the MBC₅₀, MBC₈₀, and MBC₁₀₀ in alkaline PBS (pH 8–10) were all lower than those determined in normal or

Fig. 1 – Culturability of *L. pneumophila* in PBS at room temperature (white bar) and at 42 °C for 10 min (diagonal lines) and 60 min (cross-hatch) contact. **: No detectable culturability.

Fig. 2 – Culturability of *L. pneumophila* in four kinds of hot springs classified as: acid sulfate (A), acid hydrogen carbonate (B), acid sulfate chlorine (C), and alkaline hydrogen carbonate (D) springs. Results are shown in pure spring waters (a) or in springs diluted with 1:1 sterile tap water (b) at room temperature (white bar) and at 42 °C for 10 (hatched bar) and 60 min (cross-hatched bar) contact.

acid PBS (pH 3–7). Regardless of pH, almost all the MBC values decreased with increasing the contact time with cinnamon oil. However, this MBC reduction was more evident at alkaline pH: By taking MBC₅₀ and MBC₈₀ values determined at 10-min

contact as the references, the relative percentages for MBC₅₀ and MBC₈₀ values determined at 60 min with respective pH were calculated. It was revealed that the relative percentages of MBC₅₀ and MBC₈₀ at pH 4–6 were 70%–84% and 66%–86%, respectively, and 27%–35% and 27%–36%, respectively, at pH 8–10. Lower percentages represented more MBC reductions at 60 min.

As our statistical results indicated that culturability of *L. pneumophila* was significantly affected by pH and contact time, cell culturability was included as a covariate in the univariate analysis of variance and as one of the predictors in the stepwise regression model to determine the factors significantly affecting the anti-*L. pneumophila* activity of cinnamon oil. Analysis of 255 data revealed pH, concentration of cinnamon oil, and contact time were significant determinants of *L. pneumophila* inactivation ($P \leq 0.007$). Cell culturability was neither statistically significant ($P = 0.3$) in the univariate analysis of variance nor included in the final regression model.

3.5. Anti-*L. pneumophila* activity of cinnamon oil in springs

MBC values of cinnamon oil against *L. pneumophila* in four types of springs are presented in Table 3. For non-diluted springs, the highest MBC₅₀, MBC₈₀, and MBC₁₀₀ values were always obtained in Spring A at 10- and 60-min contact with cinnamon oil. An increase in contact time from 10 to 60 min resulted in a consistent reduction of MBC₅₀ and MBC₈₀ values in all four springs. The most apparent decrease was shown in Spring D where MBC₅₀ and MBC₈₀ values at 60-min contact were 24% and 40% of that determined at 10 min, respectively.

Table 2 – MBC values of cinnamon oil against *L. pneumophila* of 4 log CFU ml⁻¹ in PBS at various pH^a

Contact time (min)	pH	MBC (μg ml ⁻¹)		
		MBC ₅₀	MBC ₈₀	MBC ₁₀₀
10	3	371	402	750
	4	545	646	750
	5	507	711	>1000
	6	561	584	1000
	7	381	447	1000
	8	165	205	500
	9	148	192	500
	10	41	44	100
60	3	161	192	250
	4	390	429	750
	5	427	499	1000
	6	395	500	750
	7	208	311	750
	8	57	74	200
	9	40	57	125
	10	11	12	20

^a MBC₁₀₀ was the minimal bactericidal concentration of cinnamon oil that resulted in no colony detected on BCYEα agar (i.e., 99.9% inactivation of *L. pneumophila*). MBC₅₀ and MBC₈₀, determined from the best-fit inactivation model against concentrations of cinnamon oil, represented the minimal concentration of oil that inactivated 50% and 80% of *L. pneumophila*, respectively.

Table 3 – MBC values of cinnamon oil against *L. pneumophila* of 4 log CFU ml⁻¹ in four types of spring waters (A–D)

Springs	Contact time (min)	MBC (μg ml ⁻¹)		
		MBC ₅₀	MBC ₈₀	MBC ₁₀₀
<i>Springs without dilution</i>				
A	10	464	571	1200
B		282	370	500
C		268	285	400
D		295	459	750
A	60	165	300	750
B		144	228	500
C		134	172	400
D		70	185	500
<i>Springs diluted with 1:1 sterile tap water</i>				
A	10	478	564	1200
B		373	545	1000
C		259	271	400
D		242	306	750
A	60	262	346	750
B		182	289	400
C		258	272	300
D		119	180	500

For diluted springs, the highest MBC values were again observed in Spring A regardless of contact time. Moreover, the MBC₁₀₀ values determined in diluted springs at 60-min contact were the same as or less than those in non-diluted springs, indicating dilution treatment caused no increase in MBC₁₀₀ at 60-min contact for all four springs. In fact, *L. pneumophila* in diluted springs was completely inactivated after 60-min contact with cinnamon oil at 300–750 μg ml⁻¹.

Univariate analysis of variance on 282 inactivation data showed concentration of cinnamon oil, contact time, and spring type was the first statistically significant factor associated with anti-*L. pneumophila* activity of cinnamon oil (all of $P \leq 0.001$), whereas the dilution treatment was not ($P = 0.49$). Concentration of cinnamon oil was the most significant determinant positively affecting the anti-*L. pneumophila* ability of cinnamon oil in every spring (all of $P < 0.001$, by stepwise regression analysis). Contact time was the second statistically significant factor for Springs A, B, and D ($P < 0.001$, $=0.007$ and 0.004 , respectively). Further pairwise comparison indicated *L. pneumophila* inactivation was more effective in Spring D and less effective in Spring A than in any other three springs (all of $P < 0.01$) at 60-min contact with cinnamon oil.

4. Discussion

Characterization of cell culturability in response to pH and spring variation provides important information in assessing the exposure risk to *L. pneumophila* during bathing activities. The present study demonstrated the culturability of *L. pneumophila* decreased more evidently in alkaline PBS than in weak acid PBS with a complete culturability loss at pH 2. These findings seem to suggest that the risk of acquiring *Legionella* infections is insignificant when bathing in alkaline or strong

acidic warm springs. However, this implication is improper as there was a much less reduction of cell culturability in spring water than in PBS. As observed in Fig. 2, a comparable culturability was identified in Springs A, B and D (pH 6.3–7.7) at 42 °C within 60 min, whereas an increased culturability loss occurred in PBS with increasing pH from 6 to 8 (Fig. 1). Moreover, there was a rapid decrease of $\geq 55,333$ CFU ml⁻¹ at pH 2 in PBS within 10 min but only a decline of 12,538 and 11,063 CFU ml⁻¹ in diluted and non-diluted Spring C (pH 2.1), respectively. In addition, the present study revealed an increase of contact time at 42 °C had less detrimental effects on cell culturability in Springs C and D than in PBS at a comparable pH of 2 and 8, respectively. Increased cell culturability represents the higher exposure risk to *L. pneumophila* in springs than in PBS, and could be attributable to the presence of a variety of abundant minerals. Certain minerals present in acid sulfate chlorine spring and hydrogen carbonate spring, e.g., K⁺, Mg²⁺, and Fe²⁺ (Song and Liu, 2003), are known to be essential for *L. pneumophila* to survive in tap water (States et al., 1985) and multiply on media (Reeves et al., 1981). Indeed, culturable *L. pneumophila* has been detected from acidic, neutral and alkaline spring waters collected at spring sources, piping systems and bathing facilities (Furuhata et al., 2004; Verissimo et al., 1991). A positive detection rate of *Legionella* between 24% and 66.7% was also revealed in spring waters with temperature above 40 °C and pH at 7–11, which were collected from spring recreational facilities in Taiwan (Hsu et al., 2006).

It is very common to blend spring water with tap water during bathing activities. Therefore, in addition to *Legionella* contamination in hot spring supply system, the infection risk of LD could also be derived from *L. pneumophila* originally present in tap water plumbing, which is blended into springs and remain culturable during bathing activities. For Springs A, B, and D, the present results indicated the culturability of *L. pneumophila* was not affected by the blending treatment with tap water. Additionally, *L. pneumophila* kept culturable for 60 min in blended Springs A, B, and D at similar levels observed at 10-min contact (Fig. 2(b)). These findings suggest that the infection risk of LD could be present in bathing Springs A, B, and D if the blending water had been contaminated with viable *L. pneumophila* that probably remained viable during 60-min bathing. As for acidic Spring C, the blending practice appeared to relieve salt-induced stress against *L. pneumophila*, as observed by increasing seven times the cell culturability in diluted spring relative to non-diluted water. Acidity at pH 2.2 has been shown to cause severe shrinkage of cytoplasmic contents and increased membrane permeability of *L. pneumophila* (Harley et al., 1997), possibly resulting in less culturable cells in Spring C (pH 2.1, Table 1) than in any other three springs. Nevertheless, cellular culturability was significantly improved by the blending practice although the pH of diluted Spring C remained the same. This could be attributed, in part at least, to the dilution effects of salts (in particular, Na⁺) that are relatively abundant in Spring C (Song and Liu, 2003) and known to have detrimental impacts on the culturability of *L. pneumophila* (Catrenich and Johnson, 1989; Heller et al., 1998). Dilution of Spring C with tap water would reduce the concentrations of Na⁺ and salts, resulting in an increase in cell culturability. Consequently, the likelihood of LD

acquisition might be increased as increasing the level of culturable cells exposed during spring bathing.

Faced with the presence of culturable *L. pneumophila* in spring water, we successfully demonstrated that cinnamon oil at 300–750 $\mu\text{g ml}^{-1}$ completely inactivated *L. pneumophila* within 60 min in diluted springs with MBC_{50} values between 119 and 270 $\mu\text{g ml}^{-1}$. Furuhashi et al. (2003) investigated the antibacterial activity of grapefruit seed extract against *L. pneumophila* and reported the MBC_{50} and MBC_{90} at 1600 and 3100 $\mu\text{g ml}^{-1}$ at exposure of 60 min, respectively. They also evaluated three phenol compounds contained in coffee and found the respective MBC_{50} and MBC_{90} at 625–2500 $\mu\text{g ml}^{-1}$ and 625–5000 $\mu\text{g ml}^{-1}$ with 8-h contact (Furuhashi et al., 2002). Comparing our MBC values with these previous data strongly suggests that cinnamon oil possesses great anti-*L. pneumophila* activity. Moreover, results of statistical analyses demonstrated that an increase of concentration of cinnamon oil and contact time significantly increased the antibacterial activity of cinnamon oil in PBS and spring water. This dose–response relationship provides the bactericidal evidence of cinnamon oil against *L. pneumophila* in waters with various pH and conductivity levels. Similar bactericidal effect has also been observed in *E. coli* O157:H7 treated with cinnamaldehyde, the major component of cinnamon oil (Kim et al., 2004).

In addition to the great anti-*L. pneumophila* property, this study further demonstrated that dilution treatment with tap water did not influence the bactericidal efficacy of cinnamon oil regardless of spring type, shown by its MBC_{100} values and by the statistical results of univariate analysis of variance. These findings indicate the stability of bactericidal property of cinnamon oil against *L. pneumophila* in spring water. Furthermore, Table 3 showed 60-min contact with cinnamon oil completely inhibited *L. pneumophila* at 750 $\mu\text{g ml}^{-1}$ or less, which is at least 13 times lower than the concentration (i.e., 1%) of cinnamaldehyde (the major component of cinnamon oil) that might result in skin sensitization on humans (Cocchiara et al., 2005). Relatively low toxicity of cinnamon oil at effective antibacterial doses and no interference from the blending practice illustrate its applicability in spring bathing.

While effective against *L. pneumophila*, the present study showed pH was a significant determinant in terms of MBC of cinnamon oil. The MBC values were always lower in alkaline PBS than in acid PBS regardless of contact time. This pH effect was statistically significant after adjusting for its influence on cell culturability. Furthermore, *L. pneumophila* inactivation by cinnamon oil in alkaline Spring D was also significantly higher than in acidic Springs A and B at 60-min contact, under the condition that cell culturability among these three springs was comparable with no statistically significant difference. These consistent findings in PBS and spring waters illustrate that cinnamon oil against *L. pneumophila* was more efficiently in alkaline region. This alkaline effect became more evident when increasing the contact time with cinnamon oil. Taking MBC_{50} and MBC_{80} values of 10-min exposure as references, the present study indicated the relative MBC values of 60-min contact were apparently lower at pH 8–10 than at pH 4–6.

The less effective bioactivity (i.e., higher MBC) of cinnamon oil in acid PBS and springs could be related to the reduction

reaction of cinnamaldehyde [$\text{C}_6\text{H}_5\text{CH}=\text{CHCHO}$]. The conjugated double bond outside the carbon ring is thought to contribute to the bioactivity of cinnamaldehyde against a variety of bacteria (Chang et al., 2001). However, in acid media this double bond could be hydrogenated to form a radical [$\text{C}_6\text{H}_5\text{CH}_2\text{CHCHO}$] $\cdot$ H, which may proceed other complex reactions (Barnes and Zuman, 1969). Consequently, the quantity of the aldehydes with bioactive double bonds would be reduced, resulting in an increased MBC of cinnamon oil. In addition to chemical reactions of cinnamaldehyde, pH-induced changes in permeability of outer membrane of bacteria could also have an impact on the bioactivity of cinnamon oil. It has been suggested that cinnamaldehyde may gain access to cell periplasm via the porin proteins of bacterial outer membrane (Helander et al., 1998). A recent study has also identified that pH is one of the environmental factors regulating the production of porin proteins as the channels to take in antibacterial agents (Begic and Worobec, 2006). The reduction in the levels of porins regulated by pH stimulus would lower the transport efficiency of antibacterial agents entering the cells, resulting in an increase in MBC.

Previous investigations on the antibacterial properties of cinnamon oil and cinnamaldehyde have been mainly conducted at $\text{pH} \leq 7$ (Chang et al., 2001, 2008; Friedman et al., 2004; Helander et al., 1998). This is the first report to present the bioactive behavior of cinnamon oil at alkaline pH, which is quite distinct from that at acidic pH, and to our best knowledge, this is also the first study to demonstrate the strong activity of cinnamon oil against *L. pneumophila* in natural springs. *Legionella* have been isolated from alkaline springs (Furuhashi et al., 2004). In particular, weak alkaline carbonate spring waters are identified as the most common sources of *Legionella* in Taiwanese springs (Hsu et al., 2006). The great anti-*L. pneumophila* activity of cinnamon oil suggests its potential application in spring bathing facilities. Moreover, with greater bactericidal characteristic at alkaline pH, cinnamon oil could also be applied to other known niches such as cooling towers, tap water and hot-water tank of the plumbing systems generally at basic pH (States et al., 1987; Wadowsky et al., 1985). As chlorination, a widely used disinfection method in these facilities, is known to be less effective at alkaline pH (EWGLINET and EWGLI, 2005) and may induce resistance of *L. pneumophila* (Chang et al., 2007), cinnamon oil could be an alternative disinfectant to control the growth of *Legionella* in these facilities and warrants future investigation.

In addition to inhibiting planktonic cells (Chang et al., 2001, 2008), cinnamaldehyde and cinnamon oil have shown to decrease biofilm formation by *E. coli* as compared to biofilms grown in Luria-Bertani medium (i.e., control) (Niu and Gilbert, 2004). The structure of cinnamaldehyde-treated biofilm and control was further analyzed by Niu and Gilbert (2004) using confocal laser scanning microscopy with a digital image analysis program COMSTAT. A significant increase in average diffusion distance and decrease in maximum thickness, substratum coverage, and surface-to-biovolume ratio were revealed in cinnamaldehyde-treated biofilms. Moreover, a significantly higher percentage of dead *E. coli* was found in cinnamaldehyde-treated biofilms than in the biofilms treated with other constituents of essential oils, i.e., eugenol and citronellol (Niu and Gilbert, 2004). The interferences by

cinnamaldehyde on quorum sensing (Niu et al., 2006) and on bacterial ability to reach the substratum (Niu and Gilbert, 2004) might be part of the attributes to the reduced biofilm formation caused by cinnamon oil. These findings indicated cinnamon oil could reduce biofilm formation and inactivate sessile bacteria to a certain extent. *L. pneumophila* not only exists planktonically but also colonizes in biofilms (EWGLINET and EWGLI, 2005). The current and previous studies (Chang et al., 2008) have demonstrated cinnamon oil and cinnamaldehyde may effectively inhibit suspensions of *L. pneumophila*. In light of the bactericidal activity of cinnamon oil/cinnamaldehyde against planktonic *L. pneumophila*/sessile *E. coli* and of the ability of cinnamon oil in reducing biofilm formation, it is possible that cinnamon oil may also be effective in inactivating *L. pneumophila* in biofilms. However, it should be noted that the effective concentration of cinnamon oil against sessile *L. pneumophila* might not be the same as that shown in planktonic cells, since an increased resistance to antibacterial compounds has been reported for *Legionella* existing as attached complex consortia (Green and Pirrie, 1993; Ozlem Sanli-Yurudu et al., 2007). Future investigation on the efficacy of cinnamon oil in inhibiting biofilm-associated *L. pneumophila* is warranted.

5. Conclusion

- Our study indicated that culturable *L. pneumophila* persisted in four kinds of spring waters with various pH and conductivity levels for 60 min. Moreover, the practice of blending with tap water showed no negative effects on culturability of *L. pneumophila* in spring waters. These findings imply the exposure potential to *Legionella* and the infection risk of LD during bathing activities.
- Cinnamon oil effectively inactivated planktonic *L. pneumophila* of 4 log CFU ml⁻¹ at 750 µg ml⁻¹ or less, which is relatively low toxic and independent of the practice of blending with tap water. Besides, its efficacy against *L. pneumophila* was significantly higher in alkaline PBS and spring waters that have been recognized as the most common sources of *Legionella* in Taiwanese springs. These characteristics highlight the potential of cinnamon oil to be employed as an effective anti-*Legionella* agent in recreational hot springs.

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