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

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\textbf{A B S T R A C T}

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 mS 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 mg l\(^{-1}\), 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

\textit{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
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., 1999; 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 EWGL, 2005). While appropriate in general, the disinfection activity of chlorine is known to be significantly reduced at the alkaline pH (EWGLINET and EWGL, 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 parahemolyticus (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 BCYEex 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 chloride (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 μm 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 μS 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 ± 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 BCYEₐ 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 BCYEₐ 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 BCYEₐ 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 BCYEₐ 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 R² ≥ 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
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). Fairwise comparison revealed cell culturability was significantly lower in Spring C than in any other three springs (all of P ≤ 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

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

<table>
<thead>
<tr>
<th>Types of hot spring</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acid sulfate</td>
<td>Acid hydrogen carbonate</td>
<td>Acid sulfate chloride</td>
<td>Alkaline hydrogen carbonate</td>
</tr>
<tr>
<td>At the sampling site</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>50.1</td>
<td>53.0</td>
<td>45.1</td>
<td>61.5</td>
</tr>
<tr>
<td>pH</td>
<td>5.6</td>
<td>6.2</td>
<td>2.1</td>
<td>7.1</td>
</tr>
<tr>
<td>Conductivity (µS cm⁻¹)</td>
<td>557 ± 197a</td>
<td>2188 ± 619</td>
<td>6460 ± 537</td>
<td>2073 ± 762</td>
</tr>
<tr>
<td>Within 1 day after sample collection</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) No dilution with sterile tap water</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>6.6</td>
<td>2.1</td>
<td>7.5</td>
</tr>
<tr>
<td>Conductivity (µS cm⁻¹)</td>
<td>382 ± 48</td>
<td>1546 ± 98</td>
<td>5595 ± 526</td>
<td>1331 ± 31</td>
</tr>
<tr>
<td>(b) 1:1 dilution with sterile tap water</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>6.5</td>
<td>6.8</td>
<td>2.1</td>
<td>7.7</td>
</tr>
<tr>
<td>Conductivity (µS cm⁻¹)</td>
<td>259 ± 36</td>
<td>915 ± 20</td>
<td>2881 ± 99</td>
<td>787 ± 17</td>
</tr>
</tbody>
</table>

a Mean ± standard deviation.

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Fig. 1 – Culturability of L. pneumophila in PBS at room temperature (■) and at 42 °C for 10 (□) and 60 min (■■) contact. **: No detectable culturability.
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 ($P < 0.007$). 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 MBC$_{50}$, MBC$_{80}$, and MBC$_{100}$ 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 MBC$_{50}$ and MBC$_{80}$ values in all four springs. The most apparent decrease was shown in Spring D where MBC$_{50}$ and MBC$_{80}$ values at 60-min contact were 24% and 40% of that determined at 10 min, respectively.
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 ≥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 springs 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 loss 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 (Catenich 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

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

<table>
<thead>
<tr>
<th>Springs</th>
<th>Contact time (min)</th>
<th>MBC (µg ml⁻¹)</th>
<th>MBC50</th>
<th>MBC80</th>
<th>MBC100</th>
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<tr>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Springs without dilution</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>10</td>
<td>464</td>
<td>751</td>
<td>1200</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>282</td>
<td>370</td>
<td>500</td>
<td></td>
<td></td>
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<tr>
<td>C</td>
<td>268</td>
<td>285</td>
<td>400</td>
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<td></td>
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<tr>
<td>D</td>
<td>295</td>
<td>459</td>
<td>750</td>
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<td>A</td>
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<td>165</td>
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<td>750</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>144</td>
<td>228</td>
<td>500</td>
<td></td>
<td></td>
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<tr>
<td>C</td>
<td>134</td>
<td>172</td>
<td>400</td>
<td></td>
<td></td>
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<tr>
<td>D</td>
<td>70</td>
<td>185</td>
<td>500</td>
<td></td>
<td></td>
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<tr>
<td>Springs diluted with 1:1 sterile tap water</td>
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<tr>
<td>A</td>
<td>10</td>
<td>478</td>
<td>564</td>
<td>1200</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>373</td>
<td>545</td>
<td>1000</td>
<td></td>
<td></td>
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<tr>
<td>C</td>
<td>259</td>
<td>271</td>
<td>400</td>
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<tr>
<td>D</td>
<td>240</td>
<td>306</td>
<td>750</td>
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<tr>
<td>A</td>
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<td>262</td>
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<td>750</td>
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<tr>
<td>B</td>
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<td>289</td>
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<td></td>
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<tr>
<td>C</td>
<td>258</td>
<td>272</td>
<td>300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>119</td>
<td>180</td>
<td>500</td>
<td></td>
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</tbody>
</table>

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 < 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

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 ≥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 springs 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 loss 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 (Catenich 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 \( \mu \)g ml\(^{-1}\) completely inactivated L. pneumophila within 60 min in diluted springs with MBC\(_{50}\) values between 119 and 270 \( \mu \)g ml\(^{-1}\). Furuhat\a 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 \( \mu \)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 \( \mu \)g ml\(^{-1}\) and 625–5000 \( \mu \)g ml\(^{-1}\) with 8-h contact (Furuhat\a 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 \( \mu \)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 (Cocc\-chia\a 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\(_{90}\) 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 acidic media this double bond could be hydrogenated to form a radical \([\text{C}_6\text{H}_5\text{CHCHCHO}]\)-, 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 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 (Furuhat\a 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 constitutes 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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