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ARTICLE

Antimicrobial Activity of Oleanolic Acid on *Listeria monocytogenes* under Sublethal Stresses of NaCl and pH

Yohan Yoon and Kyoung-Hee Choi[1,*]

Department of Food and Nutrition, Sookmyung Womens's University, Seoul 140-742, Korea
[1]Department of Oral Microbiology, College of Dentistry, Wonkwang University, Iksan 570-749, Korea

Abstract

The objective of this study was to evaluate the inhibition of *Listeria monocytogenes* growth by oleanolic acid under sublethal stresses of NaCl and pH. *L. monocytogenes* ATCC15313 (6 log CFU/mL) was inoculated in microplate wells containing brain heart infusion (BHI) broth supplemented with oleanolic acid in various amounts (0, 0.25, 0.5, 1.0, 1.5, 2.0, and 4.0 μg/mL), and different pHs (5 and 7) and NaCl concentrations (0, 3, and 6%), followed by incubation under accelerated storage condition (37°C, 48 h). The optical density (OD) of the samples was measured at 0, 6, 12, 24, and 48 h at 600 nm. After the lag phase duration was observed at the early stage of incubation, the OD values of *L. monocytogenes* significantly increased ($p<0.05$) in BHI broth formulated with 0 and 3% of NaCl during accelerated storage at pH 5 and 7. However, the growth of *L. monocytogenes* in 6% NaCl and at less than 0.5 μg/mL of oleanolic acid had no growth at pH 5 and only gradual growth at pH 7. Moreover, *L. monocytogenes* generally had lower OD values as the concentrations of oleanolic acid increased. As expected, the OD values of *L. monocytogenes* were generally higher ($p<0.05$) at pH 7 than at pH 5. These results indicate that oleanolic acid should be useful in inhibiting the growth of *L. monocytogenes*.

Key words: oleanolic acid, *Listeria monocytogenes*, ready-to-eat meat, phytochemical

Introduction

Various antimicrobials have been used to ensure that manufactured foods remain safe and unspoiled, and most preservatives commonly used in food industry today are mainly from synthetic chemicals, but their safety problems have been receiving growing attention (Feng *et al*., 2010). Thus, various phytochemicals from a variety of plants known to have antimicrobial activity have been examined to control foodborne pathogens (Lee *et al*., 2007; Ros and Recio, 2005; Tassou *et al*., 2000; Valero and Salmeroj, 2003). Plants synthesize various aromatic substances, including many antimicrobial phytochemicals such as phenols and their oxygen substituted derivatives, essentials oils, terpenes and alkalids (Kurek *et al*., 2010). Oleanolic acid are triterpenoid compounds isolated from foods, medical herbs, and various other plants in free form or bound to glycosides (Somova *et al*., 2003). Oleanolic acid has shown anti-inflammatory (Ovesna *et al*., 2004), antiallergic (Banno *et al*., 2004), hepatoprotective (Liu, 1995), antimicrobial (Ngouela *et al*., 2005) and antiulcer (Ovesna *et al*., 2004) activities, and also exert an antiparasitic activity to *Trypanosoma* spp. (Cunha *et al*., 2003) and *Leishmania* spp. (Torres-Santos *et al*., 2004).

Listeria monocytogenes is a ubiquitous psychrotrophic pathogen causing listeriosis with high fatality rate (up to 30%), and it survives and grows at low pH as well as in high salt content (Farber and Peterkin, 1991; Vazquez-Bolland *et al*., 2001). Nunez de Gonzalez *et al.* (2004) suggested that most *L. monocytogenes* contamination on ready-to-eat meat and poultry products occurs after processing. In the United States, foodborne illness caused by *L. monocytogenes* has been estimated at 2,493 infections and 499 deaths annually (Mead *et al*., 1999). Even though most ready-to-eat meat and poultry products may contain various antimicrobials such as sodium chloride, nitrite and lactate/diacetate salts to inhibit *L. monocytogenes* growth, their effectiveness may vary due to type of product and other factors (Glass *et al*., 1989; Mbandi and Shelef, 2002). In addition, most consumers have recently concerned about use of the chemicals and also synthesized chemicals in processed meat because of their safety problems. Thus, novel antimicrobials especially from

*Corresponding author: Kyoung-Hee Choi, Department of Oral Microbiology, College of Dentistry, Wonkwang University, Iksan 570-749, Korea. Tel: 82-63-850-6911, Fax: 82-63-850-7313, E-mail: kheechoi@wku.ac.kr

plants to inhibit *L. monocytogenes* growth in ready-to-eat meat and poultry products may be appropriate to improve consumer acceptance to the products.

This study evaluated the antimicrobial activity of oleanolic acid on inhibition of *L. monocytogenes* growth under sublethal stresses of NaCl and pH.

Materials and Methods

Preparation of inoculum

L. monocytogenes ATCC15313 was activated in 5 mL of brain heart infusion (BHI) broth (Becton Dickinson, MD, USA) for 24 h at 37°C. Stationary phase cells were serially diluted in phosphate buffered saline (PBS, pH 7.4; 0.2 g of KH_2PO_4, 1.5 g of $Na_2HPO_4 \cdot 7H_2O$, 8.0 g of NaCl, and 0.2 g of KCl in 1 liter of distilled water) to obtain approximately 6 log CFU/mL.

Experimental design

The effects of pH (5 and 7) adjusted solely with 1 N HCl, oleanolic acid concentrations (0, 0.25, 0.5, 1, 1.5, 2, and 4 μg/mL; Sigma-Aldrich Corp., MO, USA), and three levels (0, 3, and 6%) of NaCl (Junsei Chemical Co., Ltd., Japan) on inhibition of *L. monocytogenes* growth in BHI broth were evaluated. This resulted in a total of 42 combinations in a complete factorial design.

Inoculation and microbiological analysis

Each combination was placed in wells of 96 micro plate, and *L. monocytogenes* was inoculated in each well to obtain 4 log CFU/mL. Oleanolic acid stock solution (60 μg/mL) was prepared in dimethyl sulfoxide (Sigma-Aldrich) and added to each well to obtain 0, 0.25, 0.5, 1, 1.5, 2, and 4 μg/mL. The micro plates were incubated under accelerated condition (37°C, 48 h), which is an appropriate condition to confirm antimicrobial resistance of pathogenic bacteria to antimicrobials and full recovery of the injured pathogens (Oh *et al*. 2004). The OD (Optical density) values of the samples were measured at 0, 6, 12, 24, and 48 h at 600 nm with spectrophotometer (Spectra Max250, Molecular Devices, USA).

Statistical analysis

This study was repeated twice with two samples in each replicate. A complete factorial design (pH [5 and 7] ×oleanolic acid [0, 0.25, 0.5, 1, 1.5, 2, and 4 μg/mL]× NaCl [0, 3, and 6%]) was used in this study. The OD values in interactions among pH, oleanolic acid, and NaCl concentrations were analyzed by the mixed model procedure of SAS® version 9.2 (SAS Institute, Cary, NC, USA), and random effects in the model was removed at alpha = 0.05 by the forward stepwise method with type III F-test. All least squares mean comparisons among the interactions were performed with the pairwise *t*-test at alpha = 0.05.

Results and Discussion

The OD values of *L. monocytogenes* in BHI broth prepared with different concentrations of NaCl and different levels of oleanolic acid at pH 5, which is simulating ready-to-eat meat and poultry related condition, are shown in Tables 1-3. After lag phase duration was observed at early stage of incubation, the optical density of *L. monocytogenes* significantly increased ($p<0.05$) in BHI broth (pH 5) supplemented only with 0% and 3% of NaCl and 0-2 μg/mL of oleanolic acid during accelerated storage, but no growth ($p\geq0.05$) of the pathogen was observed in BHI broth plus 6% of NaCl at all concentrations of oleanolic acid (Tables 1-3). This result indicates that when NaCl (0-6%) was combined with oleanolic acid, the antimicrobial activity of oleanolic acid on the pathogen was synergistically increased. Moreover, *L. monocytogenes* had lower OD values as oleanolic acid concentration increased (Tables 1-3). Inhibited growth of *L. monocytogenes* may be related to impaired peptidoglycan metabolism caused by oleanolic acid because the triterpenoids of oleanolic acid may influence the enzymes related to peptidoglycan metabolism such as the proteins involved in cell wall synthesis, hydrolysis and modification which might decrease peptidoglycan cross-linkage and thus enhance its susceptibility to mutanolysin, an N-acetylmuramidase (Kurek *et al*., 2010).

A study by Horiuchi *et al*. (2007) showed that oleanolic acid inhibited even growth of methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci. On this wise, antimicrobial activities of the phytochemicals extracted from various plants have been studied to control antibiotic resistant bacteria. *Mycobacterium tuberculosis* is the bacteria causing tuberculosis with very high mortality rate in world wide because this pathogen is resistant to multiple drugs, but oleanolic acid extracted from *Lantana hispida* (Verbenacease) inhibited *M. tuberculosis* growth (Jimenez-Arellanes *et al*., 2007). Use of the extract from mulberry leaves also inhibited biofilm formation of *Streptococcus mutans*, which is one of the causes related to antibiotic resistance (Islam *et al*., 2008). They suggested that the continuous studies to examine

Table 1. Optical density of *Listeria monocytogenes* ATCC15313 at 600 nm under sublethal stresses of oleanolic acid and 0% of NaCl at pH 5 during incubation at 37°C for 48 h

Oleanolic acid (μg/mL)	Incubation time (h)				
	0	6	12	24	48
0	0.000±0.000^{Ac}	0.007±0.002^{Ac}	0.009±0.001^{Ac}	0.077±0.002^{Ab}	0.106±0.011^{Aa}
0.25	0.000±0.000^{Ac}	0.006±0.001^{Ac}	0.008±0.001^{Ac}	0.057±0.002^{Bb}	0.087±0.008^{Ba}
0.5	0.000±0.000^{Ac}	0.007±0.002^{Ac}	0.008±0.001^{Ac}	0.055±0.006^{Bb}	0.084±0.011^{Ba}
1	0.000±0.000^{Ac}	0.008±0.002^{Ac}	0.008±0.002^{Ac}	0.034±0.007^{Cb}	0.082±0.011^{Ba}
1.5	0.000±0.000^{Ac}	0.007±0.002^{Ac}	0.009±0.001^{Ac}	0.023±0.002^{Db}	0.073±0.004^{Ca}
2	0.000±0.000^{Ab}	0.006±0.002^{Ab}	0.009±0.002^{Ab}	0.009±0.001^{Eb}	0.058±0.008^{Da}
4	0.000±0.000^{Aa}	0.007±0.001^{Aa}	0.008±0.001^{Aa}	0.009±0.001^{Ea}	0.007±0.001^{Ea}

$^{A-E}$: Means within a column with different superscripts are significantly different (p<0.05)
$^{a-c}$: Means within a row with different superscripts are significantly different (p<0.05)

Table 2. Optical density of *Listeria monocytogenes* ATCC15313 at 600 nm under sublethal stresses of oleanolic acid and 3% of NaCl at pH 5 during incubation at 37°C for 48 h

Oleanolic acid (μg/mL)	Incubation time (h)				
	0	6	12	24	48
0	0.000±0.000^{Aa}	0.007±0.002^{Ab}	0.008±0.001^{Ab}	0.013±0.003^{ABb}	0.095±0.008^{Aa}
0.25	0.000±0.000^{Ac}	0.007±0.002^{Ac}	0.009±0.001^{Ac}	0.019±0.005^{Ab}	0.073±0.005^{Ba}
0.5	0.000±0.000^{Ac}	0.009±0.001^{Ac}	0.009±0.001^{Ac}	0.018±0.001^{Ab}	0.070±0.006^{Ba}
1	0.000±0.000^{Ab}	0.009±0.001^{Ab}	0.010±0.001^{Ab}	0.014±0.003^{ABb}	0.067±0.006^{Ba}
1.5	0.000±0.000^{Ab}	0.009±0.001^{Ab}	0.009±0.001^{Ab}	0.011±0.000^{ABb}	0.068±0.004^{Ba}
2	0.000±0.000^{Ab}	0.007±0.002^{Ab}	0.008±0.000^{Ab}	0.010±0.005^{Bb}	0.051±0.014^{Ca}
4	0.000±0.000^{Aa}	0.008±0.002^{Aa}	0.009±0.002^{Aa}	0.009±0.001^{Ba}	0.010±0.003^{Ca}

$^{A-C}$: Means within a column with different superscripts are significantly different (p<0.05)
$^{a-b}$: Means within a row with different superscripts are significantly different (p<0.05)

Table 3. Optical density of *Listeria monocytogenes* ATCC15313 at 600 nm under sublethal stresses of oleanolic acid and 6% of NaCl at pH 5 during incubation at 37°C for 48 h

Oleanolic acid (μg/mL)	Incubation time (h)				
	0	6	12	24	48
0	0.000±0.000^{Aa}	0.007±0.001^{Aa}	0.008±0.002^{Aa}	0.008±0.001^{Aa}	0.006±0.001^{Aa}
0.25	0.000±0.000^{Aa}	0.007±0.001^{Aa}	0.008±0.001^{Aa}	0.008±0.001^{Aa}	0.007±0.002^{Aa}
0.5	0.000±0.000^{Aa}	0.007±0.001^{Aa}	0.008±0.001^{Aa}	0.009±0.001^{Aa}	0.008±0.002^{Aa}
1	0.000±0.000^{Aa}	0.008±0.002^{Aa}	0.009±0.001^{Aa}	0.010±0.001^{Aa}	0.008±0.001^{Aa}
1.5	0.000±0.000^{Aa}	0.009±0.001^{Aa}	0.009±0.000^{Aa}	0.009±0.001^{Aa}	0.008±0.001^{Aa}
2	0.000±0.000^{Aa}	0.007±0.001^{Aa}	0.008±0.001^{Aa}	0.007±0.001^{Aa}	0.007±0.001^{Aa}
4	0.000±0.000^{Aa}	0.008±0.002^{Aa}	0.008±0.002^{Aa}	0.009±0.000^{Aa}	0.008±0.001^{Aa}

$^{A, a}$: No significant differences were observed within a column and row (p≥0.05)

antimicrobial activity of phytochemicals on various foodborne pathogens are necessary.

As expected, the OD values of *L. monocytogenes* were generally higher (p<0.05) in pH 7 than in pH 5 (Tables 1-6). The optical density of *L. monocytogenes* at pH 7 increased (p<0.05) at low concentrations (0-2 μg/mL) of oleanolic acid during incubation for all NaCl concentrations, but 4 μg/mL of oleanolic acid completely inhibited *L. monocytogenes* growth at pH 7 (Tables 4-6). Significant effect of NaCl on strengthening antilisterial effect of oleanolic acid was also observed at pH 7. In the case of pH 5, no growth of *L. monocytogenes* was observed at 6 % of NaCl for all oleanolic acid concentrations, but gradual increases of the OD values were observed at 6% of NaCl for low levels (<0.5 μg/mL) of oleanolic acid at pH 7 (Tables 4-6).

The results from this study show that oleanolic acid is still effective to inhibit *L. monocytogenes* growth even under optimum growth condition for bacterial growth of the pathogen, and the effect of the phytochemical on constraining *L. monocytogenes* growth is concentration-dependent. Since the conditions used in this study were

Table 4. Optical density of *Listeria monocytogenes* ATCC15313 at 600 nm under sublethal stresses of oleanolic acid and 0% of NaCl at pH 7 during incubation at 37°C for 48 h

Oleanolic acid (μg/mL)	Incubation time (h)				
	0	6	12	24	48
0	0.000±0.000^{Ab}	0.015±0.002^{Ad}	0.139±0.012^{Ac}	0.307±0.040^{Aa}	0.187±0.012^{Bb}
0.25	0.000±0.000^{Ab}	0.015±0.002^{Ad}	0.153±0.012^{Ac}	0.286±0.037^{Bb}	0.311±0.337^{Aa}
0.5	0.000±0.000^{Ab}	0.016±0.002^{Ad}	0.153±0.020^{Ac}	0.249±0.014^{Ba}	0.187±0.023^{Bb}
1	0.000±0.000^{Ab}	0.017±0.001^{Ad}	0.159±0.012^{Aa}	0.201±0.040^{Ca}	0.184±0.051^{Ba}
1.5	0.000±0.000^{Ab}	0.015±0.001^{Ad}	0.029±0.014^{Ab}	0.192±0.036^{Ca}	0.217±0.023^{Ba}
2	0.000±0.000^{Ab}	0.012±0.002^{Ad}	0.013±0.001^{Bb}	0.206±0.079^{Ca}	0.223±0.028^{Ba}
4	0.000±0.000^{Ab}	0.013±0.002^{Aab}	0.015±0.001^{Bab}	0.015±0.001^{Da}	0.057±0.091^{Ca}

$^{A-C}$: Means within a column with different superscripts are significantly different (p<0.05)
$^{a-d}$: Means within a row with different superscripts are significantly different (p<0.05)

Table 5. Optical density of *Listeria monocytogenes* ATCC15313 at 600 nm under sublethal stresses of oleanolic acid and 3% of NaCl at pH 7 during incubation at 37°C for 48 h

Oleanolic acid (μg/mL)	Incubation time (h)				
	0	6	12	24	48
0	0.000±0.000^{Ac}	0.013±0.002^{Ac}	0.027±0.010^{Ab}	0.254±0.045^{Aa}	0.277±0.026^{Ca}
0.25	0.000±0.000^{Ab}	0.014±0.002^{Ab}	0.031±0.012^{Ab}	0.213±0.031^{Ba}	0.232±0.022^{Ba}
0.5	0.000±0.000^{Ab}	0.015±0.002^{Ab}	0.062±0.026^{Ab}	0.271±0.057^{Aa}	0.236±0.019^{Ba}
1	0.000±0.000^{Ab}	0.015±0.001^{Ab}	0.035±0.004^{Ab}	0.220±0.090^{Ba}	0.184±0.019^{Ba}
1.5	0.000±0.000^{Ab}	0.016±0.001^{Ab}	0.017±0.001^{Ab}	0.180±0.106^{Ba}	0.218±0.026^{Aa}
2	0.000±0.000^{Ab}	0.013±0.001^{Ab}	0.014±0.001^{Ab}	0.018±0.005^{Cb}	0.185±0.044^{Aa}
4	0.000±0.000^{Aa}	0.013±0.001^{Aa}	0.014±0.001^{Aa}	0.014±0.001^{Ca}	0.014±0.005^{Aa}

$^{A-C}$: Means within a column with different superscripts are significantly different (p<0.05)
$^{a-c}$: Means within a row with different superscripts are significantly different (p<0.05)

Table 6. Optical density of *Listeria monocytogenes* ATCC15313 at 600 nm under sublethal stresses of oleanolic acid and 6% of NaCl at pH 7 during incubation at 37°C for 48 h

Oleanolic acid (μg/mL)	Incubation time (h)				
	0	6	12	24	48
0	0.000±0.000^{Aa}	0.014±0.002^{Ab}	0.014±0.001^{Ab}	0.015±0.001^{Ab}	0.138±0.061^{Ba}
0.25	0.000±0.000^{Aa}	0.013±0.001^{Ab}	0.015±0.001^{Ab}	0.014±0.002^{Ab}	0.111±0.060^{Ba}
0.5	0.000±0.000^{Aa}	0.014±0.002^{Ab}	0.014±0.002^{Ab}	0.016±0.001^{Ab}	0.182±0.094^{Aa}
1	0.000±0.000^{Aa}	0.015±0.002^{Aa}	0.015±0.002^{Aa}	0.016±0.001^{Aa}	0.033±0.017^{Ca}
1.5	0.000±0.000^{Aa}	0.016±0.001^{Aa}	0.016±0.001^{Aa}	0.015±0.001^{Aa}	0.013±0.001^{Ca}
2	0.000±0.000^{Aa}	0.013±0.002^{Aa}	0.014±0.001^{Aa}	0.013±0.001^{Aa}	0.012±0.001^{Ca}
4	0.000±0.000^{Aa}	0.015±0.001^{Aa}	0.015±0.002^{Aa}	0.016±0.001^{Aa}	0.013±0.001^{Ca}

$^{A-C}$: Means within a column with different superscripts are significantly different (p<0.05)
$^{a-b}$: Means within a row with different superscripts are significantly different (p<0.05)

optimum growth condition for *L. monocytogenes*, the antilisterial effect of oleanolic acid should be more obvious and minimal inhibition concentration to *L. monocytogenes* should be lower in ready-to-eat food related conditions such as low storage temperature, high concentration of NaCl, and presence of other antimicrobials. Moreover, use of phytochemical, which is plant-originated such as oleanolic acid, as an antilisterial additive may improve consumer acceptance than bacteria-originated antimicrobials or synthetic chemicals because customers believe that phytochemicals are safer than synthetic antimicrobials and they have additional bioactivities such as a decreased risk of cancer and other chronic diseases as well as antimicrobial activity (Surles *et al*., 2004; Feng *et al*., 2010). Therefore, use of oleanolic acid in the formulation of ready-to-eat meat and poultry products may decrease bacterial populations of *L. monocytogenes* in the products without compromising consumer acceptance.

In conclusion, use of oleanolic acid should be useful in

inhibiting *L. monocytogenes* growth, and thus development of the formulations including oleanolic acid is necessary to control the pathogen in ready-to-eat meat and poultry products.

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