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Author	Su-Yeon Kim, Seok-Seong Kang
Affiliation	Department of Food Science and Biotechnology, College of Life Science and Biotechnology, Dongguk University
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ORCID (All authors must have ORCID) https://orcid.org	Su-Yeon Kim (https://orcid.org/0000-0001-5601-977X) Seok-Seong Kang (https://orcid.org/0000-0001-7029-9122)
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CORRESPONDING AUTHOR CONTACT INFORMATION

For the <u>corresponding</u> author (responsible for correspondence, proofreading, and reprints)	**Fill in information in each box below**
First name, middle initial, last name	Seok-Seong Kang
Email address – this is where your proofs will be sent	sskang@dongguk.edu
Secondary Email address	Kangelo72@gmail.com
Postal address	32 Dongguk-ro, Ilsandong-gu, Goyang-si, Gyeonggi-do, 10326, Republic of Korea
Cell phone number	+82-10-3669-6409
Office phone number	+82-31-961-5150
Fax number	

8 **Anti-biofilm activities of Manuka honey against *Escherichia coli* O157:H7**

9

10 ## Abstract

11 Manuka honey (MH) has been shown anti-bacterial activity against several pathogenic bacteria.
12 However, the inhibitory effect of MH on biofilm formation by *Escherichia coli* O157:H7 has
13 not yet been examined. In this study, MH significantly reduced *E. coli* O157:H7 biofilm.
14 Moreover, pre- and post-treatment with MH also significantly reduced *E. coli* O157:H7 biofilm.
15 Cellular metabolic activities exhibited that the viability of *E. coli* O157:H7 biofilm cells was
16 reduced in the presence of MH. Further, colony forming unit of MH-treated *E. coli* O157:H7
17 biofilm was significantly reduced by over 70%. Collectively, this study suggests the potential
18 of anti-biofilm properties of MH which could be applied to control *E. coli* O157:H7.

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20 **Keywords:** Manuka honey, *Escherichia coli* O157:H7, biofilm, anti-bacterial

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Introduction

Honey has been consumed for the nutraceutical values and various health benefits, including anti-oxidative, anti-inflammatory, and anti-bacterial properties, in addition to wound-healing ability (Alvarez-Suarez et al., 2013). With regard to biological functions, honey is an interesting natural source for medicinal uses (Mandal and Mandal, 2011). Among the different categories of honey, Manuka honey (MH) has predominantly attracted attention owing to its biological functions including anti-bacterial activity (Alvarez-Suarez et al., 2013). MH is derived from the Manuka tree (*Leptospermum scoparium*) growing throughout New Zealand and eastern Australia and has long been used for treating infections, including those associated with abscesses, surgical wounds, traumatic wounds, and burns (Kato et al., 2012; Patel and Cichello, 2013).

E. coli O157:H7 is a serotype of *E. coli* producing Shiga toxins 1 and 2 as important virulence factors and causes significant disorders such as hemorrhagic colitis and bloody diarrhea (Mohawk et al., 2010). This pathogen is able to attach, colonize, and form biofilm, which is more than 100 times resistant than planktonic cells, on abiotic surfaces (e.g., steel, plastic, and glass) and biotic surfaces (e.g., fruits, vegetables, and meat) (Jefferson, 2004; Uhlich et al., 2006). Consequently, biofilms have become problematic in various food industries, including breweries, dairy, poultry, and meat processing, because bacteria readily form biofilms on the surface of food and food-related facilities (Srey et al., 2013). Hence, this study demonstrates the anti-biofilm property of MH against *E. coli* O157:H7.

Materials and methods

Bacterial culture conditions and honey sample

E. coli O157:H7 ATCC 35150 was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Luria-Bertani (LB) medium (LPS solution, Daejeon, Korea) at 37°C. For the experiments, *E. coli* O157:H7 was cultured at 37°C for 8 h and was diluted to 1×10^8 colony forming unit (CFU) per mL corresponding to 0.2 at 600 nm of optical density (OD) in fresh LB broth. MH with Unique Manuka Factor (UMF) 5+ (Comvita, Paengaroa, New Zealand) was purchased from a local shop in Seoul, Korea, and was diluted in phosphate-buffered saline (PBS), filtered through a 0.2-μm filter.

Biofilm formation assay

Biofilm formation assay was performed as described previously (Kim et al., 2019). Briefly, *E. coli* O157:H7 (100 μL; 1×10^8 CFU/mL) was cultured with or without MH (0, 0.1, and 0.2 g/mL) in a microtiter plate for 24 h at 37°C. After washing the microtiter plate with PBS, biofilm was stained 0.1% crystal violet for 30 min. For quantification of biofilm, 0.1% acetic acid and 95% ethanol were added to dissolve the bacterial cells bound crystal violet and the absorbance was measured at a wavelength of 595 nm to determine biofilm formation. Additionally, at 24-h incubation, *E. coli* O157:H7 biofilm cells were serially diluted and CFU of *E. coli* O157:H7 were determined by plating on LB agar. For the effect of pre- or post-treatment with MH on the biofilm of *E. coli* O157:H7, MH (0, 0.1, and 0.2 g/mL) was treated to a microtiter plate for 24 h. The bacterial suspension was then added and further incubated at 37°C for 24 h. Conversely, the bacterial suspension was treated to a microtiter plate at 37°C

for 24 h followed by the addition of MH (0, 0.1, and 0.2 g/mL) and further incubation at 37°C for 24 h. *E. coli* O157:H7 biofilm was then assessed as described above.

XTT reduction assay

To examine the viability of cells in *E. coli* O157:H7 biofilm, reduction assay was performed using XTT (2,3-Bis [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) (Biotium, Fremont, CA, USA). In brief, after the formation biofilm of *E. coli* O157:H7 with or without MH in a microtiter plate for 24 h, planktonic *E. coli* O157:H7 cells were removed by washing with PBS. Subsequently, PBS (200 µl) and XTT solution (100 µL) were added to the microtiter plate and incubated at 37°C for 2 h. The absorbance of developed color was measured at a wavelength of 492 nm, while the absorbance of background was detected at a wavelength of 630 nm. Normalization for the consequential absorbance was obtained by the subtraction of the background absorbance values.

Adenosine triphosphate (ATP) production assay

To examine ATP production in biofilm cells, *E. coli* O157:H7 was formed biofilm with or without MH for 24 h. Thereafter, ATP production was assessed using BacTitier-Glo microbial cell viability assay kit (Promega, Madison, WI, USA). Bioluminescence was determined at 560 nm using VICTOR X4 multi-label plate reader (Perkin Elmer, Waltham, MA, USA).

Growth inhibition of *E. coli* O157:H7 planktonic cells

The inhibitory effect of MH on the growth of *E. coli* O157:H7 planktonic cells was determined. Briefly, *E. coli* O157:H7 was incubated with or without MH (0.1 and 0.2 g/mL) for 1, 3, 6, 12, and 24 h. Following incubation, the bacterial growth was measured at a wavelength of 595 nm.

Statistical analysis

Results are expressed as mean ± standard deviation of triplicate samples obtained from independent three experiments. Statistically significant difference was determined in comparison with controls by conducting an unpaired two-tailed *t*-test and one-way analysis of variance (ANOVA) using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA) and IBM SPSS Statistics 23 software (IBM, Armonk, NY, USA), respectively.

Results and Discussion

MH inhibits *E. coli* O157:H7 biofilm formation

MH significantly inhibited biofilm formation by *E. coli* O157:H7 (Fig. 1A). Further, to examine the preventive effect of MH on biofilm formation, MH was added 24 h prior to the inoculation of *E. coli* O157:H7. Fig. 1B showed that MH markedly reduced the biofilm formation by *E. coli* O157:H7. Moreover, MH effectively disrupted *E. coli* O157:H7 biofilm (Fig. 1C); however, the inhibitory effect was not dose-dependent similar to that related to pre-treatment with MH. Although honey has been used as a traditional medication for microbial infections, the anti-bacterial properties of honey, including MH, have been mostly focused against clinical isolates in chronic wounds. Ulmo honey and MH exhibited optimal anti-

115 bacterial activities against methicillin-resistant *Staphylococcus aureus* isolates as well as *E.*
116 *coli* and *Pseudomonas aeruginosa* according to agar diffusion assay analysis (Sherlock et al.,
117 2010). Another study also showed similar results that MH eradicated methicillin-resistant *S.*
118 *aureus* in a synergistic manner with antibiotics (Jenkins and Cooper, 2012). More recently, it
119 was demonstrated that a multispecies biofilm consortium of wound pathogens, including *S.*
120 *aureus*, *Streptococcus agalactiae*, *P. aeruginosa*, and *Enterococcus faecalis*, was attenuated by
121 MH and honeydew honey (Sojka et al., 2016). Although these previous studies honey including
122 MH revealed effective anti-bacterial agents, this study showed for the first time that MH
123 significantly reduced the biofilm formation as well as the disruption of *E. coli* O157:H7 biofilm.

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125 **MH decreases the viability of *E. coli* O157:H7 biofilm cells**

126 Metabolically active cells are able to enhance XTT reduction, reflecting an increase of viability
127 of biofilm mass (Nett et al., 2011; Sivaranjani et al., 2016). As shown in Fig. 2A, XTT reduction
128 assay indicated that the viability of *E. coli* O157:H7 biofilm cells was significantly reduced by
129 approximately 80% when treated with 0.1 g/mL MH, and treatment with 0.2 g/mL MH also
130 significantly decreased viability (> 70% reduction). Various anti-microbial agents prevented
131 the biofilm formation of foodborne pathogens by decreasing the cellular metabolic activity of
132 biofilm cells (Khan et al., 2017; Luis et al., 2014; Sivaranjani et al., 2016). Gallic acid, caffeic
133 acid and chlorogenic acid significantly inhibited the XTT reduction of *S. aureus*, consequently
134 preventing the biofilm formation (Luis et al., 2014). Furthermore, ATP production was
135 measured in *E. coli* O157:H7 biofilm cells treated with or without MH. As expected, MH dose-
136 dependently inhibited ATP production in *E. coli* O157:H7 biofilm (Fig. 2B). Therefore, these
137 results indicate that MH suppressed the *E. coli* O157:H7 biofilm by decreasing the cellular

138 metabolic activities such as XTT reduction and ATP production. Additionally, the viability of
139 *E. coli* O157:H7 biofilm cells with or without MH was evaluated by counting CFU after 24-h
140 incubation. Treatment with 0.1 g/mL MH was highly effective against *E. coli* O157:H7 viability,
141 facilitating significant removal of bacteria (> 70% reduction); furthermore, 0.2 g/mL MH
142 demonstrated approximately 90% reduction in the viability of *E. coli* O157:H7 (Fig. 2C).

143

144 **MH suppresses the growth of *E. coli* O157:H7 planktonic cells**

145 As can be seen in Fig. 3, the presence of 0.1 g/mL MH significantly inhibited the growth of *E.*
146 *coli* O157:H7 planktonic cells even after 1-h incubation. A significant extent of inhibition was
147 observed for another 24 h as opposed to the control culture of *E. coli* O157:H7. Similarly, a
148 higher inhibitory effect on the growth of *E. coli* O157:H7 planktonic cells was exerted by 0.2
149 g/mL MH throughout the incubation time (1-24 h), suggesting that MH is effective against the
150 growth of *E. coli* O157:H7 planktonic cells. Similar results were given in previous reports that
151 cell-free supernatants of *Pediococcus acidilactici* HW01 suppressed the growth of *Candida*
152 *albicans* as well as the biofilm formation (Kim and Kang, 2019). In addition, bacteriocin of
153 *Lactobacillus brevis* DF01 inhibited the growth of *E. coli*, resulting in the reduction of biofilm
154 formation (Kim et al., 2019). In accordance with the previous studies, this study also
155 demonstrated that MH effectively inhibited the biofilm formation by decreasing the growth and
156 viability of *E. coli* O157:H7. However, ginseng extract significantly prevented the biofilm
157 formation by *P. aeruginosa*, whereas it did not reduce the growth of *P. aeruginosa* planktonic
158 cells (Wu et al., 2011). Therefore, it can be speculated that the anti-biofilm ability against
159 pathogenic bacteria may be differently regulated by decreasing the growth and viability of
160 pathogenic bacteria, which result in the obstruction of biofilm formation at the initial stage, or

by disrupting the established biofilm.

Conclusion

In conclusion, this study noticeably demonstrated that MH suppressed the biofilm formation of *E. coli* O157:H7 by decreasing bacterial growth and viability. Several studies have shown that MH has anti-bacterial activity against foodborne pathogens. This study, however, demonstrated that MH exerts anti-biofilm activity against *E. coli* O157:H7. Although extensive studies would be needed to establish the precise mechanism(s) of inhibitory action, results from this study suggest that MH may be promising a natural anti-bacterial agent for controlling *E. coli* O157:H7.

Conflict of interest

The authors have no conflicts of interest to declare.

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Author contributions

Conceptualization: Kang SS. Data curation: Kim SY, Kang SS. Formal analysis: Kim SY, Kang SS. Methodology: Kim SY, Kang SS. Software: Kim SY. Validation: Kim SY, Kang SS.

Investigation: Kim SY, Kang SS. Writing-original draft: Kim SY, Kang SS. Writing-review & editing: Kim SY, Kang SS.

Ethics approval

This article does not require IRB/IACUC approval because there are no human and animal participants.

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Figure legends

Fig. 1. Effect of MH on biofilm formation by *E. coli* O157:H7. (A) *E. coli* O157:H7 was co-incubated with MH for 24 h. (B) MH was pre-treated for 24 h and *E. coli* O157:H7 was then added and incubated for further 24 h. (C) *E. coli* O157:H7 was pre-treated for 24 h and MH was then added and incubated for further 24 h. After incubation, biofilm formation was determined using crystal violet staining. *E. coli* O157:H7 biofilm incubated without MH was set as 100%. The results are shown as mean ± standard deviations.

Fig. 2. Effect of MH on the viability of *E. coli* O157:H7 biofilm cells. After biofilm formation for 24 h, (A) biofilm cells were subjected to XTT reduction assay and (B) ATP production was measured. (C) colony-forming units of *E. coli* O157:H7 biofilm cells were enumerated by plating on LB agar. The results are shown as mean ± standard deviations.

Fig. 3. Effect of MH on the growth of *E. coli* O157:H7 planktonic cells. *E. coli* O157:H7 was incubated with or without MH for 1, 3, 6, 12, or 24 h. At each time point, the bacterial growth was measured at 595 nm. (*) indicates $P < 0.05$.

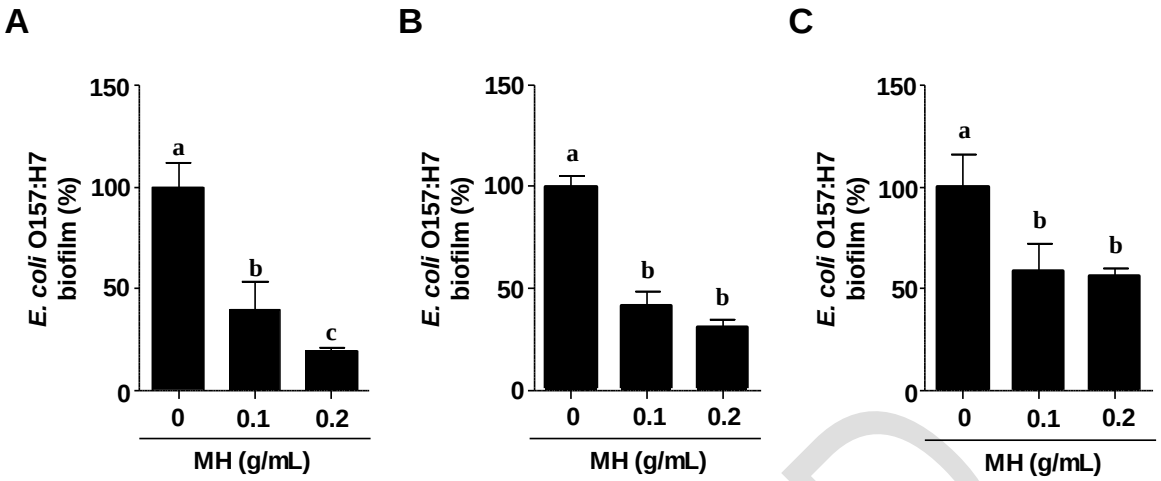


Figure 1

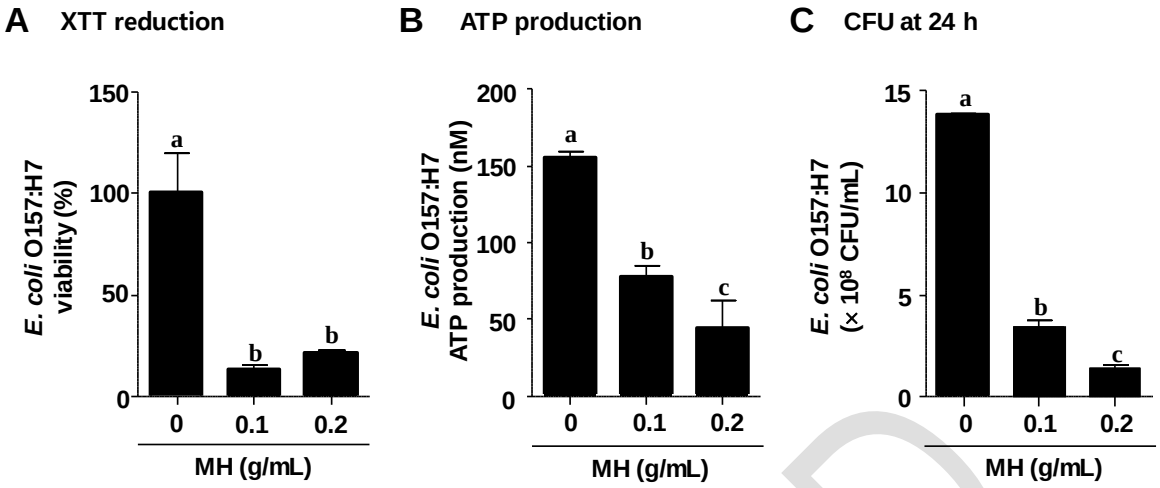
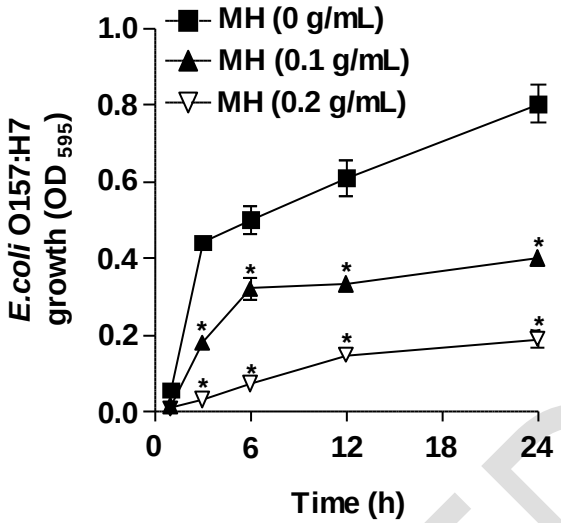


Figure 2



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305 Figure 3

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