Food Science of Animal Resources

Food Sci. Anim. Resour. 2020 July 40(4):668~674
DOI https://doi.org/10.5851/kosfa.2020.e42

pISSN : 2636-0772 eISSN : 2636-0780
http://www.kosfaj.org

SHORT COMMUNICATION

Anti-Biofilm Activities of Manuka Honey against *Escherichia coli* O157:H7

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OPEN ACCESS

Received March 25, 2020
Revised May 18, 2020
Accepted May 31, 2020

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

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

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℃. For the experiments, *E. coli* O157:H7 was cultured at 37℃ 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℃. 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℃ for 24 h. Conversely, the bacterial suspension was treated to a microtiter plate at 37℃ for 24 h followed by the addition of MH (0, 0.1, and 0.2 g/mL) and further incubation at 37℃ 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℃ 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 adenosine triphosphate (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±SD 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, 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-bacterial activities against methicillin-resistant *Staphylococcus aureus* isolates as well as *E. coli* and *Pseudomonas aeruginosa*

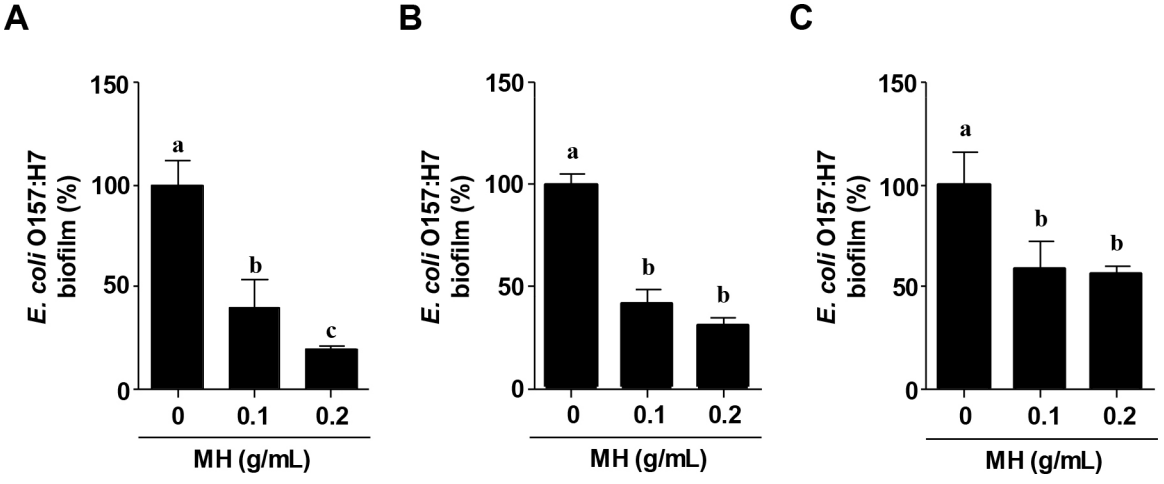

Fig. 1. Effect of MH on biofilm formation by *E. coli* O157:H7. (A) *E. coli* O157:H7 was co-incubated with MH for 24 h. (B) MH was pre-treated for 24 h and *E. coli* O157:H7 was then added and incubated for further 24 h. (C) *E. coli* O157:H7 was pre-treated for 24 h and MH was then added and incubated 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±SDs. MH, Manuka honey.

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

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

Metabolically active cells are able to enhance XTT reduction, reflecting an increase of viability of biofilm mass (Nett et al., 2011; Sivaranjani et al., 2016). As shown in Fig. 2A, XTT reduction assay indicated that the viability of *E. coli* O157:H7 biofilm cells was significantly reduced by approximately 80% when treated with 0.1 g/mL MH, and treatment with 0.2 g/mL MH also significantly decreased viability (>70% reduction). Various anti-microbial agents prevented the biofilm formation of foodborne pathogens by decreasing the cellular metabolic activity of biofilm cells (Khan et al., 2017; Luís et al., 2014; Sivaranjani et al., 2016). Gallic acid, caffeic acid and chlorogenic acid significantly inhibited the XTT reduction of *S. aureus*, consequently preventing the biofilm formation (Luís et al., 2014). Furthermore, ATP production was measured in *E. coli* O157:H7 biofilm cells treated with or without MH. As expected, MH dose-dependently inhibited ATP production in *E. coli* O157:H7 biofilm (Fig. 2B). Therefore, these results indicate that MH suppressed the *E. coli* O157:H7 biofilm by decreasing the cellular metabolic activities such as XTT reduction and ATP production. Additionally, the viability of *E. coli* O157:H7 biofilm cells with or without MH was evaluated by counting CFU after 24-h incubation. Treatment with 0.1 g/mL MH was highly effective against *E. coli* O157:H7 viability, facilitating significant removal of bacteria (>70% reduction); furthermore, 0.2 g/mL MH demonstrated approximately 90% reduction in the viability of *E. coli* O157:H7 (Fig. 2C).

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

As can be seen in Fig. 3, the presence of 0.1 g/mL MH significantly inhibited the growth of *E. coli* O157:H7 planktonic cells even after 1-h incubation. A significant extent of inhibition was observed for another 24 h as opposed to the control

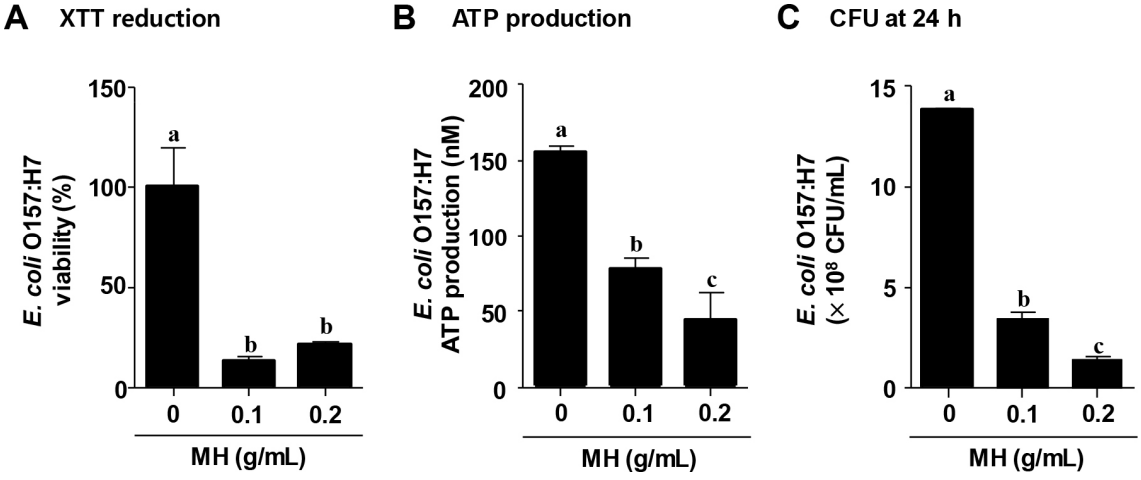

Fig. 2. Effect of MH on the viability of *E. coli* O157:H7 biofilm cells. After biofilm formation for 24 h, (A) biofilm cells were subjected to XTT reduction assay and (B) ATP production was measured. (C) colony-forming units of *E. coli* O157:H7 biofilm cells were enumerated by plating on LB agar. The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

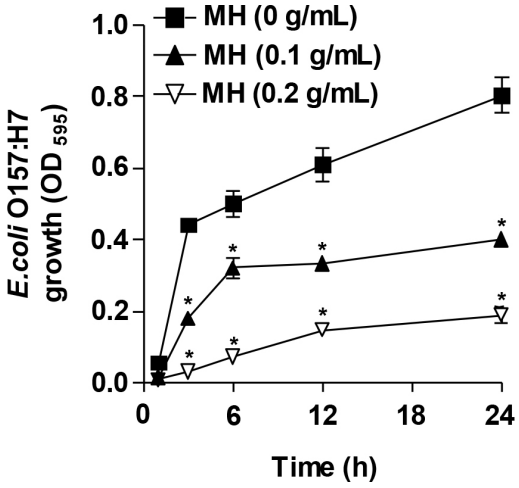

Fig. 3. Effect of MH on the growth of *E. coli* O157:H7 planktonic cells. *E. coli* O157:H7 was incubated with or without MH for 1, 3, 6, 12, or 24 h. At each time point, the bacterial growth was measured at 595 nm. (*) indicates $p<0.05$. MH, Manuka honey.

culture of *E. coli* O157:H7. Similarly, a higher inhibitory effect on the growth of *E. coli* O157:H7 planktonic cells was exerted by 0.2 g/mL MH throughout the incubation time (1–24 h), suggesting that MH is effective against the growth of *E. coli* O157:H7 planktonic cells. Similar results were given in previous reports that cell-free supernatants of *Pediococcus acidilactici* HW01 suppressed the growth of *Candida albicans* as well as the biofilm formation (Kim and Kang, 2019). In addition, bacteriocin of *Lactobacillus brevis* DF01 inhibited the growth of *E. coli*, resulting in the reduction of biofilm formation (Kim et al., 2019). In accordance with the previous studies, this study also demonstrated that MH effectively inhibited the biofilm formation by decreasing the growth and viability of *E. coli* O157:H7. However, ginseng extract significantly prevented the biofilm formation by *P. aeruginosa*, whereas it did not reduce the growth of *P. aeruginosa* planktonic cells (Wu et al., 2011). Therefore, it can be speculated that the anti-biofilm ability against pathogenic bacteria may be differently regulated by decreasing the growth and viability of 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.

Conflicts of Interest

The authors declare no potential conflicts of interest.

Acknowledgements

This work is supported by a grant from the National Research Foundation of Korea, which is funded by the Korean

government (NRF-2017R1D1A1B03028730).

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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