



Piper betle Leaf Extract Inhibits *Escherichia coli* O157:H7 Biofilms and Prevents Bacterial Adhesion on Beef during Storage

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Abstract

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is one of a major foodborne pathogen concern worldwide. This study aimed to investigate the antibacterial, antibiofilm, and anti-adhesion properties of *Piper betle* extract against EHEC O157:H7 during storage. The suspected isolates were identified as EHEC O157:H7 through 16S rRNA sequence alignment, showing 100% homology. The extract exhibited antibacterial activity against all isolates, with similar MIC and MBC values as 0.5–1.0 mg/mL. The extract at 1/2×MIC significantly inhibited biofilm formation in all isolates with 66–75% inhibition ($P < 0.05$). Established biofilms were disrupted by 63–76% when treated with *Piper betle* extract at 8×MIC. Adhered cells on beef were reduced by 3 logs with the extract at 8×MIC within 3 and 6 h at 37°C and 4°C, respectively. A significant reduction in bacterial adhesion on beef was observed when treated with the extract at 8 and 4×MIC during 7-day storage at –18°C. At 7-day storage, beef treated with 8×MIC did not show a significant difference in hardness, cohesiveness, springiness, gumminess, and chewiness of the beef, compared with the control. These findings suggest that *Piper betle* leaf extract inhibits *E. coli* O157:H7 biofilms and prevents the bacterial adhesion on beef during storage.

Keywords: *Escherichia coli* O157:H7, *Piper betle* extract, Biofilm, Bacterial adhesion, Storage.

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1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is responsible for bloody and non-bloody diarrhoea, haemorrhagic colitis, and haemolytic uremic syndrome in humans and animals. It is considered the most predominant and virulent serotype of pathogenic *E. coli*.^[1] EHEC O157:H7 possesses virulence factors, such as Shiga-like toxins 1 and 2 (encoded by *stx1* and *stx2*, respectively), intimin (encoded by *eaeA*), and EHEC haemolysin (encoded by *Ehly*).^[2] Shigatoxigenic *E. coli* (STEC) produces Shiga toxins, whereas verotoxigenic *E. coli* (VTEC) produces Shiga-like toxins (verotoxins).^[2] The consumption of food products contaminated with EHEC O157:H7 from animal origins,

contact with infected animals, or exposure to a contaminated environment can result in human infection. Beef is a common type of meat, which is mostly contaminated with EHEC O157:H7.^[3] EHEC O157:H7 contamination is found in raw meat, cooked food, and ready-to-eat food.^[4] Hence, the presence of EHEC O157:H7 in raw meat, including beef, is a major risk factor for human infection.

Bacterial biofilms are associated with chronic infections in humans and animals owing to their high antimicrobial resistance and properties that help them evade the host's immune system.^[5] Notably, bacterial biofilms are organized microbial communities of aggregated cells that can adhere to surfaces and embed in a moist matrix consisting of their own extracellular polymeric materials. EHEC O157:H7 isolated from meat possesses a high biofilm-forming ability with antibiotic resistance.^[6] EHEC O157 strain 43895 was responsible for an outbreak in Hamburger. It can form biofilms at 37°C, invade epithelial cells, and last longer in cattle compared with a biofilm-negative strain.^[7] Additionally, it can be transferred from contact surfaces to meat, poultry, ready-to-eat deli, and produces owing to its ability to form biofilms.^[8]

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Table 1: Molecular detection of *E. coli* O157:H7 and their biofilm-related genes and phenotypes.

Isolates	Detection of <i>eaeA</i> gene	Detection of biofilms		
		Biofilm related genes		Biofilm forming ability
		<i>pgaA</i>	<i>pgaC</i>	
A1.9	+, OQ910327 ^a	+, OQ910332 ^a	+, OQ910337 ^a	Strong
E5.3	+, OQ910328 ^a	+, OQ910333 ^a	+, OQ910338 ^a	Strong
E5.4	+, OQ910329 ^a	+, OQ910334 ^a	+, OQ910339 ^a	Strong
E5.8	+, OQ910330 ^a	+, OQ910335 ^a	+, OQ910340 ^a	Strong
J5.9	+, OQ910331 ^a	+, OQ910336 ^a	+, OQ910341 ^a	Strong
ATCC25922	-	+	+	Strong

+ = Positive, - = Negative, ^a = Accession no.

E. coli forms biofilms via several steps. The *pgaABCD* locus is responsible for various pathogenic traits, such as the development of *E. coli* biofilm. Some genes located at this locus, including *pgaA* and *pgaC*, have been identified to determine the bacteria's ability to form biofilms and contribute 1,6-N-acetyl-d-glucosamine production, surface adhesion, intracellular adhesion, and biofilms.^[9]

Natural resources, such as medicinal plants, can be used as alternative remedies for the treatment of bacterial infections. In the present study, we focused on the ethanol extract of the *Piper betle* leaf, a member of the Piperaceae family and a native of South and Southeast Asia, for its potential antibacterial activity against EHEC O157:H7. *Piper betle* leaf has been used as an alternate medicine for the treatment of many infections due to its antibacterial properties.^[10] It contains several chemical compounds with potential antifungal and antibacterial properties, such as hydroxychavicol, chavicol, eugenol, and phenols.^[10] *Piper betle* extract can inhibit the growth of avian pathogenic *Escherichia coli* (APEC)^[11] and *Salmonella* spp.^[12] and reduce the adhesion and biofilm production of APEC.^[11]

Based on the aforementioned information, we aimed to investigate the pathotype EHEC O157:H7 based on the detection of *eaeA* and evaluate the antibacterial and antibiofilm activities of *Piper betle* extract against EHEC O157:H7. Additionally, we also investigated the reduction in bacterial adhesion on beef.

2. Materials and methods

2.1. Bacterial strains and growth condition

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All experiments were conducted in accordance with the biosafety regulations for scientific experiments (Ref. No. WU-IBC-66-016) at Walailak University, Nakhon Si Thammarat, Thailand. Five isolates of *E. coli* O157 from ruminants and *E. coli* ATCC25922 (American Type Culture Collection, Manassas) were used. These isolates were provided by the Faculty of Veterinary Science, Prince of Songkla University, Thailand and characterized as sorbitol non-fermenting STEC.^[13] Five colonies of each bacterium, cultured on Tryptic Soy Agar (TSA) (Difco, Claix, France), were inoculated into tryptic soy broth (TSB) (Himedia, Nashik, India) at 37°C for 18–24 h. The cultures were then stored in TSB containing 25% glycerol at –80°C until further use.

2.2. Preparation of *Piper betle* leaf extract

Dry powder of *Piper betle* leaves was prepared from mature leaves collected in Phatthalung province, Thailand. Then, 50 g of the dry powder was soaked in 200 mL of 95% ethanol (powder ratio of 1:4, w/v) for 7 days at 25°C.^[12] The liquid extracts were then filtered through Whatman qualitative filter paper grade 1 (Whatman International Ltd., Maidstone, UK). The filtered sample was evaporated under low pressure at 40°C using a rotary evaporator (Buchi Labortechnik AG, Flawil, Switzerland) to concentrate the extract. The extract was subsequently air-dried at room temperature for 3 days. For stock solutions, the extract was dissolved in 100% dimethyl sulfoxide (DMSO) and/or absolute ethanol at a concentration of 200 mg/mL. Cefotaxime (Hoechst Marion Roussel, Bridgewater, USA), used as a positive control, was dissolved in 100% DMSO. All agents were stored at –20°C until further use.

2.3. Characterization of EHEC O157:H7 and biofilm-related genes

To detect virulence genes, ten colonies of each isolate were suspended in distilled water and heated at 100°C for 10 min. The samples were then centrifuged at 5,000 rpm for 5 min and the supernatant was collected to be used as the DNA template. These samples were stored at –80°C until further use. The bacteria were identified as EHEC O157:H7 using *eae*AO157:H7 primers (Forward primer:

Table 2: MIC values of antibiotics against the isolates of *E. coli* O157H7.

Drug classes	Drug names	MIC ($\mu\text{g/mL}$)					
		Isolates of <i>E. coli</i> O157H7					
		A1.9	E5.3	E5.4	E5.8	J5.9	ATCC25922
Aminopenicillin	Ampicillin	4	≤ 2	≤ 2	≤ 2	4	8
	Amoxicillin/Clavulanic Acid	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	4
Cephalosporin	Cefalexin	≤ 4	≤ 4	≤ 4	≤ 4	8	8
	Cefalotin	4	4	4	4	4	4
	Cefpodoxime	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	0.5
	Cefovecin	1	≤ 0.5	≤ 0.5	≤ 0.5	1	≤ 0.5
	Ceftiofur	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
Aminoglycoside	Amikacin	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2
	Gentamicin	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
	Neomycin	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2
Fluoroquinolone	Enrofloxacin	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12
	Marbofloxacin	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5
	Pradofloxacin	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12
Tetracycline	Doxycycline	1	1	1	1	1	1
	Tetracycline	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
Phenicol	Chloramphenicol	4	4	≤ 2	4	4	8
Folate pathway inhibitors	Trimethoprim/Sulfamethoxazole	≤ 20	≤ 20	≤ 20	≤ 20	≤ 20	≤ 20

5'-AAGCGACTGAGGTCACT-3' and reverse primer: 5'-ACGCTGCTCACTAGATGT-3'), with the polymerase chain reaction (PCR) conditions described in a previous study.^[14]

Biofilm-related genes were detected using primers targeting *pgaA* (Forward primer: 5'-GGCTTTGAACTTCTTACTGC-3' and Reverse primer: 5'-CCTGTTTATCTTGCCCGGCC-3') and *pgaC* (Forward primer: 5'-ATGATTAATCGCATCGTATCG-3' and Reverse primer: 5'-CATCGGTTCCACAATATATGC-3'), following the PCR conditions outlined in a previous study.^[9]

A 25 μL PCR reaction mixture was prepared containing 5U/ μL *Taq* DNA polymerase (Excel*Taq*TM series, SMOBIO Technology Inc., Hsinchu, Taiwan), 0.2 mM of each dNTP (abm[®], Applied Biological Materials Inc., Richmond, Canada), 1X *Taq* PCR buffer, 4 pmol/ μL of each primer (Macrogen, Seoul, Korea), 0.8% dimethyl sulfoxide, and 3 μL of the DNA template. Nuclease-free water and *E. coli* ATCC25922 DNA served as the negative and positive controls, respectively for all reactions. The PCR amplification was performed using a thermal cycler (Eppendorf Vapo Protect, Hamburg, Germany). The PCR products were then subjected to 1.5% agarose gel electrophoresis (Vivantis, Shah Alam, Malaysia), stained with anucleic acid staining reagent (SafeViewTM Nucleic Acid Stain, Applied Biological Materials Inc., Richmond, Canada) at 135 volts for 30 mins. A 100-bp DNA ladder (ExcelBandTM series, SMOBIO Technology Inc., Hsinchu, Taiwan) was used as a DNA size reference. The gel was visualised under a UV transilluminator (ChemiDocTM with Image LabTM Touch Software, Bio-Rad Laboratories, Hercules, CA, USA). The suspected bands were purified using a commercial DNA purification kit (BioFACTTM Gel and PCR Purification System,

BIOFACT, Daejeon, Korea), and the purified DNA was sequenced using Sanger's sequencing technology (Macrogen, Seoul, Korea).

The obtained nucleotide sequences were edited and analysed using SnapGene[®] Viewer version 5.3.2 software (<https://www.snapgene.com>) and the Basic Local Alignment Search Tool (BLAST) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), respectively. The phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis (MEGA) version 11 software (<https://www.megasoftware.net>) with the neighbour-joining (NJ) algorithm and 1000 bootstrapping replications. All positive nucleotide sequences were submitted to GenBank of NCBI with accession numbers as described in Table 1.

2.4. Antibacterial activity

i) Antibiotic sensitivity of EHEC O157:H7 was determined by performing agar dilution method based on minimum inhibitory concentration (MIC) using the VITEK[®] 2 card for Gram-negative microorganisms and the VITEK[®] 2 COMPACT machine (bioMérieux, Marcy l'Etoile, France). All tested antibiotics tested are listed in Table 2.

ii) Antibacterial activity of *Piper betle* extract against EHEC O157:H7 was determined by performing a microdilution assay based on the Clinical and Laboratory Standards Institute guidelines,^[15] with slight modifications as previously described.^[12] Briefly, a final concentration of 2.5 mg/disk was achieved by adding 12.5 μL of *Piper betle* extract (200 mg/mL) to sterile filter paper disks (6 mm in diameter). Then, 3–5 colonies of EHEC O157:H7 isolates cultured on Mueller Hinton agar (MHA) (HiMedia Laboratories, Mumbai, India)

Table 3: Antibacterial activity of *Piper betle* extract against the isolates of *E. coli* O157:H7.

Isolates	Inhibition zone (mm)			MIC/MBC (mg/mL)	
	<i>Piper betle</i> extract	Ampicillin	Gentamicin	<i>Piper betle</i> extract	Cefotaxime
A1.9	17.00 ± 0.82	S	S	1.0/1.0	0.00025/0.00025
E5.3	15.00 ± 0.82	S	S	1.0/1.0	0.000125/0.00025
E5.4	13.00 ± 0.00	S	S	0.5/0.5	0.00025/0.00025
E5.8	14.50 ± 0.41	S	S	1.0/1.0	0.000125/0.000125
J5.9	16.33 ± 0.24	S	S	1.0/1.0	0.00025/0.00025
ATCC25922	20.00 ± 0.00	S	S	0.5/1.0	0.00025/0.00025

were added to 0.1 M sterile normal saline solution, and the turbidity was adjusted to 0.5 McFarland's standard. The aliquots were then spread on MHA. The disks were placed on the MHA plates and incubated at 37 °C for 18 h. Positive controls were ampicillin and gentamicin (Oxoid, Hampshire, UK), whereas negative control was a disk loaded with 100% DMSO. The inhibition zones (mm) were measured and presented as mean ± standard deviation (SD).

iii) The MIC and minimum bactericidal concentration (MBC) of the extract against the isolates were evaluated by a broth microdilution assay as previously described.^[16] The extract was diluted to a final concentration of 2.0 to 0.125 mg/mL in a 96-well plate containing Mulier Hinton broth (MHB)

(HiMedia Laboratories, Mumbai, India). Cefotaxime and 1% DMSO were used as positive and negative controls, respectively. Then, 100 µL of the bacterial suspension (1×10^6 CFU/mL) was added to the microtiter plate containing the antibiotic substances and incubated at 37°C for 18 h. Resazurin (0.03%) (Thermo Fisher Scientific, Lancashire, UK) was added to the wells to determine the MIC by evaluating the colour change as previously described.^[17] The lowest concentration that completely inhibited bacterial growth and produced a blue colour was considered as the MIC (Fig. 1D). Samples producing blue colour were subsequently cultivated on TSA plates and incubated at 37°C for 24 h to evaluate the MBC.

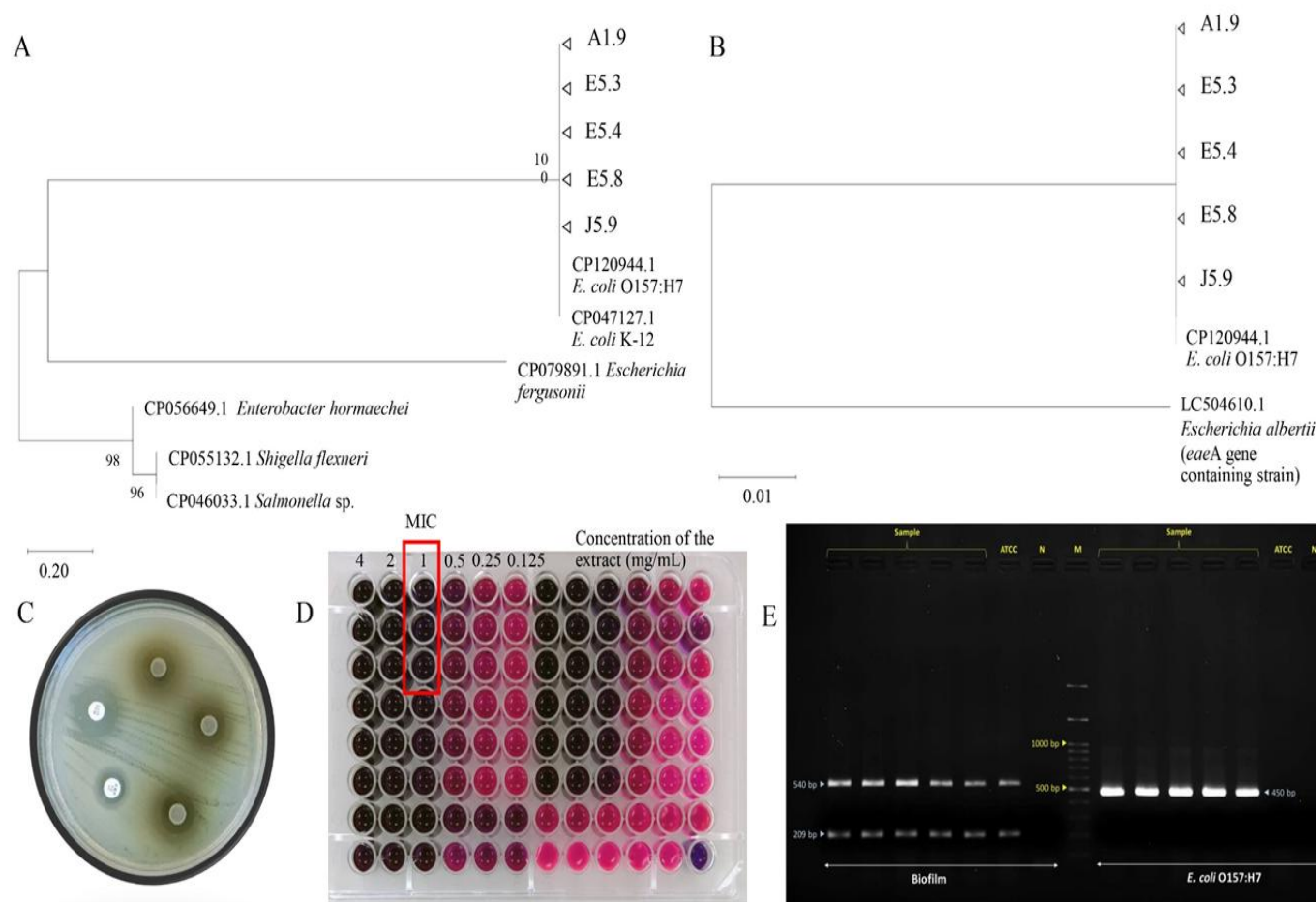


Fig. 1: Molecular identification of *Escherichia coli* O157:H7 (A), detection of *eaeA* associated with *E. coli* O157:H7 (B and E), and detection of biofilm-related genes in the isolates (E). The activity of *Piper betle* extract against the isolates by disc diffusion assay (C) and broth microdilution assay (D).

Table 4: Effects of *Piper betle* leaf extract on the viability of contaminated bacteria on beef.

Time	Types of bacteria	Control	Concentrations of the extract			
			1×MIC	2×MIC	4×MIC	8×MIC
Day 0	Total bacteria	6.44 ± 0.02 ^a	6.21 ± 0.19 ^b	6.44 ± 0.03 ^a	6.37 ± 0.13 ^{ab}	6.30 ± 0.09 ^{ab}
	Suspected <i>E. coli</i>	2.88 ± 0.06 ^a	2.92 ± 0.13 ^a	2.84 ± 0.12 ^a	2.84 ± 0.06 ^a	2.86 ± 0.07 ^a
	Suspected <i>Salmonella</i> spp.	ND	ND	ND	ND	ND
	Suspected <i>Listeria</i> sp.	ND	ND	ND	ND	ND
Day 3	Total bacteria	6.52 ± 0.01 ^a	6.44 ± 0.04 ^a	5.33 ± 0.03 ^b	5.24 ± 0.06 ^c	5.36 ± 0.09 ^{bc}
	Suspected <i>E. coli</i>	2.95 ± 0.10 ^a	2.84 ± 0.06 ^a	2.66 ± 0.10 ^b	ND	ND
	Suspected <i>Salmonella</i> spp.	ND	ND	ND	ND	ND
	Suspected <i>Listeria</i> sp.	ND	ND	ND	ND	ND
Day 7	Total bacteria	7.35 ± 0.15 ^a	6.44 ± 0.04 ^b	6.44 ± 0.11 ^{bc}	5.97 ± 0.03 ^{cd}	5.70 ± 0.24 ^d
	Suspected <i>E. coli</i>	3.11 ± 0.03 ^a	2.80 ± 0.04 ^b	2.48 ± 0.00 ^c	ND	ND
	Suspected <i>Salmonella</i> spp.	ND	ND	ND	ND	ND
	Suspected <i>Listeria</i> sp.	ND	ND	ND	ND	ND

2.5. Inhibition of EHEC O157:H7 biofilms by *Piper betle*

i) Biofilm formation: Biofilms were detected as previously described.^[5] Culture suspension (200 µL, 2×10⁶ CFU/mL) of overnight culture of bacteria grown in TSB containing 1% glucose was added into 96-well plates and incubated at 37°C for 24 h. After discarding the medium, phosphate-buffered saline solution (PBS) was used to wash the wells containing the biofilms. The crystal violet assay was performed to visualise biofilms. The biofilms were air dried, stained for 30 min using 0.1% (w/v) crystal violet, and then washed twice. The biofilms were air-dried again, and dissolved in 100% DMSO, and their absorbance was detected at OD 570 nm. The biofilm-forming ability was determined as previously described.^[5]

ii) Activity of the extract against established biofilm: The bacterial suspension (200 µL, 2×10⁶ CFU/mL) was cultured in the supplemented medium containing sub-MICs of the extract and incubated at 37°C for 24 h as described above. Additionally, 1% DMSO was used as a negative control. The inhibitory activity of the extract on the growth was detected at OD 600 nm. Crystal violet assay as described above was performed to evaluate the anti-biofilm activity of the extract. The relative percentage of biofilm formation was calculated as follows: (mean OD₅₇₀ of treated well/mean OD₅₇₀ of control well) × 100.

iii) Effects of the extract on established biofilm: Briefly, 200 µL the culture was added to a 96-well microtiter plate and incubated at 37°C for 2 days (young established biofilm) and 5 days (mature established biofilm) as previously described.^[18] Planktonic cells were removed every 48 h and fresh medium was added. The established biofilms were treated with *Piper betle* extract at concentrations of 2–8×MICs and/or 1% DMSO (negative control) and incubated at 37°C for 24 h. The medium was discarded, replaced with 200 mL of PBS supplemented with 10 µL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT), and incubated at 37°C for 2 h. Live bacterial cells present in biofilms produced an insoluble purple formazan by cleaving MTT using the

dehydrogenase enzyme. This formazan was dissolved in DMSO and its concentration was detected at OD 570 nm. The relative percentage of viable biofilm was calculated as described before.

iv) Effects of the extract on adhesion of the bacteria to glass slides: The bacteria were grown in 24 well plates containing a sterile glass (0.5 cm × 0.5 cm) and the extract at different concentrations, and incubated for 24 h at 37°C. The samples were stained with crystal violet as described above. The bacterial adhesion on the glass surface was observed under the light microscope (Nikon, Tokyo, Japan) at 200X.

v) Fluorescence microscopy: To confirm killing activity of the extract against bacteria embedded in the established biofilm, the samples were stained using 100 µL Acridine orange (AO) and Propidium iodide (PI) staining solution for 10 min at room temperature in a dark condition. Live and dead cells were observed by a fluorescence microscopy that presented in green and red color, respectively.

2.6. Inhibitory activity of *Piper betle* extract on adhesion of EHEC O157:H7 on beef

The effects of *Piper betle* extract on bacterial adhesion were evaluated on imported Australian striploin (GourMeat, Australia) obtained from a supermarket. The beef was sliced under sterile conditions into approximately 1 cm³ pieces. The meat was washed with distilled water followed by a 10-min soak in 70% ethyl alcohol to remove any contaminating bacteria. A suspension of the representative isolate E5.4 was prepared in PBS to obtain a concentration of 2×10⁶ CFU/mL as described above. The beef samples (25 g each) were co-incubated with the bacterial suspension and the *Piper betle* extract at concentrations of 4 and 8×MIC, dissolved in absolute ethanol. The samples were then incubated at −18°C, 4°C, and 37°C for different incubation periods (0, 3, 6, and 12 h). We found that the activity of the extract on bacterial adhesion at −18°C was also assessed over 7 days. A 1% ethanol solution served as the negative control. After each treatment, the beef samples were transferred to individual

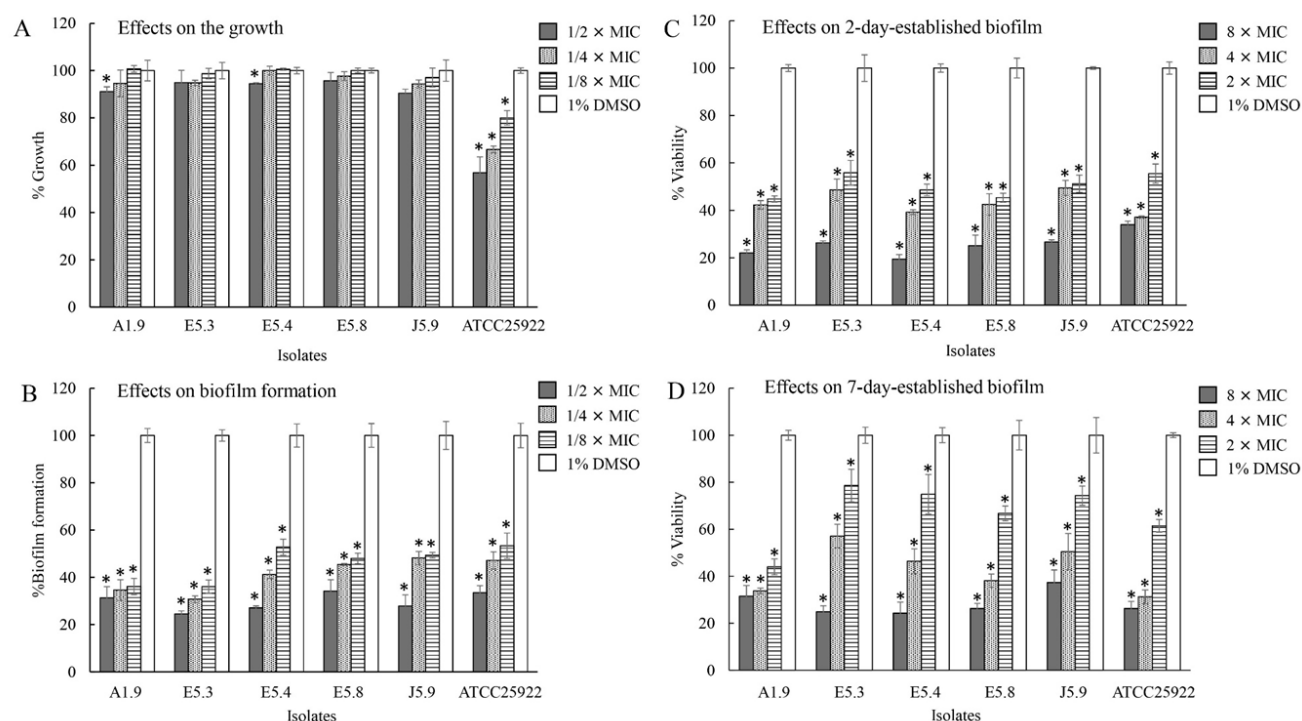


Fig. 2: Effects of *Piper betle* extract at different concentrations on *E. coli* O157:H7 growth (A), biofilm formation (B), 2-day-established biofilm (C), and 7-day-established biofilm (D). Differences were considered significant at $P < 0.05$ by the comparisons between the means.

tubes containing 9 mL of PBS and mixed using a vortex for 5 min. Bacterial numbers were determined by performing a viable cell count method on eosin methylene blue (EMB) agar (HiMedia Laboratories, Mumbai, India).

2.7. Detection of bacteria on beef after treatment with *Piper betle* extract

Detection of contaminated bacteria on beef after treatment with *Piper betle* extract was carried out. Briefly, 25 grams of the beef was weighted, and put in a tube containing *Piper betle* extract at concentrations of 1, 2, 4 and 8×MIC. The samples were kept at 4°C for different incubation periods (0, 3, and 7 days). A 1% ethanol solution served as the negative control. After treatment, the samples were then detected the survival bacteria including total bacteria, suspected *E. coli*, suspected *Salmonella* spp., and suspected *Listeria* spp. on the selective agar. The experiment was performed in triplicates. The results were presented as mean±sd.

2.8. Physical characteristics of beef during storage

Physical characteristics of beef after treatment with *Piper betle* extract were determined. Briefly, beef was sliced under sterile conditions into approximately 40 mm × 40 mm × 20 mm. The samples were treated with the extract at 2, 4 and 8×MIC, kept at 4°C for different incubation periods (0, 3, and 7 days). A texture analyzer (LLOYD Instrument LR5K, Lloyd Instruments Ltd., Bognor Regis, UK) was used to analyze using a 1/4' spherical stainless probe. The test was performed with 60% compression and a crosshead speed of 1 mm/s, as

described.^[19] A holding time of 30 seconds was applied after penetration. The hardness (N), cohesiveness, springiness (mm), gumminess (N), and chewiness (Nmm) of the beef were recorded.

We assessed the color of fresh beef treated with *Piper betle* extract while it was being stored at 4°C by assessing L^* , a^* , b^* determination. At 0, 3 and 7 days of storage, beef in each group were measured color by using colorimeter (ColorFlex EZ). It is well-accepted that L^* represents the color between black (0) and white (100), a^* value towards the positive end indicates red and negative end indicates green, and b^* value towards the positive end indicates yellow and negative end indicates blue.

2.9. Statistical analysis

All experiments were performed in triplicate and the data are presented as mean ± SD. Statistical analysis was determined by One-Way ANOVA (SPSS Inc version 15, Chicago, IL, USA). Comparisons between means were performed using Duncan's test. In addition, the two tailed unpaired Student's t test was also used. P-value of < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Molecular detection of EHEC O157:H7 and biofilm-forming ability

EHEC O157:H7 is an important food- and water-borne pathogen that causes haemorrhagic colitis and haemolytic uremic syndrome in humans, leading to major morbidity and

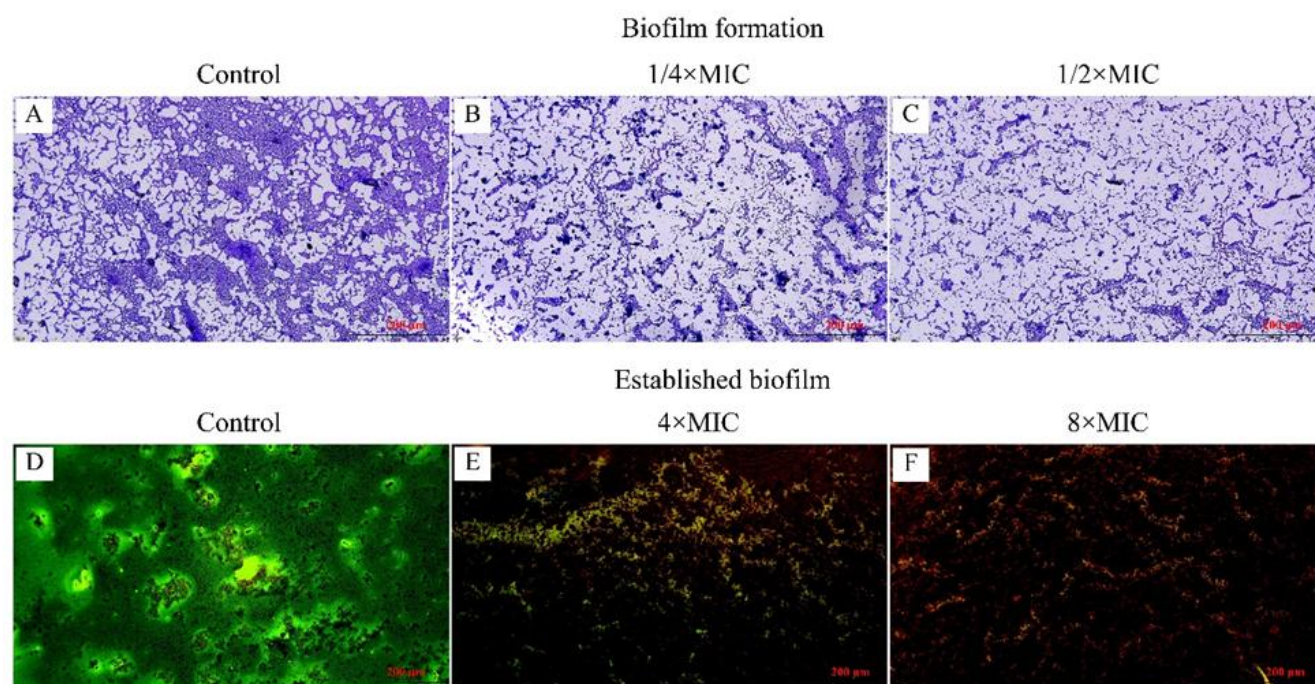


Fig. 3: Biofilm formation of *E. coli* O157:H7 isolate E5.4 cells on a surface in the presence of sub-MICs of *P. betle* extract (B and C) and one percent DMSO (A) as observed by light microscope. Fluorescence microscopy visualization of established biofilms treated with the extract at different concentrations (E and F) and one percent DMSO as the control (D). Magnifications were observed at 200X.

significant outbreaks worldwide.^[20] Meat and meat-based food products, such as beef and ready-to-eat food, can be contaminated with EHEC O157:H7.^[4] In our previous study, our research team investigated the characteristics of EHEC O157:H7 isolated from ruminants.^[13] Sorbitol non-fermenting Shiga toxin-producing *E. coli* was isolated. In the present study, we further determined a pathotype EHEC O157:H7 based on the detection of *eaeA*. We obtained 5 isolates that were *eaeA* gene positive and classified them as EHEC O157:H7 (Table 1). Furthermore, the 16S rRNA sequence alignment findings based on a GenBank database revealed that the homology of these bacteria was 100% (Fig. 1A and 2A). It has been reported that EHEC O157:H7 possesses virulence factors, such as Shiga-like toxins (encoded by *stx1* and *stx2*), intimin (encoded by *eaeA*), and EHEC haemolysin (encoded by *Ehly*).^[2] However, characterization of Shiga-like toxins and haemolysin did not investigate in this study.

In the present study, we performed a crystal violet assay and found that 5 isolates and a reference strain could form biofilms (Table 1). Additionally, biofilm-related genes, such as *pgaA* and *pgaC*, were also detected in all the isolates (Fig. 1E). These results showed that all isolates could form biofilms and possessed other abilities such as adherence to the meat surface. It has been reported that EHEC O157 (strain 43895) isolated from the outbreak associated with hamburger showed an ability to form strong biofilms and invade epithelial cells.^[7]

3.2. The MIC values of antibiotics against the isolates

The effectiveness of most currently available antibiotics for

treating bacterial infections has significantly decreased due to the emergence of several resistant strains.^[2] In the present study, the antibacterial susceptibility of antibiotics against the isolates was assessed by determining the MIC values using the agar microdilution assay. The results showed that all isolates were sensitive to all tested antibiotics, with low MIC values as presented in Table 2. However, antibiotic-resistant *E. coli* O157 has been detected in various sources, including meat^[2] and faces of ruminants.^[13]

3.3. Antibacterial activity of *Piper betle* extract against the isolates of EHEC O157:H7

Medicinal herbs and other natural products, owing to their secondary metabolites, can be effective complementary treatments for bacterial infections.^[21,22] To determine the antibacterial potency of the ethanolic extract of *Piper betle* on *E. coli* O157:H7, we performed susceptibility tests using disk diffusion and broth microdilution. The extract exhibited antibacterial activity against *E. coli* O157:H7, as preliminarily determined by the disk diffusion method (Fig. 1C). The inhibition zones of the extract against the isolates ranged from 13.00 ± 0.00 to 17.00 ± 0.82 mm (Table 3), whereas the inhibition zone against the reference strain was 20.00 ± 0.00 mm. All isolates were sensitive to the positive controls, ampicillin and gentamicin, as observed in the disk diffusion assay. Disc diffusion assay has been used to screen antibacterial activity of medicinal plant extract including *Piper betle* leaf extract.^[11] Recently, our research team has reported the antibacterial activity *Piper betle* ethanol extract

Table 5: Effects of *Piper betle* extract on texture parameters of beef during storage.

Texture parameters	Concentration	Storage (days)		
		Day 0	Day 3	Day 7
Hardness1 (N)	Control	13.762 ± 0.986 ^a	15.702 ± 2.332 ^a	13.963 ± 2.990 ^a
	2×MIC	27.644 ± 8.469 ^b	15.045 ± 0.862 ^a	15.209 ± 4.187 ^a
	4×MIC	24.009 ± 5.195 ^{ab}	18.093 ± 6.367 ^a	16.572 ± 0.569 ^a
	8×MIC	23.904 ± 5.939 ^{ab}	12.829 ± 2.734 ^a	16.085 ± 1.427 ^a
Hardness2 (N)	Control	12.789 ± 1.173 ^a	14.409 ± 1.936 ^a	13.046 ± 2.203 ^a
	2×MIC	24.927 ± 7.392 ^b	14.089 ± 1.074 ^a	14.214 ± 3.275 ^a
	4×MIC	22.071 ± 4.307 ^{ab}	16.365 ± 5.406 ^a	15.029 ± 0.267 ^a
	8×MIC	22.164 ± 5.358 ^{ab}	10.690 ± 2.032 ^a	15.245 ± 1.743 ^a
Cohesiveness	Control	0.388 ± 0.029 ^a	0.514 ± 0.068 ^a	0.537 ± 0.076 ^a
	2×MIC	0.406 ± 0.009 ^a	0.601 ± 0.035 ^{bc}	0.533 ± 0.060 ^a
	4×MIC	0.386 ± 0.005 ^a	0.535 ± 0.031 ^{ab}	0.507 ± 0.042 ^a
	8×MIC	0.393 ± 0.009 ^a	0.425 ± 0.010 ^c	0.575 ± 0.074 ^a
Springiness Index	Control	0.647 ± 0.066 ^a	0.720 ± 0.119 ^a	0.728 ± 0.006 ^a
	2×MIC	0.622 ± 0.047 ^a	0.689 ± 0.020 ^a	0.726 ± 0.040 ^a
	4×MIC	0.583 ± 0.039 ^a	0.690 ± 0.052 ^a	0.726 ± 0.019 ^a
	8×MIC	0.589 ± 0.053 ^a	0.622 ± 0.001 ^a	0.773 ± 0.016 ^a
Gumminess (N)	Control	5.340 ± 0.722 ^a	8.225 ± 2.265 ^a	7.352 ± 0.750 ^a
	2×MIC	11.289 ± 3.677 ^b	9.079 ± 1.053 ^a	7.965 ± 1.414 ^a
	4×MIC	9.256 ± 1.895 ^{ab}	9.724 ± 3.700 ^a	8.373 ± 0.410 ^a
	8×MIC	9.372 ± 2.288 ^{ab}	5.424 ± 1.031 ^a	9.323 ± 2.018 ^a
Chewiness (Nmm)	Control	34.823 ± 7.605 ^a	61.996 ± 26.130 ^a	53.669 ± 5.660 ^a
	2×MIC	68.589 ± 17.587 ^b	62.844 ± 9.042 ^a	57.527 ± 6.773 ^a
	4×MIC	53.797 ± 9.485 ^{ab}	68.484 ± 31.264 ^a	60.984 ± 4.586 ^a
	8×MIC	53.252 ± 17.296 ^{ab}	33.829 ± 6.370 ^a	72.254 ± 15.702 ^a

based on the inhibition zone against APEC (18.67±1.53 – 21.33±0.58)^[11] and *Salmonella* spp (15.11±0.34 – 20.30±0.50).^[12]

The antibacterial activity of the extract against EHEC O157:H7 was further confirmed by performing a broth microdilution assay. The MIC and MBC values of *Piper betle* extract against the clinical isolates were between 0.5–1.0 mg/mL (Table 3). The MIC and MBC values of the extract against the reference strain were within the same range as those of the tested clinical isolates. The antibacterial activity of the positive control, cefotaxime, against the isolates is shown in Table 3. *Piper betle* extract possesses broad-spectrum antimicrobial activity against several pathogens including APEC,^[11] *Salmonella* spp.,^[12] extended-spectrum β -lactamase-producing *Enterobacteriaceae*, and *Streptococcus* spp.^[10] In the present study, *Piper betle* extract was found to be a promising candidate for an antibacterial agent. It could inhibit the adhesion of EHEC O157:H7, as indicated by its MIC and MBC values. Additionally, the extract showed a bactericidal effect against pathogens because the MBC/MIC ratio was less than 2.^[11] It has been reported by our research group that hydroxychavicol and eugenol are the two main phytochemicals present in the ethanol extract of *Piper betle* leaves.^[11] These two compounds can function as bioactive compounds, possessing antimicrobial activity in *Piper betle* extract. The effects of hydroxychavicol on bacterial cells include DNA damage, the release of reactive oxygen species,

and the inhibition of cell division.^[23] Additionally, hydroxychavicol suppressed the formation of the FtsZ ring of *E. coli*, thereby inhibiting cell division.^[24] Compared with the control, APEC cells treated with *Piper betle* leaf extract exhibited longer cells without a septum, similar to cells with FtsZ, which was indicated by scanning electron microscopy.^[11] Eugenol targets the bacterial cell membrane to destroy microorganisms.^[25] This mechanism is a plausible explanation for the inhibitory effect of extract against pathogens.

3.4. Antibiofilm activity and elimination of established biofilm by *Piper betle* extract

The antibiofilm-forming activity of *Piper betle* extract EHEC O157:H7 isolates was investigated by performing a crystal violet assay. The results showed that *Piper betle* extract at 1/2, 1/4, and 1/18×MIC significantly inhibited the biofilm formation ($P < 0.05$) (Fig. 2B) of all isolates, except isolates A1.9 and E5.4, without inhibiting the growth (Fig. 2A). Furthermore, 66–75% of biofilms of all isolates were inhibited by the extract at 1/2×MIC. Nevertheless, the tested concentrations of extract inhibited the biofilm formation in the reference strain, but this concentration also inhibited bacterial growth, which may be attributed to the bioactive compounds in the extract. It is well known that adhesion is the first step of biofilm formation. The results showed that the isolate E5.4 cells treated with sub-MICs of the extract (Fig. 3B and 3C) reduced the adhesion on the surface, compared

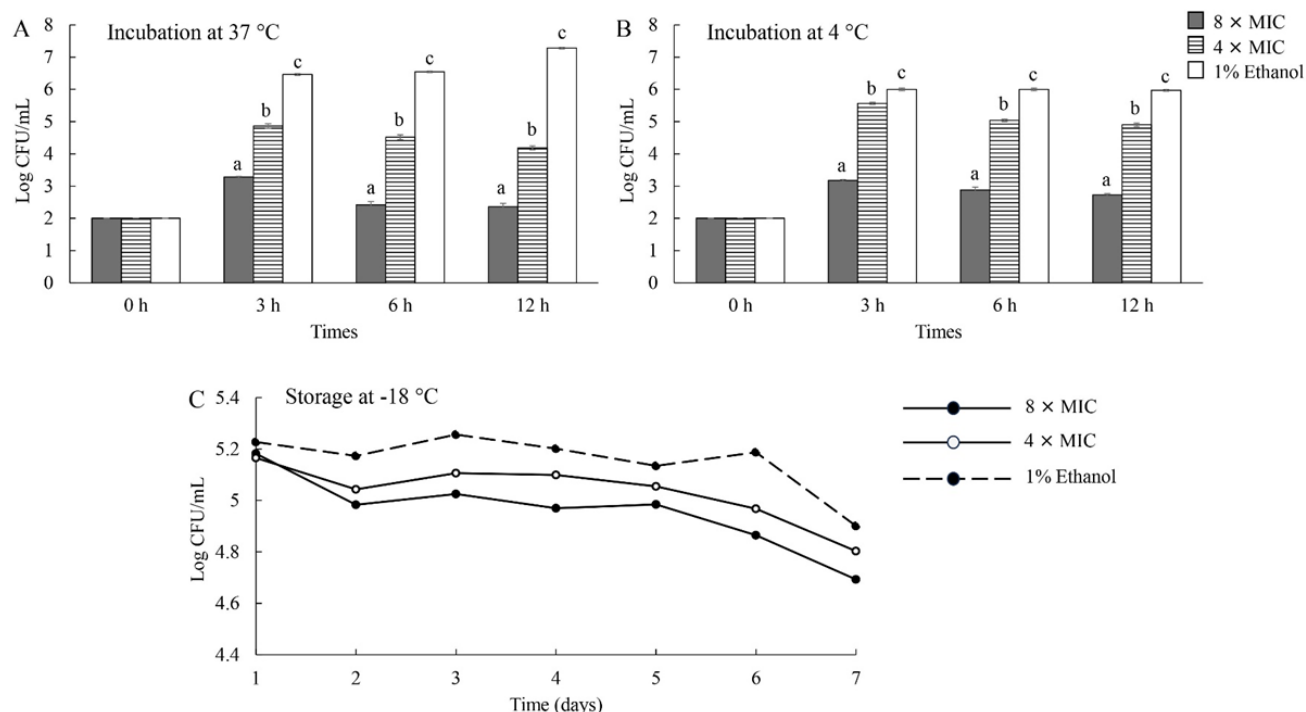


Fig. 4: Effects of *Piper betle* extract on the adhesion of *E. coli* O157:H7 on beef. The isolate E5.4 was mixed on beef, and incubated with the extract at different concentrations, stored at different temperatures. Comparisons between means were calculated using post-hoc test. Differences were considered significant at $P < 0.05$. (The viable cells at time 0 h could not be detected. However, based on the limitation of the spread plate method, the value was presented as 10^2 [2 log₁₀]).

with the control (Fig. 3A).

The inhibitory activity of the plant extract against established biofilm was determined by performing an MTT assay. The result revealed a significant decrease in the cell viability of 2-day-established biofilm (Fig. 2A) and 5-day-established biofilm of EHEC O157:H7 when treated with the extracts at 8, 4, and 2×MIC compared with that of the control ($P < 0.05$; Fig. 2B). At 8×MIC of the extract, 73–81% and 63–76% of 2-day-old and 7-day-old biofilm were eliminated, respectively. Killing activity of the extract against bacteria embedded in the established biofilm was confirmed by fluorescent microscopy images. As shown in Fig. 3D–3F, a control sample (Fig. 3D) demonstrated viable biofilms with intense green fluorescence. In contrast, the treatment of the established biofilm by 4×MIC (Fig. 3E) and 8×MIC (Fig. 3F) showed a marked reduction in green fluorescence and an increase in red fluorescence, suggesting the bacterial cell death.

Similarly, in our previous study, we reported the activity of *Piper betle* extract against APEC biofilms.^[11] The possible antibiofilm mechanisms of plant-derived compounds may include the inhibition of bacterial quorum sensing, inhibition of biofilm aggregation and build-up, and lysis of the bacteria owing to their high antibacterial effects.^[18] In our previous study, we reported that APEC cells treated with *Piper betle* extract showed longer cells without septum, similar to cells with FtsZ defect.^[11] The inhibition of MreB proteins in *E. coli* reduced *E. coli* biofilm formation.^[26] Furthermore, eugenol,

the pure compound in *Piper betle*, can inhibit cell-to-cell connections, resulting in the inhibition of biofilm formation.^[25] Eugenol suppresses the expression of genes involved in biofilm formation and the attachment and effacement phenotype including curli genes (*csgABDFG*), type I fimbriae genes (*fimCDH*), and *ler*-controlled toxin genes (*espD*, *escJ*, *escR*, and *tir*) in EHEC O157:H7.^[27] Additionally, the mature biofilm that the bacteria produced was also disrupted and detached by eugenol. Reduction in fimbria formation by eugenol may result in the detachment of the established biofilm produced by EHEC O157:H7.^[27]

3.5. Reduction of EHEC O157:H7 on beef

Adhesion is the initial step in biofilm formation. In the present study, we report the antibiofilm activity of *Piper betle* extract against *E. coli* O157:H7, leading to the exploration of its anti-adhesion activity on beef. A significant reduction in bacterial adhesion on beef was observed when the representative isolate E5.4 was treated with *Piper betle* extract at 8 × and 4 × MIC (Fig. 4A and 4B). At the optimum temperature for bacterial growth, the bacterial viability decreased by 3 logs (99.9%) within 3 h at 37°C when treated with extract at 8×MIC compared with that when treated with the control (Fig. 4A). Notably, viable cells were undetectable at 0 h; however, due to the limitations of the spread plate method, the value was presented as 10^2 (2 log₁₀). During storage at 4°C, a 3 logs reduction in bacterial growth was detected within 6 h when treated with the extract at 8×MIC compared with when treated

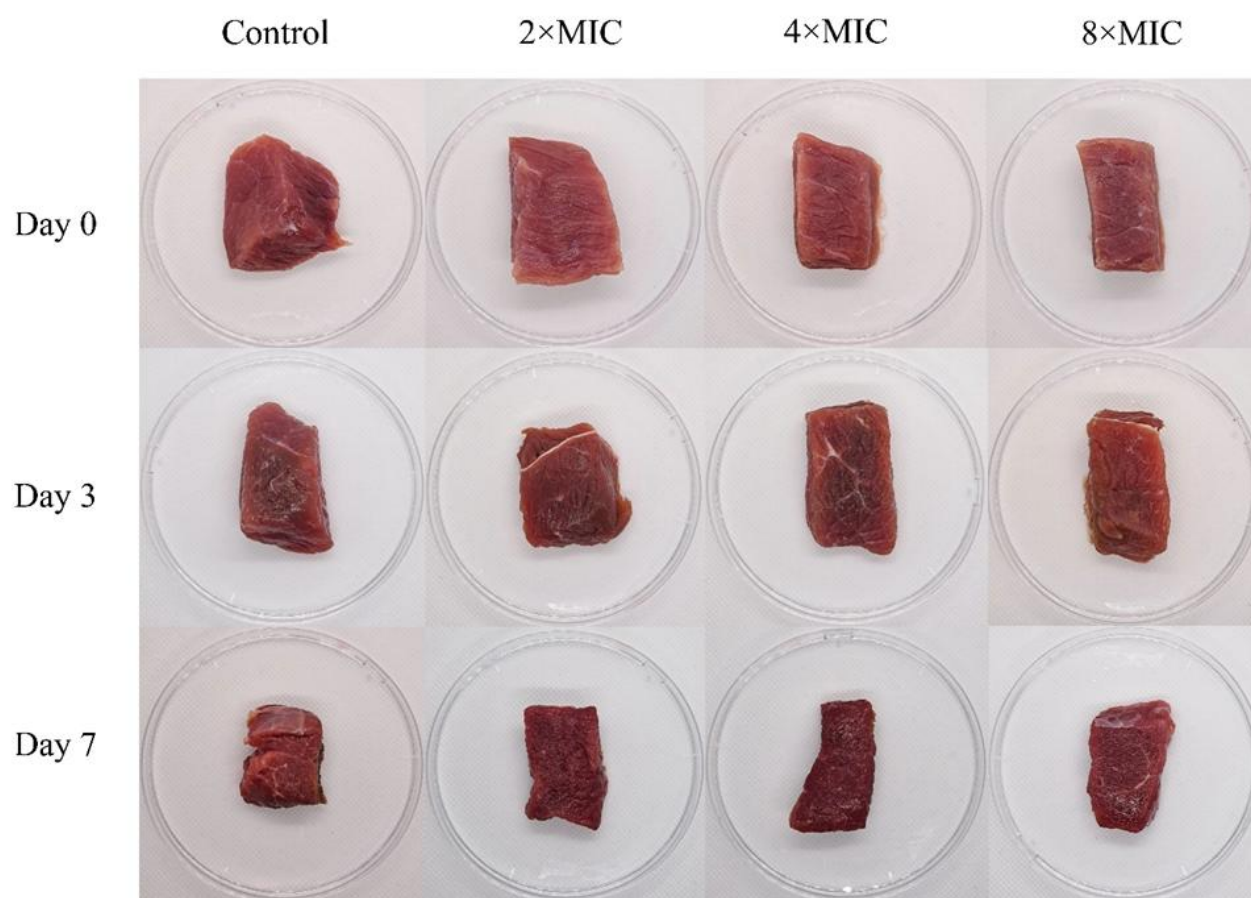


Fig. 5: Appearances of meat after treatment with the extract at different concentrations during storage.

with control. Additionally, *Piper betle* extract at 4×MIC reduced bacterial adhesion by 2.8 logs within 3 h compared with that by the control. Similarly, in our previous study, we reported the anti-adhesion activity of *Piper betle* extract against APEC adhesion.^[11] This mechanism may be attributed to bioactive compounds, including eugenol, present in the extract. Eugenol has been shown to reduce fimbriae formation in EHEC O157:H7, which decreases their biofilms and adhesion.^[27]

The addition of *Piper betle* extract to beef during storage can be an alternative strategy to reduce the adhesion of *E. coli* O157:H7 on beef. The results indicated a significant reduction in bacterial adhesion on beef when treated with the extract at 8 and 4×MIC during 7-day storage (Fig. 4C). Mixed extracts containing clove, sage, pomegranate, and *Cassia fistula* have similarly been reported to reduce *E. coli* O157:H7 adhesion on beef and fillet-fish throughout 8 days of cold storage at 4°C.^[28] Additionally, a combination of *Lactobacillus reuteri* or *Lactobacillus casei*, black pepper extract, and EDTA decreased the growth of EHEC O157:H7 on ground beef stored at 10°C for 12 days.^[29] Recently, the application of bacteriophage has been widely studied to reduce pathogens in meat and meat products.^[30] Phage biocontrol can significantly reduce EHEC O157:H7, improving food safety due to the specificity between phage and bacteria.^[30] It was noticed that

high concentrations of the extract were used in the present study. Hence, it should be recommended to evaluate the safety in consumer when the extract is used in the beef.

3.6. *Piper betle* extract reduced contaminated bacteria on beef

It is well-known that *Piper betle* leaf extract possesses antibacterial activity against a wide range of bacteria including both Gram-positive and Gram-negative bacteria. The results demonstrated that reduction of the bacterial contamination on beef after treatment with the extract was observed during the storage (Table 4). At 3-day storage, a significant reduction in viability of both total bacteria and suspected *E. coli* was detected after treatment with 2×MIC of the extract. Furthermore, non-bacterial detection was observed in the beef treated with the extract at 4×MIC during the store in 3 days. However, suspected *Salmonella* spp. and suspected *Listeria* sp. was not detected in this study.

Foodborne pathogens and food spoilage bacteria can be detected in beef, and classified as bacterial contamination in beef. *E. coli* is one of common bacteria detected on beef because it is a normal flora in gastrointestinal tract in animal. However, the presence of *E. coli* in beef is considered as poor sanitation. A reduction of the bacteria including *E. coli* in beef after treatment with *Piper betle* extract may a powerful

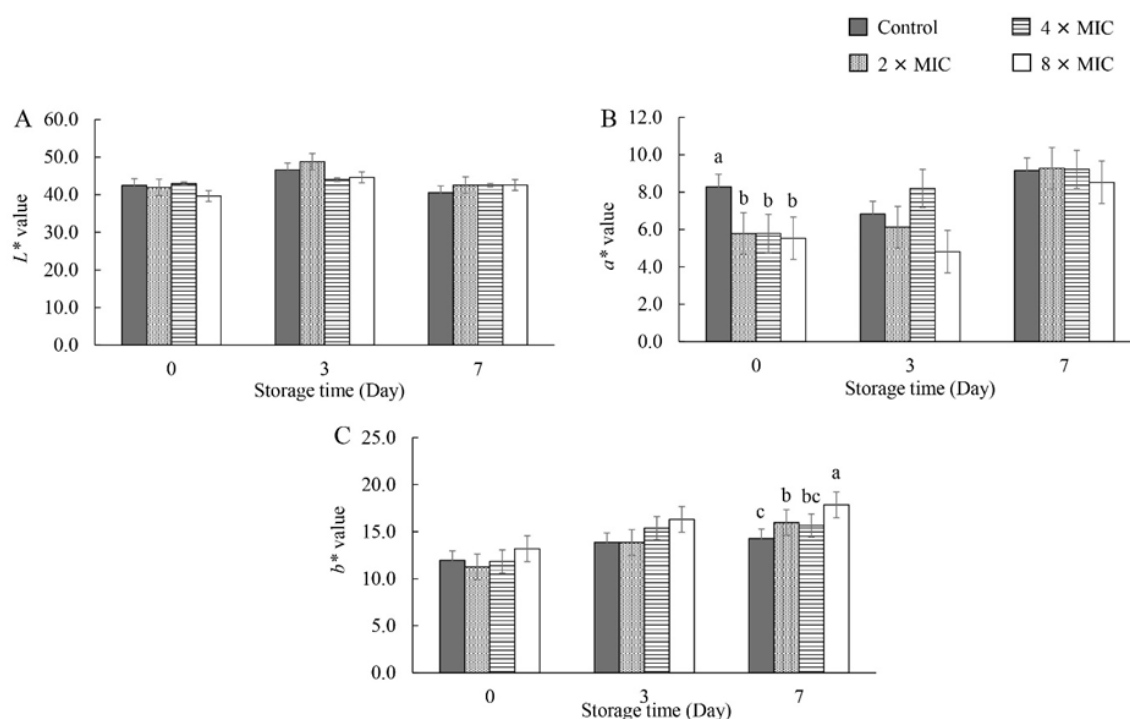


Fig. 6: Lightness (L^* ; (A)), redness (a^* ; (B)), and yellowness (b^* ; (C)) values obtained from beef treated with *Piper betle* extract during storage at 0, 3, and 7 days. Data are least-squares mean $\pm$ SE. Differences were considered significant at $P < 0.05$.

strategy to apply in food industry. In meatballs, pomegranate and cranberry extracts have been used either by themselves or in conjunction with two essential oils (oregano and thyme) to prevent the bacterial pathogens.^[31] In addition, active packet is one of a strategy to prolong the product's shelf life and protect the quality of the food based on the direct interaction between the food and bioactive compounds purposefully added to the package.^[32] According to the results, active packet containing *Piper betle* extract could be suggested to further carry out.

3.7. Effects of *Piper betle* extract on texture parameters of meat during storage

The activity of natural products including plant extract to maintain the quality of meat has attracted attention because of worries regarding the safety risks posed by synthetic food additives.^[33] The plant extracts may use as a natural food additive in meat, however, effects of the extract on meat quality and appearance should be tested. The appearances of beef after treatment with the extract at different concentrations during storage were presented in Fig. 5. A naturally red beef was observed in the control and the treatment groups at day 0. However, deep red to dark red beef was observed after the beef was treated with the extract during storage at 3 and 7 days. The deep red to dark red appearance in beef in the treatment groups may affect by the color of *Piper betle* extract that shows dark brown to black color. In addition, we assessed the color of fresh beef treated with *Piper betle* extract while it was being stored at 4°C by assessing L^* , a^* , b^* determination. It was observed that *Piper betle* extract did not show a significant

difference in the L^* parameter of beef during the storage (Fig. 6A). For a^* parameter, a significant reduction was detected at day 0 but non chance was observed during 7 days (Fig. 6B). However, the b^* parameter of the beef after treatment with the extract was a significant difference on day 7 (Fig. 6C). In addition, effects of *Piper betle* extract on texture parameters of the beef were presented in Table 5. At 7-day storage, treatment of the beef of the extract at 8×MIC did not show a significant difference in hardness, cohesiveness, springiness, gumminess, and chewiness of the beef, compared with the control. Recently, the uses of natural bioactive substances derived from *Piper betle* leaves in food preservation has been reported.^[34] Furthermore, the extract of *Piper betle* demonstrated oxidative stability and enhanced the phytochemical, textural, sensory, and antimicrobial properties of sausages produced by buffalo meat.^[35] In addition, the buffalo meat sausages incorporated the extract received great sensory ratings.^[35] However, consumers' sensory evaluation including taste and smell are not determined in this study. Hence, it should be recommended to evaluate the sensory test in consumer when the extract is used in the beef.

Limitation and recommendation: The limitations of this study include the lack of testing the bioactivity of individual compounds, specifically hydroxychavicol and eugenol, against *E. coli* O157:H7. Additionally, the mechanisms of action of the plant extract and its pure compounds warrant further investigation. Future research will focus on extending storage times and assessing consumer satisfaction with the use of *Piper betle* extract to reduce bacterial adhesion on beef.

4. Conclusion

In the present study, we investigated the antibacterial, antibiofilm, and anti-adhesion activities of *Piper betle* extract against biofilm-positive EHEC O157:H7. In addition, effects of the extract on quality and appearance of beef during storage was evaluated. The bactericidal activity of the extract was evident, with an MBC/MIC ratio of less than 2. The extract significantly inhibited and eliminated biofilm formation and established biofilms in all isolates. Furthermore, the extract reduced bacterial cells adhered to beef by 99.9% at 8×MIC within 3 and 6 h of storage at 37°C and 4°C, respectively. Additionally, a significant reduction in bacterial adhesion on beef was observed when treated with the extract during 7-day storage at −18°C. The beef treated with 8×MIC did not show a significant difference in hardness, cohesiveness, springiness, gumminess, and chewiness of the beef, compared with the control. These findings suggest that *Piper betle* leaf extract inhibits *E. coli* O157:H7 biofilms and prevents bacterial adhesion on beef during storage. Future research will be focused on consumer satisfaction of sensory aspects of the beef after treatment with *Piper betle* extract.

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Conflict of Interest

The authors declare no conflict of interest.

Supporting Information

Not applicable.

CRedit Statement

Ratchakul Wiriaprom, Phirabhat Saengsawang, Sakaoporn Prachantasena, Krittika Kabploy, Yukio Morita, and Watcharapong Mitsuwan: conceived and designed the experiments. **Ratchakul Wiriaprom, Phirabhat Saengsawang, Natawat Sittireak, Kunchaphorn Ratchasong, Krittika Kabploy, and Watcharapong Mitsuwan:** performed the experiments. **Ratchakul Wiriaprom, Phirabhat Saengsawang, Natawat Sittireak, Kunchaphorn Ratchasong, Arif Cheseng, and Watcharapong Mitsuwan:** analysed and interpreted the data. **Phirabhat Saengsawang, Kunchaphorn Ratchasong, Arif Cheseng, and Watcharapong Mitsuwan:** analysed statistical analysis. **Phirabhat Saengsawang and Yukio Morita:** contributed to reagents, materials, analysis tools or data. **Ratchakul Wiriaprom, Phirabhat Saengsawang, Sakaoporn Prachantasena, Yukio Morita, and Watcharapong Mitsuwan:** wrote the paper. All authors have

read, reviewed, and approved the final manuscript.

References

- [1] M. A. Sebsibe, E. T. Asfaw, Occurrence of multi-drug resistant *Escherichia coli* and *Escherichia coli* O157: H7 in meat and swab samples of various contact surfaces at abattoir and butcher shops in Jimma town, Southwest district of Ethiopia, *Infection and Drug Resistance*, 2020, **13**, 3853-3862, doi:10.2147/IDR.S277890.
- [2] K. I. Sallam, Y. Abd-Elrazik, M. T. Raslan, K. Imre, A. Morar, V. Herman, H. A. Zaher, Cefotaxime-, Ciprofloxacin-, and Extensively Drug-Resistant *Escherichia coli* O157: H7 and O55: H7 in Camel Meat, *Foods*, 2023, **12**, 1443, doi:10.3390/foods12071443.
- [3] L. J. Gansheroff, A. D. O'Brien, *Escherichia coli* O157: H7 in beef cattle presented for slaughter in the US: higher prevalence rates than previously estimated, *Proceedings of the National Academy of Sciences*, 2000, **97**, 2959-2961, doi: 10.1073/pnas.97.7.2959.
- [4] E. Abebe, G. Gugsu, M. Ahmed, N. Awol, Y. Tefera, S. Abegaz, T. Sisay, Occurrence and antimicrobial resistance pattern of *E. coli* O157: H7 isolated from foods of bovine origin in Dessie and Kombolcha towns, Ethiopia, *PLOS Neglected Tropical Diseases*, 2023, **17**, e0010706, doi:10.1371/journal.pntd.0010706.
- [5] J. Chuprom, K. Kidsin, S. Sangkanu, V. Nissapatorn, C. Wiart, M. D. L. Pereira, T. Wongtawan, M. Daus, D. S. Sotthibandhu, V. Tipmanee, A. K. Paul, C.N. Scholfield, M. B. Zulkipli, N. H. Abdulllah, W. Mitsuwan, *Knema retusa* is antibacterial and antibiofilm against antibiotic resistant *Staphylococcus aureus* and *S. haemolyticus* isolated in bovine mastitis, *Veterinary Research Communications*, 2022, **47**, 523-538, doi:10.1007/s11259-022-09999-0.
- [6] A. Dishan, H. Hizlisoy, M. Barel, H. B. Disli, C. Gungor, N. E. Onmaz, Z. Gonulalan, S. Al, Y. Yildirim, Biofilm formation, antibiotic resistance and genotyping of Shiga toxin-producing *Escherichia coli* isolated from retail chicken meats, *British Poultry Science*, 2023, **64**, 63-73, doi:10.1080/00071668.2022.2116697.
- [7] H. Sheng, J. Wang, J. Y. Lim, C. Davitt, S. A. Minnich, C. J. Hovde, Internalization of *Escherichia coli* O157: H7 by bovine rectal epithelial cells, *Frontiers in microbiology*, 2011, **2**, 32, doi:10.3389/fmicb.2011.00032.
- [8] K. Silagyi, S. H. Kim, Y. M. Lo, C. I. Wei, Production of biofilm and quorum sensing by *Escherichia coli* O157: H7 and its transfer from contact surfaces to meat, poultry, ready-to-eat deli, and produce products, *Food microbiology*, 2009, **26**, 514-519, doi:10.1016/j.fm.2009.03.004.
- [9] R. Shrestha, S. Khanal, P. Poudel, K. Khadayat, S. Ghaju, A. Bhandari, S. Lekhak, N. D. Pant, M. Sharma, B. P. Marasini, Extended spectrum β -lactamase producing uropathogenic *Escherichia coli* and the correlation of biofilm with antibiotics resistance in Nepal, *Annals of clinical microbiology and antimicrobials*, 2019, **18**, 1-6, doi:10.1186/s12941-019-0340-y.
- [10] N. M. D. M. W. Nayaka, M. M. V. Sasadara, D. A. Sanjaya, P. E. S. K. Yuda, N. L. K. A. A. Dewi, E. Cahyaningsih, R. Hartati,

- Piper betle* (L): Recent review of antibacterial and antifungal properties, safety profiles, and commercial applications, *Molecules*, 2021, **26**, 2321, doi:10.3390/molecules26082321.
- [11] P. Kulnaran, J. Chuprom, T. Thomrongsuwanakij, C. Romyasamit, S. Sangkanu, N. Manin, V. Nissapatorn, M. D. L. Pereira, P. Wilairatana, W. Kitpipit, W. Mitsuwan, Antibacterial, antibiofilm, and anti-adhesion activities of *Piper betle* leaf extract against avian pathogenic *Escherichia coli*, *Archives of Microbiology*, 2022, **204**, 49, doi:10.1007/s00203-021-02701-z.
- [12] R. Boripun, P. Paopradit, J. Prampramote, R. Narinthorn, S. Intongead, S. Sangkanu, T. Narongrit, W. Mitsuwan, Bactericidal activity of *Piper betle* L. extract against antibiotic resistant *Salmonella* spp. isolated from pig farms in Southern Thailand, *Veterinary Integrative Sciences*, 2022, **20**, 557-569, doi:10.12982/VIS.2022.042.
- [13] R. Wiriaprom, R. Ngasaman, D. Kaewnoi, S. Prachantasena, Prevalence and virulent gene profiles of sorbitol non-fermenting Shiga toxin-producing *Escherichia coli* isolated from goats in Southern Thailand, *Tropical Medicine and Infectious Disease*, 2022, **7**, 357, doi:10.3390/tropicalmed7110357.
- [14] T. Azimi, L. Azimi, F. Fallah, M. R. Pourmand, A. Ostadtaghizadeh, M. R. Abai, A. R. Foroushani, Detection and characterization of Enterobacteriaceae family members carried by commensal *Rattus norvegicus* from Tehran, Iran, *Archives of Microbiology*, 2021, **203**, 1321-1334, doi:10.1007/s00203-020-02126-0.
- [15] Clinical and Laboratory Standards Institute, Performance Standards for Antimicrobial Susceptibility Testing CLSI supplement M100. 29th ed. Clinical and Laboratory Standards Institute, Pennsylvania, USA (2019).
- [16] W. Mitsuwan, A. Olaya-Abril, M. Calderón-Santiago, I. Jiménez-Munguía, J. A. González-Reyes, F. Priego-Capote, S. P. Voravuthikunchai, M. J. Rodríguez-Ortega, Integrated proteomic and metabolomic analysis reveals that rhodomirtone reduces the capsule in *Streptococcus pneumoniae*, *Scientific Reports*, 2017, **7**, 2715, doi:10.1038/s41598-017-02996-3.
- [17] S. S. Gajbhiye, M. V. Salve, M. D. Gaikwad, A. R. Rasage, M. S. Khater, V. H. Ghadage, N. B. Chaure, Influence of synthesis conditions on the physical characteristics and antibacterial activities of cerium oxide nanoparticles in biomedical applications, *Engineered Science*, 2024, **32**, 1254, doi:10.30919/es1254
- [18] W. Mitsuwan, P. Wintachai, S. P. Voravuthikunchai, *Rhodymyrtus tomentosa* leaf extract and Rhodomirtone combat *Streptococcus pneumoniae* biofilm and inhibit invasiveness to human lung epithelial and enhance pneumococcal phagocytosis by macrophage, *Current Microbiology*, 2022, **77**, 3546-3554, doi:10.1007/s00284-020-02164-3.
- [19] V. Bulgaru, L. Popescu, N. Netreba, A. Ghendov-Mosanu, R. Sturza, Assessment of quality indices and their influence on the texture profile in the dry-aging process of beef, *Foods*, 2022, **11**, 1526, doi:10.3390/foods11101526.
- [20] S. M. Gambushe, O. T. Zishiri, M. E. E. Zowalaty, Review of *Escherichia coli* O157: H7 prevalence, pathogenicity, heavy metal and antimicrobial resistance, African perspective, *Infection and Drug Resistance*, 2022, **15**, 4645-4673, doi:10.2147/IDR.S365269.
- [21] W. Mitsuwan, I. Jiménez-Munguía, M. Visutthi, W. Sianglum, REIPI/GEIH Study Group, M. J. Rodríguez-Ortega, S. P. Voravuthikunchai, Rhodomirtone decreases *Staphylococcus aureus* SigB activity during exponentially growing phase and inhibits haemolytic activity within membrane vesicles, *Microbial pathogenesis*, 2019, **128**, 112-118, doi:10.1016/j.micpath.2018.12.019.
- [22] W. Kitpipit, C. N. Scholfield, S. Sangkanu, V. Nissapatorn, M. D. L. Pereira, A. K. Paul, W. Mitsuwan, Virulence factors and quorum sensing as targets of new therapeutic options by plant-derived compounds against bacterial infections caused by human and animal pathogens, *Veterinary World*, 2023, **16**, 1346-1355, doi:10.14202/vetworld.2023.1346-1355.
- [23] D. Singh, S. Narayanamoorthy, S. Gamre, A. G. Majumdar, M. Goswami, U. Gami, S. Cherian, M. Subramanian, Hydroxychavicol, a key ingredient of *Piper betle* induces bacterial cell death by DNA damage and inhibition of cell division, *Free Radical Biology and Medicine*, 2018, **120**, 62-71, doi:10.1016/j.freeradbiomed.2018.03.021.
- [24] A. Mukherjee, C. Cao, J. Lutkenhaus, Inhibition of FtsZ polymerization by SulA, an inhibitor of septation in *Escherichia coli*, *Proceedings of the National Academy of Sciences*, 1998, **95**, 2885-2890, doi:10.1073/pnas.95.6.2885.
- [25] M. K. Yadav, S. W. Chae, G. J. Im, J. W. Chung, J. J. Song, Eugenol: a phyto-compound effective against methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* clinical strain biofilms, *PLoS One*, 2015, **10**, e0119564, doi:10.1371/journal.pone.0119564.
- [26] E. Charles, Inhibition of MreB and ftsZ proteins to minimize *E. coli* biofilms formation, *bioRxiv*, 2019, 523167, doi:10.1101/523167.
- [27] Y. G. Kim, J. H. Lee, G. Gwon, S. I. Kim, J. G. Park, J. Lee, Essential oils and eugenols inhibit biofilm formation and the virulence of *Escherichia coli* O157: H7, *Scientific Reports*, 2016, **6**, 36377, doi:10.1038/srep36377.
- [28] G.M. Hamad, N. M. Abdelmotilib, S. M. Abdel-Fattah, A. M. Zeitoun, Anti-*Escherichia coli* O157: H7 as natural preservative to control and prevent food contamination in meat and fish products, *Pakistan Journal of Biological Sciences: PJBS*, 2020, **23**, 674-684, doi: 10.3923/pjbs.2020.674.684.
- [29] S. Mohammadi, J. Aliakbarlu, H. Tajik, L. Manafi, N. Mortazavi, Inhibition of *E. coli* and bio-preservation of ground beef by *Lactobacillus*, black pepper extract and EDTA, *Food Bioscience*, 2022, **47**, 101635, doi:10.1016/j.fbio.2022.101635.
- [30] P. Puligundla, S. Lim, Biocontrol approaches against *Escherichia coli* O157: H7 in foods, *Foods*, 2022, **11**, 5, 756, doi:10.3390/foods11050756.
- [31] I. Mantzourani, M. Daoutidou, M. Dasenaki, A. Nikolaou, A. Alexopoulos, A. Terpou, N. Thomaidis, S. Plessas, Plant extract and essential oil application against food-borne pathogens in raw pork meat, *Foods*, 2022, **11**, 861, doi: 10.3390/foods11060861.
- [32] L. Pinto, M. R. Tapia-Rodríguez, F. Baruzzi, J. F. Ayala-Zavala, Plant antimicrobials for food quality and safety: Recent

views and future challenges, *Foods*, 2023, **12**, 2315, doi: 10.3390/foods12122315.

[33] T. Zhou, J. Wu, M. Zhang, W. Ke, K. Shan, D. Zhao, C. Li, Effect of natural plant extracts on the quality of meat products: A meta-analysis, *Food Materials Research*, 2023, **3**, 15, doi:10.48130/FMR-2023-0015.

[34] V. T. Tran, T. B. Nguyen, H. C. Nguyen, N. H. Do, P. K. Le, Recent applications of natural bioactive compounds from *Piper betle* (L.) leaves in food preservation, *Food Control*, 2023, **154**, 110026, doi: 10.1016/j.foodcont.2023.110026.

[35] A. Manzoor, A. Haque, S. Ahmad, D. L. Hopkins, Incorporation of betel leaf extract provides oxidative stability and improves phytochemical, textural, sensory and antimicrobial activities of buffalo meat sausages, *Meat Science*, 2023, **200**, 109157, doi: 10.1016/j.meatsci.2023.109157.

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