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ORIGINAL PAPER

Antimicrobial effect of lauroyl arginate ethyl on *Escherichia coli* O157:H7 and *Listeria monocytogenes* on red oak leaf lettuce

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Abstract The objective of the present study was to determine the antimicrobial activity of lauroyl arginate ethyl (LAE) against enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 and *Listeria monocytogenes* on oak leaf lettuce. Thus, bacterial suspensions of both pathogens containing 9.0 $\log_{10}$ CFU/mL were spot inoculated on the upper surfaces of lettuce leaves and the leaves were washed with water containing LAE in a final concentration of 100 mg/L. The viable counts of *L. monocytogenes* DSM 20600^{T} and *Escherichia coli* O157:H7 strain EDL933 were reduced with this treatment by ~3.5 $\log_{10}$ CFU/g and ~2.5 $\log_{10}$ CFU/g, respectively. The microbial load of the wash water was reduced by more than 4.1 $\log_{10}$ CFU/mL and was below the detection limit. The second objective was to study whether adherence factors of EHEC O157:H7 strain EDL933 influence the ability to adhere on the lettuce surface, as well as the effectivity of the washing processes. Therefore, the flagellin gene *fliC* and the pilin subunit encoding gene *hcpA* of the hemorrhagic coli pilus were deleted. Based on the initial inoculation level, and without any washing step, the *hcpA* mutant was recovered 18 % less than the wild type from the leaf surface, the recovery of the *fliC* mutant was approximately 30 % higher as observed for the wild type. Both mutants could be washed from the lettuce leaves to a similar level as the wild type (~2.7 $\log_{10}$ CFU/g with LAE treatment and ~1.0 $\log_{10}$ CFU/g without LAE treatment). The findings of this study help to develop novel intervention strategies for fresh produce processing and washing treatments.

Keywords Antimicrobial agent · Lauroyl arginate ethyl (LAE) · Oak leaf lettuce · Enterohemorrhagic *E. coli* · *Listeria monocytogenes*

Introduction

Outbreaks of foodborne illnesses, which are related to the consumption of fresh produce, have rapidly increased during recent years and were frequently caused by *Listeria monocytogenes* and enterohemorrhagic *E. coli* (EHEC) [6, 31, 38]. Rangel et al. [25] identified fresh produce as a transmission vehicle for *E. coli* O157:H7 in 21 % of the foodborne outbreaks in the United States from 1982 to 2002. From 2000 to 2004, *E. coli* O157:H7 was identified as the second most common pathogen in fresh produce-related illness outbreaks [2]. Beuchat et al. [6] reported that *E. coli* O157 outbreaks have been linked to the consumption of apple cider, lettuce, radish, alfalfa sprouts, and other mixed salads. The largest outbreak of illness in Germany occurred in May–July 2011 and was caused by *E. coli* O104:H4-contaminated fenugreek sprouts with a total of 3126 cases of illness [13]. In 2013, 6043 confirmed cases of EHEC infections were reported in the European Union, which was 5.9 % higher than in 2012. The most commonly reported EHEC serogroup in 2013 was, as in previous years, O157 with 48.9 % of cases [12].

Listeria monocytogenes is widely distributed on raw fruits and vegetables and on plant material [7]. It is able to survive fresh produce processing and can grow on fresh produce stored at refrigerated temperatures [8, 18]. While *L. monocytogenes* causes moderate gastroenteritis in

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healthy adults, listeriosis can be severe in susceptible individuals including young, elderly, and immunocompromised people as well as pregnant women [33]. In 2013, the European Union reported 1763 confirmed human cases of listeriosis, which represented an 8.6 % increase compared to 2012, with a total of 13 *L. monocytogenes* outbreaks resulting from the consumption of ready-to-eat foods [12].

These outbreaks show that processing of fresh produce, as well as the washing steps before produce consumption, could contribute to decrease the potential of infection and to maintain human health. Washing procedures aim at removing dirt, soil, pesticide residues and other debris, reducing microorganisms responsible for quality loss, limiting browning, lowering product temperature and removing plant cell exudates that may support microbial growth [16, 30, 43]. Many studies have been published regarding the washing and sanitizing treatments for reduction of the microbial load on fresh-cut produce. A wide range of chemical sanitizers have been tested with various degrees of effectiveness. Fett et al. [15] achieved the reduction of *E. coli* O157:H7, *Salmonella enterica* sv. Anatum F4317, *S. enterica* sv. Infantis F4319, *S. enterica* sv. Newport H1275, and *S. enterica* sv. Stanley HO558 on laboratory-inoculated mung bean seed by chlorine treatment. In another study, the effectiveness of lactic acid and hydrogen peroxide against *E. coli* O157:H7, *Salmonella enterica* sv. Enteritidis and *L. monocytogenes* was demonstrated [19]. Furthermore, chlorine (100 mg/L), citric acid [0.5 % (v/v)], and ascorbic acid [0.5 % (v/v)] were applied against *E. coli* and *L. monocytogenes* on iceberg lettuce and resulted in a reduction of approximately 1.0 $\log_{10}$ colony forming units (CFU)/g [1].

Lauroyl arginate ethyl (N^{α}-lauryl-L-arginine ethyl ester, LAE®) is a surfactant produced synthetically through the reaction of L-arginine, hydrochloric acid, ethanol, thionyl chloride, sodium hydroxide, lauryl chloride, and deionized water [22, 28]. Due to its cationic surfactant nature, it damages the bacterial cell membrane by reacting with oppositely charged proteins or enzymatic systems, causing their denaturation. This results in growth inhibition of the microorganisms or finally in bacterial cell death [22, 27]. In the food industry, it is mainly used as an antimicrobial agent against molds, Gram-positive and Gram-negative bacteria. For example, Porto-Fett et al. [23] treated frankfurters with LAE and investigated the effect on *L. monocytogenes* in the sausage packages. When frankfurters were treated with LAE, numbers of *L. monocytogenes* decreased by approximately 2.0 $\log_{10}$ CFU/package within 2 h [23]. In another study, chocolate and pasteurized milk were treated with different concentrations of LAE and the reduction of the bacterial growth was observed [39]. LAE has been approved by the United States Food and Drug Administration [36] and the European Food Safety Agency [14] as "Generally Recognized as Safe" (GRAS) and represents a potential alternative food preservative to inhibit microbial growth in selected applications.

Currently, there are no reports on the effectiveness of LAE used as wash water additive in lettuce processing. Consequently, we investigated the use of LAE for the reduction of *L. monocytogenes* and *E. coli* O157:H7 on the surfaces of ready-to eat lettuce. As a model, we used oak leaf lettuce, because it is a frequently consumed lettuce cultivar in Germany, and in addition it is commercially available throughout the whole year.

In this context, we evaluated the attachment of *E. coli* O157:H7 on oak leaf lettuce surfaces after deletion of the *hcpA* and *fliC* genes, which are known to be required for attachment of EHEC on baby spinach leaves [29]. The *hcpA* gene encodes the pilin subunit of the hemorrhagic coli pilus (HcpA) [42]. The *fliC* gene encodes the major subunit of flagellin, which polymerizes to form the filaments of bacterial flagella. In addition to their role in motility, flagella in general mediate other functions associated with pathogenicity such as adherence and invasion of epithelial cells [17, 40]. Besides colonization of the intestinal tract, EHEC can also employ their flagella to survive in the environment, making leafy vegetables a unique vehicle in the human disease process [40]. Currently, there is no information about their impact on the attachment of EHEC on the leaf surfaces of oak leaf lettuce.

Materials and methods

Preparation of the lauroyl arginate ethyl solution

Lauroyl arginate ethyl (N^{α}-lauryl-L-arginine ethyl ester, LAE®, CAS registration number 60372-77-2), was distributed by VEDEQSA SA (Barcelona, Spain) as Mirenat NSM®, a white powder consisting of 14.5 % (w/w) LAE and 85.5 % (w/w) maltodextrin. 68.9 mg of Mirenat NSM® was diluted in 100 mL of autoclaved ultra-pure water (Milli-Q system, Millipore, Bedford, MA) to obtain a final LAE concentration of 100 mg/L.

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The *E. coli* strains were routinely cultivated in Luria–Bertani (LB) broth composed of 10 g/L Bacto™ tryptone, 5 g/L yeast extract, and 10 g/L NaCl, pH 7.5, under aeration at 37 °C with 180 rpm unless otherwise mentioned. For the solid medium, 1.5 % (w/v) Bacto™ agar was added to the broth prior to autoclaving. When needed, ampicillin-sodium salt (Carl Roth GmbH & Co KG) and kanamycin-sulfate (Carl Roth GmbH & Co KG) were added after autoclaving in final concentrations of

Table 1 Bacterial strains and plasmids used in this study

	Relevant characteristics	References
E. coli strains		
EDL933	Serotype O157:H7, stx_1, stx_2, *eae-γ*	[21]
EDL933Δ*flic*	Deletion of *fliC*	This study
EDL933Δ*hcpA*	Deletion of *hcpA*	This study
EDL933/pWRG435	Wild type containing pWRG435, Amp^R	This study
EDL933Δ*flic*/pWRG435	*ΔfliC* mutant containing pWRG435, Amp^R	This study
EDL933Δ*hcpA*/pWRG435	*ΔhcpA* mutant containing pWRG435, Amp^R	This study
L. monocytogenes strain		
DSM 20600^T	Serovar 1/2a	DSMZ[a]
Plasmids		
pK46	λ Red recombinase expression, Amp^R	[10]
pKD4	*aph* cassette, Amp^R, Kan^R	[10]
pCP20	FLP recombinase expression, Amp^R, Chl^R	[10]
pWRG435	*PrpsM::tagrfp-t* in pFPV25, Amp^R	[4]

[a] DSMZ: Leibniz-Institut DSMZ—Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

Kan^R = resistant against kanamycin; Amp^R = resistant against ampicillin; Chl^R = resistant against chloramphenicol

100 and 50 µg/mL, respectively. *L. monocytogenes* DSM 20600^T was routinely grown in Brain Heart Infusion (BHI) broth in a rotary shaker at 180 rpm or on BHI agar (both Merck KGaA) at 37 °C for 48 h under aerobic conditions. Polymyxin–acriflavine–lithium chloride–ceftazidime–aesculin–mannitol (PALCAM) agar plates (Merck KGaA) were used for selective growth of *L. monocytogenes.*

Construction of *E. coli* EDL933Δ*fliC* and EDL933Δ*hcpA* deletion mutants

The deletion of the *fliC* and *hcpA* genes in EHEC strain EDL933 was carried out by site directed mutagenesis according to Datsenko and Wanner [10]. The target genes were exchanged by an *aph* kanamycin resistance cassette using plasmids pKD4 and pKD46. Subsequently, the *aph* kanamycin resistance cassette was removed using the temperature-sensitive plasmid pCP20 (Table 1).

Electrocompetent EDL933 cells were prepared as described in the pulse controller instruction manual (Bio-Rad Laboratories GmbH). For transformation, 40 µL of the electrocompetent cells was thawed and mixed with 30-300 ng of respective plasmid DNA. The mixture was transferred in an ice-cold Gene Pulser/MicroPulser Cuvette (Bio-Rad Laboratories GmbH) and pulsed in the Gene Pulser Xcell™ Electroporation Systems device (Bio-Rad Laboratories GmbH) at 25 µF, 200 Ω, 2.5 kW for 5 ms. Following this, 1 mL of pre-warmed SOC-medium was added immediately. SOC-medium contained 20 g/L Bacto™ tryptone, 5 g/L yeast extract, 0.5 g/L NaCl and 2.5 mM KCl, pH 7. After autoclaving, the medium was adjusted to final concentrations of 5 mM $MgCl_2$, 5 mM $MgSO_4$, and 20 mM glucose (filter sterilized). The bacterial suspension was incubated for 1 h at 37 °C, and then spread plated on LB agar containing appropriate antibiotics. The deletions were confirmed by PCR with primer pairs surrounding the position were the deletion had taken place (Table 2) and by DNA-sequencing using a CEQ 8000 genetic analysis system (Beckmann Coulter GmbH) according to standard protocols. Oligonucleotides and PCR programs used for deletion mutagenesis and confirmation experiments are given in Table 2.

Preparation of fluorescent *E. coli* EDL933 wild type and mutants

Electrocompetent *E. coli* EDL933 cells and the respective *fliC* and *hcpA* mutants were prepared, and then transformed with plasmid pWRG435 (Table 1) as described above. The plasmid pWRG435 was designed by Bender et al. [4] and is composed of the plasmid pFPV25.1 backbone [37], encoding an ampicillin resistance gene as selective marker [37], as well as a red fluorescence protein variant (TagRFP-T) gene as an insert [4]. Plasmid pWRG435 was used for constitutive expression of the red fluorescence protein (RFP) and for clear differentiation of *E. coli* EDL933 from the autochthonous microbiota by red fluorescence. Transformants were grown in LB broth with ampicillin (100 µg/mL) and could be detected under UV light at 302 nm as red fluorescent colonies on LB agar containing ampicillin (100 µg/mL). One transformant of each experiment was selected, and designated as EDL933/pWRG435, EDL933Δ*fliC*/pWRG435, and EDL933Δ*hcpA*/pWRG435 (Table 1).

Table 2 Oligonucleotides and PCR conditions used in this study

Primer	Sequence (5′–3′)[a]	Function	PCR product sizes and PCR conditions[b,c]	References
G72	AATATAGGATAACGAATCAT GGCACAAGTCATTAATA CCAACTGTAGGCTGGAGC TGCTTCG	*fliC* deletion	1558 bp 94 °C, 30 s; 53 °C, 60 s; 68 °C, 120 s (30 cycles)	[11]
G73	TTAATCAGGTTACAACGA TTAACCCTGCAGCAGAGAC AGAACCATATGAATATCCT CCTTA			
fliC-for	TTAACCCTGCAGCAGAGAC	Confirmation of *fliC* mutagenesis	1758 bp 94 °C, 30 s; 53 °C, 60 s; 68 °C, 120 s (30 cycles)	This study
fliC-rev	ATGGCACAAGTCATTAATACC			
G70	TGGACAAGCAACGCGGTT TTACACTTATCGAACTGAT GGTGGTGTAGGCTGGAGC TGCTTCG	*hcpA* deletion	1570 bp 95 °C, 30 s; 59 °C, 60 s; 68 °C, 90 s (30 cycles)	This study
G71	GTCATCAAAGCGGAAGAC ATCTTCGCAGGCTTGCTGC AATGCCATATGAATATCCT CCTTAG			
hcpAdel-for	ATCTCAATACGTTTGGTGG	Confirmation of *hcpA* mutagenesis	675 bp 95 °C, 30 s; 52 °C, 60 s; 68 °C, 120 s (30 cycles)	This study
hcpAdel-rev	CGAAATAAAAAACCTCGG			

[a] Capital letters show homologous sequences to target gene; underlined letters show sequence homolog to vector pKD4

[b] Conditions for conventional PCR included initial denaturing at 95 °C for 5 min and final elongation at 68 °C for 5 min

[c] Each 25 µL PCR reaction contained: 2.5 µL 10 × ThermoPol™ reaction buffer, 0.5 µL dNTPs (10 mM each), 1 µL of each primer (10 pmol/µL), 0.125 µL Taq polymerase (5 U/µL; New England BioLabs GmbH, Germany) and 1 µL template DNA

The stability of the plasmids was examined in LB medium with and without ampicillin. Overnight cultures of EDL933/pWRG435 and the respective mutants previously grown in the presence of ampicillin were inoculated into LB medium without ampicillin. After 24 h at 37 °C and 180 rpm, and the cultures were serially diluted in 0.9 % NaCl solution (w/v), and spread plated onto LB agar with and without ampicillin, respectively. The plates were incubated for 24 h at 37 °C and the total numbers of colonies as well as numbers of red fluorescent colonies were determined.

Evaluation of the antimicrobial effect of LAE as wash water additive

Heads of red oak leaf lettuce (*Lactuca sativa* L. var. *crispa*) were purchased from a local supermarket. Outer leaves were removed, and lettuce leaves were cut into pieces with a weight of approximately 10 g using a sterile scalpel.

The bacterial strains used for inoculation experiments are listed in Table 1. The inoculum preparation and the experimental design were carried out as follows: 10 mL overnight cultures of each bacterial strain were prepared as described above. After centrifugation at 4000×*g* for 10 min at 4 °C, the cell pellets were washed with 10 mL of 0.9 % NaCl solution (w/v) and centrifuged again. Each cell pellet was resuspended in 5 mL 0.9 % NaCl solution (w/v), and the optical density at 600 nm (OD_{600}) was measured. In a preliminary experiment, the cell concentration of each bacterial strain was correlated with the OD_{600}. The bacterial suspension was then serially diluted in 0.9 % NaCl (w/v) to achieve the desired cell concentration of approx. 9.0 $\log_{10}$ CFU/mL. Each leaf sample was inoculated with 10 spots of 10 µL of the respective bacterial suspension at different sites on the upper leaf surface, resulting in a final concentration of approx. 7.0 $\log_{10}$ CFU/g lettuce, and incubated for 15 min under a clean bench (NuAire, IBS Tecnomara) for drying. After that, the lettuce leaves were further processed I) without washing, II) with washing in 100 mL sterile deionized water for 120 s at 6 °C, and III) with washing in 100 mL sterile deionized water containing LAE in a final concentration of 100 mg/L for 120 s at 6 °C. LAE was subsequently removed by rinsing the leaves with 100 mL sterile deionized water for approximately 10 s (Fig. 1).

The leaves were then transferred into sterile stomacher bags with lateral filter and the nine-fold weight of a 0.9 % (w/v) NaCl solution was added. Each sample was treated for 120 s using a Stomacher 400 Circulator (Seward Ltd.). Decimal dilutions of each homogenized sample solution

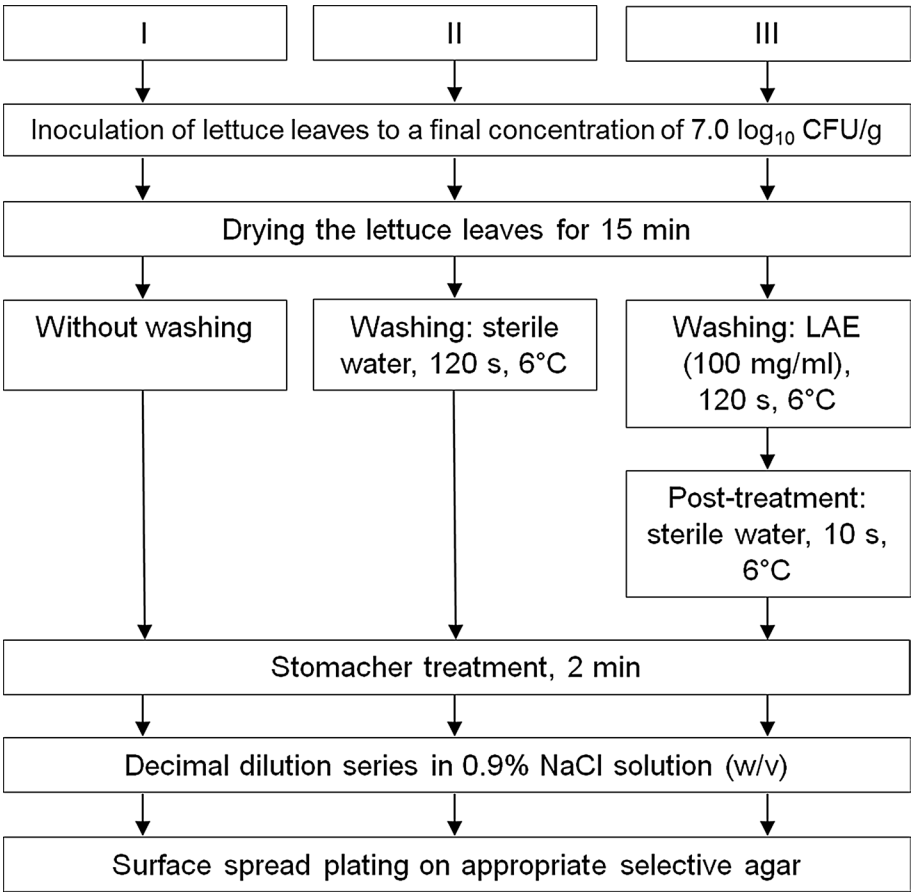

Fig. 1 Flowchart of the experimental design of this study

were prepared in the same diluent. *E. coli* EDL933 wild type and mutants were estimated by spread plating in duplicate on LB agar containing the appropriate antibiotics. Plates were incubated aerobically at 37 °C for 24 h. Furthermore, the wash water, with and without LAE, as well as the rinsing water were analyzed in duplicate on the same media directly after processing. Colonies of *E. coli* EDL933/pWRG435 and the mutants could be clearly differentiated from the autochthonous microbiota by red fluorescence after incubating for another 24 h at 4 °C.

Listeria monocytogenes viable counts were estimated by spread plating on PALCAM agar. Plates were incubated aerobically at 37 °C for 72 h before colonies were counted. *L. monocytogenes* colonies were approximately 2 mm in diameter, gray-green with a black sunken center and a black halo on a cherry-red background. As a control, the microbial loads of the lettuce leaves were determined without bacterial inoculation. In addition, each bacterial suspension used for lettuce inoculation was serially diluted in 0.9 % NaCl solution (w/v) and spread plated on the appropriate agar (see above) to ensure that the adjusted cell concentration of approx. 9.0 $\log_{10}$ CFU/mL was achieved.

Colony forming units were counted, and their weighted arithmetic means were calculated. All experiments were performed independently at least at three different times per investigated bacterial strain. The findings from representative experiments are given in the results.

Statistical evaluation

For the inoculation assays, the averaged values of three technical replicates were used for calculation of the standard error of the three biological replicates derived from three different days. Data evaluation was performed using SAS software version 9.1 (SAS Institute, Cary, NC, USA). For statistical analysis demonstrating significant differences ($\alpha = 0.05$), Tukey's multiple range test was used.

Results

Antimicrobial efficacy of lauroyl arginate ethyl against *L. monocytogenes*

Initially, the microbial load of lettuce samples was investigated. Initial total aerobic counts on unwashed, non-inoculated lettuce leaves were 6.0 ± 0.3 $\log_{10}$ CFU/g. The inoculum of *L. monocytogenes* DSM 20600^T contained

9.1 ± 8.3 $\log_{10}$ CFU/mL, resulting in a contamination of approximately 7.1 $\log_{10}$ CFU/g lettuce. Counts of *L. monocytogenes* on unwashed, non-inoculated red oak leaf lettuce were either below the detection limit of 2.0 $\log_{10}$ CFU/g (2 samples) or amounted to 2.4 $\log_{10}$ CFU/g oak leaf lettuce (one sample). After the inoculation, *L. monocytogenes* counts on red oak leaf lettuce were 6.6 ± 6.0 $\log_{10}$ CFU/g on PALCAM agar.

After inoculation and washing with cold water, counts of *Listeria* were 5.7 ± 5.1 $\log_{10}$ CFU/g on PALCAM agar. This means a reduction of 0.9 ± 0.1 $\log_{10}$ CFU/g. Washing with LAE reduced viable counts to 3.1 ± 0.3 $\log_{10}$ CFU/g oak leaf lettuce. The *L. monocytogenes* counts in the process water after washing without LAE were 5.4 ± 4.6 $\log_{10}$ CFU/mL. After washing with LAE, *L. monocytogenes* was not detected in the process water (detection limit of 1.0 $\log_{10}$ CFU/mL). In summary, it could be demonstrated that LAE is an effective antimicrobial agent to reduce viable counts of *L. monocytogenes* on red oak leaf lettuce. Therefore, LAE represents an effective alternative to traditional washing techniques for fresh-cut lettuce.

Antimicrobial efficacy of lauroyl arginate ethyl against *E. coli* strain EDL933

Members of the family *Enterobacteriaceae* are part of the lettuce phyllosphere [26]. To differentiate the inoculated EHEC strains from the autochthonous oak leaf lettuce microbiota under UV light, the strain EDL933 and its isogenic *fliC* and *hcpA* mutants were constructed to produce a red fluorescent protein (see above). This additional selection marker was necessary because additional tests had shown that there are still some ampicillin resistant bacteria in the lettuce microbiota, which could falsify the results, if ampicillin was used as sole selection marker (data not shown). In a preliminary investigation, the stability of the plasmid in the transformant was evaluated. Plasmid pWRG435 was present in all cells (100 %) under ampicillin selection. Without ampicillin, approximately 90 % of the EHEC cells retained the plasmid and the ability to form red fluorescent colonies on LB agar. Therefore, all experiments with EDL933/pWRG435 and the mutants were performed under ampicillin selection.

E. coli O157:H7 strain EDL933/pWRG435 was used for inoculation of oak leaf lettuce. The inoculum contained 9.2 ± 8.2 $\log_{10}$ CFU/mL on LB agar with ampicillin (100 µg/mL), resulting in a contamination of approximately 7.2 $\log_{10}$ CFU/g lettuce. The unwashed, non-inoculated lettuce leaves were free of red fluorescent *E. coli* (data not shown). Viable counts of EDL933/pWRG435 after inoculation without washing were 7.0 ± 5.8 $\log_{10}$ CFU/g. After inoculation and washing with cold water, counts of EHEC amounted to 6.1 ± 4.7 $\log_{10}$ CFU/g, meaning a reduction of 0.9 ± 0.0 $\log_{10}$ CFU/g. Washing with LAE reduced EHEC counts by 2.6 ± 0.2 $\log_{10}$ CFU/g. In the process water without LAE, viable counts of EHEC were 5.8 ± 3.9 $\log_{10}$ CFU/mL. After the LAE treatment EDL933/pWRG435 could not be detected in the process water (detection limit of 1.0 $\log_{10}$ CFU/mL).

Impact of gene deletion in *E. coli* O157:H7 strain EDL933 on the adherence to the lettuce leaf surface

In order to investigate the influence of particular adherence factors of EDL933/pWRG435 on the ability to adhere on the lettuce surface, as well as the effectivity of the washing process, isogenic mutants of the EHEC strain EDL933 with deletion of the *fliC* and the *hcpA* gene were constructed, transformed with pWRG435, and tested in the washing procedures.

The inoculum of EDL933Δ*fliC*/pWRG435 contained 9.2 ± 8.1 $\log_{10}$ CFU/mL on LB agar with ampicillin (100 µg/mL), resulting in a contamination of approximately 7.2 $\log_{10}$ CFU/g lettuce. Viable counts of EDL933Δ*fliC*/pWRG435 after inoculation without washing were 7.1 ± 5.8 $\log_{10}$ CFU/g. Based on the initial inoculum, the *fliC* deletion mutant adhered less than the parental strain, which is demonstrated in a ~30 % higher recovery as compared to the wild-type strain (7.0 ± 5.8 $\log_{10}$ CFU/g).

The inoculum of *E. coli* O157:H7 strain EDL933Δ*hcpA*/pWRG435 contained 9.1 ± 8.1 $\log_{10}$ CFU/mL on LB agar with ampicillin (100 µg/mL), resulting in a contamination of approximately 7.1 $\log_{10}$ CFU/g lettuce. Viable counts of EDL933Δ*fliC*/pWRG435 after inoculation without washing were 6.8 ± 5.9 $\log_{10}$ CFU/g.

Interestingly, the *hcpA* deletion mutant was detected approximately 18 % less than the wild-type strain.

The effect of washing with and without LAE to the isogenic mutants was quite similar to the results described above. Washing in sterile cold water led to a reduction between 0.7 ± 0.1 $\log_{10}$ for *E. coli* EDL933Δ*hcpA* and 1.0 ± 0.1 $\log_{10}$ for the other strains tested. The LAE treatment resulted in a reduction of approximately 2.7 $\log_{10}$ for the wild-type strain and the *hcpA* isogenic mutant. The viable counts of EDL933Δ*fliC*/pWRG435 were reduced by 3.5 ± 0.3 $\log_{10}$ CFU/g after LAE treatment.

Discussion

In the present study, with a conventional washing step the viable counts of *L. monocytogenes* and *E. coli* O157:H7 on oak leaf lettuce leaves were reduced by approximately 1.0 $\log_{10}$ CFU/g. The treatment of lettuce wash water with 100 mg/L of LAE resulted in an initial reduction of 3.5 ± 0.3 $\log_{10}$ CFU/g of *L. monocytogenes* compared to

untreated controls. The reduction of *E. coli* O157:H7 by LAE amounted to 2.7 $\log_{10}$ CFU/g. Comparable results were observed in a number of other studies, which evaluated the bactericidal efficacy of LAE on the same pathogens in other food systems. Soni et al. [32] reported a *L. monocytogenes* reduction of approximately 2 $\log_{10}$ CFU/g in Queso Fresco cheese after treatment with 200 ppm LAE. In "Lyoner style" sausages, LAE used in concentrations of 2 mg/mL reduced *L. innocua* by 2.1 ± 0.9 $\log_{10}$ CFU/g when added as aqueous solution [35]. Taormina and Dorsa [34] investigated the short-term efficacy of LAE against *L. monocytogenes* on vacuum-packaged frankfurters and noted a ~ 2 log CFU/package reduction within 48 h. In 2 % reduced fat milk the growth of *E. coli* O157:H7 was reduced by approximately 2 log CFU/mL when treated with 750 ppm LAE [20].

Besides its strong antimicrobial characteristics, LAE offers some further advantages, which were interesting for the development of new applications in food preservation [3]. LAE showed fast antimicrobial activity, which indicates that only a short exposure time is needed to reduce the viability of bacteria [3]. This is especially interesting in lettuce processing, where average washing, and thus exposure times, are approximately 2 min. Another interesting observation of this study was the effect of LAE on the wash water viable counts. These could be reduced by at least 4.1 $\log_{10}$ CFU/mL. Due to the reduction of *L. monocytogenes* and *E. coli* O157:H7 below the detection limit in the wash water, LAE represents an effective alternative to traditional washing methods for fresh-cut lettuce and could be beneficial in a bioeconomic view.

The present study was conducted to address the potential involvement of the H7 flagella from *E. coli* O157:H7 strain EDL933 and its hemorrhagic coli pilus to attach to lettuce leaf surfaces. Various studies have shown that enteric bacteria, such as *E. coli*, have many molecular and physiological mechanisms that allow them to colonize and survive in the plant environment [24]. *E. coli* O157 attached to lettuce leaves by several mechanisms involving the type III secretion system (T3SS), flagella, pilli, curli and other outer membrane proteins [5]. Our experiments on the recovery of attached *E. coli* to lettuce leaf surfaces showed that the isogenic mutants of *E. coli* EDL933 displayed a different behavior in attachment than the wild-type strain. Recovery of strain EDL933 amounted to (7.0 ± 5.8 $\log_{10}$ CFU/g), while strain EDL933Δ*fliC* was recovered at (7.1 ± 5.8 $\log_{10}$ CFU/g), which indicates an inferior attachment on the leaf surface in comparison to the parental strain. This is in accordance with Saldaña et al. [29] who reported that an EDL933Δ*fliC* isogenic mutant adhered 72 % less to spinach leaves than the wild-type strain. Xicohtencatl-Cortes [40] investigated the interaction of *E. coli* O157:H7 with baby spinach and lettuce leaves. They found that EDL933Δ*fliC* mutants were significantly hampered in their adherence to leaf surfaces. They quantified colony forming units of *E. coli* O157:H7 by plating on selective MacConkey-sorbitol agar. The Δ*fliC* mutants were reduced by approximately 2 $\log_{10}$ CFU/mL in their ability to adhere at lettuce leaves.

The hemorrhagic coli pilus (HCP) is essentially a type 4 pilus, which is associated with a number of cellular functions, like adhesion to host cells, bacterial aggregation, biofilm and microcolony formation, cell signaling and DNA uptake [9, 41]. The inactivation of the major subunit, the *hcpA* gene, in EHEC O157:H7 reduces adherence to bovine epithelial and human intestinal cells [42]. In our study, lack of *hcpA* affected the recovery of EDL933Δ*hcpA*/pWRG435 in comparison to the wild-type strain in an opposite way. Deletion of *hcpA* resulted in 18 % (6.8 ± 5.9 $\log_{10}$ CFU/g) less recovery of the mutant than the wild-type strain, meaning a tighter adherence to the surfaces of oak leaf lettuce. This was not expected and can actually not be explained. Regarding the recovery in the wash water, there were no significant differences between the wild-type strain and EDL933Δ*hcpA*/pWRG435. Saldaña et al. [29] reported that *hcpA* deletion mutants were significantly hampered (49 % reduction) in adherence to spinach leaves in comparison to the parental strain and that HCP of *E. coli* strain EDL933 was required for spinach leaf adherence. They quantified colony forming units of *E. coli* O157:H7 by plating on selective MacConkey-sorbitol agar. The use of different media could be a reason for the different recovery compared to our study.

In conclusion, the attachment of *E. coli* O157:H7 and *L. monocytogenes* to cut produce surfaces underlines the need for effective wash treatments to reduce potential contamination of pathogens. The present in vitro study revealed that LAE possesses strong and fast antimicrobial activity against both foodborne pathogens on lettuce. While LAE is known to possess an antibacterial effect in other food commodities, it was not clear if that is also the case in produce. The findings of this study indicate that LAE can enhance the food safety and extend product shelf life by inhibiting foodborne pathogens. Therefore, the knowledge gained in this study will be helpful in developing novel intervention strategies for fresh produce wash treatments.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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