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Regulation of gene transcription in *Escherichia coli* O157:H7 in response to a natural derivate peptide of esculentin-1a used in combination with essential oils from plants of the *Cymbopogon* genus

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Abstract

Introduction. We analyzed the expression of several genes implicated in the pathogenicity of *Escherichia coli* O157:H7, treating bacteria with Esc(1-21), a derivative of peptide esculentin-1 in combination with three essential oils obtained from plants from the *Cympopogon* genus.

Methods. We used the checkerboard assay to determine the antimicrobial activity of the combinations. We analyzed the expression of some genes implicated in the pathogenicity and quorum sensing system of *E. coli* O157:H7 by real-time RT-PCR technique.

Results. Treatment of the bacteria with the peptide combined with oils had an efficacious antimicrobial activity. The analysis of gene expression showed that all used combinations regulate positively the *esp*AD and *ler* genes, located in the pathogenicity island, named the locus of enterocyte effacement. None of the combinations affects the quorum sensing genes: *lsr*ABCFKR and *qse*BC.

Conclusions. This study demonstrates that the use of essential oil/peptide combinations can be effective in fighting microbial infections.

Key words

- *Escherichia coli* O157:H7
- essential oils
- esculentin-1a
- *Cymbopogon spp*
- real time RT-PCR

INTRODUCTION

The development and spread of antibiotic resistance is currently one of the main causes of death worldwide. This has induced considerable efforts in the research for new antimicrobial agents, finding, in the use of essential oils (EOs) and natural peptides, a new weapon in the fight against drug resistance and in the synergistic interactions of drugs a novel strategy.

The human enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a pathogen that causes foodborne infections and the absence of an effective therapy, to counteract its pathogenicity, makes it an interesting target in the search for new antimicrobial compounds. It is the causative agent of several outbreaks of bloody diarrhea and hemolytic-uremic syndrome throughout the world, as it colonizes the large intestine, causing attaching and effacing (AE) lesions [1]. The genes involved in the formation of the AE lesions are encoded within a chromosomal pathogenicity island named the locus of enterocyte effacement (LEE) [2]. LEE encodes a type III secretion system, an outer membrane protein called intimin, a translocated intimin receptor called Tir, and the proteins EspA, EspD, and EspB secreted by the type III secretion apparatus. EspA forms a filament-like structure, which is essential for early bacterial attachment to epithelial cells. This filament connects the needle of the secretion system to a translocation pore within the plasma membrane of the host cell, which is composed of EspD and EspB; EspB and Tir are translocated into host cells, where

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Tir serves as a receptor for intimin [3]. Nucleotide sequence analysis of the LEE region of *E. coli* O157:H7 EDL933 strain revealed the presence of 41 open reading frames, most of which are organized into five polycistronic operons named *LEE1* through *LEE4* and *tir*. The Ler protein, encoded by the gene *ler* of the *LEE1* operon, is essential for transcriptional activation of the *LEE2*, *LEE3*, and *tir* operons, which encode many of the protein factors required to produce the attaching and effacing phenotype by EHEC strains on host cells. It has been demonstrated in *E. coli* O157:H7 that Ler acts as a positive regulator of *esp* genes expression and that the quorum sensing (QS) system regulates LEE genes [4, 5].

QS is a complex mechanism of cell-to-cell signaling involving the production of hormone-like compounds called autoinducers. Through the accumulation of these autoinducers, the bacteria "sense" their population as well as the population of other bacteria in a given environment. When a concentration threshold is reached the autoinducers will bind to a transcriptional activator, which in turn will activate or repress a series of genes. Gram-negative bacteria usually produce acyl-homoserine lactones as autoinducers: autoinducer-1 and autoinducer-2 (AI-1 and AI-2); AI-2 is found in both gram-positive and-negative bacteria [5]. Two genes participate in the synthesis of AI-2, the *lux*S and *pfs* genes [6]. *lux*S positively controls the expression of *lsr*ACDBEFG operon and the flanking genes *lsr*R and *lsr*K, implicated in the AI-2 uptake and modification system [7]. Furthermore, the AI-2 regulates the expression and motility of flagella by the two-component regulatory system QseBC, where QseB is the response regulator and QseC is the sensor kinase [8].

LEE locus and QS signaling systems are considered attractive targets for new antimicrobials because they play an essential role in the pathogenesis of *E. coli* O157:H7. In this study, we used a combination of Esc(1-21), corresponding to the first 20 amino acids of frog skin-derived peptide esculentin-1a [9], with three EOs of the genus *Cymbopogon: Cymbopogon flexuosus* (Nees ex Steud.) Will.Watson, *Cymbopogon citratus* (DC.) Stapf and *Cymbopogon martinii* (Roxb.) Wats var. motia Burk. In our latest study, we observed that the three combinations, working synergistically, were able to enhance their antimicrobial effect on *E. coli* O157:H7 [10]. The aim of this work was to extend the study on the effect of these EO/peptide combinations on the expression of some LEE and QS regulated genes. Using RT-PCR technology, we analyzed the expression regulation of *ler*, *esp*A, and *esp*D genes (locus LEE) and *lux*S, *pfs*, *lsr*ABCFKR, and *qse*BC genes (QS system) in bacteria treated with EO/peptide mixtures.

MATERIALS AND METHODS

Bacteria and growth conditions

In this study, we used the enteropathogen *E. coli* O157:H7 reference strain EDL933, belonging to our laboratory collection. Bacteria were grown in modified M9 minimal medium (modM9) [11] at a standardized inoculum of $1x10^6$ CFU/ml.

Peptide

The peptide Esc(1-21) used in this study was synthesized and purified as described in Scotti *et al*. [9], but a different batch was employed.

Essential oils chemical composition

The commercial EOs *C. citratus*, *C. flexuosus* and *C. martinii*, utilized in this study were purchased from Naissance Trading (Swansea, Dyfed UK). The EOs were stored at 4 °C in the dark and the stock solutions at 10% (v/v) were prepared dissolving 50 µl of EO in 450 µl of dilution buffer (10% DMSO, 0.5% Tween 80 in PBS) before use.

Gas chromatography-mass spectrometry (GC-MS) analysis

The chemical composition of the EOs was analyzed by gas chromatography-mass spectrometry (GC-MS), using an Agilent 8890 N chromatograph (GC), equipped with a single quadrupole 5977B mass spectrometer (Santa Clara, California, USA) and an autosampler PAL RTC120 (CTC Analytics AG, Zwingen, Switzerland). The mobile phase was helium (99.999%) whereas the stationary phase was an HP-5MS (30 m x 0.25 mm, 0.1 µm i.d.) capillary column from Agilent. The analytical conditions, including injection, split mode, temperature programme, scan mode, as well as the identification and quantification of components, were the same reported by Scotti R. *et al*. [11].

Determination of MIC and FIC Index

We determined the minimum inhibitory concentration (MIC) of the peptide Esc(1-21) and the three EOs (*C. citratus, C. flexuosus* and *C. martinii*) by the broth microdilution method [11], using a two-fold dilutions of each product. An overnight inoculum was diluted in modM9 at the final concentration of $1x10^6$ CFU/ml in a total volume of 200 µl, in the absence or presence of increasing concentrations of drugs. The concentration of EOs ranged from 0.1 to 6.4% (v/v) and the concentration of the peptide ranged from 1 to 32 µM. Samples were inoculated in triplicate in a 96-well polystyrene plate and incubated at 28 °C with constant agitation for 24 h. The MIC was determined as the lowest concentration of each compound at which no visible growth was observed.

We used the checkerboard technique and the fractional inhibitory concentration (FIC) index to assess the synergy between the peptide and the three EOs. The FIC index of two compounds, A and B, is calculated using the following formula: $\Sigma FIC=FIC_A+FIC_B$ where FIC_A is obtained by the ratio between the value of MIC of compound A alone and in combination; FIC_B is obtained correspondingly [10, 12]. We inoculated a 96-well microplate with an overnight culture, in a total volume of 200 µl of modM9 per well, to obtain a final concentration of $1x10^6$ CFU/ml. Each row (x-axis) in the plate contains the same diluted concentration of the antimicrobial compound A, while the concentration in each subsequent row is half this value. Similarly, each column (y-axis) in the plate contains the same diluted concentration of the antimicrobial compound B, while the concentration in each subsequent column is half

this value. We incubated the microplate at 28 °C for 24 h. The lowest concentration of compounds in wells with no visible growth was used to calculate the FIC index. Two compounds are defined as synergistic when the FIC index value is less than 0.5 [12].

RNA isolation and quantification of gene expression

We obtained mRNA by bacteria grown in the presence or absence of Esc(1-21) combined with the three EOs as described in an our previous work [9]. Bacteria were inoculated in 8 ml of modM9, at the initial concentration of $1x10^6$ CFU/ml in the presence or absence of a concentration of ¼ FIC for the three EO/peptide mixtures. Cultures were incubated at 28 °C for 24 h with 250 rpm agitation and stabilized with RNA Protect Bacteria Reagent (Qiagen). RNA extraction was performed using Presto mini RNA bacteria kit (Geneaid), DNA contamination was eliminated using DNase I (Epicentre) for 20 min at 37 °C, and RNA was precipitated with 0.7% isopropanol and 0.3 M sodium acetate. RT-PCR was used for quantification of mRNA expression of some genes involved in pathogenesis and QS system. Data were normalized using a 16*s* rRNA gene as an internal control. The primers used for analyzing genes are listed in *Table 1*.

Statistics

The assays were performed in triplicate for at least three independent experiments. Data represented average values ± the standard deviations. The significant level was set at *P* value of 0.05.

RESULTS AND DISCUSSION

In recent years, the rapid emergence of antibiotic resistance in bacteria has induced the development of alternatives to antibiotics. One of these alternatives has been the use of combination therapies, with the employment of drugs that proved to have synergistic potential. In our previous works, we studied the antibacterial activity of EOs extracted by *Cymbopogon* spp. [11] and the frog derived peptide Esc(1-21) [9].

The components of EOs were determined by gas chromatography-mass spectrometry analysis. The main compounds of the EOs from *C. citratus* and *C. flexuo-*

Table 1
Primers used in this study for reverse transcription-quantitative polymerase chain reaction

Oligo name	Sequence 5′-3′	
	For	Rev
16*s*	CATCCACAGAACTTTCCAGAG	CCAACATTTCACAACACGAG
	For	Rev
*esp*A	CGGCACAAAAGATGGCTAAT	ATAGCCGCCTTCACTGTTTG
	For	Rev
*esp*D	AATTGTTGGCCAGGTCTTTG	GCTTGCGGCCATAGATATTC
	For	Rev
ler	TCATTGCGGTAGTAAACACCT	CAAATTGCAGTTCTACAGCAG
	For	Rev
*lsr*A	AACATCCTGTTTGGGCTGGCAA	AAACAAGCGTTCGGTTTCCGCA
	For	Rev
*lsr*B	AGCATCCTGGCTGGGAAATTGT	AAATTCTTTCACCGTGCCGCG
	For	Rev
*lsr*C	GCGGGTAACCCTTCAATCCA	TCGCGACTTTACTGCTTGGT
	For	Rev
*lsr*F	CCGGTACCCATTGTTATTGC	AGTTCATATGCCCGATCAGC
	For	Rev
*lsr*K	ACCATCCGTGACTGGAAACC	CACCTTCAGACCGCAGAGTT
	For	Rev
*lsr*R	ATTGGTTTTGGCGAGGCAAC	TATAAGAACCGACGCCACCG
	For	Rev
luxS	GGCATCACTTCTTTGTTCGG	GCTTCACAGTCGATCATACC
	For	Rev
pfs	TTGAAATTGTGGCAGACATGG	GCATTTGGTTATGAATACGGTC
	For	Rev
*qse*B	GGTTTACACAAGGTCGTCAG	ACAGATAATCGTCAGCTCCC
	For	Rev
*qse*C	GTGGGCGATAAAGATCAATGG	GTTCACGACCCAGTAGTACC

sus were the monoterpene aldehydes neral (33.84 and 33.22%, respectively) and geranial (42.83 and 42.4%, respectively); the main components of EO from *C. martinii* were the monoterpene alcohol geraniol (81.4%) and its ester geranyl acetate (11.74%). These percentages were slightly different from those previously reported [11] since different batches of EOs were employed.

The MIC values for the single component and FIC indexes for the three EO/peptide combinations were reported in *Table 2*.

MIC of Esc(1-21) was 8 µM and MIC of EOs ranged from 0.2 to 0.8%. The quantities corresponding to the MICs of the individual components EOs and the peptide were greater than those reported in previous works [9, 11] since different batches of EOs were employed. Furthermore, all FIC values obtained were less than 0.5 indicating their synergistic effect against bacteria, as already demonstrated in our latest work [10]. This translates into a greater effectiveness of the mixtures compared to the individual components, with a reduction in the dosages of the used compounds and their possible toxic effects. In the same work [10] we highlighted how the EOs and the peptide used in combination differently affected the expression of the genes analyzed, indicating that the underlying mechanism of antibacterial activity of the combinations was different compared to when the compounds were used individually. Therefore, this study focused on the analysis of gene expression due to the combined use of EO/peptide, able to produce substantial reductions in MIC values, minimizing negative side effects. However, gene expression due to the use of individual components was not taken into consideration, since it is the combined use of multiple antibacterial agents that represents one of the promising alternatives for the treatment of serious infections.

QS is a cellular mechanism, mediated by autoinducers, that allows bacteria to organize their behavior depending on their density [8]. QS system regulates different pathogenic processes like secretion of virulence factors, biofilm formation and antibiotic sensitivity [13] by producing autoinducers AI. In *E. coli* the Lsr system is responsible for the detection, uptake and signal transduction of the autoinducer AI-2. The genes *lux*S and *pfs* contribute to the AI-2 synthesis and the proteins encoded by genes of *lsr* operon (*lsr*ACDB) are involved in the transport of AI-2 inside the cell. After AI-2 is phosphorylated by LsrK kinase, it represses the activity of LsrR repressor, with the consequent activation of *lsr* operon [14]. Creating agents that interfere with the microbial QS signaling systems is considered a novel strategy to develop new antimicrobials because it does not give rise to the emergence of resistance [15]. Since the LEE locus plays an essential role in the pathogenesis of *E. coli* O157:H7 and is regulated by the QS system, we decided to study the effect of the three EO/peptide combinations on the expression of some LEE and QS regulated genes. For this purpose, we examined the expression profile of some genes implicated in the production and uptake of AI-2 (*lux*S, *pfs*, *lsr*ACBF, *lsr*K, and *lsr*R), the attaching and effacing phenotype (*ler*, *esp*A, and *esp*D) and the regulatory system *qse*BC. *Figure 1* showed that *lux*S was not affected by the treat-

Table 2

A) Minimal inhibitory concentrations (MIC); B) Fractional inhibitory concentration (FIC) index. The values are expressed in µM for peptide and in percentage (v/v) for oils

A	***C. citratus***		***C. flexuosus***		***C. martinii***		**Esc(1-21)**		
	0.40%		0.20%		0.80%		8 µM		
B	**Esc(1-21)+**	***C. citratus***	**FICI**	**Esc(1-21)+**	***C. flexuosus***	**FICI**	**Esc(1-21)+**	***C. martinii***	**FICI**
	0.5 µM	0.10%	0.312	1 µM	0.05%	0.375	0.5 µM	0.10%	0.187

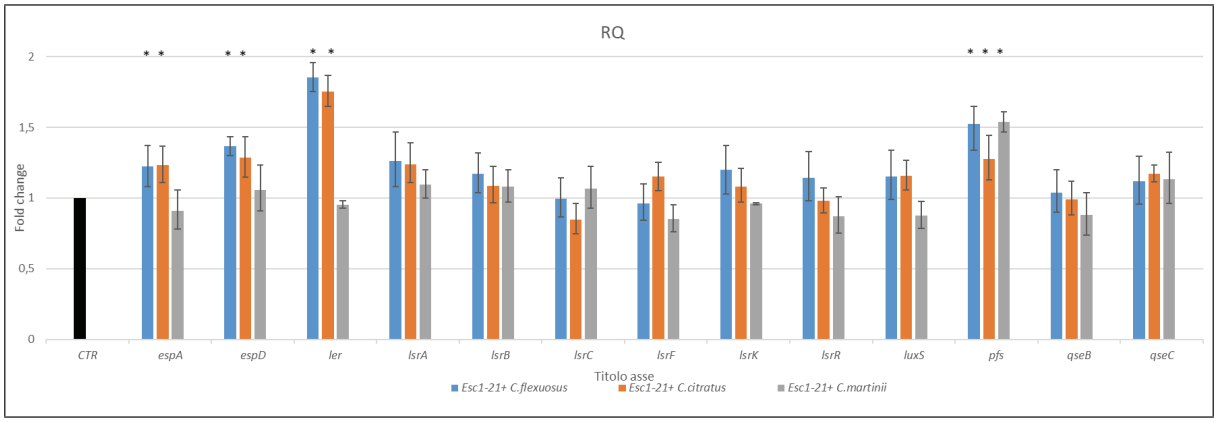

Figure 1

RT-PCR analysis of expression changes of genes related to production and uptake of AI-2, attaching and effacing phenotype and regulatory system. Relative gene expressions (RQ) represent transcriptional levels after exposure of *Escherichia coli* O157:H7 EDL933 to mixtures versus untreated control (CTR). The gene transcription level was normalized to that of the reference gene 16*s* rRNA. Data represent means ± SD of three experiments conducted in triplicate. *$P \leq 0.05$.

ment with the three EO/peptide combinations, unlike the *pfs* gene showed a significant increase.

It was also evident that the *lsr* operon genes, in the presence of the mixtures, were expressed at a similar level to the control, represented by bacteria grown in media modM9. The QS regulator genes *qse*B and *qse*C had similar behavior to the *lsr* operon, with no substantial difference from the control. These results suggested that the QS system was not involved in the response to the treatment with the three combinations and that the induction of the only *pfs* gene was not enough for the upregulation of this system.

The first gene in the LEE1 operon is *ler* and, as shown in *Figure 1*, it was significantly upregulated, 1.8-fold and 1.7-fold, by *C. flexuosus* EO/Esc(1-21) and *C. citratus* EO/Esc(1-21), respectively, but it was not by *C. martinii* EO/Esc(1-21) mixtures. We found that the *esp*A and *esp*D genes (LEE4), involved in bacterial attachment to epithelial cells, were induced by the same two mixtures, following a similar transcription profile of *ler*. This was in line with what is known from the literature, namely that Ler upregulates *esp* genes expression [4].

In summary, the three combinations did not induce the QS system. The examined LEE genes had an opposite behavior, showing to be induced by the peptide in combination with EOs from *C. flexuosus* or *C. citratus* but not in combination with that from *C. martinii* that was capable of inducing, in combination with the peptide, only the *pfs* gene. The observed different effect of the three combinations on LEE genes could be due to the composition of the EOs, which are similar in *C. flexuosus* and *C. citratus*, but different in *C. martinii*. In fact, *C. flexuosus* and *C. citratus* have as main components neral and geranial, while geraniol was the main component of *C. martinii*. However, it is also possible that other minor molecules present in the oils [16] modulate the activity of the major phytocompounds as between factors determining the activity of essential oils there are synergistic interaction of their components. In this regard, to elucidate how the different composition of oils is able to influence their activity, a new study will be carried out to better understand the action of the individual phytocompounds present in the complex mixtures of essential oils.

In conclusion, our results indicated that all EO/Esc(1-21) combinations had an efficacious antimicrobial activity with a synergistic effect on *E. coli* O157:H7 and suggested a probable mechanism of action of the compounds. Furthermore, these results should be taken into consideration when looking for new treatments capable of inhibiting the QS system and pathogenic processes, to obtain effective antimicrobial and antibiofilm activity. Understanding the mechanisms underlying the antibacterial effect of a synergistic drug combination is therefore critical to finding new and suitable therapy.

Conflict of interest statement

The Authors declare no conflict of interest.

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