BP16

Molecular basis of antimicrobial properties of green nanocoating effective against virus and bacteria

R. Brandi[1], A. Monte[1], G. Campoli[1], M. Cavalli[1], A. Fortunato[1,2], M. Di Spirito[1], M. Lipari[1], R. De Santis[1], A. Ciammaruconi[1], M. Bortone[1,3], F. Arduini[4], F. Lista[1], S. Fillo[1]

[1] Defence institute for medical Biosciences, Rome, Italy; [2] National Council of Research – Institute of Electronics, Information Engineering and Telecommunications, Milan, Italy; [3] Department of Public Health and Infectious Diseases, Sapienza University of Rome, Rome, Italy; [4] University of Rome "Tor Vergata", Department of Chemical Science and Technologies, Rome, Italy

Introduction

Microbial colonization of surfaces constitutes a dangerous reservoir of pathogens that contribute to spread of infections with repercussions on human health and a heavy economic burden. Several antimicrobial coatings exist in the market; however, they are mainly based on the leaching of environmentally unfriendly chemicals and are formulated considering synthetic, non-biobased polymers as binders. These antimicrobial coatings frequently show serious concerns linked to antibiotic resistance, complex chemical synthesis, environmental pollution, non-biodegradability, low product performance, toxicity and extremely low sustainability. The present project aims to analyze the antimicrobial properties of an innovative self-disinfecting nanocoating based on copper nanoparticles doped with natural peptides such as essential oils (EO). This nanocoating is expected to be highly effective, have good environmental safety requirements, and exhibit long-term self-disinfection. Thymol, carvacrol, and *menthol* are EO compounds with broad-spectrum antimicrobial activities against pathogens, including *Escherichia coli.* The aim of the present study is to investigate the molecular mechanisms of *E. coli* in response to different concentrations of carvacrol (Ca) by studying differentially expressed genes with RNA-seq analysis.

Materials and Methods

Determination of MIC: Standard procedures to evaluate the Minimum EO concentration that inhibited microbial growth after 1h of exposure were applied. The Serial 2-fold dilutions of Ca were prepared in Luria broth (LB) with 0.5% of DMSO in a sterile 96-well plate, and equal volumes (100 µl) of EO and the bacterial solution were mixed. The final microbial inoculum level was 10^5 CFU/ml and final EO concentration range was 0.08 to 10.0 mg/ml. After 24 h of incubation at 37°C *E. coli* cells were collected and RNA was extracted. *E. coli* in LB with 0.5% of DMSO was used as negative control. A MIC of 0.04% of carvacrol was evaluated.

RNA extraction: RNA extraction was performed using RNeasy minikit (Qiagen, Germany) with on-column DNase digestion according to the manufacturer's protocols. The RNA concentration and quality were verified using NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific, USA) and 4200 TapeStation System (Agilent Technologies, USA).

RNA-Seq: total RNA (500 ng) was rRNA depleted with a Ribo-Zero bacterial rRNA removal kit (Illumina, USA) and used for RNA-Seq libraries preparation with TruSeq Stranded total RNA library preparation protocol (Illumina). The libraries were sequenced on Illumina NextSeq 2000 platform (Illumina) using a paired-end protocol and a read length of 2 × 75 bp. The raw reads were quality filtered, adaptors were removed, and any reads below 50 bp were discarded. The reads were then mapped onto the *E. coli* ATCC8739 strain reference genome (https://genomes.atcc.org/). Statistical analysis was performed using the DESeq2 and edgeR package. A negative binomial test was used to identify differentially expressed genes, with cutoff criteria set at an adjusted P value <0.01 and a log2 fold change of ≥2.

Real-time PCR: thirteen genes previously described in the literature were evaluated by RT-qPCR. The RT-qPCR reactions were performed according to the manufacturer's instructions using SYBR green qPCR 2X Master mix (BioRad, USA). Real-time PCR was performed on a QS5 real-time PCR system (Applied Biosystems). The specificity of the PCR was determined with a melting curve analysis. Normalization of amplification curves of genes was determined using *E. coli* (16S) housekeeping reference genes. The relative changes in gene expression were calculated using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis: all experiments were performed in triplicates. The results are represented as means ± standard deviations, and significant differences ($P < 0.05$) were determined by one-way analysis of variance (ANOVA).

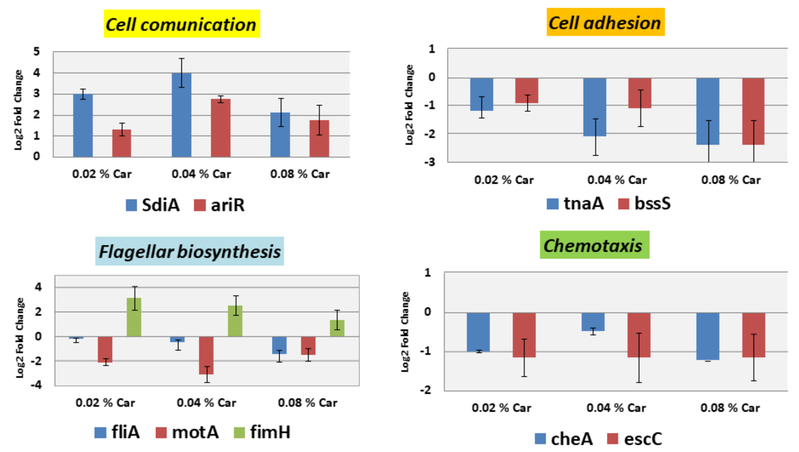
Results and Discussion

To understand transcriptional response of *E. coli* adapting to EO stress, a comparative transcriptomic analysis between *E. coli* exposed for 1h at different concentration of carvacrol (0.02%,0.04%, 0.08% Ca) and the control (CTL) was performed. Our preliminary results were based on the study of a subset of genes previously described in the literature (**Figure 1**). Data analysis showed that Ca was found to have high efficacy against biofilm formation increasing the expression of genes encoding inhibitors of cell communication pathway (e.g., *sdiA* and *ariR*)**.** Furthermore, a negative modulation of genes involved in cell adhesion mechanism (e.g., *tnaA* and *bssS*) was observed. Several virulence genes related to flagellar biosynthesis (e.g., *fliA* and *motA*) were under expressed where a gene involved in repression motility (*fimH*) was upregulated. Among the significant genes associated with virulence, chemotaxis (e.g., *cheA* and *cheZ*), and secretion system T3SS (e.g., *sepD* and *escC*) were downregulated by carvacrol at all exposure concentrations. Moreover, it was observed that adaptation of *E. coli* to sublethal concentrations of Car induced the upregulation of genes encoding multidrug efflux pumps (e.g., *marA* and *acrB*) (**Figure 2**).

From RNA-Seq investigations on E. coli treated with 0.02% of Ca, differential expressed genes (DEGs) were obtained. Data analysis was performed by two distinct methods: edgeR and DESeq2 (p-value < 0.01 and log2 Fold Change). We obtained 13 Down-regulated DEGs and 42 Up-regulated DEGs from edgeR and 16 Down-regulated DEGs 17 Up-regulated DEGs from DESeq2. A partial transcripts overlap is showed among the two analysis tools with 26 genes in common (**Figure 3)**. The first pathway analysis of these genes revealed mainly the modulation of oxidative stress, efflux pumps induced and membrane integrity (**Figure 4**).

Name	Description	Pathway
SdiA	DNA-binding transcriptional dual regulator SdiA	cell comunication
ariR	putative two-component system connector protein AriR	
tnaA	tryptophanase	cell adhesion
bssS	regulator of biofilm formation	
fliA	RNA polymerase sigma factor Fli	flagellar biosynthesis
motA	motility protein A	
fimH	type 1 fimbriae D-mannose specific adhesin	
cheA	chemotaxis protein CheA	chemotaxis
cheZ	chemotaxis protein CheZ	
sepD	T3SS secretion switching protein SepD	secretion
escC	T3SS structure protein EscC	
marA	DNA-binding transcriptional dual regulator MarA	multidrug efflux pumps
acrB	multidrug efflux pump RND permease AcrB	

Figure 1. Selected genes for this study with their description and pathway specification.



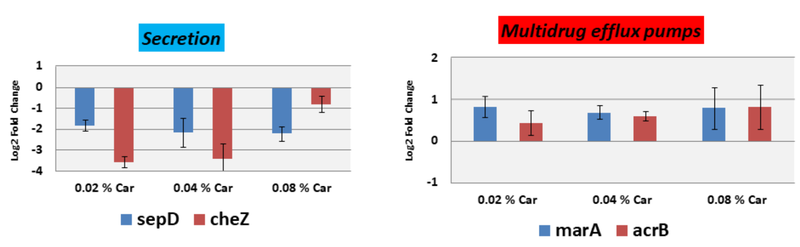
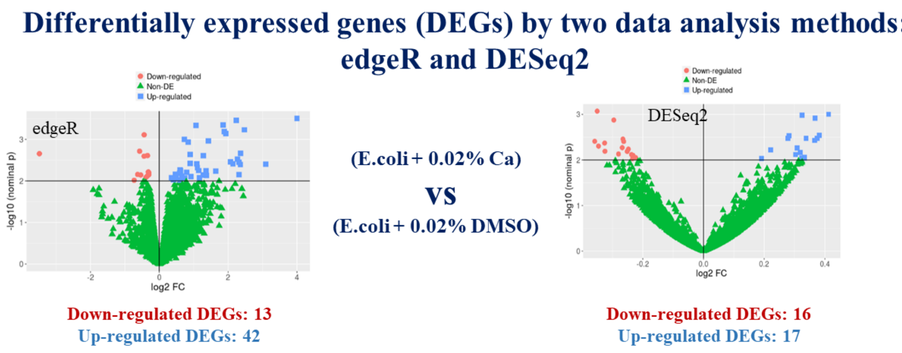


Figure 2. Expression levels of selected genes by RT-qPCR in *E. coli* exposed for 1 hour to different concentrations of carvacrol (0.02%, 0.04%, 0.08% Ca) compared to the control (CTL); (p-value<0.05).



A partial transcripts overlapping is showed among the two analysis tools

p-value<0.01	No. DEGs
edgeR	55
DESeq2	33
edgeR & DESeq2	26

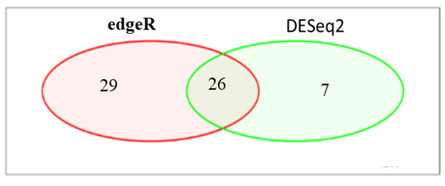


Figure 3. Differentially expressed genes by two data analysis methods (edgeR and DESeq2) after E.coli exposure at 0.02 % of carvacrol; (p-value <0.01 and log2 fold change of ≥2).

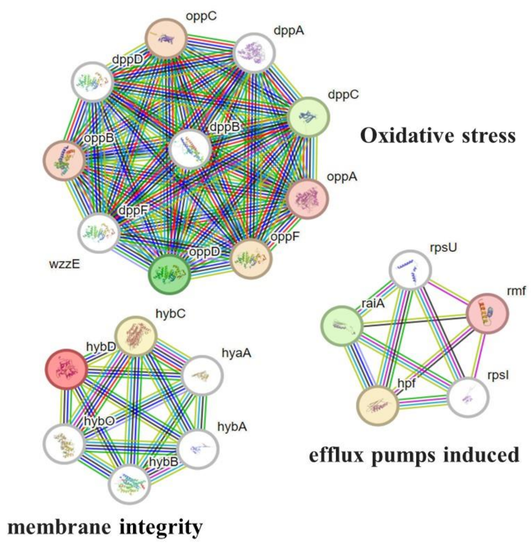


Figure 4. Pathway analysis of 26 overlapping genes by STRING DB database

Conclusions

These initial findings suggest that carvacrol may affect biological pathways associated with the inhibition of biofilm formation, and virulence of E. coli at subinhibitory concentrations and could be a promising candidate as a natural antibacterial compound.

Since the antimicrobial coatings frequently used show serious concerns linked to low product performance, pollution, toxicity and antibiotic resistance, further RNA-Seq analysis will be performed to gain new insights. The knowledge of molecular mechanisms that underlie this innovative intervention strategy, could highlight whether one or a combination of several mechanisms at structural, genomic, transcriptomic and proteomic level could trigger possible resistance mechanisms against antimicrobial nanoparticles.

References

- Yuan W, Seng ZJ, Kohli GS, Yang L, Yuk HG. Stress Resistance Development and Genome-Wide Transcriptional Response of Escherichia coli O157:H7 Adapted to Sublethal Thymol, Carvacrol, and *trans*-Cinnamaldehyde. Appl Environ Microbiol. 2018 Oct 30;84(22):e01616-18. doi: 10.1128/AEM.01616-18.
- Martínez A, Stashenko EE, Sáez RT, Zafra G, Ortiz C. Effect of Essential Oil from *Lippia origanoides* on the Transcriptional Expression of Genes Related to Quorum Sensing, Biofilm Formation, and Virulence of *Escherichia coli* and *Staphylococcus aureus*. Antibiotics (Basel). 2023 May 3;12(5):845. doi: 10.3390/antibiotics12050845.

Acknowledgments: This project has received funding from the European Union's Horizon Europe research and innovation program, grant agreement N° 101058570.