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ORIGINAL ARTICLE

Comparative transcriptomic analysis of *Listeria monocytogenes* reveals upregulation of stress genes and downregulation of virulence genes in response to essential oil extracted from *Baccharis psiadioides*

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Abstract *Listeria monocytogenes* is a pathogenic microorganism in humans and is frequently transmitted by food. Methods to control the presence of Listeria in foods are necessary. In the present study, transcriptomics of *L. monocytogenes* grown in the presence of essential oil extracted from *Baccharis psiadioides* were studied by RNA sequencing and reverse transcription quantitative polymerase chain reaction (RT-qPCR) experiments. The results obtained indicate that essential oil of *B. psiadioides* has potential bacteriostatic activity at the concentration tested, affecting *Listeria* cells functioning and development. Responses of the microorganism included upregulation of stress genes and downregulation of virulence genes, such as *actA*, *hly* and *prfA*, indicating a decrease in virulence and in the capacity of the microorganism to cause infection. Thus, the results presented here allow us to conclude that *B. psiadioides* essential oil may be an alternative means of controlling microorganisms proliferating in foods.

Keywords Bacteriostasis · Essential oil · *Listeria monocytogenes* · Virulence · RNA sequencing · RT-qPCR

Introduction

Among studies involving food safety, *Listeria monocytogenes* stands out because of its high pathogenicity, mainly related to immunocompromised individuals, such as the elderly and neonates, and the high risk of its transplacental transmission in pregnant women (Allerberger and Wagner 2010; Girard et al. 2014). The microorganism has the ability to survive and proliferate at refrigeration temperatures, which is a major problem related to food production that extensively uses the cold chain in the processing and storage of products (Farber and Peterkin 1991). Moreover, increased transcription of several *L. monocytogenes* genes involved in virulence and stress

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responses has already been demonstrated at 7 °C compared to 37 °C (Pieta et al. 2014). Among the 13 described serotypes of *L. monocytogenes*, 1/2a, 1/2b and 4b are responsible for 95% of human infections, called listeriosis (Montero et al. 2015). Historically, serotype 4b has caused the greatest proportion of listeriosis outbreaks and the largest number of cases per outbreak in the United States (Cartwright et al. 2013).

Essential oils (EO) are secondary metabolites produced by several plants, and can function as antimicrobials, antivirals, antimycotics, antipsoriatics, insecticides and in cancer treatments (Cowan 1999; Edris 2007; Reichling et al. 2009). The EO present in the *Asteraceae* plant family, with emphasis on *Baccharis psiadioides* (Less.) Joch. Müller (= *Heterothalamus psiadioides* Less.) (Giuliano and Freire 2011), has important anti-inflammatory properties (Fabri et al. 2011) and the ability to inhibit the growth of antibiotic resistant microorganisms, also reducing biofilm formation in abiotic surfaces (Negreiros et al. 2016). Natural compounds present in the essential oil of *B. psiadioides* (EO*Bp*) are classified as terpenes, and can be divided into two fractions: (1) monoterpenes with a significant percentage composed of β-pinene; and (2) sesquiterpenes with Ar-curcumene as the major component.

Transcriptomic, proteomic, genetic and physiological analyses can identify *L. monocytogenes* molecular stress adaptation responses, by global expression changes in a large number of the cellular components (Soni et al. 2011). In addition to EO, nisin—a bacteriocin produced by several lactic acid bacteria (Delves-Broughton 1990)—presents antimicrobial potential against food pathogens. Proteomic analyses of *L. monocytogenes* cells treated with a sub-lethal concentration of nisin displayed an overexpression of proteins related to oxidative stress and production of cell membrane lipids (Miyamoto et al. 2015). Experiments carried out with the Gram-positive pathogenic bacterium *Staphylococcus aureus*, showed transcriptional alterations induced by tea tree oil produced as a steam distillate of *Melaleuca alternifolia*, which has broad-spectrum antibacterial activity, including altered regulation of genes involved in heat shock and cell wall metabolism (Cuaron et al. 2013). Furthermore, the mechanism of biofilm inhibition and virulence attenuation in enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) treated with eugenol and eugenol-rich oil was shown through transcriptional and phenotypic assays (Kim et al. 2016).

The use of natural compounds with antimicrobial potential represents an alternative means to combat pathogen growth; therefore, the present work aimed to analyze the differential transcriptome profile of *L. monocytogenes* grown in the presence of EO*Bp* using RNA sequencing (RNA-Seq) and reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Materials and methods

Bacterial strain

The *L. monocytogenes* 55 (*Lm*55) strain was isolated from cheese by the National Agricultural Laboratory of Rio Grande do Sul State (LANAGRO/RS) of the Ministry of Agriculture, Livestock and Food Supply (MAPA/Brazil), and serotyped at the Oswaldo Cruz Institute (State of Rio de Janeiro, RJ, Brazil) as serotype 1/2a (de Mello et al. 2008; Nes et al. 2010).

Characterization of EO of *B. psiadioides*

EO*Bp* was obtained from the Laboratory of Chemical Ecology and Chemotaxonomy [Department of Botany, Federal University of Rio Grande do Sul (UFRGS)]. Leaves of *B. psiadiodes* were collected from populations located in Porto Alegre, RS, and subjected to drying at room temperature, with subsequent extraction of EO in a modified Clevenger apparatus (Gottleib and Taveira-Magalhães 1960). EO*Bp* was fractionated according to Kulisic et al. (2004) with some modifications, by column chromatography (40 cm in length; 2 cm diameter) with silica (21 g, 63–200 μm, 60° pore; Sigma-Aldrich, St. Louis, MO), using pentane and diethyl ether to obtain fractions containing only non-polar and polar hydrocarbons, respectively. Fractions obtained were analyzed using gas chromatography–mass spectrometry (GC-MS). For the experiments, the whole extract (both fractions) was used in *L. monocytogenes* cultures.

Experimental design, RNA sequencing and statistical analyses

The *Lm*55 strain was cultivated in tryptone soy broth (TSB; HiMedia, Mumbai, Maharashtra, India) at 37 °C under agitation. The MIC/2 of EO*Bp* (Negreiros et al. 2016) was added in the exponential growth phase, when the microorganism had reached an optical density ($OD_{600\ nm}$) between 0.3 and 0.4, measured with an ultraviolet/visible spectrophotometer (Ultrospec 3100 Pro; Amersham Biosciences, Little Chalfont, UK). After 20 min, growth was interrupted and cells were washed with 300 μL 1X TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0; reagents from Sigma-Aldrich) and resuspended in 100 μL 1X TE buffer. As control conditions, a parallel experiment was conducted without EO*Bp*. Total RNA samples from *Lm*55 were isolated using the TRIzol® Reagent kit (Thermo Fisher Scientific, Waltham, MA), and spectrophotometer readings (ratio $OD_{260\ nm}/OD_{280\ nm}$) comprised values between 1.8 and 2.0 for all samples. Experiments were performed in biological triplicates and experimental quadruplicates.

Total RNA samples were prepared using the TruSeq Stranded mRNA Sample Preparation—Low Sample (LS) protocol from the TruSeq Stranded mRNA Library Preparation Kit (Illumina, San Diego, CA), and a pool of libraries was prepared for subsequent sequencing according to the TruSeq Stranded mRNA Sample Preparation Guide (Illumina). Sequencing of the pooled libraries was performed on MiSeq Gene and Small Genome Sequencer equipment (Illumina) using the MiSeq Reagent kit v3 150 cycles (Illumina) according to the manufacturer's instructions. Finally, 600 μL [570 μL of the pooled libraries and 30 μL (5%) of PhiX control solution] was added to the cartridge for subsequent sequencing.

The presence of adapters and quality of reads produced by RNA-Seq were determined for each library using FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Based on these data, the Trim Galore! software (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) was used to eliminate sequences of reads with a quality below 30, as well as the sequences of the Illumina adapters. The cleaned reads were then anchored with TopHat2 (Kim et al. 2013) to the reference genome of *Lm*55 (Pieta et al. 2015; deposited in GenBank under the accession no. LKHO00000000), and the fragments per kilobase million (FPKM) values for all genes were calculated using Cufflinks (Trapnell et al. 2012). The counting tables of the reads mapped to each gene were generated by the featureCounts module of Subread software (Liao et al. 2013) , for sequence alignment files generated by TopHat2. To perform statistical analyses for differential expression, the counting tables were analyzed in the R Bioconductor DESeq2 package v.1.12.3 (Love et al. 2014). For each treatment comparison, all genes with $\log_2$foldchange greater than 1 and less than −1 were considered differentially expressed.. The protein sequences of these two groups of genes were functionally annotated with Blast2GO (Conesa et al. 2005), and the functional categories were visualized with the WEGO program (Ye et al. 2006). Sequences of the proteins were compared to the UniRef Enriched KEGG Orthology (UEKO) database (Guedes et al. 2011) using local BlastX (Altschul et al. 1997). The BlastX results were processed in the MySQL software (Oracle, Cupertino, CA), and the KEGG Orthology (KO) codes obtained were viewed on the iPATH2 web server (Yamada et al. 2011).

Relative gene expression

From total RNA, complementary DNA (cDNA) synthesis, recommended by Bustin et al. (2009), was performed according to Pieta et al. (2014), and relative gene expression was determined using RT-qPCR. Primers were using the GenScript tool (https://www.genscript.com/tools/real-time-pcr-tagman-primer-design-tool) based on the genes that were differentially expressed and related to virulence, stress response and transcription factors of the microorganism. Genes chosen for analysis in the present study were *actA*, *agrA*, *crp*, *degU*, fri, *fur*, *hly*, *iscR*, *malR*, *prfA*, *sigB*, and *sod* (Table 1 and Table S1 for functions of the coded proteins).

For RT-qPCR experiments, a solution containing 0.01–0.1 μM of each primer; 25 μM dNTPs (Promega, Madison, WI); 1X reaction buffer; 3 mM $MgCl_2$; 1X SYBR Green (Bio-Rad, Hercules, CA); 0.25 U Platinum *Taq* DNA polymerase (Thermo Fisher Scientific); and ultrapure Milli-Q water to complete the final volume of 10 μL was prepared. Standard curves were constructed with four points in twofold dilutions starting from a 1:50 cDNA concentration for each of the study primers to verify reaction efficiency in RT-qPCR experiments, determined with the StepOne v. 2.3 software based on slopes of plots and crossing points (Cps) versus log input of cDNA. For amplification, StepOnePlus™ Real Time–PCR System (Thermo Fisher Scientific) and 96-wells polystyrene microplates (Axygen Scientific, Union City, CA) were used. PCR was conducted at 94 °C for 5 min; 40 cycles at 94 °C for 15 s, 60 °C for 10 s, 72 °C for 15 s and 60 °C for 35 s; and a final melting curve between 50 and 99 °C (Δ0.1 °C/s). All experiments were performed in biological triplicates and experimental quadruplicates. The total volume present in each well was 20 μL, consisting of 10 μL diluted cDNA (1:50) and 10 μL reaction solution, and in the case of the negative control, a total volume consisting of 20 μL reaction solution.

Housekeeping genes *gap*, *rpoB* and *16SrRNA* (Table S2) were tested as candidates for RT-qPCR data normalization using the NormFinder algorithm (Andersen et al. 2004) and geNorm v. 3.5 software (Vandesompele et al. 2002). Relative expression of the genes was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), considering the efficiency (E) of RT-qPCR reactions for each of the primers in the calculation of relative expression ($E^{-\Delta\Delta Ct}$), and statistical analyses were performed using one-way analysis of variance (ANOVA), at a significance level of 5%, using Statistica software (Statsoft, Tulsa, OK). When there was a statistically significant difference ($P < 0.05$) between C_t (threshold cycle) values of the control and study conditions, the genes were considered to be more transcribed ($E^{-\Delta\Delta Ct} > 1$) or less transcribed ($E^{-\Delta\Delta Ct} < 1$) during growth in the presence of *Bp*EO.

Results and discussion

Determination of EOBp composition by GC-MS

Total EO*Bp* was used to perform our analysis and the EO*Bp* fractions obtained were divided into two groups: one fraction was composed predominantly of monoterpenes and the other predominantly of sesquiterpenes. Results of GC-MS indicated the presence of a complex mixture of terpenes in the two

Table 1 Sequences of primers used in the transcriptional analysis by RT-qPCR, with respective sizes of amplification fragments and annealing temperatures

Gene	Nucleotide sequence	Amplicon size (bp)	Annealing temperature (°C)
actA	5′ AGAAATCATCCGGGAAACAG 3′	147	58.98
	5′ CCTCTCCCGTTCAACTCTTC 3′		58.87
agrA	5′ CGGGTACTTGCCTGTATGAA 3′	149	58.65
	5′ TGAATAGTTGGCGCTGTCTC 3′		59.03
crp	5′ ATTCACAGTTTGCGAATGCT 3’	117	58.86
	5′ TTTGCAAATCAACATCACGA 3’		59.02
degU	5′ GGCGCGTATATTCATCCAC 3’	150	58.96
	5′ TACCTCGCACTCTCTATGCG 3’		59.20
fri	5′ GGCGAACAAATGGATGAAGT 3’	108	59.94
	5′ ATAAGGCGCTTCTTCTACGC 3’		58.77
fur	5′ TTTAGCGCCTTCTTGTCTCA 3’	114	58.80
	5′ GGCCTTGCAACCGTTTATAG 3’		59.61
hly	5′ AGCTCATTTCACATCGTCCA 3’	124	59.24
	5′ TGGTAAGTTCCGGTCATCAA 3’		58.97
iscR	5′ ATCGGACCTCTTCGTAATGC 3’	106	59.15
	5′ CGTATGATATCACCCGCAGT 3’		58.48
malR	5′ GAATCGTCTGGACCGGTAAT 3’	110	58.86
	5′ AACGTGAGCCAAGTCCTTCT 3’		58.94
prfA	5′ GGAAGCTTGGCTCTATTTGC 3’	145	59.07
	5′ ACAGCTGAGCTATGTGCGAT 3’		58.65
sigB	5′ TGGTGTCACGGAAGAAGAAG 3’	135	58.85
	5′ TCCGTACCACCAACAACATC 3’		59.27
sod	5′ CCACCATTTGGGCTAAGAAT 3’	94	58.90
	5′ GCGTTCCTGAAGATATTCGC 3’		59.81

fractions. The fraction composed predominantly of monoterpenes revealed the presence of 20 compounds (Table 2); monoterpenes represented 71.82% of this fraction, with β-pinene as the major compound (43.81%). Other compounds present in significant amounts were δ-3-carene (14.92%) and limonene (10.82%)—both monoterpenes. In relation to the fraction composed predominantly by sesquiterpenes, the presence of 14 compounds was verified (Table 3), where the sesquiterpenes represented 93.59% of this fraction, Ar-curcumene being the major compound (40.12%). In this fraction, other compounds were also found in significant concentrations, such as bicyclogermacrene (15.89%) and γ-muurolene (15.68%)—both sesquiterpenes.

Transcriptomic analysis

In total, 333 genes presented a log_2foldchange > −1 (−2 fold change cut off), being considered downregulated in the T4 sample (untreated with EO*Bp*), and, consequently, upregulated in the O6 sample (treated with MIC/2 EO*Bp*); and 273 genes presented a log_2foldchange > 1 (2 fold change cut off), which means they were upregulated in the T4 and downregulated in the O6 samples (Table S3).

Based on these data, functional categories were visualized with the WEGO program, and the results regarding the effect of EO*Bp* on differential genes expression in *Lm*55 strain are shown in Fig. 1 and Table 4 for the three categories listed: Biological Process (BP), Cellular Component (CC) and Molecular Function (MF).

With regard to the BP group (Fig. 1a), several processes presented a greater number of upregulated genes, such as biological regulation; cell cycle; catabolic process; amino acid and nitrogen compound, carbohydrate, cofactor, lipid, organic acid and sulfur metabolism; and response to stress. According to Bich et al. (2016), “biological regulation is what allows an organism to handle the effects of a perturbation, modulating its own constitutive dynamics in response to particular changes in internal and external conditions”. As the results showed 12 upregulated genes and 4 downregulated genes in this category, indicating that EO*Bp* can affect homeostasis causing changes in *L. monocytogenes* cells function and development. In support of this statement, growth in the presence of EO*Bp* upregulated 22 genes and downregulated 5 genes related to stress response. In addition, several genes related to cofactor and sulfur metabolism were upregulated, and it should be noted that the iron-sulfur ([Fe-S]) clusters or cofactors (widely distributed in nature) are of great importance in several biological processes (Johnson et al. 2005).

Table 2 Chemical composition of *Baccharis psiadioides* essential oil (EO*Bp*) fraction composed predominantly by monoterpenes. The relative percentage of each component was obtained directly from the peak areas of the chromatogram, considering 100% the sum of all evaluated peaks

Component	IK cal[a]	IK tab[b]	Yield (%)
Monoterpenes			
α-pinene	930	939	0.59
β-pinene	978	979	43.81
Mircene	993	990	0.93
δ-3-carene	1012	1011	14.92
p-cymene	1024	1024	0.75
Limonene	1029	1029	10.82
Total			71.82
Sesquiterpenes			
β-elemene	1383	1390	1.65
β-caryophyllene	1407	1419	1.15
Aromadendrene	1426	1441	1.80
Dehydro-aromadendrene	1434	1462	2.36
Allo-aromadendrene	1446	1460	3.34
γ-gurjunene	1457	1477	1.06
γ-muurolene	1462	1479	1.09
Germacrene D	1466	1481	0.80
Ar-curcumene + β-selinene	1470	1480/1490	5.32
Valencene	1473	1496	0.87
α-selinene	1480	1498	4.28
α-muurolene	1485	1500	0.90
γ-cadinene	1496	1513	1.53
δ-cadinene	1506	1523	2.03
Total			28.18

[a] Calculated Kováts retention index

[b] Tabulated Kováts retention index

Table 3 Chemical composition of EO*Bp* fraction composed predominantly by sesquiterpenes. The relative percentage of each component was obtained directly from the peak areas of the chromatogram, considering 100% the sum of all evaluated peaks

Component	IK cal[a]	IK tab[b]	Yield (%)
Monoterpenes			
β-pinene	973	979	1.41
p-cymene	1023	1024	0.64
Limonene	1027	1029	3.14
(E)-β-ocimene	1047	1050	1.22
Total			6.41
Sesquiterpenes			
β-elemene	1383	1390	4.30
β-caryophyllene	1407	1419	1.12
α-humulene	1440	1454	6.56
Allo-aromadendrene	1446	1460	4.91
γ-muurolene	1468	1479	15.68
Ar-curcumene	1477	1480	40.12
Bicyclogermacrene	1485	1500	15.89
Germacrene A	1491	1509	2.07
γ-cadinene	1498	1513	1.30
δ-cadinene	1508	1523	1.64
Total			93.59

[a] Calculated Kováts retention index

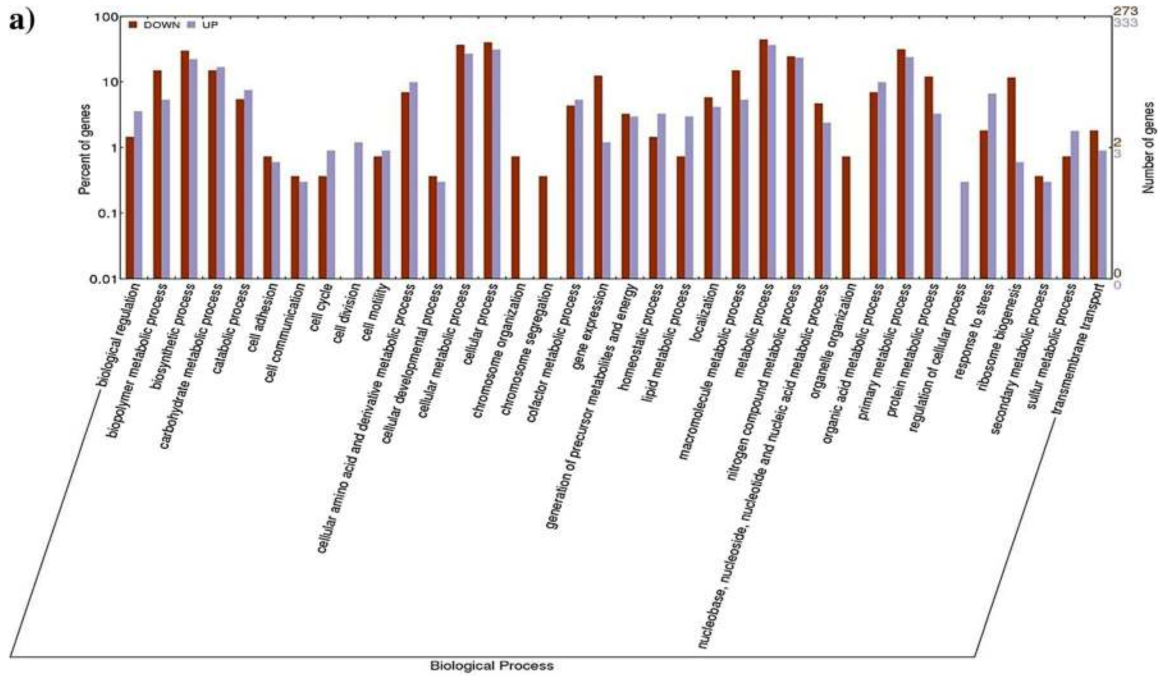
[b] Tabulated Kováts retention index

Carbohydrate and lipid metabolism indicate energy generation, and may be considered catabolic processes, which refer to the assimilation or processing of organic compounds to obtain energy. Positive regulation of genes involved in the metabolism of several compounds may be related to the EO composition, since EO are complex mixtures of volatile substances, usually lipophilic, whose components include terpene hydrocarbons, simple alcohols, aldehydes, ketones, phenols, esters and fixed organic acids (Simões and Spitzer 1999). Araújo et al. (2016) analyzed the effects of argentilactone, a constituent of the EO from *Hyptis ovalifolia*, on the transcriptional profile, cell wall and oxidative stress of *Paracoccidioides* spp., a dimorphic pathogenic fungus. Their results demonstrated that the upregulated genes were related to metabolism; cell rescue, defense and virulence; energy and cell cycle; and DNA processing. The downregulated genes were related to metabolism, transcription, protein fate, and cell cycling and DNA processing.

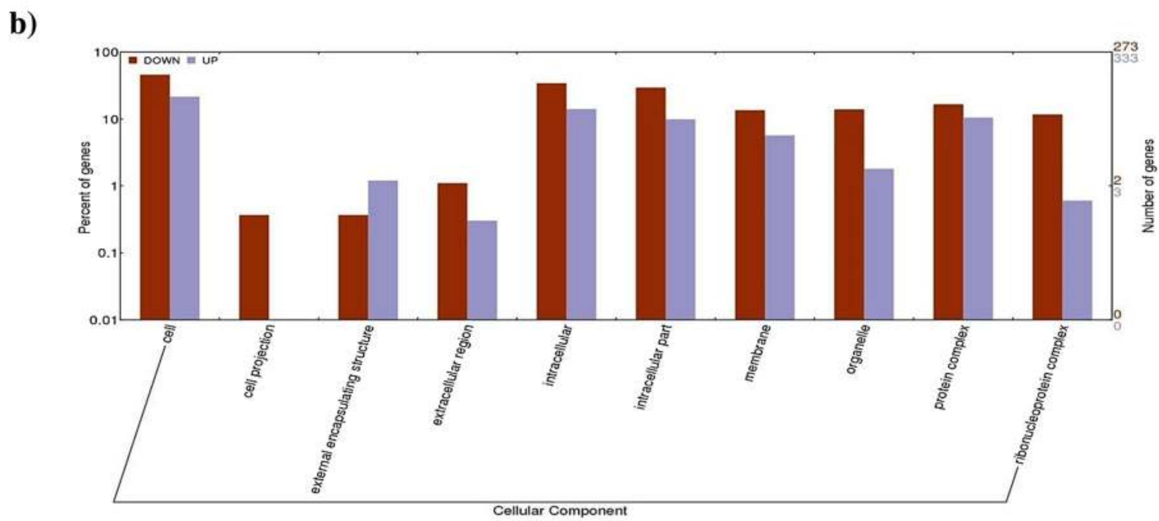
A larger number of downregulated genes related to BP were identified for categories such as biopolymers, macromolecules and protein metabolism; cell division; gene expression; ribosome biogenesis; and transmembrane transport. Biopolymer metabolism includes proteins, DNA and RNA production, and its downregulation may consequently affect ribosome biogenesis (32 downregulated versus two upregulated genes) and gene expression (34 downregulated versus four upregulated genes). The antimicrobial effect of EO may be responsible for downregulation of genes related to cell division, indicating the difficulty that the microorganism has, in the presence of the EO, to complete its binary fission and increase the microbial population.

All the categories related to CC (Fig. 1b) presented a larger number of downregulated genes, except for the external encapsulating structure. Some of those belonging to MF (Fig. 1c), such as structural constituent of ribosomes, translation regulators and transmembrane transporters, were also mostly downregulated. These data suggest an inverse correlation with the results for higher numbers of downregulated genes involved in BP, such as ribosome biogenesis, biopolymer (DNA, RNA, proteins) production, and transmembrane transport.

a)



b)



c)

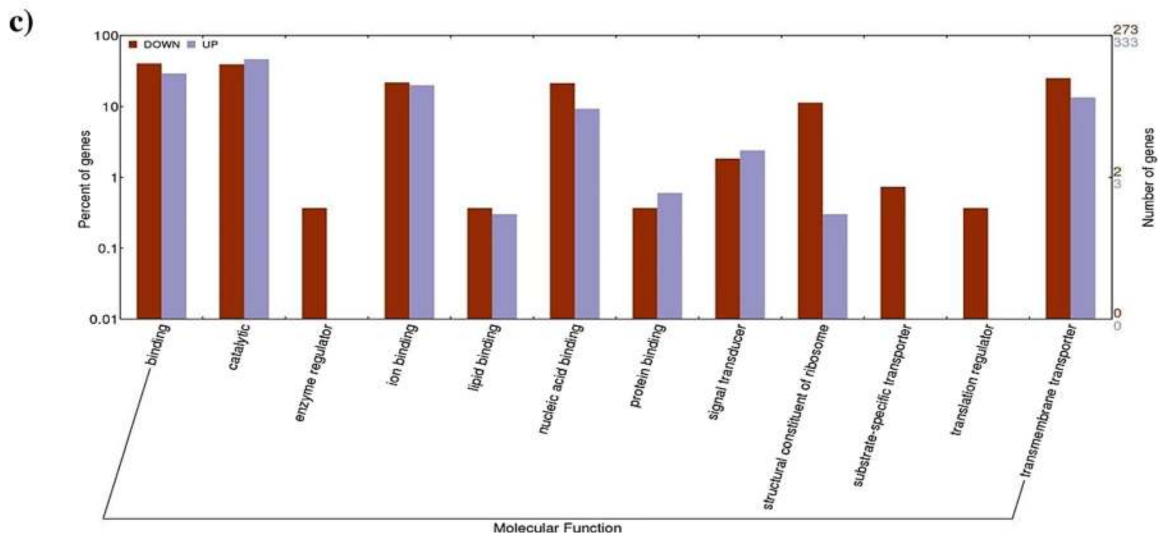


Fig. 1a–c Transcriptomic analysis results. Differential expression of genes related to functional categories **a** biological process (BP), **b** cellular component (CC), and **c** molecular function (MF) of *Listeria monocytogenes* 55 grown in the presence of *Baccharis psiadioides* essential oil (EO*Bp*). Graphical representation generated using the WEGO program

Transcriptional analysis of virulence genes and stress response genes

First, to determine the reliability of the amplification data, the efficiency of the study primers was determined (Table S4), and the housekeeping genes *gap*, *rpoB* and *16SrRNA* were tested as candidates for RT-qPCR data normalization using the NormFinder algorithm and geNorm v. 3.5 software. Both programs indicated *rpoB* and *16SrRNA* as the most stable genes and recommendable for data analysis, while *gap* was demonstrated as the least stable gene (Fig. S1 and Table S5). Results of relative gene expression for *Lm*55 strain cultivated in the presence of EO*Bp* are shown in Fig. 2. The data shown here concur with the differential expression obtained with RNA-Seq, which allowed us to validate our experiments (Table S6).

Downregulation ($P < 0.05$) was observed in virulence genes, such as *prfA*, *fur*, *hly*, *actA* and *agrA*, in the presence of EO*Bp*. Previous research has already demonstrated the antimicrobial and antibiofilm potential of plant-extracted EO against several food-borne pathogens, such as *S. aureus*, *E. coli* and *L. monocytogenes* (Upadhyay et al. 2013; Lopez-Romero et al. 2015) and the relation between the EO concentration and its bactericidal and/or bacteriostatic effect against these bacteria (Burt 2004; Mazzarrino et al. 2015). In addition, the extracted of EO*Bp* showed a high concentration of β-pinene—a monoterpene that has been reported as one of the main chemicals responsible for the antimicrobial activity of several EOs.

Both PrfA and Fur are regulators involved in *L. monocytogenes* virulence and pathogenicity. PrfA controls the transcription of several virulence genes involved in the infection process, such as *actA*, which is responsible for the polymerization of actin tails, which propels the microorganism to neighboring cells, and the *hly* gene that codifies listeriolysin O (LLO), which is critical to survival of the microorganism in the phagocytes during the infection process (Xayarath and Freitag 2012). Thus, the significantly reduced transcription of *prfA* corroborates the reduced transcription of the *hly* gene. The *agr* system of *S. aureus*, widely conserved among Gram-positive bacteria, is involved in biofilm formation (Lyon and Novick 2004), and the AgrA-AgrC two-component system has been studied extensively because of its control of virulence factors (Novick 2000). In *L. monocytogenes*, as in *S. aureus*, *agrB*, *agrD*, *agrC* and *agrA* genes are organized in a unique operon, regulating microorganism adhesion to surfaces, fundamental for a proper biofilm formation, in addition to its involvement in the *Listeria* infection process in mammals (Riedel et al. 2009). An earlier in vivo study showed that the virulence of a Δ*agrA* *L. monocytogenes* strain was attenuated, demonstrating the role of the *agr* locus in the virulence of this microorganism, and its influence in the production of several secreted proteins, such as LLO (Autret et al. 2003).

Iron, an abundant element in nature, acts as a cofactor for several enzymes involved in microorganism metabolism, being required by almost all bacteria. However, iron concentrations above physiological levels can be toxic for microorganisms. A regulator of ferric iron uptake in many bacteria, Fur is involved with *L. monocytogenes* virulence and survival in the host (Rea et al. 2004). Mutations in the *fur* gene reduced microorganism pathogenicity in mice, indicating that disruption of intracellular iron homeostasis contributes to a lower ability of this pathogen to successfully establish infection (Newton et al. 2005; Olsen et al. 2005). In agreement with this, McLaughlin et al. (2012) demonstrated that deregulation of iron uptake through the elimination of Fur significantly impacts upon virulence potential in several pathogenic bacteria, including *L. monocytogenes*, as mutants in Fur-regulated loci resulted in a significant reduction in virulence potential relative to the wild-type. A recent study characterized the composition of an EO extracted from the leaf of *Rhaphiodon echinus* GC-MS experiments revealed the presence of monoterpenes, sesquiterpenes, and the metal chelation potential of this oil (Duarte et al. 2016). As the EO*Bp* constitutes by both monoterpenes and sesquiterpenes, this may explain the significantly decreased transcription of *fur*, which is downregulated under iron-limited conditions (Ledala et al. 2010).

While some genes associated with virulence were downregulated, genes correlated with stress response such as *degU*, *sigB*, *crp*, *fri*, *iscR*, *sod* and *malR* were upregulated in the presence of EO*Bp*. An upregulation gene example was a stress response transcription factor named sigma B (σ^B), which contributes to the microorganism's resistance to several conditions unsuitable to its development, such as acidic, osmotic and energy stresses (O'Byrne and Karatzas 2008).

DegU is a regulator of the expression of flagellar and chemotaxis genes in *L. monocytogenes*, involved in microorganism motility but not required for its virulence (Williams et al. 2005). Burke et al. (2014) demonstrated that *L. monocytogenes* uses different enzymes and regulators of gene expression, such as DegU, to resist the bactericidal activity of lysozymes, which degrade the bacterial cell wall, resulting in bacteriolysis. In addition, they suggested that DegU is one of the major regulators of lysozyme resistance in *L. monocytogenes*, a mechanism commonly found in other pathogens. Members of the Crp/Fnr transcription factor family have several related functions in microorganisms, such as regulation of virulence, metabolic pathways and stress response.

Table 4 Number of down and upregulated genes related to processes belonging to the functional categories studied [Biological Process (BP); Cellular Component (CC); Molecular Function MF)] in *Listeria monocytogenes* 55 grown in the presence of EO*Bp*

	Down	Up
BP		
Biological regulation	4	12
Biopolymer metabolic process	41	18
Biosynthetic process	82	74
Carbohydrate metabolic process	41	56
Catabolic process	15	25
Cell adhesion	2	2
Cell communication	1	1
Cell cycle	0	4
Cell division	45	35
Cell motility	2	3
Cellular amino acid and derivative metabolic process	19	33
Cellular developmental process	1	1
Cellular metabolic process	101	90
Cellular process	110	104
Chromosome organization	2	0
Chromosome segregation	1	0
Cofactor metabolic process	12	18
Gene expression	34	4
Generation of precursor metabolites and energy	9	10
Homeostatic process	4	11
Lipid metabolic process	2	10
Localization	16	14
Macromolecule metabolic process	41	18
Metabolic process	122	122
Nitrogen compound metabolic process	67	78
Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	13	8
Organelle organization	2	0
Organic acid metabolic process	19	33
Primary metabolic process	86	80
Protein metabolic process	33	11
Regulation of cellular process	0	1
Response to stress	5	22
Ribosome biogenesis	32	2
Secondary metabolic process	1	1
Sulfur metabolic process	2	6
Transmembrane transport	5	3
Cellular component		
Cell	124	71
Cell projection	1	0
External encapsulating structure	1	4
Extracellular region	3	1
Intracellular	93	47
Intracellular part	80	33
Membrane	37	19
Organelle	38	6
Protein complex	45	35
Ribonucleoprotein complex	32	2
Molecular function		
Binding	110	97
Catalytic	107	154
Enzyme regulator	1	0
Ion binding	59	66
Lipid binding	1	1
Nucleic acid binding	58	31
Protein binding	1	2
Signal transducer	5	8
Structural constituent of ribossome	31	1
Substrate-specific transporter	2	0
Translation regulator	1	0
Transmembrane transporter	68	45

Crp, the cyclic AMP receptor protein, affects the metabolism of sugars or amino acids, transport processes, protein folding, as well as toxin production or pilus synthesis (Körner et al. 2003). In addition, the Crp family of transcription factors is

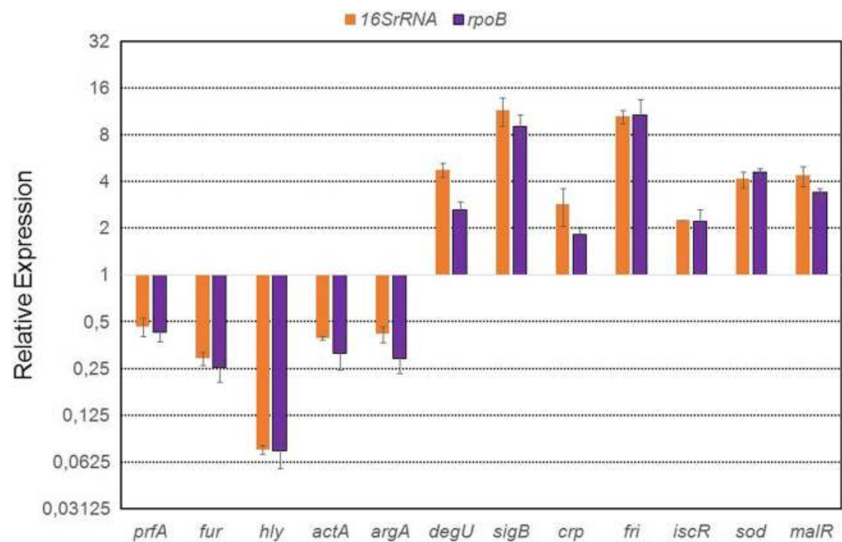


Fig. 2 Transcriptional analysis results. Relative expression of *actA*, *agrA*, *crp*, *degU*, *fri*, *fur*, *hly*, *iscR*, *malR*, *prfA*, *sigB* and *sod*, normalized with *rpoB* and *16SrRNA*, for *Listeria monocytogenes* 55 grown in the presence of EO*Bp*, and respective bars indicating the standard deviation values. All genes were statistically less or more transcribed ($P < 0.05$); graphical representation obtained with Microsoft Office Excel 2007

involved in various metabolic pathways in bacteria, acting in response to environmental changes. It has been shown that Crp acts as a transcription regulator in response to stresses in *Deinococcus radiodurans* (Yang et al. 2016). This Gram-positive bacterium is characterized by its efficient DNA repair ability and extreme stress resistance (Makarova et al. 2001) and generally considered to be an ideal model organism for studying bacterial resistance mechanisms under various stress conditions. This recent study demonstrated that the transcription levels of *crp* genes were increased to different extents when the bacteria were exposed to oxidizing agents. The Crp mutants were more susceptible to hydrogen peroxide (H_2O_2) than the wild-type strain, proving the important role of these proteins in stress resistance of *D. radiodurans*.

The *fri* gene encodes an iron-binding ferritin-like protein (Fri) that belongs to the Dps (DNA-binding proteins from starved cells) family of proteins (Haikarainen and Papageorgiou 2010). Ferritin is the most important iron reserve protein, found in all cells, especially in those involved in ferric compound synthesis, iron reserves and metabolism, which is required by several bacteria. It has been shown that the *fri* gene is repressed by Fur (Fiorini et al. 2008), being upregulated under several conditions, such as iron restriction, heat and cold shock (Hébraud and Guzzo 2000). The results obtained in the present study confirm this, since the *fur* gene was downregulated, and, consequently, the *fri* gene was upregulated in the presence of EO*Bp*. A recent study demonstrated that the cell-envelope stress response in *L. monocytogenes* is linked to the osmotic stress response, confirming the results obtained in the present work, because active terpenes compounds present in EO*Bp* act by binding the cell membrane of microorganisms (Milecka et al. 2015). Several studies suggest that Fri has a global impact on the *L. monocytogenes* regulatory network (Dussurget et al. 2005; Olsen et al. 2005), and this protein is also a mediator of beta-lactam tolerance and resistance to antibiotics such as cephalosporins (Krawczyk-Balska et al. 2012).

Iron is also necessary for cellular growth, development and survival, thus the [Fe-S] clusters—*isc*—are cofactors of enzymes involved in several biological processes related to respiration, DNA repair, carbon/nitrogen metabolism and regulation of gene expression (Py and Barras 2010). The *isc* operon encodes IscR, a [2Fe-2S] transcription factor that is involved in [Fe-S] cluster biogenesis, being a regulator responsible for governing various physiological processes during growth and stress responses (Mettert and Kiley 2014). IscR is widely conserved among proteobacteria (Rodionov et al. 2006); however, in Gram-positive bacteria, it is not well characterized. A relevant study performed by Santos et al. (2014) demonstrated that a gene from the unique Gram-positive dissimilatory metal-reducing bacterium *Thermincola potens*, which belongs to the *Firmicutes* phylum, the same as *Listeria* species, encodes a functional IscR homolog that is likely involved in the regulation of iron-sulfur cluster biogenesis.

Catalase (Kat) and superoxide dismutase (Sod) are the two major proteins implicated in protection against superoxides and reactive oxygen species (ROS) (Camejo et al. 2009), as the *sod* gene acts by dismutating the superoxide radical anion $O_2^{\bullet}$- to H_2O_2, which is transformed into H_2O by the *kat* gene (Imlay 2003). Sod proteins can be classified into different types according to their metal cofactors, but only manganese-dependent superoxide dismutase (MnSod) is found in *L. monocytogenes* (Vasconcelos and Deneer 1994). In the present study, the *sod* gene was upregulated in the presence of EO*Bp*, in agreement with others studies related to the oxidative stress response. In addition to providing bacterial resistance against host-generated toxic oxygen species, *sod* gene induction has also been demonstrated during biofilm formation (Trémoulet et al. 2002), which is related to

oxidative stress in several bacteria as a response to changes in environmental conditions (Arce Miranda et al. 2011; Bitoun et al. 2011). As well as EO, ozone also has antimicrobial potential, being widely used in food processing due to its significant disinfection and ability to degrade rapidly. Both catalase and superoxide dismutase were found to protect pathogenic *L. monocytogenes* cells from ozone attack (Fisher et al. 2000).

Listeria species are widespread in the environment and soils, which are rich in complex carbohydrates like starch and its degradation products maltodextrins and maltose, requiring efficient uptake mechanisms for these compounds (Gopal et al. 2010). The maltose repressor protein (MalR) is a member of the LacI/GalR regulatory family, which is responsible for controlling a broad range of bacterial metabolic processes, from selective carbon source utilization to nucleotide synthesis and amino acid catabolism (Nguyen and Saier 1995; Swint-Kruse and Matthews 2009).

In conclusion, the use of natural compounds provides a new way for the scientific community to control the growth of microorganisms in food products. Results obtained in the present study on the antimicrobial effect of EO*Bp* on *Lm*55 isolated from dairy products (cheese), indicate a downregulation of virulence genes and upregulation of stress response genes, which results in destabilization of bacteria. *L. monocytogenes* is considered one of the pathogens with higher mortality rates involved in foodborne outbreaks, thus the possibility of reducing its pathogenicity becomes of great relevance for future research.

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Compliance with ethical standards

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

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