UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIA E TECNOLOGIA DE ALIMENTOS

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Luiza Pieta

GENÔMICA DE *Listeria monocytogenes* E TRANSCRIPTÔMICA DO MICRORGANISMO NA PRESENÇA DE ÓLEO ESSENCIAL EXTRAÍDO DE *Baccharis psiadioides*

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Tese de Doutorado apresentada ao Curso de Pós-Graduação em Ciência e Tecnologia de Alimentos como um dos requisitos para obtenção do grau de Doutora em Ciência e Tecnologia de Alimentos.

Orientador: Prof. Dr. Jeverson Frazzon

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TESE

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Genômica de *Listeria monocytogenes* e transcriptômica do microrganismo na presença de óleo essencial extraído de *Baccharis psiadioides*

Autora: Luiza Pieta

Orientador: Jeverson Frazzon

RESUMO

Listeria monocytogenes é um bastonete Gram-positivo, anaeróbio facultativo, psicrotrófico, patogênico a humanos e transmitido por alimentos. É causador da listeriose, doença severa que acomete grupos de risco específicos, tais como idosos, imunocomprometidos, gestantes, crianças e recém-nascidos. Neste trabalho foi investigada a expressão diferencial de *L. monocytogenes* na presença de óleo essencial extraído de *Baccharis psiadioides,* planta da família *Asteraceae* popularmente chamada de “alecrim-do-campo”, “vassoura” ou “erva formiga”, utilizada pela população como planta medicinal. Além disso, os genomas de dois diferentes sorotipos de *L. monocytogenes*, frequentemente associados a surtos de listeriose, foram sequenciados através de plataforma MiSeq Illumina, sequências estas depositadas no *GenBank*, e comparados com genomas de referência. Anteriormente à execução das análises genômica e transcriptômica, foi determinada a composição do óleo essencial extraído de *B. psiadioides* utilizado nos experimentos, através de cromatografia gasosa com espectrômetro de massa (GC – MS), a qual demonstrou uma maior quantidade de β-pineno na fração composta majoritariamente por monoterpenos, composto este frequentemente encontrado em plantas medicinais aromáticas e apontado como um dos responsáveis pelo potencial antimicrobiano das mesmas. Os demais resultados obtidos no presente trabalho indicam que o óleo essencial testado apresenta potencial ação bacteriostática na concentração estudada, sendo que genes relacionados à virulência do microrganismo foram menos transcritos na sua presença, ao contrário do que foi observado para genes de resposta ao estresse, que apresentaram maiores níveis de transcrição nesta condição. A comparação genômica entre os genomas bacterianos sequenciados neste trabalho e as cepas referência sugere um maior número de proteínas expressas em *L. monocytogenes* do sorotipo 4b relacionadas à defesa e metabolismo do microrganismo, indicando mecanismos que podem estar envolvidos com a capacidade deste sorotipo estar mais envolvido nos casos humanos de listeriose.

Palavras-chave: *Listeria monocytogenes*; *Baccharis psiadioides*; bacteriostasia; virulência; genômica; transcriptômica.

Genomics of Listeria monocytogenes and transcriptomics of the microorganism in the presence of essential oil extracted from Baccharis psiadioides

Author: Luiza Pieta

Advisor: Jeverson Frazzon

ABSTRACT

Listeria monocytogenes is a Gram-positive rod-shaped microorganism, facultative anaerobic, psychrotrophic, pathogenic to humans and transmitted by food. It causes listeriosis, a severe disease that affects specific risk groups such as elderly, immunocompromised, pregnant women, children and newborns. In this study, differential expression of L. monocytogenes in the presence of essential oil extracted from Baccharis psiadioides, a plant from Asteraceae family popularly named as "alecrim-do-campo", "vassoura" or "erva formiga" used by population as a medicinal plant, was investigated. In addition, the genomes of two different L. monocytogenes serotypes, often associated with listeriosis outbreaks, were sequenced through the MiSeq Illumina platform. These sequences were deposited in GenBank and compared with reference genomes. Prior to the execution of genomic and transcriptomic analyzes, composition of the essential oil extracted from B. psiadioides used in the experiments was determined by gas chromatography with mass spectrometer (GC-MS), which demonstrated a higher amount of β-pinene in the fraction composed mainly by monoterpenes. This compound is often found in aromatic medicinal plants and also pointed as one of those responsible for their antimicrobial potential. The other results obtained in the present study indicate that the essential oil tested has a potential bacteriostatic activity at the concentration studied, and genes related to the virulence of the microorganism were less transcribed in its presence, contrary to what was observed for stress response genes, which presented higher transcription levels on that condition. Comparative genomics between the bacterial genomes sequenced in this work and the reference strains suggests a higher number of proteins expressed in L. monocytogenes serotype 4b related to the defense and metabolism of the microorganism, indicating mechanisms that may be involved with the greater ability of this serotype to cause human listeriosis.

Keywords: *Listeria monocytogenes; Baccharis psiadioides; bacteriostasis; virulence; genomics, transcriptomics.*

SUMÁRIO

1. INTRODUÇÃO

O gênero *Listeria* é composto principalmente por 6 espécies (*Listeria monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri* e *L. grayi*), ubíquas e altamente adaptadas ao meio ambiente (HAWKER *et al.,* 2012; WEIS & SEELIGER, 1975), porém 9 novas espécies (e subespécies) foram introduzidas ao gênero, sendo elas *L. rocourtiae*, *L. marthii*, e mais recentemente descobertas *L. weihenstephanensis*, *L. fleischmannii*, *L. fleischmannii* subsp. *coloradonensis*, *L. fleischmannii* subsp. *fleischmannii, L. floridensis*, *L. aquatica*, *L. cornellensis*, *L. grandensis* e *L. riparia* (BERTSCH *et al*. (a), 2013; den BAKKER *et al.,* 2013; den BAKKER *et al.,* 2014; GRAVES *et al.,* 2010; LANG, NEUHAUS & SCHERER, 2013; LECLERCQ *et al.,* 2010).

Dentre as espécies do gênero *Listeria, L. monocytogenes* se destaca em função de sua patogenicidade e complicações relacionadas aos humanos (REBAGLIATI *et al.,* 2009). Este microrganismo dificilmente infecta a população em geral, acometendo principalmente neonatos, idosos, gestantes e demais indivíduos imunocomprometidos, nos quais causa uma doença chamada listeriose, considerada um grande problema de saúde pública (SLUTSKER & SCHUCHAT, 1999). A listeriose é caracterizada muitas vezes por infecções entéricas, com sintomas também bastante similares aos de uma gripe, mas pode progredir para complicações mais severas, tais como meningoencefalite, septicemia e aborto em mulheres grávidas, uma vez que possui a capacidade de atravessar as barreiras hematoencefálica e fetoplacentária (CHOI & HONG, 2003; COSSART & LEBRETON, 2014), possuindo taxas de mortalidade de até 30% para estes grupos de risco bem definidos (DEMETRIOS, BORI & ANTONIOS, 1996).

Listeria também possui a capacidade de infectar animais, sendo que ruminantes e raramente os suínos desenvolvem a listeriose, porém a transmissão do microrganismo aos humanos é considerada rara e não foi evidenciada corretamente ainda (ROBERTS & WIEDMANN, 2003). *Listeria monocytogenes* é

transmitida aos humanos principalmente via consumo de alimentos contaminados, com destaque para os alimentos prontos para o consumo (ALLERBERGER & WAGNER, 2010; TODD & NOTERMANS, 2011). Além de ser um microrganismo ubíquo, este possui a habilidade de sobreviver e se proliferar em temperaturas de refrigeração, sendo um grande problema relacionado à produção de alimentos, que utiliza extensivamente a cadeia do frio no processamento e armazenamento de produtos alimentícios (FARBER & PETERKIN, 1991).

Os óleos essenciais são metabólitos secundários produzidos por diversas plantas e que possuem funções como antibacterianos, antivirais, antimicóticos, antiparasitários, inseticidas, dentre outras relacionadas à área médica, inclusive no tratamento contra o câncer (COWAN, 1999; EDRIS, 2007; REICHLING *et al*., 2009; SULTANBAWA, 2011). O potencial antimicrobiano destes óleos frente a bactérias Gram-positivas e Gram-negativas vem sendo extensivamente estudado, porém os mecanismos envolvidos nesse processo ainda não foram totalmente elucidados (LOPEZ-ROMERO *et al.,* 2015). Contudo, sabe-se que estes compostos atuam diretamente na parede celular dos microrganismos, que acaba sendo rompida juntamente com a membrana citoplasmática, ocasionando assim a lise das células e o extravazamento de seus compostos intracelulares (BURT, 2004). A pesquisa relacionada aos óleos voláteis da família *Asteraceae* de plantas se torna de grande importância, com destaque para a espécie *Baccharis psiadioides* (Less.) Joch. Müller (= *Heterothalamus psiadioides* Less.) (GIULIANO & FREIRE, 2011), também conhecida como “alecrim do campo”, “coralina” ou “erva formiga”, encontrada no sul do Brasil (Rio Grande do Sul e Santa Catarina), Uruguai e Argentina, e que possui, dentre outras funções, importante propriedade anti-inflamatória e antimicrobiana (DEBLE *et al.*, 2005; FABRI *et al.*, 2011). Os terpenos, compostos mais comumente encontrados em óleos essenciais são, em sua maioria, classificados em monoterpenos e sesquiterpenos, pela quantidade de unidades de isopreno presentes (NERIO, OLIVERO-VERBEL & STASHENKO, 2010), tendo já sido comprovado por estudos anteriores a presença destes em óleo essencial de *B. psiadioides* (SUYENAGA *et al.,* 2004).

O sequenciamento de DNA vem sendo amplamente utilizado, e técnicas que envolvem o sequenciamento e o mapeamento de genomas inteiros podem ser utilizadas em diversos casos, como, por exemplo, na comparação entre genomas bacterianos de microrganismos isolados com suas cepas de referência. O entendimento da filogenia de bactérias patogênicas se torna de grande importância no estudo da Microbiologia, facilitando assim a compreensão da evolução destes microrganismos e o surgimento de novos e potenciais agentes patogênicos (NYHOLM *et al*., 2015). Ainda sobre o sequenciamento de genomas inteiros, este consegue também detectar alterações que ocorreram na sequência, tais como variações de nucleotídeo único (SNV) (LUPSKI *et al*., 2010; SOBREIRA *et al.,* 2010), inserções e deleções de nucleotídeos (LEE *et al*., 2010, McKERNAN *et al*., 2009), rearranjos cromossômicos (CAMPBELL *et al*., 2008; CHEN *et al*., 2008), variações no número de cópias (CNV) (CHIANG *et al.,* 2009; PARK *et al*., 2010), dentre outras aplicações importantes no estudo de Genética Microbiana.

A nível transcricional, é possível verificar mudanças na expressão de genes em função de diferentes condições ambientais (LIU *et al*., 2016), o que facilita o entendimento do comportamento de microrganismos frente a situações adversas ao seu desenvolvimento. A técnica de sequenciamento de RNA (do inglês *RNA sequencing – RNA-Seq*) acaba sendo uma ferramenta adequada para verificar estas mudanças, tendo como importante opção para validação dos seus resultados a técnica de PCR quantitativo em tempo real (do inglês *Reverse Transcription quantitative Polymerase Chain Reaction* - RT-qPCR), que vem sendo amplamente utilizada para a identificação de perfis de transcrição de genes principalmente relacionados a mecanismos de resistência e virulência dos microrganismos (ZHANG *et al*., 2014).

2. OBJETIVOS

2.1. Objetivo geral

O objetivo do presente trabalho foi realizar uma análise genômica de duas cepas de *Listeria monocytogenes* concomitantemente com a análise transcriptômica do microrganismo quando cultivado em meio de cultura adicionado de óleo essencial extraído de *Baccharis psiadioides.*

2.2. Objetivos específicos

- Realizar anotação genômica das duas cepas de *L. monocytogenes* sequenciadas, dos sorotipos 1/2a e 4b, isoladas de alimentos;
- Comparar os genomas das cepas de *L. monocytogenes* sequenciadas entre si e com genomas de referência depositados em bancos de dados;
- Traçar um panorama de genes relacionados à virulência em *L. monocytogenes;*
- Estudar os níveis de transcrição das informações contidas nos genes de cepa de *L. monocytogenes* na presença de óleo essencial extraído de *B. psiadioides*, através de análise transcriptômica.

3. REVISÃO BIBLIOGRÁFICA

3.1. *Listeria monocytogenes*

Dentre as espécies do gênero *Listeria, Listeria monocytogenes* se destaca por ser a única espécie patogênica a humanos que pode ser transmitida por alimentos. São bastonetes Gram-positivos, anaeróbios facultativos, psicrotróficos, isto é, capazes de se desenvolver em um intervalo de temperaturas que varia entre -1ºC e 45ºC, podendo se multiplicar em temperaturas de refrigeração amplamente utilizadas em indústrias de alimentos e serviços de alimentação, principalmente para a conservação dos produtos alimentícios (RAMASWAMY *et al.*, 2007). O microrganismo possui ainda flagelos peritríquios que auxiliam na sua motilidade em diversos meios, expressos geralmente em temperaturas que variam entre 20ºC e 25°C, mas não em temperaturas próximas a 37ºC (GRÜNDLING *et al.,* 2004; WAY *et al.*, 2004).

Este microrganismo é causador da listeriose, doença severa que possui alta taxa de mortalidade, considerada inclusive a mais alta taxa dentre os principais patógenos envolvidos em infecções alimentares, podendo chegar até 30% (EFSA, 2012). A taxa de hospitalizações pela doença também apresenta valores altos, que se mantém em torno de 91%, sendo que cerca de 50% dos neonatos acometidos vão a óbito (RAMASWAMY *et al.,* 2007), e devido ao aumento da incidência da doença nas últimas duas décadas, o estudo acerca da *L. monocytogenes* e complicações relacionadas ao microrganismo apresenta cada vez maior relevância (BERTSCH *et al.* (b), 2013). *L. monocytogenes* é considerada uma bactéria ubíqua, podendo estar presente em diferentes ambientes, sobrevivendo muitas vezes a condições adversas ao desenvolvimento bacteriano, tais como baixas temperaturas e baixos valores de pH, altas concentrações de sais e bile, estresses oxidativos e ausência de fontes de carbono (RAZAVILAR & GENIGEORGIS, 1998; WU *et al*., 2015). Além da frequente contaminação de alimentos processados prontos para consumo, carnes e queijos, produtos estes que necessitam de estocagem a baixas

temperaturas, o microrganismo também pode ser encontrado em diversos alimentos crus, com destaque para vegetais, leite e pescado (BÜLA, BILLE & GLAUSER, 1995; MARTÍN *et al*., 2014; RUCKERL *et al*., 2014), sendo que linhagens resistentes a antibióticos, algumas inclusive multirresistentes, já foram isoladas não só de ambientes e amostras clínicas, mas também de alimentos contaminados (KORSAK *et al*., 2012; SHEN *et al*., 2006; SONI *et al*., 2013). Importante ainda ressaltar que cerca de 99%, isto é, quase a totalidade dos casos de listeriose em humanos, ocorrem em função da ingestão de alimentos contaminados com o microrganismo (ALLERBERGER & WAGNER, 2010; SCALLAN *et al*., 2011).

Ao total, a espécie possui 13 diferentes sorotipos descritos, sendo os sorotipos 1/2a, 1/2b e 4b os principais causadores da listeriose em humanos, estando este último relacionado com a maioria dos casos da doença (SWAMINATHAN & GERNER-SMIDT, 2007). Dessa forma, os sorotipos 1/2a, 1/2b e 4b estão envolvidos em mais de 95% dos casos de listeriose em humanos (KATHARIOU, 2002). Já foram descritas 4 linhagens evolutivas para o microrganismo, a I, II, III e IV. As linhagens I e II abrigam os sorotipos mais relacionados com os casos de listeriose em humanos, como os sorotipos 1/2b e 4b, pertencentes a linhagem I, e o sorotipo 1/2a, pertencente a linhagem II.

Este microrganismo possui também a capacidade de formar biofilmes, sendo capaz de colonizar e persistir em plantas processadoras de alimentos, ameaçando assim as indústrias alimentícias e sendo um risco à saúde dos consumidores de seus produtos (CARPENTIER & CERF, 2011; MONTERO *et al.*, 2015). Tais biofilmes proporcionam maior resistência destas bactérias frente a diversos agentes físicos e químicos de controle do crescimento microbiano, tais como desinfetantes, luz ultravioleta e dessecação (MULLAPUDI, SILETZKY & KATHARIOU, 2008), e autores buscam criar uma correlação entre sorotipos de *L. monocytogenes* e sua capacidade de formação de biofilmes, tolerância a estresses e resistência a desinfetantes e antimicrobianos, porém os resultados ainda são inconclusivos (DOIJAD *et al*., 2015; KADAM *et al*., 2013). Além disso, este microrganismo não produz quantidade suficiente de substâncias extrapoliméricas que auxiliam na formação de biofilmes, assim como ocorre em *Pseudomonas* sp. e *Staphylococcus* sp., considerados ótimos formadores de

biofilme (RENIER, HÉBRAUD & DESVAUX, 2011). Assim, fica claro que *L. monocytogenes* possui outros mecanismos que auxiliam na formação destes biofilmes, não evidenciados ainda (DOIJAD *et al*., 2015). Alguns autores sugerem que a presença e quantidade de ácidos graxos nos microrganismos pode influenciar neste processo (CHAO, WOLFAARDT & ARTS, 2010; DAVIES & MARQUES, 2009; DOIJAD *et al*., 2015; PEREZ *et al*., 2012), sendo que estes também já foram estudados quanto à sua relação com a adaptação de *L. monocytogenes* a baixas temperaturas (CHIHIB *et al*., 2003).

3.2. Importantes genes envolvidos na resistência e patogenicidade do microrganismo

Diversas são as etapas envolvidas no processo infeccioso por *L. monocytogenes.* Num primeiro momento é necessária a adesão e internalização do microrganismo em células do hospedeiro, nas quais este vai sobreviver e se multiplicar. Importantes proteínas do grupo das internalinas são as principais responsáveis por este processo, denominadas InlA, InlB, InlC e InlJ, codificadas pelos genes *inlA*, *inlB, inlC* e *inlJ*, envolvidas na patogênese da listeriose (LIU *et al*., 2007; SANT'ANA *et al*., 2012). Estas são consideradas proteínas de superfície que, além de mediar a entrada da bactéria em células epiteliais, também auxiliam na passagem do microrganismo pelas barreiras intestinal e placentária (DISSON *et al*., 2008; NIKITAS *et al*., 2011).

Após a entrada e internalização da bactéria nas células, é necessário que ocorra o rompimento do vacúolo fagocítico que a abriga, de forma que o microrganismo consiga acessar o citosol. Esta etapa é mediada pela proteína Listeriolisina O (LLO), codificada pelo gene *hly*, fundamental para a identificação do microrganismo e fator de virulência que permite a sobrevivência deste nos fagócitos (COSSART *et al*., 1989). Esta toxina secretada por *L. monocytogenes* é considerada uma citolisina que pertence a família das Citolisinas Dependentes de Colesterol (CDC = *Cholesterol Dependent Cytolysins*), que se ligam a membranas ricas em colesterol, sendo produzidas por uma importante gama de bactérias patogênicas Gram-positivas devido ao seu envolvimento também com

a sobrevivência do microrganismo no citoplasma das células infectadas (VÁZQUEZ-BOLAND *et al*., 2005). Vem sendo desenvolvidos diferentes protocolos de purificação desta citolisina, com destaque para a utilização de métodos envolvendo o uso de linhagens recombinantes de *Escherichia coli,* para o seu uso em Biotecnologia, por exemplo, no desenvolvimento de novas vacinas (CHURCHILL, LEE & HALL, 2005; GIAMMARINI *et al*., 2004). Recentemente foi estudado o potencial da LLO em erradicar células causadoras de leucemia, e o estudo demonstrou que esta proteína possui uma importante atividade citotóxica frente a leucócitos humanos, que foi facilmente regulada *in vitro* e pôde ser restringida a áreas que continham células malignas, ressaltando assim a possibilidade de uma futura aplicação clínica desta no tratamento da doença (STACHOWIAK *et al*. 2014). Outras pesquisas também têm sugerido que LLO participa de etapas adicionais durante o ciclo de vida intracelular da bactéria, podendo esta atuar inclusive como uma invasina que estimula a internalização de *L. monocytogenes* e afeta a atividade transcricional das células infectadas (HAMON *et al*., 2007; HAMON *et al*., 2012).

Uma vez que a bactéria se encontra no citosol, para dar continuidade ao processo infeccioso esta deve contaminar as demais células vizinhas. Para que isto ocorra, é necessária a polimerização de uma cauda de actina no microrganismo que vai impulsionar o mesmo, e isto ocorre pela ação de uma proteína de superfície que se concentra em um dos polos da célula, denominada ActA, codificada pelo gene *actA* (KOCKS *et al*., 1992). Uma vez que o microrganismo invade outra célula, este é alojado em um novo vacúolo fagocítico de duas membranas, do qual consegue escapar novamente pela ação da proteína LLO, e o rompimento dos vacúolos fagocíticos por esta proteína ocorre sempre com a ajuda de duas fosfolipases, denominadas PI-PLC e PC-PLC, codificadas pelos genes *plcA* e *plcB*, respectivamente (GEDDE *et al*., 2000; SMITH *et al*., 1995). Já foi demonstrado anteriormente também que a proteína ActA pode estar envolvida no escape do microrganismo em processos de autofagia (YOSHIKAWA *et al*., 2009), e uma nova função foi descrita para esta proteína. ActA, regulada pelo fator de transcrição PrfA, promove a agregação bacteriana via interação direta ActA-ActA, aumentando assim a persistência de *L. monocytogenes* no intestino e sua presença em excreção fecal pelo indivíduo

contaminado, facilitando dessa forma a transmissão do microrganismo (TRAVIER *et al*., 2013). Relacionando ainda o gene *actA* com a virulência de *L. monocytogenes*, estudos anteriores verificaram que variações neste gene sugerem um decréscimo na patogenicidade do microrganismo, principalmente em função da deleção de 35 aminoácidos da proteína ActA que representam duas regiões ricas em prolina (PRR = *Proline Rich Regions*) requeridas para a ligação com a proteína da síndrome Wiskott-Aldrich (WASP) e proteínas Mena. Esses passos são fundamentais para desencadear a mobilidade das células pela cauda de actina no citoplasma da célula hospedeira. Porém, a invasão e a capacidade de multiplicação do microrganismo não parecem ser afetadas por esta deleção (CONTER *et al*., 2010).

Importantes fatores de transcrição estão envolvidos na regulação de genes relacionados à virulência e resistência a estresses pelo microrganismo. PrfA e Fur são ambos reguladores envolvidos na virulência de *L. monocytogenes*, sendo este último considerado uma proteína de regulação da absorção de ferro ou zinco envolvida no metabolismo microbiano, responsável pela repressão do gene *fri*, que codifica uma proteína semelhante à ferritina ligada ao ferro. A ferritina é considerada a mais importante proteína de reserva de ferro encontrada em células, uma vez que a síntese de compostos férricos e a reserva deste metal são necessárias para o metabolismo de diversas bactérias (FIORINI *et al*., 2008). Outros fatores de transcrição também estão envolvidos no metabolismo do ferro, tais como IscR, codificado pelo primeiro gene do operon *iscRSUA*, considerado o principal regulador da homeostase de Fe-S em microrganismos tais como *Escherichia coli* (FLEISCHHACKER *et al*., 2012). Em relação ao metabolismo de carboidratos, destacam-se os reguladores pertencentes a família Lac-I de proteínas, como MalR, considerado um centro de repressão de maltose, o qual é capaz de reprimir a expressão do regulon maltose (*mal)* na ausência deste carboidrato em microrganismos tais como *Streptococcus pneumomiae* (AFZAL *et al*., 2015).

O fator de transcrição PrfA, membro da família Crp/Fnr de reguladores, expressa fatores de virulência necessários para a infecção de humanos por *L. monocytogenes* (COSSART & LEBRETON, 2014). É um ativador transcricional que regula a transcrição de 9 genes envolvidos na patogenicidade de *L.*

monocytogenes, tais como *actA, inlA, inlB, hly, plcA, plcB* e *mpl,* transcritos durante a fase exponencial de crescimento do microrganismo até o início de sua fase estacionária, em temperaturas acima de 30ºC, ocorrendo o ápice de sua transcrição em temperaturas próximas ou iguais a 37ºC (de LAS HERAS *et al.*, 2011; JOHANSSON *et al*., 2002; LEIMEISTER-WÄCHTER, DOMANN & CHAKRABORTY, 1992; SCORTTI *et al*., 2007), tendo já sido demonstrado que cepas de *L. monocytogenes* sem o gene *prfA* são avirulentas (CHAKRABORTY *et al*., 1992; XAYARATH & FREITAG, 2012).

A ativação de PrfA, que ocorre somente no citosol da célula hospedeira, está envolvida na passagem do microrganismo de um estado saprofítico para a sua forma de patógeno intracelular, uma vez que este é ativado após a entrada de *L. monocytogenes* nas células do hospedeiro (XAYARATH & FREITAG, 2012). Porém, se existe algum sinal que estimula a sua ativação, este ainda não foi totalmente elucidado. Um estudo recente sugere o envolvimento de um pequeno peptídeo, um ferormônio denominado pPplA, que após ser secretado auxilia no escape do microrganismo das células do hospedeiro, facilitando assim a ativação de PrfA (XAYARATH, ALONZO & FREITAG, 2015). Ao mesmo tempo, outro trabalho indica que a glutationa (antioxidante), tanto bacteriana quanto derivada do hospedeiro, é requerida para a ativação do fator PrfA, tendo sido identificada neste estudo uma cepa mutante em relação a produção da enzima glutationa-sintase que apresentou redução na transcrição de genes de virulência (RENIERE *et al*., 2015).

Outras pesquisas avaliaram a relação entre o fator PrfA e demais proteínas que podem influenciar no processo infeccioso causado por *L. monocytogenes*. PrsA2 faz parte de uma família de lipoproteínas associadas à membrana do microrganismo que contribuem para a estabilidade das proteínas secretadas, durante a passagem destas pela membrana bacteriana (FORSTER & MARQUIS, 2012). Além disso, elas contribuem para a integridade da parede celular de *L. monocytogenes*, para a sua motilidade nos meios diversos e para a resistência ao estresse osmótico. Principalmente se destaca o seu requerimento e influência na virulência e viabilidade de *L. monocytogenes* dentro da célula hospedeira, uma vez que *L. monocytogenes prsA2* foi primeiramente identificado via análise

transcriptômica como um gene cuja transcrição aumentou em função da ativação do fator PrfA (CAHOON & FREITAG, 2014).

Já o fator de transcrição SigmaB regula os genes envolvidos na resposta do microrganismo a condições de estresse, sendo de extrema importância tanto para a sobrevivência da bactéria durante o processamento de alimentos quanto para o desenvolvimento da infecção no hospedeiro. Auxilia na sobrevivência de *L. monocytogenes* em baixos valores de pH do trato gastrointestinal, condições de alta pressão osmótica e estresses energéticos, possuindo também envolvimento na sobrevivência do microrganismo durante a fase estacionária (LEE *et al*., 2013; O'BYRNE & KARATZAS, 2008). Foi anteriormente demonstrado que cepas de *L. monocytogenes* mutantes Δ*sigB* (deleção do gene *sigB*) apresentaram menor resistência a condições ácidas, ambientes salinos, variações de temperatura, antimicrobianos e também estresses causados pela falta de carbono (BECKER *et al*., 1998; SHIN, BRODY & PRICE, 2010; SHIN *et al*., 2010, WIEDMANN *et al*., 1998). Existem interações entre reguladores que auxiliam na transcrição de genes pelo microrganismo, quando este é submetido a diferentes condições ambientais. Um exemplo disso seria o envolvimento do fator de transcrição SigmaB na regulação da transcrição do fator PrfA e de outros diversos genes de virulência de *L. monocytogenes* (LOBEL *et al*., 2012; OLLINGER *et al*., 2009). Além disso, SigmaB e PrfA co-regulam a transcrição de ao menos 3 genes, incluindo os que codificam as internalinas InlA e InlB, denominados *inlA* e *inlB, e bsh,* que codifica uma hidrolase de sais biliares, a qual contribui para a defesa do microrganismo frente a estes sais (GUARIGLIA-OROPEZA *et al*., 2014).

Exemplos de proteínas dependentes de SigmaB incluem GadB, que controla a expressão de glutamato descarboxilase, promovendo resistência do microrganismo ao estresse ácido, e OpuCA, importante para a resistência causada pelo estresse osmótico (FRASER *et al*., 2003; HAIN *et al*., 2008; KAZMIERCZAK *et al*., 2003). O fator de transcrição SigmaB é amplamente estudado em bactérias Gram-positivas, e as estruturas de operons do gene *sigB* são idênticas em *L. monocytogenes* e *Bacillus subtilis*, apesar das vias de transdução de sinal serem diferentes nos dois microrganismos (KAZMIERCZAK, WIEDMANN & BOOR, 2005; WIEDMANN *et al*., 1998). Em *L. monocytogenes*, a

ativação de SigmaB por estressses energéticos e ambientais ocorre através de uma via única, que inclui RsbT, RsbU, RsbV e RsbW. Já foi demonstrado que estresses ácidos, por antimicrobianos, pelo frio, pelo calor, pela presença de etanol, estresses osmóticos e também por falta de nutrientes requerem RsbU para ativar SigmaB (CHATURONGAKUL & BOOR, 2004; SHIN, BRODY & PRICE, 2010). Como resposta a estresses oxidativos, a proteína superóxido dismutase, codificada pelo gene *sod*, apresenta importante papel ao eliminar espécies reativas de oxigênio (EROs) que são naturalmente produzidas durante o metabolismo aeróbio (ERDAL *et al*., 2014).

Em função da transferência de material genético que pode ocorrer entre as bactérias, seja por processos de conjugação ou transformação, *L. monocytogenes* possui semelhanças com outros gêneros de bactérias Gram-positivas, tais como *Bacillus* sp. e *Staphylococcus* sp.. Em *B. subtilis*, o sistema de dois componentes DegS-DegU auxilia a transição do microrganismo de sua fase exponencial para a fase estacionária de crescimento, além de estar envolvido na regulação da competência das células, formação de DNA recombinante, excreção de enzimas degradativas e motilidade do patógeno (HAMOEN *et al*., 2000; OGURA & TANAKA, 2002; TOKUNAGA *et al*., 1994). Em um estudo realizado por Knudsen, Olsen e Dons (2004) foi caracterizado um regulador DegU em *L. monocytogenes*, codificado pelo gene *degU*, com 63% de identidade de aminoácidos quando comparado ao regulador DegU de *B. subtilis*. Além disso, foi demonstrado que a deleção desse gene em cepas de *L. monocytogenes* afetou a transcrição do gene *flaA*, codificador da flagelina e responsável pela produção de flagelo no microrganismo (DONS *et al*., 2004), diminuindo também a virulência deste quando comparado a uma cepa selvagem. Foi sugerido inclusive pelos autores que este regulador ativa a transcrição do gene *flaA* em temperaturas em torno de 25°C, temperatura na qual a transcrição deste gene é acentuada, uma vez que sua detecção não ocorre a 37°C (DONS, OLSEN & RASMUSSEN, 1994).

O sistema *agr* de *S. aureus* e outros estafilococos, que é amplamente conservado em bactérias Gram-positivas com grande quantidade de pares de base Guanina-Citosina, como no caso da *L. monocytogenes* (LYON & NOVICK, 2004), está envolvido no mecanismo de formação de biofilme pelo

microrganismo. Em *L. monocytogenes* os genes *agrB*, *agrD*, *agrC* e *agrA* estão organizados na forma de um *operon*, e este sistema, assim como em *S. aureus*, regula a adesão do microrganismo a superfícies e auxilia na posterior formação de biofilme (RIEDEL *et al*., 2009; RIEU *et al*., 2007). Também o sistema *agr* está envolvido no processo infeccioso causado pelo microrganismo em hospedeiros mamíferos (AUTRET *et al*., 2003; RIEDEL *et al*., 2009). Dentre os diversos reguladores de transcrição presentes no genoma de *L. monocytogenes*, AgrA é o componente responsável pelo sistema AgrC/AgrA (VIVANT *et al*., 2014), e já foi demonstrado que a deleção do gene que codifica este regulador diminuiu a habilidade de *L. monocytogenes* EGD-e aderir-se a superfícies abióticas (RIEU *et al*., 2007).

3.3. Oléo essencial de *Baccharis psiadioides*

Os óleos essenciais são extratos complexos altamente voláteis produzidos por plantas aromáticas na forma de metabólitos secundários, que possuem forte odor, são insolúveis em água, obtidos por hidro ou vapor-destilação (BAKKALI *et al.*, 2008; NASIR, TAFESS & ABATE, 2015) e de ampla utilização nas indústrias de perfumaria, cosméticos, farmacêutica, de alimentos, entre outras (DAHANUKAR, KULKARNI & REGE, 2000). Estes são geralmente compostos por terpenos e/ou fenilpropenos (BURT & REINDERS, 2003; KALEMBA & KUNICKA, 2003), e dentre o grupo dos terpenos destacam-se os monoterpenos e os sesquiterpenos, substâncias naturais cuja origem biossintética deriva de unidades de isopreno (HYLDGAARD, MYGIND & MEYER, 2012; ROBBERS *et al*., 1997). Monoterpenos apresentam 10 carbonos em sua estrutura química, enquanto que os sesquiterpenos apresentam peso molecular mais elevado, sendo constituídos por 15 carbonos. Os terpenos são formados a partir de um precursor de 5 carbonos denominado isopentenil-difosfato (IPP) e seu isômero, dimetilalil-difosfato (DMAPP), tendo o envolvimento neste processo das enzimas terpeno sintases (TPS) (THOLL, 2006; YAHYAA *et al*., 2015).

A eficaz ação antimicrobiana e antibiofilme destes compostos frente a importantes patógenos alimentares, tais como *S. aureus*, *E. coli* e *L.*

monocytogenes, tem sido demonstrada. São componentes de diversos óleos essenciais extraídos de plantas, tais como carveol (hortelã), carvona (alcarávia, hortelã), citronelol (citronela, gerânio, rosas), citronelal (capim-limão, citronela, eucalipto citriodora), eugenol (cravo, louro, noz-moscada), timol (alecrim-pimenta, tomilho, orégano), carvacrol (tomilho, orégano), *trans*-cinamaldeído (canela), limoneno (frutas cítricas), entre outros (LOPEZ-ROMERO *et al*., 2015; UPADHYAY *et al*., 2013). Devido a tais propriedades antimicrobianas já relatadas previamente, se busca descobrir novas moléculas nas classes químicas de antifúngicos e medicamentos antibacterianos que possam atacar linhagens altamente resistentes a demais drogas que vem sendo utilizadas no controle de infecções (ALI *et al*., 2001; ANGIONI *et al.*, 2006).

A família *Asteraceae*, considerada uma das maiores famílias de plantas floríferas no mundo, possui um número estimado de 25.000 espécies (FUNK *et al.*, 2009), sendo que algumas estão envolvidas no desenvolvimento de novos fármacos, inseticidas, além de propriedades citotóxicas e alelopáticas (influência positiva ou negativa no crescimento de sistemas biológicos) já descritas anteriormente (SCHMIDT-SILVA *et al*., 2011). Esta família compreende o gênero *Baccharis,* com destaque para a espécie *B. psiadioides* Less., que se desenvolve no sul do Brasil, Uruguai e Argentina (DEBLE *et al*., 2005), tendo como nomes populares “alecrim-do-campo”, “vassoura” ou “erva formiga”, sendo já utilizada pela população como planta medicinal (SUYENAGA *et al*., 2004).

Em relação à constituição do óleo essencial de *B. psiadioides*, este é composto tanto por monoterpenos como por sesquiterpenos (SUYENAGA *et al*., 2004), com destaque para o β-pineno, considerado um monoterpeno álcool cíclico e composto majoritário não só deste, mas também do óleo essencial extraído de diversas outras espécies de plantas medicinais aromáticas (DUSCHATZKY *et al*., 2007; MOREIRA *et al*., 2013; SCHMIDT-SILVA, 2012). Recentemente, estudos comprovaram também a eficácia das atividades antimicrobiana e antibiofilme de óleo essencial extraído de *B. psiadioides* frente a microrganismos patogênicos, tais como *Enterococcus faecalis* e *L. monocytogenes* (ELLWANGER, 2013; NEGREIROS, 2016).

3.4. Ciências ômicas

Quando se fala em ciências ômicas, subentende-se que o objetivo das técnicas relacionadas é a análise global dos sistemas biológicos, integrando conhecimentos de diferentes áreas como bioquímica, genética, fisiologia, e inclusive computação, em função da utilização de ferramentas de bioinformática para análise dos dados gerados. Após o sequenciamento do genoma humano, diz-se que iniciou a "era pós-genômica", com o aperfeiçoamento e desenvolvimento de novas tecnologias, tais como a transcriptômica, a proteômica e a metabolômica, objetivando isolar e caracterizar o RNA, as proteínas e os metabólitos relacionados (ESPINDOLA *et al*., 2010). Ao invés de focar no estudo de somente uma única ciência ômica, a integração entre as diferentes ciências permite descrever a complexidade e a funcionalidade de sistemas biológicos, tais como interações entre patógenos e seus hospedeiros (MARCELINO *et al*., 2012; VAN ASSCHE *et al.,* 2015), e a descoberta de novos alvos para a prevenção e controle de doenças infecciosas (de LA FUENTE & MERINO, 2013).

A análise genômica, através da utilização de técnicas para sequenciamento de DNA, por exemplo, vem sendo amplamente realizada na área de Microbiologia. Hoje em dia são encontradas diversas sequências genômicas de microrganismos depositadas no *GenBank* (http://www.ncbi.nlm.nih.gov/genbank/), facilitando assim as pesquisas nas áreas de Genética e Biologia Molecular relacionadas a microrganismos. Duas importantes plataformas de *Next-Generation Sequencing* (NGS) vêm sendo mais utilizadas, sendo elas o sistema Illumina MiSeq e a tecnologia Ion Torrent Personal Gene Machine (PGM) Life Technologies, e tem sido demonstrado menores falhas em sequenciamentos utilizando-se o sistema MiSeq em comparação a tecnologia PGM (STROM *et al.,* 2015). Análises genômicas comparativas entre diferentes cepas são frequentemente realizadas para identificar diferenças que podem, muitas vezes, explicar a habilidade de microrganismos causarem infecções e os mecanismos envolvidos nestes processos de virulência (LIU *et al.,* 2015). Além disso, a verificação de mutações se torna fundamental para o entendimento do comportamento diferente dos microrganismos. Um exemplo disso é o trabalho realizado por Bécavin e

colaboradores (2014), que demonstrou diferenças genômicas entre importantes cepas referência de *L. monocytogenes* utilizadas em pesquisas, ressaltando uma mutação no fator de transcrição PrfA de *L. monocytogenes* EGD. Esta mutação induz uma superexpressão de genes de virulência, indicando uma maior capacidade de invasão do microrganismo em culturas de células e diferenças em relação à sua virulência quando aplicado em modelos animais.

Outras ciências ômicas aplicadas pós-genômica, como a proteômica e a transcriptômica, têm auxiliado na compreensão de interações existentes entre patógenos. Além disso, a investigação de sistemas biológicos através da integração entre as análises metabolômica, transcriptômica e proteômica pode resultar na descoberta de novas rotas metabólicas que acabam sendo afetadas num processo infeccioso, por exemplo (VILLAR *et al*., 2015). Para entender a relação entre organismos diferentes colonizando um mesmo ambiente, se torna de extrema importância o monitoramento dos perfis de transcrição de ambos, de forma simultânea. Nesse caso pode-se fazer uso de uma abordagem transcriptômica mista (do inglês *mixed transcriptomics*), capaz de revelar a transcrição gênica em determinados momentos durante a interação entre um patógeno/parasita e seu hospedeiro (de BEKKER *et al*., 2015). Assim, a análise transcriptômica se torna de grande valia, uma vez que consegue demonstrar mudanças específicas em padrões de transcrição gênica em organismos submetidos a variadas condições, incluindo as utilizadas para o seu crescimento (KOURIST *et al*., 2015).

4. ARTIGOS CIENTÍFICOS

4.1. Artigo Científico 1

Artigo científico publicado no periódico *"Genome Announcements"* em dezembro de 2015.

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Complete Genome Sequences of Two *Listeria monocytogenes* Serovars, 1/2a and 4b, Isolated from Dairy Products in Brazil

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Listeria monocytogenes **is the foodborne pathogen responsible for a bacterial infection called listeriosis. Here, we present the whole-genome sequences of two *L. monocytogenes* serovars, 1/2a and 4b, which are considered the most prevalent in food processing plants and listeriosis outbreaks, respectively.**

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Complete Genome Sequence of two *Listeria monocytogenes* Serovars 1/2a and 4b, Isolated from Dairy Products in Brazil

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Running Title: Genome Sequence of *Listeria monocytogenes*

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Abstract

Listeria monocytogenes is the foodborne pathogen responsible for a bacterial infection called Listeriosis. Here, we present the whole genome sequence of two *L. monocytogenes* serovars 1/2a and 4b, which are considered the most prevalent in food processing plants and related to listeriosis outbreaks, respectively

Keywords: *Listeria monocytogenes*, serovars 1/2a and 4b, food contamination, genomics.

Genome Announcement

Listeria monocytogenes is a psychrotrophic microorganism, widely distributed in the environment. Thirteen different serovars were already described for this bacterium, but three serovars denominated 1/2a, 1/2b and 4b are responsible for most human cases of listeriosis (1). Four evolutive lineages (I, II, III and IV) have been described for the microorganism, and serovar 1/2a belongs to lineage II, most involved with food contamination (2), being frequently isolated from food sources or food processing plants (3). Moreover, the serotype 4b is more related to outbreaks of the disease (37 to 64% of cases), and in addition to the frequent contamination of processed "ready-to-eat foods", meats and cheeses, which require storage at low temperatures, the microorganism can also be found in many raw foods, especially vegetables, milk and fish (4, 5)

In this study, we report the complete genome sequence of two *L. monocytogenes* strains serovars 1/2a and 4b isolated from dairy products in Brazil, which had some genes related to biofilm formation, stress-response and virulence tested by RT-qPCR when growing at different temperatures (6).

Library of *L. monocytogenes* genomic DNA was prepared using the Nextera DNA Library Preparation Kit 24 samples (Illumina, San Diego – California, USA; Cat.#FC-121-1030), and the paired-end sequencing was performed on the Illumina MiSeq Platform

(Illumina, San Diego – California, USA) using the MiSeq Reagent kit v3 150 cycles (Illumina, San Diego – California, USA; Cat.#MS-102-3001). The reads were subjected to *de novo* assembly using Andrew and Aaron's Awesome Assembly pipeline (A5) and ORFs were predicted using rapid prokaryotic genome annotation (PROKKA). After assembly, a total of 49 contigs and 14 scaffolds were generated to *L. monocytogenes* serovar 1/2a, while a total of 78 contigs and 28 scaffolds were generated to the serovar 4b.

Sequence assembly yielded a 2.990.228 bp to *L. monocytogenes* serovar 1/2a complete genome with G+C content of 37.8%, and the longest scaffolds size was of 1.477.456 bp, with N50 of 509.790 bp and raw coverage of 236x. At the same time, to *L. monocytogenes* serovar 4b, sequence assembly yielded a 3.001.292 bp complete genome with G+C content of 37.8%, and the longest scaffolds size was of 481.612 bp, with N50 of 308.327 bp and raw coverage of 430x.

Nucleotide sequence accession numbers. These Whole Genome Shotgun projects have been deposited in GenBank under the accession no. LKHO00000000 and LKCY00000000 to serovars 1/2a and 4b, respectively. The versions described in this paper are the first versions, LKHO00000000 (serovar 1/2a) and LKCY00000000 (serovar 4b).

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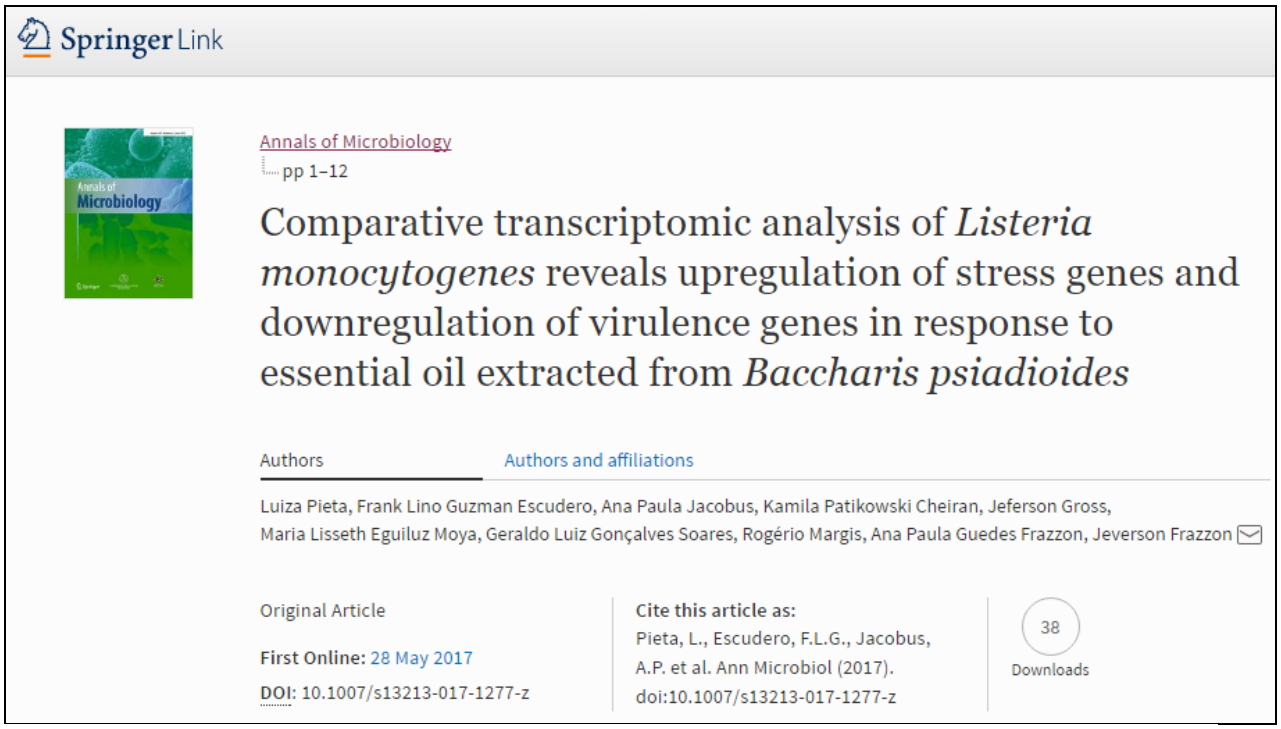
4.2 Artigo Científico 2

Artigo científico publicado no periódico *"Annals of Microbiology"* em maio de 2017.



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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 had 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 the virulence and 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 to control microorganisms proliferating in foods.

Keywords: *Bacteriostasis, essential oil, Listeria monocytogenes, virulence, RNA sequencing, RT-qPCR*

1. 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 responses has already been demonstrated at 7 ºC compared to 37 ºC (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 USA (Cartwright et al. 2013).

Essential oils (EO) are secondary metabolites produced by several plants and may 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* plants 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: (i) monoterpenes with a significant percentage composed of β-pinene; and (ii) 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). Comparable 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 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 was shown through transcriptional and phenotypic assay in enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) treated with eugenol and eugenol-rich oil (Kim et al. 2016).

The use of natural compounds with antimicrobial potential has been an alternative against 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).

2. Materials and Methods

2.1. 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) from 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).

2.2. Characterization of the Essential Oil of *Baccharis psiadioides*

The EO*Bp* was obtained from the Laboratory of Chemical Ecology and Chemotaxonomy (Department of Botany, 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, USA), using pentane and diethyl ether to obtain the fraction 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.

2.3. 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 of 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 of 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, USA), 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, USA), 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) were added to 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 the sequences of reads with a quality below 30 as well as the sequences of the Illumina adapters. Then, the cleaned reads were 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 the *Subread* software (Liao et al. 2013), for sequence alignment files generated by *TopHat2*. To perform the

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, USA) and the *KEGG Orthology* (KO) codes obtained were viewed on the *iPATH2* web server (Yamada et al., 2011).

2.4. 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. The design of the primers was performed using the *GenScript* tool (https://www.genscript.com/tools/real-time-pcr-tagman-primer-design-tool) and 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 Supplementary Table 1 for functions of the coded proteins).

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

Gene	Nucleotide sequence	Amplicon size (bp)	Annealing temperature (ºC)
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

Supplementary Table 1 Functions of the proteins encoded by genes analyzed in the present study

Gene	Protein function
actA	Responsible for the actin tail formation, necessary for bacteria intracellular mobility and cell-to-cell spread
agrA	Influences the microorganism adhesion to surfaces and subsequent biofilm formation
crp	Transcriptional regulator
degU	Involved in the flagella synthesis, motility, virulence and biofilm formation
fri	Binding of ferritin - antioxidant protein
fur	Iron uptake regulatory protein
hly	Essential protein for *L. monocytogenes* virulence, important in the process of host cell invasion
iscR	Iron-Sulfur centers regulator
malR	Maltose repressor center
prfA	Factor that regulates the transcription of genes involved in *L. monocytogenes* virulence
sigB	Factor that regulates the transcription of genes involved in *L. monocytogenes* response to environmental stress
sod	Manganese superoxide dismutase - oxidative stress response gene

For RT-qPCR experiments a solution containing 0.01 - 0.1 µM of each primer; 25 µM dNTPs (Promega, Madison, WI, USA); 1X reaction buffer; 3 mM $MgCl_2$; 1X SYBR Green (Bio-Rad, Hercules, CA, USA); 0.25 U of 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*^{TM} Real Time – PCR System (Thermo Fisher Scientific) and 96-wells polystyrene microplates (Axygen Scientific, Union City, CA, USA) 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* (Supplementary Table 2) 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, USA). 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.

Supplementary Table 2 Sequences of primers tested for RT-qPCR data normalization with its respective sizes of amplification fragments

Gene	Nucleotide sequence	Amplicon size (bp)
gap	5' CGCTTACACTGGTGACCAAA 3'	119
	5' CGATAGCTTTAGCAGCACCA 3'	
rpoB	5' CGTCGTCTTCGTTCTGTTGG 3'	82
	5' GTTCACGAACCACACGTTCC 3'	
16SrRNA	5' GATGCATAGCCGACCTGAGA 3'	116
	5' TGCTCCGTCAGACTTTCGTC 3'	

3. Results and Discussion

3.1. Determination of EOBp Composition by GC-MS

Total EO*Bp* was used to perform our analysis and obtained EO*Bp* fractions were divided into two groups: one fraction predominantly composed of monoterpenes and the other predominantly composed of sesquiterpenes. Results of GC-MS indicated the presence of a complex mixture of terpenes in the two fractions. The fraction composed predominantly of monoterpenes revealed the presence of 20 compounds (Table 2), the 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.

Table 2 Chemical composition of *Baccharis psiadioides* essential oil fraction composed predominantly by monoterpenes

Components	**IK *cal***	**IK *tab***	**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***

IK *cal*, calculated Kováts retention index; IK *tab*, tabulated Kováts retention index.

The relative percentage of each component was obtained directly from the peak areas of the chromatogram, considering 100% the sum of all evaluated peaks.

Table 3 Chemical composition of *Baccharis psiadioides* essential oil fraction composed predominantly by sesquiterpenes

Components	IK *cal*	IK *tab*	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***

IK *cal*, calculated Kováts retention index; IK *tab*, tabulated Kováts retention index.

The relative percentage of each component was obtained directly from the peak areas of the chromatogram, considering 100% the sum of all evaluated peaks.

3.2. Transcriptomic Analysis

In total, 333 genes presented a $\log_2$foldchange > -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 (Supplementary Table 3).

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).

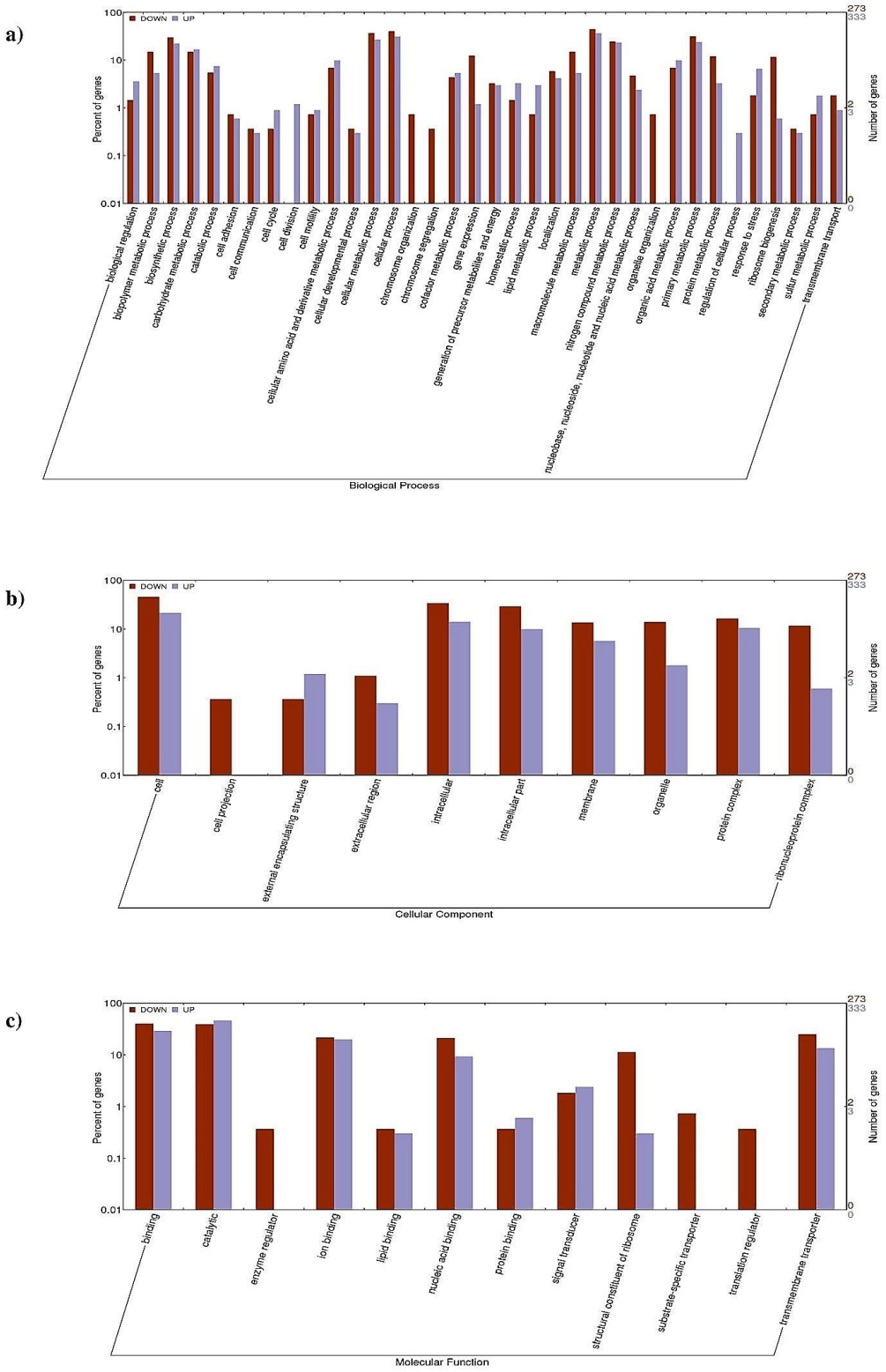


Fig. 1 Transcriptomic analysis results. Differential expression of genes related to functional categories **a)** Biological Process; **b)** Cellular Component; and **c)** Molecular Function of *Listeria monocytogenes* 55 grown in the presence of *Baccharis psiadioides* essential oil. Graphical representation obtained with *WEGO* program.

Table 4 Number of down and upregulated genes related to processes belonging to the functional categories studied (Biological Process; Cellular Component; Molecular Function) in *Listeria monocytogenes* 55 grown in the presence of *Baccharis psiadioides* essential oil

	DOWN	**UP**
BIOLOGICAL PROCESS		
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

In 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, it indicates 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 five 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).

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 ribosome, translation regulator and transmembrane transporter, 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.

3.3. 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 (Supplementary Table 4), 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 (Supplementary Fig. 1 and Supplementary Table 5). 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 of our experiments (Supplementary Table 6).

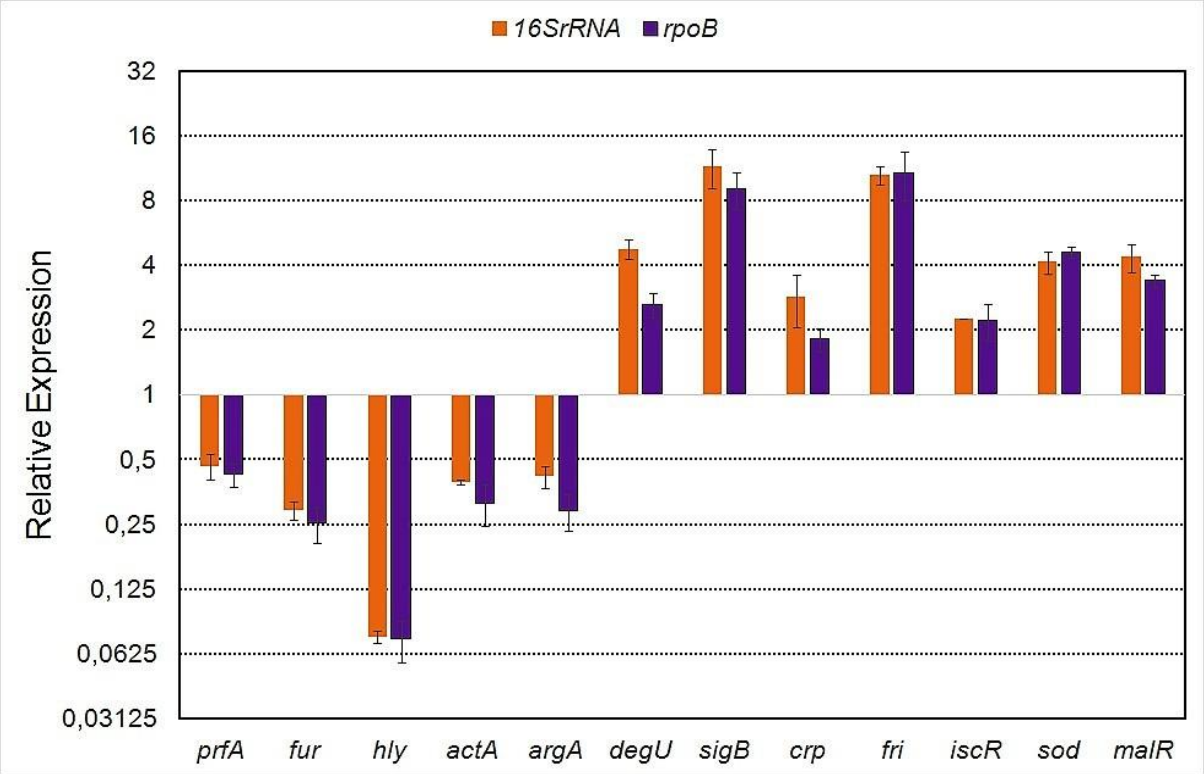
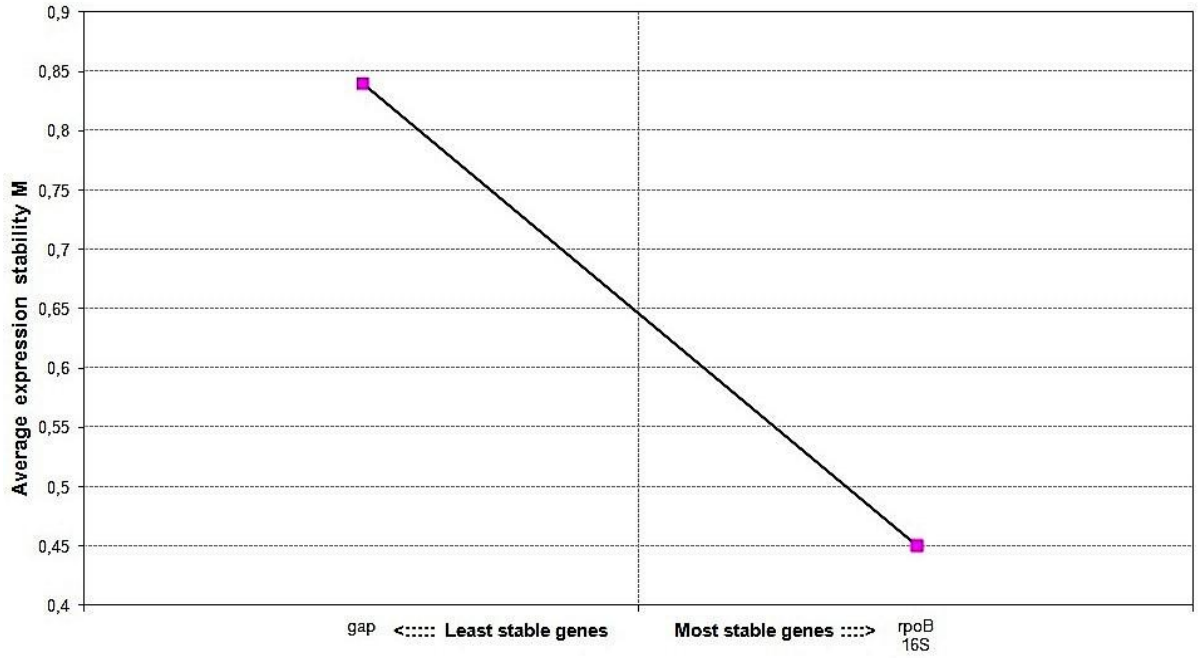


Fig. 2 Transcriptional analysis results. Relative expression of *actA, agrA, crp, degU, fri, fur, hly, iscR, malR, prfA, sigB* and *sod*, normalized with *rpoB* and *16SrRNA*, for *Listeria monocytogenes* 55 grown in the presence of *Baccharis psiadioides* essential oil, and respective bars indicating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



Supplementary Fig. 1 Housekeeping genes expression stability tested with *geNorm v. 3.5* software. Average expression stability (M) for *gap*, *rpoB* and *16SrRNA*, tested as candidates for RT-qPCR data normalization, calculated by *geNorm.*

Supplementary Table 4 Work solution primer concentration, efficiency (E) and slope value for each RT-qPCR reaction

Primer	[Primer] (µM)	E (%)	Slope
actA	10	98.75	-3.35
agrA	10	101.01	-3.30
Crp	10	108.20	-3.14
degU	10	103.01	-3.25
Fri	1	105.03	-3.21
Fur	10	97.84	-3.38
Gap	10	100.68	-3.31
Hly	10	100.04	-3.32
iscR	5	92.38	-3.52
malR	10	106.69	-3.17
prfA	10	107.11	-3.16
rpoB	10	102.64	-3.26
sigB	10	101.10	-3.30
Sod	5	107.24	-3.16
16SrRNA	10	93.38	-3.49

Supplementary Table 5 Stability values of housekeeping genes *gap*, *rpoB* and *16SrRNA*, tested as candidates for RT-qPCR data normalization, calculated by *NormFinder* algorithm

Gene	Stability value
gap	0.714
rpoB	0.113
16SrRNA	0.263

Supplementary Table 6 Results of relative and differential expression ($E^{-\Delta\Delta Ct}$ and $\log_2$foldchange, respectively) of *Listeria monocytogenes* 55 grown in the presence of *Baccharis psiadiodes* essential oil. Relative gene expression and standard deviation (SD) values are demonstrated for both *rpoB* and *16SrRNA*, used in the normalization of RT-qPCR data.

Gene	$E^{-\Delta\Delta Ct}_{rpoB}$ ± SD	$E^{-\Delta\Delta Ct}_{16SrRNA}$ ± SD	Row name	$\log_2$foldchange
actA	0.314 ± 0.068	0.394 ± 0.010	**Lis_764**	1.312774275
agrA	0.289 ± 0.055	0.416 ± 0.051	**Lis_610**	1.008035617
crp	1.804 ± 0.220	2.838 ± 0.777	**Lis_1807**	-0.538133889
degU	2.607 ± 0.335	4.758 ± 0.474	**Lis_256**	-1.428870192
fri	10.753 ± 2.749	10.454 ± 0.976	**Lis_1515**	-1.606631991
fur	0.252 ± 0.047	0.290 ± 0.027	**Lis_2515**	0.135372973
hly	0.073 ± 0.016	0.075 ± 0.005	**Lis_762**	1.939291415
iscR	2.210 ± 0.422	2.251 ± 0.007	**Lis_2072**	-0.145386033
malR	3.391 ± 0.223	4.355 ± 0.641	**Lis_2285**	-0.019951
prfA	0.426 ± 0.052	0.464 ± 0.062	**Lis_760**	0.296081716
sigB	9.054 ± 1.787	11.467 ± 2.383	**Lis_1465**	-0.959589558
sod	4.587 ± 0.277	4.158 ± 0.486	**Lis_1995**	-0.265592761

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 plants-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 EO.

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*, responsible for the polymerization of actin tails, which propels the microorganism to neighboring cells, and the *hly* gene, that codifies listeriolysin O (LLO), 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 with the reduced transcription of *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 extensively studied 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 wasattenuated, 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 the 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 factors family have several related functions in microorganisms, such as regulation of virulence, metabolic pathways and stress response. 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 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 an 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 microorganisms cell membrane (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 *Bp*EO, in agreement with other 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 one of the LacI/GalR regulatory family members, which are 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 for the scientific community provides a new way to control the growth of microorganisms in food products. Results obtained in the present study for 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, the possibility of reducing its pathogenicity becomes of great relevance for future researcher.

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

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4.3 Artigo Científico 3

Artigo científico que será submetido ao periódico *“Journal of Biotechnology”*.

Comparative Genomics suggests differences between food-isolated *Listeria monocytogenes* serotypes 1/2a and 4b that influence host infection

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Abstract

Among the four lineages described for Listeria monocytogenes (I, II, III and IV), lineages I and II harbor the serotypes most related to listeriosis in humans, such as serotypes 1/2b and 4b, associated with the majority of listeriosis outbreaks, and serotype 1/2a, frequently involved with food contamination. Based on this information, the present study aimed to analyze genomic differences between two L. monocytogenes strains isolated from cheeses in southern Brazil, serotypes 1/2a and 4b, with known reference strains L. monocytogenes EGD-e and L. monocytogenes Scott A, choosen previously by a phylogenetic analysis. Illumina Miseq platform was used to perform the genomes sequencing, and a cluster analysis of orthologs provided investigation of similarities and differences between the two serotypes studied regarding proteins expression. In comparison with serotype 1/2a, 4b strain expressed a major number of proteins related to defense and metabolism, indicating mechanisms that can be involved with the greater ability of this serovar to cause infections in humans.

Keywords: *Listeriosis; Next Generation Sequencing; Orthologs; Defense mechanisms; Metabolism*

1. Introduction

Listeria monocytogenes is a psychrotrophic foodborne pathogen, capable of growing during the storage and processing of refrigerated foods, causative of a disease called listeriosis, which presents a high mortality rate through specific risk-groups **(Ramaswamy et al. 2007; Schlech 2000).** In addition, the microorganism is considered an ubiquitous bacterium, often surviving in conditions that are adverse to bacterial development, such as low temperatures and low pH values, high salt and bile concentrations, oxidative stresses and absence of carbon sources (**Razavilar and Genigeorgis 1998; Wu et al. 2015**), which ends up contributing to its widespread in nature and presence in food processing environments (**Taormina and Beuchat 2001**). It is important to note that approximately 99%, i.e., almost all cases of listeriosis in humans, occur due to the ingestion of food contaminated with the microorganism (**Allerberg and Wagner 2010; Scallan et al. 2011**), mainly by serotypes 1/2a, 1/2b and 4b (**Swaminathan and Gerner-Smidt 2007**).

Four evolutionary lineages have been described for the microorganism (I, II, III and IV), with emphasis on lineages I and II, which harbor the serotypes most related to listeriosis in humans, such as serotypes 1/2b and 4b, belonging to lineage I, and serotype 1/2a belonging to lineage II. Most of listeriosis outbreaks are associated with lineage I serotypes, while lineage II appears to be more involved in food contamination (**Orsi et al. 2011)**.

Besides the frequent contamination of ready-to-eat foods, meats and cheeses, the microorganism can also be found in other several raw products that also require storage at low temperatures, especially vegetables, milk and fish (**Büla et al. 1995; Martín et al. 2014; Ruckerl et al. 2014**). *Listeria* sp. also have animals as reservoir that facilitate its presence in milk, and consequently on dairy products (**Quigley et al. 2013**), and antimicrobial-resistant strains, some of which are multidrug-resistant and commonly found in clinical specimens, have already been isolated from contaminated food (**Korsak et al. 2012; Soni et al. 2013**).

Genomic analysis, through the use of techniques for whole genome sequencing, has been widely performed in microbiology facilitating, for example, new insights into *L. monocytogenes* genomics and the understanding of aspects related to virulence and stress response of the microorganism (**den Bakker et al. 2010; Maury et al. 2016; Muller et al. 2013; Nightingale et al. 2005**). Comparative genomic analyses are performed to identify differences that may explain the ability of microorganisms to cause infections and the mechanisms involved in virulence processes, which vary from one strain to another (**Liu et al. 2015**). In addition, the verification of mutations becomes critical for understanding the

behavior of microorganisms, as shown by **Bécavin et al. (2014)**, which demonstrated genomic differences between important reference strains of *L. monocytogenes*, highlighting a mutation in the transcription factor PrfA of *L. monocytogenes* EGD that induces an overexpression of virulence genes, indicating a greater capacity of the microorganism to invade cell cultures.

In this way, the present study aimed to analyze genomic differences between two *L. monocytogenes* strains isolated from cheeses in southern Brazil, serotypes 1/2a and 4b, with other known reference strains widely used for researchers, named *L. monocytogenes* EGD-e (serotype 1/2a) and *L. monocytogenes* Scott A (serotype 4b).

2. Materials and Methods

2.1 Bacterial strains

Two *L. monocytogenes* strains serotypes 1/2a and 4b were used, named *Lm*55G and *Lm*47G respectively, both isolated from cheese samples by the National Agricultural Laboratory of Rio Grande do Sul State (LANAGRO/RS), from the Ministry of Agriculture, Livestock and Food Supply (MAPA/Brazil), and serotyped at the Oswaldo Cruz Institute (State of Rio de Janeiro - RJ, Brazil). For bacterial cells enrichment, Brain Heart Infusion (BHI; HiMedia, Mumbai, Maharashtra, India) broth was used, with subsequent culture on selective media plates containing Listeria Enrichment Broth (LEB; Thermo Fisher Scientific, Waltham, MA, USA) and bacteriological agar (HiMedia) at 37ºC **(Pieta et al. 2014)**.

2.2 Genome sequencing and assembly

Extraction of genomic DNA occurred through the PureLinkTM Genomic DNA kit (Thermo Fisher Scientific), according to the manufacturer's instructions for Gram Positive bacterial cells, and parameters such as extraction yield and quality of genomic DNA were measured in spectrophotometer (Ultrospec 3100 Pro; Amersham Biosciences, Little Chalfont, UK) at 260 and 280 nm. Previous to library preparation with the Nextera DNA Library Preparation kit 24 samples (Illumina, San Diego, CA, USA), genomic DNA samples were quantified on a fluorimeter (Qubit® 2.0; Thermo Fisher Scientific). Genome sequencing was

performed on MiSeq Gene and Small Genome Sequencer (Illumina) equipment using the MiSeq Reagent kit v3 150 cycles (Illumina). One paired-end library of 76 bp reads was generated from each strain, and the quality of sequencing was analyzed using *FastQC* software (**Andrews 2010**), while *Trim Galore!* software (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) was used to simultaneously remove Illumina adapter sequences and trim ends of reads of low quality.

The filtered reads from *Lm*55G and *Lm*47G were assembled *de novo* with *ABYSS* software (**Simpson et al. 2009**) using kmer=31 and kmer=65 parameter, respectively. The obtained scaffolds were orientated using alignment script NUCmer from *MUMmer3* package (**Kurtz et al. 2004**), using *L. monocytogenes* EGD-e (*Lm*EGD-e) as reference genome.

2.3 Genome annotation

*Lm*55G and *Lm*47G genomes were annotated with Rapid Annotation using Subsystems Technology (*RAST*) (**Aziz et al. 2008**) and Gene Ontology (GO) were assigned using *Blast2GO* (**Conesa et al. 2005**). Whole gene sequences in *RAST* were doubled checked using *BLASTx* against *Lm*EGD-e as reference genome.

2.4 Phylogenetic analysis

The listeriolysin O (*hly*) gene sequences retrieved from *Lm*55G and *Lm*47G were compared against the same gene present in other available complete genomes (Supplementary Material 1). The sequences were aligned and manually edited using *BioEdit v. 7.2.5* software (http://www.mbio.ncsu.edu/bioedit/bioedit.html). A phylogenetic tree was constructed by the neighbor-joining method (**Saitou and Nei 1987**) using *MEGA version 6* (**Tamura et al. 2013**). The robustness of the tree topology was evaluated by bootstrap analysis based in 1000 replicates.

Supplementary Material 1 List of complete genomes with the accession number from NCBI used in the phylogenetic analysis

ID	Strain	NCBI accession number
10-1047	*Listeria monocytogenes* serotype 1/2a str. 10-1047	NZ_CP006861.1
88-0478	*Listeria monocytogenes* serotype 1/2a str. 88-0478	NZ_CP006862.1
10-0815	*Listeria monocytogenes* serotype 1/2a str. 10-0815	NZ_CP006860.1
08-6997	*Listeria monocytogenes* serotype 1/2a str. 08-6997	NZ_CP006859.1
01-1280	*Listeria monocytogenes* serotype 1/2a str. 01-1280	CP006940.1
08-6569	*Listeria monocytogenes* serotype 1/2a str. 08-6569	NZ_CP006858
F6854	*Listeria monocytogenes* serotype 1/2a str. F6854	NZ_AADQ01000133.1
EGD	*Listeria monocytogenes* EGD	NC_022568.1
EGD-e	*Listeria monocytogenes* EGD-e	NC_003210.1
SHL001	*Listeria monocytogenes* SHL001	NZ_APIB01000009
ScottA	*Listeria monocytogenes* str. Scott A	NZ_CM001159.1
SLCC2482	*Listeria monocytogenes* serotype 7 str. SLCC2482	NC_018591.1
CLIP80459	*Listeria monocytogenes* Clip80459 serotype 4b	NC_012488.1
F2365	*Listeria monocytogenes* str. 4b F2365	NC_002973.6
Listeria ivanovii	*Listeria ivanovii* FSL F6-596	CM001050.1

2.5 Cluster analysis of orthologs

OrthoMCL v.2.0.5 program was used to identify the orthologous groups (**Li et al. 2003**). The algorithm pairs sequences using an all-versus-all *BLAST* and then clusters the pairs to orthologues groups using the Markov Clustering Algorithm (*MCL*) program. Aminoacid sequences obtained from *RAST* annotation (*Lm*55G and *Lm*47G) and from NCBI (*Lm*EGD-e and *L. monocytogenes* Scott A; *Lm*ScottA) and all standard parameters (a percent match cutoff =50 and E-value exponent cutoff=10^{-5}) were used in *OrthoMCL*. The graphical representation of relationships between the different strains was generated using *VennDiagram* package from R (**Chen and Boutros 2011**).

2.6 Data access

The whole genome shotgun projects for *Lm*55G and *Lm*47G have been deposited in *GenBank* (**Pieta et al. 2015**).

3. Results and Discussion

3.1 Genome sequencing and gene annotation

The genome assembly statistics of the two *L. monocytogenes* strains studied are shown in Table 1. *Lm*55G presented a higher number of scaffolds and mapping-rate percentage, with a media contig size equal to 439.603, in comparison to 258.151 for *Lm*47G. In contrast, *Lm*47G demonstrated greater values for genome coverage and number of reads. Also *Lm*47G comprised an incresead amount of genes equal to 3.026 in comparison with *Lm*55G, *Lm*EDG-e and *Lm*ScottA, which presented 2.873, 2.867 and 2.969 genes, respectively (Table 2). For all the four strains, the total coding region was about 89% of the full draft genome sequence.

Table 1 *Listeria monocytogenes* 55G and *Listeria monocytogenes* 47G genome assembly statistics

	***Lm*55G**	***Lm*47G**
Total scaffolds	30	24
Total bases in scaffolds	3.026.679	3.036.711
Scaffolds N50 (bases)	439.603	258.151
Scaffolds max (bases)	865.036	483.808
Total reads	4.780.984*x*2	9.107.687*x*2
Fold-coverage	123.4	227
Mapping-rate (%)	94	90
G+C content (%)	37.8	37.9

Table 2 Draft genome annotation statistics

	*Lm*55G	*Lm*47G	*Lm*EGD-e	*Lm*ScottA
CDS	2.873	3.026	2.867	2.969
tRNA	24	65	67	67
rRNA	3	6	18	18
CDS (length)	2.593.612	2.728.182	2.629.341	2.688.456
CDS (%)	89.77%	89.47%	89.29%	88.96%

CDS, Coding Sequence

Based on the phylogenetic tree obtained with the *hly* gene (Fig. 1), *Lm*EGD-e was selected for a comparative genetic analysis with *Lm*55G, and *Lm*ScottA choosen for comparison with *Lm*47G, these two strains being considered important references widely used in research involving *L. monocytogenes* (**Bécavin et al. 2014; Briers et al. 2011; Guenther et al. 2009)**.

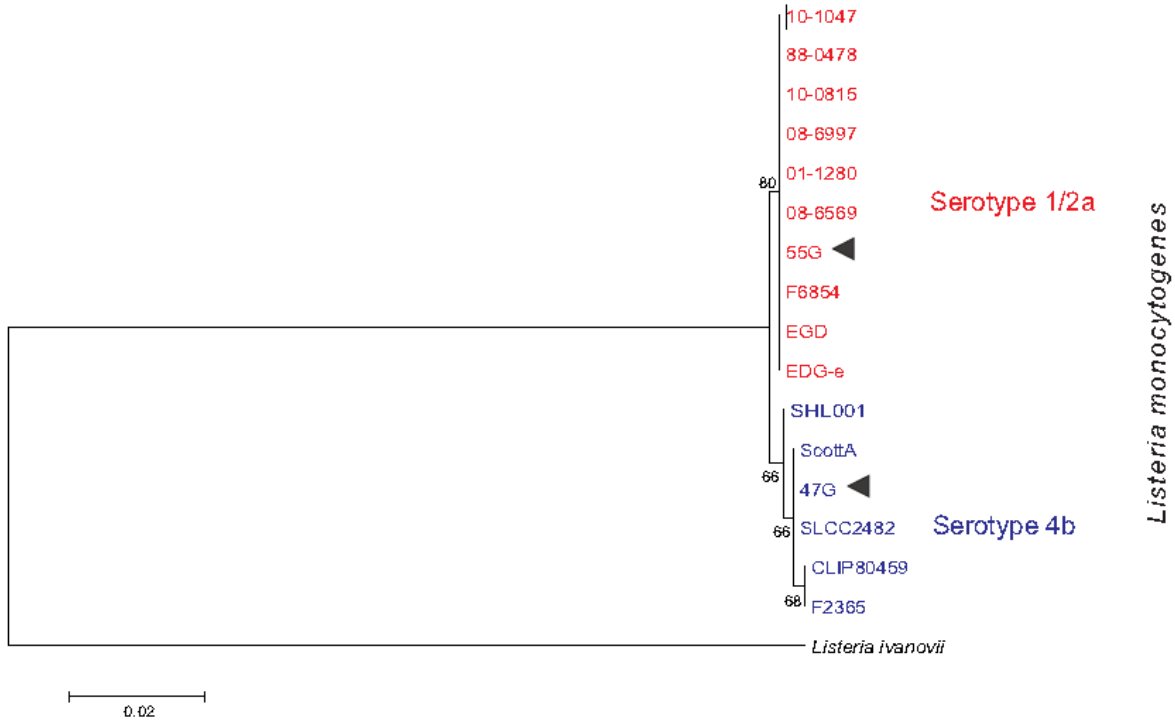

Fig. 1 Neighbor-joining phylogenetic tree based on *hly* gene sequences showing the relationships of *Listeria monocytogenes* serovars. Bootstrap values (>60%) based on 1,000 resamplings are shown above the nodes. Bar, 0.02 substitution per nucleotide position. *Listeria ivanovii* was used as the outgroup.

3.2 Orthologous genes and diferences between serotypes 1/2a and 4b

To investigate orthologous genes between *Lm*55G, *Lm*47G, *Lm*EGD-e and *Lm*ScottA, *OrthoMCL* (**Li et al. 2003**) was used, and a total of 2.587 genes were identified as shared between the four *L. monocytogenes* strains, based on a 10 aa *cutoff* (Fig. 2). In relation to serotype 4b strains, *Lm*47G and *Lm*ScottA presented 162 orthologous genes in common, while the comparison between *Lm*55G and *Lm*EGD-e strains, serotype 1/2a, indicated a lower number equal to 153 orthologous. Differences between serotypes of cheese-isolated strains were also studied, indicating a major number of *Lm*47G proteins that were not shared with *Lm*55G (Table 3 and Table 4).

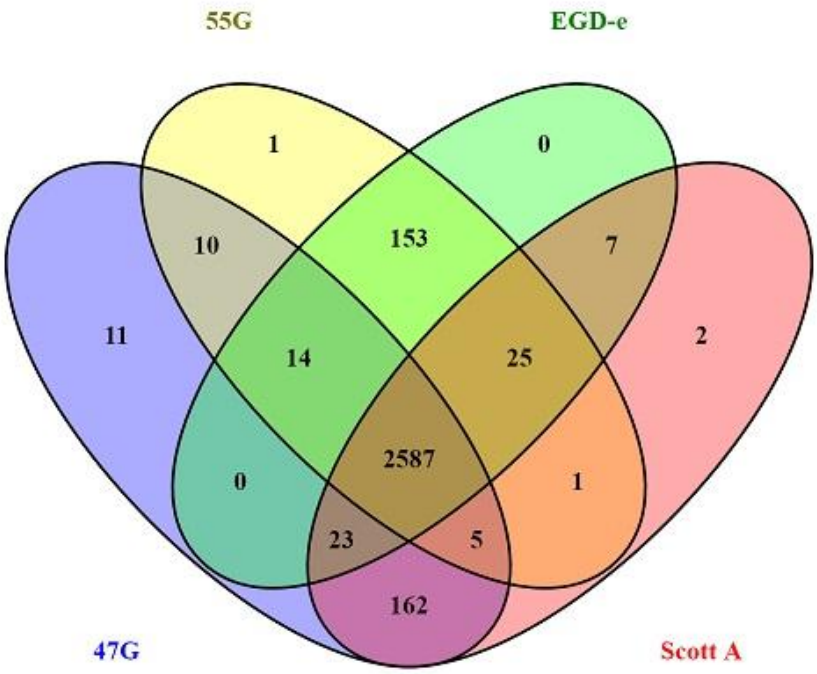

Fig. 2 Venn Diagram as a graphical representation of relationshis between the different strains of *Listeria monocytogenes* studied

Table 3 Proteins present only in *Listeria monocytogenes* 47G in comparison with *Listeria monocytogenes* 55G

***GenBank* accesion number**	**Protein**	**Note**
EGJ25784.1	ArsA	ArsA ATPase function as an efflux pump located on the inner membrane of the cell. This ATP-driven oxyanion pump catalyzes the extrusion of arsenite, antimonite and arsenate
EGJ25782.1	ArsD	Arsenical resistance operon trans-acting repressor ArsD
EGJ24594.1	YeeA	Type II restriction/modification system, DNA methylase subunit YeeA [Defense mechanisms]
EGJ24596.1	YeeC	This entry represents the putative helicase A859L; smart00984
EGJ23545.1	Dcm	Site-specific DNA-cytosine methylase [Replication, recombination and repair]
EGJ24402.1	Ybia	*Escherichia coli* swarming motility protein YbiA and related proteins
EGJ24436.1	ArnT	4-amino-4-deoxy-L-arabinose transferase or related glycosyltransferase of PMT family [Cell wall/membrane/envelope biogenesis]
EGJ25195.1	MurQ	N-acetylmuramic acid-6-phosphate etherase
EGJ25194.1	MurP	PTS system N-acetylmuramic acid transporter subunits EIIBC
EGJ23561.1	XtmA	Phage terminase, small subunit [Mobilome:prophages, transposons]
EGJ23817.1	HsdS	Restriction endonuclease S subunit [Defense mechanisms]
EGJ23819.1	Mrr	Restriction endonuclease Mrr [Defense mechanisms]
EGJ23942.1	RhaS	Uncharacterized conserved protein RhaS, contains 28 RHS repeats [General function prediction only]

Table 4 Proteins present only in *Listeria monocytogenes* 55G in comparison with *Listeria monocytogenes* 47G

NCBI reference sequence	Protein	Note
NP_463783.1	Penicillinase_R	Penicillinase repressor
NP_464296.1	COG4833	Predicted alpha-1,6-mannanase, GH76 family [Carbohydrate transport and metabolism]
NP_464605.1	GgaB	Teichoic acid biosynthesis protein GgaB
NP_464606.1	RmlA	Glucose-1-phosphate thymidylyltransferase, short form
NP_464608.1	RfbB	dTDP-D-glucose 4,6-dehydratase [Cell wall/membrane/envelope biogenesis]
NP_463600.1	DraG	ADP-ribosylglycohydrolase [Posttranslational modification, protein turnover, chaperones]
NP_464607.1	dTDP_sugar_isom	dTDP-4-dehydrorhamnose 3,5-epimerase
WP_003733010.1	TatC	Sec-independent protein secretion pathway component TatC [Intracellular trafficking, secretion, and vesicular transport]
NP_463952.1	SigC	RNA polymerase factor sigma C
NP_463599.1	YeeF	Predicted ribonuclease, toxin component of the YeeF-YezG toxin-antitoxin module [Defense mechanisms]

As an environment contaminant of water and soils, arsenic enters in biosphere primarily through geological formations and by anthropomorphic sources that include arsenic-containing fungicides, pesticides and herbicides application (**Rosen 1999; Rosen 2002**). Defense mechanisms against this metal are found in prokaryotic and eukaryotic cells, and at least nine genes are known to be involved in resistance, including regulatory (*arsR*), export (*arsA, arsB, acr3*) and reduction (*arsC*) of arsenic compounds, organized in several organisms as *ars* operons (**Dheer et al. 2015; Stolz et al. 2006**). *Lm*47G demonstrated the presence of *arsABD* genes, while only *arsB* gene was present in *Lm*55G. The Arsenical pump-driving ATPase (ArsA) function as an efflux pump on the inner membrane of the cell, which catalyzes the extrusion of arsenite, antimonite and arsenate, participating in the detoxification of arsenic-containing substances, and ArsB is similar to Arsenite efflux pump Acr3, being involved in arsenite transport. Much of bacteria use ArsB, found in most *ars* operons, to expel

arsenit, but ArsA can be co-expressed with ArsB, and when this happens, they act together as an ArsAB pump to eliminate arsenic compounds from cells (**Rosen 2002**). The trans-acting repressor ArsD is related to responses to arsenic-containing substances as arsenates, arsenites, and arsenides that can affect several processes of the organisms, in terms of movement, secretion, enzyme production and gene expression. As results obtained show, *Lm*47G has an ACR cluster in comparison to only ArsB protein found in *Lm*55G, which may confer a greater arsenic resistance to the serotype 4b strain**.** Other proteins related to defense mechanisms of the microorganism were present only in *Lm*47G, such as Dcm, HsdS and Mrr.

Involved on the regulation of many important biological processes in such prokaryotic and eukaryotic organisms, DNA methylation is catalyzed by solitary methyltransferases and those associated with R-M systems in bacteria, that include the N6-adenine methyltransferases Dam and CcrM, and the C5-cytosine methyltransferase Dcm. The Dam methylase is considered the best characterized methyltransferase in bacteria, and in *Escherichia coli* this protein is involved in multiple processes including chromosomal replication, DNA repair, and also pilus expression (**Li et al. 2016; van der Woude et al. 1992; Wion and Casadesús 2006**). In prokaryotic organisms, the R-M systems are composed by the majority of DNA methyltransferases and endonucleases, being responsible for a defense mechanism against invasion of foreign DNA, particularly bacteriophages (**Loenen et al. 2014**). A typical type-II R-M system is composed by a DNA endonuclease (HsdR) and a methyltransferase (HsdM), while a type-I R-M system contains a HsdS, named a sequence specificity protein, responsible for sequence recognition function of the other proteins HsdM and HsdR of this system (**Dryden et al. 1997**). In *Lm*ScottA, the region that contains HsdS, characterized as a restriction endonuclease S subunit involved in defense mechanisms of the microorganism, contain two methylases Methylases_S, considered Type I restriction modification DNA specificity domains, and also Dam and Dcm are present in the microorganism, involved in DNA replication, recombination and repair. Results of the present study demonstrated that *Lm*47G contains HsdS and methyltransferases Dam and Dcm, while only Dam was expressed in *Lm*55G. In the same way, the presence of a restriction endonuclease Mrr, involved in defense mechanisms by catalysis of the hydrolysis of ester linkages within nucleic acids by creating internal breaks, was verified in *Lm*47G. The microorganism *E. coli* K12 encodes a number of three type-IV restriction endonucleases, named McrA, McrBC, which have previously shown to be involved in restricting phage infection, and Mrr. In this microorganism, Mrr was primarily discovered as an enzyme that confers genotoxicity on heterologous expression of some exotic type-II methyltransferases

(**Waite-Rees et al. 1991**). However, subsequent research revealed other functions to this protein, that include generation of double-strand breaks in the host chromosome specifically in the presence of sublethal high (hydrostatic) pressure (**Aertsen and Michiels 2005**), indicating that host DNA integrity is deliberately affected in response to stress by Mrr activation.

Bacteria cell wall, mainly composed by peptidoglycan, is frequently remodeled, degraded and rebuilt during bacterial growth and cell division (**Höltje 1998; Mayer 2012**). The metabolic process of peptidoglycan recycling by Gram-negative bacteria such as *E. coli* is widely known (**Goodell 1985**), while in Gram-positive bacteria it is not so well characterized. The first evidence for a muropeptide catabolic pathway for cell wall recovery in a Gram-positive organism occurred in **2010, by Litzinger et al.**, this pathway showing to be different from that performed by *E. coli* and other Gram-negative bacteria. It was identified a pathway in *Bacillus subtilis* used for recovery of *N*-acetylglucosamine (GlcNAc)-*N*-acetylmuramic acid (MurNAc) peptides (muropeptides) derived from the peptidoglycan of the cell wall, which was encoded by a cluster of six genes. The first three genes are orthologs of *E. coli* genes and are involved in *N*-acetylmuramic acid dissimilation that encode a MurNAc-6-phosphate etherase (MurQ), a MurNAc-6-phosphate-specific transcriptional regulator (MurR), and a MurNAc-specific phosphotransferase system (MurP). A recent study performed by **Borisova et al. (2016)** showed that three Gram-positive model organisms, including *Staphylococcus aureus* and *B. subtilis*, are able to recycle the sugar *N*-acetylmuramic acid (MurNAc) of their peptidoglycan during growth in rich medium by presence of the MurNAc-6-phosphate (MurNAc-6P) etherase (MurQ in *E. coli*) enzymes. Quantification of MurNAc-6P in Δ*murQ* cells of *S. aureus* and *B. subtilis* revealed a small amount during exponential growth phase but, a large amount during transition and stationary phases, thus indicating that recycling occurs predominantly during the transition to stationary phase, providing a benefit for long-term survival of these microorganisms. Interestingly, MurQ and MurQ were only present in *Lm*47G, not being found in *Lm*55G.

*3.3 Lm*47G *single proteins*

Among the four *L. monocytogenes* strains studied, *Lm*47G expressed the greatest number of single proteins. In total, 11 proteins were found only in this strain, including a DNA recombination protein RecT and gp47 [*Listeria monocytogenes* serotype 1/2a str. F6854], a putative phage-type endonuclease.

Recombineering is an option to engineer DNA molecules *in vivo* without the *in vitro* use of restriction enzymes and DNA ligase, being considered in bacteria a powerful technique for genome reconstruction (**Binder et al. 2013; Copeland et al. 2001**). In *E. coli*, phage-derived proteins are involved in this process, as the Red proteins of phage λ, encoded by *gam*, *bet* and *exo* (**Court et al. 2002; Murphy 1998**) or RecET proteins, encoded by *recE* and *recT* (**Clark et al. 1993; Zhang et al. 1998**). Previous work performed by **Datta et al. (2008)** has identified and characterized genes similar to *bet* or *recT* from Gram-positive and other Gram-negative bacteria and their phages, such as *B. subtilis, L. monocytogenes, Lactococcus lactis, S. aureus, Enterococcus faecalis and Vibrio cholerae.* **Iyer et al. (2002)** demonstrated by phylogenetic analyses that RecT family is predominantly seen in the low-GC Gram-positive bacteria, such as *Bacillus* sp., *Streptococcus* sp., *Lactococcus* sp. *and Listeria* sp., and their phages. Corroborating this, other studies reported Bet and RecT proteins facilitating recombineering in microorganisms such as *Salmonella enterica*, *Lactobacillus* sp. and *B. subtilis* (**Sawitzke et al. 2007; van Pijkeren et al. 2012; Wang et al. 2012**). It has already been shown that the extensive genome recombineering achieved by *E. coli* is also possible in *Corynebacterium glutamicum*, a Gram-positive rod-shaped bacteria such as *L. monocytogenes*, facilitating the creation of productive mutants genetically different (**Binder et al. 2013).**

A major number of proteins expressed in *Lm*47G related to defense and metabolism of the microorganism important for DNA repair and recombineering, response to stresses such as pressure and heavy metals presence, which can promote a long survival during stationary phase allowing a greater adaptation of the bacterium in adverse environments, agree with research that has already reported this serotype as the most related to listeriosis outbreaks (**Swaminathan and Gerner-Smidt 2007**), indicating mechanisms that can be involved with its greater ability to cause infections in humans.

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

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5. PERSPECTIVAS E CONSIDERAÇÕES FINAIS

A continuidade dos experimentos para verificação da possível citotoxicidade e capacidade de lise celular do óleo essencial de *B. psiadioides*, assim como o seu mecanismo de ação em células de *L. monocytogenes*, é de grande valia para enriquecer os dados aqui expostos. Além disso, para aplicação deste óleo como ferramenta contra microrganismos em ambientes processadores/industrializadores de alimentos, se faz necessária uma análise acerca da composição total deste óleo, de forma a verificar, por exemplo, se este produz algum metabólito que possa ser tóxico ao organismo humano.

O desenvolvimento de novas embalagens para alimentos se apresenta como um grande mercado de atuação, sendo uma tendência a busca por produtos alimentícios que possuam durabilidade maior. Em função das necessidades do consumidor, se torna necessária a pesquisa e o maior desenvolvimento de tecnologias como a aplicação de compostos com potencial antimicrobiano nanoencapsulados em filmes e superfícies de embalagens que entram em contato com os alimentos. Além disso, a utilização de compostos de origem natural tem sido preferida pelos consumidores em substituição a componentes obtidos sinteticamente, que apresentam historicamente maiores riscos de intoxicações e relação com doenças a longo prazo, como o câncer.

As chamadas “ciências ômicas” proporcionam uma visão global dos sistemas, gerando grandes quantidades de dados a serem analisados. Neste trabalho foram realizadas as técnicas de genômica e transcriptômica, cujos resultados podem ser complementados através da realização de análises proteômica e metabolômica. A possibilidade de realização destes experimentos também com outros sorovares isolados de alimentos pode auxiliar numa melhor compreensão das linhagens existentes de *Listeria* sp. que causam listeriose.

Listeria monocytogenes tem causado recorrentes surtos alimentares em diversos países, seja em produtos de origem animal ou vegetal, refrigerados/congelados ou não. Contudo, se faz necessária uma maior investigação dos seus mecanismos de virulência e de que formas estes podem

ser controlados. O embasamento científico é fundamental para que ocorram maiores exigências acerca de sua ausência nos alimentos pelos órgãos fiscalizadores, uma vez que no Brasil, por exemplo, a ausência deste microrganismo só é exigida em queijos de média e alta umidade, mesmo tendo sido já relatada a presença desta bactéria em variados produtos alimentícios.

Tratando-se de um microrganismo que apresenta altas taxas de mortalidade, considerada uma das maiores entre os patógenos alimentares, e que possui comportamento ubíquo, podendo permanecer em ambientes diversos, são necessárias medidas de controle eficazes que impeçam a sua multiplicação e desenvolvimento em condições diversas nas quais consegue sobreviver e causar infecções que frequentemente levam a óbito.

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7. ANEXOS

7.1 Publicação 1

Colaboração em artigo científico publicado no periódico "*Genome Announcements*" em janeiro de 2016.

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Complete Genome Sequence of *Enterococcus faecalis* Strain P8-1 Isolated from Wild Magellanic Penguin (*Spheniscus magellanicus*) Feces on the South Coast of Brazil

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***Enterococcus faecalis* strains have a ubiquitous nature that allows them to survive in different niches. Studies involving enterococci isolated from marine animals are scarce. Therefore, in this study, we report the complete genome sequence of *E. faecalis* strain P8-1 isolated from feces of a Magellanic penguin on the south coast of Brazil.**

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Citation Prichula J, Campos FS, Pereira RI, Cardoso LA, Wachholz GR, Pieta L, Mariot RF, de Moura TM, Tavares M, d'Azevedo PA, Frazzon J, Frazzon APG. 2016. Complete genome sequence of *Enterococcus faecalis* strain P8-1 isolated from wild Magellanic penguin (*Spheniscus magellanicus*) feces on the south coast of Brazil. Genome Announc 4(1):e01531-15. doi:10.1128/genomeA.01531-15.

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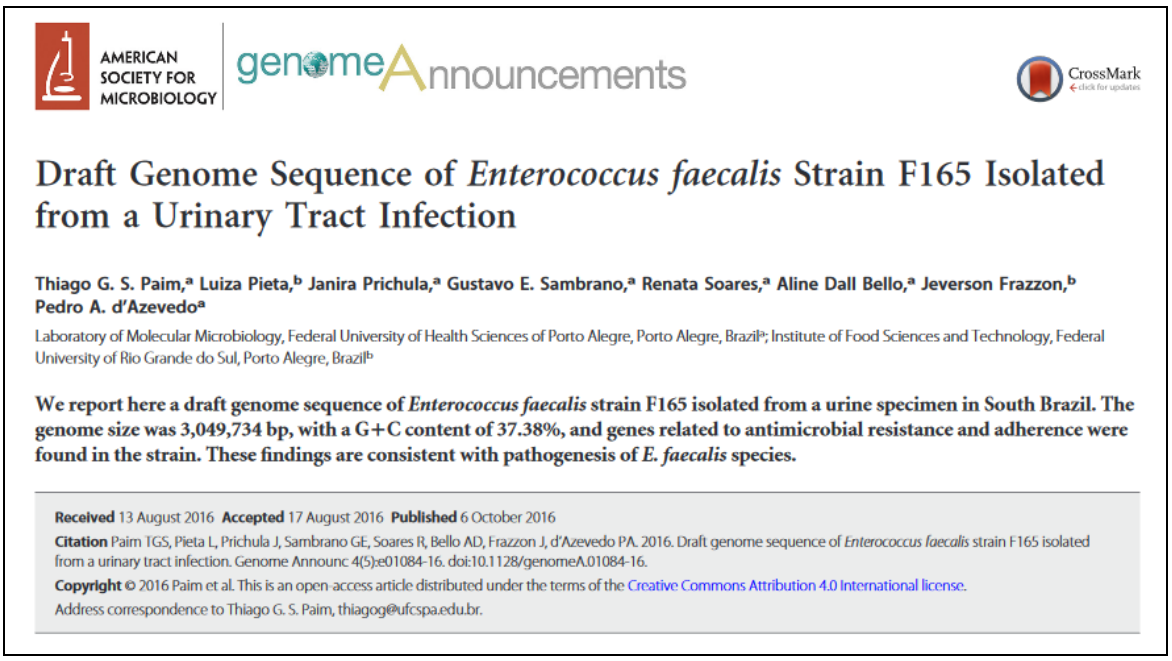
7.2 Publicação 2

Colaboração em artigo científico publicado no periódico "*Genome Announcements*" em outubro de 2016.



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Draft Genome Sequence of *Enterococcus faecalis* Strain F165 Isolated from a Urinary Tract Infection

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We report here a draft genome sequence of *Enterococcus faecalis* strain F165 isolated from a urine specimen in South Brazil. The genome size was 3,049,734 bp, with a G+C content of 37.38%, and genes related to antimicrobial resistance and adherence were found in the strain. These findings are consistent with pathogenesis of *E. faecalis* species.

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Citation Paim TGS, Pieta L, Prichula J, Sambrano GE, Soares R, Bello AD, Frazzon J, d'Azevedo PA. 2016. Draft genome sequence of *Enterococcus faecalis* strain F165 isolated from a urinary tract infection. Genome Announc 4(5):e01084-16. doi:10.1128/genomeA.01084-16.

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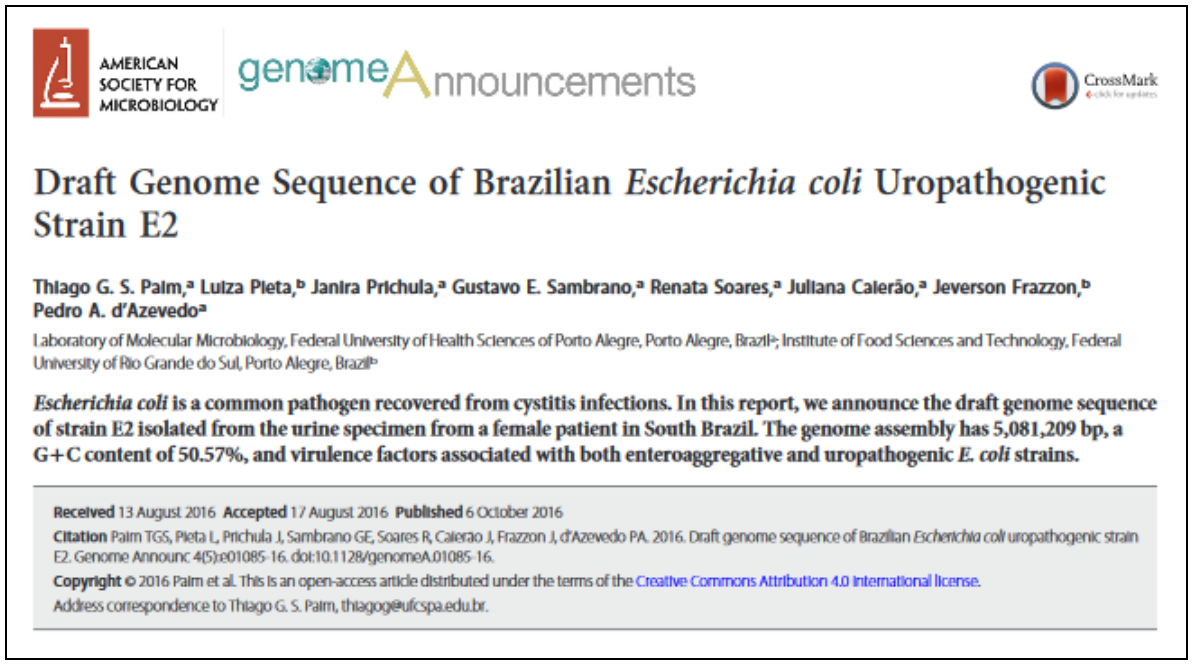
7.3 Publicação 3

Colaboração em artigo científico publicado no periódico "*Genome Announcements*" em outubro de 2016.



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Draft Genome Sequence of Brazilian *Escherichia coli* Uropathogenic Strain E2

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***Escherichia coli* is a common pathogen recovered from cystitis infections. In this report, we announce the draft genome sequence of strain E2 isolated from the urine specimen from a female patient in South Brazil. The genome assembly has 5,081,209 bp, a G+C content of 50.57%, and virulence factors associated with both enteroaggregative and uropathogenic *E. coli* strains.**

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Citation Palm TGS, Pieta L, Prichula J, Sambrano GE, Soares R, Calerão J, Frazzon J, d'Azevedo PA. 2016. Draft genome sequence of Brazilian *Escherichia coli* uropathogenic strain E2. Genome Announc 4(5):e01085-16. doi:10.1128/genomeA.01085-16.

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