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Perfil Proteico Global de Células Planctônicas e de Células Aderidas de *L. monocytogenes* por 1D-LC/tandem MS.

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PERFIL PROTEICO GLOBAL DE CÉLULAS PLANCTÔNICAS E DE CÉLULAS ADERIDAS DE *L. monocytogenes* POR 1D-LC/tandem MS.

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Co-Orientador(es): Dr. John Bowman

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Resumo

MATA, Marcia Magalhães. **Perfil Proteico Global de Células Planctônicas e de Células Aderidas de *L. monocytogenes* por 1D-LC/tandem MS.** 2013. 110f. Tese (Doutorado) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

L. monocytogenes é o agente etiológico da listeriose, uma doença severa de origem alimentar. Esse patógeno também é capaz de se aderir a uma grande variedade de superfícies do processamento de alimentos. Sendo assim, os objetivos deste estudo foram primeiramente, avaliar a influência da temperatura (04-10-25 e 37°C) e tempo de incubação (24-48-168h) na formação de células aderidas de cepas de *L. monocytogenes* de diferentes origens, sorotipos e linhagens utilizando o método colorimétrico em placas de microtitulação. Após, experimentos de proteômica abrangentes que não utilizam marcadores, como a Cromatografia líquida de 1D/ espectrometria de massa em tandem (1D-LC/tandem MS) foram realizadas para determinar se o perfil proteico global de células planctônicas e de células aderidas de cepas de *L. monocytogenes* (Siliken e F2365) foi alterado significativamente quando os meios de crescimento e de temperatura de incubação foram os mesmos. A partir dos resultados obtidos verificou-se que as células aderidas formadas por *L. monocytogenes* de diferentes origens não sofreram alterações significativas quando submetidas às condições experimentais, diferentemente do que foi observado com as células aderidas formadas por *L. monocytogenes* de diferentes sorotipos e linhagens, as quais foram claramente afetadas pelas condições do ambiente. A habilidade da linhagem II e dos sorotipos 1/2a e 1/2b de formar grande quantidade de células aderidas quando comparadas com as demais, em condições específicas, indica alto risco de contaminação e disseminação da listeriose, bem como a sobrevivência e persistência deste micro-organismo no ambiente. Com base na análise proteômica, apenas oito proteínas demonstraram alterações substanciais em comum entre ambas as cepas e temperaturas em células aderidas comparadas com suas respectivas células planctônicas. São elas: GroEL, DnaK, PtsH, PdxS, Pgi, RpsB, RpsD, and RpsP. Verificou-se também que a abundância da proteína de superfície celular BapL, embora baixa, não foi aumentada em células aderidas sugerindo que o seu papel na adesão pode ser uma contribuição generalizada para a hidrofobicidade da parede celular. De forma muito interessante, nosso experimento sugere que células aderidas a 25°C por ambas as cepas levam a uma síntese de repressão flagelar. E ainda, Sig B Regulon pode estar associado com o aumento em geral da resposta ao estresse ocorrido na cepa de linhagem II (Siliken) mas não na cepa de linhagem I (F2365) o que pode estar relacionado com as consequências da adesão. A técnica utilizada no experimento demonstrou claramente que com alta abrangência é possível estudar proteomas bacterianos representando assim uma ferramenta poderosa para investigar respostas dinâmicas em *L. monocytogenes* através de uma perspectiva de genômica funcional.

Palavras-chave: Perfil protéico. Células planctônicas. Células aderidas. *L. Monocytogenes.* Proteômica. Adesão celular

Abstract

MATA, Marcia Magalhães**. Perfil Proteico Global de Células Planctônicas e de Células Aderidas de *L. monocytogenes* por 1D-LC/Tandem MS.** 2013. 110f. Tese (Doutorado) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

L. monocytogenes is the etiologic agent of listeriosis, a severe food-borne disease. This pathogen has also variable ability to adhere to food-processing surfaces. Thus, the aims of this study was at first to evaluate the influence of the temperature (04-10-25-37°C) and time of incubation (24-48-168h) on the formation of attached cells by *L. monocytogenes* strains of diverse origins, serotypes and lineages using a colorimetric microtitre plate method. After this, comprehensive proteomics experiments using label-free 1D- liquid chromatography/tandem mass spectrometry (1D-LC/tandem MS) were performed to determine if the global proteomic responses of *L. monocytogenes* strains (Siliken and F2365) is altered markedly as attached cells compared to its planktonic state when growth media and temperature are the same. Our results showed that attached cells produced by different origins of *L. monocytogenes* did not change significantly when subjected to experimental conditions, unlike what was observed with attached cells produced by different serotypes and lineages of *L. monocytogenes*, which were clearly affected by environmental conditions.such as temperature and time of incubation. The ability of lineage II and serotype 1/2a and 1/2b to form large amount of attached cells when compared with the others in specific conditions indicates that risks from *Listeria* adherence must be taken seriously in sensitive food environments in order to find safer alternatives to prevent contamination and further dissemination of listeriosis. Only 8 proteins demonstrated substantial changes in common between both strains and temperatures in attached cells compared to their planktonic counterparts. They are: GroEL, DnaK, PtsH, PdxS, Pgi, RpsB, RpsD, and RpsP. Moreover, it was observed that the cell surface protein BapL abundance, though low, was not enhanced in attached cells suggesting its role in adherence could be a generalized contribution to the cell wall hydrophobicity. Interestingly, our experiment suggest that at 25°C the attached cells in both strains undergo flagella synthesis repression. Also, Sig B Regulon can be associate with an enhanced general stress response occurs in lineage II Strain (Siliken) but not in lineage I Strain (F2365) and could relate to the consequences of attachment. The temporal survey-based approach demonstrates clearly that high coverage represents a powerful means to investigate dynamic responses in *L. monocytogenes* from a functional genomics perspective.

Keywords: Proteomic profile. Planktonic cells. Adherent cells. *L. monocytogenes.* Proteomic. Cellular attachment.

Sumário

1 INTRODUÇÃO GERAL

Listeria monocytogenes emergiu como um patógeno alimentar a mais de 30 anos, e tem provocado diversos surtos e casos esporádicos de listeriose ligados ao consumo de alimentos contaminados. Isto tem gerado inúmeras discussões em todo o mundo e, consequentemente, estimula pesquisadores a buscar respostas para as várias questões que têm surgido sobre a relação entre *L. monocytogenes*, listeriose e os fatores de virulência envolvidos na relação patógeno-hospedeiro, bem como a interação patógeno/superfícies.

Este patógeno tolera altas concentrações de NaCl e valores de pH relativamente baixos, e juntamente com a sua capacidade de multiplicação em temperaturas de refrigeração, torna-se uma das principais ameaças à seguridade alimentar.

Uma vez que pode sobreviver por longos períodos sob condições ambientais adversas, resistir a diversos processamentos subsequentes adotados na indústria de alimentos, além de apresentar alta capacidade de colonização de superfícies e de formação de biofilmes, pode tornar-se endêmico em plantas de processamento, podendo contaminar o produto final. Para efetivo controle da contaminação por *Listeria* spp. em indústrias de alimentos, é fundamental a compreensão de como esse micro-organismo consegue expressar a sua capacidade de adesão e formação de biofilmes. Nesse sentido, a associação entre a capacidade de adesão em superfícies abióticas e a virulência de diferentes isolados de *L. monocytogenes* é fundamental para avaliar a relevância desse micro-organismo como patógeno e propor mecanismos para seu controle e eliminação.

A sequência completa dos genomas desse micro-organismo tem revelado muito no que se refere à sua estrutura genética, auxiliando na diferenciação de bactérias patogênicas capazes de causar a doença através da pesquisa da expressão e identificação de genes de virulência e da quantificação de toxinas envolvidas no processo de infecção . Sendo assim, a análise proteômica pode trazer novos rumos para colaborar com uma melhor compreensão dos mecanismos de sobrevivência desta bactéria, no ambiente e em alimentos, bem como de seus fatores de virulência e mecanismos de patogenicidade.

Desta forma, a proposta desse estudo foi primeiramente, avaliar se há influência de temperatura e tempo de incubação no desenvolvimento de células aderidas de isolados de *L. monocytogenes* de diferentes origens, sorotipos e linhagens. Após, através da técnica de proteômica comparativa, livre de gel e marcadores através de 1D-LC/MS-MS, avaliar o perfil proteico global de células planctônicas e de células aderidas de cepas de *L. monocytogenes*, quando o meio de cultura e a temperatura de incubação são os mesmos, a fim de contribuir com conhecimentos sobre a versatilidade adaptativa deste importante micro-organismo.

2 REVISÃO DE LITERATURA

2.1 *Listeria monocytogenes*

2.1.1 Filogenia e Taxonomia

L. monocytogenes foi descrita inicialmente em 1926, sendo denominada de *Bacterium monocytogenes*, devido à monocitose apresentada em animais infectados (MURRAY et al., 1926 apud LEDERMANN, 2008). No ano seguinte, a bactéria começou a ser isolada em humanos e em animais por James Hunter Harvey Pirie, o qual a nomeou de *Listerella hepatolytica*, em homenagem a Joseph Lister, médico e pioneiro na área de bacteriologia (PIRIE, 1927 apud LEDERMANN, 2008). Sucessivos estudos e trocas de nomes levaram a descoberta de uma identidade única para os bacilos encontrados em diferentes estudos, passando por *Bacterium monocytogenes hominis,* de Nyfeldt (1932), *Corynebacterium parvulum* de Shultz (1934), *Erysipelothrix monocytogenes* de Miles e Wilson (1946), *Corynebacterium infantisepticum* de Potel (1950), para terminar, em 1957, como *Listeria monocytogenes* através de Heinz Seeliger e Cherry, dois apaixonados pela filogenia e taxonomia bacteriana (LEDERMANN, 2008).

A posição filogenética do gênero *Listeria* atualmente é estável e está relacionada aos gêneros *Bacillus*, *Clostridium*, *Staphylococcus, Streptococcus, Lactobacillus* e *Brochotrix.* Esta posição filogenética é consistente com o baixo conteúdo de moléculas G+C em seu DNA, na média, cerca de 38% (ALLERBERGER, 2003; KHELEF et al., 2006; MCLAUCHLIN; REES, 2009).

Inicialmente, para a comunidade científica, *L. monocytogenes* era a única espécie representante deste gênero (HOF, 2003). Contudo, atualmente o gênero *Listeria* é formado por oito espécies: *L. monocytogenes*, *L. ivanovii*, *L. seeligeri, L. innocua, L. welshimeri, L. grayi, L. rocourtiae* e *L. marthii* (GRAVES et al., 2010; LECLERCQ et al., 2009). Dentre essas espécies, somente *L. monocytogenes* e

L. ivanovii são patogênicas, sendo a primeira patogênica para humanos e animais (LIU et al., 2006), a segunda, apenas para animais (VÁZQUEZ-BOLAND et al., 2001).

L. monocytogenes exibe um grande nível de heterogeneidade entre cepas, o que, segundo Jay (2005), pode estar relacionada com o grande número de hospedeiros animais nos quais é capaz de multiplicar-se. Sendo assim várias técnicas têm sido desenvolvidas ao longo dos anos para discriminar os isolados deste micro-organismo (CHEN; KNABEL, 2008). Entre os métodos fenotípicos, a sorotipagem foi o mais usado e pode diferenciar 4 sorogrupos e pelo menos 13 distintos sorotipos, nomeados 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e, 7 (VASQUEZ-BOLAND et al., 2001; DOUMITH et al., 2004; MEINERSMANN et al., 2004; LIU et al., 2006; ORSI et al., 2008). Destes, os sorotipos 1/2a, 1/2b e 4b são responsáveis por mais de 95% dos surtos em humanos (ROBERTS et al., 2005). Esta classificação é baseada em reações sorológicas dos 15 antígenos somáticos (Fator O subtipado de I a XV) e 5 antígenos flagelares (Fator H subtipado de A –E) com seus específicos anti-soros (SEELIGER; HOHNE, 1979; SEELIGER; JONES, 1996).

Estas técnicas tradicionais como a sorologia, embora confiáveis e eficientes, requerem vários dias ou mesmo semanas para que se obtenham os resultados. Isso pôde ser contornado com o desenvolvimento de métodos moleculares que têm aplicação direta na detecção e caracterização de bactérias patogênicas (CASTELANI; DUARTE, 2011).

Através da utilização de métodos baseados em ferramentas moleculares, tais como, ribotipagem, eletroforese em campo pulsado (PFGE) e sequenciamento por Multilocus (do Inglês, Multilocus Sequence Typing – MLST), pode-se afirmar que essa espécie bacteriana está representada por pelo menos quatro linhagens filogenéticas (linhagens I, II, III e IV) em que cada uma delas possui características distintas das demais (RASMUSSEN et al., 1995; ROBERTS et al., 2006; WARD et al., 2008; ORSI et al., 2011). Segundo Cheng et al. (2008), os diferentes sorotipos parecem estar associados com linhagens específicas: (i) linhagem I contém os sorotipos 1/2b, 3b, 4b; (ii) linhagem II contém os sorotipos 1/2a, 1/2c, 3c; (iii) linhagem III contém os sorotipos 4a, 4c e atípicas 4b; (iv) linhagem IV contém os sorotipos 4a, 4c e atípicas 4b. Enquanto vários estudos evolucionários relevantes na caracterização de *L. monocytogenes* mostram claramente que há pelo menos três principais linhagens filogenéticas (linhagem I, II, III) (MEINERSMANN et al., 2004; NIGHTINGALE et al., 2005;

RAGON et al., 2008; ZHANG et al., 2004), as cepas da linhagem IV aparentemente são raras e não tem sido incluídas na maioria dos estudos (ORSI et al., 2011).

A distribuição entre sorotipos e linhagens não deve ser considerada de forma estrita e absoluta, tendo em vista que alguns sorotipos são pouco representados ou, ainda, não são representados ou são distribuídos entre as diferentes linhagens. Cepas 4b, por exemplo, são distribuídas nas linhagens I, III e IV (LIU et al., 2006; Cheng et al., 2008).

2.1.2 Características do Patógeno

L. monocytogenes é uma bactéria, Gram-positiva, que tem a capacidade de se multiplicar e sobreviver em ambientes diversos. É ubíqua na natureza e pode ser isolada do solo, água, vegetais em decomposição e, talvez, como integrante transitório de muitos mamíferos, incluindo humanos adultos saudáveis (LUKINMAA et al., 2004; SCHLECH et al., 2005; ABRAM et al, 2008). Tem sido isolada de uma grande variedade de alimentos, incluindo vegetais (ELLS; TRUELSTRUP HANSEN, 2006; 2010; CORDANO; JACQUET, 2009), leite (PANAGOU; NYCHAS, 2008), queijos macios e artesanais (MATARAGAS et al., 2008; O'BRIEN et al., 2009; PINTADO et al., 2009), peixes e produtos cárneos (KUSHWAHA; MURIANA, 2009; LIMA et al., 2005), bem como de produtos prontos para consumo (TAKAHASHI et al., 2009; LITTLE et al., 2010).

A temperatura ótima para o crescimento desse micro-organismo está situada entre 30 e 37ºC (AUTIO, 2003), embora seu desenvolvimento possa ocorrer em temperaturas que variam entre -0,4 a 45ºC (VASQUEZ-BOLAND et al., 2001; BELL; KYRIAKIDES, 2005). Esta é uma característica particular de *L. monocytogenes*, no que se refere à manipulação de alimentos, pois pode crescer em temperaturas de refrigeração (4ºC a 10ºC), normalmente utilizadas para o controle microbiano em alimentos. O congelamento também provoca poucos efeitos sobre essa bactéria e, falhas em alcançar a temperatura de congelamento no interior das embalagens podem levar a multiplicação do micro-organismo (BORTOLUSSI, 2008).

Sendo assim, a capacidade de se multiplicar em temperaturas de refrigeração, bem como de tolerar altas concentrações de sal e valores de pH relativamente baixos, faz com que *L. monocytogenes* seja considerada um dos principais perigos biológicos no que diz respeito à seguridade alimentar, uma vez que pode sobreviver por longos períodos sob condições ambientais adversas e

resistir a vários processamentos subsequentes adotados na indústria de alimentos (VÁZQUEZ-BOLAND et al., 2001; LUKINMAA et al., 2004; ILSI RSI, 2005).

2.1.3 Listeriose

L. monocytogenes é um patógeno intracelular facultativo que é capaz de invadir e se multiplicar em células epiteliais e macrófagos, podendo causar a listeriose, uma doença grave de origem alimentar (OLIER et al., 2002; DUSSURGET, et al., 2004; TRABULSI; ALTERTHUM, 2004; GILBRETH et al., 2005).

Antes dos anos 80, a listeriose era uma enfermidade considerada exclusivamente de animais, provocando abortos e encefalite em ovinos e bovinos. No entanto, evidências comprovaram que a listeriose veterinária era, na verdade, uma doença de origem alimentar, devido ao consumo de silagem contaminada, seguida do adoecimento dos animais e disseminação do agente através das fezes e material proveniente de aborto. Como consequência, a bactéria distribuiu-se no ambiente, e tornou-se hábil em sobreviver por longos períodos de tempo sob condições adversas e de se desenvolver a baixas temperaturas (INSTITUTE OF FOOD TECHNOLOGISTS, 2004).

Atualmente, a principal forma de transmissão da doença para humanos é através do consumo de alimentos contaminados (JAY; LOESSNER; GOLDEN., 2005). As fontes potenciais de listeriose a partir de alimentos contaminados têm aumentado em função de mudanças fundamentais na produção, preservação e consumo de alimentos (YDE; GENICOT, 2004). Além disso, *L. monocytogenes* consegue se multiplicar em uma ampla variedade de reservatórios nas plantas de processamento de alimentos, contaminando diversos destes, incluindo produtos prontos para o consumo (*ready-to-eat products*) (CHEN et al., 2005). Isso pode ser evidenciado pela alta ocorrência do patógeno em diferentes tipos de alimentos e ambientes de indústrias de alimentos (CORDANO; ROCOURT, 2001; PECCIO et al., 2003; GUDBJÖRNSDÓTTIR et al., 2004; SILVA et al., 2006; VON LAER et al., 2005; BARROS et al., 2007; NALÉRIO et al., 2009).

Diversos alimentos de origem animal e vegetal têm sido relacionados a casos esporádicos e a surtos de listeriose no mundo. Embora *L. monocytogenes* tenha sido reconhecida como um patógeno animal há mais de 80 anos atrás (MURRAY et al.,1926 apud LEDERMANN, 2008), o primeiro surto confirmado da transmissão indireta de animais para humanos ocorreu em 1981, na Província

Marítima do Canadá (SCHLECH et al., 1983). Este surto resultou em 41 doentes e 11 mortos e foi relacionado com a ingestão de *coleslaw* (produto fermentado de repolho) contaminado a partir de adubo *in natura* proveniente de ovinos com histórico da doença (IVANEK et al., 2006). Um surto subsequente confirmou o papel dos alimentos na disseminação da listeriose. Ocorrido no sul da Califórnia, Estados Unidos, com 142 casos e 48 mortes, o surto foi causado por um tipo de queijo macio, denominado *Mexican-style*, provavelmente produzido a partir de leite cru ou proveniente de processo de pasteurização inadequada (LYNCH et al., 2006).

Listeriose humana é uma doença relativamente rara quando comparada com outras doenças alimentares (2-10 casos reportados por milhão de pessoas/ano) e que inclui como grupo de risco, indivíduos imunocomprometidos e imunosuprimidos, incluindo mulheres grávidas, recém nascidos, crianças e idosos (KIMURA, 2006; FELÍCIO et al., 2007; RAMASWAMY et al, 2007; COSSART; TOLEDO-ARANA, 2008). Segundo Voetsch et al. (2007), a cada ano, 3,1 casos da doença por milhão populacional são registrados nos Estados Unidos. Isso pode ser devido aos rígidos controles atualmente utilizados na produção de alimentos, bem como à população suscetível a forma mais severa da doença, que é formada por um restrito grupo de risco (ALLERBERGER; WAGNER, 2010; BARBUDDHE et al., 2011). Entretanto, devido ao alto índice de letalidade (20-30%), a listeriose encontra-se em segundo lugar no ranking das causas mais frequentes de morte por consumo de alimentos contaminados, ficando atrás apenas da salmonelose nos EUA e na França (DUSSURGET, 2004; WALLS; BUCHANAM, 2005; VAILLANT et al, 2005; FRATAMICO et al., 2005; RIEMANN; CLIVER, 2006; LYNCH et al., 2006; WATSON, R., 2009).

Índices de casos fatais variam muito de país para país, mas invariavelmente a maior taxa de letalidade está entre recém-nascidos com infecção adquirida através de suas mães (25-50%) e entre as pessoas com mais de 60 anos (10-20%) (BORTOLUSSI, 2008). Devido à severidade da doença, os Estados Unidos mantêm uma política de tolerância zero em relação à contaminação de alimentos prontos para o consumo (BORUCKI et al., 2004). Já a Europa estipulou um limite de contaminação por *L. monocytogenes* de até 100 unidades formadoras de colônia por grama de alimento ($UFC.g^{-1}$) para produtos prontos para o consumo, enquanto estiverem em sua vida útil (EUROPEAN COMISSION REGULATION, 2005). Mesmo assim, provavelmente por uma combinação de vários fatores, os casos de listeriose têm

aumentado em vários países europeus nos últimos anos (ALLERBERGER; WAGNER, 2010).

Com relação à listeriose causada por ingestão de produtos cárneos contaminados, o primeiro surto comprovado envolveu um tipo de patê importado pelo Reino Unido e resultou em 366 doentes e 63 mortes (MCLAUCHLIN et al., 1991), e o primeiro relato nos Estados Unidos foi de um caso esporádico relacionado ao consumo de embutido de carne de peru, por uma paciente com câncer (MMWR, 1989). Uma grande variedade de carnes e produtos cárneos, além de plantas de processamento, tem sido associada à contaminação por *L. monocytogenes* (CORDANO; ROCOURT, 2001; PECCIO et al., 2003; GUDBJÖRNSDÓTTIR et al., 2004; SILVA et al., 2006; BARBALHO et al., 2005; VON LAER et al., 2005, NALÉRIO, et al., 2009). Além de produtos cárneos, vegetais frescos como alface, milho, brotos, pimentas entre outros também tem sido alvo de *recalls* nos Estados Unidos (FDA, 2010a; 2010b; 2010c).

A listeriose pode ser transmitida, ainda, pelo contato direto com animais infectados, mas essa via de transmissão é considerada rara. Nesta forma de doença surgem lesões cutâneas, especialmente sobre os braços de pessoas que manipulam animais. McLauchlin (1996) relata a ocorrência de 17 casos descritos na literatura mundial. Outra forma de transmissão é por contaminação cruzada durante o período neonatal, com casos de infecção nosocomial descritos (HOF; LAMPIDIS, 2001; COLODNER et al., 2003).

No Brasil, os primeiros casos relatados de listeriose ocorreram em 1989, no Distrito Federal, envolvendo três pessoas, as quais desenvolveram meningite bacteriana: um recém-nascido com 10 dias de idade, uma portadora de lúpus eritematoso sistêmico, e uma criança com 8 anos. Destes, somente a mulher portadora da doença imunossupressora veio a óbito. Contudo, não foi possível estabelecer qual foi a origem de transmissão de *L. monocytogenes* a estes indivíduos (HOFER et al., 1998). No Brasil ainda não existem relatos de listeriose humana associada ao consumo de alimentos contaminados, no entanto, *L. monocytogenes* tem sido isolada de uma ampla variedade de alimentos (DESTRO et al., 1991; LIMA et al., 2005; VON LAER et al., 2005; DESTRO, M.T., 2006; NALÉRIO et al., 2009).

L. monocytogenes é considerada uma bactéria oportunista que provoca a doença por ser apta a atravessar a barreira epitelial intestinal, tendo acesso aos tecidos mais profundos do hospedeiro (VASQUEZ-BOLAND et al., 2001; FREITAG et al., 2009). Nestes hospedeiros, é capaz de cruzar a barreira hemato-encefálica para

afetar o sistema nervoso central, bem como a barreira feto-placentária, infectando o feto em mulheres grávidas, o que pode provocar aborto espontâneo, natimorto, ou nascimento de um bebê gravemente enfermo (BURKHOLDER; BHUNIA, 2010).

A incidência de listeriose é difícil de ser estabelecida primeiramente porque é difícil obter a relação entre o alimento e o caso clínico, haja vista o longo período de incubação da doença que pode variar de 2 a 70 dias após a ingestão de produtos contaminados. E também porque os sintomas se assemelham muito a uma gastroenterite comum, no caso de indivíduos que não pertencem ao grupo de risco, por isso a identificação e investigação de surtos de listeriose humana requerem conhecimento das características microbiológicas de isolados clínicos e ambientais (VASQUEZ-BOLAND et al, 2001; AUTIO, 2003; INSTITUTE OF FOOD TECHNOLOGISTS, 2004; TRABULSI; ALTERTHUM, 2004; WALLS; BUCHANAM, 2005; YDE; GENICOT, 2004; BORTOLUSSI, 2008).

2.1.4 Biofilmes

Biofilmes podem ser definidos genericamente, como uma comunidade de micro-organismos aderentes a uma superfície (COSTERTON; LEWANDOWSKI, 1995). São comunidades altamente organizadas e sua formação e desenvolvimentos podem ser estimuladas por diferentes fatores ambientais sobre as células bacterianas. Situações de estresse, como baixa disponibilidade de nutrientes, baixas temperaturas e presença de sanitizantes, são considerados os principais deles (JEFFERSON, 2004).

L. monocytogenes se tornou um grande problema para a indústria de alimentos. Uma das principais evidências disso é que esse micro-organismo possui comprovada capacidade de adesão em superfícies de diferentes tipos de materiais, como borracha, plástico, vidro e aço inoxidável, frequentemente utilizados em utensílios e equipamentos nessas indústrias (BERESFORD et al., 2001; PALMER et al., 2007; GANDHI ; CHIKINDAS, 2007).

A capacidade de adesão ou formação de biofilmes são as principais razões para a persistência da bactéria em superfícies que entram em contato com alimentos, uma vez que as células bacterianas em biofilmes são muito mais resistentes a detergentes, biocidas e antibióticos do que em sua forma livre (planctônica).

Os mecanismos de formação de biofilmes por *L. monocytogenes* são basicamente os mesmos geralmente utilizados por outros micro-organismos e se resume a cinco etapas que didaticamente, os quais podem ser colocadas na seguinte ordem: condicionamento da superfície pela adsorção de material orgânico; transportes de células e nutrientes para o sítio de aderência; início do processo de adesão bacteriana ("bactéria pioneira"), ainda reversível, por atração eletrostática; crescimento celular, colonização e adesão irreversível (colonizadores secundários); desenvolvimento e maturação do biofilme (CHMIELEWSKI; FRANK, 2003; MØRETRØ; LANGSRUD, 2004; MCLANDSBOROUGH et al., 2006; GANDHI; CHIKINDAS, 2007).

Acredita-se que moléculas de sinalização extracelular (auto indutores de *quorum-sensing*) estejam envolvidas na regulação de fatores de transcrição específicos, os quais facilitam o controle global da expressão do genoma bacteriano, alterando a configuração bacteriana da forma planctônica para a forma séssil, formadora de biofilme (KONG et al., 2006; RENIER et al., 2011).

Biofilmes que contenham *L. monocytogenes* são considerados como as principais formas de contaminação de alimentos por esse patógeno durante o processamento industrial. Isso estimula o desenvolvimento de vários estudos que procuram elucidar os fatores e mecanismos de adesão desse micro-organismo, visando principalmente o conhecimento de sua fisiologia e, consequentemente, formas de controle adequadas (JEFFERSON, 2004; STEPANOVIC et al., 2004; HARVEY et al., 2007; DI BONAVENTURA et al., 2008).

Superfícies cobertas por biofilmes são particularmente difíceis de descontaminar, uma vez que estes protegem as bactérias contra a dessecação, antimicrobianos e agentes sanitizantes (FOLSOM; FRANK, 2006; TESSEMA et al., 2009). Essa habilidade permite a persistência de *L. monocytogenes* por longos períodos de tempo no ambiente de processamento e, por isso, é considerada um problema para a segurança dos alimentos, uma vez que biofilmes são importantes fontes de contaminação quando produtos alimentícios entram em contato com eles (MORETRO; LANGSRUD, 2004; GOUNADAKI et al., 2008; POIMENIDOU et al., 2009; KOUTSOUMANIS et al., 2010; RENIER et al., 2011).

Estudos demonstraram que sorotipos de *L. monocytogenes* apresentam diferenças significativas na sua habilidade de se aderir às superfícies do processamento de alimentos. Dessa forma, a hipótese de que a capacidade de adesão e de formação de biofilmes possa ser conservada entre as linhagens

filogenéticas foi então formulada. Investigações subsequentes encontraram uma correlação entre sorotipos e linhagens e a habilidade de formar biofilmes, mas suas conclusões são divergentes (DJORDJEVIC et al., 2002; BORUCKI et al., 2003; PAN et al., 2009). Em função dos diferentes resultados apresentados, nenhuma conclusão no que se refere a correlação entre a habilidade de formar biofilmes e a linhagem bacteriana pode ser estabelecida. Isso não é surpreendente considerando que *L. monocytogenes* ocorre como membro de comunidades bacterianas complexas no meio ambiente ou nas plantas de processamento de alimentos (RENIER et al., 2011).

2.1.5 Proteômica de *L. monocytogenes* na Formação de Biofilme

Bactérias secretam inúmeras proteínas, permitindo que elas se adaptem e sobrevivam em seu ambiente. As proteínas secretadas possuem vários papéis funcionais como a degradação de substratos e a alteração de volume e sensoriamento da parede celular. Além disso, as proteínas secretadas são a linha de frente das interações patógeno-hospedeiro (TROST et al., 2005).

A grande evolução nas técnicas utilizadas nas pesquisas com micro-organismos tem permitido um rápido avanço na compreensão de muitos aspectos ligados a sua fisiologia, metabolismo e genética. O sequenciamento completo de genomas tem revelado novas perspectivas de estudo sobre a estrutura genética de um grande número de espécies bacterianas (DUMAS et al, 2008). Utilizando-se ferramentas pós-genômica (proteômica), vários fatores de virulência que não haviam sido descritos têm sido identificados e parcialmente caracterizados (DUSSURGET et al., 2002; CABANES et al., 2005; TROST et al., 2005; ARCHAMBAUD et al., 2006; BIERNE et al., 2007; BUBLITZ et al., 2008; SABET et al., 2008).

A capacidade de *L. monocytogenes* de se adaptar e responder as mudanças ambientais aparentemente está relacionada ao grande repertório de proteínas regulatórias preditas, o que inclui diferentes fatores RNA polimerase Sigma, fatores de transcrição e sistemas de fosforilação de proteínas (BUCHRIESER et al., 2003).

A formação do biofilme bacteriano é um processo complexo que envolve um grande número de vias biossintéticas e, aparentemente, é submetido a regulação através de diferentes vias de transdução de sinais (WATERS; BASSLER, 2005; VON BODMAN et al., 2008). Entre os sistemas de sinalização célula-célula, *quorum sensing*

(QS) tem sido o mais investigado e se refere a densidade celular coordenada com a expressão gênica que correlaciona a concentração de sinais que induz uma resposta sincronizada da população. Em outras palavras, o QS é somente um exemplo do comportamento multicelular de procariotos que leva a regulação de diversos processos fisiológicos, que somente são induzidos quando a bactéria está em alta densidade populacional (RENIER et al., 2011).

Em *L. monocytogenes*, muitos genes ou a expressão de produtos gênicos estão envolvidos na formação de biofilmes. Primeiramente, proteínas responsáveis por motilidade, como as flagelinas, são importantes para adesão bacteriana em superfícies, o passo inicial para formação de biofilmes (VATANYOOPAISAN et al., 2000; TRESSE et al., 2006; 2009; GUERIRI et al., 2008). Segundo, duas vias de transdução de sinais bacterianos foram mostradas como reguladoras da formação de biofilme em *L. monocytogenes* (REN et al., 2004; BELVAL et al., 2006; RIEU et al., 2007). Uma envolve o autoindutor 2 (AI-2)- Furanosil Borato Diéster, uma molécula universal de sinalização de QS que é sintetizada e transportada por vias conservadas na bactéria; a outra via de transdução de sinais envolve o sistema de gene regulador acessório (*agr*), que controla a expressão de vários fatores de virulência, que é muito estudada em *Staphylococcus aureus* (SHIRTLIFF et al., 2002), mas ocorre em outras bactérias Gram-positivas, como *L. monocytogenes* (WATERS; BASSLER, 2005).

Durante o desenvolvimento e maturação do biofilme, mecanismos do complexo celular envolvidos requerem uma coordenação na regulação da expressão gênica pela comunicação célula-célula. A matriz extracelular, que é responsável pela comunicação entre células de biofilme, é uma mistura complexa de exopolissacarídeos, DNA, proteínas e outras substâncias poliméricas extracelulares que têm um papel de estabilizadores da estrutura e de proteção do biofilme (SUTHERLAND, 2001). A parte das proteínas diretamente envolvidas na adesão bacteriana e colonização de ambas, superfícies bióticas e abióticas, a caracterização de genes determinantes responsáveis pela regulação da formação de biofilmes ainda é um desafio chave no estudo de *L. monocytogenes*.

No contexto da segurança de alimentos, a influência das condições ambientais na formação do biofilme e na regulação da rede no curso do crescimento séssil é de crucial importância. Desta forma, a compreensão destes fatores e/ou condições ambientais ideais ao estabelecimento e formação do biofilme poderá auxiliar no desenvolvimento de práticas e políticas para prevenir a contaminação nas

plantas de processamento de alimentos e consequentemente nos produtos finais. Além disso, a análise global, bem como a comparação de proteínas expressas em diferentes cepas submetidas a diferentes condições de formação de biofilme, pode trazer novas informações para colaborar com uma melhor compreensão dos mecanismos de sobrevivência desta bactéria, no ambiente e em alimentos, bem como de seus fatores de virulência.

3 ARTIGO

3.1 Artigo 1

Influence of the temperature and time of incubation on the development of adherent cells by *L. monocytogenes* strains of diverse origins, serotypes and lineages

(Artigo formatado segundo as normas do Periódico International Journal of Food Microbiology)

Influence of the temperature and time of incubation on the formation of adherent cells by *L. monocytogenes* isolates of diverse origins, serotypes and lineages

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Abstract

Listeria monocytogenes is the etiologic agent of listeriosis, a severe food-borne disease. This pathogen has also variable ability to adhere to food-processing surfaces. Thus, the aim of this study was to evaluate the influence of the temperature (04-10-25-37°C) and time of incubation (24-48-168h) on the formation of attached cells by *L. monocytogenes* isolates of diverse origins, serotypes and lineages. A total of 31 *L. monocytogenes* isolates were studied and attachment was measured *in vitro* using a colorimetric microtitre plate method. Our results showed that attached cells produced by different origins of *L. monocytogenes* did not change significantly when subjected to experimental conditions, unlike what was observed with attached cells produced by different serotypes and lineages of *L. monocytogenes*, which were clearly affected by environmental conditions.such as temperature and time of incubation. At 25°C and 37°C the 4b isolates produced much less attached cells than other 1/2a and 1/2b isolates and lineage I isolates produced less attached cells than lineage II *L. monocytogenes* isolates. The ability of lineage II and serotype 1/2a and 1/2b to form large amount of attached cells when compared with the others in specific conditions indicates that risks from *Listeria* adherence must be taken seriously in sensitive food environments in order to find safer alternatives to prevent contamination and further dissemination of listeriosis.

Keywords: environmental conditions, adherent cells, cellular attachment, listeriosis

1 Introduction

L. monocytogenes is the etiologic agent of listeriosis, a severe food-borne disease, that affects mainly immunocompromised individuals, the elderly, pregnant women, and neonates (Khelef et al., 2006; Ramaswamy et al., 2007; Donalsdson et al., 2009). Although rare when compared to other food-borne diseases, a significant feature of listeriosis is its high lethality rate (about 30%), which makes *L. monocytogenes* an important human pathogen (Severino et al., 2007; Watson, 2009; Allerberger and Wagner, 2010).

L. monocytogenes exhibits a high level of heterogeneity from one strain to another, having several techniques been developed over the years to discriminate isolates (Chen and Knabel, 2008). Among phenotypic methods, serotyping has been the most widely used method and it can differentiate 4 serogroups and 13 distinct serotypes, named 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e and 7 (Seeliger and Hohne, 1979; Seeliger and Jones, 1996). The development of more reliable methods based on molecular tools, allowed the identification of four evolutionary lineages (I, II, III, and IV) for this bacterial species, with coincident niches. Most isolates of this bacteria belong to lineages I and II, being them the serotypes most commonly associated to human clinical cases: serotype 1/2a (lineage II) and serotypes 1/2b and 4b (lineage I). Lineage II strains are common in foods, widespread in the natural and farm environments, and are also commonly isolated from animal listeriosis cases and sporadic human clinical cases. However, most human listeriosis outbreaks are associated with lineage I isolates though (Orsi et al. 2011).

Biofilm formation allows bacteria to better persist in the environment and resist to different kinds of stresses, such as desiccation, UV light, antimicrobial and sanitizing agents (Pan et al., 2006; Adrião et al., 2008; Belessi et al., 2011; Nostro et al., 2010; Takahashi et al., 2010; Vaid et al., 2010; Chorianopoulos et al., 2011; Van der Veen and Abee, 2011). The population of bacteria found in biofilms can reach 10^4–10^7 CFU/cm^2 (Gram et al., 2007). Attention should be called to the fact that *L. monocytogenes* is unable to form thick multilayer biofilms being its population of 10^9–10^{12}CFU/cm^2, so in this study instead of using the term “biofilm” we will refer to *L. monocytogenes* adhered cells as “attached cells”. Therefore, the presence of biofilms raises

health and safety concerns in the food industry, mainly because biofilms facilitate the survival of pathogenic bacteria such as *L. monocytogenes* by holding these bacterial colonies in place and protecting them from being damaged by these agents. In addition to what was stated above, several studies have reported that *L. monocytogenes* strains show significant differences in their ability to adhere to food-processing surfaces, such as glassware, metal (stainless steel), rubber and plasticware (polystyrene), where it can further establish a biofilm (Chae et al., 2006; Gudmundsdóttir et al., 2006; Chambel et al., 2007; Keto-Timonen et al., 2007; Palmer et al., 2007; Adriao et al., 2008; Di Bonaventura et al., 2008; Rieu et al., 2008; Ortiz et al., 2010; Takahashi et al., 2010; Van der veen and Abee, 2010).

Attached cells formed by *L. monocytogenes* varies among isolates, being the reasons for this variation still unclear (Borucki et al., 2003; Harvey et al., 2007). It is known, however, that environmental stress influences attachment by *L. monocytogenes* (Folsom and Frank, 2006; Pan et al., 2006; Begley et al., 2009). A better understanding regarding the factors that contribute to the variation in attached cells formation by *L. monocytogenes* strains is needed to optimize preventive measures and minimize the risk that biofilm production by *L. monocytogenes* presents to food industries.

This study was designed to evaluate the influence of the temperature and time of incubation on the formation of attached cells by *L. monocytogenes* isolates of diverse origins, serotypes and lineages.

2 Material and Methods

2.1 Bacterial strains

Thirty one *L. monocytogenes* isolates (Tasmania Collection) obtained from different lineages (I and II), serotypes (1/2a, 1/2b, 4b) and origins (industry, animals, foods and clinical cases) were studied. Details of all the strains are presented in Table 1.

2.2 Microtitre plate attachment cells formation assay

2.2.1 Effect of time and temperature on attachment cells formation

The formation of attached cells for each of four temperatures (04-10-25 and 37℃) was measured in triplicate (3 biological replicates and 3 technical replicates) for each of the 31 *L. monocytogenes* isolates using the colorimetric 96-well microtitre plate method described by Djordjevic et al. (2002) with the modifications recommended by Borucki et al. (2003). Briefly, all isolates were recovered from frozen storage by culture on Brain–Heart Infusion (BHI) agar (Oxoid CM0225B, Oxoid Australia, Adelaide, with 1.5% agar) and incubated at 25 ℃ for 24 h. Following this, each strain was transferred into four 10mL BHI broths (pH 7.3 ± 0.1) and incubated for 24 h at 4℃, 10℃, 25℃ and 37℃. After incubation, concentrations of the inoculum were adjusted to 10^8 CFU. ml $^{-1}$ and then, 100µL of each culture was added to fresh 9.9mL BHI broths, gently mixed, and 200µL aliquots were transferred to wells of four 96-well polystyrene (PS) microtitre plates (Greiner Scientific, Sigma-Aldrich, Australia). The four plates were incubated statically at their corresponding treatment temperature (4ºC, 10ºC, 25ºC and 37℃) and time of incubation (24 h, 48 h and 168 h) to allow attachment and growth to occur, Further, the culture media were discarded and the procedures of washing plates, fixation and distaining with crystal violet solution of 1% of the attached cells were carried out. Optical density at 595 nm (OD_{595}) was measured.

2.3 Statistical analysis

The significance of differences in the relative amount of attached cells produced by each of the *L. monocytogenes* isolates under each test condition and as a function of the origin, serotype, and lineage were assessed by Univariate Analysis of Variance (ANOVA) coupled with tukey-test high significant difference (t-HSD) post-hoc testing. Significance was assigned at $P<0.05$. Logistic Regression was performed to determine which of the

qualitative traits (origin, serotype and lineage) correlation was considered significant at $P<0.05$.

3 Results

In order to identify the three target groups (origin, serotype and lineage), all the isolates were classified and had their frequency and percentual estimated (Table 2). There was no significant difference along time in the attached cells formation within the temperatures (Fig.1). The variables of time and temperature of incubation, when subjected to the logistic regression test, showed no degree of statistical significance between them (Logistic Regression Test). However some variation was observed in these same periods of incubation at 37°C. In contrast, the correlation coefficient (R^2) determination and reliability of the results at 4°C at different incubation times is higher when compared to the data generated at 37°C probably due to less variation between the indices obtained (Fig. 2).

When the origin of the strains was analyzed, there was no statistically significant difference between then and the formation of attached cells, as well as between the times and temperatures of incubation and the origin (t-HSD)(Fig. 3; Fig. 4). However, we observed a statistically significant difference when comparing the formation of attached cells of two lineages of *L. monocytogenes* (Fig. 5; Fig. 6). The lineage II strains produced significantly more attached cells than those belonging to the lineage I when cultured at 25°C and 37°C on all three different times of incubation (Fig. 6).

The results observed after analysis of the serotypes variable showed a statistically significant difference related to the ability of cell adhesion of *L. monocytogenes* isolates used in the study (Fig. 7; Fig. 8). At 25°C and 37°C isolates of 4b serotype produced much less attached cells than other 1/2a and 1/2b strains (Fig. 8).

4. Discussion

The formation of attached cells by *L. monocytogenes* isolates were observed at all standardized conditions in this experiment, suggesting a refined regulatory system enabling the microorganism to rapidly adapt to changing environmental conditions (Gandhi and Chikindas, 2007). The ability of *L. monocytogenes* to colonize surfaces at low temperatures found in the food industry during processing and storing, together with the finding that attached cells of *L. monocytogenes* could mantain at 4 or 10ºC for at least 5 days (Somers and Wong, 2004), increase the likelihood of cross-contamination. Furthermore, a study suggested that the biofilms formed at 4ºC, despite the low amount of biomass, are metabolically more active than those formed at 37ºC, in an attempt to adapt to the adversity imposed by the low temperatures (Rodrigues et al., 2009). These authors also explain that the low amount of biomass may be due to the fact that the bacteria grows slower and accumulates lower amounts of exopolymers at 4ºC.

The absence of a significant difference between incubation times and temperatures may be the result of a large deviation in the individual profile of adhesion of each *L. monocytogenes* strain, which allowed the diagnosis of a high inter-strain variability (Fig. 1). This inter-strain variability has also been observed in other studies (Folsom and Frank, 2006; Harvey et al. 2007; Di Bonaventura et al., 2008, Pan et al., 2009, Nilsson et al., 2011) and it has probably been caused by the stimuli used in the experiment and that somehow interfered in the metabolic activity of attached-forming cells and, consequently, on the final profile of attached cells formation by each *L. monocytogenes* strain. The degree of variability observed was proportional to the incubation temperature, with the largest variation been observed when the strains were submitted to 37℃ while the lowest variation was observed at the temperature of 4℃ (Fig. 2). A possible explanation for this behavior may be in the gene expression profile of individual strains, which allowed a better response and adaptation of the microorganism to the stress generated by the low temperature, what would increase the metabolism of the cells, thereby reducing bacterial adhesion and biofilm production among strains. Otherwise, when the temperature was higher and it was considered optimal for bacterial growth, the

metabolism rate decreased and allowed higher bacterial adhesion and biofilm production.

In our study we found no evidence of a significant correlation between the ability to form attached cells when submitted to different temperatures and time of incubation and the origins of isolation of the strains. However, we found an expressive significant correlation among the attached cells formation when incubated in different temperatures and time of incubation with lineages and serotypes. We found that serotype 4b produces much less attached cells than the other two serotypes (mainly 1/2a) which agree with experiments conducted by Nilsson et al. (2011) and Di Bonaventura et al. (2008), but differ with Liu et al. (2006) who said that the distribution between serotypes and lineages should not be considered so strictly and absolutely once some serotypes were under-represented or not represented.

As Combrouse et al. (2013), our results showed that lineage II strains produce more attached cells than strains of lineage I. In contrast Djordjevic et al. (2002) and Takahashi et al. (2009) observed an opposite trend. Such discrepancies, often found in the literature (Borucki et al. 2003; Harvey et al. 2007; Takahashi et al. 2009), may be related to differences in the experimental designs and in the methods used to study biofilm. Indeed, both strains and culture conditions (medium, temperature, incubation time and surface) differed between the studies reported above. However, all agreed that *L. monocytogenes* biofilms are significantly influenced by the temperature (Moltz and Martin 2005; Mai and Conner 2007; Di Bonaventura et al. 2008), strain (Borucki et al. 2003; Tresse et al. 2006) and the incubation time (Harvey et al. 2007).

Another interesting result was seen in the graphs of cultivation of *L. monocytogenes* of different lineages and serotypes (Fig. 4 and Fig. 5) at 37°C for 168h. The decrease in the concentration of attached cells under these conditions suggests a probable reduction in the concentration of nutrients in the medium and the consequent metabolic depletion of bacterial cell.

5 Conclusions

To conclude, our results showed that attached cells produced by different origins of *L. monocytogenes* did not change significantly when subjected to experimental conditions, unlike what was observed with attached cells produced by different serotypes and lineages of *L. monocytogenes*, which were clearly affected by environmental conditions.such as temperature and time of incubation.

The ability of lineage II and serotype 1/2a and 1/2b to form large amount of attached cells when compared with the others in specific conditions indicates that the risks originated from *Listeria* adherence must be taken seriously in consideration in different food environments in order to find safer alternatives to prevent the contamination by *L. monocytogenes* and the further dissemination of listeriosis caused by this bacteria.

Tables

Table 1- *Listeria monocytogenes* isolates used in the current study

Strains	Origin	Lineage	Serotype
Scott A	Clinical	I	4b
FW03/0035	Food	I	4b
F2365	Clinical	I	4b
84-1886	Animal	I	4b
78-2183	Animal	I	4b
92-0305	Animal	I	4b
80-4798	Animal	I	4b
77-2294	Animal	I	4b
101M	Clinical	I	4b
JBL 2365	Clinical	I	4b
79-0869	Animal	I	4b
79-1828	Animal	I	4b
L2 (S2657)	Animal	I	1/2b
79-3194	Animal	I	1/2b
FW04/0019	Clinical	I	1/2b
ATCC 19111	Animal	II	1/2a
FW04/0025	Food	II	1/2a
FW03/0034	Food	II	1/2a
TF/F6854	Food	II	1/2a
L1 (S2542)	Animal	II	1/2a
H776	Industry	II	1/2a
Hal-21MFS.2	Industry	II	1/2a
Livev	Clinical	II	1/2a
Joyce	Animal	II	1/2a
87-0041	Animal	II	1/2a
80-3354	Animal	II	1/2a
90-0053	Animal	II	1/2a
86-3009	Animal	II	1/2a
78-1098	Animal	II	1/2a
80-3453	Animal	II	1/2a
Fw04/0017	Food	II	1/2a

Table 2 – Frequency distribution of *Listeria monocytogenes* isolates (n=31)

	Qualitative Assignment	Percentual (%)
origin	Animal (n=17)	54.8
	Clinical (n=6)	19.4
	Food (n=6)	19.4
	Industry(n=2)	6.5
serotype	1/2a (n=16)	51.6
	1/2b (n=3)	9.7
	4b (n=12)	38.7
lineage	I (n=15)	48.4
	II (n=16)	51.6

Figures Captions

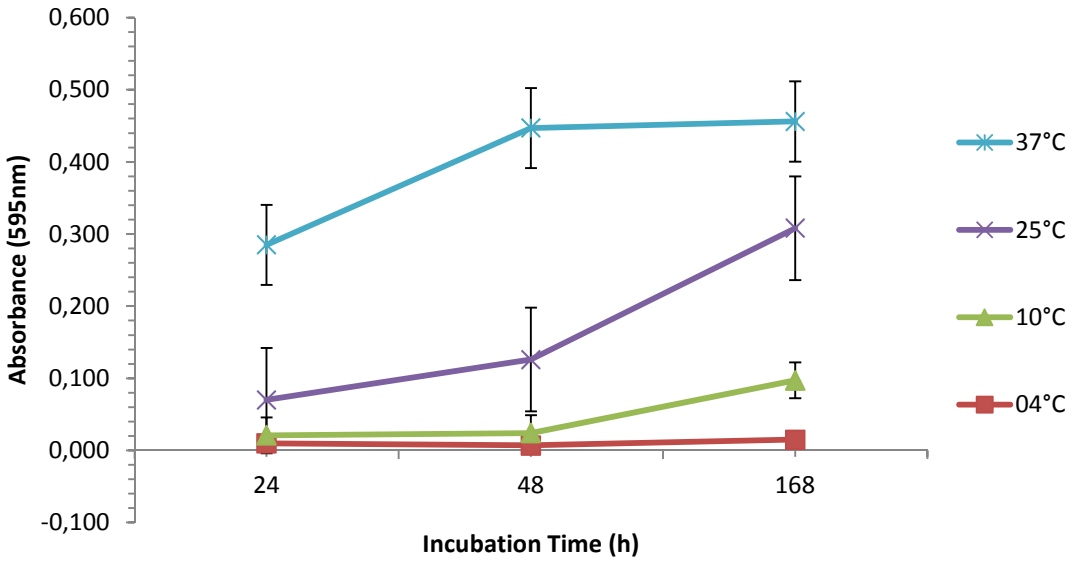

Figure. 1 - Attachment assay results for all *L. monocytogenes* isolates (n=31) cultured in BHI Medium. The mean of three independent measurements at three incubation time and four incubation temperatures are presented. Error bars indicate the standard deviation.

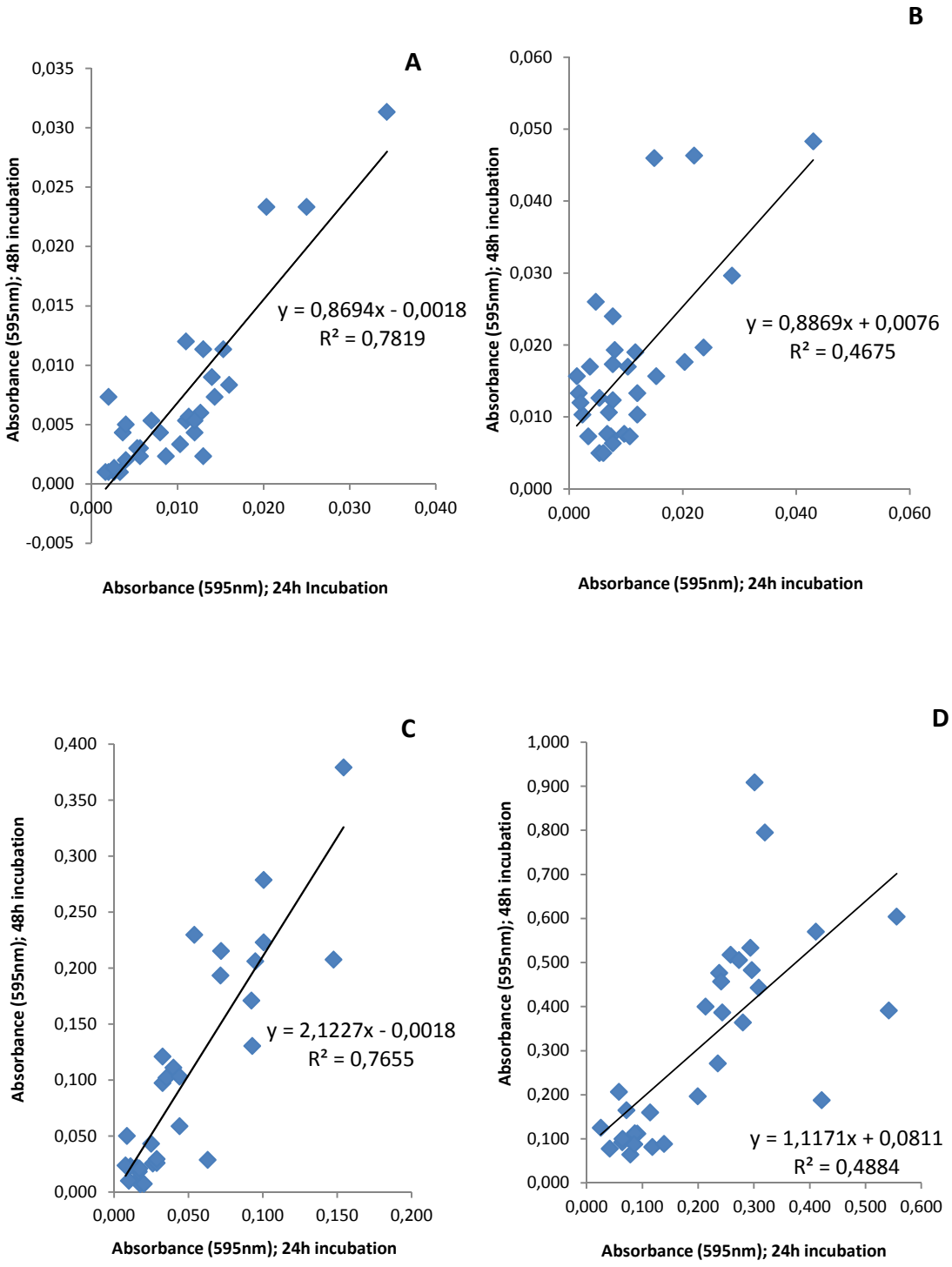

Figure. 2. Attached cells formation by *L. monocytogenes* isolates (n=31) grown in BHI medium and incubated at 4℃ (A), 10℃ (B), 25℃ (C) or 37℃ (D) measured at 24 and 48 h.

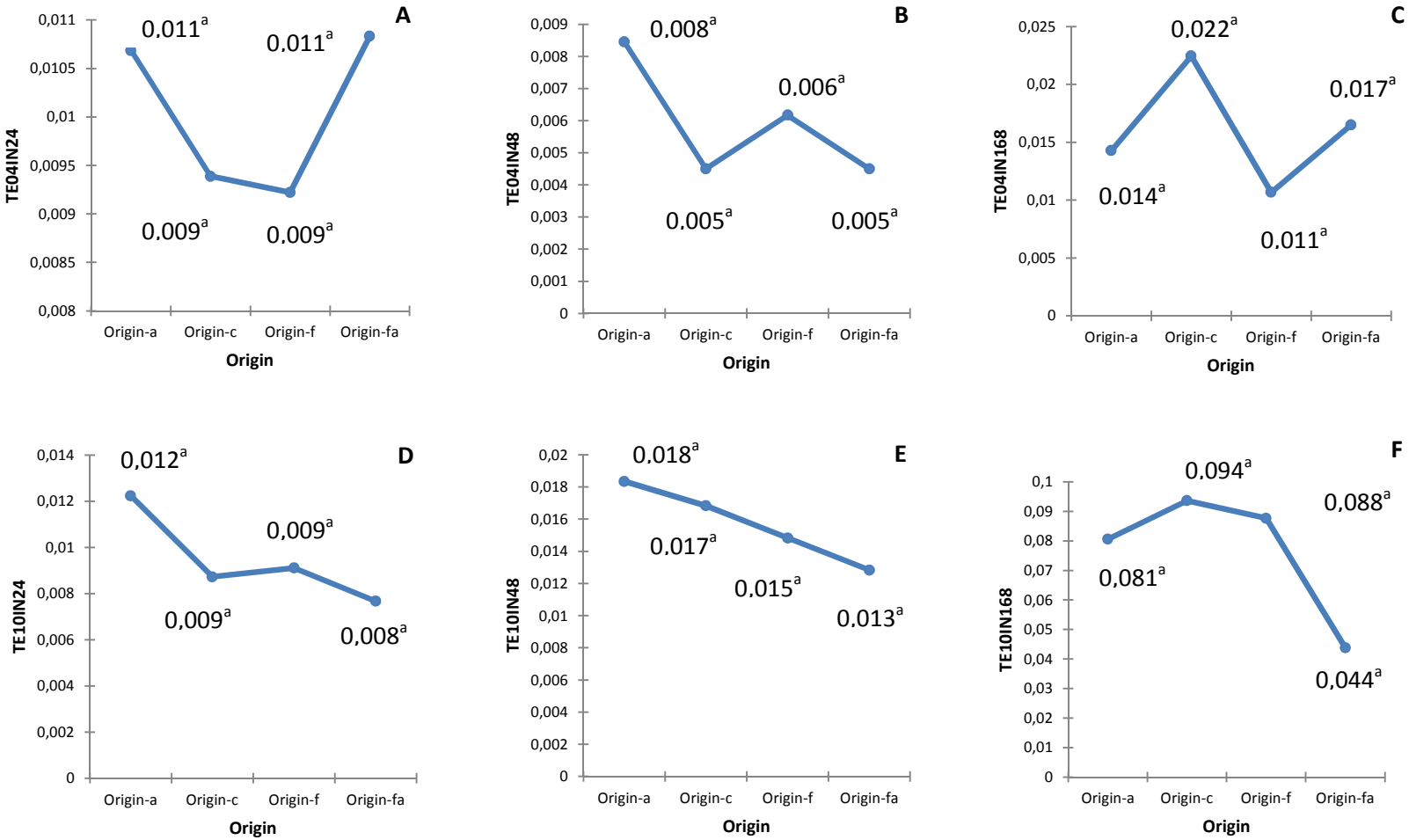

Figure 3- Analysis of the differences in the absorbance mean (OD_{595}) between the origins categories at different temperatures and time of incubation with a confidence interval of 95% (tukey – HSD); (A) 04℃-24h; (B) 04℃-48h; (C) 04℃-168h; (D) 10℃-24h; (E) 10℃-48h; (F) 10℃-168h. Homogeneous groups within each qualitative assignment were assigned the same subscript letter within the Figure. Values with a unique subscript letter differ significantly at $p \leq 0.05$.

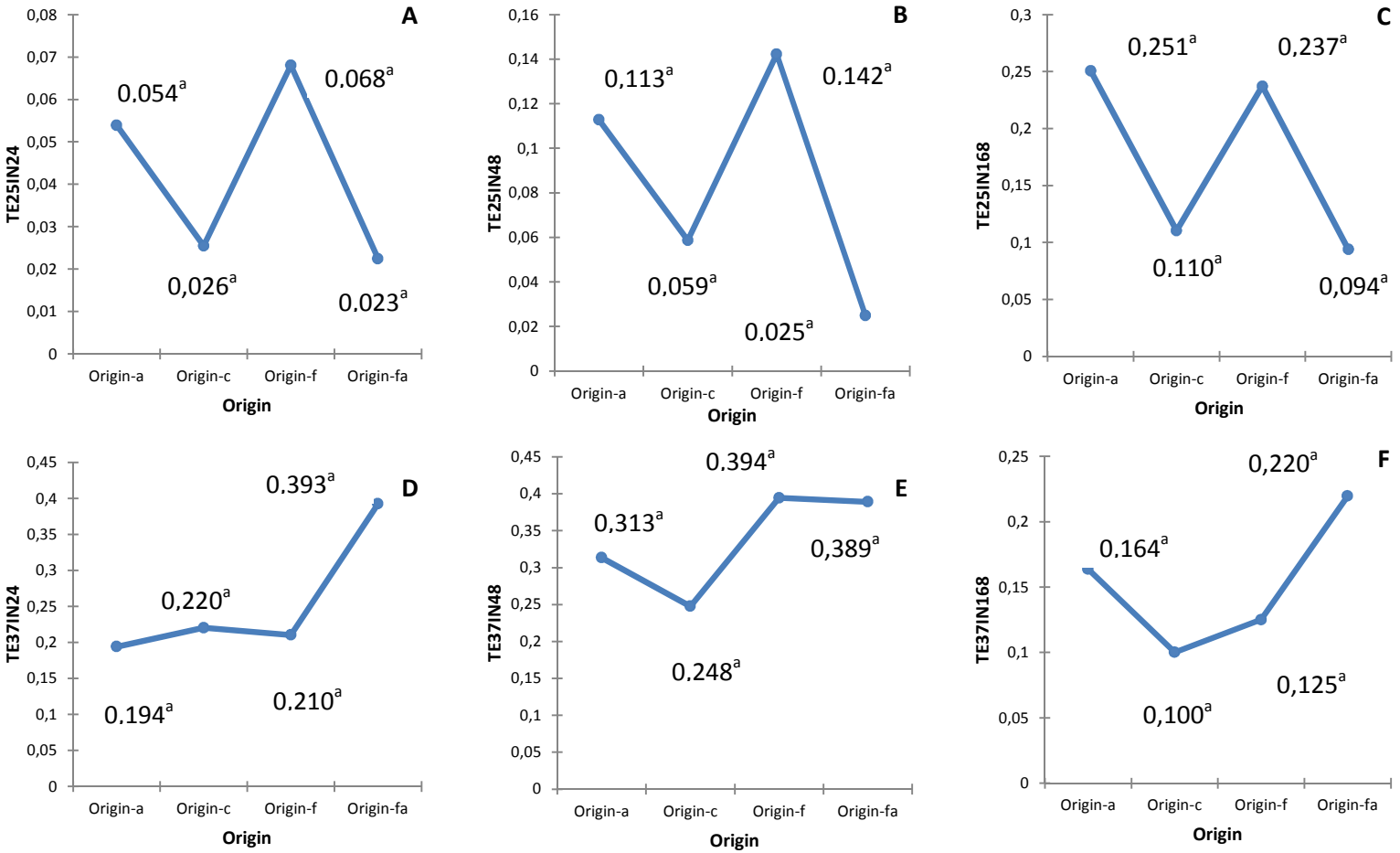

Figure 4- Analysis of the differences in the absorbance mean (OD_{595}) between the origins categories at different temperatures and time of incubation with a confidence interval of 95% (tukey – HSD); (A) 25℃-24h; (B) 25℃-48h; (C) 25℃-168h; (D) 37℃-24h; (E) 37℃-48h; (F) 37℃-168h. Homogeneous groups within each qualitative assignment were assigned the same subscript letter within the Figure. Values with a unique subscript letter differ significantly at $p \leq 0.05$.

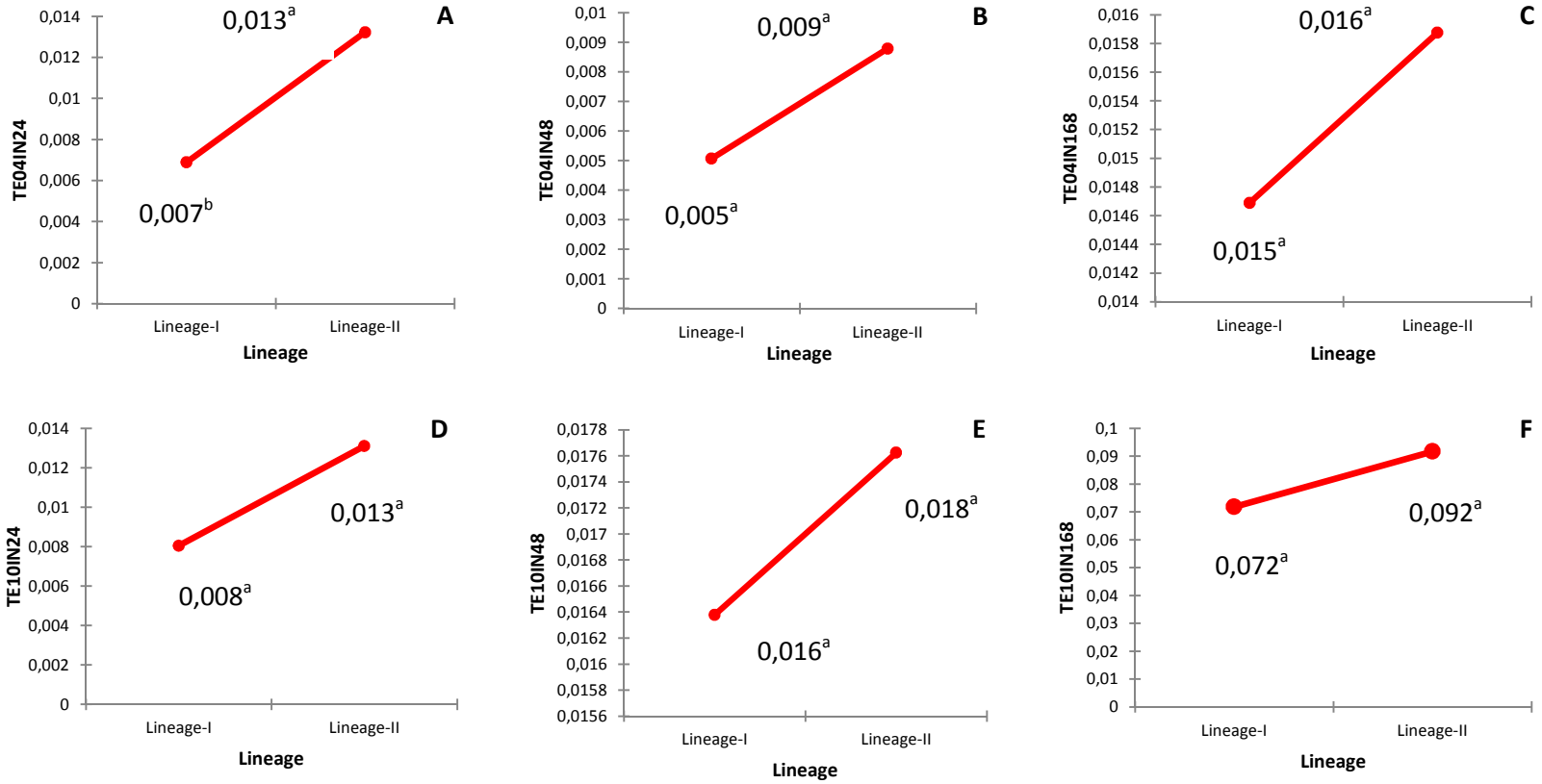

Figure. 5 Analysis of the differences in the absorbance mean (OD_{595}) between the lineages categories (I and II): (A) at 04℃-24h; (B) at 04℃-48h; (C) at 04℃-168h; (D) at 10℃-24h; (E) at 10℃-48h and (F) at 10℃-168h of incubation time with a confidence interval of 95% (t– HSD). Homogeneous groups within each qualitative assignment were assigned the same subscript letter within the Figure. Values with a unique subscript letter differ significantly at p≤0.05.

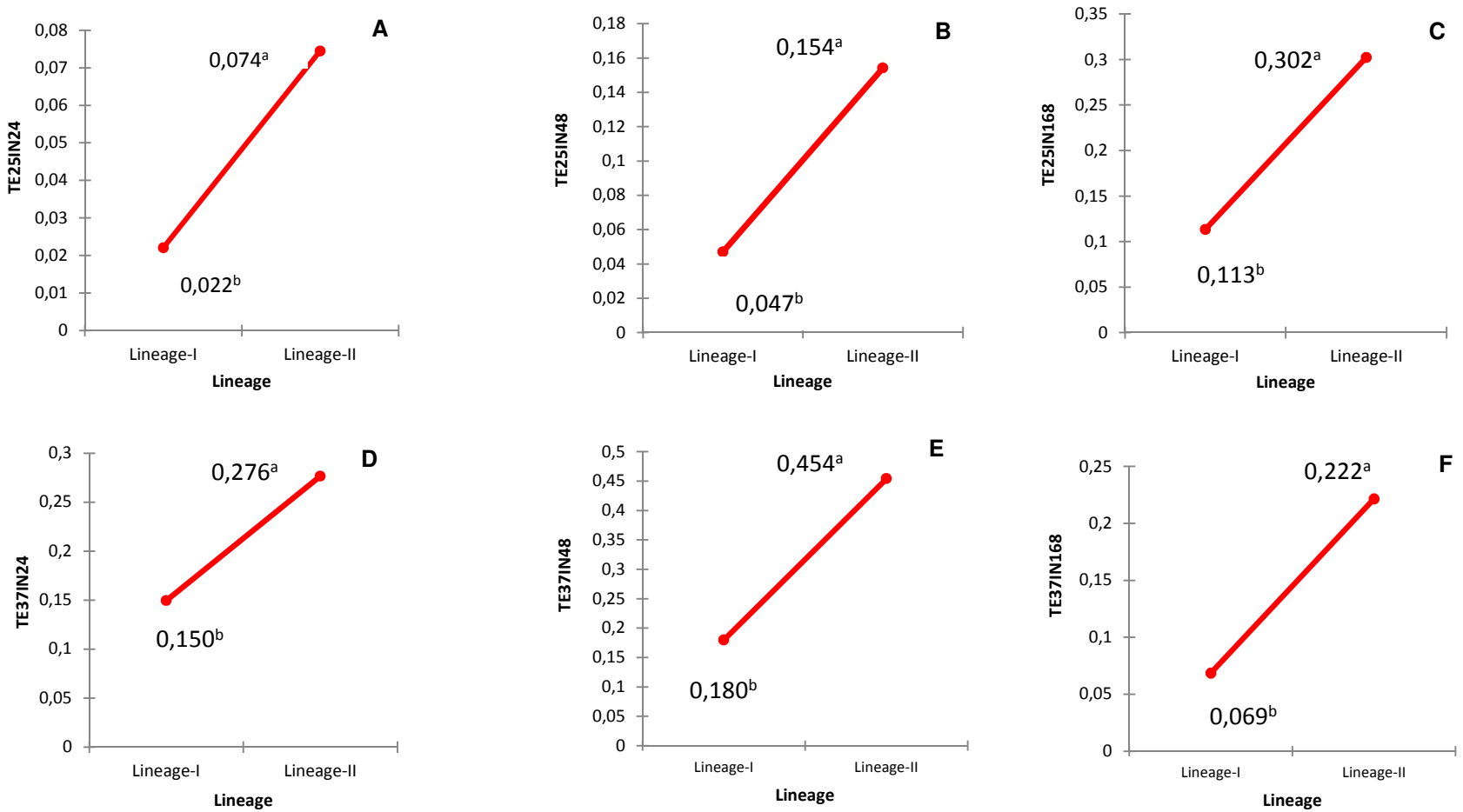

Figure. 6 Analysis of the differences in the absorbance mean (OD_{595}) between the lineages categories (I and II): (A) at 25℃-24h; (B) at 25℃-48h; (C) at 25℃-168h; (D) at 37℃-24h; (E) at 37℃-48h and (F) at 37℃-168h of incubation time with a confidence interval of 95% (t– HSD). Homogeneous groups within each qualitative assignment were assigned the same subscript letter within the Figure. Values with a unique subscript letter differ significantly at $p \leq 0.05$.

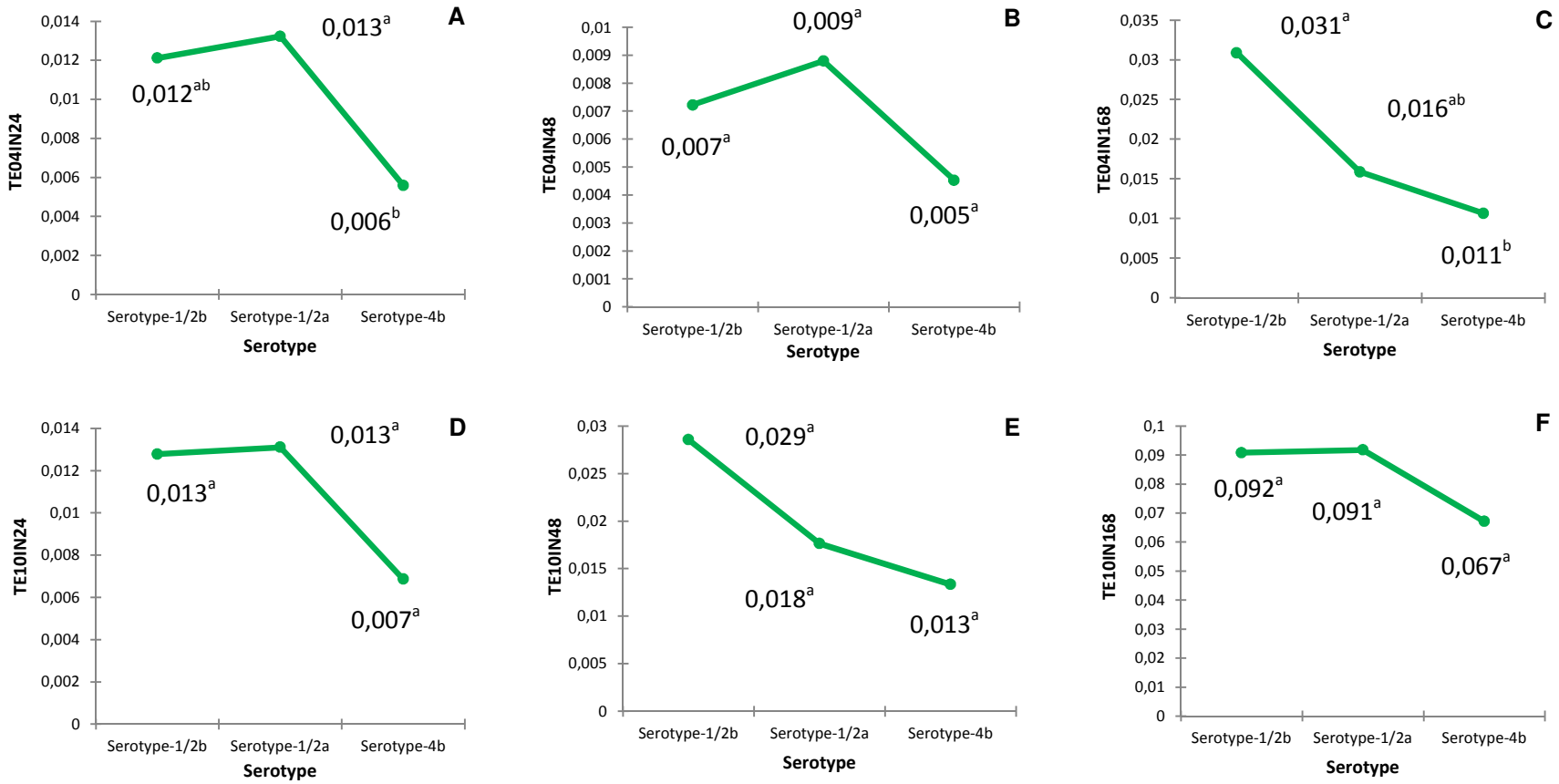

Figure 7. Analysis of the differences in the absorbance mean (OD_{595}) between the serotype categories (1/2a; 1/2b and 4b): (A) at 04℃-24h; (B) at 04℃-48h; (C) at 04℃-168h; (D) at 10℃-24h; (E) at 10℃-48h and (F) at 10℃-168h of incubation time with a confidence interval of 95% (t– HSD). Homogeneous groups within each qualitative assignment were assigned the same subscript letter within the Figure. Values with a unique subscript letter differ significantly at $p \leq 0.05$.

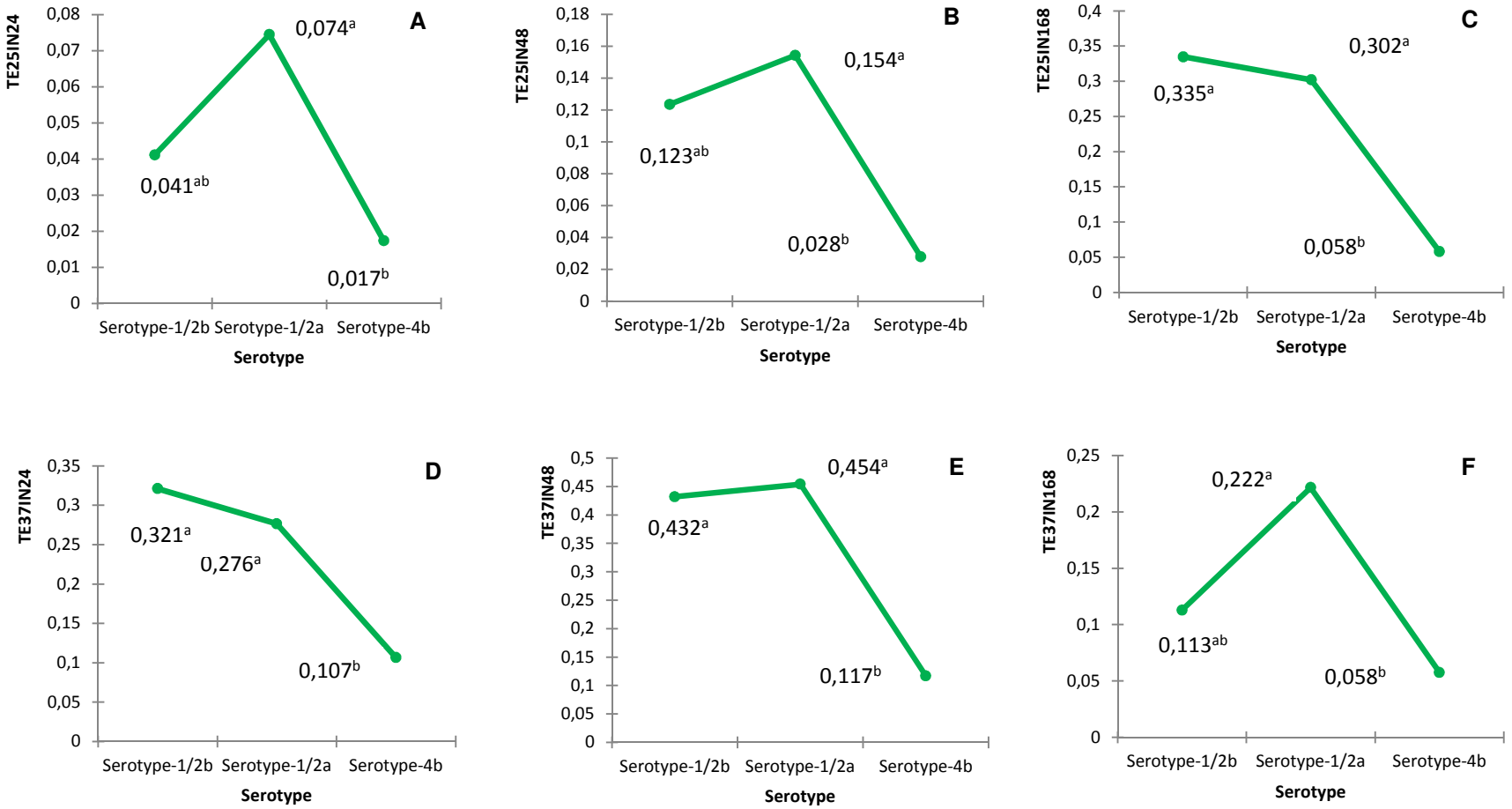

Figure 8. Analysis of the differences in absorbance mean (OD_{595}) between the serotype categories (1/2a; 1/2b and 4b): (A) at 25℃-24h; (B) at 25℃-48h; (C) at 25℃-168h; (D) at 37℃-24h; (E) at 37℃-48h and (F) at 37℃-168h of incubation time with a confidence interval of 95% (t– HSD). Homogeneous groups within each qualitative assignment were assigned the same subscript letter within the Figure. Values with a unique subscript letter differ significantly at p≤0.05.

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3.2 Artigo 2

Global proteomic responses of *L. monocytogenes* growing as planktonic and attached cells when growth media and temperature are the same

(Artigo formatado segundo as normas do Periódico Journal of Proteome Research)

Global proteomic responses of *L. monocytogenes* growing as planktonic and attached cells when growth media and temperature are the same

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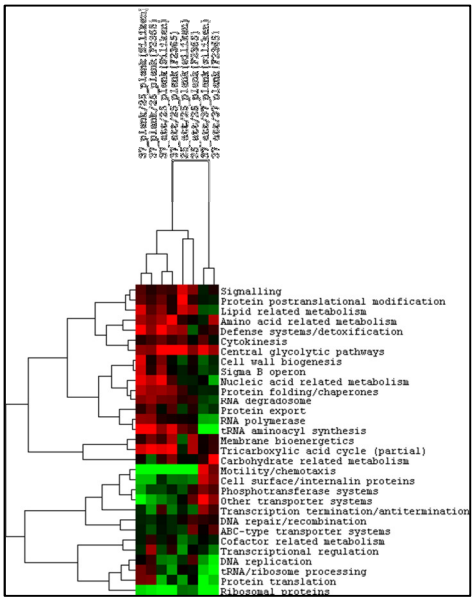
ABSTRACT: *L. monocytogenes* is able to adhere to a variety of food processing surfaces and form attached cells. Thus, there is a considerable risk of disseminating endemic *L. monocytogenes* throughout food industry environments and into food products so that persistent industry contamination by *L. monocytogenes* can have serious economic and public health implications. Comprehensive proteomics experiments using label-free 1D- liquid chromatography/tandem mass spectrometry (1D-LC/tandem MS) were performed to determine if the physiology of *L. monocytogenes* strains (Siliken and F2365) is altered markedly as attached cells compared to its planktonic state when growth media and temperature are the same. Only 8 proteins demonstrated substantial changes in common between both strains and temperatures in attached cells compared to their planktonic counterparts. They are: GroEL, DnaK, PtsH, PdxS, Pgi, RpsB, RpsD, and RpsP. Moreover, it was observed that the cell surface protein BapL abundance, though low, was not enhanced in attached cells suggesting its role in adherence could be a generalized contribution to the cell wall hydrophobicity. Interestingly, our experiment suggest that at 25°C the attached cells in both strains undergo flagella synthesis repression. Also, Sig B Regulon can be associate with an enhanced general stress response occurs in lineage II Strain (Siliken) but not in lineage I Strain (F2365) and could relate to the consequences of attachment. The temporal survey-based approach demonstrates clearly that high coverage represents a powerful means to investigate dynamic responses in *L. monocytogenes* from a functional genomics perspective.



Keywords: Protein profile, Plaktonic cells, Attached cells, 1D-LC/Tandem MS

INTRODUCTION

Listeria monocytogenes is a Gram-positive pathogenic bacterium responsible for human and animal listeriosis. Invasive listeriosis predominantly affects immuno-compromised individuals including pregnant women, elderly and patients whose immunity is compromised by drug treatment and/or an underlying disease.[1,2]

L. monocytogenes is notorious for its ability to survive under a diverse array of deleterious and harsh environmental conditions, such as acidic food, gastric secretions, freezing temperature, high salinity, following phagocytosis. When exposed to those adverse environments, *L. monocytogenes* is particularly adept at initiating an acid-tolerance response (ATR) that allows it to survive and cross-protects itself against those forms of stress.[3,4,5,6] In addition, *L. monocytogenes* is able to adhere to a variety of surfaces where food is in contact with, including polystyrene, polypropylene, glass, stainless steel, quartz, marble, granite and bunan rubber (materials commonly used in food processing equipment) and form biofilms.[7,8] Thus, there is a considerable risk of disseminating endemic *L. monocytogenes* throughout the industry and food products, being the persistent industry contamination by *L. monocytogenes* a serious economic and public health problem.[9,10]

The biofilms structures confer protection to bacterial cells and decrease the efficiency of cleaning and disinfection procedures.[11] Attention should be called to the fact that *L. monocytogenes* is unable to form thick multilayer biofilms with 10^9–10^{12}CFU/cm^2 in the way it occurs with other bacteria commonly found in biofilms, as it adheres to surfaces with populations reaching 10^4–10^7 CFU/cm^2 [12], so in this study instead of using the term “biofilm” we will refer to *L. monocytogenes* adhered cells as “attached cells”. The formation of biofilms in association with surfaces is comprised of five steps: (1) bacterial cells in the planktonic form adhere to a particular solid surface by means of van der Waals forces, electrostatic forces, and hydrophobic interactions, (2) cellular proliferation and production of extracellular polymeric substances (EPS) occurs, (3) more complex structures are constructed, (4) a mature biofilm containing channels for flow of nutrients and excreta is formed, and (5) cell dispersion occurs, with subsequent colonization of other surfaces.[13]

Several studies have showed that the main factors that influence the ability of *L. monocytogenes* to adhere and to form biofilms are the physicochemical properties of the contact surface (electrostatic charge and hydrophobicity), the diversity of

serotypes, the expression of flagella, the EPS composition, and the environmental conditions, such as pH, temperature, and culture medium.[14,15,16,17,18,19]However, molecular mechanisms of how environmental and intrinsic factors modulate *L. monocytogenes* biofilm formation are still not well understood. Consequently, there is considerable interest in determining the mechanisms of the initial attachment and subsequent biofilm formation of *L. monocytogenes*.

The aim of the present study was to compare the global proteomic responses of *L. monocytogenes* growing as planktonic and attached cells when growth media and temperature were the same, using label-free 1D - liquid chromatography/tandem mass spectrometry (1D-LC/tandem MS).

MATERIAL AND METHODS

L. monocytogenes Strains and Experimental Growth Conditions

For these experiments two *L. monocytogenes* strains were chosen. Siliken 204231/1 (referred from here on as simply “Siliken”), a serotype 1/2c industry isolate, was selected because it proficiently attaches to inanimate surfaces including polystyrene and glass wool (personal communication). The second strain, F2365, was chosen for poorly attaching to surfaces (personal communication). This strain is of serotype 4b and has a complete genome sequencing.[20] The experiment was conducted at two temperatures 25ºC and 37ºC and focused on cultures in the stationary growth phase, as attachment is subject to cellular physiological state and growth temperature.[21,22,23]

To prepare physiologically homogeneous experimental inoculum of both strains, cells replicates were grown in 10 mL of Brain-Heart Infusion broth (CM225, ‘BHI’; OXOID, Australia), incubated at 25ºC and 37ºC overnight. The culture conditions were repeated for a period of five days by transferring (daily) a 100 µL aliquot of the cultures to 9.9 mL of fresh BHI broth.

Harvesting Biomass from Planktonic and Attached Cells

The methodology for harvesting biomass was adapted for use in this experiment.[24,25]

The substratum used in the attachment experiments was glass wool (GW), which provides enough surface area for harvesting sufficient biomass for protein extraction. The density of the GW used in this study was experimentally estimated, a mean diameter of 11 µm indicates a surface of 290 cm^2 per gram of GW.

For the establishment of Planktonic Glass Wool Cells (PGW) and the Attached Glass Wool Cells (AGW), 3 mL of 24 hours culture approximately (OD_{600} of 1.3 to 1.4 - cell concentrations ~5×10^9 CFU/mL for all strains- growth curve) was added to 300 mL of BHI containing 6 g of glass wool. All strains were grown without agitation and harvested after 24h at 25°C and 37°C. PGW cells were recovered from the culture medium (ten 15mL tubes with 12 mL PGW culture each) by centrifugation at 5000 x *g* for 15 min and pellets were washed twice in PBS. The cells were concentrated in one final tube. Then to AGW harvesting, glass wool was aseptically removed from the BHI after incubation by using sterile tweezers, sterile pipette, and gently washed twice in PBS (30mL each time) and blotted dry on filter paper to remove planktonic cells. The dried glass wool was placed in a sterile flask containing 60g of glass beads (6 mm in diameter) and 30 mL of PBS, and shaken vigorously by hand for 10 min to detach the cells attached to the glass wool. The liquid phase was collected in three 15 mL tubes with 10 mL AGW biomass harvesting and centrifuged at 5000 x g for 15 min to pellet the detached cells. The pellets were washed twice in sterile PBS and the cells were concentrated in one final tube. Both PGW and AGW cells were stored at -20°C until protein extraction. One of the replicate pellets were used to the quantification of biomass.

Quantification of Biomass

The growth of PGW cells was characterized by determining vegetative cell as colony forming units (CFU) per mL by using the pour plate technique. The pour plete technique was also used to estimate attached cells (AGW) counts as CFU.cm^2 of GW. All determinations were performed on three separate samples.

Proteomics Analysis

Protein Extraction

PGW and AGW cells were collected for protein extraction as described before[24,25]. These cells were used to prepare total protein extraction. Two hundred microliter of cell suspension were lysed by beating with 0.5 g of glass beads (0.1 mm) using a mini-bead beater (MBB-8; Biospec Products) for 3-5 min with cooling on ice for 5 min. Cellular debris was removed by centrifugation and the collected supernatant was centrifuged at 15. 000 x g for 30 min at 4ºC.[26] Protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Victoria, Australia) according to the manufacturer's instructions.

Protein Digestion

Volumes of protein extract containing 50 µg of protein were transferred to clean Lobind microcentrifuge tubes, frozen with liquid nitrogen, and freeze-dried for 48 h using a Dynavac mini ultra-cold vacuum freeze drier (Technolab, Kingston, Tasmania, Australia). The concentrated protein samples were digested with tripsin as described previously.[27] After digestion, the samples were transferred to high pressure liquid chromatography vials (Waters, USA).[10]

Protein Analysis

1D-LC/tandem MS analysis was performed as done previously[6, 28] using a ThermoFinnegan LTQ Orbitrap tandem mass spectrometer with a nano-electrospray ion source operated with a fragment-ion mass tolerance of 0.5 Da.

Proteins in the sample were identified by matching the peptides predicted from the tandem mass spectra data against the complete *L. monocytogenes* non-redundant database of the National Centre for Biotechnology Institute (NCBI) using

the Computational Proteomics Analysis System (CPAS) Version 8.1 (www.labkey.org).

Relative protein abundances between growth conditions were determined using the spectra counting method[29] and expressed as a fold-change (FC). Statistical significance of the differences in spectra abundances for protein identifications between samples were assessed using a likelihood ratio test for independence (G-test) adjusted using the William's correction (Gadj) to reduce false positive rates.[30,31] Finally, a scatter plot of fold-change (2.5), determined to be significant at $p < 0.05$ by adapting the LOESS method[32] was prepared and only proteins greater than or equal to both criteria were considered to be biologically relevant and further discussed.[10] In order to gain a broad understanding of the dataset the individual biological replicates were spatially analyzed. Firstly the inter sample group variability and overlap of treatments was assessed using non-parametric multi-dimensional scaling (nMDS) plots using Primer-6. The global comparisons were also examined at the functional level. Identified proteins were classified, on the basis of ontology, into distinct functional classes as performed previously.[6, 33]

RESULTS AND DISCUSSION

Proteomic analysis of *L. monocytogenes* strains to examine planktonic and attached state cellular physiology

Comprehensive proteomics experiments were performed to determine if the physiology of attached *L. monocytogenes* is markedly altered when compared to its planktonic state when growth media and temperature are the same.

The total number of spectra analyzed and non-singleton proteins obtained from strains Siliken and F2365 across all conditions tested that passed the filtration criteria are shown in Table 1.

Global protein-level comparisons of attached and planktonic cells of *L. monocytogenes* strains

The results indicated that both strains when grown at 37°C in the planktonic phase produce apparently indistinguishable protein profiles when replicate variability

is considered (R=0.1, p=0.18). Secondly, attached populations grown at 37°C had similar profiles to their planktonic counterparts, based on distance within the MDS spatial analysis. However, still at 37°C, considerable variability was found for the Siliken derived samples as compared to those from strain F2365. There was more dispersion and distinctiveness on protein profiles of the individual treatments from 25°C grown cultures (from those grown at 37°C), however attached and planktonic cell derived protein profiles showed considerable overlap for both strains (R=0.30-0.35, p=0.05-0.07) (Fig. 1).

A heat map based on hierarchically clustered t-values is shown below (Fig. 2), providing both an indication of functional trends but also unsupervised comparisons of the strains and treatments in relation to a control state, in which cells were grown at 25°C and harvested as planktonic cells. A t-value of >+3 and <-3 can be considered statistically significant ($p<0.05$).

Hierarchical cluster analysis of the functional data (Fig. 2) revealed similar treatment relationships to that of the MDS plots (Fig.1), where the strain genetic differences had the limited influence, instead the physicochemical treatments were much more important in defining protein abundance patterns. The temperature was the most influential parameter, with 37°C grown cultures being separated from the 25°C grown attached cultures in the T-value-based heat map. Attachment status was also clearly separated for cultures grown at 37°C but not at 25°C.

Based on the heat map t-value data the changes in protein profiles associated to attachment (in comparison to planktonic cells) are subtle and the associated trends are usually weaker statistically (Fig. 2). However, at 37°C attachment promotes carbohydrate related metabolism and central glycolytic pathways while DNA replication, tRNA/ribosome processing, ribosomal proteins and tRNA aminoacyl synthesis protein groups are reduced in overall abundance. This data suggests that broad induction of stress protective responses is not associated with attachment. Still, at 37°C attachment, the changes to motility / chemotaxis and cell wall proteins likely suggest within the population that promote the attachment, a reversed link, leading to cells that readily detach, however variability between replicates were so large and these trends were not statistically significant. At 25°C the differences between attached and planktonic states apparently are on the surface, however trends suggests that there are less definable differences between

the states as compared to 37°C simply due to the greater replicate-to-replicate variability.

Overall the MDS and functional group cluster data shows a profound effect of the temperature on *L. monocytogenes* as indicated in some previous studies [34,35] while strain genetics and attachment states have discernible but more subtle influences.

Virulence proteins and the effect of attachment status

Virulence in *L. monocytogenes* requires several proteins some of which are actively involved in the initial cell invasion, evasion of the immune system, and cell-to-cell spread. From the data it is clear that though various virulence-associated factors show significantly increased abundance there is no evidence that “virulence capacity” per se is promoted outside of a host at 37°C or when attached.

At 37°C several proteins showed an increased abundance if compared to 25°C in both strains (Fig. 3) including the proteins that have a house-keeping support role for activities.

Comparisons between attached and planktonic cells otherwise showed no consistent patterns especially for cells grown at 37°C. Overall, only certain proteins associated with virulence were affected by the conditions of the experiment. Proteins with overt connection to GI tract and intracellular survival, such as bile salts hydrolase (Bsh) were found to be much more abundant in both planktonic and attached Siliken cells at 37℃ when compare with 25℃ grow (up to 20 fold) and LLO, a pore-forming thiol-activate cytolysin was more abundant, signifcantly in the case of Siliken attached cells grew at 37°C (3-6 fold). These proteins showed strain dependent and attachment dependent responses, however, the other proteins relevant to virulence presented only general support roles and are primarily promoted at 37°C due to the induction of stress response at that temperature. Other virulence proteins were not detected either due to extraction biomass or due to culture conditions acting to suppress their synthesis, in particular catabolite-suppression due to the high level of carbohydrates in the growth medium.

Comparison of attached versus planktonic cells

The numbers of proteins that increased and decreased significantly in abundance in attached Siliken and F2365 cells compared to planktonic counterparts are shown in Venn diagrams (Fig. 4) and Supl. Table 2. Only 8 proteins demonstrated substantial changes in common between both strains and temperatures. These included the following proteins: GroEL (Hsp60 chaperonin large subunit), DnaK (molecular chaperone), PtsH (phosphotransferase system phosphocarrier Hpr), PdxS (pyridoxine biosynthesis lyase subunit), and Pgi (glucose6-phosphate isomerase). RpsB, RpsD, and RpsP (small subunit ribosomal proteins) all showed decreases in abundance. These results suggest attached cells potentially have increased protein folding, and increased carbohydrate uptake and metabolism.

a) Effect on motility/chemotaxis proteins

Surface attachment is clearly associated with changes in cellular flagellation and thus related to changes in the electrostatic properties of the cell.[22] Our experiments showed that attached cells grown at 25°C with both strains have 3 to 9-fold less abundance of flagellin FlaA than planktonic cells as well as the flagella hook protein FlgE (8-10-fold less) suggesting attached cells have overall reduced flagellation. At 37°C, in both planktonic and attached samples, FlaA was either undetected or present at very low levels (Fig. 5) suggesting cells were generally denuded of flagella. Interestingly, the results suggest that at 25°C the attached cells undergo flagella synthesis repression. At either 25°C or 37°C the abundance of flagella motor switch protein FliM remained relatively constant in both strains suggesting the flagella motor protein complex itself does not seem diminished in attached cells.

In Siliken samples at 37°C grow, the chemotaxis sensor proteins CheA and CheY were reduced in abundance when compared with 25°C grow cells while in F2365 the putative flagellar hook-length control protein (lmo0695) was reduced (10-fold reduction) at 25°C (Fig. 5).

b) Cell wall biogenesis related changes

In biomass grown at 25°C there were little consistent changes in cell wall biogenesis-related protein abundances, either between attachment states or between strains. The results suggest that, in general, peptidoglycan biosynthesis seems to be repressed in attached cells, which may reflect in the reduction of the growth rate (see below part g). D-alanylation of Teichoic Acid (TA) is carried out by 4 proteins DltABCD. DltB, being a membrane protein, was underrepresented in the dataset. DltA showed also low abundance, but was most abundant in Siliken planktonic cells grown at 37°C but not detected in attached cells, while in F2365 highest abundance of DltA occurred in attached cells grown at 25°C. In both strains, especially Siliken, increased DltD and DltC abundance at 37°C than at 25°C (Fig. 6) could promote adherence due to potentially increased D-alanylation of TA (as observed in other related bacteria).[36] Elimination of the gene *dltD* has been shown to reduce adherence by as much as 80%.[37] The difference in the abundance of DltD, which promotes the transfer of D-alanine to the ligation site[38] could be a factor in the greater attachment of Siliken compared to F2365 to polystyrene and glass wool surfaces. Studies into how Wall Teichoic Acid (WTA) and TA influences surface charge specifically as well as strain-to-strain variation are required to confirm these observations.

Assuming D-alanine residues are surface exposed, this could reduce cell surface electrostatic interactions, which would render less adherent cells due to less interaction with eDNA surface deposits or other cells. The chemical distribution of D-alanine esterification and the effect of glycosylation of TA needs further study since it is unclear to what degree it influences adherence and whether this is reelected in physiologically or genetically imposed differences in cellular adhesion.

c) Cell surface proteins

Besides the proteins involved in cell wall biogenesis and protein secretion, *Listeria* secretes a range of proteins that are covalently anchored to peptidoglycan via sortases or has membrane insertions that are exposed partially on the cell surface. Some of these surface exposed proteins have possible adhesin-like roles.[39] Besides well studied internalins (such as InlA and InlB) that have roles in host cell

adhesion and invasion, little information is available about contributions of surface proteins to adherence to inanimate surfaces. One peptidoglycan-bound protein, termed BapL (biofilm-associated protein, lmo0435) has been linked to surface adherence in strains belonging to lineage II (such as Siliken).[40] Here it was observed that BapL abundance, though low, was not enhanced in attached cells, suggesting its role in adherence could be a generalized contribution to the cell wall hydrophobicity. No other cell surface protein (as defined) [39] were found to be enriched in the attached cell samples of either strain.

d) Extracellular DNA

Extracellular DNA (eDNA) of high molecular weight interacting with cell wall-derived N-acetylglucosamine (NAG) has been shown to promote early attachment of *Listeria*.[19] In another recent study eDNA deposition seems to be carried out by a DNA translocase FstK-like protein normally also involved in cell division.[41] Based on the protein profiles there was no convincing evidence that eDNA synthesis itself is more active in attached cells. In addition due to the membrane-bound nature of FtsK (lmo1386), it was hard to be certain that it was more abundant in attached cells since few spectra were detected.

e) Quorum sensing related proteins

Adherence to glass has also been linked to the *agr* quorum sensing gene cluster that encodes a two component sensory system (AgrAC) and a putative quorum sensory peptide and its exporter (AgrDB).[42] The actual function of the agr system is still poorly understood but is known that *agr* gene expression is enhanced in attached cells in a growth phase dependent manner while this is not the case in planktonic cells. Deletion of genes *agrA* or *agrD* results in *L. monocytogenes* strain EGD-e having poorer adherence.[42] We observed that, at both temperatures, Siliken attached cells showed substantially more abundance of the exporter AgrB (12-19 fold) while in F2365 this was significant only at 25°C (Fig. 7). AgrB is an integral membrane endopeptidase that is putatively required for production of the mature quorum sensory peptide as determined in an analogous version of the agr system in *Staphylococcus aureus*.[43]

The enzyme Mtn converts S-adenosylhomocysteine, a toxic metabolite derived from S-adenosylmethyionine, to SRH. The enzyme LuxS degrades SRH. This compound seems to induce, by an unknown mechanism, adherence.[44] We observed Mtn levels were higher at 37°C then 25°C in both strains while LuxS abundance was not substantially altered (Fig. 7). The proteomics-based results do circumstantially suggest a possible role of the agr system quorum sensing and also the Mtn/LuxS systems in attachment in *Listeria*, however the downstream gene cascade of the regulatory responses and associated physiological links remains to be determined. Furthermore, the data suggests the Mtn/LuxS levels is temperature dependent and could partly explain temperature-dependent influences on rates of attachment observed for various strains of *L. monocytogenes* [23], however further work is needed to understand the mechanism by which SRH enhances adherence and whether it acts as a chemical messenger or has another unknown capacity.

f) Metabolic impact of attachment

We observed that attached cells have a potentially temperature-dependent altered metabolism based on changes in proteins associated with transport, catabolism, and various aspects of intermediary metabolism. The overall data suggests crowding of sessile cells on the glass wool substratum creates a quasi-acid stress tolerance response. This response however was strain and temperature – dependent suggesting that acidic stress itself was not likely to occur but rather protein changes represents a broader metabolic adjustment beyond what occurs in the stationary growth phase.

SigB regulon is enhanced in attached Siliken cells when grown at 25°C as well as at 37°C unlike F2365. It is possible there is a link within the regulon members, which include many uncharacterized proteins to either adherence or physiologcial traits possessed by attached cells.

The SigB core regulon[46] associated proteins overall were found to be statistically elevated in abundance at 25°C for attached Siliken cells compared to planktonic cells (Fig. 8). Promotion of SigB regulated proteins was equal between planktonic and attached cells cultured at 37°C.

The data suggests an enhanced general stress response occurs in Siliken (but not F2365) and could be related to the consequences of the attachment. SigB-dependent glutamate decarboxylase GadB2 (lmo2434), which aids in acid shock protection was more abundant at 37°C than at 25°. However, no difference was found in terms of cell attachment state suggesting that specifically acid shock survival is not necessarily affected but potentially broad stress cross protection could occur. Acid habituated cells have shown to become more adherent [21,23] and the changes observed here could reflect this occurrence.

Certain SigB-*independent* proteins associated with pH homeostasis were promoted in attached cells at 25°C. Interestingly, the response was stronger in F2365. Glutamate decarboxylase GadB (lmo2363) had 7-fold and 180-fold greater abundance in Siliken and F2365, respectively. The apparent increase in GadB could be due to localised H^+ and organic acid accumulation in the vicinity of the attached cells. The lack of detection of AlsD (acetolactate decarboxylase, required for acetoin production) and reduced abundance of both phosphotransacetylase and acetate kinase (which lead to acetate formation), suggests acetoin and acetate production could be minimal or produced at reduced levels by attached cells.

g) Growth related and stress induction in attached cells

A large number of proteins involved with lipid-related metabolism, nucleic acid and nucleotide metabolism, DNA replication, transcription, mRNA turnover and protein translation have their abundance reduced in attached cells of both strains at both temperatures, especially at 37°C (Supl. Table 1). This data suggests attached cells show slower growth rates than planktonic cells. This agrees with the observation that cell division proteins FtsA and FtsE are also reduced in abundance in attached cells grown at 37°C. The increased abundance of the pleotrophic regulatory protein CodY at 37°C could act constraing the metabolism and carefully conserving the supplie of cellular energy. Compelling evidence of a stringent response, which would suggest amino acid starvation in attached cells[47] was not observed due to the RelA/SpoT paralogs (lmo0802, lmo0812, lmo1523) being presented at only very low abundance in attached cells.

Attached cells were found to have increased abundance of a number of stress protective proteins though the responses are clearly strain- and temperature dependent. At 25°C both strains showed increases in the non-heme iron-binding protein Fri/Dps (4-5 fold), conjugated bile salt hydrolase (3-19 fold) and a DJ-1/PfpI protein family YhbO-like protein (lmo2256) that in *E. coli* and *Bacillus subtilis* is required for either protection or repair of oxidation, pH and UV stress associated stress damage.[48,49] At 37°C stresses protective proteins are overall elevated compared to that at 25°C, so a small difference was observed between attached and planktonic cells, however catalase (lmo2785) and a OhrA/OhrB-like peroxiredoxin (lmo2199) were significantly more abundant in F2365 attached cells. At 37°C peroxide stress regulator PerR regulon associated proteins[50] are more abundant in general while the Fur regulon is more abundant in attached cells (Fig. 9). Both Fur and PerR act to restrict iron uptake since excessive levels cause oxidative stress via the Fenton reaction.[51] The results suggesting that attached cells (at 37°C) exhibit oxidative stress related responses. The increased level of Dps likely acts to protect DNA from oxidative damage.[52] These is consistent with a study that demonstrated superoxide dismutase (Sod) has an important role in *Listeria* biofilms.[53]

The overall results suggest oxidative stress protection as well as acid stress protection via the GAD system seem to be more enhanced in attached cells though the degree of protection is affected by a combination of strain and temperature factors.

CONCLUSIONS

For instance, the cultivation temperature substantially influences protein profiles above and beyond the effects of strain genetic type as well as adeptness in the ability to attach to polystyrene and to glass wool.

Consistent protein abundance changes independently from the strain or the temperature suggest that although the attached cells have an increased protein folding, and an increased carbohydrate uptake and metabolism, but they grow more slowly.

Surface attachment is clearly associated with changes in cellular flagellation. Interestingly, our experiment suggest that at 25°C the attached cells in both strains

undergo flagella synthesis repression and at 37°C, in both planktonic and attached samples, in both strains cells were generally denuded of flagella.

We find that the role of BapL in adherence could be a generalized contribution to the cell wall hydrophobicity.

Sig B Regulon can be associate with an enhanced general stress response occurs in Lineage II Strain (Siliken) but not in Lineage I Strain (F2365) and could relate to the consequences of attachment.

Further work is needed to determine exactly how Agr quorum sensing peptide and AI-2-like Mtn/LuxS systems act to promote adherence.

The label-free 1D-LC/MS/MS temporal survey-based approach demonstrates clearly that high coverage is possible of bacterial proteomes and thus represents a powerful means to investigate dynamic responses in *L. monocytogenes* from a functional genomics perspective.

Table

Table 1- Spectra and protein numbers analyzed in *L. monocytogenes* strains.

Strain	Treatment	Total spectra analyzed	Non-singleton proteins identified
Siliken			
	37°C planktonic	25944	724
	37°C attached	21523	620
	25°C planktonic	17787	475
	25°C attached	12788	419
F2365			
	37°C planktonic	16487	580
	37°C attached	20112	612
	25°C planktonic	14890	438
	25°C attached	15134	555

Figures Captions

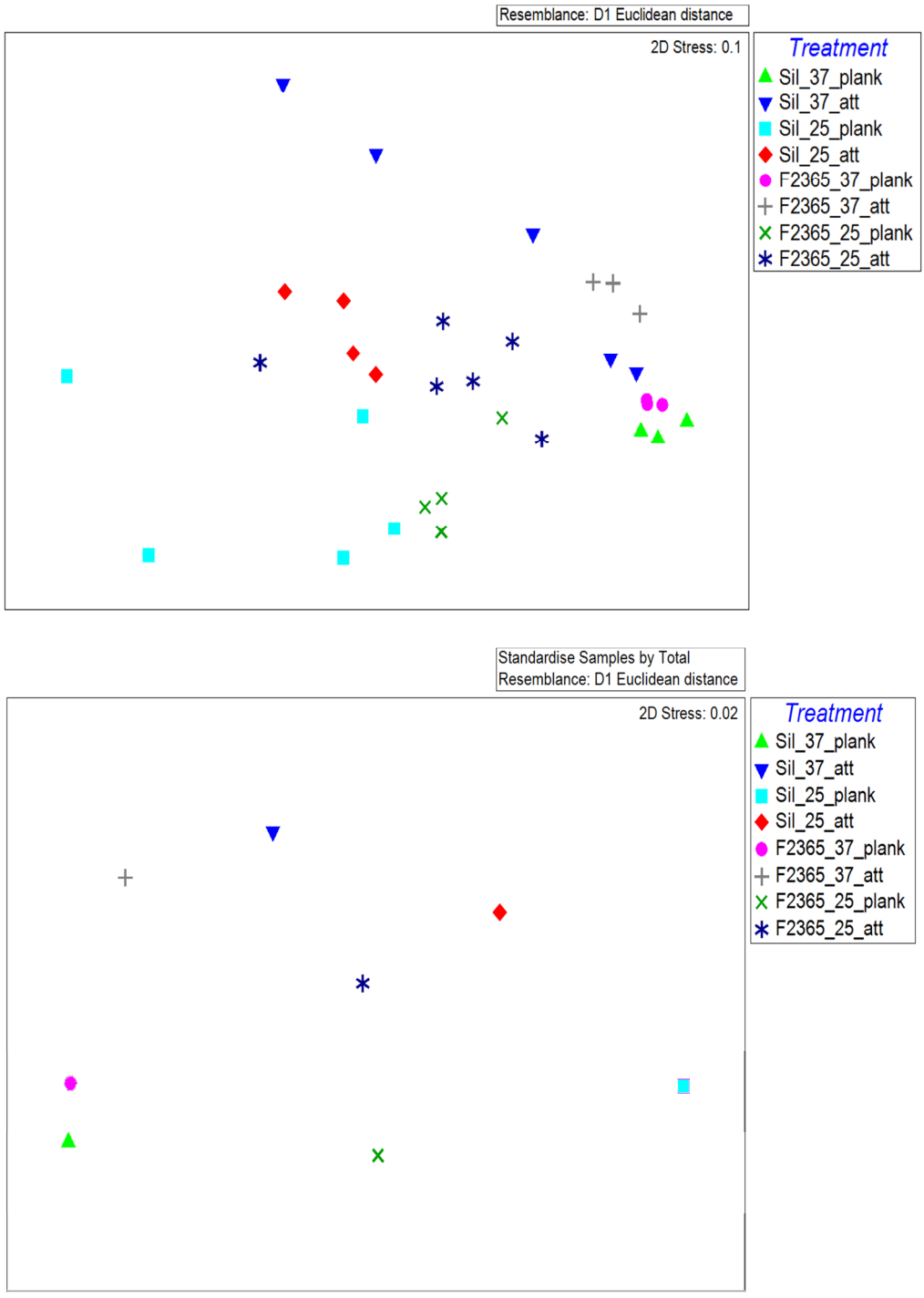


Figure 1. Non-parametric MDS plots spatially visualizing similarity of protein profiles obtained from *L. monocytogenes* strains (Sil, Siliken; F2365) grown at two different temperatures (25°C and 37°C) and when in a planktonic (plank) or attached state (att; attached to glass wool). The top graph shows variability between replicate samples while the bottom graph shows the relationship of the treatments when the spectral counts are fully pooled.

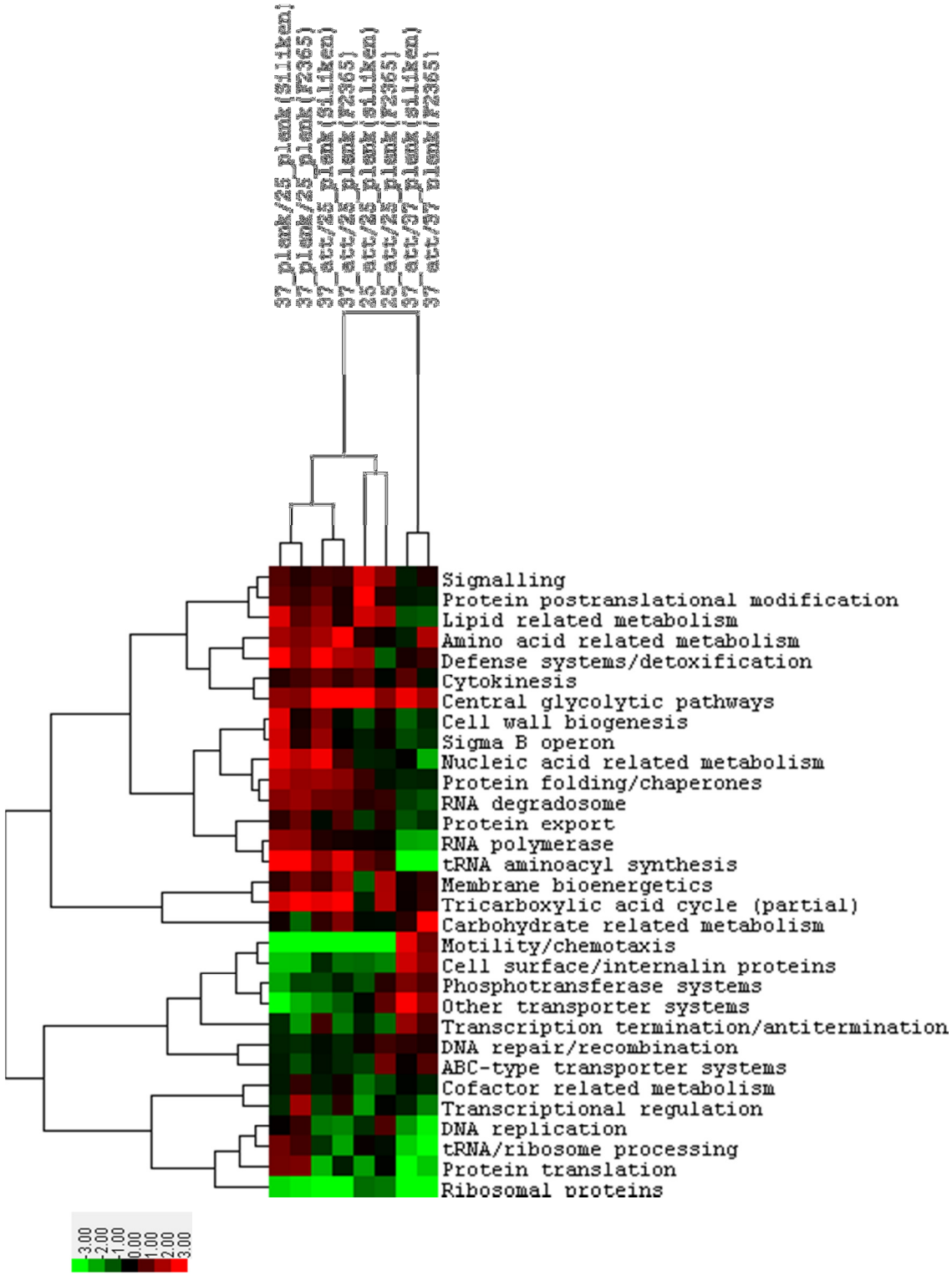


Figure 2. Heat map showing abundance change trends in functional groups of proteins in *L. monocytogenes* strains Siliken and F2365 grown in a planktonic (plank) or attached state (att; attached to glass wool),.at two different temperatures (25°C and 37°C). The log ratio scale is to a base of two.

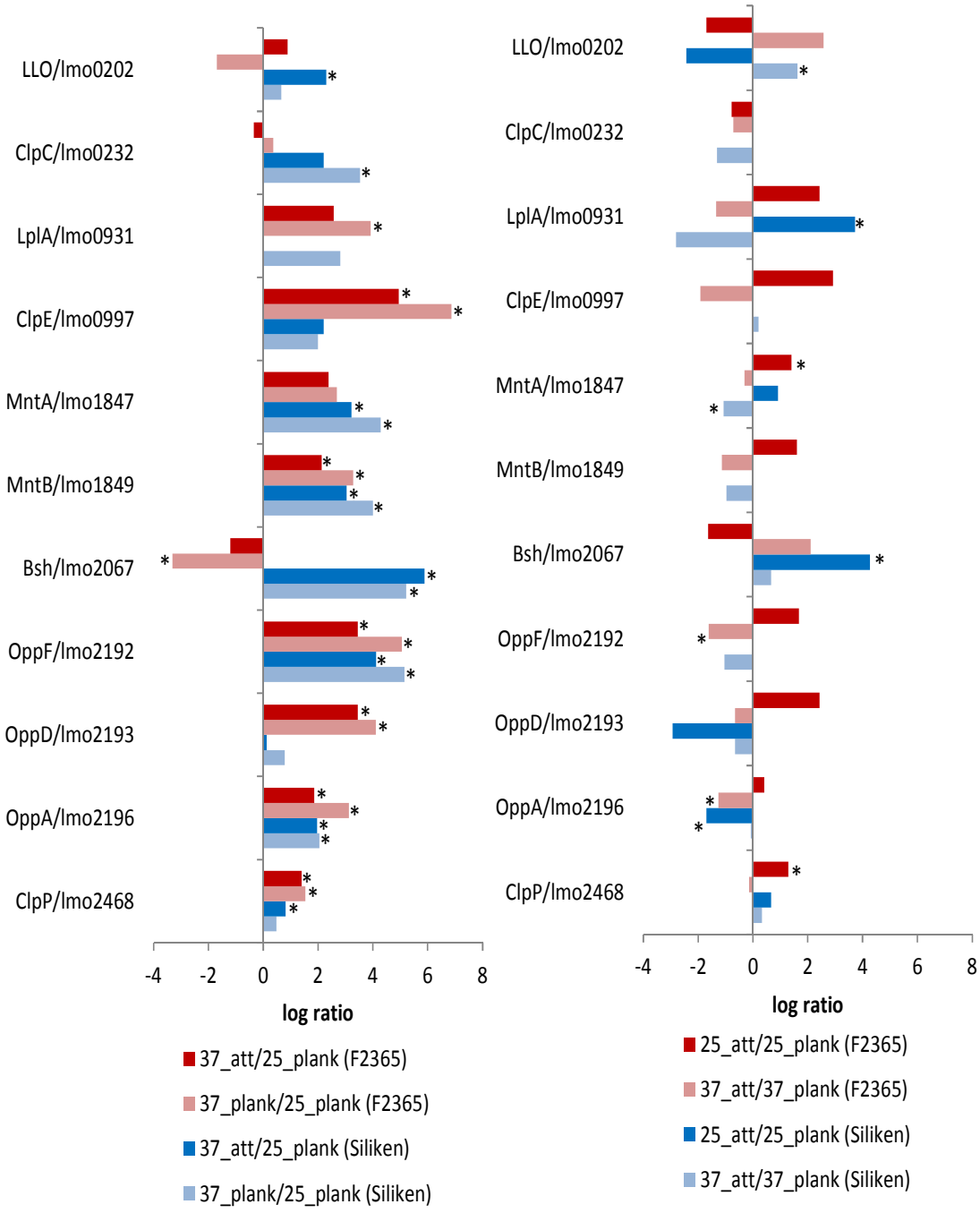
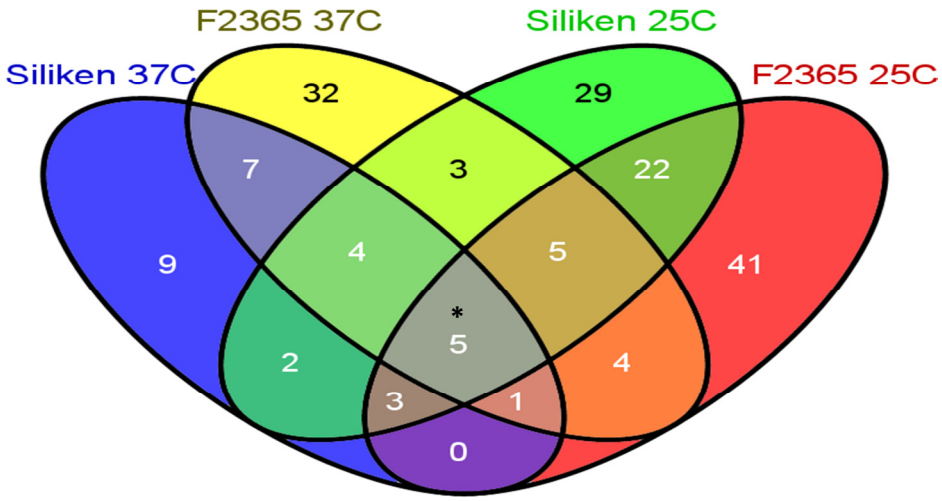


Figure 3. Fold change in virulence associated proteins in glass wool attached and planktonic cells of *L. monocytogenes* strains Siliken and F2365. Asterisk indicate a significant fold change ($p<0.05$).

Proteins with increased abundance:



Proteins with reduced abundance:

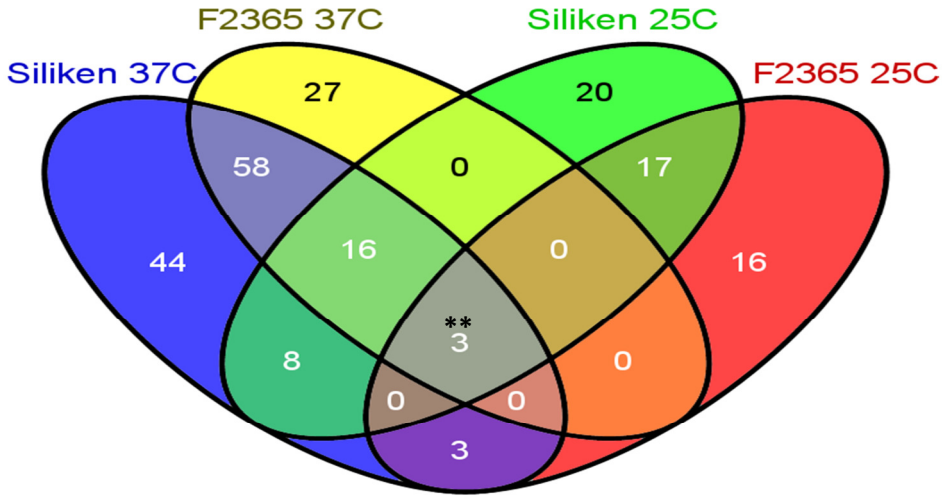


Figure 4 Venn diagrams showing the number of proteins that exhibited significant increased (top diagram) and decreased (bottom diagram) abundance (>2-fold, $p<0.05$) between two *L. monocytogenes* strains grown at either 37°C or 25°C with biomass investigated either taken from planktonic or attached fractions of cultures containing glass wool as an adherent substratum. * GroEL (Hsp60 chaperonin large subunit), DnaK (molecular chaperone), PtsH (phosphotransferase system phosphocarrier Hpr), PdxS (pyridoxine biosynthesis lyase subunit), and Pgi (glucose6-phosphate isomerase). ** RpsB, RpsD, and RpsP (small subunit ribosomal proteins).

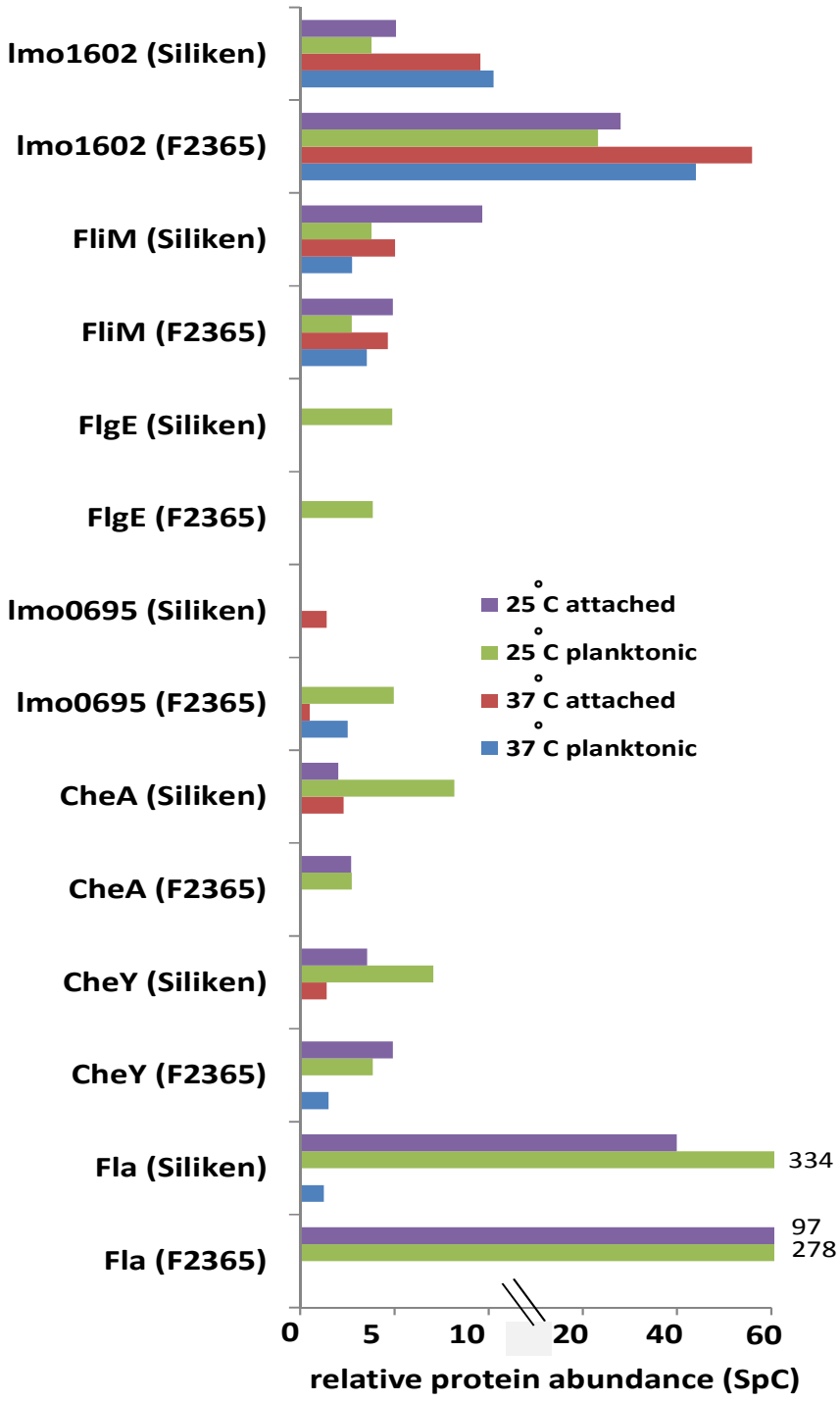


Figure 5 Relative abundance (normalized spectral counts) of proteins associated with either motility or chemotaxis processes. Data is derived from two *L. monocytogenes* strains grown at either 37°C or 25°C with biomass investigated either taken from planktonic or attached fractions of cultures containing glass wool as an adherent substratum. Values on the bars for FlaA are here out of the scale (>60) SpC values.

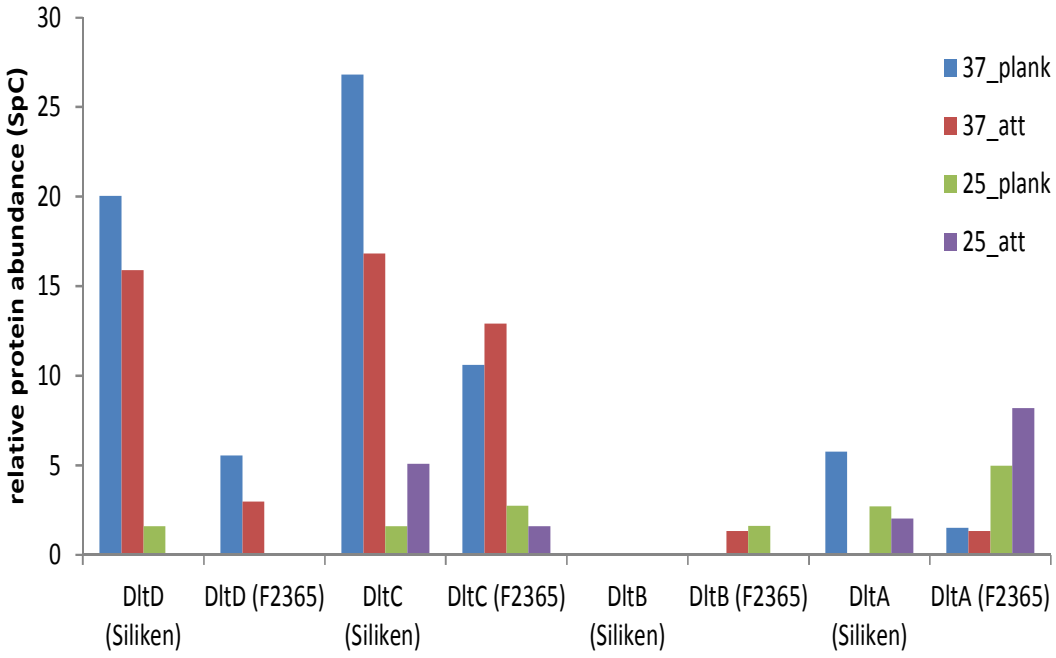


Figure 6 Relative abundance (normalised spectral counts) of proteins associated with teichoic acid D-alanylation (DltA, DltB, DltC, DltD). Data is derived from two *L. monocytogenes* strains grown at either 37°C or 25°C with biomass investigated either taken from planktonic or attached fractions of cultures containing glass wool as an adherent substratum.

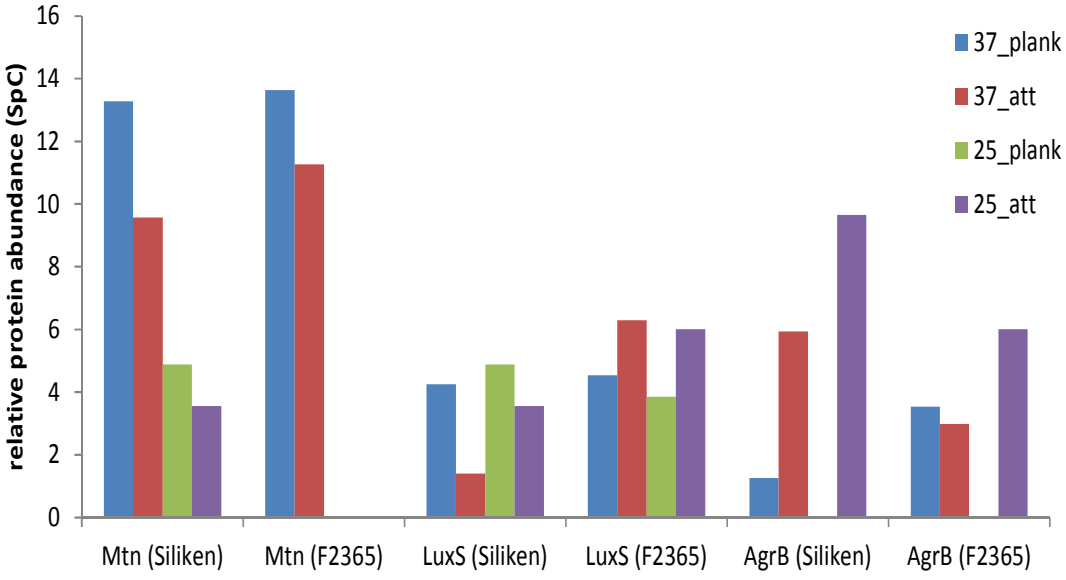


Figure 7.Relative abundance (normalised spectral counts) of proteins associated with quorum sensing (AgrB of the accessory gene system) as well as proteins associated with putative chemical messenger like activities including Mtn (5-methylthioribose kinase) and LuxS (S-ribsyl-homocysteine lyase). Data is derived from two *L. monocytogenes* strains grown at either 37°C or 25°C with biomass investigated either taken from planktonic or attached fractions of cultures containing glass wool as an adherent substratum.

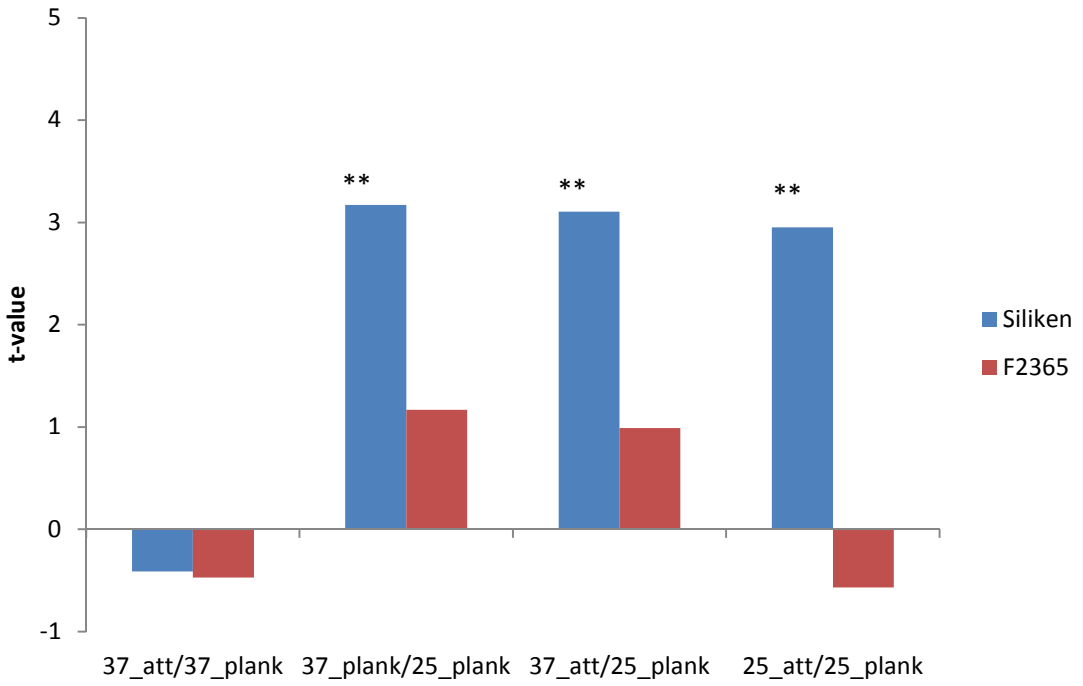


Figure 8 Statistical trends for the abundance changes associated with proteins coded by genes belonging to the SigB core regulon. Asterisks indicate significance (** $p<0.01$).

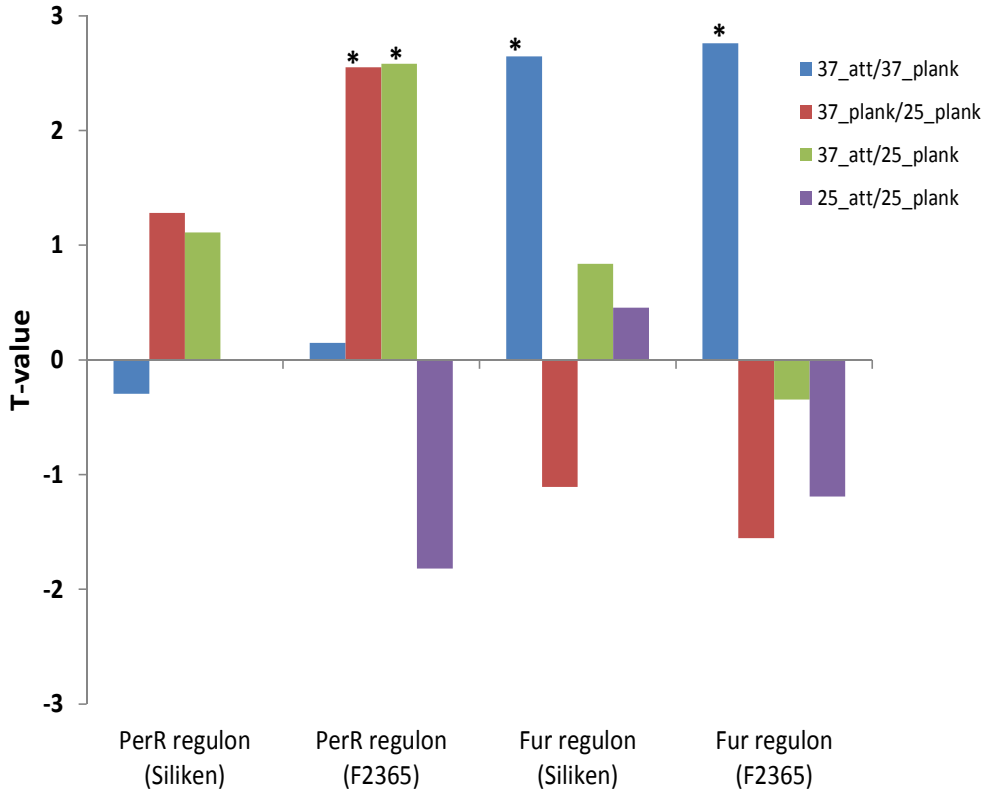


Figure 9.Statistical trends for the abundance changes associated with proteins coded by genes belonging to the PerR and Fur regulons. Asterisks indicate significance (* $P<0.05$).

Associated Content

Appendix A

Supplementary Table 1

EGD-e locus	Siliken_1/2c Fold change (37°C)	significance (G-test)	F2365_4b Fold change (37°C)	significance (G-test)	Predicted/known function	Functional group
lmo2373	**1,84**	0,03138	1,39	0,29108	PTS system (Lactose/DACB/beta-glucoside family) IIB	phosphotransferase systems
lmo1002	**1,70**	0,00000	**1,61**	0,00000	PTS system phosphocarrier HPr component PtsH	phosphotransferase systems
lmo0613	1,09	0,85312	**2,56**	0,00934	quinone oxidoreductase, NADPH-dependent	membrane bionergetics
lmo0690	**5,47**	0,03922	ND	ND	flagellin Fla	Motility
lmo0196	0,97	0,84364	**1,54**	0,00203	regulator of septum location	cell division
lmo1804	2,06	0,38650	**9,28**	0,04984	chromosome condensation and segregation protein	cell division
lmo0183	ND	ND	**1,53**	0,00399	alpha-1,4-glucosidase, GH31_glucosidase_II_MalA family	carbohydrate metabolism
lmo0401	1,31	0,56809	**1,96**	0,00000	alpha-mannosidase	carbohydrate metabolism
lmo0271	0,98	0,94632	**2,09**	0,00841	aryl-phospho-beta-glucosidase	carbohydrate metabolism
lmo0539	**1,42**	0,00922	**2,41**	0,00430	LacD/DeoC-like aldolase, TIM_phosphate_binding superfamily	carbohydrate metabolism
lmo0078	0,72	0,60159	**3,39**	0,01240	mannoside-phospho-beta-D-glucosidase	carbohydrate metabolism
lmo2094	**1,62**	0,04982	**3,67**	0,00268	putative type II aldolase, aldolase_II superfamily	carbohydrate metabolism
lmo1254	1,07	0,78582	**3,68**	0,00045	alpha,alpha-phosphotrehalase	carbohydrate metabolism
lmo0574	ND	ND	**10,94**	0,02784	mannoside-phospho-beta-D-glucosidase	carbohydrate metabolism
lmo1179	2,03	0,21644	**12,59**	0,01557	aldehyde dehydrogenase EutE	carbohydrate metabolism
lmo1177	2,03	0,21644	**29,16**	0,00005	carboxysome shell protein EutL	carbohydrate metabolism

EGD-e locus	Siliken_1/2c Fold change (37 C)	significance (G-test)	F2365_4b Fold change (37 C)	significance (G-test)	Predicted/known function	Functional group
lmo0348	1,67	0,20299	**1,66**	0,02471	fructose 1,6-bisphosphate aldolase type II	central glycolytic pathways/glycolysis
lmo1571	**1,77**	0,00001	**1,55**	0,00660	6-phosphofructokinase PfkA	central glycolytic pathways/glycolysis
lmo1571	**1,77**	0,00001	**1,55**	0,00660	glucose-6-phosphate isomerase	central glycolytic pathways/glycolysis
lmo2367	**1,38**	0,00003	**1,45**	0,00005	dihydroxyacetone kinase, N-terminal domain	central glycolytic pathways/glycolysis
lmo2455	**1,64**	0,00000	**1,57**	0,00000	enolase Eno	central glycolytic pathways/glycolysis
lmo2457	**1,97**	0,00000	1,19	0,23659	triosephosphate isomerase TpiA	central glycolytic pathways/glycolysis
lmo2459	**3,23**	0,00000	**1,21**	0,00059	glyceraldehyde-3-phosphate dehydrogenase Gap	central glycolytic pathways/glycolysis
lmo2556	**1,57**	0,00000	**1,53**	0,00000	6-phosphofructokinase	central glycolytic pathways/glycolysis
lmo1305	**1,66**	0,00010	**1,57**	0,00181	transketolase Tkt	central glycolytic pathways/pentose phosphate pathway
lmo1995	**1,95**	0,00015	1,22	0,49377	deoxyribose-phosphate aldolase DeoC	central glycolytic pathways/pentose phosphate pathway
lmo2663	1,15	0,88175	**2,58**	0,01928	putative (6-phospho)gluconolactonase	central glycolytic pathways/pentose phosphate pathway
lmo2674	**2,56**	0,02314	**3,48**	0,05533	ribose 5-phosphate isomerase B RpiB	central glycolytic pathways/pentose phosphate pathway
lmo2743	ND	ND	**15,91**	0,00488	fructose 6-phosphate transaldolase, transaldolase_FSA Family	central glycolytic pathways/pentose phosphate pathway
lmo1611	1,00	0,98870	**1,52**	0,02955	putative glutamyl aminopeptidase, M20_dimer superfamily	amino acid metabolism
lmo1578	1,41	0,34411	**1,66**	0,03254	putative PepP_like Xaa-Pro aminopeptidase, APP_like family	amino acid metabolism
lmo1620	1,20	0,33169	**1,69**	0,00664	putative PepV-like dipeptidase, M20_dimer superfamily	amino acid metabolism
lmo1217	**1,78**	0,05071	**1,72**	0,02103	aspartate-semialdehyde dehydrogenase Asd	amino acid metabolism
lmo1711	1,20	0,52233	**1,74**	0,03623	putative leucyl aminopeptidase	amino acid metabolism
lmo1437	1,44	0,09789	**1,87**	0,01165	putative glutamyl aminopeptidase, M20_dimer superfamily	amino acid metabolism
lmo1350	1,30	0,40112	**2,15**	0,00067	glycine dehydrogenase subunit 2 GcvP	amino acid metabolism
lmo2425	1,32	0,41305	**3,39**	0,01240	glycine cleavage system lipoylprotein GcvH	amino acid metabolism
lmo2006	0,32	0,10090	**4,80**	0,00901	2-acetolactate synthase AlaS	amino acid metabolismo

EGD-e locus	Siliken_1/2c Fold change (37 C)	significance (G-test)	F2365_4b Fold change (37 C)	significance (G-test)	Predicted/known function	Functional group
lmo0265	1,40	0,44298	**9,28**	0,04984	succinyl-diaminopimelate desuccinylase DapE	amino acid metabolism
lmo2338	2,81	0,50284	**9,28**	0,04984	putative PepC-like cysteinyl aminopeptidase, peptidase C1 family	amino acid metabolism
lmo0223	1,32	0,09487	**2,54**	0,00000	cysteine synthase CysK	amino acid metabolism
lmo0560	1,34	0,57018	**3,07**	0,01151	NADP-specific glutamate dehydrogenase	amino acid metabolism
lmo2363	1,08	0,50260	**2,01**	0,00000	glutamate decarboxylase GadB	amino acid metabolism
lmo1993	1,20	0,43990	**1,50**	0,02469	pyrimidine-nucleoside phosphorylase Pdp	nucleic acid/nucleotide metabolism
lmo1765	1,18	0,77929	**5,27**	0,02828	phosphoribosylaminoimidazole carboxamide formyltransferase/inosine-monophosphate cyclohydrolase PurH	nucleic acid/nucleotide metabolism
lmo1775	6,44	0,13623	**9,28**	0,04984	phosphoribosylaminoimidazole carboxylase, catalytic subunit PurE	nucleic acid/nucleotide metabolism
lmo2101	**1,65**	0,00050	**1,53**	0,01333	pyridoxine synthesis lyase subunit	cofactor metabolism
lmo1675	1,44	0,44569	**3,58**	0,00823	2-oxoglutarate decarboxylase / 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase MenD	cofactor metabolism
lmo2101	**1,65**	0,00050	**1,53**	0,01333	pyridoxine synthesis lyase subunit PdxS	cofactor metabolism
lmo2652	**10,06**	0,03783	1,00		MtlR-like PTS system transcriptional activator	transcriptional regulation (PTS system)
lmo1364	**1,68**	0,00693	**1,59**	0,02628	cold shock protein CspL	transcription associated
lmo1473	**1,81**	0,00000	**1,45**	0,00004	class I heat-shock protein (molecular chaperone) DnaK	protein folding
lmo0251	**2,01**	0,00000	**1,95**	0,00000	ribosomal protein L7/L12 RplL	ribosomal proteins
lmo2624	**2,38**	0,00016	1,14	0,69913	ribosomal protein L29 RpmC	ribosomal proteins
lmo1756	2,73	0,08769	**6,91**	0,00619	aspartyl/glutamyl-tRNA amidotransferase subunit C	tRNA amino-acyl synthesis
lmo1059	1,30	0,30046	**1,81**	0,02066	DsbG-like protein-disulfide isomerase, thioredoxin-like superfamily	posttranslational modification
lmo1233	**2,03**	0,00421	1,30	0,36129	thioredoxin 1 TrxA	posttranslational modification
lmo1473	**1,81**	0,00000	**1,45**	0,00004	class I heat-shock protein (molecular chaperone) DnaK	protein folding
lmo2069	**1,38**	0,00073	**1,92**	0,00000	class I heat-shock protein (chaperonin) GroEL	protein folding
lmo0202	**3,10**	0,02855	5,97	0,16103	listeriolysin O LLO	virulence

EGD-e locus	Siliken_1/2c Fold change (37 C)	significance (G-test)	F2365_4b Fold change (37 C)	significance (G-test)	Predicted/known function	Functional group
lmo0943	**1,90**	0,00003	1,09	0,67703	non-heme iron-binding ferritin Fri/Dps	iron homeostasis
lmo2785	1,28	0,53985	**1,66**	0,02256	catalase (hydroperoxidase) Kat	defence mechanisms (oxidative stress)
lmo2199	-1,42	0,74563	**9,28**	0,04984	OhrA/OhrB-like lipoyl-dependent Cys-based peroxiredoxin	defence mechanisms (oxidative stress)
lmo2471	**2,12**	0,02774	1,26	0,59066	NADH:flavin oxidoreductase, OYE family NemA-like	defence mechanisms
lmo2564	**3,95**	0,00467	2,52	0,31026	4-oxalocrotonate tautomerase family protein	general prediction only
lmo2094	**1,62**	0,04982	**3,67**	0,00268	putative type II aldolase, aldolase_II superfamily	general prediction only
lmo2565	ND	ND	**9,28**	0,04984	HD superfamily phosphohydrolase	general prediction only
lmo2800	ND	ND	**9,28**	0,04984	putative dehydrogenase, NADP-binding, Gfo/Idh/MocA family	general prediction only
lmo0534	1,40	0,44298	**2,47**	0,03953	Lin0512_fam superfamily protein	unknown function
lmo2426	0,54	0,21309	**3,19**	0,03921	DUF711 (RNR_PFL-like) superfamily protein	unknown function
lmo0512	2,51	0,24581	**9,28**	0,04984	TPR domain containing protein, PEP_CTERM family	unknown function
lmo1510	0,97	0,94614	**10,94**	0,02784	DUF1292 superfamily protein	unknown function
lmo2823	ND	ND	**12,59**	0,01557	ArsC-like family protein	unknown function
lmo1522	1,18	0,82295	**14,25**	0,00872	DUF1015 superfamily protein	unknown function
lmo2223	**2,00**	0,00023	1,39	0,14581	DUF964 superfamily protein	unknown function
lmo0198	**-5,29**	0,00130	**-3,16**	0,01956	glucosamine-1-phosphate N-acetyltransferase / UDP-N-acetylglucosamine pyrophosphorylase GlmU	cell wall biogenesis
lmo0727	**-2,91**	0,00000	**-2,91**	0,00091	L-glutamine:D-fructose-6-phosphate aminotransferase GlmS	cell wall biogenesis
lmo0974	**-11,53**	0,02263	-0,19	0,91390	D-alanine-poly(phosphoribitol) ligase DltA	cell wall biogenesis
lmo1078	ND	ND	**-2,15**	0,00842	UTP-glucose-1-phosphate uridylyltransferase GtaB	cell wall biogenesis
lmo1083	**-2,35**	0,01573	absent	absent	dTDP-glucose 4,6-dehydratase RbfB	cell wall biogenesis
lmo1084	**-10,51**	0,00033	absent	absent	dTDP-4-dehydrorhamnose reductase RbfC	cell wall biogenesis
lmo2036	**-11,05**	0,00020	**-3,37**	0,01200	UDP-N-acetylmuramoyl-L-alanine:D-glutamate ligase MurD	cell wall biogenesis

EGD-e locus	Siliken_1/2c Fold change (37 C)	significance (G-test)	F2365_4b Fold change (37 C)	significance (G-test)	Predicted/known function	Functional group
lmo2526	ND	ND	**-7,22**	0,00791	UDP-N-acetylglucosamine 1-carboxyvinyltransferase MurAA	cell wall biogenesis
lmo2691	**-10,02**	0,03838	-5,04	0,22432	gamma-D-glutamate-meso-diaminopimelate muropeptidase	cell wall biogenesis
lmo1073	ND	ND	**-5,70**	0,02785	putative teichoic acid-related ABC transporter, substrate binding protein BtuF-like	ABC-type transporters (teichoic acid)
lmo2192	ND	ND	**-3,05**	0,01486	oligopeptide ABC transporter, ATP binding protein OppF	ABC-type transporters (oligopeptides)
lmo2196	**-1,69**	0,00423	**-2,39**	0,00001	oligopeptide ABC transporter, substrate binding protein OppA	ABC-type transporters (oligopeptides)
lmo1847	**-2,10**	0,00163	-1,23	0,18503	manganese uptake ABC transporter, substrate binding protein LpeA	ABC-type transporters (manganese)
lmo1948	ND	ND	**-5,25**	0,00212	anaerobic sensing response regulator ResD	signal transduction
lmo0287	**-3,39**	0,00860	-1,73	0,16614	cell wall metabolism response regulator WalR/CpxR-like	signal transduction
lmo0515	**-5,09**	0,00836	-2,66	0,30879	UspA-like universal stress protein, Usp_like family	signal transduction
lmo2673	**-13,03**	0,01335	-3,42	0,17271	UspA-like universal stress protein, Usp_like family	signal transduction
lmo1801	**-10,02**	0,03838	-0,84	0,84314	signal recognition particle, subunit SRP54 Ffh	protein export
lmo2510	**-5,74**	0,00350	-1,64	0,56245	preprotein translocase trafficking protein SecA	protein export
lmo2507	4,05	0,32025	**-13,12**	0,01294	cell division protein; ABC Transporter, ATP-binding FtsE	cell division
lmo2033	**-2,74**	0,02515	**-2,91**	0,00853	cell divisome complex ATP-binding protein FtsA	cell division
lmo0344	4,05	0,32025	**-2,59**	0,01147	putative ribitol/xylitol (phosphate) dehydrogenase, SDRc family	carbohydrate metabolism
lmo1171	**-3,79**	0,04517	**-2,06**	0,09507	alcohol dehydrogenase, iron-dependent EutG	carbohydrate metabolism
lmo0722	**-3,63**	0,00214	-3,02	0,46594	pyruvate oxidase, thiamin-dependent, FAD-binding PoxB-like	central glycolytic pathways/fermentation
lmo1052	**-2,78**	0,00001	**-1,83**	0,01351	pyruvate dehydrogenase E1 component, alpha subunit PdhA	central glycolytic pathways/fermentation
lmo1053	**-2,25**	0,00045	-1,14	0,62795	pyruvate dehydrogenase E1 component, beta subunit PdhB	central glycolytic pathways/fermentation

EGD-e locus	Siliken_1/2c Fold change (37 C)	significance (G-test)	F2365_4b Fold change (37 C)	significance (G-test)	Predicted/known function	Functional group
lmo1054	**-2,49**	0,00000	**-2,12**	0,00315	pyruvate dehydrogenase E2 component (dihydrolipoamide acetyltransferase) PdhC	central glycolytic pathways/fermentation
lmo1072	**-3,09**	0,00000	**-3,02**	0,00000	pyruvate carboxylase PycA	central glycolytic pathways/TCA cycle
lmo1406	**-3,39**	0,00001	**-1,47**	0,01627	pyruvate-formate lyase PflB	central glycolytic pathways/fermentation
lmo1634	**-2,11**	0,00000	**-1,83**	0,00000	acetaldehyde-CoA dehydrogenase/iron-dependent alcohol dehydrogenase/pyruvate-formate lyase deactivase AdhE-like	central glycolytic pathways/fermentation
lmo1435	**-2,97**	0,00480	-1,51	0,23450	dihydrodipicolinate synthase DapA	amino acid metabolism
lmo2547	-1,13	0,52290	**-8,74**	0,00220	homoserine dehydrogenase Hom	amino acid metabolism
lmo1663	**-4,31**	0,00244	**-4,45**	0,02413	asparagine synthase (glutamine-hydrolysing) AsnB	amino acid metabolism
lmo2462	**-4,77**	0,01285	1,02	0,97372	zinc-dependent dipeptidase, rDP_like family	amino acid metabolism
lmo2546	**-8,38**	0,00226	-1,41	0,29932	threonine synthase ThrC	amino acid metabolism
lmo1331	**-2,42**	0,00032	**-1,78**	0,03674	polyribonucleotide nucleotidyltransferase PnpA	nucleic acid/nucleotide metabolism
lmo1874	**-4,12**	0,02989	**-11,10**	0,02627	thymidylate synthase ThyA	nucleic acid/nucleotide metabolism
lmo1939	**-3,79**	0,04517	**-3,98**	0,04249	cytidylate kinase Cmk	nucleic acid/nucleotide metabolism
lmo2155	**-3,43**	0,03340	**-15,14**	0,00638	ribonucleoside reductase, alpha subunit NrdE	nucleic acid/nucleotide metabolism
lmo2340	**-2,23**	0,04152	-1,29	0,49862	putative pseudouridine 5'-phosphate glycosidase, indigoidine superfamily PscG-like	nucleic acid/nucleotide metabolism
lmo2559	**-2,09**	0,00077	1,09	0,70004	CTP synthase PyrG	nucleic acid/nucleotide metabolism
lmo2693	-7,10	0,10763	**-13,12**	0,01294	thymidylate kinase Tmk	nucleic acid/nucleotide metabolism
lmo2758	**1,64**	0,00212	**-2,05**	0,00000	inosine monophosphate dehydrogenase GuaB	nucleic acid/nucleotide metabolism
lmo0814	-3,19	0,43731	**-3,05**	0,04207	putative enoyl-(acyl-carrier-protein) reductase II, NPD_like family	lipid-related metabolism
lmo0978	**-5,29**	0,00130	-0,43	0,37710	branched-chain amino acid aminotransferase IlvE	lipid-related metabolism
lmo1357	**-8,02**	0,00015	**-5,43**	0,00035	acetyl-CoA carboxylase subunit (biotin carboxylase subunit) AccC	lipid-related metabolism
lmo1374	**-7,84**	0,00363	-2,82	0,10922	branched-chain alpha-keto acid dehydrogenase E2 subunit BkdB	lipid-related metabolism
lmo1538	**-6,05**	0,01137	**-2,27**	0,02116	glycerol kinase GlpK	lipid-related metabolism

EGD-e locus	Siliken_1/2c Fold change (37 C)	significance (G-test)	F2365_4b Fold change (37 C)	significance (G-test)	Predicted/known function	Functional group
lmo1572	**-3,66**	0,02267	**-13,12**	0,01294	acetyl-CoA carboxylase carboxyl transferase subunit alpha AccA	lipid-related metabolism
lmo1573	**-11,53**	0,02263	-7,06	0,10922	acetyl-CoA carboxylase carboxyl transferase subunit beta AccD	lipid-related metabolism
lmo1688	**-13,03**	0,01335	-4,31	0,29142	enoyl-[acyl carrier protein] reductase III FabL	lipid-related metabolism
lmo1807	**-2,03**	0,00840	-1,47	0,11353	3-oxoacyl-[acyl-carrier protein] reductase FabG	lipid-related metabolism
lmo1936	**-2,33**	0,00554	-1,36	0,38538	glycerol-3-phosphate dehydrogenase GpsA	lipid-related metabolism
lmo1586	**-19,05**	0,00163	-1,20	0,75261	inorganic polyphosphate/NAD+ kinase PpnK	cofactor metabolism
lmo1592	**-10,02**	0,03838	**-11,10**	0,02627	thiamine biosyn. ATP pyrophosphatase/tRNA sulfurtransferase ThiL	cofactor metabolism
lmo2411	**-2,40**	0,02312	**-4,03**	0,00261	FeS cluster assembly protein SufB	cofactor metabolism
lmo2415	**-8,33**	0,00000	**-2,36**	0,01927	Fe-S cluster assembly, ATP-binding proteinSufC	cofactor metabolism
lmo0002	**-2,75**	0,00185	**-2,02**	0,01674	DNA polymerase III beta subunit DnaN	DNA replication
lmo0006	**-10,62**	0,00000	**-5,25**	0,00212	DNA gyrase, B subunit GyrB	DNA replication
lmo0007	**-11,05**	0,00020	**-5,85**	0,00418	DNA gyrase, A subunit GyrA	DNA replication
lmo1275	**-6,24**	0,01485	**-4,18**	0,09507	topoisomerase IA TopA	DNA replication
lmo1287	1,27	0,82155	**-13,12**	0,01294	topoisomerase IV, A subunit ParC	DNA replication
lmo2488	**-11,53**	0,02263	**-5,39**	0,00755	excinuclease ATPase subunit UvrA	DNA repair
lmo0258	**-3,98**	0,00000	**-2,26**	0,00000	RNA polymerase, beta subunit RpoB	RNA polymerase
lmo0259	**-3,48**	0,00000	**-2,19**	0,00000	RNA polymerase, beta' subunit RpoC	RNA polymerase
lmo2606	**-2,13**	0,00030	-1,46	0,09000	RNA polymerase, alpha subunit RpoA	RNA polymerase
lmo0402	1,27	0,82155	**-3,89**	0,01958	LicR-like PTS system transcriptional activator	transcriptional regulation (PTS system)
lmo1515	**-3,66**	0,02267	**-7,22**	0,00791	putative transcriptional cysteine metabolism repressor CymR	transcriptional regulation (cysteine metabolism)
lmo1599	**-3,39**	0,00001	-1,63	0,39933	catabolite control protein A CcpA	transcriptional regulation (sugar metabolism)
lmo2072	**-3,04**	0,00132	-1,94	0,08521	redox (NADH)-sensing transcriptional repressor Rex-like	transcriptional regulation (NADH levels)
lmo2083	**-10,02**	0,03838	-7,06	0,10922	putative transcriptional activator, AraC_E_bind superfamily	transcriptional regulation
lmo2792	**-3,35**	0,00034	**-2,37**	0,01438	putative transc. reg.HTH_XRE domain	transcriptional regulation

EGD-e locus	Siliken_1/2c Fold change (37 C)	significance (G-test)	F2365_4b Fold change (37 C)	significance (G-test)	Predicted/known function	Functional group
lmo1434	1,34	0,66520	**-4,45**	0,02413	ribonuclease J2 RnjB	RNA degradation
lmo1322	**-6,50**	0,00002	**-3,97**	0,00004	transcription elongation factor NusA	transcription (elongation)
lmo0218	**-5,17**	0,03730	-2,20	0,24118	putative RNA binding protein, S1_like superfamily YabR	tRNA/ribosome processing/assembly
lmo0256	ND	ND	**-11,10**	0,02627	16S rRNA methyltransferase RsmC	tRNA processing
lmo0866	**-5,48**	0,00000	**-2,45**	0,00005	ATP-dependent RNA helicase CshA	tRNA/ribosome processing/assembly
lmo1462	**-14,54**	0,00789	**-2,30**	0,05170	16S rRNA-binding GTPase Era	tRNA/ribosome processing/assembly
lmo1937	**-4,36**	0,00684	-1,41	0,56510	GTP binding protein EngA	tRNA/ribosome processing/assembly
lmo1821	**-11,53**	0,02263	-1,14	0,91390	phosphoprotein phosphatase Stp	posttranslational modification
lmo1935	**-17,54**	0,00276	-5,04	0,22432	putative protein-tyrosine/serine phosphatase, PTPc superfamily	posttranslational modification
lmo1268	**-17,54**	0,00276	**-5,70**	0,02785	Clp protease ATP-binding subunit ClpX	protein turnover
lmo2206	**-3,76**	0,00000	**-2,04**	0,00067	Clp endopeptidase ATP-binding subunit ClpC	protein turnover
lmo2597	-1,35	0,15828	**-1,93**	0,01166	ribosomal protein L13 RplM	ribosomal proteins
lmo2615	**-2,12**	0,00002	**-2,00**	0,02218	ribosomal protein S5 RpsE	ribosomal proteins
lmo0046	**-3,00**	0,01279	-2,01	0,08083	ribosomal protein S18 RpsR	ribosomal proteins
lmo0053	**-4,50**	0,00163	-2,16	0,09635	ribosomal protein L9 RplI	ribosomal proteins
lmo1335	**-3,19**	0,04887	absent	absent	50S ribosomal protein L33 RpmG	ribosomal proteins
lmo1480	**-11,53**	0,02263	-5,04	0,22432	ribosomal protein S20 RpsT	ribosomal proteins
lmo1540	**-3,89**	0,01529	-1,78	0,16161	ribosomal protein L27 RpmA	ribosomal proteins
lmo1596	**-4,21**	0,00000	**-1,65**	0,03387	ribosomal protein S4 RpsD	ribosomal proteins
lmo1658	**-2,35**	0,00000	**-1,47**	0,03039	ribosomal protein S2 RpsB	ribosomal proteins
lmo1784	**-3,04**	0,03503	-1,90	0,11512	50S ribosomal protein L35	ribosomal proteins
lmo1787	**-2,01**	0,00781	-1,46	0,18219	ribosomal protein L19 RplS	ribosomal proteins
lmo1797	**-6,93**	0,00006	**-2,26**	0,02842	ribosomal protein S16 RpsP	ribosomal proteins
lmo1938	**-2,16**	0,00099	-1,16	0,43486	ribosomal protein S1 RpsA	ribosomal proteins
lmo2596	**-3,45**	0,00000	**-1,59**	0,02333	ribosomal protein S9 RpsI	ribosomal proteins
lmo2605	**-3,06**	0,00069	-1,47	0,18251	ribosomal protein L17 RplQ	ribosomal proteins
lmo2607	**-2,70**	0,00071	**-2,34**	0,00162	ribosomal protein S11 RpsK	ribosomal proteins
lmo2608	**-4,25**	0,00000	**-2,22**	0,00120	ribosomal protein S13 RpsM	ribosomal proteins
lmo2609	**-16,04**	0,00466	-1,14	0,91390	ribosomal protein L36 RpmJ	ribosomal proteins

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lmo2616	**-7,63**	0,00002	**-5,39**	0,00755	ribosomal protein L18 RplR	ribosomal proteins
lmo2617	**-2,01**	0,00021	-1,32	0,18311	ribosomal protein L6 RplF	ribosomal proteins
lmo2618	**-2,07**	0,00098	**-1,72**	0,02510	ribosomal protein S8 RpsH	ribosomal proteins
lmo2621	**-3,63**	0,00214	-2,06	0,10350	ribosomal protein L24 RplX	ribosomal proteins
lmo2623	**-2,78**	0,00929	-1,91	0,14972	ribosomal protein S17 RpsQ	ribosomal proteins
lmo2625	**-2,55**	0,00005	**-2,19**	0,00046	ribosomal protein L16 RplP	ribosomal proteins
lmo2627	**-2,73**	0,00134	**-2,24**	0,00965	ribosomal protein L22 RplV	ribosomal proteins
lmo2628	**-4,82**	0,00000	**-1,91**	0,01530	ribosomal protein S19 RpsS	ribosomal proteins
lmo2629	**-3,06**	0,00000	**-1,79**	0,00081	ribosomal protein L2 RplB	ribosomal proteins
lmo2632	**-2,85**	0,00000	**-1,76**	0,00087	ribosomal protein L3 RplC	ribosomal proteins
lmo2655	**-2,36**	0,00151	-1,09	0,71182	ribosomal protein S7 RpsG	ribosomal proteins
lmo2656	**-2,71**	0,01793	**-2,08**	0,04441	ribosomal protein S12 RpsL	ribosomal proteins
lmo1459	-3,19	0,43731	**-6,32**	0,00229	glycyl-tRNA synthetase alpha subunit GlyQ	tRNA amino-acyl synthesis
lmo0177	**-2,34**	0,00399	**-1,72**	0,03943	methionyl-tRNA synthetase MetS	tRNA amino-acyl synthesis
lmo0228	**-4,21**	0,00000	**-2,02**	0,01011	lysyl-tRNA synthetase LysS	tRNA amino-acyl synthesis
lmo0239	**-7,04**	0,00059	**-2,78**	0,06809	cysteinyl-tRNA synthetase CysS	tRNA amino-acyl synthesis
lmo1221	**-3,19**	0,04887	**-11,10**	0,02627	phenylalanyl-tRNA synthetase alpha subunit PheS	tRNA amino-acyl synthesis
lmo1222	**-7,37**	0,00037	**-6,79**	0,00125	phenylalanyl-tRNA synthetase beta subunit PheT	tRNA amino-acyl synthesis
lmo1458	**-5,40**	0,00001	**-5,43**	0,00035	glycyl-tRNA synthetase, beta subunit GlyS	tRNA amino-acyl synthesis
lmo1504	**-5,78**	0,00000	**-5,78**	0,00000	alanyl-tRNA synthetase AlaS	tRNA amino-acyl synthesis
lmo1520	**-4,26**	0,00032	**-3,37**	0,00104	histidyl-tRNA synthetase HisS	tRNA amino-acyl synthesis
lmo1552	**-3,08**	0,00001	**-2,16**	0,00105	valyl-tRNA synthetase ValS	tRNA amino-acyl synthesis
lmo1559	**-2,63**	0,00476	**-5,99**	0,00002	threonyl-tRNA synthetase ThrS	tRNA amino-acyl synthesis
lmo1660	**-6,72**	0,00092	**-4,61**	0,00002	leucyl-tRNA synthetase LeuS	tRNA amino-acyl synthesis
lmo1755	**-2,02**	0,00248	**-1,67**	0,01723	aspartyl/glutamyl-tRNA amidotransferase subunit A GatA	tRNA amino-acyl synthesis
lmo2198	**-28,07**	0,00007	**-7,73**	0,00037	tryptophanyl-tRNA synthetase TrpS	tRNA amino-acyl synthesis
lmo1325	**-67,18**	0,00000	**-6,42**	0,00001	translation initiation factor IF2 InfB	protein translation (initiation)
lmo1785	**-2,04**	0,01977	-1,65	0,10648	translation initiation factor IF3 InfC	protein translation (initiation)
lmo1067	**-3,22**	0,02436	**-4,23**	0,01139	ribosome-assoc. GTP-binding elongation factor BipA	protein translation (elongation)

EGD-e locus	Siliken_1/2c Fold change (37 C)	significance (G-test)	F2365_4b Fold change (37 C)	significance (G-test)	Predicted/known function	Functional group
lmo2654	**-2,70**	0,00000	**-1,76**	0,00001	elongation factor EF-G Fus	protein translation (elongation)
lmo1051	**-2,74**	0,02515	**-2,49**	0,05763	polypeptide deformylase Def	protein translation (release)
lmo1709	**-5,09**	0,00836	**-2,13**	0,05599	methionyl aminopeptidase Map	protein translation (release)
lmo2509	**-5,74**	0,00350	4,31	0,29142	peptide chain release factor RF2 PrfB	protein translation (release)
lmo2779	**-6,77**	0,00931	**-7,98**	0,00418	putative GTP-and nucleic acid- binding protein EngD	protein translation (release)
lmo0997	ND	ND	**-3,77**	0,00000	Clp endopeptidase ATP-binding subunit ClpE	protein turnover
lmo1279	-2,41	0,26914	**-6,26**	0,00037	protease ATP-binding subunit ClpY/HslU	protein turnover
lmo1439	-1,59	0,18712	**-4,36**	0,00000	superoxide dismutase SodA	defence mechanisms (oxidative stress)
lmo1604	**-3,79**	0,04517	ND	ND	putative peroxiredoxin, PRX_Typ2cys family YkuU	defence mechanisms (oxidative stress)
lmo0554	**-2,22**	0,00961	4,31	0,29142	putative Fe- and NAD(P)-dependent butanol (butyraldehyde) dehydrogenase acting against short chain aldehydes, DHQ_Fe-ADH superfamily YugJ	defence mechanisms
lmo2700	**-11,58**	0,00013	**-4,03**	0,00261	putative multifunctional methylglyoxal reductase DkgA-like	defence mechanisms
lmo0786	**-10,02**	0,03838	**-8,20**	0,00020	putative NADH-azoreductase, FMN-dependent, FMN_red superfamily AzoR-like	general prediction only
lmo0273	ND	ND	**-11,10**	0,02627	putative acetyltransferase, GNAT superfamily	general prediction only
lmo2474	-5,39	0,19816	**-11,10**	0,02627	P-loop-containing ATPase family protein	general prediction only
lmo1065	ND	ND	**-15,14**	0,00638	DUF1054 superfamily protein	unknown function
lmo2360	1,66	0,36650	**-5,69**	0,00000	membrane protein, Pip_Yhge superfamily	unknown function
lmo0170	**-10,02**	0,03838	-1,14	0,91390	DUF1254/DUF1214 multidomain superfamily	unknown function
lmo0553	**-6,07**	0,00225	-2,00	0,14198	CBS pair domain superfamily protein	unknown function
lmo1814	**-3,66**	0,02267	-1,17	0,86620	Dak2 domain fusion protein YloV	unknown function
lmo2707	**-3,40**	0,01683	**-2,97**	0,01127	uncharacterised protein	unknown function

ND = protein not detected; absent, gene for protein absent from genome; bold type indicates a significant change in abundance ($p<0.05$)

Appendix B

Supplementary Table 2

EGD-e locus	Siliken_1/2c Fold change (25°C/37°)	significance (G-test)	F2365_4b Fold change (25°C/37°C)	significance (G-test)	Predicted/known function	Functional group
DIFFERENTIAL ABUNDANCES						
lmo0727	**-2,79**	0,00169	**2,61**	0,03318	L-glutamine:D-fructose-6-phosphate aminotransferase GlmS	Cell wall biogenesis
lmo2650	**-14,16**	0,00899	**3,20**	0,43616	PTS system (L-Ascorbate family) IIB-1	Phosphotransferase systems
lmo0135	**-2,11**	0,03399	**3,55**	0,01550	cysteine/cystine uptake ABC transporter, substrate binding protein	ABC-type transporter systems
lmo2196	**-3,23**	0,00423	**3,20**	0,01415	oligopeptide ABC transporter, substrate binding protein OppA	ABC-type transporter systems
lmo1917	**-1,78**	0,04197	**2,22**	0,00228	pyruvate-formate lyase PflB	Carbohydrate-related metabolismo
lmo1600	**-16,36**	0,00418	**2,79**	0,02050	bifunctional phospho-2-dehydro-3-deoxyheptonate aldolase/chorismate mutase AroG	Amino acid-related metabolismo
lmo0053	**5,85**	0,00138	**-1,72**	0,00472	ribosomal protein L9 RplI	Ribosomal proteins
lmo0211	**1,72**	0,01305	**-1,48**	0,03815	ribosomal protein L25 Ctc/RplY	Ribosomal proteins
INCREASED ABUNDANCE						
lmo1086	**2,95**	0,00037	1,56	0,49238	D-ribitol-5-phosphate cytidylyltransferase TarI	Cell wall biogenesis
lmo1002	**2,26**	0,00000	**2,46**	0,00000	putative PTS system phosphocarrier HPr component PtsH	Phosphotransferase systems
lmo1255	-1,87	0,46072	**14,21**	0,00885	PTS (Glucose/Glucoside/Trehalose family) IIBC TreP	Phosphotransferase systems
lmo2373	**1,52**	0,05653	**3,88**	0,00029	PTS system (Lactose/DACB/beta-glucoside family) IIB LicB-like	Phosphotransferase systems
lmo0181	1,27	0,82155	**7,74**	0,00000	dextrin/maltose ABC transporter, substrate binding protein	ABC-type transporter systems

EGD-e locus	Siliken_1/2c Fold change (25°C/37°)	significance (G-test)	F2365_4b Fold change (25°C/37°C)	significance (G-test)	Predicted/known function	Functional group
lmo1422	2,45	0,19283	**9,80**	0,04147	bile efflux ABC transporter, substrate binding protein BileB	ABC-type transporter systems
lmo1847	1,88	0,38875	**100,05**	0,00000	manganese uptake ABC transporter, substrate binding protein LpeA	ABC-type transporter systems
lmo1697	**10,15**	0,03667	1,39	0,67653	Zn/Cd/Fe cation exporter, cation_efflux superfamily	Other transporter proteins
lmo0048	**19,31**	0,00149	**12,01**	0,01914	accessory ArgD processing/exporting protein AgrB	Signal transduction
lmo0515	**10,15**	0,03667	ND	ND	UspA-like universal stress protein, Usp_like family	Signal transduction
lmo1580	**2,47**	0,00000	**13,81**	0,00000	UspA-like universal stress protein, Usp_like family	Signal transduction
lmo2529	ND	ND	**2,73**	0,00005	F0F1-type ATP synthase, beta subunit AtpD	Membrane bioenergetics
lmo2531	-3,19	0,43731	**3,07**	0,00065	F0F1-type ATP synthase, alpha subunit AtpA	Membrane bioenergetics
lmo1548	-1,08	0,83426	**2,43**	0,05291	cell-shape determining protein MreB	Cytokinesis
lmo0106	**10,15**	0,03667	1,67	0,60054	N-acetyl-D-glucosamine kinase NagK-like	Carbohydrate-related metabolismo
lmo0344	4,05	0,32025	**3,40**	0,05173	putative ribitol/xylitol (phosphate) dehydrogenase, SDRc family	Carbohydrate-related metabolismo
lmo0401	**10,15**	0,03667	2,35	0,34153	alpha-mannosidase MngB-like	Carbohydrate-related metabolismo
lmo0539	**1,60**	0,00087	1,79	0,43029	LacD/DeoC-like aldolase, TIM_phosphate_binding superfamily	Carbohydrate-related metabolismo
lmo0956	**16,26**	0,00432	ND	ND	N-acetylglucosamine-6-phosphate deacetylase NagA	Carbohydrate-related metabolismo
lmo0957	-3,19	0,43731	**9,80**	0,04147	glucosamine-6 phosphate isomerase NagB	Carbohydrate-related metabolism
lmo1871	-3,19	0,43731	**12,01**	0,01914	phosphomanno(gluco)mutase, PMM_PGM family	Carbohydrate-related metabolism
lmo1998	4,05	0,32025	**12,01**	0,01914	putative fructoselysine 6-phosphate deglycase FrlB-like	Carbohydrate-related metabolism
lmo2094	**10,15**	0,03667	3,20	0,43616	putative type II aldolase, aldolase_II superfamily	Carbohydrate-related metabolism
lmo2761	**10,15**	0,03667	-3,24	0,43045	aryl-6-phospho-beta-glucosidase BglC-like	Carbohydrate-related metabolism
lmo1634	1,07	0,22891	**4,50**	0,00000	acetaldehyde-CoA dehydrogenase/iron-dependent alcohol dehydrogenase/pyruvate-formate lyase deactivase AdhE	Central glycolytic/fermentation
lmo0343	7,10	0,10763	**12,01**	0,01914	fructose 6-phosphate transaldolase, transaldolase_FSA family	Central glycolytic/glycolysis
lmo0348	**5,09**	0,02852	**23,01**	0,00041	dihydroxyacetone kinase, N-terminal domain DhaK	Central glycolytic/glycolysis

EGD-e locus	Siliken_1/2c Fold change (25°C/37°)	significance (G-test)	F2365_4b Fold change (25°C/37°C)	significance (G-test)	Predicted/known function	Functional group
lmo1571	**2,70**	0,00000	**4,63**	0,00000	6-phosphofructokinase PfkA	Central glycolytic/glycolysis
lmo2205	**6,60**	0,00000	**38,42**	0,00000	phosphoglycerate mutase 1 GpmA	Central glycolytic/glycolysis
lmo2367	**1,79**	0,00000	1,29	0,06727	glucose-6-phosphate isomerase Pgi	Central glycolytic/glycolysis
lmo2475	**2,63**	0,03284	**3,42**	0,00832	alpha-phosphoglucomutase Pgm	Central glycolytic/glycolysis
lmo2696	**4,16**	0,00143	**9,80**	0,04147	dihydroxyacetone kinase, C-terminal domain DhaL	Central glycolytic/glycolysis
lmo2743	ND	ND	**14,21**	0,00885	fructose 6-phosphate transaldolase, transaldolase_FSA family	Central glycolytic/glycolysis
lmo0558	**1,77**	0,00332	1,05	0,80723	(6-phospho)gluconolactonase Pgl	Central glycolytic/pentose phosphate pathway
lmo1376	**2,05**	0,00037	1,36	0,12057	6-phosphogluconate dehydrogenase Gnd	Central glycolytic/pentose phosphate pathway
lmo0355	**3,09**	0,00000	**4,21**	0,00000	fumarate reductase flavoprotein FrdA	Tricarboxylic acid pathway
lmo1072	-1,39	0,56453	**3,27**	0,02687	pyruvate carboxylase PycA	Tricarboxylic acid pathway
lmo1641	1,32	0,73107	**12,01**	0,01914	aconitate hydratase CitB	Tricarboxylic acid pathway
lmo0223	1,06	0,68701	**1,96**	0,00007	cysteine synthase CysK	Amino acid-related metabolism
lmo0396	**10,15**	0,03667	ND	ND	pyrroline-5-carboxylate reductase ProC	Amino acid-related metabolism
lmo1348	1,27	0,82155	**36,22**	0,00000	aminomethyltransferase GcvT	Amino acid-related metabolism
lmo1349	**3,35**	0,02414	**31,81**	0,00002	glycine dehydrogenase subunit 1 GcvPA	Amino acid-related metabolism
lmo1350	**3,65**	0,00193	**11,55**	0,00000	glycine dehydrogenase subunit 2 GcvPB	Amino acid-related metabolism
lmo1435	**4,13**	0,07006	**16,41**	0,00410	dihydrodipicolinate synthase DapA	Amino acid-related metabolism
lmo1493	1,66	0,36650	**4,39**	0,05369	oligoendopeptidase, M3B_PepF_2 PepF-like	Amino acid-related metabolism
lmo1578	-1,33	0,75804	**2,61**	0,03318	PepP_like Xaa-Pro aminopeptidase, APP_like family	Amino acid-related metabolism
lmo1579	1,58	0,33546	**5,41**	0,00252	L-alanine dehydrogenase Ald	Amino acid-related metabolism
lmo1897	1,27	0,82155	**14,21**	0,00885	aspartate aminotransferase AspB	Amino acid-related metabolism
lmo2006	**8,91**	0,00066	**27,41**	0,00009	alpha-acetolactate synthase AlsS	Amino acid-related metabolism
lmo2363	**6,96**	0,00000	**181,48**	0,00000	glutamate decarboxylase GadB	Amino acid-related metabolism
lmo2425	**2,66**	0,01891	**1,87**	0,24810	glycine cleavage system lipoylprotein GcvH	Amino acid-related metabolism

EGD-e locus	Siliken_1/2c Fold change (25°C/37°)	significance (G-test)	F2365_4b Fold change (25°C/37°C)	significance (G-test)	Predicted/known function	Functional group
lmo1832	7,10	0,10763	**9,80**	0,04147	orotidine-5'-phosphate decarboxylase PyrF	Nucleic acid/nucleotide metabolism
lmo2559	1,44	0,14973	**3,88**	0,00029	CTP synthase PyrG	Nucleic acid/nucleotide metabolism
lmo2758	**1,64**	0,00212	**1,70**	0,01745	inosine monophosphate dehydrogenase GuaB	Nucleic acid/nucleotide metabolism
lmo0825	**10,15**	0,03667	**3,20**	0,43616	hydroxymethylglutaryl-CoA reductase MvaA	Lipid-related metabolism
lmo0978	1,25	0,54835	**2,44**	0,02300	branched-chain amino acid aminotransferase IlvE	Lipid-related metabolism
lmo1373	3,18	0,16724	**14,21**	0,00885	2-oxoisovalerate dehydrogenase beta subunit, E1 component BkdAB	Lipid-related metabolism
lmo1538	**6,05**	0,01137	ND	ND	glycerol kinase GlpK	Lipid-related metabolism
lmo1807	**2,67**	0,01099	1,41	0,33888	3-oxoacyl-[acyl-carrier protein] reductase FabG	Lipid-related metabolism
lmo1936	**5,09**	0,02852	**9,80**	0,04147	glycerol-3-phosphate dehydrogenase GpsA	Lipid-related metabolism
lmo1673	**1,92**	0,02545	**6,62**	0,00037	naphthoate synthase MenB	Cofactor-related metabolism
lmo1877	**4,16**	0,00503	**12,55**	0,00001	formate-tetrahydrofolate ligase, Ras_GTPase_like superfamily	Cofactor-related metabolism
lmo2101	**3,53**	0,00000	**2,96**	0,00000	pyridoxine synthesis lyase subunit PdxS	Cofactor-related metabolism
lmo0001	**13,21**	0,01257	-1,01	0,99198	chromosomal replication initiator protein DnaA	DNA replication-related
lmo1286	**13,21**	0,01257	ND	ND	topoisomerase IV, B subunit ParE	DNA replication-related
lmo0918	**10,15**	0,03667	ND	ND	LicR-like PTS system transcriptional antiterminator	Transcriptional regulation
lmo1956	-1,39	0,56453	**4,39**	0,05369	ferric uptake regulator Fur	Transcriptional regulation
lmo2792	**16,26**	0,00432	ND	ND	putative transcriptional regulator, HTH_XRE domain	Transcriptional regulation
lmo0888	**3,58**	0,04131	ND	ND	putative MazF-like toxin ribonuclease, PemK superfamily EndoA	Addiction module
lmo1359	**10,15**	0,03667	-1,71	0,58306	transcription termination factor NusB	Transcription-associated proteins
lmo2016	**1,57**	0,00000	1,22	0,07339	cold shock protein CspB	Transcription-associated proteins
lmo1977	10,15	0,03667	ND	ND	ribonuclease Z Rbn	tRNA/Ribosome assembly/processing
lmo2511	**1,77**	0,00332	**9,8**	0,04147	ribosome-associated sigma 54 modulation protein Hpf	tRNA/Ribosome assembly/processing

EGD-e locus	Siliken_1/2c Fold change (25°C/37°)	significance (G-test)	F2365_4b Fold change (25°C/37°C)	significance (G-test)	Predicted/known function	Functional group
lmo0237	1,34	0,08826	**1,47**	0,01879	glutamyl-/glutaminyl-tRNA synthetase GltX	tRNA aminoacyl synthesis
lmo1319	**1,96**	0,02548	**25,21**	0,00019	prolyl-tRNA synthetase ProS	tRNA aminoacyl synthesis
lmo1519	-1,42	0,40528	**12,01**	0,01914	aspartyl-tRNA synthetase AspS	tRNA aminoacyl synthesis
lmo1755	-1,68	0,23323	**1,78**	0,03159	aspartyl/glutamyl-tRNA amidotransferase subunit A GatA	tRNA aminoacyl synthesis
lmo1520	2,29	0,11161	**1,84**	0,02287	histidyl-tRNA synthetase HisS	tRNA aminoacyl synthesis
lmo0931	**13,21**	0,01257	5,40	0,19714	lipoate-protein ligase LplA	Posttranslational modification
lmo1059	**74,23**	0,00000	1,68	0,19713	DsbG-like protein-disulfide isomerase, thioredoxin-like superfamily	Posttranslational modification
lmo1233	**2,49**	0,00269	1,16	0,70299	thioredoxin 1 TrxA	Posttranslational modification
lmo1138	**34,57**	0,00001	**2,51**	0,02770	ATP-dependent Clp protease protease subunit ClpP2	Protein folding/turnover
lmo1473	**2,26**	0,00000	**1,57**	0,00003	class I heat-shock protein (molecular chaperone) DnaK	Protein folding/turnover
lmo2068	**2,68**	0,00000	**2,61**	0,00000	class I heat-shock protein (chaperonin) GroEL	Protein folding/turnover
lmo2069	**4,42**	0,00000	**3,77**	0,00000	class I heat-shock protein (chaperonin) GroES	Protein folding/turnover
lmo2206	**3,35**	0,02414	**7,60**	0,09016	Clp endopeptidase ATP-binding subunit ClpC	Protein folding/turnover
lmo2468	**1,59**	0,08289	**3,27**	0,00243	ATP-dependent Clp protease proteolytic subunit ClpP	Protein folding/turnover
lmo0889	**16,26**	0,00432	3,20	0,43616	positive (anti-anti-sigma B) regulatory factor RsbR	SigmaB operon
lmo0943	**4,17**	0,00000	**5,19**	0,00008	non-heme iron-binding ferritin Fri/Dps	Cell defense/detoxification
lmo2067	**19,31**	0,00149	-2,61	0,00000	conjugated bile salt acid hydrolase Bsh	Cell defense/detoxification
lmo2256	**4,42**	0,00019	**3,20**	0,01415	YhbO-like protein with general role in stress management	Cell defense/detoxification
lmo0134	**10,15**	0,03667	ND	ND	putative acyltransferase, NAT_SF superfamily	General prediction only
lmo0392	1,04	0,95214	**4,39**	0,05369	YdfA immunity superfamily protein	General prediction only
lmo0907	1,04	0,95214	**3,80**	0,02896	putative phosphatase, HP_PGM_like family	General prediction only
lmo2391	**18,47**	0,00000	ND	ND	putative oxidoreductase, atypical SDR subfamily 5 protein	General prediction only
lmo2473	**10,15**	0,03667	-1,01	0,99198	putative transferase, YvcK-like family protein	General prediction only

EGD-e locus	Siliken_1/2c Fold change (25°C/37°)	significance (G-test)	F2365_4b Fold change (25°C/37°C)	significance (G-test)	Predicted/known function	Functional group
lmo2564	**10,82**	0,00010	-2,16	0,14505	4-oxalocrotonate tautomerase (keto-enol tautomerisation) family protein	General prediction only
lmo0377	**13,21**	0,01257	5,40	0,19714	uncharacterised protein	Unknown/uncharacterized
lmo1028	**3,54**	0,00672	**2,76**	0,03936	DUF 1447 superfamily protein	Unknown/uncharacterized
lmo1468	**5,85**	0,00138	**2,43**	0,05291	GatB_YqeY superfamily protein	Unknown/uncharacterized
lmo1522	1,11	0,71923	**4,53**	0,00050	DUF1292 superfamily protein	Unknown/uncharacterized
lmo2426	**13,21**	0,01257	-1,43	0,65120	ArsC-like family protein	Unknown/uncharacterized
lmo2692	**10,15**	0,03667	-1,01	0,99198	DUF970 family protein	Unknown/uncharacterized
DECREASED ABUNDANCE						
lmo1083	**-14,16**	0,00899	absent	absent	dTDP-glucose 4,6-dehydratase RfbB	Cell wall biogenesis
lmo2335	**-4,58**	0,02480	1,53	0,41140	PTS (Fructose/Mannitol family) IIABC-6 FruA	Phosphotransferase systems
lmo2638	**-2,05**	0,01585	-1,16	0,67066	respiratory NAD(P)H-disulfide dehydrogenase, pyr_redox superfamily Ndh	Membrane bioenergetics
lmo0690	**-9,38**	0,00000	**-2,85**	0,00000	flagellin Fla	Motility/chemotaxis
lmo0692	**-4,04**	0,04617	-1,01	0,98766	chemotaxis sensor histidine kinase CheA	Motility/chemotaxis
lmo0695	ND	ND	**-9,95**	0,03942	flagellar hook-length control protein, Flg_hook superfamily	Motility/chemotaxis
lmo0697	**-9,78**	0,04189	**-7,71**	0,08677	flagellar hook protein FlgE	Motility/chemotaxis
lmo0197	-1,17	0,29113	**-1,57**	0,00238	regulator of septum location SpoVG	Cytokinesis
lmo0182	-3,19	0,43731	**-1,39**	0,02552	putative limit dextrinase, GH31_transferase_CtsY family	Carbohydrate-related metabolism
lmo0184	ND	ND	**-3,39**	0,00000	oligo-1,6-glucosidase/trehalase, alpha_amylase superfamily	Carbohydrate-related metabolism
lmo1175	-1,03	0,95155	**-1,77**	0,04056	ethanolamine ammonia lyase large subunit EutB	Carbohydrate-related metabolism
lmo1179	**-36,10**	0,00000	-2,11	0,06912	aldehyde dehydrogenase EutE	Carbohydrate-related metabolism
lmo1180	**-1,41**	0,04666	**1,89**	0,00209	carboxysome shell protein EutM	Carbohydrate-related metabolism
lmo1182	**-5,39**	0,19816	**-9,95**	0,03942	putative phosphotransacetylase PduL-like	Carbohydrate-related metabolism

EGD-e locus	Siliken_1/2c Fold change (25°C/37°)	significance (G-test)	F2365_4b Fold change (25°C/37°C)	significance (G-test)	Predicted/known function	Functional group
lmo1187	**-3,12**	0,01579	**-3,07**	0,04024	uncharacterised protein EutQ	Carbohydrate-related metabolism
lmo1406	**-3,15**	0,00000	1,13	0,49616	pyruvate-formate lyase PflB	Central glycolytic/fermentation
lmo1581	**-2,74**	0,00000	**-1,64**	0,00497	acetate kinase	Central glycolytic/fermentation
lmo2103	**-1,90**	0,00230	**-2,28**	0,00011	phosphate acetyltransferase	Central glycolytic/fermentation
lmo1053	**-1,39**	0,02595	1,23	0,35969	pyruvate dehydrogenase E1 component, beta subunit	Central glycolytic/fermentation
lmo2455	1,07	0,05433	**1,22**	0,00000	enolase	Central glycolytic/glycolysis
lmo2456	**-1,33**	0,04277	-1,10	0,47585	phosphoglyceromutase GpmM	Central glycolytic/glycolysis
lmo2458	-1,01	0,91668	**-1,27**	0,00092	phosphoglycerate kinase PgK	Central glycolytic/glycolysis
lmo2556	**-1,20**	0,00057	**-1,99**	0,00000	fructose 1,6-bisphosphate aldolase type II FbaA	Central glycolytic/glycolysis
lmo1354	-1,82	0,16269	**-2,92**	0,00446	PepP_like Xaa-Pro aminopeptidase, APP_like family	Amino acid-related metabolism
lmo1619	**-3,23**	0,03610	**-2,22**	0,03602	D-alanine transaminase Dat	Amino acid-related metabolism
lmo1711	-1,23	0,40744	**-1,71**	0,03915	leucyl aminopeptidase AmpS-like	Amino acid-related metabolism
lmo1780	-1,35	0,39396	**-4,51**	0,04931	peptidase T (tripeptide aminopeptidase) PepT	Amino acid-related metabolism
lmo1923	-2,95	0,15269	**-16,66**	0,00376	5-enolpyruvylshikimate-3-phosphate synthase AroA	Amino acid-related metabolism
lmo0132	**-14,16**	0,00899	**-3,24**	0,43045	inosine monophosphate dehydrogenase GuaB	Nucleic acid/nucleotide metabolism
lmo1096	**-1,64**	0,00037	-1,29	0,06982	GMP synthase GuaA	Nucleic acid/nucleotide metabolism
lmo1524	-1,61	0,39213	**-3,52**	0,00683	adenine phosphoribosyltransferase Apt	Nucleic acid/nucleotide metabolism
lmo1953	**-2,36**	0,03233	**-3,22**	0,00313	purine nucleoside phosphorylase XapA-like	Nucleic acid/nucleotide metabolism
lmo1993	**-2,11**	0,03399	1,25	0,44691	pyrimidine-nucleoside phosphorylase Pdp	Nucleic acid/nucleotide metabolism
lmo2415	**-3,57**	0,00042	1,59	0,19021	Fe-S cluster assembly, ATP-binding protein SufC	Cofactor-related metabolism
lmo0259	1,29	0,05973	**1,91**	0,00021	RNA polymerase, beta' subunit RpoC	RNA polymerase
lmo2606	**-1,57**	0,04598	-1,02	0,94933	RNA polymerase, alpha subunit RpoA	RNA polymerase
lmo2248	**-2,69**	0,04329	1,71	0,28561	putative phosphate transport regulator	Transcriptional regulation

EGD-e locus	Siliken_1/2c Fold change (25°C/37°)	significance (G-test)	F2365_4b Fold change (25°C/37°C)	significance (G-test)	Predicted/known function	Functional group
lmo1322	**-16,36**	0,00418	2,60	0,15665	transcription elongation factor NusA	Transcription-associated proteins
lmo1364	**-2,65**	0,00000	**-2,12**	0,00000	cold shock protein CspL	Transcription-associated proteins
lmo0866	**-2,51**	0,00001	-1,06	0,70830	ATP-dependent RNA helicase CshA	tRNA/Ribosome assembly/processing
lmo1937	**-6,20**	0,00361	-3,24	0,43045	GTP binding protein EngA	tRNA/Ribosome assembly/processing
lmo1949	**-9,78**	0,04189	-3,11	0,17774	23S RNA-specific pseudouridine synthase RluB	tRNA/Ribosome assembly/processing
lmo0044	**-1,46**	0,00204	**-1,72**	0,00001	ribosomal protein S6 RpsF	Ribosomal proteins
lmo0248	-1,41	0,07757	**-1,36**	0,00012	ribosomal protein L11 RplK	Ribosomal proteins
lmo0249	**-1,99**	0,00059	**-1,94**	0,00001	ribosomal protein L1 RplA	Ribosomal proteins
lmo0250	1,09	0,66486	**-14,42**	0,00821	ribosomal protein L10 RplJ	Ribosomal proteins
lmo0251	1,13	0,07282	**-2,90**	0,00866	ribosomal protein L7/L12	Ribosomal proteins
lmo1330	**-1,36**	0,03444	**-1,49**	0,00253	ribosomal protein S15 RpSO	Ribosomal proteins
lmo1480	-1,33	0,75804	**-2,32**	0,00649	ribosomal protein S20 RpsT	Ribosomal proteins
lmo1540	1,36	0,56745	**-2,21**	0,00634	ribosomal protein L27 RpmA	Ribosomal proteins
lmo1542	1,12	0,49534	**-1,65**	0,02835	ribosomal protein L21 RplU	Ribosomal proteins
lmo1596	**-2,18**	0,00249	**-5,20**	0,02541	ribosomal protein S4 RpsD	Ribosomal proteins
lmo1658	**-2,06**	0,00010	**-1,76**	0,05082	ribosomal protein S2 RpsB	Ribosomal proteins
lmo1783	**-5,32**	0,00000	**-1,51**	0,00026	ribosomal protein L20 RplT	Ribosomal proteins
lmo1797	**-2,42**	0,00319	**-1,37**	0,01562	ribosomal protein S16 RpsP	Ribosomal proteins
lmo2047	-1,02	0,95612	**2,02**	0,03452	ribosomal protein L32 RpmF	Ribosomal proteins
lmo2596	**-1,67**	0,00503	-1,24	0,71927	ribosomal protein S9 RpsI	Ribosomal proteins
lmo2605	**-1,57**	0,02548	1,62	0,07858	ribosomal protein L17 RplQ	Ribosomal proteins
lmo2615	**-2,12**	0,00002	1,15	0,64394	ribosomal protein S5 RpsE	Ribosomal proteins
lmo2617	**-1,77**	0,00017	-1,58	0,14908	ribosomal protein L6 RplF	Ribosomal proteins
lmo2618	**-8,74**	0,00000	-1,14	0,51774	ribosomal protein S8 RpsH	Ribosomal proteins

EGD-e locus	Siliken_1/2c Fold change (25°C/37°)	significance (G-test)	F2365_4b Fold change (25°C/37°C)	significance (G-test)	Predicted/known function	Functional group
lmo2620	**-1,38**	0,00730	-1,02	0,94578	ribosomal protein L5 RplE	Ribosomal proteins
lmo2623	**-2,12**	0,01586	-1,18	0,40305	ribosomal protein S17 RpsQ	Ribosomal proteins
lmo2631	**-2,07**	0,00000	-1,18	0,65329	ribosomal protein L4 RplD	Ribosomal proteins
lmo2632	**-1,34**	0,02954	-1,09	0,52468	ribosomal protein L3 RplC	Ribosomal proteins
lmo1325	**-5,12**	0,01317	7,60	0,09016	translation initiation factor IF2 InfB	Protein translation (initiation)
lmo1067	**-9,45**	0,00006	**1,35**	0,00009	ribosome-assoc. GTP-binding elongation factor BipA	Protein translation (elongation)
lmo1657	**-1,53**	0,00000	-1,01	0,98169	elongation factor EF-Ts Tsf	Protein translation (elongation)
lmo2653	**-2,00**	0,00000	1,06	0,54034	elongation factor EF-Tu Tuf	Protein translation (elongation)
lmo2654	**-3,67**	0,00000	1,19	0,24948	elongation factor EF-G Fus	Protein translation (elongation)
lmo1314	**2,02**	0,00960	1,12	0,68196	ribosome recycling factor Frr	Protein translation (peptide release)
lmo2779	**-20,74**	0,00090	-3,24	0,43045	GTP-and nucleic acid- binding protein EngD-like	Protein translation (peptide release)
lmo1267	**-1,50**	0,00101	-1,14	0,33790	trigger factor (prolyl isomerase) Tig	Protein folding/turnover
lmo2219	**-9,78**	0,04189	-1,84	0,40840	post-translocation molecular chaperone/foldase PrsA	Protein folding/turnover
lmo2376	**-2,20**	0,02286	**-1,95**	0,01465	peptidyl-prolyl cis-trans isomerase PpiB	Protein folding/turnover
lmo1604	**-9,78**	0,04189	ND	ND	putative peroxiredoxin, PRX_Typ2cys family	Cell defense/detoxification
lmo0663	**-9,78**	0,04189	-1,01	0,99198	putative sugar phosphatase, Cof subfamily	General prediction only
lmo2157	**-14,16**	0,00899	3,20	0,43616	beta-lactamase superfamily protein SepA	General prediction only
lmo2223	**-2,04**	0,00000	**-2,04**	0,00001	DUF964 superfamily protein	Unknown/uncharacterized

ND = protein not detected; absent, gene for protein absent from genome; bold type indicates a significant change in abundance ($p<0.05$)

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5 CONCLUSÕES GERAIS

A temperatura de cultivo influencia substancialmente o perfil de proteínas quando comparado com o tipo genético das cepas e a capacidade de aderências das mesmas.

As consistentes alterações na abundância de proteínas expressas de uma cepa ou de uma determinada temperatura sugerem que embora as células aderidas apresentem um aumento no percentual proteico e um aumento nas taxas de carboidrato e metabolismo, elas crescem de forma mais lenta.

A superfície de adesão está claramente associada a alterações flagelares da célula. De forma muito interessante, nosso experimento sugere que células aderidas a 25℃ por ambas as cepas levam a uma síntese de repressão flagelar.

A abundância da proteína de superfície celular BapL, embora baixa, não foi aumentada em células aderidas sugerindo que o seu papel na adesão pode ser uma contribuição generalizada para a hidrofobicidade da parede celular.

O Sig B Regulon pode estar associado com o aumento em geral da resposta ao stress ocorrido na cepa de Linhagem II (Siliken) mas não na cepa de Linhagem I (F2365) o que pode estar relacionado com as consequências da adesão.

Os resultados baseados na proteômica circunstancialmente sugerem um possível papel do sistema de *agr*, de *quorum sensing* e também os sistemas Mtn / LuxS no processo de adesão por *Listeria* mas mais estudos são necessários para determinar os detalhes exatos de como esses sistemas *agr* promovem a adesão.

A técnica 1D-LC/Tandem MS livre de marcador demonstrou claramente que com alta abrangência é possível estudar proteomas bacterianos e, sendo assim, representa uma ferramenta poderosa para investigar *L. monocytogenes* através de uma perspectiva de genômica funcional.

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