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ALEX FIORI DA SILVA

# Biofilm of *Salmonella* Typhimurium on polypropylene: effect of cinnamaldehyde and proteomic analysis

Maringá

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Dissertação apresentada ao programa de Pós-Graduação em Ciências da Saúde do Centro de Ciências da Saúde da Universidade Estadual de Maringá, como requisito para obtenção do título de Mestre em Ciências da Saúde.

Área de concentração: Saúde Humana

Orientadora: Profª. Drª. Jane Martha Graton Mikcha

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# FOLHA DE APROVAÇÃO

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Biofilm of *Salmonella* Typhimurium on polypropylene: effect of cinnamaldehyde and proteomic analysis

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# EPÍGRAFE

“Todas as vitórias ocultam uma abdicação”

(Simone de Beauvoir)

# Biofilme de *Salmonella* Typhimurium em polipropileno: efeito de cinamaldeído e análise proteômica


**RESUMO**

*Salmonella* spp. é o patógeno transmitido por alimentos mais importante, causando consequências, especialmente para saúde pública e para indústria de alimentos. O efeito do cinamaldeído contra células planctônicas e em biofilme de *Salmonella* Typhimurium em polipropileno foi avaliado. Além disso, a diferença no padrão de expressão proteica por bactérias planctônicas e em biofilme, bem como a expressão de proteínas de biofilmes tratados com cinamaldeído também foram avaliados. A concentração inibitória mínima (CIM) e concentração bactericida mínima sobre células planctônicas foi 312 e 624 µg/mL, respectivamente. O ensaio de curva de morte mostrou que o cinamaldeído apresenta atividade bacteriostática em concentrações subinibitórias e concentração inibitória mínima, eliminando completamente as células bacterianas com concentração de 624 µg/mL após 12 h de exposição. *S.* Typhimurium formou biofilme em polipropileno, observado pelas contagens de colônia (8.03 log UFC/cm$^2$) e microscopia eletrônica de varredura. O cinamaldeído a 234 µg/mL (0.75 x CIM) reduziu 1.3 log UFC/cm$^2$ durante 48 h de formação de biofilme, enquanto que o tratamento por 1 h com cinamaldeído a 624 µg/mL reduziu 2.33 log UFC/cm$^2$ em biofilme formado há dois dias. Nas análises de proteômica, comparando células em biofilme e planctônicas, 16 proteínas foram positivamente reguladas e 5 foram negativamente reguladas. As Lipoproteína Lpp, proteína 30S ribossômica e proteína 50S ribossomal L3 foram identificadas apenas em células sésseis, sugerindo a importância delas na formação de biofilme. No biofilme tratado com cinamaldeído, foram identificadas 8 e 11 proteínas reguladas positivamente e negativamente, respectivamente. Dentre as proteínas reguladas negativamente, serina hidroximetil transferase apresentou a maior redução de expressão (23.8-fold). O cinamaldeído apresentou atividade antimicrobiana contra células planctônicas, no entanto não foi efetivo para eliminar completamente o biofilme em polipropileno. As análises de proteômica mostraram que as células em biofilme expressam diferencialmente proteínas comparadas às células planctônicas homólogas, especialmente proteínas envolvidas no metabolismo celular. Além disso, foi observado que o perfil proteico de células em biofilme é afetado pelo cinamaldeído.



**Palavras-chave:** *Salmonella* Typhimurium; biofilme; polipropileno; proteômica; curva de morte; cinamaldeído.

# Biofilm of *Salmonella* Typhimurium on polypropylene: effect of cinnamaldehyde and proteomic analysis


## ABSTRACT

*Salmonella* spp. is the most important foodborne pathogen, leading to consequences, especially to public health and food industry. The effect of cinnamaldehyde against *Salmonella enterica* serotype Typhimurium ATCC 14028 planktonic and biofilm cells on polypropylene was evaluated. Additionally, differential protein pattern expressed by planktonic and biofilm bacteria, as well protein expression on biofilms treated with cinnamaldehyde, was also evaluated. Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of cinnamaldehyde were 312 and 624 µg/mL, respectively. Time-kill curve assay demonstrated that cinnamaldehyde presented bacteriostatic activity at sub-MIC and MIC concentrations and completely eliminated bacterial cells at 624 µg/mL after 12 h of exposure. *S.* Typhimurium formed biofilm on polypropylene, observed by colony counts (8.03 log CFU/cm$^2$) and scanning electron microscopy. Cinnamaldehyde at 234 µg/mL (0.75 x MIC) decreased 1.3 log CFU/cm$^2$ during 48 h of biofilm formation, while treatment for 1 h with cinnamaldehyde at 624 µg/mL reduced 2.33 log CFU/cm$^2$ of 2-days-old biofilm. In proteomic analysis of biofilm compared to planktonic cells, 16 proteins were up and 5 were down-regulated. Lipoprotein Lpp, 30S ribosomal protein S2 and 50S ribosomal protein L3 were indentified only in biofilm cells, suggesting their importance on biofilm formation. In cinnamaldehyde-treated biofilm, it was identified 8 up and 11 down-regulated proteins. Among down-regulated, serine hydroxymethyltransferase presented the major decrease (23.8-fold). Cinnamaldehyde showed antimicrobial activity against planktonic cells, nevertheless it was not effective to completely eliminate the biofilm on polypropylene. Proteomic analysis showed that biofilm cells presented differentially expressed proteins compared to its planktonic counterparts, especially proteins involved in cell metabolism. Moreover, it was also observed that proteomic profile of biofilm-treated cells is affect by cinnamaldehyde.



**Keywords:** *Salmonella* Typhimurium; biofilm; polypropylene; proteome; time-kill curve; cinnamaldehyde.

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**SUMÁRIO**

# CAPÍTULO I

As doenças transmitidas por alimentos (DTA) resultam em altas taxas de morbidade e mortalidade, significativas perdas econômicas e, apesar dos esforços realizados para prevenilas, elas ainda são consideradas um problema de saúde pública em todo o mundo (WHO, *online*, 2015). Nos Estados Unidos estima-se que os gastos relacionados com DTA ultrapassem 50 bilhões de dólares com 48 milhões de pessoas afetadas (BERMÚDEZ-AGUIRRE E BARBOSA-CANOVAS, 2013). Aproximadamente 250 agentes causadores de DTA foram identificados, dentre eles *Salmonella* spp. (LINSCOTT, 2011).

*Salmonella* spp. está entre as principais bactérias transmitidas por alimentos e em vários países é associada a surtos de DTA. De acordo com o *Centers for Disease Control and Prevention* (*online*, 2015), nos Estados Unidos são estimados 1,2 milhões de casos de salmonelose anualmente, com 19,000 hospitalizações e quase 400 mortes. *Salmonella* spp. foi o agente causador de aproximadamente 40% dos surtos de DTA causados por microorganismos no Brasil (BRASIL, *online*, 2013). No estado do Paraná, entre 1998 e 2010, os dados disponíveis mostram que esta bactéria foi responsável por 57,1% dos casos de surtos de DTA (KOTTWITZ et al., 2010).

Muitos estudos têm demonstrado a capacidade de *Salmonella* spp. formar biofilme em superfícies utilizadas nos ambientes de processamento de alimentos, sendo, portanto, potencial fonte de contaminação e fonte de surtos de DTA (CHELVAM et al., 2014; PIOVEZAN et al., 2014; ZHANG et al., 2014). As ocorrências de surtos de DTA associadas à importância dos biofilmes bacterianos demonstram a necessidade de se compreender os aspectos que envolvem a formação do bifilme, bem como do desenvolvimento de novas estratégias de controle. Nesse sentido, a utilização de métodos de biologia molecular, como análise proteômica, é uma ferramenta que permite avaliar as mudanças no perfil proteico de *Salmonella* spp. na formação de biofilmes e o uso de substâncias naturais com propriedades antimicrobianas é uma alternativa para controle de biofilmes (ZHANG et al., 2014; GIAOURIS et al., 2013).

## *Salmonella* spp.

*Salmonella* spp. são bactérias da família *Enterobacteriaceae*, Gram-negativas, móveis e aeróbias facultativas. O gênero compreende duas espécies: *Salmonella enterica* e

*Salmonela bongori*, existindo mais de 2600 sorotipos identificados amplamente distribuídos na natureza (GUIBOURDENCHE et al., 2010; BRENNER et al., 2000).

Salmonelose é o nome da infecção causada por salmonela, sendo caracterizada por gastroenterite, com diarreia, náusea, vômito e febre de 12-72 horas após a infecção. Podem ser necessários de 25 até $1x10^{10}$ células bacterianas para causar a doença. Normalmente apresenta quadro autolimitado com duração de 4 a 7 dias em adultos saudáveis. Crianças, idosos e pacientes imunocomprometidos compreendem um grupo de risco, podendo desenvolver septicemia, meningite e até mesmo levar à morte (BOYLE et al., 2007; INGRAHAM e INGRAHAM, 2012).

Nesse sentido, a contaminação de alimentos por *Salmonella* spp. é uma preocupação tanto para saúde pública mundial, quanto para industria de alimentos, uma vez que a formação de biofilmes por este micro-organismos torna-se uma potencial fonte de contaminação e acarreta prejuízos para indústrias de alimentos.

## BIOFILMES

Biofilmes são células microbianas sésseis que se organizam em comunidades, fixadas a superfícies, bióticas ou abióticas, inclusas em uma matriz de substâncias poliméricas extracelulares, agregadas em camada única ou tridimensional (NIKOLAEV e PLANKUNOV, 2007; STOODLEY et al., 2002; COSTERTON et al., 1999). De acordo com Lynch e Robertson (2008), entre 95 a 99% dos micróbios vivem na forma de biofilmes, em alternância entre os estados de vida livre e séssil.

Os biofilmes são compostos pelos micro-organismos, água e, em maior parte, por polissacarídeos. Em menor parte também são encontradas proteínas, lipídeos, vitaminas e ácidos nucleicos. Em biofilmes de *Salmonella* spp. o principal componente da matriz exopolissacarídica é a celulose (COLLINSON, 1996; MACÊDO, 2000).

A capacidade do micro-organismo em formar biofilme é uma estratégia para sua sobrevivência em condições intempéries, e esta capacidade é influenciada pelas características do micro-organismo, da superfície colonizada e pelas características ambientais (SIMÕES et al., 2010; SREY et al., 2013). A principal característica conferida pelo estado séssil é a resistência aos antimicrobianos, tornando a eliminação de biofilmes um grande desafio (MORETRO et al., 2009; JAHID e HA, 2012).

A resistência aos agentes desinfetantes é conferida pela expressão de genes envolvidos com a resistência aos antimicrobianos (CAPPITELLI et al., 2014). Além disso, a resistência também pode ser conferida pela atividade metabólica reduzida dos micro-organismos, a presença de enzimas que degradam os biocidas e pela matriz exopolissacarídica, que atua como barreira física contra os agentes desinfetantes e pela interação destes com componentes da matriz (STEENACKERS et al., 2012; STOODLEY et al., 2002; MAH e O'TOOLE, 2001).

Uma vez que *Salmonella* spp. forma biofilme em superfícies de processamento de alimentos (KUDA et al., 2015) e os agentes sanitizantes comumente usados nos ciclos de limpeza são muitas vezes ineficientes na sua eliminação (CORCORAN et al., 2014), o uso de novas estratégias de controle, como o uso de substâncias de origem natural, torna-se imprescindível na tentativa de eliminar patógenos neste estilo de vida.

## ÓLEOS ESSENCIAS

Os óleos essenciais (OE) são misturas complexas de substâncias voláteis, com características lipofílicas e odoríferas. São considerados metabólitos secundários, formados como resultado de reações envolvendo o metabolismo vegetal. Eles podem ser produzidos em todos os órgãos de plantas e sua complexa composição é atribuída a vários fatores, como o estágio de desenvolvimento da planta, composição química do solo e fatores ambientais (BAKKALI et al., 2008; SIMÕES e SPITZER, 2004).

Os OE exercem papel protetor para plantas contra bactérias, fungos, insetos e até mesmo herbívoros. Além disso, eles podem atuar como propagadores de espécie, uma vez que, em alguns casos, favorecem a dispersão de pólen (BAKKALI et al., 2008).

A atividade antimicrobiana de óleos essenciais e seus constituintes, tanto contra bactérias Gram-positivas, Gram-negativas e fungos, estão amplamente difundidos na literatura, onde vários trabalhos comprovam tais propriedades (AIDA et al., 2015; HERCULANO et al., 2015; SUN et al., 2015). Além disso, a atividade antimicrobiana e antibiofilme de compostos naturais, como óleo essencial de canela (*Cinnamomum zeylanicum*) e seu principal constituinte (cinamaldeído) tem sido relatado na liteura (PIOVEZAN et al., 2015; ZHANG et al., 2014).

O cinamaldeído, composto majoritário do OE de canela, apresenta aspecto viscoso, coloração amarelada e odor forte idêntico ao de canela, sendo muito aplicado na indústria cosmecêutica em fragrâncias (COCCHIARA et al., 2005). Esta substância é conhecida por seu potente efeito antimicrobiano contra bactérias e fungos e também por apresentar atividade anti-inflamatória, antioxidante e vasodilatadora (SHEN et al., 2015; RAFFAI et al., 2008; CHAO et al., 2008; CHENG et al., 2008).

## PROTEÔMICA

A introdução do termo proteômica ocorreu em 1995 e foi definido como a caracterização do conjunto de proteínas expressas por célula em uma situação pontual (WILKINS et al., 1996). Enquanto na genômica é possível fazer análises de situações estáticas, na proteômica é possível avaliar se as proteínas codificadas por determinados genes estão realmente sendo expressas em uma gama de situações diferentes (PENG e GYGI, 2001). Uma característica importante dessa técnica é a possibilidade de se fazer a identificação de proteínas que sofrem mudanças pós-transducionais, que não podem ser detectadas pelas técnicas de genômica (ABHILASH, 2009).

A identificação de proteínas na escala proteômica fornece não só um rol do conjunto de proteínas expressas, mas sim uma abordagem eficaz da dinâmica das funções celulares, podendo responder a questões basais sobre os mecanismos biológicos que envolvem o metabolismo de um organismo (GROMOV e CELIS, 2000; CASH, 1998).

A eletroforese bidimensional é o método mais utilizado para caracterização de um proteoma, possibilitando a caracterização de um grande número de proteínas e suas isoformas (LOW et al., 2002; HAYNES e YATES, 2000). Esta técnica associada à focalização isoelétrica acompanhada pela eletroforese em gel de poliacrilamida (primeira e segunda dimensão, respectivamente), permite a separação de polipeptídeos pelos seus pontos isoelétricos e de massas moleculares (BJELLQVIST et al., 1982; GORG et al., 1999; CELIS et al., 2000; GORG et al., 2000). Na sequência, as proteínas separadas podem então ser identificadas por espectrometria de massas, pela técnica de MALDI-TOF (MANN et al., 2001).

Nesse sentido, a utilização da análise proteômica é um instrumento valioso, que pode auxiliar na compreensão dos aspectos biológicos que conduzem a formação de biofilme pelos

micro-organismos e também compreender de que forma os agentes antimicrobianos alteram o perfil fisiológico de células sésseis.

## JUSTIFICATIVA

A presença de biofilmes formados por micro-organismos patogênicos em ambientes de processamento de alimentos está intimamente relacionada com a contaminação do produto acabado, gerando custos à saúde humana e elevados prejuízos econômicos às indústrias alimentícias.

O conceito de biofilme vem sendo amplamente estudado com o intuito de compreender esse estilo de vida e muitas técnias são aplicadas na tentativa de controlar e eliminar bactérias aderidas às diversas superfícies.

A fim de controlar biofilmes e diminuir a aplicação de agentes biocidas sintéticos, que potencialmente podem contaminar os alimentos e, consequentemente, influenciar negativamente a aceitação desses produtos pelos consumidores, a aplicação de substâncias de origem natural tem sido sugerida como alternativa em muitas pesquisas.

## OBJETIVOS

### GERAL

Avaliar o efeito de cinamaldeído contra células de *Salmonella* Typhimurium planctônicas e em biofilme formado em polipropileno, bem como o perfil proteico expresso por este micro-organismo em células planctônicas e em biofilme.

### ESPECÍFICOS

Determinar a concentração inibitória mínima e concentração bactericida mínima de cinamaldeído contra *S.* Typhimurium.

Avaliar a cinética de ação do cinamaldeído frente a células planctônicas de *S.* Typhimurium.

Avaliar a formação *in vitro* de biofilme por *S.* Typhimurium em polipropileno.

Avaliar a ação de cinamaldeído durante a formação de biofilme em polipropileno por *S*. Typhimurium.

Verificar o efeito de cinamaldeído em biofilme formado por *S*. Typhimurium em polipropileno.

Caracterizar a diferença de expressão proteica entre células planctônicas e sésseis de *S*. Typhimurium.

Determinar a diferença de expressão proteica entre biofilme de *S*. Typhimurium não expostos e expostos ao cinamaldeído.

## REFERÊNCIAS

ABHILASH, M. Applications of proteomics. **International Journal of Genomics and Proteomics**, v. 4, p. 1-10, 2009.

AIDA, A.; ALI, A.A.M.S.; BEHROOZ, M.V. Chemical composition and antimicrobial effect of the essential oil of *Zataria multiflora* Boiss endemic in Khorasan-Iran. **Pacific Journal of Tropical Disease**, v. 5, p. 181-185, 2015.

BAKKALI, F.; AVERBECK, S.; AVERBECK, D.; IDAOMAR, M. Biological effects of essential oils – A review. **Food and Chemical Toxicology**, v. 46, p. 446–475, 2008.

BERMÚDEZ-AGUIRRE, D.; BARBOSA-CÁNOVAS, G.V. Disinfection of selected vegetable under nonthermal treatments: Chlorine, acid, ultraviolet light and ozone. **Food control**, v. 29, p. 82-90, 2013.

BJELLQVIST, B.; EK, K.; RIGHETTI, P.G.; GINAZZA, E.; GORG, A.; WESTERMEIER, R.; POSTEL, W. Isoelectric focusing in immobilized pH gradients: Principle, methodology and some applications. **Journal of Biochemical and Biophysical Methods**, v. 6, p. 317-339, 1982.

BOYLE, E.C.; BISHOP. J.L.; GRASSL, G.A.; FINLAY, B.B. *Salmonella:* from Pathogenesis to Therapeutics. **Journal of Bacteriology**, v. 189, p. 1489–1495, 2007.

BRASIL. Ministério da Saúde. Secretaria de Vigilância em Saúde. Vigilância epidemiológica das doenças transmitidas por alimentos. Ministério da Saúde, Secretaria de Vigilância em

Saúde. Brasília: Ministério da Saúde, 2013. Disponível em: www.saude.gov.br/svs; (Acesso em 16 de Janeiro de 2015).

BRENNER, F.W.; VILLAR, R.G.; ANGULO, F.J.; TAUXE, R.; SWAMINATHAN, B. *Salmonella* Nomenclature. **Journal of Clinical Microbiology,** v. 38, p. 2465–2467, 2000.

CAPPITELLI, F.; POLO, A.; VILLA, F. Biofilm formation in food processing environments is still poorly understood and controlled. **Food Engineering Reviews,** v. 6, p. 29-42, 2014.

CASH, P. Characterization of bacterial proteomes by two-dimensional electrophoresis. **Analytica Chimica Acta**, v. 372, p. 121-145, 1998.

CELIS, J.E.; KRUHOFFER, M.; GROMOVA, I.; FREDERIKSEN, C.; OSTERGAARD, M.; THYKJAER, T.; GROMOV, P.; YU, J.; PÁLSDÓTTIR, H.; MAGNUSSON, N.; ORNTOFT, T.F. Gene expression profiling: monitoring transcription and translation products using DNA microarrays and proteomics. **Federation of European Biochemical Societies**, v. 480, p. 2-16, 2000.

Centers for Disease Control and Prevention. What is Salmonellosis? [Internet]. Atlanta, Geórgia: CDC; 2012. Disponível em: http://www.cdc.gov/salmonella/general/index.html; (Acesso em 17 de Janeiro de 2014).

CHAO, L.K.; HUA, K.F.; SHU, H.Y.; CHENG, S.S; LIN, F.; CHEN, C.J.; CHEN, S.T.; CHANG, S.T. Cinnamaldehyde inhibits pro-inflammatory cytokines secretion from monocytes/ macrophages through suppression of intracellular signaling. **Food and Chemical Toxicology**, v. 46, p. 220–231, 2008.

CHELVAM, K.K.; CHAI, L.C.; THONG, K.L. Variations in motility and biofilm formation of *Salmonella enterica* serovar Typhi. **Gut Pathogens**, v. 2, p. 1-10, 2014.

CHENG, S.S.; LIU, J.Y.; CHANG, E.H.; CHANG, S.T. Antifungal activity of cinnamaldehyde and eugenol congeners against wood-rot fungi. **Bioresource Technology**, v. 99, p. 5145–5149, 2008.

COCCHIARA, J.; LETIZIA, C.S.; LALKO, J.; LAPCZYNSKI, A.; API, A.M. Fragrance material review on cinnamaldehyde. **Food and Chemical Toxicology**, v. 43, p. 867–923, 2005.

COLLINSIN, S.K.; CLOUTHIER, S.C.; DORAN, J.L.; BANSER, P.A.; KAY, A.W. *Salmonella enteritidis* agfBAC operon encoding thin, aggregative fimbriae. **Journal of Bacteriology**, v. 178, p. 662-667, 1996.

CORCORAN, M., MORRIS, D., LAPPE, N.DE., O'CONNOR, J., LALOR, P., DOKERY, P., CORMICANA, M. Commonly used disinfectants fail to eradicate *Salmonella* enterica biofilms from food contact surface materials. **Applied and Environmental Microbiology**, v. 80, p. 1507-1514, 2013.

COSTERTON, J.W.; GEESEY, G.G; CHENG, K.J. Bacterial biofilms: A common cause of persistent infections. **Science**, v. 284, p. 1318–1322, 1999.

GIAOURIS, E.; SAMOILIS, G.; CHORIANOPOULOS, N.; ERCOLINI, D.; NYCHAS, GEORGE-JOHN. Differential protein expression patterns between planktonic and biofilm cells of *Salmonella enterica* serovar Enteritidis PT4 on stainless steel surface. **International Journal of Food Microbiology**, v. 162, p. 105–113, 2013.

GORG, A.; OBERMAIER, C.; BOGUTH, G.; SCHEIBE, B.; WILDGRUBER, R.; WEISS, W. The current state of two-dimensional electrophoresis with immobilized pH gradients. **Electrophoresis,** v. 21, p. 1037-1053.

GORG, A.; OBERMAIER, C.; BOGUTH, G.; WEISS, W. Recent developments in two-dimensional gel electrophoresis with immobilized pH gradients: Wide pH gradients up to pH 12, longer separation distances and simplified procedures. Electrophoresis, v. 20, p. 712-717, 1999.

GROMOV, P.S.; CELIS, J.E. From genomics to proteomics. **Molecular Biology**, v. 34, p. 508-520, 2000.

GUIBOURDENCHE, M.; ROGGENTIN, P.; MIKOLEIT, M.; FIELDS, P.; BOCKEMUHL, J.; GRIMONT, P.A.D.; WEILL, F.X. Supplement 2003–2007 (No. 47) to the White-Kauffmann-Le Minor scheme. **Research in Microbiology**, v. 161, p. 26-29, 2010.

HAYNES, P.A.; YATES, J.R. Proteome profiling – pitfalls and progress. **Yeast**, v. 17, p. 81-87, 2000.

HERCULANO, E.D.; PAULA, H.C.B.; FIGUEIREDO, E.A.T.; DIAS, F.G.B.; PEREIRA, V.A. Physicochemical and antimicrobial properties of nanoencapsulated *Eucalyptus staigeriana* essential oil. **LWT - Food Science and Technology**, v. 61, p. 484-491, 2015.

INGRAHAM, J.; INGRAHAM, C.A.. **Introdução a Microbiologia** – Uma abordagem baseada em estudos de casos. São Paulo: Cengage Learning. 2011

JAHID, I.K.; HA, S.D. A Review of Microbial Biofilms of Produce: Future Challenge to Food Safety. **Food Science and Biotechnology**, v. 21, p. 299-316, 2012.

KOTTWITZ, L.B.M., OLIVEIRA, T.C.R.M., ALCOCER, I.; FARAH, S.M.S.S.; ABRAHAO, W.S.M.; RODRIGUES, D.P. Avaliação epidemiológica de surtos de salmonelose ocorridos no período de 1999 a 2008 no estado do Paraná, Brasil. **Acta Science Health Science**, v. 32, p. 9–15, 2010.

KUDA, T.; SHIBATA, G.; TAKAHASHI, H.; KIMURA, B. Effect of quantity of food residues on resistance to desiccation of food-related pathogens adhered to a stainless steel surface. **Food Microbiology**, v. 46, p. 234-238, 2015.

LINSCOTT, A.J. Food-Borne Illnesses. **Clinical Microbiology Newsletter**, v. 33, p. 41-45, 2011.

LOW, T.Y.; SEOW, T.K.; CHUNG, M.C. Separation of human erythrocyte membrane associated proteins with one-dimensional and two-dimensional gel electrophoresis followed by identification with matrix-assisted laser desorption/ionization-time of flight mass spectrometry. **Proteomics,** v. 2, p.1229-1239, 2002.

LYNCH, A.S.; ROBERSTON, G.T. Bacterial and Fungal Biofilm. **Annual Review of Medicine**, v. 59, p. 415-428, 2008.

MACÊDO, J.A.B. Biofilmes bacterianos, uma preocupação da indústria farmacêutica. **Revista de Fármacos e Medicamentos**, v. 2, p. 19-24, 2000.

MAH, THIEN-FAH, C.; O'TOOLE, G.A. Mechanisms of biofilm resistance to antimicrobial agents. **Trends in Microbiology,** v. 9, p. 34-39, 2001.

MANN, M.; HENDRIKSON, R.C.; PANDEY, A. Analysis of proteins and proteomes by mass spectrometry. **Annual Review of Biochemistry**, v. 70, p. 437-473, 2001.

MØRETRØ, T.; VESTBY, L.K.; STORHEIM, S.E.; KOLTLARZ, K.; LANGSRUD, S. Evaluation of efficacy of disinfectants against *Salmonella* from the feed industry. **Journal of Applied Microbiology**, v. 106, p. 1005–1012, 2009.

NIKOLAEV, Y.A.; PLAKUNOV, V.K. Biofilm—"City of Microbes" or an Analogue of Multicellular Organisms. **Microbiology,** v. 76, p. 125-138, 2007.

PENG, J.; GYGI, S.P. Proteomics: the move to mixtures. **Journal of Mass Spectrometry,** v. 36, p. 1083 – 1091, 2001.

PIOVEZAN, M.; UCHIDA, N.S.; SILVA, A.F.;, GRESPAN, R.; SANTOS, P.S.; SILVA, E.L.; CUMAN, R.K.N.; MACHISNKI JR, M.; MIKCHA, J.M.G. Effect of cinnamon essential oil and cinnamaldehyde on *Salmonella* Saintpaul biofilm on a stainless steel surface. **Journal of General and Applied Microbiology,** v. 60, p. 119–121, 2014.

RAFFAI, G.; KIM, B.; PARK, S.; KHANG, G.; LEE, D.; VANHOUTEE, P.M. Cinnamaldehyde and cinnamaldehyde-containing micelles induce relaxation of isolated porcine coronary arteries: role of nitric oxide and calcium. **International Journal of Nanomedicine**, v. 4, p. 2557-2566, 2014.

SHEN, S., ZHANG, T., YUAH, Y., LIN, S., XU, J., YE, H. Effects of cinnamaldehyde on *Escherichia coli* and *Staphylococcus aureus* membrane. **Food Control**, v. 47, p. 196-202, 2015.

SIMÕES, C. M. O.; SPITZER, V. Óleos voláteis. In: **Farmacognosia:** da planta ao medicamento. 2 ed. Porto Alegre: Editora da UFRGS, 2000, p. 394-412.

SIMÕES, M.; SIMÕES, L. C.; VIEIRA, M. J. A review of current and emergent biofilm control strategies**. LWT - Food Science and Technology**, v. 43, p. 573-583, 2010.

SREY, S.; JAHID, I.K.J.; HA, SANG-DO. Biofilm formation in food industries: a food safety concern. **Food Control**, v. 31, p. 572-585, 2013.

STEENACKERS, H.; HERMANS, K.; VANDERLEYDEN, J.; KEERSMAECKER, S.C.J. *Salmonella* biofilms: An overview on occurrence, structure, regulation and eradication. **Food Research International**, v. 45, p. 502–531, 2012.

STOODLEY, P.; SAUER, K.; DAVIES, D.G.; COSTERTON, J.W. Biofilms as complex differentiated communities. **Annual Review of Microbiology**, v. 56, p. 187-209, 2002.

SUN, J.; WANG, X.; WANG, P.; LI, L.; QU, W.; LIANG, J. Antimicrobial, antioxidant and cytotoxic properties of essential oil from *Dictamnus angustifoliu*. **Journal of Ethnopharmacology**, v. 159, p. 296–300, 2015.

WHO -World Health Organization, Health topics: *Salmonella*. [Internet]. Disponível em: http://www.who.int/topics/salmonella/en/; (Acesso em 18 de Janeiro de 2015).

WILKINS, M.R.; PASQUALI, C.; APPEL, R.D.; OU, K.; GOLAZ, O., SANCHEZ, J.C.; YAN, J.X.; GOOLEY, A.A.; HUGHES, F.; HUMPHERY0SMITH, I.; WILLIAMS, K.L., HOCHSTRASSER, D.F. From proteins to proteome: large scale protein identification by two-dimensional electrophoresis and amino acid analysis. **Biotechnology**, v. 14, p. 61-65, 1996.

ZHANG, G.; ZHOU, W.; ZANG, W.; YANG, A.; LIU, Y.; JIANG, Y.; HUANG, S.; SU, J. Inhibitory effects of citral, cinnamaldehyde, and tea polyphenols on mixed biofilm formation by foodborne *Staphylococcus aureus* and *Salmonell*a Enteritidis. **Journal of Food Protection,** v. 77, p. 927-933, 2014.

# CAPÍTULO II

**Artigo 1: "Biofilm of *Salmonella* Typhimurium on polypropylene: effect of cinnamaldehyde and proteomic analysis"**

# Biofilm of *Salmonella* Typhimurium on polypropylene: effect of cinnamaldehyde and proteomic analysis

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## ABSTRACT

*Salmonella* spp. is the most important foodborne pathogen, leading to consequences, especially to public health and food industry. The effect of cinnamaldehyde against *Salmonella enterica* serotype Typhimurium ATCC 14028 planktonic and biofilm cells on polypropylene was evaluated. Additionally, differential protein pattern expressed by planktonic and biofilm bacteria, as well protein expression on biofilms treated with cinnamaldehyde, was also evaluated. Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of cinnamaldehyde were 312 and 624 µg/mL, respectively. Time-kill curve assay demonstrated that cinnamaldehyde presented bacteriostatic activity at sub-MIC and MIC concentrations and completely eliminated bacterial cells at 624 µg/mL after 12 h of exposure. *S.* Typhimurium formed biofilm on polypropylene, observed by colony counts (8.03 log CFU/cm$^2$) and scanning electron microscopy. Cinnamaldehyde at 234 µg/mL (0.75 x MIC) decreased 1.3 log CFU/cm$^2$ during 48 h of biofilm formation, while treatment

for 1 h with cinnamaldehyde at 624 µg/mL reduced 2.33 log CFU/cm$^2$ of 2-days-old biofilm. In proteomic analysis of biofilm compared to planktonic cells, 16 proteins were up and 5 were down-regulated. Lipoprotein Lpp, 30S ribosomal protein S2 and 50S ribosomal protein L3 were indentified only in biofilm-cells, suggesting their importance on biofilm formation. In cinnamaldehyde-treated biofilm, it was identified 8 up and 11 down-regulated proteins. Among down-regulated, serine hydroxymethyltransferase presented the major decrease (23.8-fold). Cinnamaldehyde showed antimicrobial activity against planktonic cells, nevertheless it was not effective to eliminate completely the biofilm on polypropylene. Proteomic analysis showed that biofilm cells presented differentially expressed proteins compared to its planktonic counterparts, especially proteins involved in cell metabolism. Moreover, it was also observed that proteomic profile of biofilm-treated cells is affect by cinnamaldehyde.

**Highlights**

Cinnamaldehyde presented dose-dependent activity against *Salmonella* Typhimurium.

*Salmonella* Typhimurium formed biofilm on polypropylene surface.

Cinnamaldehyde showed antibiofilm activity, but did not eliminate attached all cells.

There are differences in proteome profile between planktonic and sessile cells.

Cinnamaldehyde changed cells protein expression on biofilm formation by *S.* Typhimurium.

**Keywords:** *Salmonella* Typhimurium; biofilm; polypropylene; proteome; time-kill curve; cinnamaldehyde.

## 1. Introduction

Salmonellosis is the most common occurring bacterial foodborne illness, reaching to 42 thousands of cases and approximately 400 deaths per year in United States (CDC, 2015). Food-contact surfaces are recognized to be one of the most important source of food-bacterial contamination (Cappitelli et al., 2014) and several studies have demonstrated that *Salmonella* spp. are able to form biofilm onto surfaces commonly found in food processing environments (Wang et al., 2013; Corcoran et al., 2014; Nguyen et al., 2014), however few studies have demonstrated biofilm formation of *Salmonella* spp. on polypropylene, a surface commonly used in food processing environments (Oliveira et al., 2006; Iibuchi et al., 2010).

Bacteria in biofilm are more difficult to mechanical removal and exhibit more resistance to disinfectants than their planktonic counterparts (Pan et al., 2006; Simões et al., 2010; Nguyen et al., 2014). The presence of sessile pathogenic bacteria is life-threatening and the development of new strategies, as the use of natural compounds to control biofilms, is extremely desirable (Shi and Zhu, 2009). In this context, cinnamaldehyde, a major compound presented in cinnamon essential oil, is well-known for its antimicrobial properties and has been demonstrated to be effective against bacterial biofilms (Jia et al., 2011; Oliveira et al., 2012; Piovezan et al., 2014; Zhang et al., 2014).

Biofilm formation is a dynamic event and understanding the physiological aspects that assembly the adhesion of bacteria to surfaces and how antimicrobial substances influence it, is a fundamental step to its effective control. Proteomic analysis is a powerful technique that potentially fulfills this requirement. Global protein expression is an excellent approach that allows us to comprehend the pattern and level of protein expression in several conditions (Sauer, 2003).

Considering the above, the purpose of this study was to evaluate the effect of cinnamaldehyde against planktonic and biofilm cells of *Salmonella enterica* serotype Typhimurium on polypropylene. In addition, for the first time, the proteomic techniques were used to identify changes in protein expression between sessile and planktonic cells of *S.* Typhimurium, as well as the changes in the protein expression of biofilm cells exposed to cinnamaldehyde.

## 2. Materials and methods

### 2.1. Bacterial strain and culture conditions

Bacterial strain used was *Salmonella enterica* serotype Typhimurium ATCC 14028. The culture was maintained in Tryptic Soy Broth (TSB; Difco, Le Pont-de-Claix, France)

supplemented with 20% of glycerol at -20 ºC and recovered in Brain Heart Infusion Broth (BHI; Difco, Le Pont-de-Claix, France) for 24 h at 37 ºC. The culture was transferred to Hektoen Agar (Difco, Le Pont-de-Claix, France) and incubated under the same conditions.

### 2.2. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of cinnamaldehyde (purity 93%, Sigma Aldrich, China) were determined by broth microdilution method in 96-well microtiter plates (TTP, Trasadingen, Switzerland) according to Clinical and Laboratory Standards Institute (CLSI, 2012). Bacterial culture was grown on Mueller-Hinton Broth (MHB; Difco, Le Pont-de-Claix, France) for 6 h at 37 ºC, standardized accord to tube 0.5 McFarland Scale and diluted to obtain $5x10^6$ CFU/mL (colony forming units per milliliter). Then, 10 µL of this culture were added into each well containing 100 µL of cinnamaldehyde diluted in MHB (19 to 5000 µg/mL), obtaining a final inoculum of $5x10^5$ CFU/ mL. Microtiter plates were incubated for 24 h at 37 ºC and MIC was visually determined as the lowest concentration of cinnamaldehyde required to inhibit bacterial growth.

Minimum Bactericidal Concentration (MBC) was determined by subcultivation of 10 µL from each well with no bacterial growth on Hektoen Agar for 24 h at 37 ºC. MBC was defined as the lowest concentration that did not allow bacterial growth. Each test was performed in duplicate and the results correspond to three experiments.

### 2.3. Time-kill curve assay

The test was performed as recommended by Isenberg (2004) with slight modifications. Overnight culture of *S.* Typhimurium ATCC 14028 was standardized according to 1.0 McFarland Standard and diluted to obtain final inoculums of $6x10^7$ CFU/ mL. Then, 100 µL of bacterial suspension was transferred to tubes containing 10 mL of MHB supplemented with cinnamaldehyde (0.5 x MIC, 0.75 x MIC, MIC and 2 x MIC), obtaining a final inoculum of $6x10^5$ CFU/ mL, and incubated for 24 h at 37 ºC. At selected time interval for sub-MIC concentrations (0, 6, 12 and 24 h) and MIC and 2 x MIC concentrations (0, 1, 2, 3, 4, 5, 6 12 and 24 h) a sample of 100 µL was removed of each test suspension, serially diluted and plated on Mueller-Hinton Agar (MHA; Difco, Le Pont-de-Claix, France). Plates were incubated for 24 h at 37 ºC and CFU counted. Each test was performed in duplicate and repeated three times.

## 2.4. Biofilm assays

### 2.4.1. Biofilm formation

Biofilm formation on polypropylene was performed according to Uchida et al. (2014). Briefly, overnight culture of *S.* Typhimurium ATCC 14028 was diluted in TSB ($10^7$ CFU/mL), placed into microtubes containing polypropylene (PP) coupons (1 x 8 x 8 mm), previously cleaned according to Bayoumi et al. (2010), and incubated for 24 h at 37 ºC. The content were gently replaced for a fresh TSB and incubated again under the same conditions. Afterwards, attached cells were released from the coupons using an ultrasonic bath for 5 min at 25 kHz (Ultra Cleaner 750A, Unique). The content of microtubes were serially diluted in saline solution, plated on MHA, incubated for 24 h at 37 ºC and CFU were counted. The experiments were performed in triplicate and repeat three times.

### 2.4.2. Effect of cinnamaldehyde on pre-formed biofilms

The biofilm was formed on PP surface as described in the subsection 2.4.1. Coupons were rinsed with saline solution and treated with cinnamaldehyde at concentration of 312 and 624 µg/mL (MIC and 2 x MIC, respectively) for 60 min. The coupons were washed with saline solution and CFU were quantified, as described previously in the subsection 2.4.1.

### 2.4.3. Effect of cinnamaldehyde on biofilm formation

Polypropylene coupons were incubated with overnight culture of *S.* Typhimurium ATCC 14028 diluted in TSB ($10^7$ CFU/mL) supplemented with cinnamaldehyde at concentrations of 234 and 156 µg/ mL (0.75 and 0.50 MIC) for 24 h at 37 ºC. After this period, the medium was gently replaced with a fresh broth supplemented with the same concentrations of cinnamaldehyde and incubated once more for 24 h at 37 ºC. The viable cells were quantified, as described previously in the subsection 2.4.1.

## 2.5. Scanning Electron Microscopy (SEM)

This assay was performed as described by Wong et al. (2009) with slight modifications. Bacterial biofilms on PP coupons were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at 4 ºC for 48h. The coupons were washed two times with 0.1 M cacodylate buffer and dehydrated in a graded ethanol series (50, 70, 80, 90 and 100% twice). Coupons were critical-point dried in $CO_2$, coated with gold and the images were obtained with a Shimadzu SS-550 scanning electron microscope.

## 2.6. Proteomic analyses

### 2.6.1. Recovery of planktonic cells

*S.* Typhimurium ATCC 14028 was incubated in TSB for 24 h at 37 ºC. After this period, the bacterial culture was centrifuged at 4500 x *g* for 5 min and the pellet was incubated in TSB medium at the same conditions. Then, bacterial culture was collected at 4500 x *g* for 5 min, washed twice with saline solution and the pellet obtained was used directly for protein extraction.

### 2.6.2. Recovery of biofilm cells

Without-treated and treated with cinnamaldehyde *S.* Typhimurium biofilm cells on PP surface (90 x 15 x 1 mm) (during and after biofilm formation) was performed as described in subsection 2.4.2 and 2.4.3. Biofilm cells were removed using a cell scrapper and subsequently submitted to ultrasonic bath 25 kHz for 10 min, to recovery the maximum number of cells. Bacterial suspension were centrifuged at 4500 x *g* for 5 min and washed twice with saline solution. The pellets obtained from each treatment were used directly for protein extraction.

### 2.6.3. Protein extraction

Pellet cells were resuspended directly in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS {3—[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 0.5% IPG buffer and 0.5% DTT {dithiotretiol}) and 10 µL of protease inhibitor and submitted to sonication on ice with 12 bursts of 3 s ON, 9 s OFF with 100% amplitude. The suspension was centrifuged 14000 x *g* for 10 min at 4 ºC to remove unlysed and cell debris (Giaouris et al., 2013). Protein solution was treated with 2D Clean-up Kit (Amersham Biosciences) according to manufacture instructions. The determination of total protein was verified according to Bradford method (Bradford, 1976).

### 2.6.4. Two dimensional (2-D) gel electrophoresis

The procedures were carried out according to Giaouris et al. (2013) with some modifications. First dimension separation was carried out using 13 cm Immobiline DryStrip gels (IPG strips) pH gradient 4-7 (GE Helthcare Life Science, USA). Samples corresponding to 400 µg/mL of protein were suspended in Rehydtratation Solution (GE Helthcare Life Science, USA) with 0.6% IPG Buffer and applied to IPG strips. Rehydtratation of IPG strips were performed using Ettan IPGphor II Isoeletric focusing system (GE Helthcare Life

Science, USA) according to the following parameters: (i) 50 V/h, (ii) 500 V/h, (iii) 1000 V/h, (iv) 8000 V/h, (v) 8000 V/h.

After isoelectric focusing, IPG strips were incubated for 30 min in the equilibration solution (75 mM Tris-HCl, pH 8.8, 6M urea, 30% glycerol, SDS {sodium dodecyl sulfate}, 2.5% bromophenol blue) supplemented with 1% of DTT and then another 30 min with equilibration solution containing 2.5% iodoacetamide.

The second dimensional separation was carried out in SDS-PAGE (12.5% acrylamide/Bis-Acrylamide [30:0.8]). Equilibrated IPG strips were sealed on top of the SDS-PAGE gel and vertical separation was performed in two-steps: 16.5 mA per gel during the stacking period and 40 mA per gel during separation period. After electrophoresis, proteins were fixed (1.3% ortho-phosphoric acid and 20% methanol) and stained overnight with Coomassie Brilliant Blue G-250.

Gels were digitalized and spot detection, quantification and analysis were performed using the ImageMaster 2D Platinum software 6.0 (Amersham Biosciences). Differential protein expression was defined as an increase or decrease of spot volume of 2-fold or more.

### 2.6.5. Mass spectrometry

Blue-Coomassie-stained protein spots differentially expressed were manually excised from 2-D gels and destained with 400 µL acetonitrile (50%) in ammonium bicarbonate (25 mmol/L) until the solution became colorless. The sample preparation for mass spectrometry was performed according to Shevchenko et al. (1996) with slight modifications. Trypsin Gold Mass Spectrometry Grade 20 ng/µL (Promega) was used to digestion in gel. The peptides were mixed 1:1 with freshly prepared α-cyano-4-hydroxycinnamic acid matrix (Sigma Aldrich), in 50% acetonitrile and 0.1% trifluoroacetic acid, and 1 µL was spotted on MALDI target plate. Peptide mass spectra were obtained on a mass spectrometer MALDI-TOF/TOF *Autoflex II* (Bruker Daltonics) and analyzed on *Flex Analysis* 2.0 (Bruker Daltonics).

### 2.6.6. Protein identification

Peptide mass fingerprint and MS data were matched to the National Center for Biotechnology Information non-redundant (NCBInr), entries against proteins from *Salmonella* spp., using the *Mascot* program (http://www.matrixscience.com) employing the Biotools software (Bruker Daltonics). The search parameters were fixed: trypsin as enzyme, one incomplete cleavage, 100 ppm error tolerance, and carbamidomethylation and methionine oxidation as fixed and variable modification, respectively. Some of the identified proteins that

had a significant score matching, a hypothetical or predicted listing were subsequently subjected to a homology search using BLAST against NCBInr, in order to assign identities to other proteins.

### 3. Statistics

Results of assays were performed using GraphPad Prism software (version 5.0). The data were analyzed using one-way analysis of variance (ANOVA) to determine significant differences between means for each group, followed by Tukey's test. Values of $p < 0.05$ were considered statistically significant.

### 4. Results

#### 4.1. Minimum inhibitory concentration and Minimum bactericidal concentration

The MIC value of cinnamaldehyde against *S.* Typhimurium ATCC 14028 was 312 µg/mL while the MBC was one-fold higher than MIC values (624 µg/mL).

#### 4.2. Time-kill curve assay

The growth curves of *S.* Typhimurium ATCC 14028 are shown in Figure 1.

After 24 h of growth, bacterial population without cinnamaldehyde reached to ≅ 9 $\log_{10}$ CFU/mL and the number of viable cells decreased gradually with the increased concentration of cinnamaldehyde. Treatment with 0.5 x MIC of cinnamaldehyde caused a growth delay at initial time period of incubation, with an increase of 2 $\log_{10}$ CFU/mL, compared to initial population, after 24 h of incubation. Treatment with 0.75 x MIC of cinnamaldehyde showed a slight reduction of ≅ 1 log CFU/mL after 24 h, while treatment with MIC concentration decreased about 2 log CFU/mL ($p<0.05$) compared to initial population. After treating *S.* Typhimurium ATCC 14028 with cinnamaldehyde (2 x MIC), no viable cells were detected after 12 h of incubation.

#### 4.3. Biofilm formation on polypropylene surface

After 48 h of contact with PP surface, the number of viable cells of *S.* Typhimurium ATCC 14028 recovered from coupons reached to 8.03 log CFU/cm$^2$ (Table 1). The SEM images (Figure 2A and 2B) showed the presence of microcolonies demonstrating biofilm formation. It was also visualized a large number of tightly associated bacteria cells covering PP.

### 4.4. Effect of cinnamaldehyde against *Salmonella* Typhimurium biofilm.

Numbers of *S.* Typhimurium ATCC 14028 attached cells on PP were significantly decreased ($p < 0.05$) from 8.03 log CFU/cm$^2$ to 6.90 and 5.70 log CFU/cm$^2$, after being exposed for 1 h to cinnamaldehyde (MIC and 2 x MIC, respectively) (Table 1). Images of SEM (Figure 2B) demonstrated that biofilm structure was disrupted by cinnamaldehyde; showing diffuse and lower adherence of cells to PP than untreated biofilm and moreover, absence of cells aggregates.

The presence of cinnamaldehyde at 0.5 x MIC during biofilm formation on PP did not reduced significantly ($p>0.05$) the number of cells of *S.* Typhimurium ATCC 14028. A decrease from 8.03 to 6.70 log CFU/cm$^2$ was observed in treatment with 0.75 MIC ($p<0.05$) (Table 1). The effect of these treatments was also observed by SEM (data not shown).

### 4.5. Differential protein expression between planktonic and biofilm cells

The expression of proteins in *S.* Typhimurium ATCC 14028 biofilm cells was compared with the protein expression profile of its planktonic state. Of 37 proteins identified, 16 proteins were up-regulated while 5 proteins were down-regulated in biofilm cells. Thirteen proteins were expressed only in free-living cells, while 3 proteins were expressed in sessile cells. Most of the proteins identified to be up or down-regulated are involved in energy and DNA metabolism, stress response, nutrient and environmental conditions. Three proteins expressed only in biofilm cells were identified: Lipoprotein Lpp, 30S ribosomal protein S2 and 50S ribosomal protein L3 (Table 2).

### 4.6. Differential protein expression between untreated and cinnamaldehyde-treated biofilm cells

The protein expression profile of biofilm cells treated with cinnamaldehyde at sub-inhibitory concentrations was similar, likewise, treatments at MIC and 2 x MIC concentrations. Therefore, the results of proteomic analysis are reported only for 0.75 MIC and 2 x MIC concentrations. During biofilm formation in the presence of cinnamaldehyde (234 µg/mL), 6 and 9 proteins were up and down-regulated, respectively, and 9 proteins were detected only in untreated-cells. In pre-formed biofilms exposed to cinnamaldehyde (624 µg/mL), it was observed that 2 and 7 proteins were up and down-regulated, respectively. Among all proteins down-regulated, 5 were decrease for both treatments (Table 3).

## 5. Discussion

It is well known that foodborne bacteria are able to form biofilms on different surfaces commonly used in food processing environments and several studies proposing strategies to control biofilms have been reported (Niemira et al., 2014; Lee et al., 2013). This study confirmed the capacity of *S.* Typhimurium ATCC 14028 to form biofilm on PP and we purposed the use of cinnamaldehyde to its control. We also applied proteomic techniques in order to compare the protein expression between planktonic and sessile cells, as well, the effect of cinnamaldehyde on *S.* Typhimurium biofilm cells.

The antimicrobial properties of cinnamaldehyde were evaluated by *in vitro* susceptibility and time-kill curve assay. Cinnamaldehyde showed important antimicrobial activity against *S.* Typhimurium planktonic cells and this result is consistent with previous studies that obtained similar MIC and MBC values for *Salmonella* spp. (Piovezan et al., 2014; Becerril et al., 2012; Palaniapan and Holley, 2010). Lower MIC value (6.25 µg/mL) was presented by Sanla-Ead et al. (2011) against *Salmonella* EnteritidisThe mode of action of cinnamaldehyde against *Salmonella* spp. are not yet completely understood, nevertheless Shen et al. (2015) suggested that cinnamaldehyde permeabilizes cell membrane of *Escherichia coli*, leading to a leakage of cytoplasmic content, which could be associated with antimicrobial properties of this compound. Considering the structural and physiological similarities between *E.coli* and *Salmonella* spp., it is possible that the same mechanism could be happening in the present study.

Time-kill curve assay allows assessment of kinetic action of the compound and infer its bactericidal or bacteriostatic effect. According to Pournara et al. (2011) the tested substance is considered to be bactericidal when it is able to reduce $\geq 3$ $\log_{10}$ compared to initial inoculums, and bacteriostatic when it maintains or reduces less than 3 $\log_{10}$. Our results showed bacteriostatic activity of cinnamaldehyde at 312 and 234 µg/mL (MIC and 0.75 x MIC). A bactericidal effect was observed on treatment with cinnamaldehyde at 624 µg/mL (2 x MIC) after 12 h of exposure, since no viable cells could be recovered and remained until the end of 24 h. Yossa et al. (2012) observed a bactericidal effect in nalidixic acid-resistant *Salmonella enterica* after 1 h of exposure to cinnamaldehyde at 800 µg/mL, however after 5 h of exposure it was observed a slight bacterial growth.

In this study, the number of S. Typhimurium ATCC 14028 viable cells recovered from PP and SEM images (Figure 2A and 2B) clearly show multilayer bacterial cells attached to PP and microcolonies, which are characteristics of biofilm structure (Bridier et al., 2015). These results indicate that *S.* Typhimurium ATCC 14028 formed biofilm on PP previously

demonstrated by another studies (Iibuchi et al., 2012; Bayoumi et al., 2010; Oliveira et al., 2006), highlighting the necessity of developing new strategies for biofilm control, once PP is commonly used in food processing environments.

Despite of the great number of studies related to an excellent antimicrobial activity of cinnamaldehyde against planktonic cells of *Salmonella* spp., few studies have demonstrated the effect of cinnamaldehyde against bacterial biofilms (Piovezan et al., 2014; Zhang et al., 2014; Oliveira et al., 2012; Jia et al., 2011) and according to our knowledge, no data relating the effectiveness of cinnamaldehyde against *Salmonella* spp. biofilms on PP are currently available in literature. Our results demonstrated that cinnamaldehyde was not effective to eliminate viable cells, slight reducing *S.* Typhimurium ATCC 14028 attached cells on PP was verified; SEM images showed the presence of remaining cells, as observed by colony counts. However, a significant event was observed, the classic structure of biofilm was disrupted; therefore adhered bacteria could be easily eliminated (Table 1; Figure 1B). Piovezan et al. (2014) also evaluated the effect of cinnamaldehyde against *Salmonella* Saintpaul on stainless by quantifying viable cells and SEM, and similar to ours results, authors recovered about 7 log CFU/cm$^2$ even after treatments with cinnamaldehyde at sub-MIC, MIC and 2 x MIC. Zhang et al. (2014) demonstrated that 0.25 x MIC of cinnamaldehyde reduced less than 1 log CFU/cm$^2$ of *S.* Enteritidis attached to stainless steel.

The results obtained in this study showed that cinnamaldehyde presents bacteriostatic and bactericidal activity against planktonic *S.* Typhimurium, nevertheless it was not so effective against bacteria in sessile cells, confirming that microorganisms in biofilm state are more resistant to antimicrobial agents. This behavior is attributed to the changes in growth rate and phenotype of these biofilm cells and especially to the presence of EPS, which limits the transport of antimicrobial agent through structured biofilm (Mah and O'Toole, 2001).

The mechanism of action of cinnamaldehyde against *Salmonella* spp. sessile bacteria has not been completely elucidated. Some studies have demonstrated that cinnamaldehyde interfered with quorum-sensing on Gram-negative bacilli, an important mechanism of cell-to-cell communication that is directly involved on biofilm formation (Steenackers et al., 2012; Truchado et al., 2012; Niu et al., 2006).

Understanding the interactions between bacteria and food contact surfaces is a critical step in the process of food safety. In this study, proteomic analysis were applied as a tool to gain further insight into physiological changes between free-living and biofilm *S.*

Typhimurium on PP, as well to understand how changes in metabolism occurs when sessile cells are expose to antimicrobial agent cinnamaldehyde.

One of the majors protein up-regulated in this study on *S.* Typhimurium biofilm cells was peroxiredoxin (7.6-fold), an important enzyme involved in bacterial defense against toxic peroxides (Zhang et al., 2005). Once in bacterial biofilms there is accumulation of toxic metabolites, the expression of protective proteins is necessary for bacterial survival. Our results are supported by Condell et al. (2012), which observed an up-regulation of this protein in *S.* Typhimurium exposed to triclosan (stress condition) and by Giaouris et al. (2013) that evaluated the proteomic of *S.* Typhimurium biofilms, and observed the expression of antioxidant proteins only in sessile state.

Lipoprotein Lpp, a membrane protein, was one of the three proteins expressed only in biofilm cells, indicating that it is involved in the pathways for biofilm formation. It was reported that deletion of *lpp* gene (encodes lipoprotein Lpp) in *E. coli* O157:H7 and *S.* Typhimurium reduced biofilm formation by these microorganisms (Uhlich et al., 2009; Sha et al., 2004). Ribosomal protein L3 and 30S ribosomal protein S2, both involved in protein synthesis, were also indentified only in biofilm mode of growth. In other studies, it were observed that expression of *rplC* (gene encodes ribosomal L3 protein) was increased in *E. coli* biofilm and ribosomal protein S2 was also up-regulated in *Staphylococcus xylosus* biofilm, indicating that these proteins are important for biofilm formation (Planchon et al., 2008; Schembri et al, 2003).

On biofilm-treated cells, serine hydroxymethyltransferase (SHMT), that catalyses reactions providing amino acids to metabolism, was down-regulated (23.8-fold). SHMT production is positively regulated by CsgD, an important regulator of matrix components production, and has been demonstrated to stimulate curli production and biofilm formation (Grantcharova et al., 2010; Chirwa and Herrington, 2003). Our findings indicate that the decreased on SHMT could be attributed to cinnamaldehyde.

It was also observed a decrease on expression of peroxiredoxin on biofilm cells treated with cinnamaldehyde both in pre-formed and during its formation (Table 3). As described above, this enzyme confers protection and was up-regulated on biofilm cells compared with their planktonic counterparts. A down-regulation of peroxiredoxin in the presence of cinnamaldehyde could become biofilm-bacterial cells more susceptible to peroxides and other chemical agents.

The stringent starvation protein A was up-regulated on biofilm compared to planktonic cells (2.8-fold) while Giaouris et al. (2013) observed that this protein was expressed only in *S.* Typhimurium biofilm cells. This protein acts as transcriptional activator and has been suggested to play important role in stress response and starvation in *E. coli*. The increase in expression of this protein is in accordance to biofilm stage, once this lifestyle is associated to a reduction in nutrient availability. In treatments with cinnamaldehyde (both during biofilm formation and pre-formed biofilms), this protein was not expressed in the presence of compound, suggesting cinnamaldehyde may affect biofilm formation by reducing biofilm-cell defenses.

## 6. Conclusion

This is the first report, to best our knowledge, about the effect of cinnamaldehyde against *S.* Typhimurium biofilm on polypropylene and the proteomic analysis of that microorganism under this condition. Cinnamaldehyde showed dose-dependent antimicrobial activity against planktonic cells and moreover, reduced the number of cells attached to surface, but was not effective to eliminate the totality of the cells at time period evaluated. Differential protein expression was found between planktonic and sessile cells, as well between untreated and cinnamaldehyde-treated biofilm, especially proteins involved in cellular metabolism and stress response.

## 7. References

Bayoumi, M.A., Kamal, R.M., Abd El Aal, S.F., Awad, E.I., 2012. Assessment of a regulatory sanitization process in Egyptian dairy plants in regard to the adherence of some food-borne pathogens and their biofilms. International Journal of Food Microbiology 158, 225–231.

Becerril, R., Nerín, C., Gómez-Lus, R., 2012. Evaluation of bacterial resistance to essential oils and antibiotics after exposure to oregano and cinnamon essential oils. Foodborne Pathogens and Disease 9, 699-705.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248-254.

Bridier, A., Sanchesz-Vizuete, P., Guilbaud, M., Piard, C., Naïtali, M., Briandet, R., 2015. Biofilm-associated persistence of food-borne pathogens. Food Microbiology 45, 167-178.

Cappitelli, F., Polo, A., Villa, F., 2014. Biofilm formation in food processing environments is still poorly understood and controlled. Food Engineering Reviews 6, 29-42.

Chirwa, N.T., Herrington, M.B., 2003. CsgD, a regulator of curli and cellulose synthesis, also regulates serine hydroxymethyltransferase synthesis in *Escherichia coli* K-12. Microbiology 149, 525–535.

Clinical Laboratory Standards Institute. 2012. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically: Approved Standard, 9th ed. NCCLS document M7-A9. Wayne, PA: Clinical and Laboratory Standards Institute.

Condell, O., Sheridan, Á.; Power, K.A., Bonilla-Santiago, R., Sergeant., K. Renaut, J., Burgess, C. 2012. Comparative proteomic analysis of *Salmonella* tolerance to the biocide active agent triclosan. Journal of Proteomics 75, 4505–4519.

Corcoran, M., Morris, D., Lappe, N.De., O'Connor, J., Lalor, P., Dokery, P., Cormicana, M., 2013. Commonly used disinfectants fail to eradicate *Salmonella* enterica biofilms from food contact surface materials. Applied and Environmental Microbiology 80, 1507-1514.

Giaouris, E., Samoilis, G., Chorianopoulos, N., Ercolini, D., Nychas, George-John., 2013. Differential protein expression patterns between planktonic and biofilm cells of *Salmonella enterica* serovar Enteritidis PT4 on stainless steel surface. International Journal of Food Microbiology 162, 105–113.

Grantcharova, N., Peters, V., Monteiro, C., Zakikhany, K., Römling, U., 2010. Bistable expression of CsgD in biofilm development of *Salmonella enterica* serovar Typhimurium. Journal of Bacteriology 192, 456–466.

Iibuchi, R., Hara-Kudo, Y., Hasegawa, A., Kumagai, S., 2010. Survival of *Salmonella* on a polypropylene surface under dry conditions in relation to biofilm formation capability. Journal of Food Protection 73, 1506–1510.

Isenberg HD., 2004. Clinical Microbiology Procedures Handbook ASM Press, Washington, pp. 5.10.2.

Jia, P., Xue, Y.J., Shao, S.H., 2011. Effect of cinnamaldehyde on biofilm formation and sarA expression by methicillin-resistant *Staphylococcus aureus*. Letters in Applied Microbiology 53, 409-416.

Lee, Young-Duck., Kim, Jin-Young., Park, Jong-Hyun., 2013. Characteristics of coliphage ECP4 and potential use as a sanitizing agent for biocontrol of *Escherichia coli* O157:H7. Food Control 34, 255-260.

Mah, Thien-Fah, C., O'Toole, G.A., 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends in Microbiology 9, 34-39.

Nguyen, H.D.N., Yang, Y.S., Yuk, H.G., 2014. Biofilm formation of *Salmonella* Typhimurium on stainless steel and acrylic surfaces as affected by temperature and pH level. LWT - Food Science and Technology 55, 383-388.

Niemira, B.A., Boyd, G., Sites, J., 2014. Cold plasma rapid decontamination of food contact surfaces contaminated with *Salmonella* biofilms. Journal of Food Science 79, 917-922.

Niu, C., Afre, S., Gilbert, E.S. Subinhibitory concentrations of cinnamaldehyde interfere with quorum sensing. Letters in Applied Microbiology 43, 489–494.

Oliveira, K., Oliveira, T., Teixeira, P., Azeredo, J., Henriques, M., Oliveira, R., 2006. Comparison of the adhesion ability of different *Salmonella* Enteritidis serotypes to materials used in kitchens. Journal of Food Protection 69, 2352–2356.

Oliveira, M.M.M., Brugnera, D.F., Nascimento, J.A., Batista, N.N., Picolli, R.H., 2012. Cinnamon essential oil and cinnamaldehyde in the control of bacterial biofilms formed on stainless steel surfaces. European Food Research and Technology 234, 821–832.

Palaniappan, K., Holley, A., 2010. Use of natural antimicrobials to increase antibiotic susceptibility of drug resistant bacteria. International Journal of Food Microbiology 140, 164–168.

Pan, Y., Breidt, Jr., F., Kathariou, S., 2006. Food processing environment simulated biofilms to sanitizing agents in a *Listeria monocytogenes* resistance. Applied and Environmental Microbiology 72, 7711–7717.

Piovezan, M., Uchida, N.S., Silva, A.F., Grespan, R., Santos, P.S., Silva, E.L., Cuman, R.K.N., Machisnki Jr, M., Mikcha, J.M.G., 2014. Effect of cinnamon essential oil and cinnamaldehyde on *Salmonella* Saintpaul biofilm on a stainless steel surface. Journal of General and Applied Microbiology 60, 119-121.

Planchon,S., Desvaux, M., Chafsey, I., Chambon, C., Leroy, S., Hébraud, M., Talon, R., 2009. Comparative subproteome analyses of planktonic and sessile *Staphylococcus xylosus* C2a: new insight in cell physiology of a coagulase-negative S*taphylococcus* in biofilm. Journal of Proteome 8, 1797–1809.

Pournaras, S., Vrioni, G., Neou, E.; Dendrinos, J., Dimitroulia, E., Poulou, A., Tsakris, A., 2011. Activity of tigecycline alone and in combination with colistin and meropenem against *Klebsiella pneumoniae* carbapenemase (KPC)-producing Enterobacteriaceae strains by time–kill assay. International Journal of Antimicrobial Agents 37, 244–247.

Sanla-Ead, N., Jangchud, A., Chonhechob, V., Suppakul, P., 2012. Antimicrobial activity of cinnamaldehyde and eugenol and their activity after incorporation into cellulose-based packaging films. Pakaging Techonology and Science 25, 7-17.

Sauer, K., 2003. The genomics and proteomics of biofilm formation. Genome Biology, 4, 219.

Sha, J., Fadl, A.A., Klimpel, G.R., Niesel, D.W., Popov, V.L., Chopra, A.K., 2004. The two murein lipoproteins of *Salmonella enterica* serovar Typhimurium contribute to the virulence of the organism. Infection and Immunity 72, 3987–4003.

Shembri, M., Kjaergaard K., Klemm, P., 2003. Global gene expression in *Escherichia coli* biofilms. Molecular Microbiology 48, 253–267.

Shen, S., Zhang, T., Yuah, Y., Lin, S., Xu, J., Ye, H., 2015. Effects of cinnamaldehyde on *Escherichia coli* and *Staphylococcus aureus* membrane. Food Control 47, 196-202.

Shevchenko, A., Wilm, M., Vorm, O., Mann, M., 1996. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Analytical Chemistry 68, 850-858.

Shi, X., Zhou, X., 2009. Biofilm formation and food safety in food industries. Trends in Food Science & Technology 20, 407-413.

Simoões, M., Simões, L.C., Vieira, M.J., 2010. A review of current and emergent biofilm control strategies. LWT - Food Science and Technology 43,573–583.

Steenackers, H., Hermans, K., Vanderleyden, J., Keersmaecker, S.C.J. De., 2012. *Salmonella* biofilms: An overview on occurrence, structure, regulation and eradication. Food Research International 45, 502–531.

Truchado, P., Tomás-Barberán, F.A., Larrosa, M., Allende, A. Food phytochemicals act as Quorum Sensing inhibitors reducing production and/ or degrading autoinducers of *Yersinia enterocolitica* and *Erwinia carotovora*. Food Control 24, 78-85.

Uchida, N.S., Grespan, R., Piovezan, M,., Ferreira, E.C., Machinski Jr., M. Cuman., R.K.N., Mikcha, J.M.G., 2014. Effect of carvacrol on *Salmonella* Saintpaul biofilms on stainless Steel Surface. Tropical Journal of Pharmaceutical Research 13, 2021-2025.

Uhlich, G.A., Gunther IV, N.W., Bayles, D.O., Mosier, D.A., 2009. The CsgA and Lpp proteins of an *Escherichia coli* O157:H7 strain affect HEp-2 cell invasion, motility, and biofilm formation. Infection and Immunity 77, 1543–1552.

Wang, Hu-Hu., Ye, Ke-Ping., Zhang, Qiu-Qin., Dong, Y., Xu, Xing-Lian., Zhou, Guang-Hong., 2013. Biofilm formation of meat-borne *Salmonella enterica* and inhibition by the cell-free supernatant from *Pseudomonas aeruginosa*. Food Control 32, 650-658.

Wong, H.S., Townsend, K.M., Fenwick, S.G., Trengove, R.D., O'Handley, R.M., 2010. Comparative susceptibility of planktonic and 3-day-old *Salmonella* Typhimurium biofilms to disinfectants. Journal of Applied Microbiology 108, 2222-2228.

Yossa, N., Patel, J., Macarisin, D., Millner, P., Murphy, C., Bauchan, G., Lo, Y.M., 2012. Antibacterial activity of cinnamaldehyde and sporan against *Escherichia coli* O157:H7 and *Salmonella*. Journal of Food Processing and Preservation 10, 87-96.

Zhang, B., Su, Y., Ai, G., Wang, Y., Wang, T., Wang, F., 2005. Involvement of peroxiredoxin in protecting cells from radiation-induced death. Journal of Radiation Research 46, 305–312.

Zhang, G., Zhou, W., ZAng, W., Yang, A., Liu, Y., Jiang, Y., Huang, S., Su, J., 2014. Inhibitory effects of citral, cinnamaldehyde, and tea polyphenols on mixed biofilm formation by foodborne *Staphylococcus aureus* and *Salmonella* Enteritidis. Journal of Food Protection 77, 927-933.

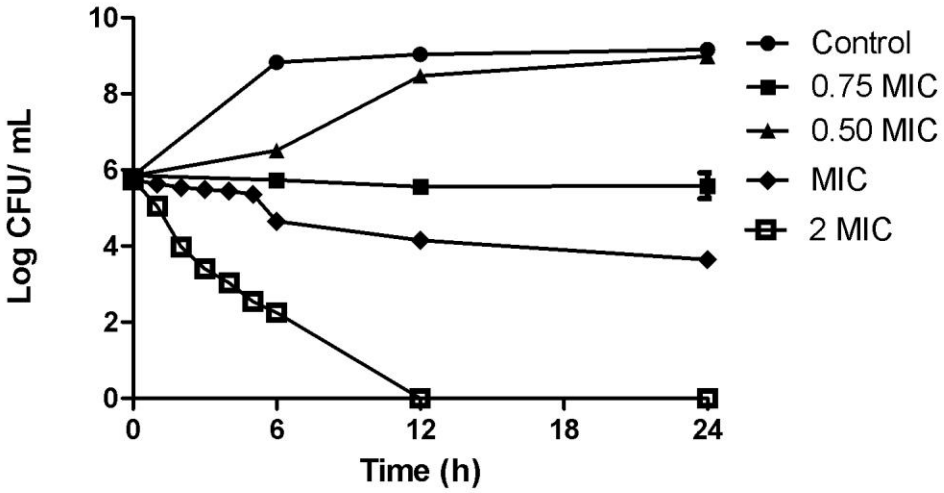


Figure 1. Time-kill curve assay of cinnamaldehyde against planktonic cells of *Salmonella* Typhimurium ATCC 14028. Cinnamaldehyde at sub-inhibitory concentrations (• control; ■ 234µg/mL; ▲156 µg/mL) B) Cinnamaldehyde at MIC and 2 x MIC concentrations (♦312µg/mL; □624 µg/mL).

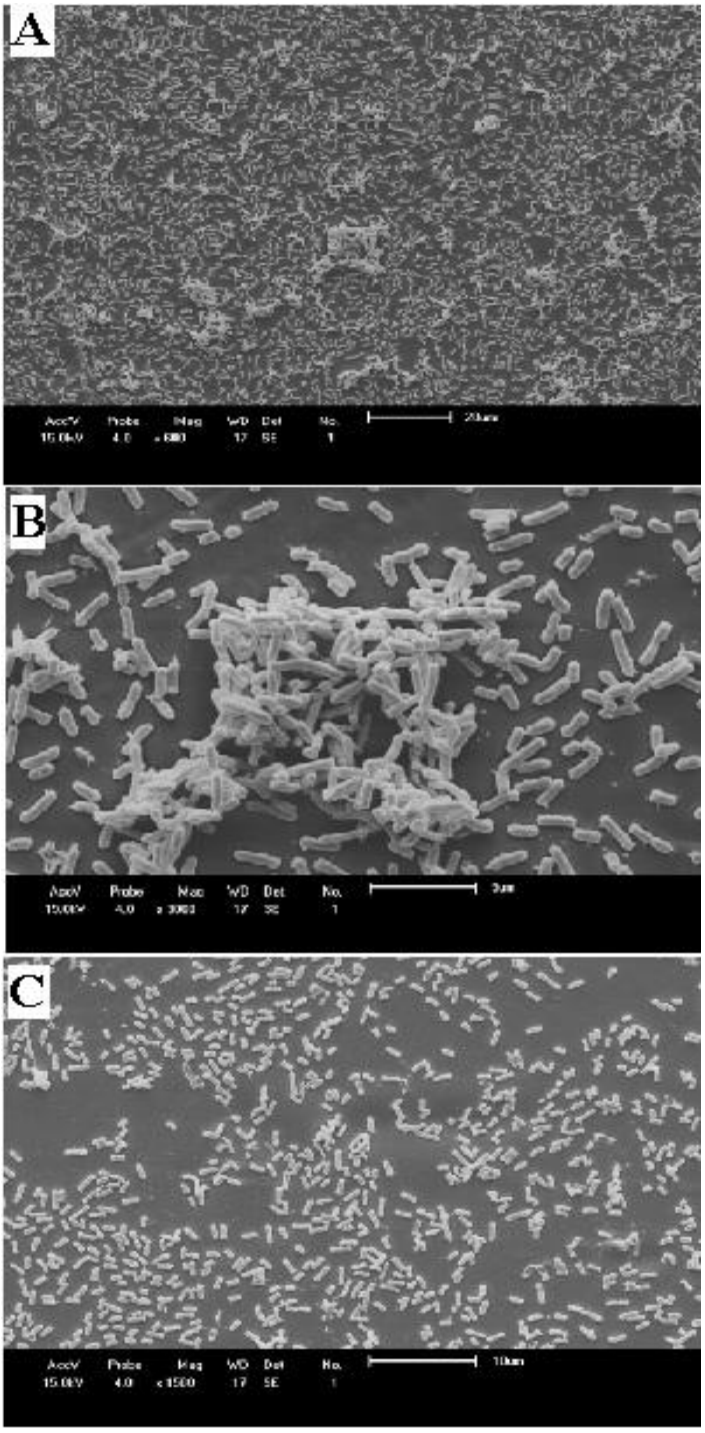


Figure 2. Scanning electron microscopy of *Salmonella* Typhimurium ATCC 14028 biofilm on polypropylene surface. A) No treatment (Magnification 600x); B) No treatment, demonstrating the presence of microcolonies and tridimensional structure of biofilm (magnification 3000x); C) Treatment with cinnamaldehyde at 2 x MIC (624 µg/mL), evidencing lower adherent cells and de absence of cell aggregates (Magnification 600x).

Table 1. Effect of different concentrations of cinnamaldehyde against biofilm of *Salmonella* Typhimurium ATCC 14028 on polypropylene.

| Cinnamaldehyde concentration (µg/mL) | Bacterial count [Mean CFU/ $cm^2$ ±(SD)] |
|---|---|
| 0 | 8.03 (±0.05) |
| 234 (0.75 MIC) [a] | 6.70 (±0.09)* |
| 156 (0.50 MIC) [a] | 7.80 (±0.06) |
| 312 (MIC)[b] | 6.90 (±0.13)* |
| 624 (2 x MIC)[b] | 5.70 (±0.28)* |

Values represent the mean ± SD of at least two independent experiments performed in triplicate.

*$p < 0.05$, compared with no treatment; a) biofilm formation; b) pre-formed biofilm.

Table 2. List of identified proteins altered in *Salmonella* Typhimurium ATCC 14028 biofilm compared to planktonic cells.

| Protein name | Class or function(s) | Fold change | Accession number | Mascot score (>69) | Sequence coverage % | Mass weight (kDa) / pI | |
|---|---|---|---|---|---|---|---|
| | | | | | | Theoretical | Experimental |
| **Up-regulated** | | | | | | | |
| Conjugal transfer nickase/helicase TraI | Replication, recombination and repair | 7.6 | gi\|555237268 | 97 | 21 | 61374/6.84 | 84000/5.32 |
| Elongation factor G | Protein biosynthesis | 7.0 | gi\|446046836 | 253 | 35 | 77723/5.14 | 88000/5.34 |
| Enolase | Energy metabolism | 4.5 | gi\|445958879 | 161 | 40 | 45627/5.25 | 50000/5.6 |
| Membrane protein | Multifunctional | 5.9 | gi\|446875542 | 121 | 29 | 40035/5.67 | 34000/5.38 |
| Peroxiredoxin | Oxidative stress protection | 7.6 | gi\|685254235 | 123 | 53 | 16598/5.8 | 25000/5.34 |
| 6-phosphofructokinase | Carbohydrate transport and metabolism | 2.8 | gi\|685379745 | 251 | 57 | 34959/5.57 | 36000/5.85 |

Table 2. continued

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| FKBP-type 22KD peptidyl-prolyl cis-trans isomerase | Unknown | 3.1 | gi\|62130474 | 131 | 36 | 26363/4.92 | 14000/4.77 |
| Fructose-bisphosphate aldolase | Energy metabolism | 2.2 | gi\|557277093 | 89 | 29 | 39251/5.68 | 40000/5.88 |
| Stringent starvation protein A | Transcriptional activator | 2.8 | gi\|529293610 | 159 | 43 | 24666/5.13 | 25000/5.50 |
| ATP synthase alpha chain protein | Production of ATP from ADP | 6.6 | gi\|513039469 | 204 | 38 | 55341/5.71 | 59000/6.12 |
| Nuclease | DNA function | 4.2 | gi\|446269153 | 78 | 45 | 16507/5.91 | 25000/4.76 |
| Hypothetical protein | Hypothetical function | 4.6 | gi\|640137554 | 96 | 34 | 24208/10.0 | 27000/4.84 |
| Spermidine/putrescine ABC transporter periplasmic substrate-binding protein | Transport of spermidine into the cell | 2.3 | gi\|554659294 | 166 | 66 | 23700/4.82 | 34000/4.83 |
| Peptidyl-prolyl cis-trans isomerase | Catalyze the cis-trans conversion of peptidyl proline bonds | 2.9 | gi\|446178138 | 103 | 46 | 18243/5.52 | 11000/5.77 |

Table 2. Continued

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Heat shock protein | Involved in anti-apoptotic signaling and in cell survival promotion and proliferation | 2.1 | gi\|487702030 | 151 | 66 | 16218/5.08 | 11000/5.75 |
| Hypothetical protein Sb36 | Unknown | 2.3 | gi\|94317625 | 84 | 48 | 15963/ 5.16 | 45000/5.28 |
| **Down-regulated** | | | | | | | |
| Molecular chaperone DnaK | Involved in heat-shock and similar stress response conditions | 2.0 | gi\|555240340 | 185 | 33 | 69274/4.83 | 69000/4.80 |
| 30S ribosomal protein S1 | Induced under various stress conditions, including starvation, heat, ethanol and salt stress | 3.1 | gi\|554471219 | 201 | 35 | 57475/4.93 | 64000/4.91 |

Table 2. Continued

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Aspartate ammonia-lyase | Evolved with amino acid uptake | 9.8 | gi\|689474738 | 89 | 22 | 50257/5.07 | 52000/5.24 |
| Phosphoglycerate kinase | Energy metabolism | 6.6 | gi\|553429808 | 156 | 40 | 37533/5.5 | 60000/5.31 |
| Transketolase | Carbohydrate transport and metabolism | 3.0 | gi\|599858687 | 99 | 18 | 67943/5.56 | 74000/5.65 |
| **Expressed only in plancktonic** | | | | | | | |
| Beta-lactamase | Hypothetical function | * | gi\|685230326 | 118 | 22 | 59494/6.4 | 55000/5.72 |
| Hypothetical protein | Hypothetical function | * | gi\|640137554 | 96 | 34 | 24250/10.3 | 27000/4.8 |
| Phosphoglyceromutase | Carbohydrate degradation (glycolysis) | * | gi\|553429808 | 156 | 40 | 37533/5.5 | 60000/5.31 |
| PhoP family transcriptional regulator | Transcription factor | * | gi\|446909264 | 131 | 54 | 25574/5.18 | 37000/5.61 |

Table 2. Continued

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Uracil phosphoribosyltransferase | Nucleotide transport and metabolism | * | gi\|323210183 | 104 | 37 | 15820/5.01 | 25000/5.62 |
| Phosphoribosylaminoimidazole-succinocarboxamide synthase | Purine and pyrimidine metabolism | * | gi\|353569742 | 170 | 44 | 26989/5.22 | 26000/5.46 |
| Inosine 5'-monophosphate dehydrogenase | Catalyses the conversion of inosine 5′phosphate to xanthosine 5′-phosphate | * | gi\|486165162 | 214 | 37 | 52158/6.06 | 60000/6.69 |
| Lysyl-tRNA synthetase | Involved to stress conditions | * | gi\|487702088 | 140 | 31 | 55495/4.93 | 70000/5.40 |
| DNA-directed RNA polymerase subunit beta | Role in assembly of subunits in DNA-directed RNA polymerase. | * | gi\|487553622 | 127 | 17 | 112866/5.2 | 179000/5.66 |

Table 2. Continued

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Ribose-phosphate pyrophosphokinase | Nucleotide transport and metabolism | * | gi\|447042128 | 190 | 45 | 36886/5.48 | 35000/5.53 |
| Biodegradative arginine decarboxylase | Cell metabolism | * | gi\|553494639 | 93 | 15 | 81658/5.19 | 91000/5.50 |
| Phosphate acetyltransferase | Cell metabolism | * | gi\|685251835 | 249 | 36 | 74786/5.33 | 94000/5.61 |
| Excinuclease ABC subunit B | Involved in DNA repair | * | gi\|446025216 | 71 | 11 | 74961/5.9 | 50000/5.35 |
| **Expressed only in Biofilm** | | | | | | | |
| Lipoprotein Lpp | Membrane protein involved in virulence and adhesion factors | * | gi\|353077690 | 80 | 69 | 10482/9.83 | 32000/5.19 |
| 30S ribosomal protein S2 | Protein synthesis; stress response | * | gi\|487362214 | 83 | 30 | 25907/6.62 | 38000/6.55 |
| 50S Ribosomal protein L3 | Protein s synthesis | * | gi\|513038519 | 120 | 38 | 22333/9.79 | 35000/4.63 |

* No fold change; expressed only in one of both gels.

Table 3. List of identified proteins altered in *Salmonella* Typhimurium ATCC 14028 sessile cells treated with cinnamaldehyde during biofilm formation (0.75xMIC) and after biofilm formation (2 x MIC) compared to untreated biofilm.

| Protein name | Class or function(s) | Fold change | | Accession number | Mascot score (>69) | Sequence coverage % | Mass weight (kDa) / pI | |
|---|---|---|---|---|---|---|---|---|
| | | 0.75 x MIC[a] | 2 x MIC[b] | | | | Theoretical | Experimental |
| **Up-regulated** | | | | | | | | |
| Outer membrane protein A | Contributes to the structural integrity of a complex or assembly within or outside a cell | 2.0 | * | gi\|554628450 | 176 | 46 | 38266/5.49 | 29000/5.48 |
| Spermidine/putrescine ABC transporter periplasmic substrate-binding protein | Transport of spermidine into the cell and stress response | 5.2 | * | gi\|554659294 | 166 | 66 | 23700/4.82 | 34000/4.83 |
| Single-stranded DNA-binding protein | DNA replication, repair, recombination | 2.9 | * | gi\|554917647 | 120 | 70 | 12137/8.01 | 87000/5.41 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Elongation factor G | Protein biosynthesis | 6.2 | * | gi\|446046836 | 253 | 35 | 77723/5.14 | 88000/5.34 |
| Conjugal transfer nickase/helicase TraI | Replication, recombination and repair | 3.6 | * | gi\|555237268 | 97 | 21 | 61374/6.84 | 84000/5.32 |
| Heat shock protein 90 | Expressed in stress conditions | 4.0 | * | gi\|555252087 | 111 | 22 | 71470/5.17 | 65000/5.26 |
| Elongation factor Tu | Elongation-Translation | * | 2.1 | gi\|549591826 | 225 | 51 | 42767/5.23 | 45000/5.64 |
| RNA polymerase, alpha subunit | Gene transcription | * | 3.8 | gi\|447084838 | 190 | 46 | 36717/4.98 | 38000/5.08 |
| **Down-regulated** | | | | | | | | |
| Enolase | Energy metabolism (glycolysis) | 5.1 | 8.3 | gi\|445958879 | 161 | 40 | 45627/5.25 | 50000/5.6 |
| Serine hydroxymethyltransferase | Carbon metabolism | * | 23.8 | gi\|446841891 | 80 | 20 | 45608/6.15 | 50000/6.41 |
| Membrane protein | Multifunctional | 4.4 | 7.6 | gi\|446875542 | 121 | 29 | 40035/5.67 | 34000/5.38 |
| Phosphoglycerate kinase | Energy metabolism | 2.0 | * | gi\|553429808 | 156 | 40 | 37533/5.5 | 60000/5.31 |
| Endo-1,4-D-glucanase | Breakdown cellulose | 2.2 | * | gi\|446740867 | 195 | 50 | 30452/5.21 | 31000/5.21 |
| Peroxiredoxin | Oxidative stress protection | 4.8 | 139 | gi\|685254235 | 94 | 48 | 16598/5.8 | 15000/5.1 |

| Table 3. Continued | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| F0F1 ATP synthase subunit beta | | * | 3.8 | gi\|446112644 | 316 | 60 | 50309/4.9 | 50000/4.9 |
| Triosephosphate isomerase | Energy metabolism | 2.2 | * | gi\|557029326 | 183 | 56 | 27087/5.68 | 26000/5.94 |
| Elongation factor Tu | Translation | 2.2 | * | gi\|549591826 | 225 | 51 | 42767/5.23 | 45000/5.64 |
| Fructose-bisphosphate aldolase | Energy metabolism | 2.3 | 3.9 | gi\|557277093 | 89 | 29 | 39251/5.68 | 40000/5.88 |
| FKBP-type 22KD peptidyl-prolyl cis-trans isomerase | Unknown | 12.3 | 4.0 | gi\|62130474 | 131 | 36 | 26363/4.92 | 14000/4.77 |
| **Expressed only in biofilm untreated** | | | | | | | | |
| Stringent starvation protein A | Transcriptional activator | ▲ | ▲ | gi\|529293610 | 159 | 43 | 24666/5.13 | 25000/5.50 |
| 50S Ribosomal protein L3 | Protein synthesis | ▲ | ▲ | gi\|513038519 | 120 | 38 | 22333/9.79 | 35000/4.63 |
| 6-phosphofructokinase | Carbohydrate transport and metabolism | ▲ | ▲ | gi\|685379745 | 251 | 57 | 34959/5.57 | 36000/5.85 |
| Hypothetical protein | Hypothetical function | ▲ | ▲ | gi\|640137554 | 117 | 42 | 24208/10,3 | 95000/5,88 |
| Nuclease | DNA function | ▲ | ▲ | gi\|446269153 | 78 | 45 | 16507/5.9 | 25000/4.76 |
| Cell division inhibitor MinD | Cellular processes: cell division | ▲ | ▲ | gi\|554960902 | 109 | 38 | 27199/5.04 | 30000/5.54 |

Table 3. Continued

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ATP synthase alpha chain protein | Production of ATP through oxidative phosphorylation | ▲ | ▲ | gi\|513039469 | 204 | 38 | 55341/5.71 | 59000/6.12 |
| Serine hydroxymethyltransferase[c] | Carbon metabolism | • | • | gi\|446841891 | 80 | 20 | 45608/6.15 | 50000/6.41 |
| Phosphoglycerate kinase[d] | Energy metabolism | • | • | gi\|553429808 | 156 | 40 | 37533/5.5 | 60000/5.31 |
| Outer membrane protein A[d] | Contributes to the structural integrity | • | • | gi\|554628450 | 176 | 46 | 38266/5.49 | 29000/5.48 |
| Spermidine/putrescine ABC transporter periplasmic substrate-binding protein[d] | Transport of spermidine into the cell | • | • | gi\|554659294 | 166 | 66 | 23700/4.82 | 34000/4.83 |
| Single-stranded DNA-binding protein[d] | DNA replication, repair, recombination | • | • | gi\|554917647 | 120 | 70 | 12137/8.01 | 87000/5.41 |
| Elongation factor G[d] | Protein biosynthesis | • | • | gi\|446046836 | 253 | 35 | 77723/5.14 | 88000/5.34 |
| Conjugal transfer nickase/helicase TraI[d] | Replication, recombination and repair | • | • | gi\|555237268 | 97 | 21 | 61374/6.84 | 84000/5.32 |
| Heat shock protein 90[d] | Expressed in stress conditions | • | • | gi\|555252087 | 111 | 22 | 71470/5.17 | 65000/5.26 |

[a] Fold-change in protein expression compared to untreated biofilm; [b] Fold-change in protein expression compared to untreated biofilm; [c] Did not expressed during biofilm formation in the presence of cinnamaldehyde; [d] Did not expressed on pre-formed biofilm treated with cinnamaldehyde; ▲ No fold change; expressed only in untreated-biofilm cells; • Expressed only in one of both gels; *Did not show any change.

# CAPÍTULO III

## CONCLUSÕES

Os resultados obtidos demonstraram que:

1) O cinamaldeído apresenta atividade antimicrobiana dose-dependente contra *S.* Typhimurium;

2) *Salmonella* Typhimurium é capaz de formar biofilme em superfície de polipropileno;

3) O cinamaldeído apresentou atividade antibiofilme durante e após a sua formação em polipropileno, no entanto não foi capaz de eliminá-lo completamente no período de tempo avaliado;

4) Existem diferenças entre o perfil de proteínas expressas entre células livres e sésseis de *S.* Typhimurium;

5) A presença de cinamaldeído durante e após a formação de biofilme por *S.* Typhimurium altera a expressão de proteínas por estas células.

## PERSPECTIVAS FUTURAS

Os biofilmes bacterianos têm sido amplamente estudados e a busca por estratégias para eliminá-los ainda demanda muito esforço da comunidade científica. Uma vez que a formação de biofilme por micro-organismos patogênicos se torna um risco à saúde humana e a economia de muitas indústrias, algumas sugestões para trabalhos futuros são expostas a seguir:

1) Aumentar o tempo de exposição do biofilme ao cinamaldeído.

2) Testar o efeito de compostos naturais em biofilmes mistos;

3) Testar outras superfícies utilizadas em ambientes de processamento de alimentos;

4) Testar o uso combinado de substâncias antimicrobianas;

5) Avaliar a expressão de proteínas por células em biofilme em outras condições e expostas a outros tratamentos;

6) Utilizar outras ferramentas de biologia molecular, para compreender os aspectos genéticos que envolvem a formação de biofilme.