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UNIVERSIDADE ESTADUAL PAULISTA  
"JÚLIO DE MESQUITA FILHO"  
Campus de Botucatu



PG-BGA

## PRODUÇÃO DE BIOFILME POR *Salmonella* sp. ISOLADA DE FRANGO

**DÉBORA CRISTINA VIDAL DE OLIVEIRA**

Dissertação apresentada ao Instituto de biociências, Campus de Botucatu, UNESP, para obtenção do título de Mestre no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração Biologia de parasitas e micro-organismos.

*Vera Lúcia Mores Rall*

**BOTUCATU – SP  
2011**

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*“A mente humana é um grande teatro. Seu lugar não é na platéia, mas no palco, brilhando na sua inteligência, alegrando-se com suas vitórias, aprendendo com as suas derrotas e treinando para ser a cada dia, autor da sua história, líder de si mesmo.”*

*(Augusto Cury)*

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## RESUMO

Bactérias do gênero *Salmonella* estão entre as principais causas de enfermidades transmitidas por alimentos (ETA), sendo que os de origem animal são os maiores responsáveis pela veiculação desse micro-organismo, principalmente frangos que são portadores assintomáticos de alguns sorotipos patogênicos para o homem.

A Instrução Normativa nº 70 (2003), do Programa de Redução de Patógenos do Ministério da Agricultura e Abastecimento confere um controle minucioso sobre o processo de abate, garantindo a higiene correta dos alimentos e sua segurança em todos os estágios da produção. Porém, a presença de micro-organismos formadores de biofilmes nas indústrias de alimentos, como a *Salmonella*, é motivo de preocupação pelas falhas que podem ocorrer no processo de higienização.

As propriedades físico-químicas de uma superfície podem exercer influência sobre a adesão dos micro-organismos, os quais aderem mais facilmente às superfícies hidrofóbicas (PVCs) do que às hidrofílicas (vidro ou metais como aço inox).

Assim como outras bactérias patogênicas, as salmonelas possuem fímbrias e produzem celulose, que vão influenciar na sua adesão às superfícies e estão entre os principais componentes da matriz em biofilmes. Os genes *agf* (agregative fímbrias) estão envolvidos na biossíntese de fímbrias, enquanto o segundo componente, a celulose, é produzida pelos genes *bcsA*, *bcsB*, *bcsZ* e *bcsC* (síntese de celulose bacteriana). A produção de fímbrias, co-regulada pelo regulador tipo LuxR, o *agfD*, vai regular indiretamente a produção de celulose atuando no gene *adrA*. A expressão dos dois componentes leva à formação dessa matriz.

Sendo assim, o presente trabalho teve por objetivo a pesquisa da presença desses genes nas cepas de *Salmonella* sp., isoladas de frango e o comportamento dessas cepas quanto à produção de biofilme em diferentes temperaturas (16°, 20°, 28° e 35°C) e materiais (vidro, PVC e aço inoxidável) além da análise da morfologia característica expressa a 28°C, melhor temperatura para a produção de biofilme.

Palavras chave: *Salmonella*, frango, produção de biofilme, genes *agf* e *adrA*, morfologia *rdar*.

## ABSTRACT

Bacteria of the genus *Salmonella* are among the leading causes of foodborne disease (FBD), being, those from animals, the most responsible for placement this microorganism, especially poultry that are asymptomatic carriers of some serotypes pathogenic to humans.

Normative Instruction No. 70 (2003), of the Pathogen Reduction Program of the Ministry of Agriculture and Supply gives rigorous control over the slaughter process, ensuring proper food hygiene and safety in all stages of production. However, the presence of microorganisms forming biofilms in the food, such as *Salmonella*, is a cause of concern due to the failures that may occur in the process of cleaning.

The physicochemical properties of a surface can influence the adhesion of microorganisms, which adhere more readily to hydrophobic surfaces (PVCs) than to hydrophilic (glass or metals such as stainless steel).

Like other pathogenic bacteria, *Salmonella* possess fimbriae and produces cellulose, which will affect their adherence to surfaces and are among the main components of the matrix in biofilms. Genes *agf* (agregative fimbriae) are involved in the biosynthesis of fimbriae, while the second component, cellulose, is produced by genes *bcsA*, *bcsB*, *bcsZ* and *bcsC* (bacterial cellulose synthesis). The production of fimbriae, co-regulated by LuxR type regulator, the *agfD*, will regulate the production of cellulose indirectly acting on the gene *adrA*. The expression of these two components leads to the formation of this matrix.

Therefore, this study aimed to research the presence of these genes in strains of *Salmonella* isolated from poultry and the behavior of these strains for the production of biofilm at different temperatures (16°, 20°, 28° and 35°C) and materials (glass, PVC and stainless steel) as well as the analysis of the characteristic morphology expressed at 28°C, optimum temperature for the production of biofilm.

Key words: *Salmonella*, poultry, biofilm production, *agf* e *adrA* genes, *rdar* morphology

## 1. INTRODUÇÃO

Nos últimos anos a comercialização de frangos no Brasil aumentou pela elevação dos preços de outras carnes e como consequência da alteração dos hábitos alimentares (Carvalho, Florio e Pereira, 2002). A avicultura no país vem se destacando também no cenário internacional e a oferta de alimentos de origem animal em todo o mundo aumentou. Porém, o desenvolvimento tecnológico necessário, como o incremento da criação artificial dos animais que requer temperatura, aeração e umidade, favorece também a multiplicação de micro-organismos. Além disso, o aumento e a concentração populacional de aves podem desencadear condições propícias à infecção, instalação e à propagação de agentes patogênicos. Assim, se um controle adequado não for implementado, muitas bactérias patogênicas podem causar problemas na avicultura (Salles, 2007).

O principal objetivo no abate de animais domésticos para consumo humano é a obtenção da carne com a menor contaminação possível (Silva, Soares e Costa, 2001). Na maioria das plantas de processamento, após o abate e depena do frango, as carcaças são evisceradas e lavadas para serem processadas de acordo com a forma que chegará ao comércio (picadas, empanadas ou pré-cozidas). Finalmente são refrigeradas e os produtos são embalados e armazenados em temperatura de refrigeração (Giordano, 2004). Mas o processo de abate e manipulação das carcaças nos açougues pode acabar por aumentar a microbiota contaminante.

Segundo a legislação brasileira, carnes resfriadas devem ser armazenadas acima de 1°C, não podendo exceder 7°C. A vida útil da carne de aves sobre condições de resfriamento depende da interação de fatores intrínsecos e extrínsecos como o número e o tipo de micro-organismos presentes inicialmente, a temperatura de armazenamento, pH e o tipo de material de embalagem usado (Delazari, 1998).

A microbiota da ave viva se encontra essencialmente na superfície externa, tegumentos cutâneos, no trato digestivo e, em menor grau, no aparelho respiratório. A contaminação se dá inicialmente pela retenção das bactérias sobre a pele, a qual vai permitir que os micro-organismos possam se aderir convenientemente (Silva, 1998). Devido a grande quantidade de nutrientes nas carcaças associada ao desenvolvimento microbiano a carne pode deteriorar-se em um curto espaço de tempo. O tipo e o número de micro-organismos presentes na carne refletem o grau de higiene do abatedouro, como também as condições de

armazenamento após o abate dos animais (Silva, Soares e Costa, 2001). Assim, plantas de processamento de aves favorecem a sobrevivência e transmissão de bactérias comensais levando à deterioração e, potencialmente, a transmissão de bactérias patogênicas (Huys et al., 2005).

Na carne de aves diversos micro-organismos deteriorantes já foram detectados como *Pseudomonas*, *Acinetobacter*, *Aeromonas* sp., *Shewanella putrefacins*, *Lactobacillos* sp. e *Brochorix thermosphaca*. Entretanto, o grupo mais importante são as bactérias patogênicas como *Salmonella* sp., *Clostridium botulinum*, *C. perfringens*, *Campylobacter* sp., *Escherichia coli* e *Listeria monocytogenes* (Silva, Soares e Costa, 2001).

Dentre as fontes de contaminação de *Salmonella*, as carnes são as mais importantes, sendo a de frango, o veículo em numerosos casos de infecções humanas, gerando no homem, quadros de salmonelose (Peresi et al., 1999; Sumner, Raven e Givney, 2004). Medidas de biossegurança são empregadas na indústria avícola, incluindo o sacrifício das aves infectadas em granjas matrizes. Porém, essa enfermidade continua sendo responsável por grandes perdas econômicas para a avicultura, seja pela queda na produção ou gerando frequentes problemas de saúde pública. (Salles, 2007).

O gênero *Salmonella* compreende bacilos Gram negativos, compondo um dos grupos mais complexos da família Enterobacteriaceae, com mais de 2.501 sorotipos descritos. Essas bactérias estão amplamente dispersas na natureza e podem ser encontradas na água, frutas, grãos, flores, árvores e no trato gastrintestinal de vários animais, como insetos, homem e outros mamíferos, além de répteis, aves e insetos (Holt et al., 1994; CDC, 2002).

Segundo Popoff, Bockemühl e Hickman-Brenner (1997), o gênero *Salmonella* consiste somente em duas espécies, *Salmonella bongori* e *Salmonella enterica*, sendo esta última dividida em seis subespécies: *S. enterica* subespécie *enterica*, *S. enterica* subespécie *salamae*, *S. enterica* subespécie *arizonae*, *S. enterica* subespécie *diarizonae*, *S. enterica* subespécie *houtenae* e *S. enterica* subespécie *indica*.

As salmonelas estão entre as principais causas de enfermidades veiculadas por alimentos (D'aoust, Maurer e Bailey, 2001). Em alguns casos provoca distúrbios intestinais leves, ou sintomas mais graves como disenterias. Muitas salmonelas do grupo das infecções paratíficas têm sido isoladas de aves, causando ou não enfermidade, mas permanecendo nos animais, tornando-os portadores (Gast, 2003).

O crescimento da *Salmonella* pode ser evitado se o alimento for mantido sob refrigeração abaixo de 5°C. A temperatura ótima de crescimento está na faixa de 35-43°C e a máxima é de até 49,5°C. Assim, alimentos quentes devem ser mantidos acima dessa temperatura e, embora 55°C já seja uma temperatura segura, 63°C é a recomendada em regulamentações (ICMSF, 1996). A habilidade de crescer em temperaturas abaixo de 7°C depende do sorovar envolvido. Cepas de *S. Typhimurium* são capazes de crescer em temperaturas entre 5 e 6°C e as de *S. Agona*, abaixo de 6°C (Varnam e Evans, 1991).

Em relação aos fatores intrínsecos de um alimento, a atividade de água (aa) pode afetar o crescimento de *Salmonella*, sendo seu valor mínimo igual a 0,94 (aa ótimo: 0,99). Esse micro-organismo pode viver um ano ou mais em alimentos com baixa aa, como chocolate, pimenta e gelatina (ICMSF, 1996). Em relação ao pH, o valor mínimo para o crescimento é 3,8 e o máximo, 9,5, sendo o ótimo entre 7 e 7,5 (ICMSF, 1996).

Segundo Oliveira (1995), as salmonelas resistem meses no ambiente, mas são sensíveis à luz solar e aos desinfetantes mais usados, tais como fenóis, clorados e iodados. A presença de salmonelas na avicultura industrial é significativa. Segundo Gast (1997), para prevenir a introdução de *Salmonella* em granjas é necessário evitar a transmissão vertical, garantindo um programa eficaz de biossegurança. Assim, lotes livres de contaminação dependem de um controle rígido dentro dos programas sanitários das matrizes (Rocha et al., 2003; Tessari et al., 2003). Tessari et al. (2003), pesquisaram a incidência de *Salmonella* sp. em 103 lotes de pintos de corte recém-nascidos, dos quais 32 (24,62%) apresentaram resultado positivo para *Salmonella* sp., sendo 24 (18,46%) *Salmonella* Enteritidis e 8 (6,15%) *Salmonella enterica* subespécie *enterica*.

Surtos de salmonelose no Brasil foram relatados entre os anos de 1994 e 1995 em vários estados, com o número de pessoas afetadas variando de duas a 300 por surto (Gelli, 1995). Segundo Tavechio et al. (2002), de um total de 4.581 isolamentos de *Salmonella* sp. realizados pelo Instituto Adolfo Lutz – SP - Brasil, entre 1996 e 2000, a *Salmonella* Enteritidis foi o sorovar predominante, correspondendo a 32,7 % dos isolados.

Em 1994, o Ministério da Agricultura Pecuária e do Abastecimento implementaram o Programa Nacional de Sanidade Avícola (PNSA), que estabeleceu normas para inspeção de aves industriais em se tratando de *Salmonella*. Este programa prevê a avaliação microbiológica de todos os lotes de aves importadas e, posteriormente, de aves reprodutoras, de corte e postura, devendo ser feita logo nos primeiros dias de vida (Zancan, 2000).

A *Salmonella* Enteritidis é um sorovar muito encontrado em carne de aves. Em 1995, o Ministério da Agricultura reforçou a legislação de controle desse sorotipo nas granjas avícolas, enfatizando o Programa Nacional de Sanidade Avícola (Brasil, 1995). Entretanto, sua operacionalização tem ficado muito aquém do desejado, pois esse micro-organismo predominou entre todos os sorovares isolados entre 1994 e 1999, correspondendo a 75,6% dos 45 sorovares isolados de aves no período (Andreatti Filho, 2001).

A Instrução Normativa nº 70 (Brasil, 2003) surgiu em 2003 visando conferir um controle minucioso sobre o processo de abate e atender as exigências de segurança do alimento baseado nos princípios de Boas Práticas de Fabricação (BPF), no Procedimento Padrão de Higiene Operacional (PPHO) e na Análise de Perigos e Pontos Críticos de Controle (APPCC).

Na Suécia, regulamentos governamentais impostos desde 1961, estipulam um controle das aves importadas para que se evite a contaminação por *Salmonella* sp. Além de um certificado de origem que garanta que elas não possuem tal bactéria, após o ingresso, elas sofrem quarentena por 15 semanas, período no qual são submetidas a quatro exames bacteriológicos. Caso o micro-organismo seja isolado, as aves são sacrificadas (Ribeiro, 2004).

As bactérias presentes nos alimentos, além de favorecerem a deterioração e/ou redução da vida útil desses produtos, podem veicular patógenos, trazendo riscos à saúde do consumidor. Assim, a higiene correta no processamento e manipulação desses alimentos é necessária para garantir sua segurança em todos os estágios de produção minimizando a preocupação com a saúde pública (Cortez, 2003).

Falhas no processo de higienização permitem que resíduos aderidos aos equipamentos e superfícies transformem-se em potencial fonte de contaminação na indústria de alimentos. Os micro-organismos podem aderir às superfícies, interagindo com as mesmas e iniciando a multiplicação celular (Oliveira et al., 2006). Quando a massa bacteriana é suficientemente espessa para agregar nutrientes, resíduos e outros organismos, o biofilme está estabelecido (Zottola e Sasahara, 1994).

No século 17, Anton van Leeuwenhoek construiu um microscópio, através do qual ele observou uma placa de biofilme formada em seus dentes, denominando-a de "animalculi", unidades capazes de produzir uma comunidade microbiana. No entanto, somente na década de 1970, foi desenvolvida a idéia de que bactérias sésseis poderiam existir sob a forma de

biofilme, constituindo um dos principais componentes da biomassa bacteriana em muitos ambientes (Costerton, Geesey e Cheng, 1978). A partir dos anos 80 surgiu a concepção de que bactérias associadas entre si organizavam-se de forma elaborada (Lawrence et al., 1991).

Assim, micro-organismos podem existir no ambiente como células planctônicas ou em comunidades formando os biofilmes. Na forma de tal matriz, as células podem estar ligadas à uma superfície de forma compacta, incluídas nessa rede hidrofóbica predominantemente composta por polissacarídeos (Donlan, 2002). Essa matriz é formada por células bacterianas sésseis que foram aleatoriamente aderidas (Costerton et al., 1987), passando por um processo de diferenciação que transforma pequenos grupos de bactérias aderidas em uma comunidade de biofilme espesso, em uma superfície colonizada (Stoodley et al., 2002).

Caiazza e O'Toole (2004) sugeriram que as bactérias, em resposta às mudanças das condições ambientais são capazes de alternar entre um estado de vida livre, virulenta e um estado aderido, menos virulentos. Outros estudos indicaram que essa mudança de estado de vida livre para o modo de biofilme coincide com uma mudança no metabolismo (Hamilton et al., 2009; White et al., 2010 ).

O lipopolissacarídeo (LPS) é um importante componente da membrana externa das bactérias Gram negativas, que desempenha um papel na fixação inicial na superfície. O estado fisiológico das células também influencia a hidrofobicidade e o grau de aderência bacteriana. Bower, Mc Guire e Daeshel (1996) observaram que esporos aderem mais rapidamente que as células vegetativas nas superfícies de contato, devido à maior hidrofobicidade da superfície celular.

A matriz do biofilme é um ambiente complexo formado por proteínas, DNA, RNA, íons e polímeros polissacarídicos. Os polissacarídeos estão frequentemente envolvidos no estabelecimento de produção de biofilme por interações célula-célula (Branda et al., 2005). Apêndices extracelulares filamentosos como flagelos e fímbrias são produzidos por muitas células e desempenham um papel complementar no processo de adesão (Harbron e Kent, 1988).

Assim como outras bactérias patogênicas, as salmonelas possuem fímbrias, que consistem em apêndices de membrana mais curtos que os flagelos. São compostas por apenas uma proteína estrutural, a pilina, dispostas de maneira helicoidal na superfície das bactérias Gram negativas (Tortora, Funke e Case, 2000). Segundo Sauer et al. (2001), as fímbrias e estruturas associadas tem se mostrado importantes na adesão e colonização de superfícies,

sendo importantes na interação bactéria-hospedeiro, na persistência ambiental, na formação de biofilmes e colonização e invasão de células (Gibson et al., 2007). Tais estruturas possuem resíduos hidrofóbicos de aminoácidos (Rosenberg e Kjelleberg, 1986), contribuindo para a hidrofobicidade da célula bacteriana e auxiliando ainda mais na agregação de novas bactérias. A principal função das fímbrias é superar a barreira de repulsão eletrostática inicial que existe entre uma célula e o substrato (Corpe, 1980).

Diferentes sinais ambientais, como a osmolaridade, temperatura, O<sub>2</sub>, CO<sub>2</sub>, pH, compostos nitrogenados e disponibilidade de nutrientes, vão gerar respostas nas regulações gênicas das bactérias, como por exemplo na formação do biofilme (Guiney, 1997).

Hamilton et al. (2009) estudaram os biofilmes de *Salmonella* Typhimurium. Vários genes e proteínas envolvidas na fixação bacteriana, motilidade, detecção e resposta a disponibilidade de oxigênio, regulação gênica global, transporte e resposta ao estresse foram encontrados sendo diferentemente expressos em biofilmes quando comparados às células planctônicas. Curiosamente, também vários genes envolvidos no metabolismo de aminoácidos mostraram-se diferentemente expressos (Hamilton et al., 2009).

Fímbrias e celulose estão entre os principais componentes da matriz em biofilmes de *Salmonella*. Os genes *agf* (agregative fímbrias) envolvidos na biossíntese de fímbrias são organizados em dois operons, o *agfBAC* e o *agfDEFG* (Collinson et al., 1996). O segundo componente da matriz extracelular dos biofilmes de *Salmonella* é a celulose, produzida pelos genes *bcsA*, *bcsB*, *bcsZ* e *bcsC* (síntese de celulose bacteriana). A produção de fímbrias, co-regulada pelo regulador tipo LuxR, o *agfD*, vai regular indiretamente a produção de celulose, atuando no gene *adrA*, (Romling et al., 2000, Romling, 2002; Zakikhany et al., 2010). A co-expressão dos dois componentes leva à formação de uma rede altamente hidrofóbica com células compactadas, alinhadas em paralelo formando uma matriz rígida.

As fímbrias, associadas com a síntese de celulose em *Salmonella* na formação do biofilme leva à expressão de um fenótipo distinto das colônias, em placas de agar Luria Bertani, vermelho, seco e áspero (*rdar*, no inglês red, dry and rough) a 28°C mas não a 37°C (Solano et al., 2002; Gerstel e Romling, 2003). A formação de tais colônias se dá por uma mudança de morfologia suave para uma morfologia agregada com a produção dos componentes da matriz extracelular (White et al., 2006), produzidos em resposta a mudanças ambientais (Gerstel e Romling, 2001). A morfologia *rdar* fornece uma maior resistência à dessecação e desinfecção, permitindo uma maior capacidade de sobrevivência dos micro-



organismos (Anriany et al., 2001; Scher, Romling, Yaron, 2005; White et al., 2006; Apel et al., 2009). Hipóteses apontam que a morfologia *rdar* representa um estado crítico na transmissão de *Salmonella* entre os ambientes (Gerstel e Romling, 2001; White e Surette, 2006).

Grantcharova et al. (2010) demonstraram que o gene *agfD* é necessário para a maturação do biofilme, porém não atua durante o estabelecimento da colônia bacteriana. Tal gene pode ser visto, portanto, como um ponto de controle da formação do biofilme, regulação da expressão de todos os constituintes principais dessa matriz em *Salmonella* (em condições *rdar*) e controle da transição entre células planctônicas e comportamentos multicelulares. Porém, é importante ressaltar que a sua expressão é altamente regulada por diferentes estímulos do ambiente (temperatura, tensão de oxigênio, nutrientes, osmolaridade, ferro e pH) (Gerstel e Romling, 2001).

Segundo O'Toole, Kaplan e Kolter (2000), trabalhos com bactérias Gram negativas têm demonstrado que a formação de biofilme é prejudicada por mutações em genes envolvidos na mediação da motilidade, na síntese de exopolissacarídeos, adesinas de membrana externa, bem como os reguladores globais de expressão gênica. Quando células de uma colônia *rdar* de *S. Typhimurium* apresentaram mutações no gene *agf*, as colônias formadas se apresentaram rosas (pink), morfologia denominada *pdar*, em vez de vermelhas (Romling et al., 1998; Romling e Rohde, 1999). Por outro lado, a inserção de uma mutação no *adrA*, formou colônias marrons (brown), morfologia denominada *bdar*. Quando ocorreu deleção do *agfD*, as colônias se apresentaram brancas e lisas, morfologia denominada *saw* (smooth and white, lisas e brancas) (Romling et al., 1998; Romling e Rohde, 1999; Romling et al., 2000).

A formação do biofilme pelas células bacterianas, ao oferecer tolerância ao estresse incluindo susceptibilidade reduzida aos antibióticos e desinfetantes, torna a sua eliminação de instalações de processamento de alimentos um grande desafio (Mulcahy, Charron-Mazenod e Lewenza, 2008; Simões e Vieira, 2009).

Cada vez mais, o aumento da resistência gera um impacto negativo em várias atividades, representando perdas significativas para indústrias (Simões, Pereira e Vieira, 2003). A formação de biofilmes microbianos pode gerar estragos em equipamentos através da biocorrosão, contaminação de produtos, perdas energéticas relacionadas com o aumento de atrito, resistência acrescida à transferência de calor e perdas de pressão (Jass e Walker, 2000).

Além disso, a formação de tal matriz pode atuar como um substrato para outros micro-organismos menos propensos a formação de biofilme, aumentando a probabilidade da sobrevivência destes e sua disseminação (Lapidot, Romling e Yaron, 2006).

O tempo de formação do biofilme depende da frequência de limpeza e dos regimes de desinfecção. Superfícies de contato com o alimento devem ser limpas várias vezes por dia, enquanto as superfícies do ambiente, como paredes, geralmente, são limpas uma vez por dia. Gibson et al. (1995) relataram que um biofilme recentemente aderido a uma superfície em uma rede de processamento de alimentos foi proveniente de um biofilme antigo que se formou anteriormente em superfícies ambientais.

A limpeza imprópria, assim como a desinfecção de equipamentos realizada de maneira ineficaz estão entre as principais fontes de contaminação dos produtos em uma indústria de alimentos (Jessen e Lammert, 2003). O design dos equipamentos em uma indústria alimentícia, assim como a escolha dos materiais de superfície e revestimento são de extrema importância na prevenção da formação de biofilme. Até os programas de saneamento mais eficazes não conseguem compensar as deficiências básicas causada por design falhos de equipamentos, com cantos inacessíveis, frestas, fendas, juntas, válvulas e articulações, que são pontos vulneráveis para a acumulação de biofilme (Chmielewski e Frank, 2006). A combinação de um equipamento de alto padrão e um ambiente higienicamente concebidos (sem fendas, espaços mortos, material de superfície, etc) permitem uma limpeza eficaz. Um programa de saneamento eficaz remove todo material indesejável das superfícies, incluindo micro-organismos, corpos estranhos e resíduos provenientes de produtos de limpeza (Dosti, Guzel-Seydim e Greene, 2005).

Para que o biofilme se desenvolva, deve ocorrer a interação entre as células bacterianas, a superfície a qual elas vão se aderir e a composição do meio circundante (Davey e O'Toole, 2000; Donlan, 2002; Dunne, 2002; Stoodley et al., 2002). Quanto mais hidrofóbica e áspera a superfície, melhor a adesão bacteriana (Simões et al., 2008). Um aumento na velocidade de fluxo ou concentração de nutrientes também podem favorecer a formação da matriz (Simões et al., 2007).

Biofilmes podem se formar em tubulações do sistema de água, equipamentos industriais e em instalações de processamento de alimentos, onde essas matrizes podem ocorrer em superfícies de manipulação de alimentos ou áreas onde o alimento é armazenado

ou, ainda, correias transportadoras e equipamentos (Kumar e Anand, 1998; Wong, 1998; Donlan, 2002).

Relatos têm demonstrado que as salmonelas conseguem formar biofilmes em superfícies abióticas, como plástico, borracha, cimento, vidro e aço inoxidável (Joseph et al., 2001; Solano et al., 2002; Prouty e Gunn, 2003; Arnold e Yates, 2009; Hurrell et al., 2009; Moretro et al., 2009).

O aço inoxidável é frequentemente utilizado como material de equipamentos em uma planta de processamento de alimentos. A preferência de escolha deste material para as superfícies de trabalho e pias de cozinha por muitos anos se deve à sua força mecânica, resistência à corrosão, longevidade e facilidade de fabricação (Holah e Thorpe, 1990). Além disso, esse material é relativamente resistente ao ataque químico por oxidação e outros agentes sanitizantes (Boulangue-Peterson, 1996). Os sistemas de tubulação nos aviários normalmente são feitos de PVC e aço inoxidável. Foi relatado que a tubulação de PVC e os bicos dos bebedouros das aves podem abrigar biofilmes (Trachoo, Frank e Stern, 2002). Em açougues, revestindo reservatórios, e nas residências, constituindo superfícies de corte e potes, o vidro é amplamente utilizado.

O aumento no consumo da carne de aves, em decorrência do aumento do preço de outras fontes protéicas de origem animal e pela alteração de hábitos alimentares da população (Valeriano et al., 2003), associada a possível presença de micro-organismos patogênicos em biofilmes (Donlan e Costerton, 2002), aponta para a necessidade do estudo de cepas portadoras dos genes responsáveis pela produção de biofilmes em materiais utilizados na indústria e também das características do ambiente, fatores como pH, temperatura e nutrientes (Shi e Zhu, 2009). A presença de *Salmonella* como patógeno e produtora de biofilmes nas indústrias de alimentos é motivo de preocupação por parte dos órgãos responsáveis pela inspeção de produtos de origem animal e saúde pública (Joseph et al., 2001), mas poucas são as pesquisas realizadas com *Salmonella* para a produção de biofilme em vidro, aço inox e PVC.

## **2. OBJETIVOS**

### **2.1. Objetivo Geral**

Avaliar a produção de biofilme por cepas de *Salmonella* sp. isoladas de carne de frango, em diferentes temperaturas (16°, 20°, 28° e 35°C) e materiais (PVC, vidro e aço inox).

### **2.2. Objetivos Específicos**

-Isolamento de cepas de *Salmonella* de amostras de frango comercializadas na cidade de Botucatu.

-Pesquisa da presença dos genes envolvidos na produção de biofilme nessas cepas.

-Capacidade da expressão desses genes em diferentes temperaturas (16°, 20°, 28° e 35°C) e materiais (PVC, vidro e aço inox) e comparação das diferentes condições, a fim de se verificar quais as melhores condições de não-produção.

-Observar a morfologia das colônias, em relação à aparência *rdar* (red, dry and rough, do inglês vermelha, seca e rugosa).

## **CAPÍTULO 1**

Este trabalho deu origem ao artigo “Analysis of the capacity of producing biofilm on glass, PVC and stainless steel by *Salmonella* sp. Isolated from raw poultry.” que foi submetido para publicação no periódico “Food Science and Technology”.

ANALYSIS OF THE CAPACITY OF PRODUCING BIOFILM ON GLASS, PVC AND  
STAINLESS STEEL BY *Salmonella* sp. ISOLATED FROM RAW POULTRY

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## ABSTRACT

Bacteria of the genus *Salmonella* are among the leading causes of foodborne-disease and those of animal origin are largely responsible for transmitting this microorganism, especially poultry, since they are asymptomatic carriers. The presence of microorganisms; such as *Salmonella*, forming biofilms, a complex matrix, in the food, is a cause of concern, because of the failures that may occur in the sanitation process. The physicochemical properties of a surface can exert a strong influence on the adhesion of microorganisms, which adhere more readily to hydrophobic surfaces (PVCs) than to hydrophilic (glass or metal such as stainless steel). Thus, this study aims to investigate the production of biofilm by strains of *Salmonella* isolated from raw poultry, in different temperatures (16°, 20°, 28° and 35°C) and materials (glass, PVC and stainless steel).

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Key words: *Salmonella*, poultry, biofilm production

## 1. INTRODUCTION

35           The increased consumption of poultry leveraged, in the past years, the poultry industry, creating conditions to the installation and spread of pathogens (Salles, 2007). Poultry are carriers of many *Salmonella* serotypes, being a vehicle for numerous cases of human infections by this microorganism (Peresi et al., 1999; Sumner, Ravem and Givney, 2004).

40           The process of cleaning in a food industry must be careful, avoiding that sediment become stuck to the equipment (Oliveira et al., 2006). When this process is ineffective, bacteria can form biofilms on environment (Zottola and Sasahara, 1994). Biofilms provide tolerance to stress, including reduced susceptibility to antibiotics, and are responsible for biocorrosion of equipment, product contamination, energy losses related to increased friction, increased resistance to heat transfer and pressure loss (Jass and Walker, 2000; Mulcahy, 45 Charron-Mazenod and Lewenza, 2008). Biofilms can also serve as a substrate for other bacteria, less likely to form such a matrix (Lapidot, Romling and Yaron, 2006).

          A hydrophobic surface, such as PVC, favors bacterial adhesion (Simões et al., 2008), whereas in hydrophilic surfaces such as stainless steel and glass, the microorganisms are more difficult to adhere. *Salmonella* demonstrated the ability to form biofilms on abiotic surfaces 50 such as plastic, rubber, cement, glass and stainless steel (Joseph et al., 2001; Prouty and Gunn, 2003; Solano et al., 2002; Arnold and Yates, 2009; Hurrell et al., 2009; Moretro et al., 2009).

          In a food processing plant, the material often used is stainless steel because of its mechanical strength, resistance to corrosion, chemicals and sanitizing agents, easiness of 55 manufacturing, being widely used in sinks and machinery (Holah and Thorpe, 1990; Boulanger-Peterson, 1996). In addition to stainless steel, pipes and the Nipple from the drinkers of the birds are usually made of PVC (Trachoo, Frank and Stern., 2002). Yet, in butcher shops, coating reservoirs, and at homes, making pots and boards, the glass is widely used.

60           In recent years, demand for poultry meat increased by the search for healthy foods and the increasing prices of other meats (Valeriano et al., 2003). The possible presence of pathogenic microorganisms in biofilms and/or biofilm-forming microorganisms in the food, point the need to study the capacity of the development of biofilms on materials used in the food industry throughout the food process, involving the conditions of which meat is exposed



65 as the temperature variation (Donlan and Costerton, 2002; Shi and Zhu, 2009). *Salmonella*,  
capable of producing biofilms, is cause for concern by the bodies responsible for inspection of  
products from animals and public health (Joseph et al., 2001), but there are few studies  
conducted with this microorganism for the production of biofilm on glass, stainless steel and  
PVC.

70 Thus, the present study was aimed to analyse the behavior of *Salmonella* sp. strains  
isolated from poultry for the production of biofilm at different temperatures (16 °, 20 °, 28 °  
and 35 ° C) and materials (glass, PVC and stainless steel).

## 75 2. MATERIALS AND METHODS

### 2.1 Obtaining samples of poultry

A total of 240 samples of poultry were analyzed. These samples were purchased in  
stores in the city of Botucatu – SP, Brazil, and transported immediately in refrigerated  
isothermal box containing dry ice to the Laboratory of Food Microbiology, Institute of  
80 Biosciences, UNESP. - Botucatu.

### 2.2 Microbial Analysis

All culture media, except where especificed, were from Oxoid brand. The detection of  
*Salmonella* was performed according to Andrews and Hammack (2001). For this purpose, 25g  
85 of sample were homogenized in 225 ml of petoned water and incubated at 35°C for 24 hours.  
After this period, 1 ml was transferred to 10 ml of Tetrathionate broth (TT) to which was  
added 0.1 ml of iodine-potassium iodide immediately before use, followed by incubation at  
35°C for 24 hours. Another 0.1 ml aliquot of the sample was transferred to 10 ml Rapapport-  
Vassiliadis broth and incubated at 42°C for 24 hours. After this period, a domain of each tube  
90 was spread on Salmonella-Shigella Agar (SS) and Salmonella Chromogenic Agar Base. Plates  
were incubated at 35°C/24h. After the incubation period, the characteristics *Salmonella*  
colonies were subcultured to inclined tubes of tryptic soy agar (TSA). From these,  
biochemical screening tests were made in inclined tubes of triple sugar iron agar (TSI) and  
phenyl agar (phenylalanine agar). The colonies that showed typical reactions were subjected  
95 to identification by API-20E system (bioMérieux). The strains that showed positive  
confirmation in the API have been tested for polyvalent antisera somatic and flagellar.

## 2.3 Verification and quantification of biofilm production

100 The production of biofilm was tested in three different materials, stainless steel, glass  
and polyvinyl chloride (PVC). The first comprises almost all surfaces of slaughtering poultry  
process, the second can be used as containers in trade as support and hewers of meats and  
vegetables (replacing the "wooden boards") and the third is the main component of pipes of  
water.

105 The temperatures used were 35°C, optimum temperature for growth of *Salmonella*,  
28°C, temperature that *Salmonella* has the morphology *rdar* (red, dry and rough) featured in  
the production of biofilm, 16°C, recommended temperature of water in pre-chiller plants of  
poultry slaughterhouse (port 210 by MAPA, 1998), and 20°C, the temperature of water closer  
to reality, since large amounts of poultry carcasses at 42°C are simultaneously added at this  
stage and where the flow water is not as great, with little renovation, unlike what happens in  
110 the chiller.

### 2.3.1 Preparation of plates

Were used chips of: Circles of stainless steel with a diameter of 1 cm, squares of 1cm<sup>2</sup>  
of PVC and glass slides with a diameter of 1.3 cm. These materials have been properly  
115 washed, dried and placed in Petri dishes, which were autoclaved. Then, with the aid of sterile  
forceps, each material was placed at the bottom of a well of a 24-well plate, closing sterile  
container.

### 2.3.2 Preparation and inoculation of the culture plate

120 *Salmonella* strains were incubated in brain heart infusion broth (BHI) and incubated at  
35°C/24h. Then the culture was diluted to 10<sup>8</sup> CFU of bacteria, with the help of Densicheck  
(bioMérieux). Aliquots of 600 µl of this dilution were distributed in triplicate into the wells of  
24-well plate and incubated in the four set temperatures for 96 hours.

### 2.3.3 Quantification of biofilm production

125 Next, the chips were transferred to a new 24-well plate. This step aimed to prevent the  
quantification of biofilm that could be formed at the plastic plate (24-well plate) around the  
chips. On the new board, the chips were washed three times with buffer solution (PBS, pH

7.4) for the removal of unfixed cells and colored with 1% crystal violet for 15 minutes. The coloring was removed and the plate was washed again. Next, the biofilm was resuspended in 300  $\mu$ L of glacial acetic acid for 15 minutes, which ensures the homogeneity of the colored material. A volume of 200  $\mu$ l was transferred to a 96-well microplate and optical density (OD) was read in an ELISA reader (Babsystems, Multiskan EX) at 560 nm. The uninoculated BHI was used as a negative control and an average of three wells was used to correct the absorbance value. The BHI inoculated with *Salmonella* Typhimurium ATCC 14028, was used as a positive control (ODcp), used to compare the production of biofilm by strains analyzed.

Based on the OD of the biofilm production of the strains (ODc), these were classified according to Stepanovic et al. (2000), in no biofilm producer, weak, moderate and strong producer, according to the following formula:

$ODc \leq ODcp$  = no biofilm producer

$ODcp < ODc \leq (2 \times ODcp)$  = weak biofilm producer

$(2 \times ODcp) < ODc \leq (4 \times ODcp)$  = Moderate biofilm producer

$(4 \times ODcp) \leq ODc$  = Strong biofilm producer

#### 2.3.4 Photograph by scanning electron microscopy.

The processing occurred by the technicians of the Center of Electron Microscopy (CME) of the Institute of Biosciences, under the supervision of Prof. Dr. Daniela Carvalho dos Santos and was read by the authors. We used strains that showed no biofilm production, weak, moderate or strong biofilm production in the three materials and temperatures evaluated.

### **2.4 Evaluation of results**

The test used was the Chi-squared test with subsequent analysis to identify significant associations with  $p < 0.001$  (Haberman, 1973; Vieira, 2004).

### 3. RESULTS AND DISCUSSION

Out of 240 samples of poultry carcasses analyzed, 112 (46,7%) strains of *Salmonella* sp. were isolated, which, added to the other 62 previously isolated from the same type of food, totalized 174 strains, which were tested for the production of biofilm.

Of the 174 strains analyzed, only three (1,7%) were not able to produce biofilm in any temperature and in any material. Thus, 98,3% of the strains were able to produce the matrix in a temperature and/or material analyzed (Figure 1). This result agrees with that found by Solano et al. (2002) that observed 97% of strains positive for the production of biofilm. Lu et al. (2011) found smaller value, 63%.

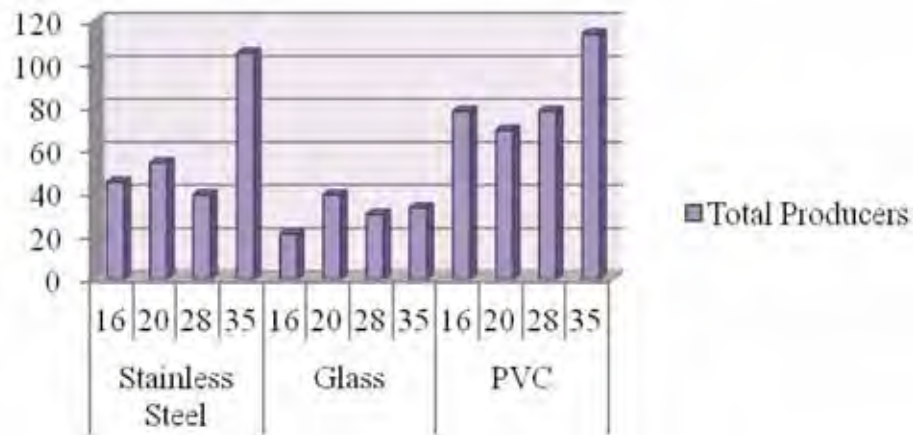


Figure 1. Quantification of biofilm-producing strains by temperature in each material

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According to the classification of Stepanovic et al. (2000), the strains were analyzed for their OD and subsequently classified as not producing biofilm, weak, moderate and strong biofilm producer (Figure 2).

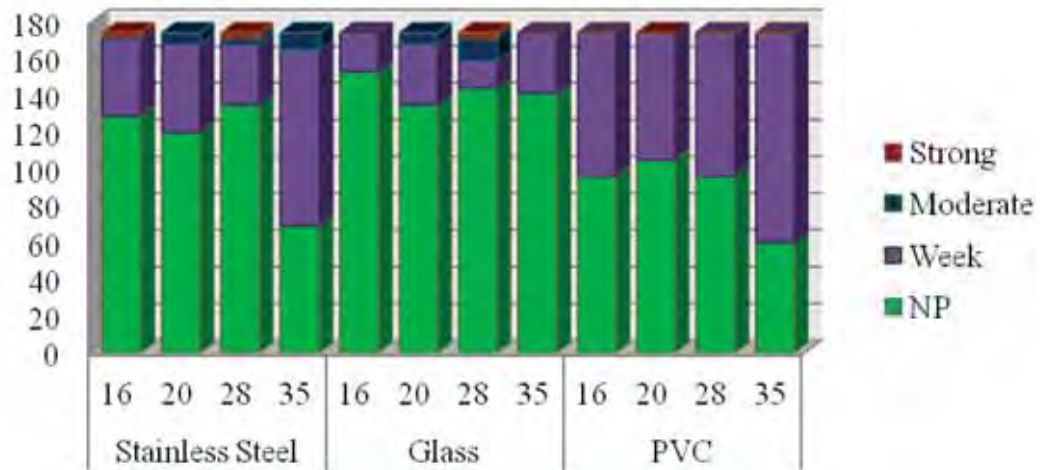


Figure 2. Comparative analysis of biofilm production for temperature in each material

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The production was dependent on temperature and the material analyzed in accordance with Table 1.

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**Table 1. Production of biofilm by *Salmonella* sp. isolated from poultry by material at different temperatures.**

Material	Temperature	NP (%)	Week (%)	Moderate (%)	Strong (%)	Total of Producers (%)
Stainless Steel	16°C	129 (74,2)	42 (24,1)	0	3 (1,7)	45 (25,8)
	20°C	120 (69)	48 (27,6)	6 (3,4)	0	54 (31)
	28°C	135 (77,6)*	33 (19)	3 (1,7)	3 (1,7)	39 (22,4)
	35°C	69 (39,7)	96 (55,2)	9 (5,2)	0	105 (60,3)
Glass	16°C	153 (88)*	21 (12)	0	0	21 (12)
	20°C	135 (77,6)	33 (19)	6 (3,4)	0	39 (22,4)
	28°C	144 (82,8)	15 (8,6)	12 (6,9)	3 (1,7)	30 (17,2)
	35°C	141 (81)	33 (19)	0	0	33 (19)
PVC	16°C	96 (55,2)	78 (44,8)	0	0	78 (44,8)
	20°C	105 (60,3)*	69 (39,7)	0	0	69 (39,7)
	28°C	96 (55,2)	78 (44,8)	0	0	78 (44,8)
	35°C	60 (34,5)	114 (65,5)	0	0	114 (65,5)

NP: Not producer;

\*p<0,001

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Analyzing the material (stainless steel, glass and PVC) according to the temperature, one can observe that none stood out as the best temperature for the absence of biofilm production, dependent on the material being analyzed. For stainless steel, the absence of biofilm was statistically significant at 28°C. For glass, the largest number of strains that did

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not form biofilm occurred at 16°C, statistically significant result. For PVC, the absence of the matrix production occurred statistically significantly at 20°C. No material had a higher number of strains statistically significantly not forming biofilm at 35°C.

200 Sinde and Carballo (2000) comparing stainless steel with other materials more hydrophobics found that *Salmonella* is more easily to adhere to these materials than to steel, also observed in the present work that demonstrates that the stainless steel is better (lower bacterial adhesion) than PVC (more hydrophobic).

205 Although the differences were not statistically significant, in Table 1 it can be observed that, independent of temperature, that *Salmonella* strains adhere more to the PVC (hydrophobic) compared to stainless steel and glass (hydrophilic). Joseph et al. (2001) also observed an increased production of biofilm on plastic when compared to stainless steel by strains of *Salmonella*.

210 Stepanovic et al. (2003) tested 30 strains of *Salmonella* sp. They observed that 97% of the isolates produced biofilm at 30°C after 48h of incubation, followed by temperatures of 37°C (93%) and 22°C (90%) in polystyrene plates. The authors also found higher percentages of strong biofilm producer strains in relation to this work. Interestingly, despite the temperature of 22°C showed the less amount of producing strains (90%), this temperature had the highest number of strong biofilm producer strains (30%). Either way, it can be seen in Table 1, that biofilm formation occurred at all temperatures tested, regardless of the material and the intensity of production. Results are worrying because 16°C can be considered a  
215 relatively low temperature compared to the optimum temperature for growth of *Salmonella* and, even in these conditions, 42 strains (24,1%) were producing this matrix in stainless steel, material used in pre-chiller tanks. Also, at 20°C there was production of biofilm in all materials. The temperature of 16°C is recommended for the entry of water into the pre-chiller.  
220 However, it must be considered unlikely that this temperature is maintained due to continuous inflow of carcasses at 42°C, and the temperature of 20°C is closer to real conditions. Furthermore, it should be noted that 20°C is the average temperature of the environment in poultry slaughtering plants, emphasizing the need for proper and regular cleaning (Stepanovic et al., 2003).

225 The differences between the results of Stepanovic et al. (2003) can be explained by the difference in methods and different materials used in both studies and the origin of the strains, since while the current study used only *Salmonella* strains isolated from poultry, Stepanovic

et al. (2003) tested strains isolated from humans, other animals and food not specified. However it should be noted that Stepanovic et al. in 2004, used 122 strains of *Salmonella* isolated from the same sources cited in the work of 2003, as research production of biofilms in polystyrene boards, but taking into account different culture media, and found only 1.6% of the strains producing strong biofilm, when BHI broth was used at 28 ° C, the same used in the present work, where was observed the percentage of 1,7%, but in stainless steel and in glass, not in PVC. In that same culture medium, the production was only moderate in 1 (0,8%) among the 122 tested. Results close to 0% for moderate production were also seen at this work in 6 situations, varying temperatures and materials. However, at 28°C, this production was moderate in 12 (6,9%) strains when adhered to glass and 3 (1,7%) in stainless steel.

The discussion of the results found in this work was strongly affected by the choice of materials. As already explained, were chosen stainless steel (hydrophilic material), by the presence in most of the superficies at a poultry slaughtering plant; PVC (hydrophobic material), being in the main pipes, in the network of water supply, and the nipple from the drinkers of the birds, which provides water to the birds; The glass (hydrophilic material), was also tested because it is present in points of sale, lining the counter and its doors, and used at homes, as cutting surfaces. However, most published works, included in broad literature review, used polystyrene, hydrophobic matter such as PVC, not being possible a comparison between the polystyrene and hydrophilic materials such as stainless steel and glass. In addition, the works do not clarify the degree of hydrophobicity of PVC and polystyrene, not being possible a real comparison between the materials, as the production of biofilm by *Salmonella* strains.

Changes in adhesion capacity (the first step in the production of biofilm) in the same type of material, were observed by Stepanovic et al. (2003), who tested four different brands of polystyrene plates and three of them had treated surface, to allow better cell adhesion, if the order was the formation of a pad cell, using cell culture, but it also facilitated the formation of biofilm.

In addition, studies that tested the stainless steel, or glass used few strains, normally 2 or 3 and the main purpose of these articles was the genetic manipulation of strains known to be positive, noting changes or not in their behavior, as the production of biofilm (Latasa et al. 2005; Malcova et al., 2008; Kim and Wei, 2009), or sensitivity to sanitizers producing strains

260 compared to planktonic bacteria (Sinde and Carballo, 2000; Ramesh et al., 2002; Asséré,  
 265 Oulahl and Carpentier, 2008; Marin, Hernandez and Lainez, 2009).

The physicochemical properties of the surface of different materials can influence the  
 adhesion of microorganisms (Donlan and Costerton, 2002). The glass and stainless steel  
 materials are considered hydrophilic while rubber, plastic and PVC are hydrophobic and  
 several authors have noted that *Salmonella* adheres better on hydrophobic materials (Sinde  
 265 and Carballo, 2000; Donlan, 2002). This remark can be observed in Table 2, which shows the  
 capacity or not of producing biofilm, classified by different temperatures. Note that the glass,  
 with hydrophilic characteristics, was the material that presented less biofilm formation in 3 of  
 4 temperatures tested (16, 28 and 35°C). At 20°C, although there is no statistical difference  
 between different materials, glass was the material which had the higher number of no biofilm  
 270 producer strains, with 77,6%, followed by steel (also hydrophilic), 69%, and PVC, 60,3%.

**Table 2. Production of biofilm by *Salmonella* strains isolated from chicken carcasses by temperature in three different materials.**

Temp.	Material	NP (%)	Weak (%)	Moderate (%)	Strong (%)	Total of Producers (%)
16°C	Stainless steel	129 (74,2)	42 (24,1)	0	3 (1,7)	45 (25,9)
	Glass	153 (88)*	21 (12)	0	0	21 (12)
	PVC	96 (55,2)	78 (44,8)	0	0	78 (44,8)
20°C	Stainless steel	120 (69)	48 (27,6)	6 (3,4)	0	54 (31)
	Glass	135 (77,6)	33 (19)	6 (3,4)	0	39 (22,4)
	PVC	105 (60,3)	69 (39,7)	0	0	69 (39,7)
28°C	Stainless steel	135 (77,6)	33 (19)	3 (1,7)	3 (1,7)	39 (22,4)
	Glass	144 (82,8)*	15 (8,6)	12 (6,9)	3 (1,7)	30 (17,2)
	PVC	96 (55,2)	78 (44,8)	0	0	78 (44,8)
35°C	Stainless steel	69 (39,7)	96 (55,2)	9 (5,1)	0	105 (60,3)
	Glass	141 (81,)*	33 (19)	0	0	33 (19)
	PVC	60 (34,5)	114 (65,5)	0	0	114 (65,5)

NP: Not producer

\* p < 0,001.

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Turki et al. (2011), despite having checked the production of biofilm on glass by  
 another methodology, that is, by viewing a film on the surface of LB and M9 broth, found that  
 96.5% of 57 *Salmonella* strains analyzed, did not produce biofilm, at a temperature of 4°C. In



280 the present work, the lower temperature used was 16°C, but also found high numbers of  
strains not producing this matrix in this material, 88%, as shown in Table 2.

Photos by scanning electron microscopy confirmed the formation of biofilm by strains  
analyzed, as shown in Figures 3, 4 and 5.

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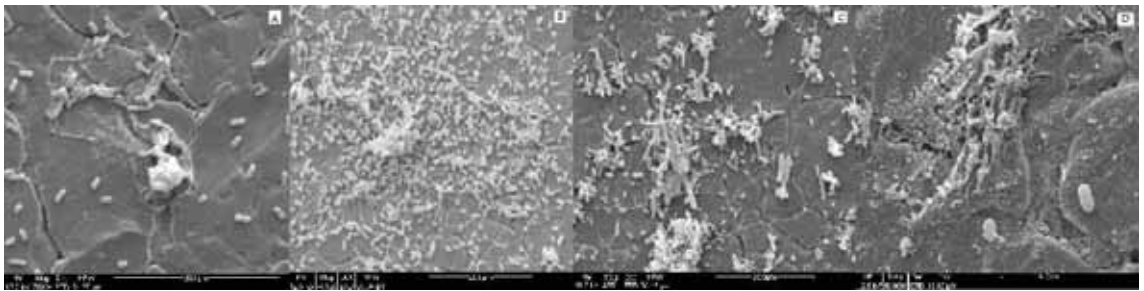


Figure 3. Biofilms of *Salmonella* sp. produced in stainless steel. (A) 16°C, (B) 20°C, (C) 28°C, (D) 35°C.

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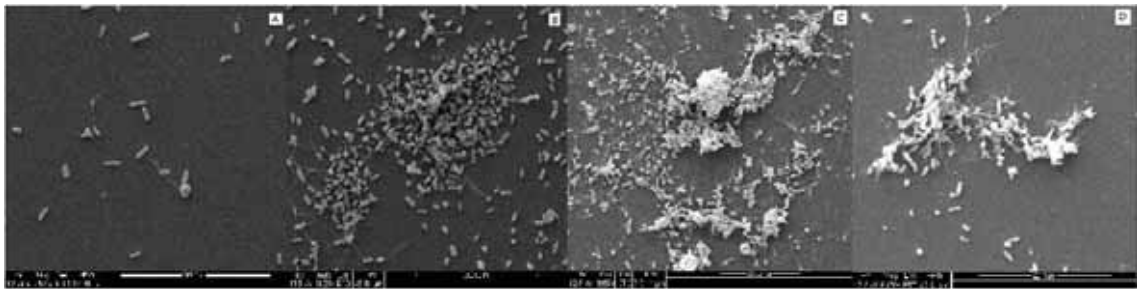


Figure 4. Biofilms of *Salmonella* sp. produced in glass. (A) 16°C, (B) 20°C, (C) 28°C, (D) 35°C.

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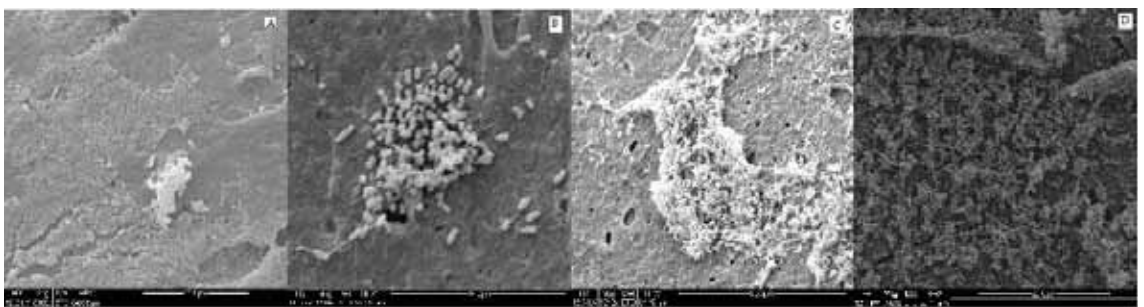


Figure 35 Biofilms of *Salmonella* sp. produced in PVC. (A) 16°C, (B) 20°C, (C) 28°C, (D) 35°C.

300 **4. CONCLUSION**

The ability of strains of *Salmonella* to produce biofilm is linked, independently, to the temperatures and materials.

305 There is a pressing need to establish the hydrophobicity of each material tested so a real evaluation and comparison can be made without any false interpretations of what is hydrophilic or hydrophobic.

The need for studies in this area grows by the day, as well as analysis of other factors that may influence bacterial adhesion in the food industry and beyond.

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## **CAPÍTULO 2**

Este trabalho deu origem ao artigo “Analysis of *rdar* morphology associated with the presence of genes *adrA* and *agfD* from strains of *Salmonella* sp. isolated from poultry”, que foi submetido para publicação no periódico “International Journal of Food Microbiology”.

ANALYSIS OF *rdar* MORPHOLOGY ASSOCIATED WITH THE PRESENCE OF GENES *adrA* AND *agfD* FROM STRAINS OF *Salmonella* sp. ISOLATED FROM RAW POULTRY

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## ABSTRACT

Bacteria of the genus *Salmonella* are among the leading causes of foodborne disease (FBD). Those from animal are most responsible for placement this microorganism, especially poultry, since these animals are asymptomatic carriers. Such microorganisms can adhere to surfaces, interacting with the same, starting cell multiplication and establishing the biofilm.

Like other pathogenic bacteria, *Salmonella* possess fimbriae and produces cellulose, the main matrix components in these bacteria biofilms. The synthesis of fimbriae and cellulose is co-regulated by a LuxR type regulator, the *agfD* (agregative fimbriae), and the cellulose is regulated indirectly through gene *adrA*. The fimbriae, associated with the synthesis of cellulose in *Salmonella* in biofilm leads to an expression of a distinctive phenotype on agar plates, a red, dry and rough morphology (*rdar*) in LB medium at 28°C, and not at 37°C. The *rdar* morphology provides a greater resistance to desiccation and disinfection, allowing greater survivability of microorganisms. Studies with cells from a *rdar* colony of *S. Typhimurium*, shown that when mutations occur in genes *agf* and *adrA* other morphologies are presented.

Therefore, this study aimed to analyze the expression of biofilm characteristic morphology of *Salmonella* sp. isolated from poultry associated with the presence of the genes *agfD* and *adrA*.

Key words: *Salmonella*, poultry, *agf* e *adrA* genes, *rdar* morphology



## 1. INTRODUCTION

35 *Salmonella* is the leading cause of foodborne illness, and poultry meat is in many cases the vehicle (Sumner, Raven and Givney, 2004). This is because the process of sanitization in a poultry industry is not always effective and/or possible sediment remain an can adhered to equipment and surfaces, which becomes potential sources of contamination because they favor the adhesion of microorganisms to surfaces with production of biofilms (Zottola and Sasahara, 1994, Oliveira et al., 2006).

40 Sessile bacterial cells randomly adhered pass through a process of metabolic differentiation to form biofilm (Stoodley et al, 2002; Hamilton et al, 2009; White et al, 2010).

*Salmonella* possesses fimbriae that play a complementary role in the adhesion process (Harbron and Kent, 1988; Tortora, Funke and Case, 2000). Along with cellulose, these two components are important in biofilm formation. Genes *agf* (agregative fimbriae) involved in the biosynthesis of fimbriae are organized into two operons, the *agfBAC* and *agfDEFG* 45 (Collinson et al, 1996). The second component of the extracellular matrix of biofilms, cellulose, is produced by genes *bcsA*, *bcsB*, *bcsZ* and *bcsC* (bacterial cellulose synthesis). The production of fimbriae, co-regulated by LuxR type regulator, the *agfD*, will regulate the production of cellulose indirectly acting on the gene *adrA* (Romling et al, 2000; Romling, 50 2002; Zakikhany et al, 2010). When cellulose synthesis is associated with the presence of fimbriae, *Salmonella* expresses a distinctive phenotype in Luria Bertani agar plates, the morphology of red, dry and rough (*rdar*) at 28°C , and not at 37°C (Solano et al., 2002; Romling and Gerstel, 2003). This morphology provides a greater resistance to desiccation and disinfection process, allowing greater survivability of microorganisms (Anriany et al, 2001; 55 Scher, Romling, Yaron, 2005; White et al., 2006).

Studies have shown that the gene *agfD* is necessary for the maturation of the biofilm, but is dispensable in the establishment of the bacterial colony and is responsible for regulating the expression of all major constituents of this matrix (under *rdar* conditions) (Grantcharova et al, 2010). Other studies have reported that this gene may be susceptible to other 60 environmental stimuli as an example, the temperature (Romling and Gerstel, 2003).

According to O'Toole, Kaplan e Kolter (2000), mutations in genes involved in biofilm formation may change the production of the components involved as well as the matrix itself. Other studies have found that mutations in the gene *agf* generated colonies with different morphologies that the *rdar*, the *pdar* (pink, dry and rough). Insertion of a mutation in *adrA*,

65 formed brown colonies, the *bdar* (brown, dry and rough) and total deletion of the *agfD*  
generate the morphology *saw* (smooth and white) (Romling et al, 1998; Romling and Rohde,  
1999; Romling et al, 2000).

The possible presence of pathogenic microorganisms in biofilms and biofilm-forming  
microorganisms in foods points the need to study strains that carry the genes responsible for  
70 producing such a matrix (Donlan and Costerton, 2002; Shi and Zhu, 2009).

Therefore, this study aimed to research the presence of *adrA* and *agfD* genes in strains  
of *Salmonella* sp. isolated from poultry and the behavior of these strains for the production of  
biofilm and expression analysis of biofilm morphology characteristic of *Salmonella*.

## 75 2. MATERIALS AND METHODS

### 2.1 Obtaining samples of poultry

A total of 240 samples of poultry were analyzed. These samples were purchased in  
stores in the city of Botucatu – SP, Brazil, and transported immediately in refrigerated  
80 isothermal box containing dry ice to the Laboratory of Food Microbiology, Institute of  
Biosciences, UNESP. - Botucatu.

### 2.2 Microbial Analysis

All culture media, except where especified, were from Oxoid brand. The detection of  
85 *Salmonella* was performed according to Andrews et al. (2001). For this purpose, 25g of  
sample were homogenized in 225 ml of petoned water and incubated at 35°C for 24 hours.  
After this period, 1 ml was transferred to 10 ml of Tetrathionate broth (TT) to which was  
added 0.1 ml of iodine-potassium iodide immediately before use, followed by incubation at  
35°C for 24 hours. Another 0.1 ml aliquot of the sample was transferred to 10 ml Rapapport-  
90 Vassiliadis broth and incubated at 42°C for 24 hours. After this period, a domain of each tube  
was spread on Salmonella-Shigella Agar (SS) and Salmonella Chromogenic Agar Base. Plates  
were incubated at 35°C/24h. After the incubation period, the characteristics *Salmonella*  
colonies were subcultured to inclined tubes of tryptic soy agar (TSA). From these,  
biochemical screening tests were made in inclined tubes of triple sugar iron agar (TSI) and  
95 phenyl agar (phenylalanine agar). The colonies that showed typical reactions were subjected

to identification by API-20E system (bioMérieux). The strains that showed positive confirmation in the API have been tested for polyvalent antisera somatic and flagellar.

### **2.3. PCR for the detection of biofilm-producing genes in *Salmonella***

100

#### 2.3.1. DNA Extraction and Purification

*Salmonella* strains were inoculated into brain heart infusion broth (BHI) at 35°C/24 h. Next, 1 mL was transferred to a microcentrifuge tube for centrifugation at 10.000g/10 minutes. The supernatant was discarded and the sediment resuspended in 1 ml of PBS (Phosphate buffered saline - 0.01 M, pH 7.2). This step was repeated twice, with centrifugation time of 5 minutes. Next, the pellet was resuspended in 200 µl of lysis buffer (50 mM Tris-Cl-H, 1 mM EDTA 0.025% Tween, 0.2 mg proteinase K), incubated in a water bath at 56°C/1hour and then at 95°C/10 minutes. New centrifugation was performed at 13.000g/5 minutes and the supernatant was used for the PCR reaction. (Arnold et al., 2004)

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#### 2.3.2. Nucleic acid amplification (PCR)

For the PCR reactions were used microcentrifuge tubes from 0.5 mL in a total volume of 25 µl per sample, composed of 2.5 µl of 10X PCR buffer (Invitrogen), 2.5 mM magnesium chloride (Invitrogen), 200 mM of each dNTP, 1.25 U of Taq DNA polymerase, 10 picomoles of each primer (Table 1), autoclaved ultrapure water (qs) (Milli-Q Plus, Millipore) and 3 µl of sample DNA. Incubation was performed in a thermocycler GeneAmp PCR System 9700 (Applied Biosystems) using the parameters of an initial cycle at 94°C for 5 minutes for initial denaturation, followed by 35 cycles of 94°C/30s, 60°C/30s and 72°C/30s. The final extension temperature was 72°C for 4 minutes. All reactions were performed using a negative control by replacing the nucleic acid in ultrapure water. As a positive control, we used a standard strain of *Salmonella* Typhimurium ATCC 14028 (Arnold et al., 2004).

120

125 Table1. Sequency of the primers and temperatures of anelling used in the PCR reaction, and the lenght of the products.

	Sequência	T°C anelamento	pb
<i>agfD</i> foward	TGCGGACTCGGTGCTGTTGT		
<i>agfD</i> reverse	CAGGAACACGTGGTCAGCGG	60°C	123
<i>adrA</i> foward	GGGCGGCGAAAGCCCTTGAT		
<i>adrA</i> reverse	GCCCATCAGCGCGATCCACA	60°C	92

Primers were designed using the program Primer Blast (<http://www.ncbi.nlm.nih.gov/nucore>):

130 *agfD*: Access number: NC 0031971; gene *agfD* 1252660; interval: 1229728 - 1230378  
*adrA*: Access number: NC 0031971; gene *adrA* 1251904; interval: 438129- 439241.

### 2.3.3. Visualization of amplified products

135 The products of PCR reactions were subjected to electrophoresis (Electrophoresis Power Supply Model 600 LTR - Amersham-Pharmacia Biotech ® Inc.) in 1.5% agarose gel in buffer Tris-boric acid-EDTA (TBE) and revealed with SYBR Green (2 µl 10x / 0.8 mL of sample - Invitrogen ®). The DNA fragments were analyzed in comparison with DNA markers of 100 bp, and analyzed and photographed in image analyzer (Alphaimager – Alpha esasy FC Software – AlphaInotech Corporation®).

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### **2.4. Checking the *rdar* colony morphology**

The colony morphology was observed in agar Luria Bertoni (LB) without salt, supplemented with Congo red (40 mg / ml) and Coomassie Brilliant Blue (20 mg / ml).

145 The *Salmonella* strains were grown in BHI broth for 18 hours at 35°C and then were sown in agar described above, and incubated at 28° and 35°C for 96 hours with daily reading on colony morphology (Parys et al. 2010).

### 3. RESULTS AND DISCUSSION

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Out of the 240 samples of poultry carcasses analyzed, 112 (46.7%) strains of *Salmonella* sp. were isolated, which added to the other 62 previously isolated from the same type of food, totalized 174 strains. All of them were positive for the *agfD* gene, Figure 1, and the *adrA* gene, Figure 2.

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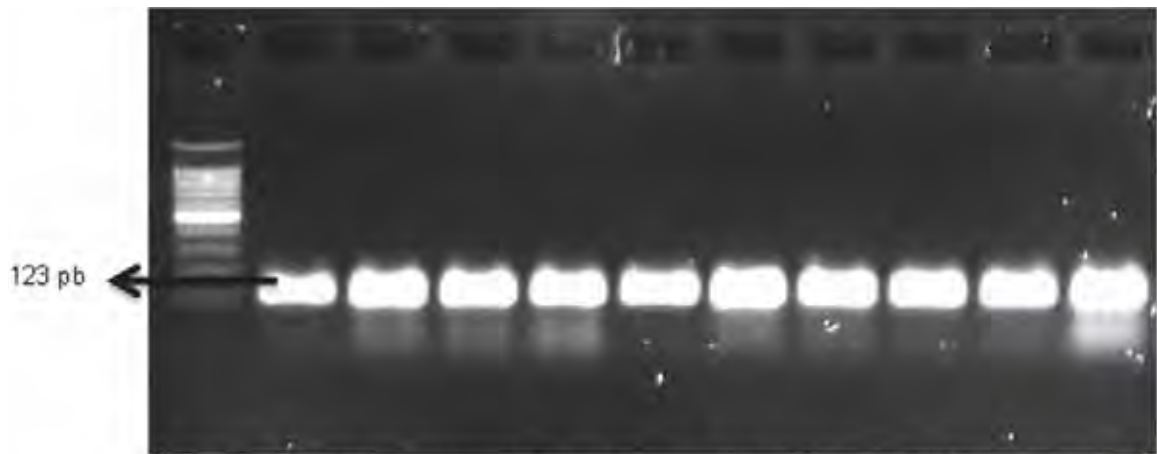


Figure 1. Result of agarose gel electrophoresis of the *agfD* gene. Well 1 corresponds to molecular weight 100bp, well 2 *Salmonella* Typhimurium ATCC 14028, wells 3-11 correspond to samples 1 to 9 positive.

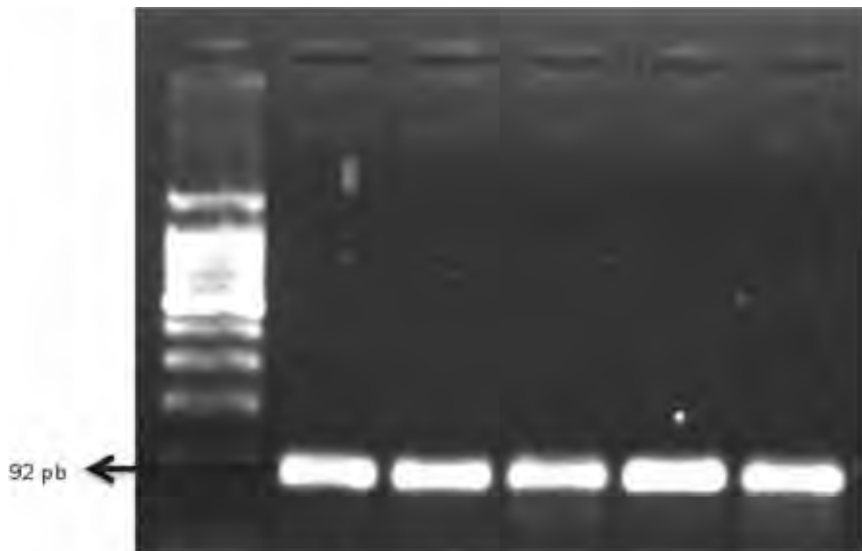


Figure 2. Result of agarose gel electrophoresis of the gene *adrA*. Well 1 corresponds to molecular weight 100bp, well 2 *Salmonella* Typhimurium ATCC 14028, wells 3-6 correspond to samples 1 to 4 positive.

160 Of the 174 strains analyzed in the temperature of 28°C, 54 (31%) were unable to  
develop any typical morphology in LB, while 120 (69%) showed one of the morphological  
types previously discussed by Romling and Rohde (1999) and Romling (2000), being 96  
(80%) the *rdar* morphology, 21 (17.5%) the *bdar* morphology, 3 (2.5%) the *saw* morphology  
and no strains presented *pdar* morphology. (Figure 3)

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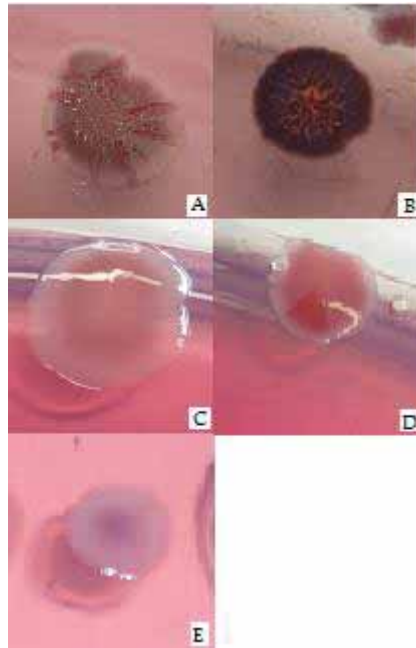


Figure 3. Morphologies displayed by strains of *Salmonella* sp. isolated from poultry at LB at 28°C. A and B represent *rdar* morphology, C and D the *bdar* morphology and E the *saw* morphology.

170 At 35°C, 111 (63.8%) were unable to develop any of the morphologies in LB agar and  
between the 63 (36.2%) positives, 57 (90.5%) showed the *rdar* morphology, 3 (4.8%) the  
*bdar* morphology, 3 (4.8%) the *saw* morphology, and, again, no *pdar* morphology was  
presented. (Figure 4)

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Figure 4. Morphologies displayed by strains of *Salmonella* sp. isolated from poultry at LB at 35°C. A represents the *rdar* morphology, B the *bdar* morphology and C the *saw* morphology.

180 Although all strains were positive for the genes studied, associated with the production  
of biofilm, not all were able to produce the characteristic morphology of the biofilm. This can  
be explained by the fact that the behavior in *Salmonella* is regulated by environmental  
conditions, which will act on the promoter *agfD* and trigger the cascade production of the  
biofilm. The ability to adapt to different habitats ensures the survival of bacteria isolated in  
changing environments. The ability to change the pattern of expression of fimbriae can be  
185 changed according to temperature (28°C expression and/or 37°C or absence of expression)  
(Romling et al, 1998).

The results obtained in this work showed that some strains of *Salmonella* sp. were able  
to produce characteristic morphologies. At 28°C, 96 (55.2%) presented the *rdar* morphology,  
21 (12%) the *bdar* and 3 (1.7%) the *saw*. At 35°C, 57 (32.76%) produced the *rdar*  
190 morphology, 3 (1.7%) the *bdar* and 3 (1.7%) the *saw*. According to Gerstel and Romling  
(2003), interference can occur in the biofilm formation of *S. Typhimurium*, such as oxygen  
and pH variables, which act directly on the expression of the morphology.

Comparing the results observed in the temperature of 28°C, Solano et al. (2002) found  
similar values with those of the present work, presenting a production of biofilm in 72.5% of  
195 isolates tested. They observed that 93% expressed the *rdar* morphology, result higher than  
found in this study that was of 80%. This result is very close to that found by White et al.  
(2006), who found 80.5% of its strains producing *rdar* morphology. Solano et al. (2002)  
found that 7% of the isolates tested showed *bdar* morphology, lower to that obtained in this  
work (17,5%). Vestby et al. (2009) found 26% strains presenting this morphology, a result  
200 higher than that found in the present paper. Solano et al. (2002) found that 27.5% of the  
strains tested expressed the *saw* morphology, far above the results observed in the present  
work (2,5%). Vestby et al. (2009) found no strain forming *saw* morphology in his work.  
Equally to Solano et al. (2002) and Vestby et al. (2009) this study found no strain presenting  
the *pdar* morphology. This result was expected because the color changing from red to pink  
205 implies on the deletion of the *agf* gene, which was observed in all strains (Romling et al.,  
1998).

Romling et al. (2003) noted that some strains failed to produce biofilm at 37°C. Kader  
et al (2006) observed that *S. Typhimurium* UMR1, depending on conditions, can expressed or  
not the matrix. The present study demonstrated that, although there was a lesser amount when  
210 compared to strains producing biofilm at 28°C (69%), at 35°C 36.2% of the isolates tested

were able to express biofilm in *rdar*, *bdar* and *saw* morphologies, but not in *pdar* morphology.

#### 4. CONCLUSION

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The *agfD* gene controlling the aggregation process, together with the action of *adrA*, induces changes in the bacterial cell, generating the biofilm. Thus, the morphology associated with the expression of genes involved in the production of biofilm is dependent on extrinsic factors such as temperature. Although all the isolates have shown both analyzed genes, their  
220 expression did not occur in 100% of them. This may be due to factors that interfere with gene regulation, not generating the characteristic morphology and other genes may also be involved in this production.

Since the morphologies express an adaptation of bacterial cells to survive in situations not favorable, further studies of lower temperatures such as refrigeration (4 ° C) and the  
225 ability to express the matrix at a temperature of 37°C is necessary for better understanding and further control in food processing plants.

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## CONCLUSÃO

Pelos resultados obtidos, pode-se concluir que a contaminação das carcaças de frango por *Salmonella* é alta, apesar dos programas de controle implementados no Brasil. O quadro se agrava pela observação da presença dos genes responsáveis pela produção de biofilme em todas as cepas isoladas e a expressão desses genes na maioria delas, inclusive em baixas temperaturas. Porém, para que a formação de tal matriz e a expressão das morfologias relacionadas com a produção do biofilme (*rdar*, *bdar*, *saw* e *pdar*) ocorra, outras variáveis a favor são necessárias, como o tipo de material ao qual a bactéria vai se aderir e a temperatura sob a qual o micro-organismo será submetido.

Mais estudos devem ser realizados com as temperaturas utilizadas na produção das carcaças e em diferentes materiais, principalmente o vidro, que foi o mais eficiente contra a adesão das células bacterianas.

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## APÊNDICES

### 1.TABELAS DO CAPÍTULO 1

**Table 1. Production of biofilm by *Salmonella* spp. isolated from poultry by material at different temperatures.**

Material	Temperature	NP (%)	Week (%)	Moderate (%)	Strong (%)	Total of Producers (%)
Stainless Steel	16°C	129 (74,2)	42 (24,1)	0	3 (1,7)	45 (25,8)
	20°C	120 (69)	48 (27,6)	6 (3,4)	0	54 (31)
	28°C	135 (77,6)*	33 (19)	3 (1,7)	3 (1,7)	39 (22,4)
	35°C	69 (39,7)	96 (55,2)	9 (5,2)	0	105 (60,3)
Glass	16°C	153 (88)*	21 (12)	0	0	21 (12)
	20°C	135 (77,6)	33 (19)	6 (3,4)	0	39 (22,4)
	28°C	144 (82,8)	15 (8,6)	12 (6,9)	3 (1,7)	30 (17,2)
	35°C	141 (81)	33 (19)	0	0	33 (19)
PVC	16°C	96 (55,2)	78 (44,8)	0	0	78 (44,8)
	20°C	105 (60,3)*	69 (39,7)	0	0	69 (39,7)
	28°C	96 (55,2)	78 (44,8)	0	0	78 (44,8)
	35°C	60 (34,5)	114 (65,5)	0	0	114 (65,5)

NP: Not producer;

\*p<0,001

**Table 2. Production of biofilm by *Salmoenlla* strains isolated from chicken carcasses by temperature in three different materials.**

Temp.	Material	NP (%)	Week (%)	Moderate (%)	Strong (%)	Total of Producers (%)
16°C	Stainless steel	129 (74,2)	42 (24,1)	0	3 (1,7)	45 (25,9)
	Glass	153 (88)*	21 (12)	0	0	21 (12)
	PVC	96 (55,2)	78 (44,8)	0	0	78 (44,8)
20°C	Stainless steel	120 (69)	48 (27,6)	6 (3,4)	0	54 (31)
	Glass	135 (77,6)	33 (19)	6 (3,4)	0	39 (22,4)
	PVC	105 (60,3)	69 (39,7)	0	0	69 (39,7)
28°C	Stainless steel	135 (77,6)	33 (19)	3 (1,7)	3 (1,7)	39 (22,4)
	Glass	144 (82,8)*	15 (8,6)	12 (6,9)	3 (1,7)	30 (17,2)
	PVC	96 (55,2)	78 (44,8)	0		78 (44,8)
35°C	Stainless steel	69 (39,7)	96 (55,2)	9 (5,1)	0	105 (60,3)
	Glass	141 (81,)*	33 (19)	0	0	33 (19)
	PVC	60 (34,5)	114 (65,5)	0	0	114 (65,5)

NP: Not producer

\*  $p < 0,001$ .

## 2. FIGURAS DO CAPÍTULO 1

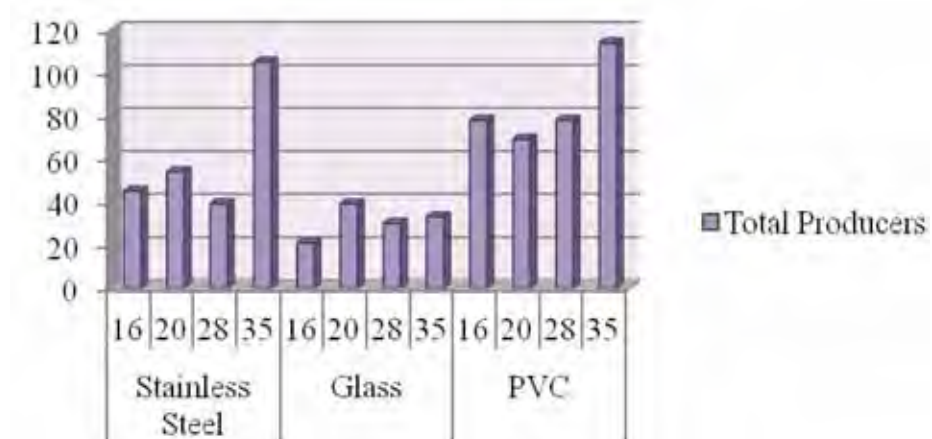


Figure 1. Quantification of biofilm-producing strains by temperature in each material

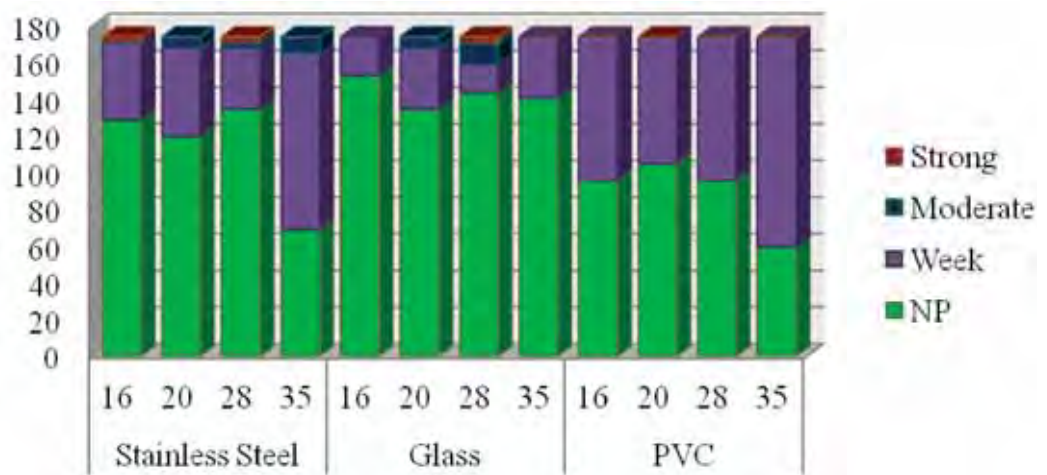


Figure 2. Comparative analysis of biofilm production for temperature in each material

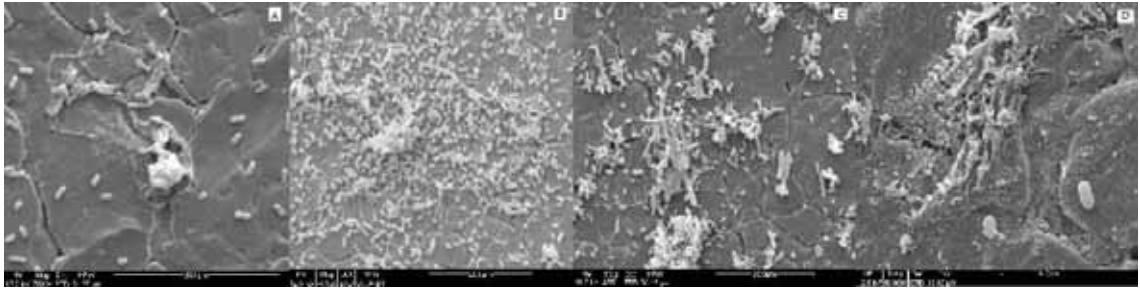


Figure 3. Biofilms of *Salmonella* sp. produced in stainless steel. (A) 16°C, (B) 20°C, (C) 28°C, (D) 35°C.

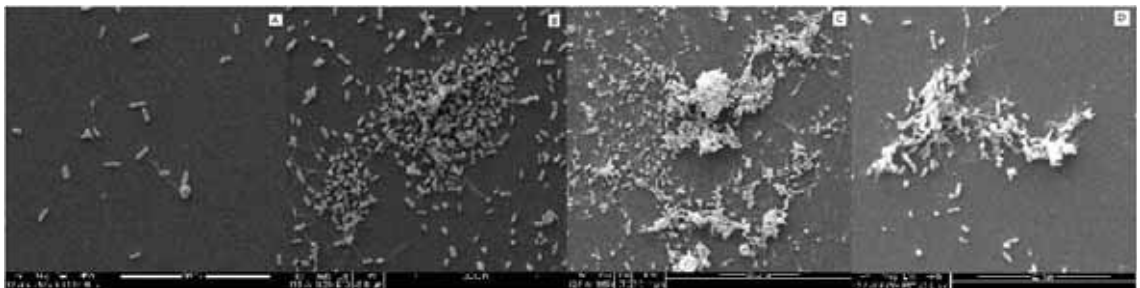


Figure 4. Biofilms of *Salmonella* sp. produced in glass. (A) 16°C, (B) 20°C, (C) 28°C, (D) 35°C.

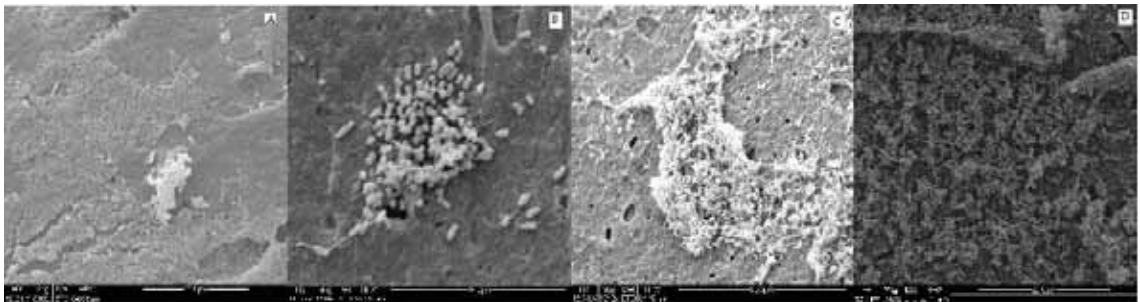


Figure 35 Biofilms of *Salmonella* sp. produced in PVC. (A) 16°C, (B) 20°C, (C) 28°C, (D) 35°C.



### 3. TABELAS DO CAPÍTULO 2

Table1. Sequency of the primers and temperatures of anelling used in the PCR reaction, and the lenght of the products.

	Sequência	T°C anelamento	pb
<i>agfD</i> foward	TGCGGACTCGGTGCTGTTGT		
<i>agfD</i> reverse	CAGGAACACGTGGTCAGCGG	60°C	123
<i>adrA</i> foward	GGGCGGCGAAAGCCCTTGAT		
<i>adrA</i> reverse	GCCCATCAGCGCGATCCACA	60°C	92

#### 4. FIGURAS DO CAPÍTULO 2

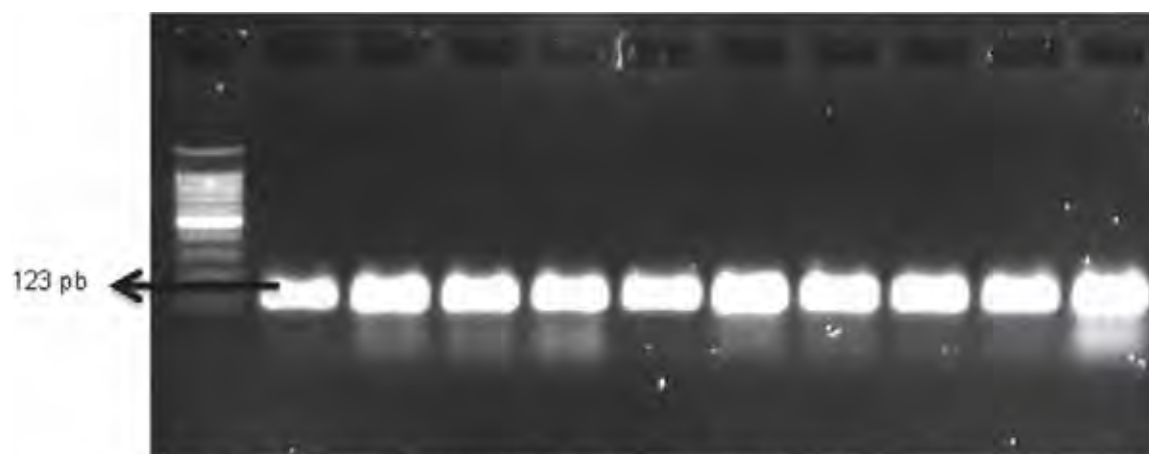


Figure 1. Result of agarose gel electrophoresis of the agfD gene. Well 1 corresponds to molecular weight 100bp, well 2 *Salmonella Typhimurium* ATCC 14028, wells 3-11 correspond to samples 1 to 9 positive.

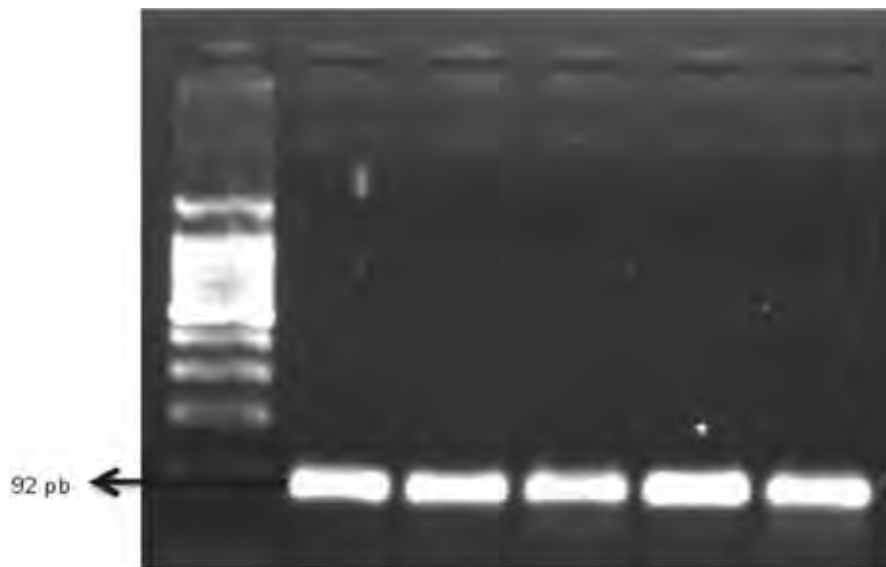


Figure 2. Result of agarose gel electrophoresis of the gene *adrA*. Well 1 corresponds to molecular weight 100bp, well 2 *Salmonella* Typhimurium ATCC 14028, wells 3-6 correspond to samples 1 to 4 positive.

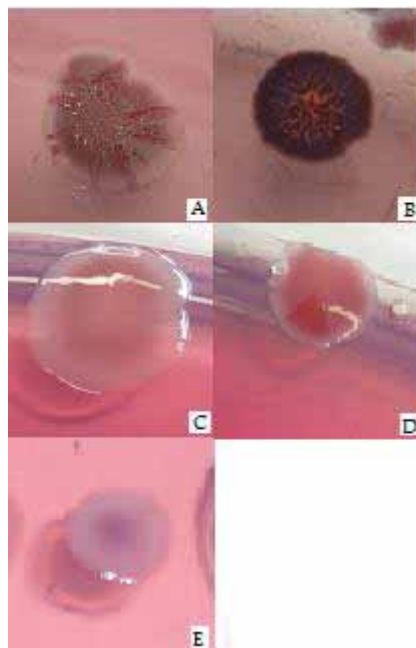


Figure 3. Morphologies displayed by strains of *Salmonella* sp. isolated from poultry at LB at 28°C. A and B represent *rdar* morphology, C and D the *bdar* morphology and E the *saw* morphology.



Figure 4. Morphologies displayed by strains of *Salmonella* sp. isolated from poultry at LB at 35°C. A represents the *rdar* morphology, B the *bdar* morphology and C the *saw* morphology.

## 5. FIGURAS

### 5.1 Geis de eletroforese de *Salmonella* sp.

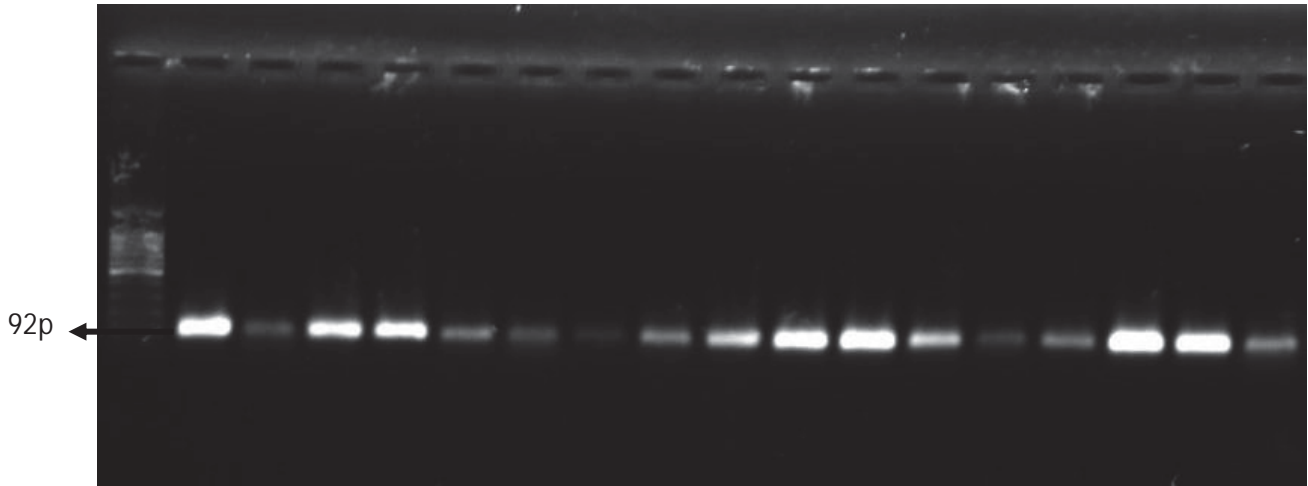


Figura A. Resultado da Eletroforese em Gel de Agarose do gene *adrA*. Poço1 corresponde ao peso molecular 100bp, poço 2 *Salmonella* Typhimurium ATCC 14028, poços 3 a 18 correspondem a amostras positivas 37 a 52.

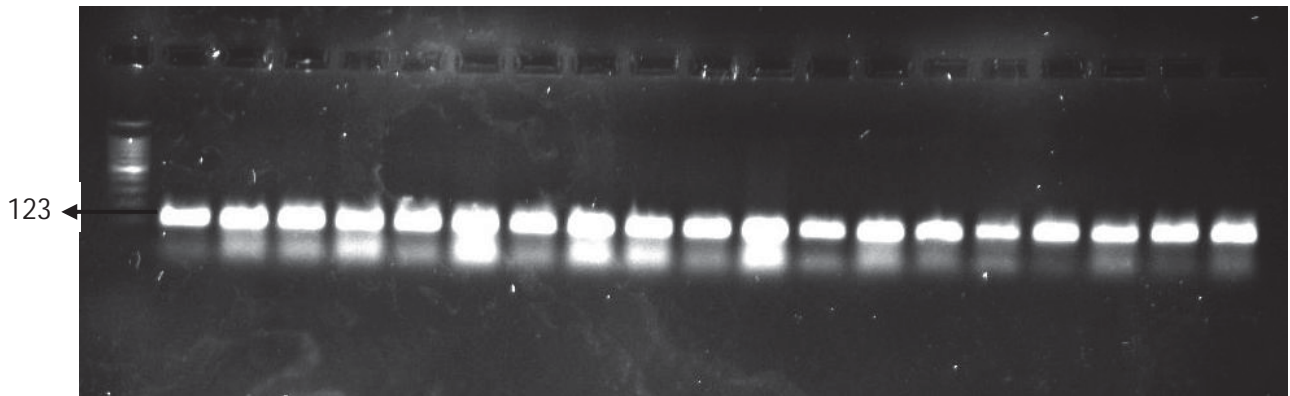
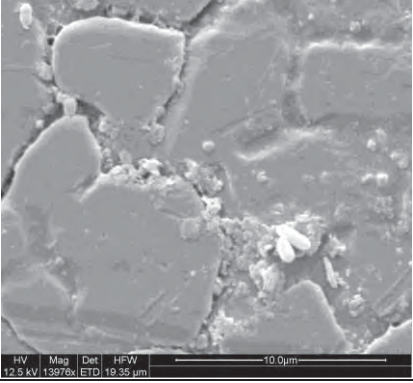


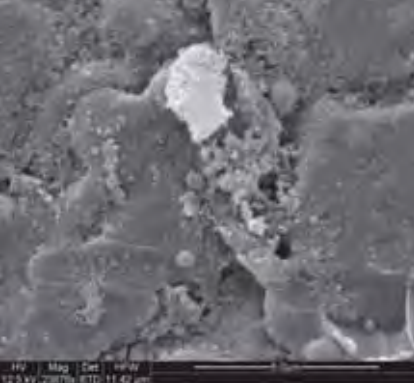
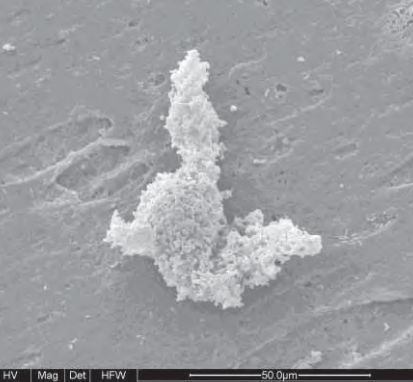
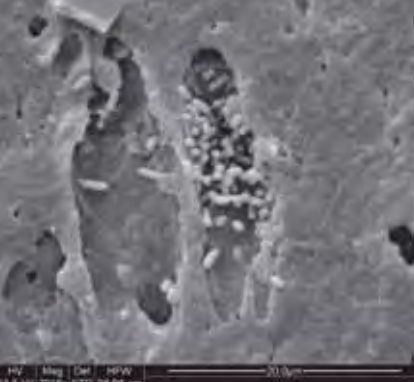

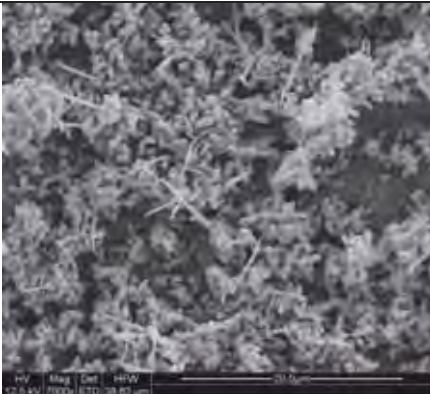

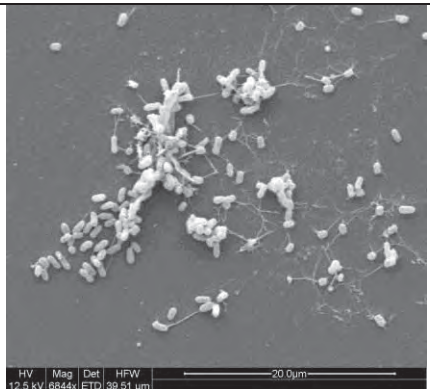
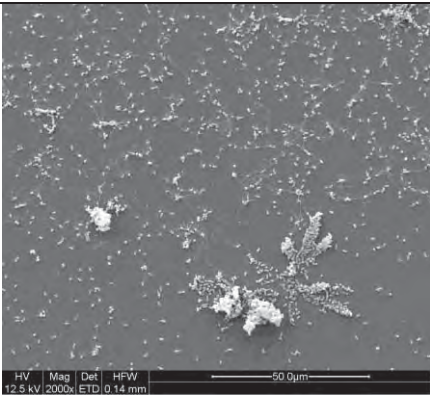



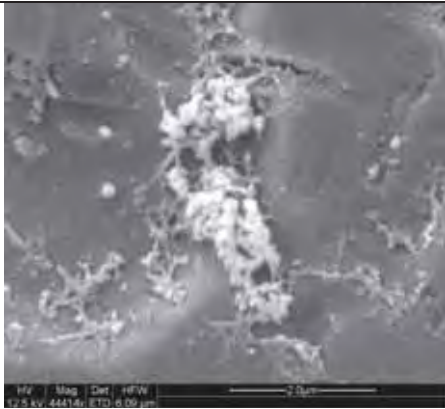
Figura B. Resultado da Eletroforese em Gel de Agarose do gene *agfD*. Poço1 corresponde ao peso molecular 100bp, poço 2 *Salmonella* Typhimurium ATCC 14028, poços 3 a 20 correspondem a amostras positivas 37 a 54.

## 5.2 Fotos por microscopia eletrônica de varredura de *Salmonella* sp.

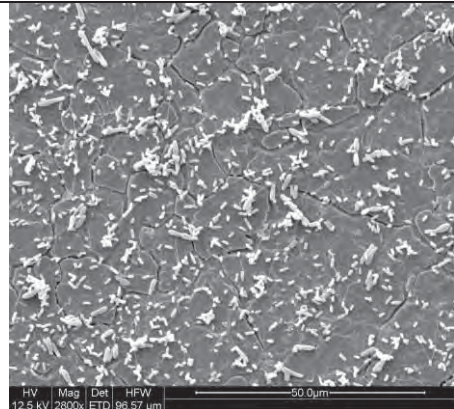
	
<p>Microscopia eletrônica de varredura da ATCC em aço inoxidável na temperatura de 16°C.</p>	<p>Microscopia eletrônica de varredura da ATCC em aço inoxidável na temperatura de 20°C.</p>
	
<p>Microscopia eletrônica de varredura da ATCC em aço inoxidável na temperatura de 28°C.</p>	<p>Microscopia eletrônica de varredura da ATCC em aço inoxidável na temperatura de 35°C.</p>
	
<p>Microscopia eletrônica de varredura da ATCC em PVC na temperatura de 16°C.</p>	<p>Microscopia eletrônica de varredura da ATCC em PVC na temperatura de 20°C.</p>

	
<p>Microscopia eletrônica de varredura da ATCC em vidro na temperatura de 16°C.</p>	<p>Microscopia eletrônica de varredura da ATCC em PVC na temperatura de 35°C.</p>
	
<p>Microscopia eletrônica de varredura da ATCC em vidro na temperatura de 16°C.</p>	<p>Microscopia eletrônica de varredura da ATCC em vidro na temperatura de 20°C.</p>
	
<p>Microscopia eletrônica de varredura da ATCC em vidro na temperatura de 28°C.</p>	<p>Microscopia eletrônica de varredura da ATCC em vidro na temperatura de 35°C.</p>

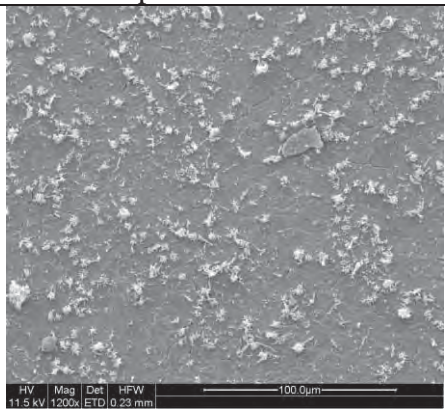




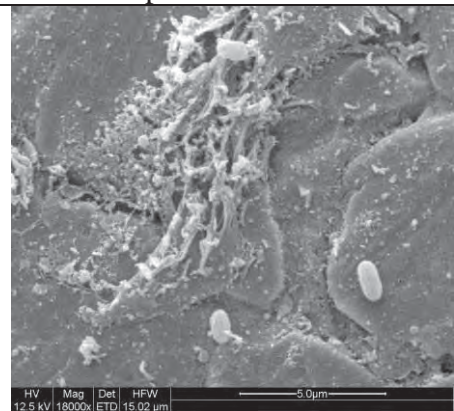
Microscopia eletrônica de varredura de uma fraca produtora em aço inoxidável na temperatura de 16°C.



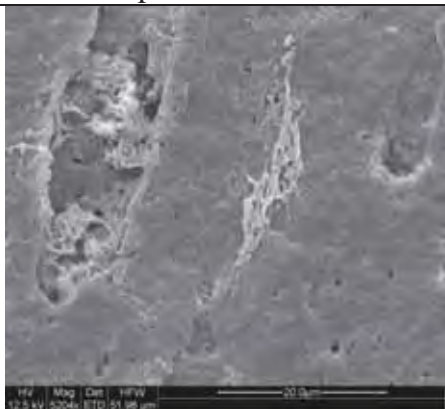
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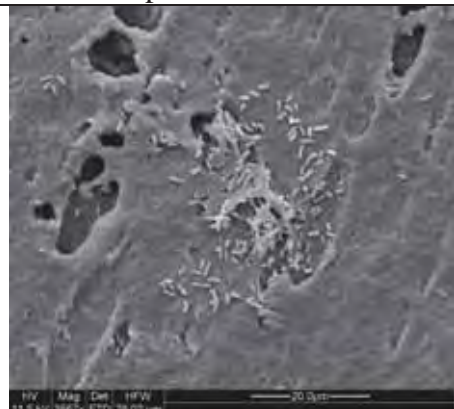
Microscopia eletrônica de varredura de uma fraca produtora em aço inoxidável na temperatura de 28°C.



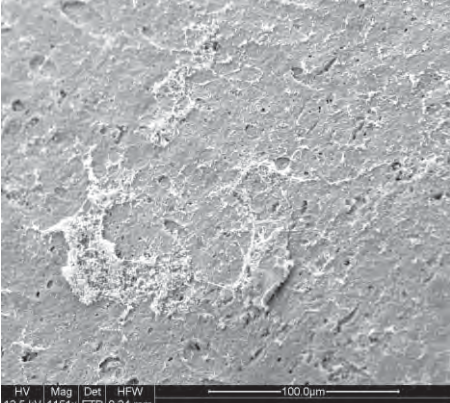
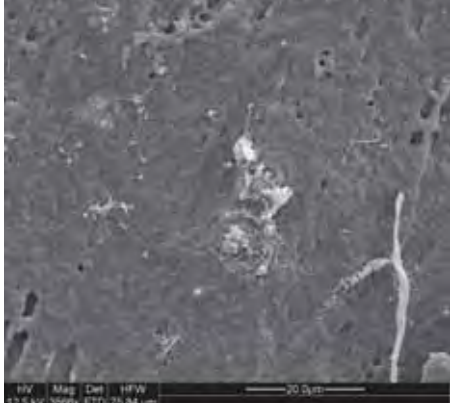
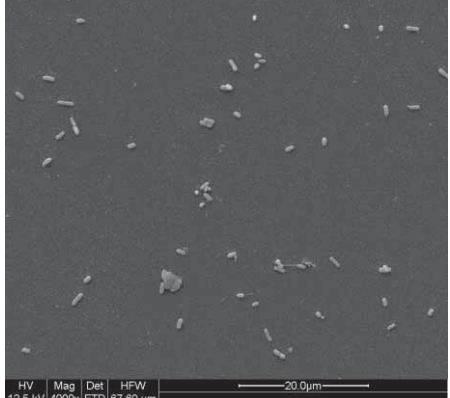
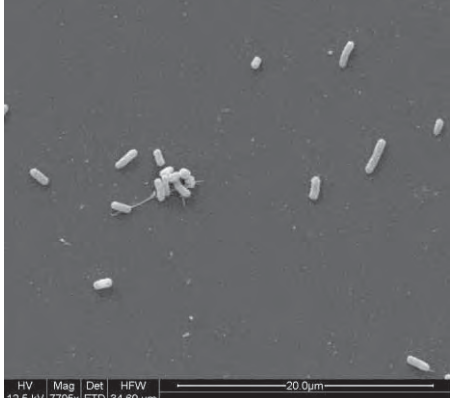

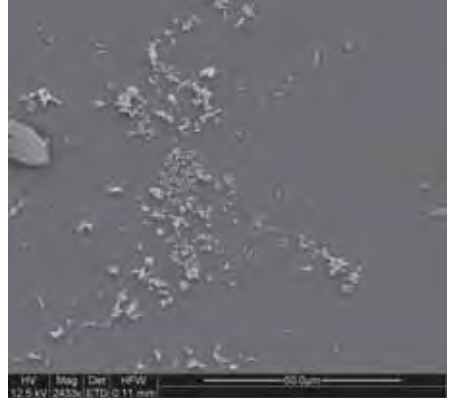
Microscopia eletrônica de varredura de uma fraca produtora em aço inoxidável na temperatura de 35°C.


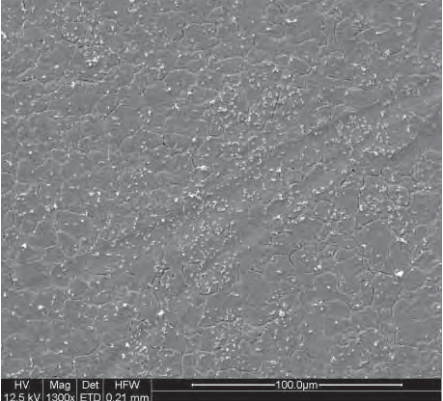
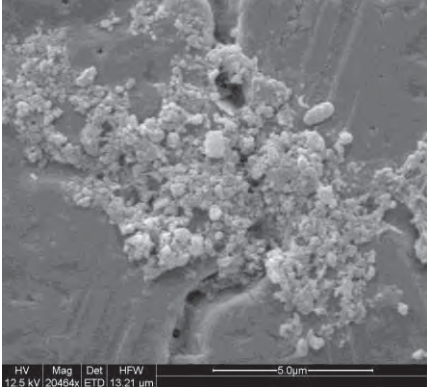
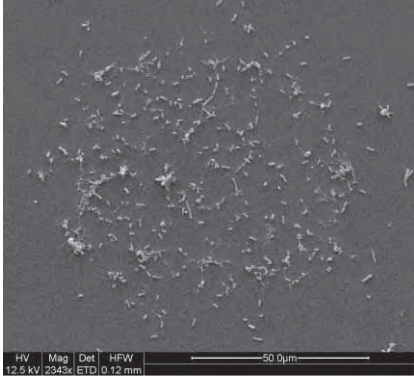
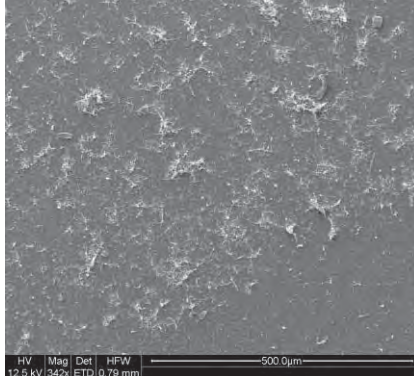


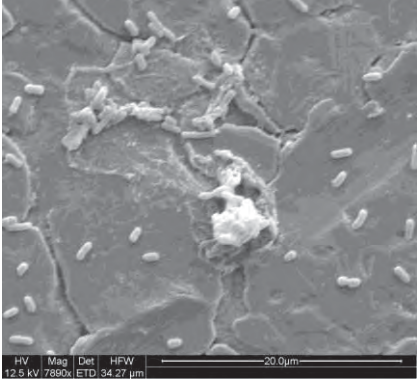
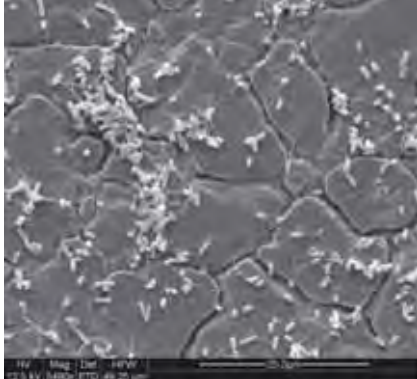
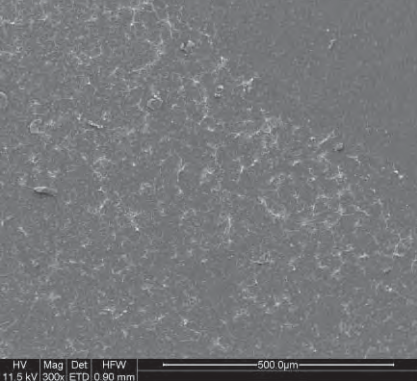
Microscopia eletrônica de varredura de uma fraca produtora em PVC na temperatura de 16°C.



Microscopia eletrônica de varredura de uma fraca produtora em PVC na temperatura de 20°C.

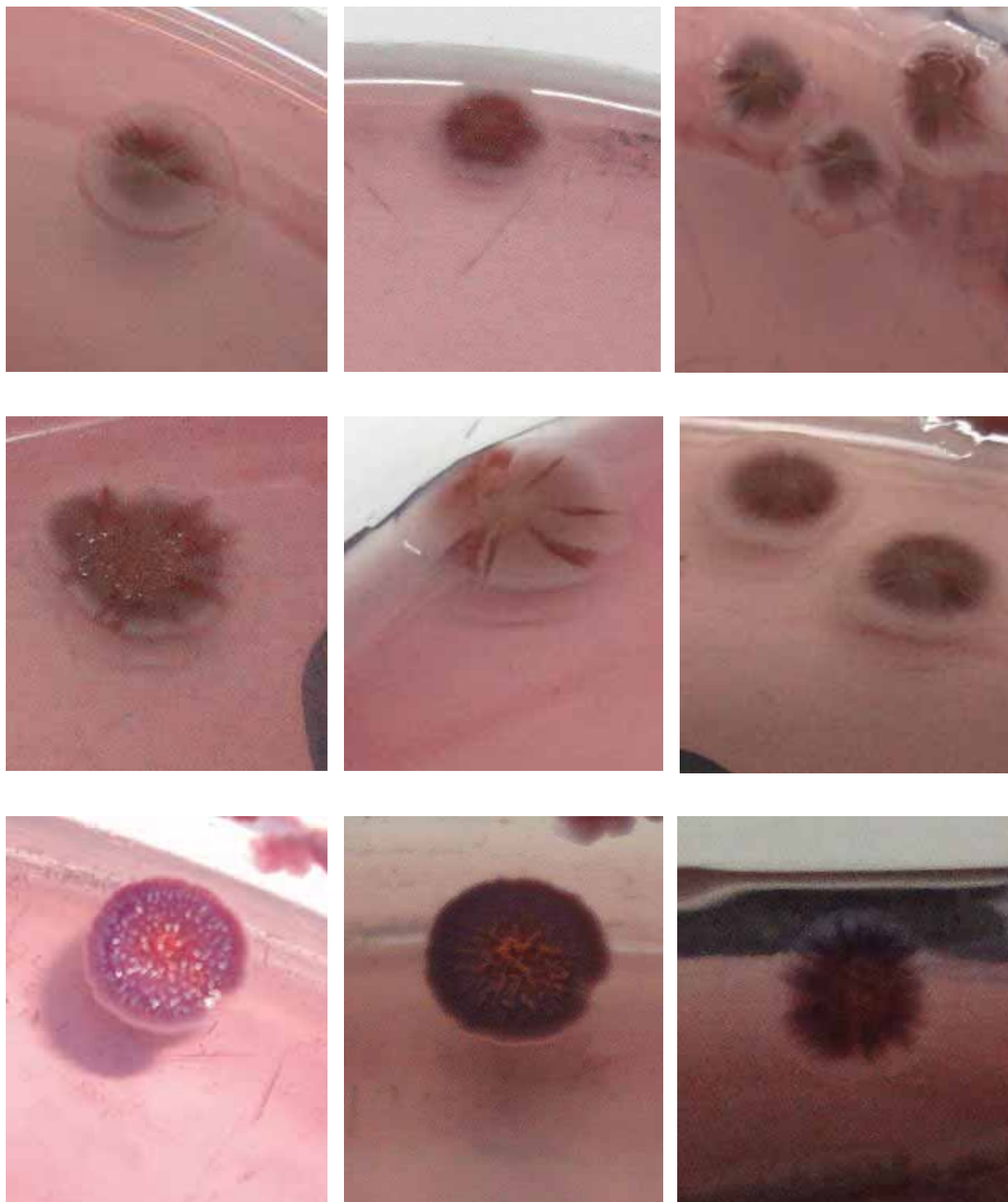
	
<p>Microscopia eletrônica de varredura de uma fraca produtora em PVC na temperatura de 28°C.</p>	<p>Microscopia eletrônica de varredura de uma fraca produtora em PVC na temperatura de 35°C.</p>
	
<p>Microscopia eletrônica de varredura de uma fraca produtora em vidro na temperatura de 16°C.</p>	<p>Microscopia eletrônica de varredura de uma fraca produtora em vidro na temperatura de 20°C.</p>
	
<p>Microscopia eletrônica de varredura de uma fraca produtora em vidro na temperatura de 28°C.</p>	<p>Microscopia eletrônica de varredura de uma fraca produtora em vidro na temperatura de 35°C.</p>

	
<p>Microscopia eletrônica de varredura de uma moderada produtora em aço inoxidável na temperatura de 20°C.</p>	<p>Microscopia eletrônica de varredura de uma moderada produtora em aço inoxidável na temperatura de 28°C.</p>
	
<p>Microscopia eletrônica de varredura de uma moderada produtora em aço inoxidável na temperatura de 35°C.</p>	
	
<p>Microscopia eletrônica de varredura de uma moderada produtora em vidro na temperatura de 20°C.</p>	<p>Microscopia eletrônica de varredura de uma moderada produtora em vidro na temperatura de 28°C.</p>

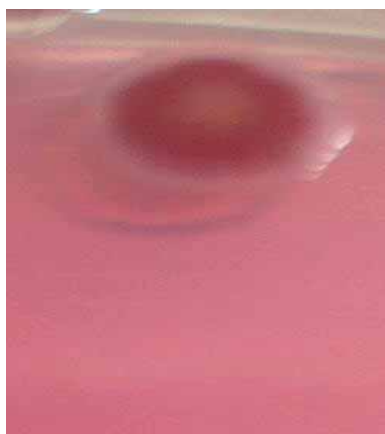
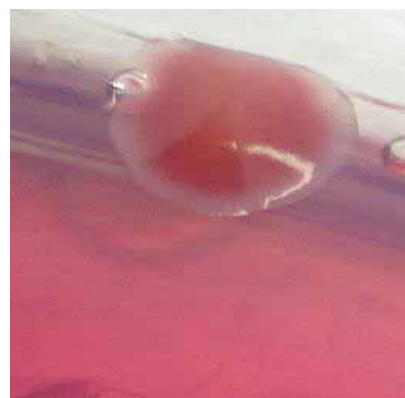
	
<p>Microscopia eletrônica de varredura de uma forte produtora em aço inoxidável na temperatura de 16°C.</p>	<p>Microscopia eletrônica de varredura de uma forte produtora em aço inoxidável na temperatura de 28°C.</p>
	
<p>Microscopia eletrônica de varredura de uma forte produtora em vidro na temperatura de 28°C.</p>	

## 6. Morfologias de *Salmonella* sp.

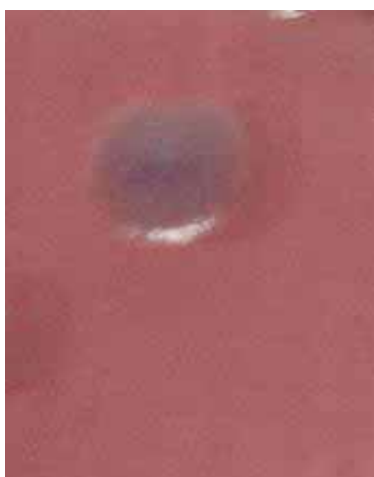
### 6.1 Morfologias *rdar* 28°C



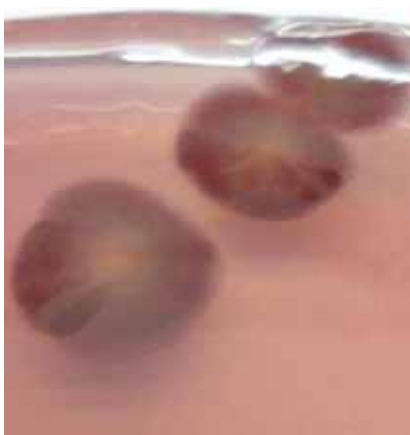
## 6.2 Morfologias *bdar* 28°C



### 6.3 Morfologia *saw* 28°C

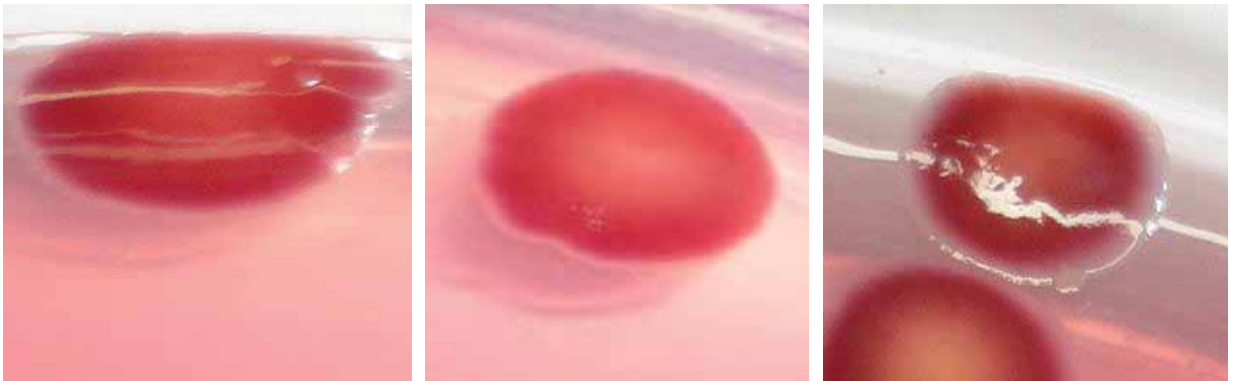


6.4 Morfologia *rdar* 35°C





### 6.5 Morfologia *bdar* 35°C



## 6.6 Morfologia *saw* 35°C



