UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL-UFRGS
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM MICROBIOLOGIA AGRÍCOLA E DO AMBIENTE

ESTUDO DO BIOSSURFACTANTE PRODUZIDO PELO PATÓGENO ALIMENTAR *Salmonella* Enteritidis SE86

Eliandra Mirlei Rossi

Porto Alegre
2015

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL-UFRGS
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM MICROBIOLOGIA AGRÍCOLA E DO AMBIENTE

ESTUDO DO BIOSSURFACTANTE PRODUZIDO PELO PATÓGENO ALIMENTAR *Salmonella* Enteritidis SE86

Eliandra Mirlei Rossi
Bacharel em Ciências Biológicas- UNOESC/SMO
Especialista em Ciência e Tecnologia de Alimentos- UPF
Mestre em Microbiologia Agrícola e do Ambiente- UFRGS

Tese apresentada ao Programa de Pós-Graduação em Microbiologia Agrícola e do Ambiente (Área de concentração em microbiologia de alimentos) como requisito para obtenção do grau de doutor em Microbiologia Agrícola e do Ambiente.

Porto Alegre, Rio Grande do Sul, Brasil.
Março de 2015

CIP - Catalogação na Publicação

Rossi, Eliandra Mirlei
ESTUDO DO BIOSSURFACTANTE PRODUZIDO PELO PATÓGENO ALIMENTAR Salmonella Enteritidis SE86 / Eliandra Mirlei Rossi. -- 2015.
120 f.

Orientador: Eduardo César Tondo.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Programa de Pós-Graduação em Microbiologia Agrícola e do Ambiente, Porto Alegre, BR-RS, 2015.

1. Salmonella Enteritidis SE86. 2. Biossurfactante. 3. Resistente. 4. Aderência. 5. Alface. I. Tondo, Eduardo César, orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

Dedico este trabalho:
Aos meus pais Elias e Miraci e
minhas irmãs Elaine e Elis, pelo seu amor, carinho,
incentivo e paciência incondicional.

AGRADECIMENTOS

Primeiramente agradeço a DEUS por ter me acompanhado e abençoado durante todos esses anos nas minhas inúmeras viagens e nos momentos de angústia e cansaço.

A minha família, meus pais Elias e Miraci, pelo amor e apoio constante e em especial para duas pessoas maravilhosas, minhas irmãs Elaine e Elis, que sempre estiveram ao meu lado, me apoiando, encorajando e me fazendo rir dos obstáculos que encontrei durante toda minha vida de estudante.

A todos os professores do Programa de pós-graduação em Microbiologia Agrícola e do Ambiente da UFRGS, em especial ao meu orientador Prof. Dr. Eduardo César Tondo.

Aos professores Dr. Adriano Brandelli e Dr.Fátima Bento pelas sugestões e conhecimento repassados durante a realização deste trabalho.

A Universidade do Oeste de Santa Catarina- UNOESC, em especial para minhas alunas Luniele Beilke, Marília Kochhann e Diana Sarzi que participaram efetivamente da realização dos experimentos.

A eterna amiga Karla J.Perez companheira de muitos momentos, uma verdadeira amiga/irmã que encontrei quando iniciei meus estudos em 2001.

A minha primeira orientada e hoje minha colega e grande amiga Luciana Senter.

As minhas amigas Leticia Sopena e Patricia Malheiros pelo carinho, conhecimento, inspiração e apoio repassados durante todo doutorado.

As minhas amigas Stela, Juliana, Fernanda, Jamile, Lurdete e Evelise apesar de estarmos distantes, sempre estiveram presentes.

A secretária mais prestativa que eu conheço, Vera Masutti, pelo carinho e todos os favores realizados.

Aos meus amigos e colegas da UNOESC que sempre me apoiaram.

A minha fisioterapeuta Richeli por todo carinho e por manter minha coluna capaz de aguentar todas as viagens entre São Miguel do Oeste-SC e Porto Alegre-RS.

Enfim, minha eterna gratidão a todas as pessoas que passaram pela minha vida durante todos esses anos de estudo e que mesmo não mencionadas aqui serão eternamente lembradas.

ESTUDO DO BIOSSURFACTANTE PRODUZIDO PELO PATÓGENO ALIMENTAR *Salmonella* Enteritidis SE86[1]

Autor: Eliandra Mirlei Rossi
Orientador: Prof. Dr. Eduardo César Tondo

RESUMO

Salmonella Enteritidis SE86 tem sido a principal responsável por surtos de salmonelose no Rio Grande do Sul, desde 1999. Essa bactéria apresenta várias características que a diferencia de outros sorovares de *Salmonella*, dentre elas a capacidade de produzir biossurfactante. Porém, a caracterização desse composto e o motivo pelo qual ele é produzido ainda não foram investigados. Assim, esse estudo teve como objetivo caracterizar o biossurfactante produzido por *S.* Enteriditis SE86 e verificar algumas de suas prováveis funções, como por exemplo, a influência desse composto na aderência e na resistência a desinfetantes, em folhas de alface. A produção de biossurfactante por *S.* Enteriditis SE86 foi avaliada em caldo infusão cérebro e coração (BHI), em meio mínimo e em folhas de alface. Foram realizados testes para caracterização do composto e *S*. Enteritidis SE86, com e sem biossurfactante, foi inoculada em folhas de alface, a fim de avaliar a influência do biossurfactante na aderência e resistência da bactéria a diversos métodos de desinfecção de vegetais folhosos. Os resultados demonstraram que o maior índice de emulsificação (IE_{24}: 62,95% após 72 horas) foi produzido em caldo BHI, mas a bactéria também foi capaz de produzir biossurfactante em meio mínimo (IE_{24}: 46% após 46 h) e em contato com as folhas de alface (IE_{24}: 52,15% após 120h). Os testes de caracterização do demonstraram que o biossurfactante é um composto polimérico que apresenta estabilidade em diferentes pH, temperatura e salinidade. O biossurfactante aumentou a aderência de *S*. Enteritidis SE86 (4,1 LogUFC/cm^2 para 7,3 Log UFC/cm^2 após 60 minutos) e contribuiu para o aumento da resistência do patógeno na superfície das folhas de alface para todos os sanitizantes testados.

---

[1] Tese de Doutorado em Microbiologia Agrícola e do Ambiente- Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (118 p.) março, 2015.

STUDY OF BIOSURFACTANT PRODUCED BY THE FOOD PATHOGEN
Salmonella Enteritidis SE86[2]

Author: Eliandra Mirlei Rossi
Advisor: Eduardo César Tondo

ABSTRACT

Salmonella Enteritidis SE86 has been recognized for outbreaks of salmonellosis in Rio Grande do Sul-RS since 1999. This bacteria shows several characteristics that differ it from other serovars, as its ability to produce biosurfactant. However, the characterization of this compound and the reason why its produced have not been investigated yet. The present study aimed to characterize the biosurfactant produced by *S*. Enteritidis SE86 and to check some of their probable functions, as checking the influence of this compound in the adherence and resistance of food pathogen on lettuce leaves. The biosurfactant production by S. Enteritidis SE86 was evaluated in Brain Heart Infusion broth (BHI), in minimal medium and on lettuce leaves. Several tests were taken to characterize the compound produced and S. Enteritidis SE86 with and without biosurfactant, was inoculated on lettuce leaves to evaluate the influence of biosurfactant in adherence and resistance to several bacterial disinfection methods of leafy vegetables. The results showed that the more emulsification index (IE24: 62.95% after 72 hours) was produced in BHI broth, but *S.*Enteritidis SE86 was also able to produced biosurfactants in minimal medium (IE24: 46% after 46 hours) and on lettuce leaves (IE24: 52.15% after 120 hours). The characterization tests showed that the biosurfactant is a polymeric compound which is stable at different pH, temperature and salinity. The biosurfactant increased adherence of S. Enteritidis SE86 (4.1 LogUFC / cm^2 to 7.3 log CFU / cm^2 after 60 minutes) and contributed to increasing pathogen resistance on surface of lettuce leaves for all tested sanitizers.

Doctoral thesis in Agricultural and Environmental Microbiology, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil (118 p.) , March, 2015.

SUMÁRIO

1 INTRODUÇÃO **1**
2 REVISÃO DE LITERATURA **4**
2.1 DOENÇAS TRANSMITIDAS POR ALIMENTOS 4
2.2 *Salmonella enterica* Enteritidis SE86 7
2.3 BIOSSURFACTANTES.. 17
2.4 ALFACE 17
3 RESULTADOS E DISCUSSÃO **20**
3.1 ARTIGO 1 21
Characterisation of a biosurfactant produced by the food pathogen *Salmonella* Enteritidis SE86 .... **21**
3.2 ARTIGO 2 57
Biosurfactant produced by *Salmonella* Enteritidis SE86 **increases the adherence and resistance to sanitizers on lettuce leaves** (*Lactuca sativa* L., *cichoraceae*) **57**
4 DISCUSSÃO GERAL **88**
5 CONCLUSÃO **98**
REFERÊNCIAS **100**

LISTA DE TABELAS

3.1.1 Characterisation of a biosurfactant produced by the food pathogen *Salmonella* Enteritidis SE86

Table 1: Mean and standard deviation of the effect of concentration of sodium chloride (NaCl), pH and temperature (Temp) on the emulsification index (IE24) of the supernatant of the culture of *Salmonella* Enteritidis SE86……………………………………………………………………………51

3.2.1 Biosurfactant produced by *Salmonella* Enteritidis SE86 **increases the adherence and resistance to sanitizers on lettuce leaves** *(Lactuca sativa L., cichoraceae)*

Table 1: *In vitro* susceptibility testing to disinfectants (Sodium hypochlorite 200 ppm and 50 ppm and vinager 2% and 20%) of *S.* Enteritidis SE86 with and without biosurfactant ……………………………………………….……… 78

Table 2: Media and standard deviation the adherence of *S.* Enteritidis SE86 with and without biosurfactant in lettuce leaves at different times……………………………………………………………………………79

Table 3: Reduction (Log10UFC / g) in counts of *S.* Enteritidis SE86 with and without bioemulsificant on lettuce after treatments ....…80

LISTA DE FIGURAS

3.1.1 Characterisation of a biosurfactant produced by the food pathogen *Salmonella* Enteritidis SE86

Figure 1: Growth, pH and emulsification index of *Salmonella* Enteritidis SE86 in BHI broth (initial pH 7.0) for 168 hours at 36 ± 1°C..46

Figure 2: Growth, pH and emulsification index of *Salmonella* Enteritidis SE86 in minimal medium (initial pH 7.0) for 120 hours at 36 ± 1°C. 47

Figure 3: Emulsification index in 24 hours (IE24h) with different concentrations of *Salmonella* Enteritidis SE86 cells. A) 6.5 log CFU/ml B) 7.7 log CFU/ml and C) 8.40 log CFU/ml...48

Figure 4: Growth pH and emulsification index of *Salmonella* Enteritidis SE86 at 36 ± 1°C for 72 h in minimal medium at different pH: A - acidified with acetic acid (initial pH 5.0); B - alkalised with sodium hydroxide solution (initial pH 8.0); C - alkalised with sodium hydroxide solution (initial pH 9.0) 49

Figure 5: Growth pH and emulsification index of *Salmonella* Enteritidis adapted SE86 acid in minimal medium with 2% glucose (initial pH 7.0) for 72 hours at 36 ± 1°C……………………………………………………………….50

3.2.1 Biosurfactant produced by *Salmonella* Enteritidis SE86 **increases the adherence and resistance to sanitizers on lettuce leaves** (*Lactuca sativa L., cichoraceae*)

Figure 1: Growth, pH and emulsification index of *Salmonella* Enteritidis SE86 on lettuce leaves for 120 hours at 36 ± 1 ° C………………..………………....75

Figure 2: Scanning electron microscopy on lettuce leaf surface…………….76

Figure 3: Mean (LogCFU/g) of *S*. Enteritidis SE86 on lettuce leaves contaminated with S. Enteritidis SE86 and biosurfactant (Experiment 1) and S. Enteritidis SE86 without biosurfactant (Experiment 2)……………………………………………………………………………….77

LISTA DE ABREVIATURAS E SIMBOLOS

%: percentual

®: Marca registrada

ºC: grau Celsius

μl:microlitro

ml: mililitro

DTA: Doenças transmitidas por alimentos

SVS: Secretaria de Vigilância em Saúde

CDC: Centers for Disease Control and Prevention

UFC: Unidade formadora de colônia

BHI: Brain Heart Infusion

PBS: Phosphate Buffered Saline

rpm: rotações por minute

sp: espécies

TSA: Tryptic soy agar

MEV: Electronic Microscopy Varredure

XLD: Xylose lysine deoxycholate agar

mN: miliNewtons

R: resistente

S: sensível

1 INTRODUÇÃO

Nos últimos anos, um aumento expressivo de Doenças Transmitidas por Alimentos (DTA) vem ocorrendo em nível mundial, afetando um grande número de indivíduos e tornando-se uma preocupação de saúde pública. *Salmonella* spp. é um dos principais microrganismos causadores de DTA no mundo (Scallan et al., 2011; Pires et al., 2014; McEntire, 2013), sendo que, no Brasil, esse patógeno foi responsável por 1564 (38,2%) surtos, no período de 2000 à 2014 (Sistema de Vigilância em Saúde- SVS, 2014).

No estado do Rio Grande do Sul, *Salmonella* Enteritidis SE86 foi responsável por mais de 95% dos casos de salmonelose entre 1999 e 2013 (Geimba et al., 2004; Oliveira et al., 2007; Capalonga et al., 2014). Esta bactéria apresenta características que a tornam diferente de outras espécies de *Salmonella*, tais como multiplicação mais rápida que outros sorovares de *Salmonella* durante as primeiras seis horas em maionese caseira armazenada em temperatura ambiente, maior capacidade de adaptação a compostos ácidos, maior resistência térmica, maior resistência a solução clorada, elevada sobrevivência em fluído gástrico simulado pH 1,5 e maior virulência em animais gnobióticos, após

adaptação ácida (Malheiros et al. 2007; Malheiros et al., 2009; Perez et al., 2010; Perez et al., 2012; Tondo et al., 2015).

Embora bactérias do gênero *Salmonella* causem surtos alimentares principalmente após o consumo de produtos animais, nos últimos anos, diversos trabalhos tem relatado salmoneloses transmitidas por vegetais folhosos, como a alface (Horby et al., 2003; Irvine et al., 2009; Nygard et al., 2008; Sagoo et al., 2003; Takkinen et al., 2005). Recentemente, Lima et al. (2013) demonstraram que *S.* Enteritidis foi capaz de se aderir em folhas de alface, destacando a importância de desenvolver estudos que investiguem os fatores envolvidos na aderência dessa bactéria em vegetais folhosos.

Dados na literatura destacam que a aderência das bactérias em diferentes superfícies pode estar associada a vários fatores, como a concentração de micro-organismos, estrutura da superfície e produção de exopolissacarídeos. De acordo com Ron & Rosenberg (2001) também pode estar associada à aderência dos microbiana a produção de biossurfactantes. Tais compostos são produzidos por diversas bactérias, como por exemplo, membros dos gêneros *Pseudomonas*, *Rhodococcus*, *Bacillus* e *Acinetobacter* (Rufino et al., 2011), contudo, até agora, ainda não foi relatada a produção de biossurfactantes por patógenos alimentares, o que justifica a realização de estudos nesta área.

Em um estudo prévio, Machado (2007) verificou que *S*. Enteritidis SE86 produziu biossurfactante quando cultivada em caldo infusão de cérebro e coração (BHI), porém não foram estudadas as características deste composto e as razões pelo qual este patógeno produz esse biossurfactante. Desse modo, o presente estudo tem o objetivo de caracterizar o biossurfactante produzido por *S.* Enteriditis SE86, bem como verificar se este composto pode influenciar na resistência a sanificantes e na aderência desse micro-organismo em folhas de alface.

2 REVISÃO DE LITERATURA

2.1 Doenças transmitidas por alimentos

Alimentos contaminados são uma das principais preocupações de saúde pública, podendo ser uma das maiores causas de doenças e mortes em nível mundial (Sarter et al., 2010; Alsayeqh, 2015; Kim et al., 2015).

As DTA são causadas por agentes químicos, como pesticidas, metais e micotoxinas, ou por agentes biológicos, como micro-organismos patogênicos, os quais são ingeridos por água ou alimentos contaminados (Piggot, 2008; Torgerson et al., 2014). Uma vez que os agentes biológicos são responsáveis pela maior parte das DTA, eles têm sido estudados intensivamente, durante décadas (Greig & Ravel, 2009).

As DTA podem originar uma variedade de sintomas clínicos, variando desde sintomas de brandos (dores abdominais e enjôos) à graves (hemorragias intestinais e septicemia) (Braden & Tauxe, 2013).

Segundo Alsayeqh (2015), muitos fatores podem afetar a segurança de um alimento, como por exemplo, a contaminação da água utilizada para o preparo dos alimentos, estocagem e transporte inadequados, a falta de práticas higiênicas sanitárias apropriadas, entre muitos outros, o que aumenta a prevalência das DTA em diversos países.

Nos últimos anos, vários alimentos foram responsáveis por surtos de DTA. De acordo com Braden & Tauxe (2013), de 2003 à 2008, de 1565 surtos

notificados no *Centers for Disease Control and Prevention* (CDC) destacaram-se aves, verduras, carne e produtos lácteos. No Brasil, de acordo com o Sistema de Vigilância em Saúde (SVS, 2014), dentre os surtos registrados no período de 2000-2014, os principais alimentos responsáveis por DTA foram alimentos mistos, ovos, produtos à base de ovos, água, doces e sobremesas. Apesar dos esforços para a sua prevenção, as DTA continuam muito frequentes, podendo apresentar elevada gravidade para um grande número de pessoas, no Brasil e no mundo (Havelaar et al., 2009; SVS, 2014).

Estudos realizados nos Estados Unidos, Austrália, Alemanha e Índia confirmaram a gravidade do problema causado pelas DTA e os dados indicam que, por ano, aproximadamente 30% da população dos países industrializados são afetados por essas síndromes (FAO/WHO, 2003).

Na França, estima-se que as DTA resultem entre 10.000 e 18.000 hospitalizações por ano, sendo *Salmonella* spp. a principal causa identificada. Na Inglaterra e no País de Gales, entre os anos de 1999 e 2000, foram registradas 1.997 internações e 687 óbitos (Adak et al., 2005).

Nos Estados Unidos, anualmente, são estimados 9,4 milhões de casos de DTA, resultando em 55.961 hospitalizações e 1.351 mortes (Scallan et al., 2011 e Scallan et al., 2012).

Nos últimos anos, um aumento expressivo de DTA vem ocorrendo em nível mundial (Havelaar et al., 2009), e isso também tem acontecido no Brasil

(Oliveira et al., 2009). Neste país, segundo a SVS, de 2000 à 2014, foram registrados 9.719 surtos de DTA, os quais acometeram 192.803 pessoas.

Estudos realizados por Oliveira et al. (2010) demonstram que *Salmonella* sp, *Staphylococcus aureus*, *Bacillus cereus* e *Escherichia coli* representam os principais agentes bacterianos causadores de DTA mundialmente. De acordo com Scallan et al. (2011), nos Estados Unidos, no ano de 2006, as bactérias foram as principais causadoras de surtos de DTA, com destaque para *Campylobacter* spp., e *Salmonella* spp., não tifóide.

De acordo com McEntire (2013), avaliar dados epidemiológicos mundiais de micro-organismos que causam DTA é um desafio, tendo em vista de que são usados diferentes sistemas de monitoramento para análise, dependendo da região. Embora as estimativas sejam baseadas em diversas e, muitas vezes, limitadas suposições, mostram que, pelo menos para um agente patogênico, a prevalência de uma determinada DTA está relacionada com certas regiões do mundo. McEntire (2013) discute que determinar o por que algumas áreas têm maiores taxas de salmonelose, requer um estudo adicional, mas afirma que é evidente que as pessoas que viajam para essas áreas têm uma chance maior de serem expostas a *Salmonella*, e provavelmente a outros agentes patogênicos, particularmente, aqueles que são transmitidos por via fecal-oral.

No Brasil, os dados da SVS (2014) reportam que *Salmonella* spp. foi o principal micro-organismo causador de DTA, no período de 2000 à 2014, causando 1564 (38,2%) dos surtos registrados.

De acordo com as pesquisas realizadas no Rio Grande do Sul, o principal patógeno causador de DTA, há mais de uma década, foi *Salmonella enterica* Enteritidis SE86 (Geimba et al. 2004; Oliveira et al., 2009; Capalonga et al., 2014).

2.2 *Salmonella enterica* Enteritidis SE86

Salmonella enterica sorovar Enteritidis denominada de SE86 foi isolada em 1999 de um repolho responsável por um surto de DTA. Trabalhos de caracterização molecular demonstram que ela continua causando surtos no RS (Geimba et al. 2004; Oliveira et al., 2009, Mürmann et al., 2008; Capalonga et al., 2014).

Nos últimos anos, a *S*. Enteritidis SE86 esteve envolvida em aproximadamente 90% dos casos investigados de salmonelose no RS (Oliveira et al., 2009, 2010; Capalonga et al., 2014), sendo motivo de diversos estudos posteriores. Como por exemplo: Geimba et al. (2004) publicaram um estudo que investigou 75 cepas de *Salmonella* envolvidas em surtos ocorridos de 1999 a 2000, no RS, onde 98% foram classificadas como *S.* Enteritidis, e, 83% apresentaram genes regulatórios de um plasmídeo de virulência de *Salmonella* (*spvR)*, demonstrando uma correlação positiva ($P<0.05$) entre *S*. Enteritidis e a presença do *spvR*. Outros trabalhos, como os de Oliveira (2007) e De Paula et al. (2010) demonstraram que, no período de 2001-2007, *S.* Enteritidis SE86 foi responsável pela maioria dos casos de salmonelose investigados pela Secretaria de Saúde do RS.

S. Enteritidis SE86 tem se destacado como patógeno alimentar por apresentar várias características que a diferencia de outros sorovares de *Salmonella*, como por exemplo, multiplicação mais rápida nas seis primeiras horas em salada de batatas com maionese caseira armazenada em temperatura ambiente, quando comparada com *S*. Bredeney e *S*. Typhimurium (Malheiros et al., 2010); maior resistência ao hipoclorito de sódio a 400 ppm, além de ser capaz de sobreviver por até 15 minutos de exposição a 200 ppm desse desinfetante (Machado et al., 2010). Perez et al. (2010, 2012) demonstraram que *S*. Enteritidis SE86 ácido-adaptada apresentou maior resistência a fluído gástrico simulado com pH 1,5 e causou mais rapidamente a morte dos camundongos *germ-free*. Ritter et al. (2012) relataram o envolvimento dos genes *rpo*S e *dps* para a resistência ao *stress* oxidativo, no qual ambos foram expressados durante a exposição ao hipoclorito de sódio 200 ppm, por 15 minutos.

Além dessas características, também foi relatado por Casarin et al. (2009) que *S*. Enteritidis SE86, quando comparada com cepas de *Staphylococcus aureus* e *Escherichia coli,* apresentou menor taxa de redução microbiana em hambúrguer de frango congelado a -18ºC, por 28 dias, sugerindo que a resistência ao congelamento pode estar associada ao efeito protetor do componentes do hambúrguer (como por exemplo a gordura) sobre as células de *S*. Enteritidis SE86.

Recentemente Casarin et al. (2014) demonstraram que *S*. Enteritidis SE86, foi capaz de se aderir em superfícies de solda e aço inoxidável formando

grumos, sendo que o tipo de solda e a rugosidade da superfície não influenciaram na adesão da bactéria nas superfícies estudadas. Além disso, esses pesquisadores afirmaram que a hidrofobicidade *S.* Enteritidis SE86 e as superfícies estudadas não demonstraram uma relação positiva com a aderência bacteriana, indicando que o processo de adesão pode ter sido influenciado por outras propriedades físico-químicas e/ou fatores biológicos não investigados pelo estudo.

Ainda, Machado (2007) verificou que *S.* Enteritidis SE86 foi capaz de produzir biossurfactante em caldo BHI, porém não foram estudadas as características desse composto e nem as razões pelas quais essa bactéria produzia essa substância.

2.3 Biossurfactantes

O termo surfactante foi caracterizado como "agentes com superfície ativa", pois descreve moléculas com atividades anfílicas capazes de atuar como detergentes, umectantes, emulsionantes, dispersantes e agentes de formação de espuma que podem ser utilizados como ingredientes de muitas formulações que vão desde os detergentes domésticos, produtos de higiene pessoal, produtos farmacêuticos e tintas (Marchant & Banat, 2012).

O grupo de surfactantes mais estudado são os biossurfactantes. Esses são compostos tensoativos produzidos por micro-organismoss (Araújo et al., 2011), classificados de acordo com a sua composição química: Glicolípidios (ramnolípidios e sorolípidios), lipopeptídios (surfactina) e poliméricos (emulsan e

alasan), ácidos graxos (3- hidroxialcano) e fosfolípidios (fosfodietanolamina) (Nitschke & Pastore, 2002; Nitschke & Costa, 2007; Aparna et al., 2012;).

De acordo com Merchant & Banat (2012), os biossurfactantes também podem ser classificados como substâncias de baixo peso molecular (glicolípidios, lipopeptídios e flavolípidios) e de alto peso molecular (polissacarídeos, proteínas, lipopolissacarídeos e lipoproteínas). Além disso, esses compostos podem possuir composição aniônica ou neutra e uma porção hidrofóbica com ácidos graxos e seus derivados e outra porção hidrofilica que podem ser carboidratos, aminoácidos, fosfatos ou peptídios (Nitschke & Costa, 2007).

Segundo Oliveira et al. (2013), os biossurfactantes podem ser produzidos extracelularmente ou estar aderidos na membrana celular de bactérias, leveduras e fungos filamentosos. Para a produção desses compostos os micro-organismos utilizam vários tipos de substratos como, por exemplo, carboidratos, óleos e alcanos (Oliveira et al., 2013).

De acordo com Fontes et al. (2008), a produção de biossurfactante pode ser espontânea ou induzida pela presença de compostos lipofílicos, por variações de pH, temperatura, aeração e velocidade de agitação.

Vários estudos (Singh & Cameotra, 2004; Hamme et al., 2007; Sriram et al., 2011; Oliveira et al., 2013; Gudiña et al., 2013) têm demonstrado as propriedades e aplicações dos biossurfactantes em diferentes áreas. Essas substâncias têm atraído a atenção dos pesquisadores, desde 1960 (Francy et al., 1991), pois possuem várias propriedades vantajosas para sua comercialização,

como por exemplo, baixa toxicidade, alta biodegrabilidade, efetividade em temperaturas extremas, pH e salinidade, atividade emulsificante e antimicrobiana (Nitschke & Costa, 2007).

Diversas pesquisas (Deleu & Paquot, 2004; Singh & Cameotra, 2004; Nitschke & Costa, 2007; Marchant & Banat, 2012; Martiet al., 2014) têm demonstrado que os biossurfactantes possuem diversas aplicações, das quais destacam-se remediação de solos, remoção de biofilmes microbianos, atividade antimicrobiana e fabricação de alimentos. O quadro 1 demonstra os diversos usos dos biossurfactantes.

Quadro 1: Usos e campos de aplicação dos biossurfactantes.

Funções	**Campos de aplicação**
Emulsionantes e dispersantes	Cosméticos, tintas, biorremediação, óleos e alimentos
Solubilizantes	Produtos farmacêuticos e de higiene
Agentes molhantes e penetrantes	Produtos farmacêuticos, têxteis e tintas
Detergentes	Produtos de limpeza, agricultura
Agentes espumantes	Produtos de higiene, cosméticos e flotação de minérios
Agentes espessantes	Tintas e alimentos
Sequestrante de metais	Mineração

Formadores de vesículas	Cosméticos
Fator de crescimento microbiano	Tratamento de resíduos oleosos
Emulsificantes	Tratamento de resíduos e recuperação de petróleo
Redutores de viscosidade	Transporte em tubulações, oleodutos
Dispersantes	Misturas de carvão, água calcáreo-água
Fungicidas	Controle biológico de fitopatógenos
Agente de recuperação	Recuperação terciária do petróleo
Agentes antitumorais	Medicina
Agentes antiadesivos	Medicina e indústria de alimentos
Agentes biocidas	Medicina e indústria de alimentos

Fonte: Nischke & Pastore (2002); Araújo et al., (2010); Marchant & Banat (2012); Gudina et al. (2013).

Originalmente os biossurfactantes atraíram a atenção por facilitar a dissolução de hidrocarbonetos, mas, nos últimos anos, o interesse pela função dessas moléculas tem aumentado consideravelmente, especialmente, na indústria de petróleo, alimentos e farmacêutica (Nitschke et al., 2009).

Marchant & Banat (2012) destacam que os biossurfactantes podem possuir a capacidade de destruir alguns tipos de células, como células vermelhas, zoósporos de fungos e bactérias. Esses pesquisadores destacam que essas

substâncias podem desempenhar um papel importante no desenvolvimento e manutenção de biofilmes de *Pseudomonas aeruginosa*, mas também podem inibir a adesão de leveduras e bactérias, impedir o estabelecimento de biofilmes formados por outras bactérias na maioria das vezes considerados patógenos alimentares ou clínicos.

Na microbiologia clínica as propriedades dos biossurfactantes têm sido estudadas no intuito de controlar diversos mecanismos ou patógenos de interesse clínico. Pode-se citar como exemplo o trabalho de Rivardo et al. (2011) que demonstrou a capacidade de um biossurfactante produzido por *Bacillus licheniformis* em aumentar a atividade antimicrobiana da ampicilina, no controle de biofilme de *Escherichia coli* CFT 073, cepa uropatógena. Ainda, Saravanakumari & Mani (2010) relataram que biossurfactantes produzidos por *Lactococcus lactis* possuem atividade antimicrobiana para patógenos como *Escherichia coli* e *Staphylococcus aureus* multirresistentes, ressaltando que esses compostos podem possuir atividade antimicrobiana, inibindo a multiplicação de diversos micro-organismos.

Uma das grandes discussões no mundo científico é qual a função fisiológica dos biossurfactantes nas células microbianas, uma vez que eles são produzidos naturalmente em diferentes ambientes (Nitschke & Pastore, 2002). De acordo com Ron & Rosenberg (2001), a capacidade de produzir biossurfactante é considerada uma vantagem particular na ecologia microbiana e nos diferentes nichos. Esses pesquisadores discutem as diversas vantagens que um micro-

organismo produtor de biossurfactante possui quando comparado com os grupos microbianos que não produzem tal substância.

Ainda, de acordo com Ron & Rosenberg (2001), a produção de biossurfactantes tem sido associada a mecanismos de comunicação microbiana tipo *quorum-sensing*, o que confere ao micro-organismo capacidade de realizar diversas reações fisiológicas, como por exemplo, a produção de biofilmes. Normalmente, o *quorum- sensing* é regulado por altas quantidades de micro-organismos presentes no meio, o que pode estar indiretamente correlacionado com um ou mais fatores fisiológicos, como a disponibilidade de energia, nitrogênio ou oxigênio, elementos estes que podem ser encontrados em alimentos e, consequentemente, facilitar a sobrevivência desses micro-organismos.

Atualmente, não há relatos de fatores de virulência associados a produção de biossurfactantes por patógenos de alimentos. Por outro lado, há relatos relacionados com produção de biossurfactante por *Pseudomonas aeruginosa* (incluída no grupo de bactérias deteriorantes de alimentos). Ron & Rosenberg (2001) afirmaram que ramnolipídeos produzidos por *Pseudomonas aeruginosa* são considerados um exoproduto associado à virulência, sendo que essa produção também carreia e regula a correlação com outros fatores de virulência como, por exemplo, a proteína AlgC, envolvida na produção de alginato (polissacarídeo capsular que permite a aderência das bactérias infectantes às superfícies epiteliais pulmonares, formando biofilmes, os quais protegem as bactérias contra ação dos antibióticos e do sistema imunológico) e

lipolissacarídeos-LPS (endotoxina causadora de síndrome de sepse: febre, choque, coagulação intravascular disseminada e leucopenia ou leucocitose).

Segundo Nitschke & Pastore (2002), a diversidade de biossurfactantes produzida é uma das características que, muitas vezes, dificulta os estudos de ecologia microbiana e, por isso, a exata função fisiológica dessas substâncias ainda não é completamente compreendida.

Segundo Bento et al. (2008), o principal papel fisiológico atribuído aos biossurfactantes é facilitar a multiplicação dos micro-organismos em substratos imiscíveis em água, pela redução da tensão superficial, tornando-os mais disponíveis para captação e metabolismo. Também, Ron & Rosenberg (2001) descrevem algumas funções fisiológicas para os biossurfactantes, dentre as quais se destacam: aumento da área de superfície hidrofóbica, ligação com metais pesados, patogenicidade, atividade antimicrobiana, regulação no *quorum-sensing*, regulação na adesão a superfícies e formação de biofilmes.

Na área de alimentos, Nitschke & Costa (2007) afirmaram que os biossurfactantes possuem diversas utilidades, que vão desde ingredientes para formulação de alimentos até a função como bioprotetores contra patógenos.

Segundo Magalhães & Nitschke (2013), o efeito bioprotetor dos biossurfactantes ocorreu quando os ramnolipídios exerceram atividade antimicrobiana contra diversas cepas de *L. monocytogenes* isoladas de alimentos e superfícies. Além disso, quando esse composto foi combinado com nisina, foi

observado um efeito sinérgico, aumentando a eficácia no controle desse patógeno.

Por outro lado, nos últimos anos, algumas pesquisas (Araújo et al., 2010; Shaheen et al. 2010; Mellor et al., 2011; Oliveira et al. 2013) têm apontado os biossurfactantes como substâncias que podem facilitar a sobrevivência de micro-organismos, bem como facilitar a decomposição dos alimentos.

Mellor et al. (2011) demonstraram que em carne de frango, aerobicamente armazenada a 4 ºC, a presença de biossurfactantes produzido por *Pseudomonas fluorescens,* além de aumentar a deterioração da carne, aumentou a contagem total de bactérias, sugerindo que a produção de biossurfactante represente uma vantagem competitiva para cepas de *Pseudomonas fluorescens* em carne de frango aerobicamente armazenada.

Já o trabalho de Shaheen et al. (2010) discute que a formação de biofilmes por *Bacillus cereus* pode estar associada à produção de surfactina (um tipo de biossurfactante). De acordo com esses pesquisadores, os resultados podem indicar uma necessidade do *B. cereus* produzir biossurfactante para formar biofilmes em silos utilizados para armazenar leite refrigerado, o que poderia influenciar na eficácia dos desinfetantes utilizados na higienização dos silos. Corroborando com essa hipótese, Peng et al. (2002) reportaram que os sanitizantes possuem uma baixa eficácia sobre biofilmes produzidos por *B. cereus* em superfícies de aço inoxidável com resíduos de leite.

Desse modo, se os biossurfactantes possuem tais atividades, sugere-se que a capacidade de produzir biossurfactante possa ser uma vantagem competitiva para os micro-organismos causadores de DTA, uma vez que todas as vantagens dos micro-organismos que produzem biossurfactantes destacadas nos parágrafos anteriores podem ser encontradas no universo dos alimentos.

A capacidade de aumentar a aderência do patógeno e formação de biofilmes pode ocorrer, tanto em superfícies de equipamentos e utensílios, quanto nos alimentos, o que dificultará a eliminação do patógeno, uma vez que esses estão entre os principais fatores responsáveis pela resistência dos micro-organismos aos desinfetantes.

Segundo Lima et al. (2013), bactérias como a *S.* Enteritidis possuem uma tendência natural de se aderir em superfícies como mecanismo de sobrevivência, incluindo as folhas de alface.

2.4 Alface

Embora os produtos de origem animal sejam os principais alimentos responsáveis por surtos de DTA, as hortaliças também têm sido identificadas como importantes veículos de patógenos alimentares, nos últimos anos (Tauxe & Braden, 2013).

Dentre os vegetais folhosos, o mais consumido no mundo, é a alface (*Lactuca sativa* L., *cichoraceae*), pois é um vegetal de fácil aquisição, que se destaca por ser uma hortaliça de baixo custo (Abreu et al., 2010; Lima et al., 2013)

e alta aceitação. Entretanto, a alface pode apresentar contaminação por diferentes micro-organismos (Anderson et al. 2011; Critzer & Doyle, 2010; Erickson et al. 2010; Santana et al., 2006; Lima et al., 2013). Essa contaminação pode ser proveniente da água de irrigação, do solo, fertilizantes orgânicos, dos processos que envolvem a colheita, a lavagem, a embalagem e transporte (Ceuppens et al., 2014; Koseki & Isobe, 2005; Barrera et al., 2012).

Quando os frutos ou vegetais são consumidos crus, como é o caso de alfaces, patógenos podem estar presentes e, consequentemente, serem ingeridos sem ser inativados, o que pode ser a causa de diversas doenças gastrintestinais (Koseki & Isobe, 2005).

Segundo Anderson et al. (2011), especificamente para alfaces, as ameaças mais graves são *Escherichia coli* enterohemorrágica e *Salmonella enterica.* Recentemente Ceuppens et al. (2014) relataram a presença de *Salmonella* sp. em alfaces produzidas no Rio Grande do Sul, demonstrando que essa bactéria pode estar presente nesses alimentos. Trabalhos como o de Patel & Sharma (2010) demonstraram que diversos sorovares de *Salmonella (S.* Thompson 2051H*, S.* Tennessee 2053N*,* e *S.* Negev *26 H)* podem se aderir após cinco minutos de contato com a superfície de folhas de alface, aumentando o número de células aderidas após 24 h, favorecendo a formação de biofilmes. Esses pesquisadores destacaram que a formação de agregados de *Salmonella* pode facilitar a aderência dessa bactéria em superfícies de vegetais folhosos

(como o alface) e consequentemente manter sua sobrevivência por longos períodos.

A aderência dos micro-organismos a superfícies e formação de biofilmes pode estar associada a diversos fatores e dentre eles a produção de biossurfactantes (Rosenberg & Ron, 1999; Ron & Rosenberg, 2001; Nitschke & Pastore, 2002).

Atualmente não existem trabalhos que demonstrem a aderência de micro-organismos produtores de biossurfactantes em alface, o que justifica a realização de estudos que caracterizem a função desses compostos nas superfícies desse vegetal folhoso.

3 RESULTADOS E DISCUSSÃO

Os resultados deste trabalho estão apresentados na forma de artigos científicos.

3.1 Characterisation of a biosurfactant produced by the food pathogen *Salmonella* Enteritidis SE86

Artigo submetido para publicação no periódico Food Microbiology.

3.2 Biosurfactant produced by *Salmonella* Enteritidis SE86 increases the adherence and resistance to sanitizers on lettuce leaves (*Lactuca sativa L., cichoraceae*)

Artigo publicado na Frontiers Microbiology

3.1 Artigo 1

Characterisation of a biosurfactant produced by the food pathogen *Salmonella* Enteritidis SE86

Eliandra Mirlei Rossi [a,c]; Marília Kochhann [a]; Luniele Beilke [a]; Diana Helena Sarzi [a]; Fátima Menezes Bento[b], Eduardo César Tondo[c *].

[a] Laboratório de Microbiologia, Departamento de Ciências Biológicas e da Saúde, Universidade do Oeste de Santa Catarina- UNOESC, Campus de São Miguel do Oeste-SC, Brasil.

[b] Departamento de Microbiologia, Federal Universidade Federal do Rio Grande do Sull, Sarmento Leite, 500, CEP 90050-170, Porto Alegre, RS, Brasil.

[c] Laboratório de Microbiologia e Controle de Alimentos, Instituto de Ciência e Tecnologia de Alimentos, Universidade Federal do Rio Grande do Sul - ICTA/UFRGS, Av. Bento Gonçalves 9500, prédio 43212, Campus do Vale, Agronomia, Porto Alegre/RS, Porto Alegre, RS, Brasil

*** Corresponding author. Tel.: 055 51 3308 6677; fax: 055 051 3308**

E-mail address: tondo@ufrgs.br

Abstract

Biosurfactants are amphipathic compounds produced by microorganisms that may have emulsifying properties and can be involved in microbial pathogenicity. *Salmonella* Enteritidis SE86 has been identified as a major cause of foodborne diseases in Southern Brazil and demonstrated the capacity to produce a biosurfactant. The aim of the present study is to characterise this biosurfactant. First, the emulsifying index and pH were evaluated in Brain Heart Infusion Broth (BHI) and minimal medium during the growth of *S.* Enteritidis SE86. After that, ninhydrin reaction, phenol-sulphuric acid assay, protein concentration, ionic charge, and the stability of the biosurfactant were analysed. The results showed that the highest emulsification ratio was 62.95% when bacterial growth reached 8.22 log CFU/ml, after 72 hours of incubation in BHI broth. *S*. Enteritidis SE86 was

also able to produce the biosurfactant in minimal medium and emulsion in this medium was stable after exposure to different pH, temperatures and salinity conditions. The biosurfactant was characterised as a polymer compound, since it presents free amines and carbohydrates in its composition. Thus, the results demonstrate that *S.* Enteritidis SE86 was able to produce a polymeric biosurfactant at high cell concentrations and the compound maintained its stability when exposed to different environmental conditions.

Key words: Biosurfactant, *Salmonella* Enteritidis SE86,Southern Brazil.

1. Introduction

The term surfactant was characterised as "surface active agents", as the described activities of molecules capable of acting as detergents, wetting, emulsifying, dispersing and foaming agents (Merchant; Banat, 2012). Several studies demonstrated the applications of different biosurfactants (Singh and Cameotra, 2004; Oliveira et al., 2013; Hamme et al., 2006; Gudiña et al., 2013; Sriram et al., 2011).

Biosurfactants are surfactant compounds produced by microorganisms (Araújo et al., 2011.), classified according to their chemical composition: glycolipids (rhamnolipids, trhealolipids, sophorolipids), lipopeptides (surfactin, viscosin, serrawettina, subtilisin, gramicidins and polymixyns), phospholipids, fatty acids, neutral lipids, polymeric surfactants (emulsan, alasan, liposan, lipomanan)

(Merchant and Banat, 2012; Aparna et al., 2012; Nitschke and Pastore, 2002; Nitschke and Costa, 2007). According to Merchant and Banat (2012), the biosurfactant can be classified as low molecular weight (glycolipids, lipopeptides and flavolipid) and high molecular weight substances (polysaccharides, proteins, lipopolysaccharides and lipoproteins). These substances are produced extracellularly from various substrates, including sugars, oils and many other alkanes, or part of the cell membrane of bacteria, yeast and filamentous fungi (Oliveira et al., 2013; Satpute et al., 2010; Singh and Cameotra, 2004).

Ron and Rosenberg (2001) showed that some physiological roles have been assigned to biosurfactants that increase the hydrophobic surface area and adherence to surfaces. In addition, biosurfactant are involved in bacterial pathogenesis, quorum sensing and biofilm formation.

The capacity to produce biosurfactants can be considered an advantage for ecological niches for microorganisms, especially for performing various functions in the microbial cells, e.g., adhesion to surfaces and antimicrobial factors that may contribute to the survival of microorganisms (Ron and Rosenberg, 2001; Nitschke and Pastore, 2002). Several microorganisms have been reported as producers of biosurfactants, among them members of the genera *Pseudomonas, Rhodococcus, Bacillus, Acinetobacter, Candida, Torulopsis, Aspergillus* and *Corynebacterium* (Ron and Rosenberg, 2001; Rufino et al., 2011; Deleu and Paquot, 2004).

Some compounds of microbial origin exhibit surfactant properties, i.e., they decrease surface tension and act as a high capacitance emulsifier. It is known that

such compounds can be involved in bacterial pathogenicity, such as *Pseudomonas aeruginosa* (Oliveira et al., 1999).

Currently, the main causative pathogen of foodborne illness in the world is *Salmonella* sp. (Scallan et al., 2011; Pires et al., 2014; McEntire, 2013). In Brazil, health surveillance registry records (SVS, 2014) show that *Salmonella* spp. were major the food pathogens in the period 2000 to 2014 (accounting for 38.2% of foodborne outbreaks).

In the State of Rio Grande do Sul (RS), Southernmost State of Brazil, *S.* Enteritidis SE86 was identified as the major agent of foodborne diseases since 1999 to 2012 and several studies were carried out in order to known this important food pathogen (Geimba et al. 2004; Oliveira et al., 2007; Oliveira et al., 2009; Mürmann et al., 2008; De Paula et al., 2010; Capalonga et al., 2014). *S.* Enteritidis SE86 distinguished itself by presenting various characteristics that differentiate it from other serovars of *Salmonella*, such as faster growth in potato salad with homemade mayonnaise stored to 30°C within the first six hours, compared with *S.* Bredeney and *S.* Typhimurium (Malheiros, De Paula and Tondo, 2007), greater resistance to sodium hypochlorite at 400 ppm, and the ability to survive for up to 15 minutes exposure to 200 ppm of this disinfectant (Machado et al., 2010). Perez et al. (2010, 2012) showed that acid-adapted *S.* Enteritidis SE86 showed higher resistance in simulated gastric fluid pH 1.5 and caused more quickly the death of germ-free mice. Ritter et al. (2012) reported the involvement of *rpoS* and *dps* gene

for resistance oxidative stress, which were both expressed during exposure in 200 ppm sodium hypochlorite for 15 minutes.

Some years ago, Machado (2007) found that *S*. Enteritidis SE86 was able to produce a biosurfactant, but the characteristics of this substance and the reasons why this microorganism produced this compound have not been studied. Thus, the aim of this study was to assess the ability of biosurfactants production by *S.* Enteritidis SE86 and study the characteristics of this compound.

2. Materials and methods

In order to increase the reliability of the study, all experiments were repeated three times and the averages were subsequently made to express the final result.

2.1 Microorganism

To develop this research, S. Enteritidis SE86 strain was used, which was isolated from a cabbage involved in an outbreak of salmonellosis in the State of RS in 1999. This microorganism was characterised by Geimba et al. (2004) as presenting the same profile and genotypic strains of *Salmonella* sp. involved in most cases of salmonellosis that occurred from 1999 to 2013 in RS (Capalonga et al., 2014). For the tests, the strain was grown in BHI broth (Himedia-India) at 36 ± 1°C, for 18 hours.

2.2 Growth curve and determination of emulsifying activity

To assess the stage when *S.* Enteritidis SE86 produces the biosurfactant, a microbial growth curve in BHI broth (pH 7.0) was performed for 168 hours at 36 ± 1°C. Initially, 20 µl of the culture SE86 was inoculated into 150 ml of BHI broth and incubated at 36 ± 1°C. The initial concentration of viable cells was approximately 4 logCFU/ml. Aliquots (6 ml) were removed at various times (every two hours for 24 hours and every 24 hours of 128 hours) to carry out emulsification index (IE24), bacterial count and pH analysis.

Bacterial counts were performed in triplicate by seeding the samples onto plates containing Tryptone Soy Agar (TSA, Merck , Darmstadt) and incubated at 36 ± 1°C for 24 hours. The pH was measured with a pH meter (PHTECK).

The emulsification index (IE_{24}) was carried out using the method of Cooper and Goldenberg (1987) which, in a test tube, involved the addition of 4 ml of xylene to 6 ml of BHI broth containing *S.* Enteritidis SE86. This solution was stirred for three minutes, and after 24 hours, the height of the column of the emulsion (the range between the aqueous phase and the hydrocarbon) was measured.

To calculate the emulsion, the following rate equation was used:

$$\text{Emulsification index } (IE_{24}) = \frac{\text{Emulsified phase(mm)}}{\text{total volume of liquid in the column (mm)}} X\ 100$$

To verify that the emulsion was produced extracellularly, this test was also performed with culture supernatants as a negative control and were put into 4 ml vials of xylene and 6 ml of BHI broth without bacterial culture.

2.3 Emulsification production in minimal medium

To evaluate the emulsifier production capacity in minimum nutritional conditions, means least phosphate buffered saline (PBS, Himedia India) containing 8.5 g/l of sodium chloride, 1.91 g/l of disodium hydrogen phosphate and 0.38 g/l of potassium dihydrogen phosphate was used. The pH of the medium was adjusted to 7.0.

Initially, *S.* Enteritidis SE86 culture was made in BHI for 24 hours at 36 ± 1°C. Subsequently, 6 ml of culture were centrifuged at 3500 rpm for 15 minutes and washed with PBS buffer three times. Then, the washed cells were inoculated in 150 ml minimal medium and incubated at 36 ± 1°C for 120 hours. The initial concentration of viable cells was approximately 4 logCFU/ml.

Subsequently, aliquots were taken from that inoculum every two hours and the pH parameters, microbial count and emulsification index were evaluated, as previously mentioned in the methodology.

Furthermore, to determine the initial concentration of inoculated bacterium, counts on TSA were performed.

The determination of biomass (cell dry weight) was performed according to the methodology described by Peixoto (2008) in which 10 ml of the initial inoculum

was centrifuged at 4500 rpm for 15 minutes, washed with distilled water, filtered through a 0.45 micron membrane (Millipore), and dried at 100°C for 5 hours. After this period, the membranes remained in a desiccator for 15 minutes, the plates were weighed and dry matter (g/l) was determined by the following formula:

$$MS = \frac{(Mcel - Mmemb + UM) \times 1000}{\text{centrifuged volume}}$$

Where: MS: Cell dry weight (g/l) Mcel: membrane with the cell mass (g) Mmemb: Membrane mass without cells (g) and a moisture contents membrane.

2.3 Changing the pH X production emulsifier

After completion of the microbial growth curve, three times (24 h, 48 h and 72 h after inoculation of the microorganism) were chosen to evaluate the production of an emulsifier in minimal medium with different pH.

The inoculum was prepared in minimal medium, as described in item 2.3, acidified with acetic acid (pH 5.0) and basified with sodium hydroxide (pH 8.0 to 9.0). We also assessed the production of an emulsifier in minimal medium supplemented with 2% glucose.

Aliquots of each inoculum were taken in three stages (24, 48 and 72 hours after incubation) to check the pH, emulsifier production and bacterial counts, using the methods already described in section 2.2.

2.4 Detection of surfactant activity of the emulsifier produced by S. Enteritidis SE86

To detect the emulsifying surfactant produced by *S.* Enteritidis SE86*,* activity tests were performed as suggested by Benedict et al. (2008), including the collapse of the drop test, the measurement of surface tension, inclined glass slide, haemolysis on the blood agar test and cetyltrimethylammonium bromide CTAB.

2.4.1 Measurement of surface tension

The measurement of surface tension was performed by the Wilhelmy plate method, using a tensiometer (brand Gilbertini-TSD-Italy) described by Bodour and Maier (2002). To achieve the surface tension, measurements used minimal medium (pH 7.0) with 8.5 log CFU/ml of *S*. Enteritidis SE86 cells incubated for 48 hours at room temperature (25°C) and 36 ± 1°C. As a control, a minimum medium without bacterial cells and distilled water was used. The media were centrifuged at 6000 rpm for 15 minutes. Subsequently, the measurement was of the surface tension of the supernatant and emulsifying index (IE24).

2.4.2 Collapse in drop test

The collapse of the drop test was performed according to the methodology proposed by Bodour and Maier (1998; 2002) with a 48 h culture of *S.* Enteritidis SE86 in minimal medium to check biosurfactant production, where 100 µl of suspensions (cells and medium with supernatant only) were inoculated into 96-well plates with 50µl of mineral oil. After 1 minute of reaction, the result was determined

visually in stereo (Bioval). The positive control was prepared using SDS (sodium dodecyl sulphate) 25%.

2.4.3 Inclined Glass sheet

In the slide test, the methodology proposed by Bodour and Maier (2002) was used. Initially, one colony of *S*. Enteritidis SE86 cultivated on BHI agar for 48 hours was mixed in a droplet of a sodium chloride solution (0.9%) and placed on a glass slide, before being tilted.

We used sodium chloride solution (0.9%) without bacterial cells as a control. The ability of the droplet to move the blade was considered a positive test.

2.4.4 Haemolytic activity

To evaluate the ability of the microorganism to produce haemolysis, methods suggested by Youssef et al. (2004) were used, wherein the strain of *S.* Enteritidis SE86 was spread on plates with sheep blood agar and then incubated for 48 hours in an oven at 36 ± 1°C.

The presence of a haemolytic halo (β-haemolysis-transparent halo and greenish-grey α- and γ-halo haemolysis no haemolysis) around the growth was considered positive for the haemolysis assay.

2.4.5 Test cetyltrimethylammonium bromide (CTAB)

To perform the test, we used the methods proposed by Benedict et al. (2005), where colonies of *S.* Enteritidis SE86 were inoculated on agar cetyltrimethylammonium bromide (CTAB) with methylene blue and incubated in an incubator at 36 ± 1°C for 72 hours. Positivity for rhamnolipids detection was performed by observing the formation of a dark blue halo around the colonies.

2.5 Characterisation of biosurfactant

For the partial characterisation of emulsifier, tests suggested by Yin et al. (2009), Monteiro et al. (2010), Zhang et al. (2012), Singh and Tripathi (2013), Ayed et al. (2014), Patil and Chopade (2001) and Rufino et al. (2014) were used.

Ninhydrin reactions were performed, along with the phenol sulphuric acid assay, protein concentration, ionic charge and determination of the emulsifying stability.

To perform all of the characterisation tests, we used the supernatant of the culture of *S.* Enteritidis SE86 on minimal medium (pH 7.0) at 36 ± 1°C for 48 hours. The supernatant was obtained by centrifugation at 3500 rpm for 15 min.

2.5.1 Reaction of Ninhydrin

To evaluate the presence of free amines in the minimal medium containing the microorganism, one ml of the supernatant was pipetted into a test tube and three drops of 0.5% ninhydrin solution were added, before being boiled for three

minutes and observing the colour change of the mixture. The purple colour was regarded as positive for the presence of free amines.

As a negative control we used the minimal medium without the presence of the microorganism.

2.5.2 Reaction of phenol sulphuric acid

To assess the overall carbohydrate concentration, two ml of the supernatant were placed into a test tube, and 1 ml of phenol and 5 ml of sulphuric acid were mixed and agitated with a vortex-type mixer for 1 min. The tubes were boiled in a water bath for 15 min. They were then cooled to room temperature and held measuring the optical density at 480 nm.

2.5.3 Determination of ionic charge

The ionic charge was determined by the double diffusion technique in 1% agar in which two rows of regularly spaced wells were made in agar. The wells of one row were filled with the biosurfactant solution and wells of the other were filled a pure compound of known ionic charge. The anionic substance chosen was sodium dodecyl sulphate (SDS) 20 mM and the cationic one was barium chloride, 50 mM. The appearance of precipitation lines between the wells, indicative of the ionic character of the biosurfactant.

The onset of precipitation was monitored for 48 hours at room temperature.

2.5.4 Concentration of total protein

The total protein concentration was estimated using the total protein kit Labtest Diagnostica SA (Brazil).

2.5.5 Stability studies of the emulsion

To assess the stability of the emulsion, the supernatant of the culture of the test organism was exposed to different conditions of pH (3, 4, 5, 6, 7, 8, 9 and 10) for 1 h at room temperature (25°C).

To investigate the heat stability of the emulsion, the supernatant was subjected to different temperatures (30, 40, 50, 60, 70, 80, 90 and 100°C) for 15 minutes.

Subsequently, the stability of the supernatant was evaluated for proteolytic enzymes (Proteinase K) and lipolytic (lipase-Sigma L0777) at a concentration of 1mg/ml.

The stability of the supernatant was also tested at different concentrations of sodium chloride (2, 3, 4, 5, 6, 7, 8, 9 and 10%) for 15 minutes at room temperature (25°C).

After all of the stability tests, supernatants were subjected to the emulsification index test, as described in the methodology Section 2.2.

Furthermore, to check the stability of the emulsion, both tubes (with *S.* Enteritidis SE86 and the other with supernatant) from the cultivation of *S.*

Enteritidis SE86 (pH 7.0) after 48 hours, were subjected to the test of emulsification index and were then incubated at 36 ± 1 ° C for 30 days.

The emulsifier was assessed daily by measuring the emulsified phase.

3. Results and discussion

The results showed that *S.* Enteritidis SE86 was able to produce emulsifier quantities above 40%, both in minimal medium and in BHI broth.

In BHI, the production of emulsifier (IE24: 18.1%) after 6 h of incubation and microbial count of 7.86 log CFU/ml and the largest IE24 (62.25%) was observed after 72 h and 8.22 log CFU/ml SE86.

Furthermore, it was found that there was an increase in the pH of the culture medium after 48 h; the pH ranged between 7.1 (48 h) and 8.9 (after 168 h). It was also demonstrated that although the *S.* Enteritidis SE86 counts decreased to 7.35 log CFU/ml, after 168 hours, the IE24 was 60% (Figure 1).

In minimal medium, the emulsifier production was detected only after 18 h of incubation with 12% IE24 and 7.70 log CFU/ml SE86. The biggest IE24 observed was 46% after 46 h with 8.4 log CFU/ml SE86. The pH was maintained (7.0) throughout the experiment (120 hours). Furthermore, it was found that the IE24 decreases to 3% after 120 h and counting of *S.* Enteritidis SE86 to 5.65 log CFU/ml (Figure 2).

Also, no differences were observed between IE24 and supernatant obtained from the media (BHI broth and minimal medium) with *S.* Enteritidis SE86, demonstrating that the emulsifier is produced extracellularly by bacteria.

A comparison of the growth curves (BHI broth and minimal medium), and the studied parameters (Figures 1 and 2) showed that the emulsifying production, besides being higher in BHI broth before it occurs (after 6 h). This result probably because of the carbon source (glucose, bovine heart infusion and calf brain infusion), in the BHI broth. Furthermore, increasing pH was found in BHI broth, which did not happen in the growth on minimal medium.

Also, the time of production of the emulsifier in BHI broth was found to be higher, remaining for 168 h. In addition, there was a greater reduction in bacterial count in minimal broth, because there were only 5.65 log CFU/ml SE86 after 120h. The growth of the bacteria may has been hindered by the fact that this medium does not have a carbon source; on the other hand, results in minimal media are important because, although such medium has no source of nutrients for microbial growth, the bacterium produces emulsifier, demonstrating that *S.* Enteritidis SE86 can grow in environments with minimal nutritional conditions.

The growth on minimal medium and the production of emulsifier only occurred when the initial concentration of *S.* Enteritidis SE86 in the growth curve was 4,5 logCFU/ml (data not shown). These results suggest that nutritional deficiencies interfere in the minimal medium growth of SE86 and consequently the production of emulsifier once during 120 h. Maximum growth was assessed 8

logCFU/ml, while bacterial growth reached a maximum quantity of 9.9 log CFU/ml in BHI broth.

These results (concentration of cells X emulsifier producing) can be explained by Ron and Rosenberg (2001) that reported the production of emulsifier occurs, especially when the cell concentrations are high. This finding may be fortuitous or may reflect an indirect correlation with one or more physiological factors, such as the availability of energy, nitrogen or oxygen. However, it is possible that the production of biosurfactant at high bacterial density has a selective advantage, because of emulsifiers produced by pathogens; it has been suggested that being virulence factors, they are produced when the cell density is high enough to cause a localized attack on the host.

Growth in the minimal medium can be explained by the notion of McMeechan et al. (2005) in which state the accumulated glycogen in the cell can provide a constant supply of carbon for metabolism and biosynthesis during periods when growth rates decreased considerably and the replenishment of glycogen and carbon sources may be intermittent. These researchers conclude that glycogen has a role in the growth of bacteria in the genus *Salmonella* in extra intestinal environment, such as water, minimal medium and faeces; therefore, it was found that even under minimum nutritional conditions, *Salmonella* strains maintained their survival by activating genes (*glgC*) responsible for the biosynthesis of glycogen and therefore the supply of carbon.

Still et al. (2012), argue that the lack of carbon source can significantly affect the growth of microorganisms, results observed in our study, when we compared the kinetics of *S.* Enteritidis SE86 growth in BHI and minimal media. According to these researchers, in carbon-free environments, the genus *Salmonella* responds to different types of physiological responses to maintain their survival, noting that this mechanism may be responsible for maintaining the survival of this bacterium in different environments with nutrient limitation.

The results for growth on minimal medium for *S.* Enteritidis SE86 may also suggest that washing the cell during inoculum preparation is insufficient to eliminate the carbon sources of the cell surface, since *S.* Enteritidis SE86 was inoculated for 24 hours in BHI broth to activate the metabolism of the bacterial cells.

Another very important factor which enhances the possibility of developing an *S*. Enteritidis SE86 mechanism to grow in medium without carbon is that although the average initial microbial count in minimal medium of 4.5 logCFU/ml biomass (dry cell weight) was only 0.32 g/l, this was the most likely factor that demonstrates that this is not the carbon source used by bacteria to grow on the medium. Also, the emulsifier production only occurred when the amount of *S*. Enteritidis SE86 cells in media (BHI broth and minimal medium) was above 7.7 log CFU/ml (Figure 3).

Several studies have shown that the production of emulsifier by microorganisms only happens when cell concentrations are high, suggesting that

this mechanism may be related to quorum-sensing (Ron and Rosenberg, 2001; Mellor et al., 2011; Hamme et al., 2006).

These results (production of emulsifier in BHI broth and minimal medium) suggest that the emulsifier production by this microorganism may occur in other environments where the nutritional conditions allow good microbial growth, such as in foods.

The emulsifier production was also observed in minimal acidified (pH 5.0) and alkaline (pH 8.0 to 9.0) medium. In pH 5.0, emulsifier production was observed after 24 h, even if the amount of *S.* Enteritidis SE86 in the medium was 8.27 logCFU/ml; however, after 48 and 72 hours, when the pH rose to 6.0, the production of emulsifier was 43.30% and 39.53%, respectively (Figure 4).

In minimal medium at pH 9.0 to 8.0, emulsifier production was observed after 24 h, yielding 33% and 36.36%, respectively. The largest amount of emulsifier was observed after 72 h at pH 8.0, where 7.5 logCFU/ml of *S*. Enteritidis SE86 production was 50.5% (Figure 4).

When the bacteria were exposed to minimal medium supplemented with 2% glucose, no emulsifier was produced at any of the times tested (24, 48 and 72 h), but a marked reduction of pH (7.0 to 4.0) was observed at 24 hours (Figure 5). The pH reduction of the medium can be explained by the ability of *S*. Enteritidis SE86 to ferment carbohydrates such as glucose present in the medium with the consequent production of acids (Mirmomeni et al., 2009).

Several studies (Youssef et al., 2004; Hamme et al. 2006; Nitschke and Costa, 2007; Mellor et al., 2011; Ismail et al., 2012; Jain et al., 2013) have demonstrated the production of biosurfactant by various microorganisms, such as bacteria of the genus *Bacillus, Pseudomonas*, *Acinetobacter, Serratia, Nocardia, Artrobacter, Corynebacterium, Halomonas*, *Sphingomonas, Flavobacterium, Klebsiella, Rhodococcus* and *Candida y*easts.

Our study is the first to demonstrate the production of emulsifier by *Salmonella* sp., since there is currently no scientific registry of the production of emulsifiers by this microorganism.

In addition, the kinetics of microbial growth and emulsifier production by *S.* Enteritidis SE86 in different growth conditions showed that this organism has the ability to increase the pH of the medium (acidification - observed in pH 5.0 medium and BHI) and then begin the production of emulsification, since higher levels of emulsification were observed at pH 7.0, 8.0 and 9.0.

It was also found that when the pH was strongly acidic (pH 4.0-4.5 growth observed with glucose 2%), the bacteria could not make the medium sufficiently alkaline to produce emulsifier, although the number of existing cells in the medium (8.41 log CFU/ml) was favourable for the production of emulsifier.

According to Benedict et al. (2008), the biosurfactants can be quantified by surface and interfacial tension measurements, providing a common tool to monitor changes in the media for microbial growth.

The measurement of the surface tension of distilled water was 69 mN/m, and the minimal medium was 62 mN/m. The surface tension values found in our work were 55.8 mN/m and 57.4 mN/m in the supernatant of *S*. Enteritidis SE86 incubated at 36±⁰C and room temperature (25°C) after 48 hours, respectively. In BHI broth the surface tension found were 45.5 mN/m after 48 hours of *S*. Enteritidis SE86 incubated at 36±⁰C.

Thus, it was observed that there was a decrease in surface tension of 6.2 mN/m to SE86 *Salmonella* supernatant incubated at 36 ± 1°C and 4.6 mN/m to one incubated at room temperature (25°C) for 24 hours. Thus, it is noted that the emulsifier produced by *S*. Enteritidis SE86 was able to reduce the surface tension of the culture medium and that these results indicate biosurfactant production, since they are below the values set by Youssef et al. (2004), where surface tension values above 60mN/m suggest the inability of microorganisms to produce biosurfactants.

According to Nitschke and Costa (2007), a good surfactant can reduce the surface tension of water from 72 to 35 mN/m. Several studies (Youssef et al., 2004; Mellor et al., 2011; Darvishi et al., 2011; Aparna et al., 2012; Ismail et al., 2013) have demonstrated that the biosurfactant production is related to the decrease in surface tension means and that the values are most often associated with the type of biosurfactant.

The rhamnolipids lipopeptide usually decreases the surface tension to values close to 30 mN/m (Benedict et al., 2008; Aparna et al., 2012; Ismail et al.,

2013), while the high mass molecular biosurfactants (Emulsan, Alasan and Liposan) have polysaccharides that are effective emulsifiers without promoting, however, sharp reductions in surface tension values (Rosenberg and Ron, 1999; Benedict et al., 2008). According to these authors, several species of bacteria produce extracellular polymeric surfactants, consisting of polysaccharides, proteins, lipopolysaccharides, lipoproteins, or a complex of these biopolymers.

The results also showed that the emulsifier was negative for the drop in breakdown test, i.e., there was no collapse formation between the culture medium containing *S.* Enteritidis SE86 and mineral oil. According to Tugrul and Cansunar (2005), this method has often been used to screen for microorganisms to verify the capacity to produce biosurfactants, although Youssef et al. (2004) stated that the drop in collapse is an easy and sensitive method for detecting the presence of biosurfactant, but is unable to detect low levels of surfactant production (50-63 mg/l).

Similar to our results, Youssef et al. (2004) showed that 7.8% of 205 environmental isolates tested were negative for the collapse in drop test, but were able to produce biosurfactant.

For the inclined glass slide test, the droplet containing 0.9% sodium chloride and *S.* Enteritidis SE86 was capable of moving the blade, which indicates a positive test. Satpute et al. (2010) stated that this test is also suitable for screening for strains producing biosurfactants, as isolated *Pseudomonas* strains producing environment surfactants were positive for this test.

Cultivation of *S.* Enteritidis SE86 showed that the microorganism is not haemolytic and is negative for the CTAB test with methylene blue. Diverse studies such as Satpute et al. (2010), Youseff et al. (2004) and Zhang et al. (2012) used this test to correlate haemolysis on blood agar with biosurfactant production. However, Benedict et al. (2008) stated that this test, when performed with the emulsifier, may indicate the production of rhamnolipids. Considering our findings, we propose that *S*. Enteritidis SE86 was not able to produce rhamnolipids, since such compounds typically decrease the surface tension below 30 mN/m (Benedict et al., 2008; Araujo et al., 2011). Corroborating that, *S*. Enteritidis SE86 was negative in the test with methylene blue, indicating that the emulsifier was not a rhamnolipid, since this test relies on the formation of an ion pair insoluble anionic surfactants with CTAB (cetyltrimethylammonium bromide) cationic surfactant and basic dye methylene blue, and are detected when rhamnolipids form dark blue halos around the colonies (Heyd et al., 2008). Cetyltrimethylammonium bromide cationic surfactant and basic dye methylene blue, and are detected when rhamnolipids formed dark blue halos around the colonies (Heyd et al., 2008).

The characterisation of emulsifier using the ninhydrin reaction tests and the phenol sulphuric acid reaction showed that the emulsifier produced by *S.* Enteritidis SE86 presented free amines and sugars in its constitution since both were positive. The biosurfactant showed 1.39 g/dl total protein in constitution and 1.8 mg/ml glucose.

The emulsion stability tests showed that the emulsifier produced by *S.* Enteritidis SE86 is resistant to high temperatures (30-100°C), pH (3.0 to 10.0) and different concentrations of NaCl (2-10%). In these tests, the high temperatures (80-100°C) and salt concentrations (7-10%), as well as a reduced emulsification rate, were observed (Table 1).

Furthermore, it was observed that the emulsifier produced with *S.* Enteritidis SE86 cells from the cultivation for 48 h in minimal medium (pH 7.0), was stable at 36 ± 1°C for 30 days, whereas the emulsion derived from the supernatant produced under the same conditions was stable for only 48 hours. The stability of the emulsion found for different temperatures, salt concentration and acidity and alkalinity conditions have been reported elsewhere (Ayed et al., 2014; Barros et al., 2008) and assume importance in this work, as these conditions are often used in food preparation as barriers to control microbial growth, which suggests that these bacteria have the ability to produce emulsifier in food, meaning that such compounds may not be destroyed and will remain in the food.

In tests with proteolytic and lipolytic enzymes, it was observed that in the presence of proteinase K (proteolytic enzymes) the production of emulsifier did not occur. In the presence of lipase observed a high production emulsifier (57.50% IE24) and more solid visual appearance compared to the emulsions that are usually produced.

The ionic load test showed that the biosurfactant produced by *S*. Enteritidis SE86 was neutral because it was not precipitated in the presence of SDS (anionic character) and barium chloride (cationic character).

Thus, the findings of this study suggest that *S.* Enteritidis SE86 is a producer of a polymeric biosurfactant because these polymers may exhibit large chains of carbohydrates or protein and may have a lipid fraction (Benedict et al., 2008). Rosenberg and Ron (1999) demonstrated that these compounds exhibit high emulsifying activity and, in most cases, do not significantly reduce surface tension. Similar results were found in the present study.

4. Conclusion

These results support the conclusion that *S*. Enteritidis SE86 has the ability to produce emulsifier in BHI broth and minimal medium with features that suggest a polymeric compound.

Furthermore, it can be concluded that the pathogen to produce emulsifier alkalised the medium and that this substance is produced only when the cell concentration is high (over 7.7 log CFU/ml). However, when produced, the biosurfactant is resistant to different temperatures, pH and NaCl concentrations, which may be considered an important feature, as *S*. Enteritidis SE86 is a food pathogen and factors such as pH, temperature and osmotic pressure are used to control the growth of microorganisms in food.

The conditions in which *S*. Enteritidis SE86 produced emulsifier and emulsifying characteristics (resistance to different conditions of pH, temperature and salinity) may suggest that this pathogen uses this substance to keep their survival or attack the host, given that the biosurfactant is produced only at high cell concentrations. On the other hand, further studies are needed to determine the importance of the production of that substance for microorganisms of the genus *Salmonella*.

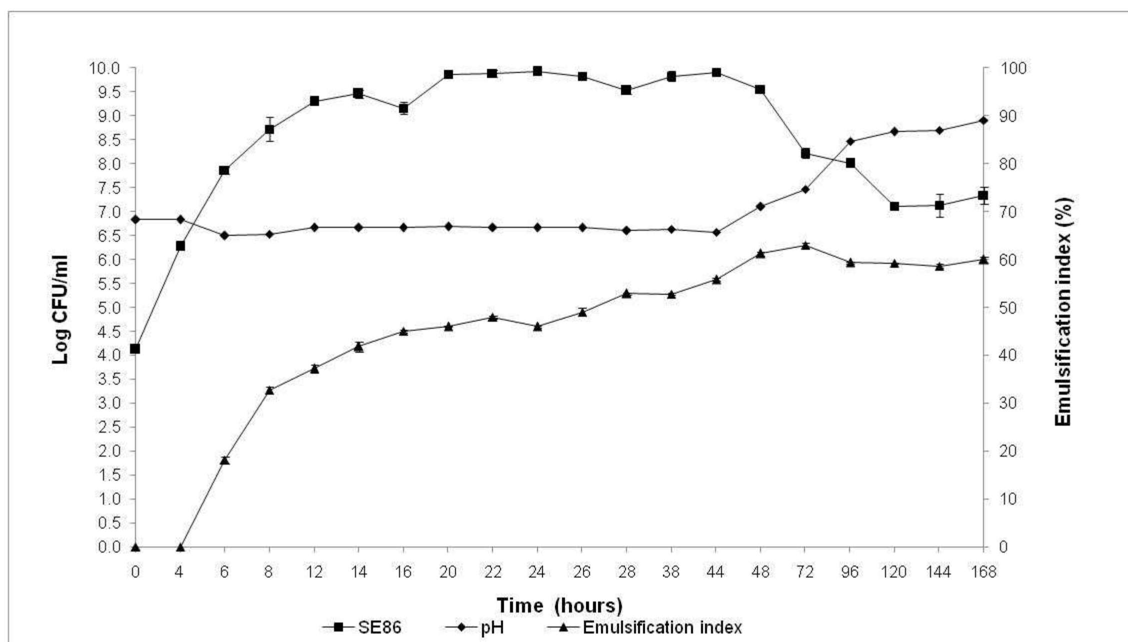

Figure 1: Growth, pH and emulsification index of *Salmonella* Enteritidis SE86 cultivated in BHI broth (initial pH 7.0) for 168 hours at 36 ± 1°C.

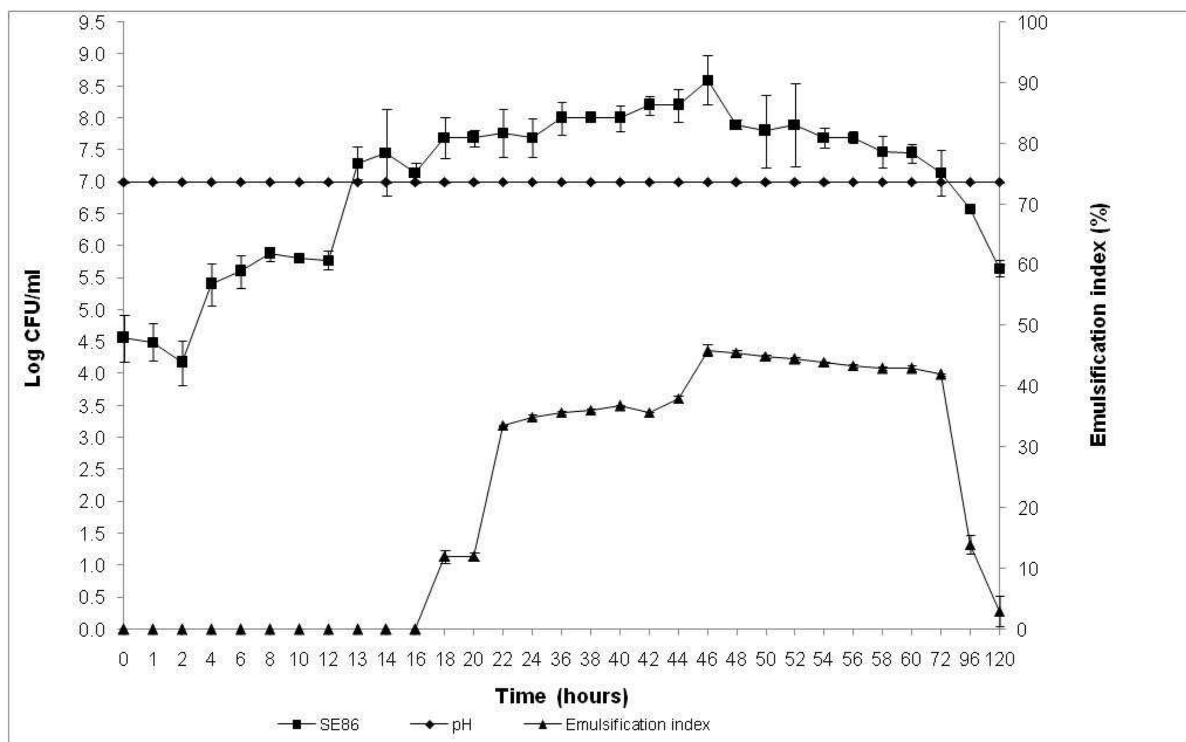

Figure 2: Growth, pH and emulsification index of *Salmonella* Enteritidis SE86 cultivated in minimal medium (initial pH 7.0) for 120 hours at 36 ± 1°C.

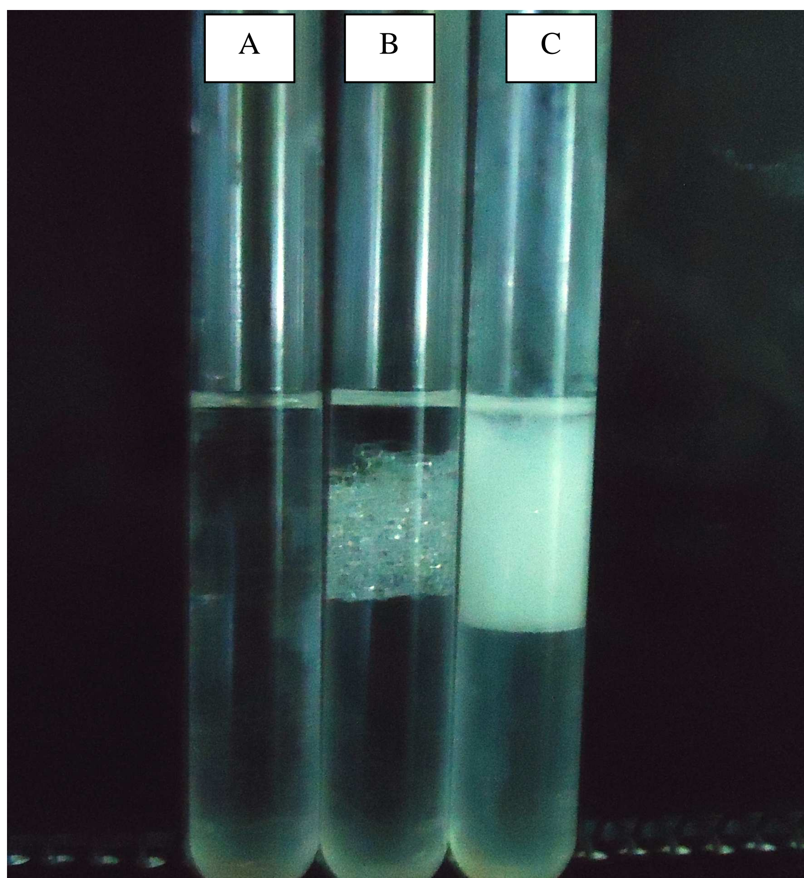

Figure 3: Emulsification index in 24 hours (IE24h) with different concentrations of *Salmonella* Enteritidis SE86 cells. A) 6.5 log CFU/ml B) 7.7 log CFU/ml and C) 8.4 log CFU/ml.

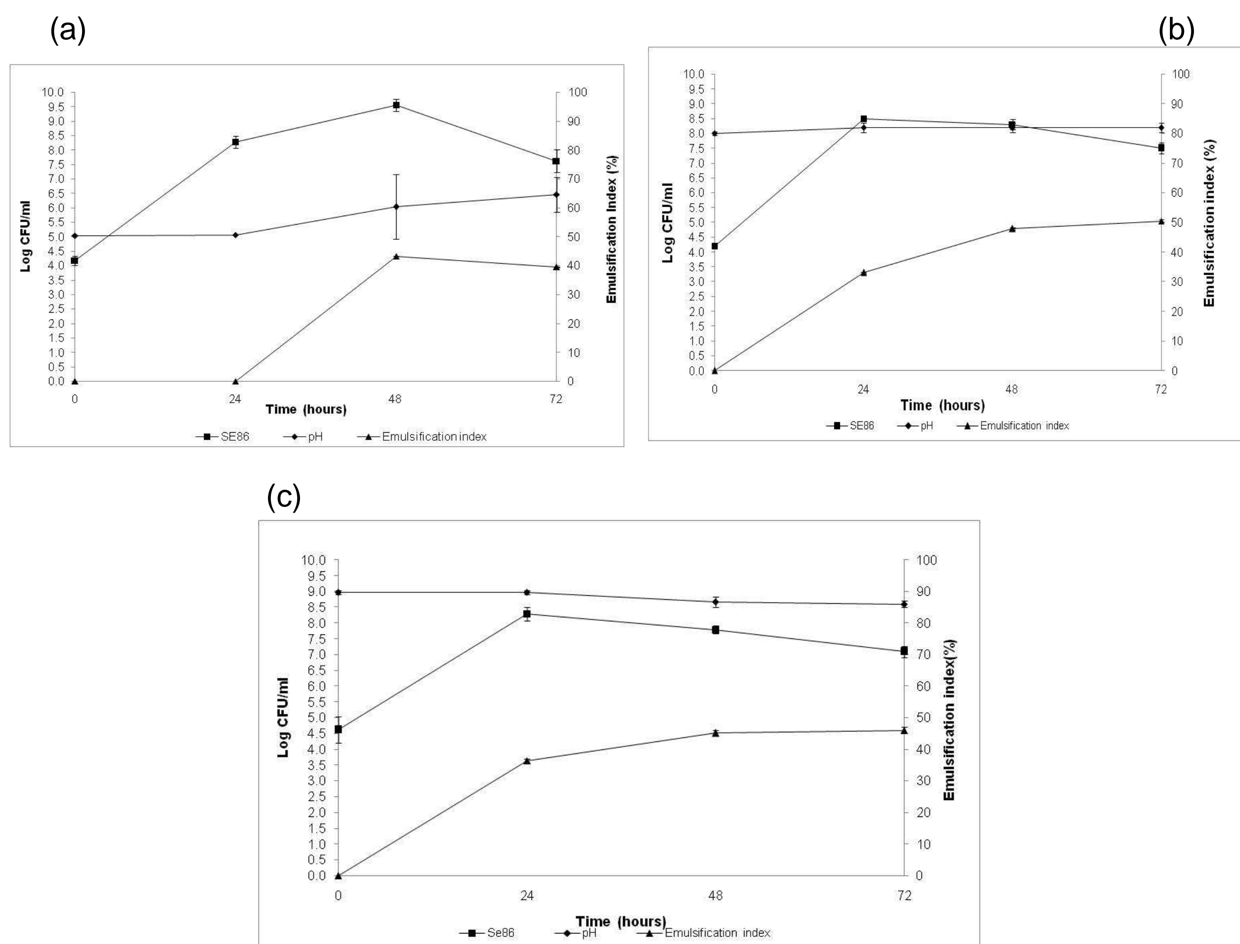

Figure 4: Growth, pH and emulsification index of *Salmonella* Enteritidis SE86 cultivated in minimal medium at 36 ± 1°C for 72 h at different pH: A - acidified with acetic acid (initial pH 5.0); B - alkalised with sodium hydroxide solution (initial pH 8.0); C - alkalised with sodium hydroxide solution (initial pH 9.0).

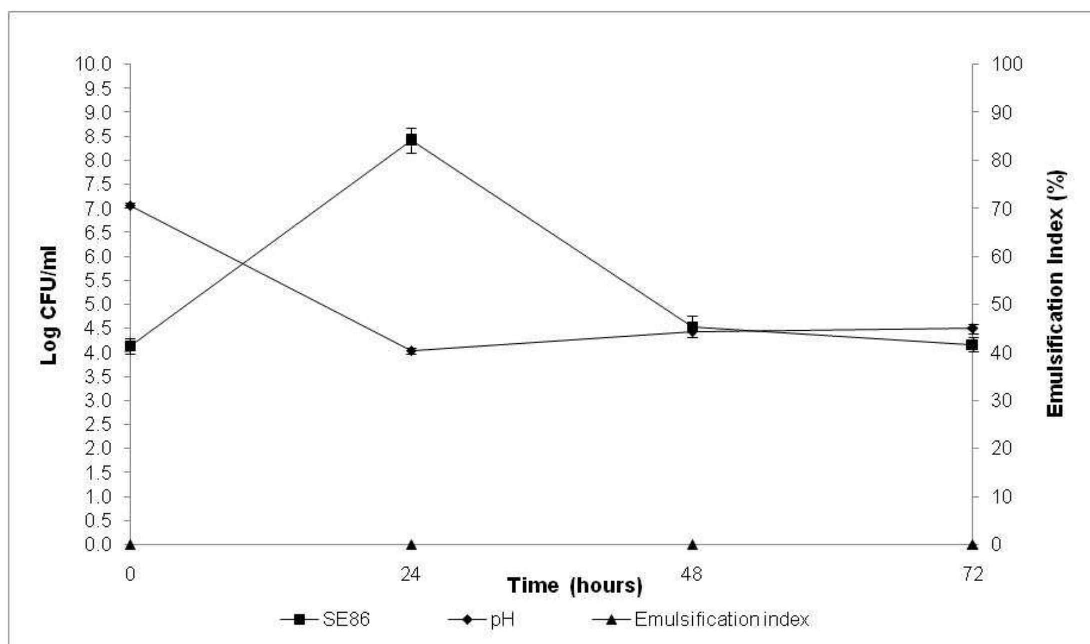

Figure 5: Growth, pH and emulsification index of *Salmonella* Enteritidis SE86 cultivated in minimal medium with 2% glucose (initial pH 7.0) for 72 hours at 36 ± 1°C.

Table 1: Mean and standard deviation of the effect of concentration of sodium chloride (NaCl), pH and temperature (Temp) on the emulsification index (IE24) of the supernatant of culture of *Salmonella* Enteritidis SE86.

Salinity		**Acidity and alkalinity**			
NaCl	**IE24 (%)**	**pH**	**IE24 (%)**	**Temp. (ºC)**	**IE24 (%)**
2%	35.9 ± 0.9	3	32.4 ± 0.6	30	40.6 ± 0.5
3%	36.1 ± 0.3	4	39.4 ± 1.3	40	35.1 ± 0.7
4%	36.4 ± 0.4	5	39.9 ± 0.3	50	33.4 ± 0.5
5%	32.7 ± 0.3	6	38.4 ± 0.7	60	32.1 ± 0.1
6%	30.4 ± 0.6	7	37.2 ± 2.3	70	32.3 ± 0.3
7%	22.0 ± 0.2	8	35.6 ± 0.4	80	32.0 ± 0.1
8%	18.0 ± 0.2	9	28.6 ± 0.6	90	30.3 ± 0.2
9%	6.7 ± 1.5	10	17.2 ± 2.0	100	30.2 ± 0.5
10%	3.5 ± 0.6	-	-	-	-

References

Aparna, A., Srinikethan, G., Smitha, H. 2012. Production and characterisation of biosurfactant produced by a novel *Pseudomonas* sp. 2B. Colloids Surf., B**.** 95, 23-29.

Araujo, L. V., Abreu, F., Lins, U., Anna, L. M. M. S. Nitschke, M. Freire, D.M.G. 2011. Rhamnolipid and a surfactin inhibit *Listeria monocytogenes* adhesion. Food Res. Int. 44, 481-488.

Ayed, H.B. , Himet, N., Béchet, M., Chollet, M., Chataigné, G., Leclére, V., Jacques, P., Nasri, M. 2014. Identification and biochemical characteristics of lipopeptides from *Bacillus mojavensis* A21. Process Biochem. 49, 1699–1707.

Bento, F. M., Camargo, F. A. O., Gaylarde, C. C. Biossurfactantes. In: De Melo, I. S., De Azevedo, J. L. [Org.] 2008. Microbiologia Ambiental. second edition . Jaguariúna, Embrapa Meio Ambiente,. Cap.7. pp. 152-184.

Bodour, A. A., Miller-Maier, R. M. 1998. Application of a modified drop collapse technique for surfactant quantitation and screening of bosurfactant-procucing microorganisms. J Microbiol Meth**.** 32, 273-300.

Bodour, A. A., Maier, R. M. 2002. Biosurfactants: Types, Screening Methods, and Applications. Encyclopedia of Environmental Microbiology, Wiley, NY pp. 750–770.

Capalonga, R., Ramos, R. C., Both, J. M. C., Soeiro, M. L. T., Longaray S. M., Haas, S., Tondo, E. C. 2014. *Salmonella* serotypes, resistance patterns, and food vehicles of salmonellosis in southern Brazil between 2007 and 2012. J. Infect. Dev. Ctries. 8, 811-817.

Cooper, D. G., Goldenberg, A. B. G. 1987. Surface-active agents from two *Bacilllus* species. Appl. Environ. Microbiol**.** 53, 224-229**.**

Darvishi, P., Ayatollahi, S., Mowla, D., Niazi, A. 2011. Biosurfactant production under extreme environmental conditions by an efficient microbial consortium, ERCPPI-2. Colloids Surf., B. 84, 292-300.

De Paula, C. M. D., Geimba, M. P., Do Amaral, P. H., Tondo, E. C. 2010. Antimicrobial resistance and PCR-ribotyping of *Shigella* responsible for foodborne outbreaks occurred in southern Brazil. Braz. J. Microbiol. 41, 966–977.

Geimba, M. P. Tondo E. C., de Oliveira F.A., Canal C.W., Brandelli A. 2004. Serological Characterisation and prevalence of spvR genes in *Salmonella* isolated from foods involved in outbreaks in Brazil. J. Food. Prot. 67, 1229-1233.

Gudiña, E. J., Pereira, J. F. B., Costa, R., Coutinho, J. A. P., Teixeira, J. A., Rodrigues, L. R. 2013. Biosurfactant-producing and oil-degreasing *Bacillus subtilis* strains enhance oil recovery in laboratory sand-packed columns. J. Hazard. Mater., 216. 106-113.

Heyd, M., Kohnert, A., Tan, T. H., Nusser, M., Kirschhöfer, F., Brenner-Weiss, G., Franzreb, M., Berensmeier, S. 2008. Development and trends of biosurfactant analysis and purification using rhamnolipids as an example. Anal. Bioanal. Chem.391, 1579-1590.

Hamme, J. D. V., Singh, A., Ward, O. P. 2006. Physiological aspects: Part 1 in a series of papers devoted to surfactants in microbiology and biotechnology. Biotechnol. Adv. 24, 604–620.

Ismail, W., Al-Rowaihi, I. S., Al-Human, A., Hamza, R. Y., Nayal, A. M. E., Bououdina, M. 2013. Characterisation of a lipopeptide biosurfactant produced by a crude-oil-emulsifying *Bacillus* sp. I-15. Int. Biodeterior. Biodegrad. 84, 168-178.

Jain, R. M., Mody, K., Joshi, N., Mishra, A., Jha, B. 2013. Production and structural characterisation of biosurfactant produced by an alkaphilic bacterium, *Klebsiella* sp.: Evaluation of different carbon sources. Colloids Surf., B**.** 108, 199-204.

Machado, T. R. M., Malheiros, P. S., Brandelli, A., Tondo, E. C. 2010. Avaliação da resistência de *Salmonella* à ação de desinfetantes ácido peracético, quaternário de amônio e hipoclorito de sódio. Rev. Inst. Adolfo Lutz. 69, 475-481.

Machado, T. R. M. 2007. Avaliação da aderência ao aço inoxidável e ao polietileno por três sorovares de *Salmonella* e da capacidade de desinfecção dessas superfícies. Dissertação (Mestrado em Microbiologia Agrícola e do Ambiente)- Universidade Federal do Rio Grande do Sul- UFRGS, Porto Alegre, 143 f.

Malheiros, P. S., De Paula, C. M. D, Tondo, E. C. 2007. Cinética de crescimento de *Salmonella* Enteritidis envolvida em surtos alimentares no RS: uma comparação com linhagens de outros sorovares. Ciênc. Tecnol. Aliment. [online]. 27, 751-755.

Mcmeechan, A., Lovell, M. A., Cogan, T. A., Marston, K. L., Humphrey, T. J., Barrow, P. A. 2005. Glycogen production by different *Salmonella enterica*

serotypes: contribution of functional *glgC* to virulence, intestinal colonisation and environmental survival. Microbiology. 151, 3969–3977.
Mcentire, J. 2013. Foodborne Disease: The Global Movement of Food and People. Infect. Dis. Clin. N. Am. 27, 687-693.

Mellor, G. E., Bentley, J. A., Dykes, G. A. 2011. Evidence for a role of biosurfactants produced by *Pseudomonas fluorescens* in the spoilage of fresh aerobically store chicken meat. Food Microbiol. 28, 1101-1104.

Merchant, R., Banat, I. M. 2012. Biosurfactants: a sustainable replacement for chemical surfactants? Biotechnol. Lett. 34, 1597–1605.

Mirmomeni, M.H., Naderi, A., Hosseinzadeh, C., Sisakhtnezhad, S. 2009. Isolation of *Salmonella* Enteritidis using biochemical tests and diagnostic potential os *Sdfl* amplified gene. Res. J. Biol. Sci. 4, 656-661.

Monteiro, A. S., Bonfim, M.R.Q., Domingues, V.S., Côrrea Jr, A., Siqueira, E. P., Zani, C.L., Santos, V. L. 2010. Identification and characterisation of bioemulsifier-producing yeasts isolated from effluents of a dairy industry. Bioresour. Technol. 101, 5186-5193.

Mürmann, L., Dos Santos, M. C., Longaray, S.M., Both, J.M.C., Cardoso, M. 2008. Quantification and molecular characterization of *Salmonella* isolated from food samples involved in salmonellosis outbreaks in Rio Grande do Sul, Brazil. Braz. J. Microbiol. 39, 529-534.

Nitschke, M., Costa, S. G. V. A. O. 2007. Biosurfactants in food industry. Trends Food Sci. Technol. 18, 252-259.

Nitschke, M., Pastore, G. M. 2002. Biossurfactantes: propriedades e aplicações. Quim. Nova [online]. 25, 772-776.

Oliveira, D. W. F., França, I.W.L., Félix, A.K.N., Martins, J.J.L., Giro, M. E.A., Melo, V.M.M., Gonçalves, L.R.B. 2013. Kinetic study of biosurfactant production by *Bacillus subtilis* LAMI005 grown in clarified cashew apple juice. Colloids Surf., B. 101, 34-43.

Oliveira, F. A., Frazzon, A.P., Brandelli, A., Tondo, E.C. 2007. Use of PCR-ribotyping, RAPD, and antimicrobial resistance for typing of *Salmonella* Enteritidis involved in food-borne outbreaks in Southern Brazil. J. Infect. Dev. Countries. 1, 170-176.

Oliveira, F. A., et al. Geimba, M. P. Pasqualotto, A.P., Brandelli, A., Pasquali, G., Da Silva, W.P., Tondo, E.C. 2009. Clonal relationship among S*almonella enterica* serovar Enteritidis involved in foodborne outbreaks in Southern Brazil. Food Control. 20, 606–610.

Oliveira, F. A., Pasqualotto, A. P., Padilha, W., Tondo, E. C. 2012. Characterization of *Salmonella* Enteritidis isolated from human samples. Food Res. Int. 45, 1000-1003.

Olveira, C., Goldberg, J.B., Sánchez, R., Soberón-Chávez, G. 1999. The *Pseudomonas aeruginosa algC* gene product participates in rhamnolipid biosynthesis. FEMS Microbiol Lett.. 179, 85–90.

Patil, J. R., Chopade, B. A. 2001. Studies on bioemulsifier production by *Acinetobacter* strains isolated from healthy human skin. J. Appl. Microbiol. 91, 290-298.

Perez, P. K. Ceccon, R.V. Malheiros, P.S., Jong, E.V., Tondo, E.C. 2010. Influence of acid adaptation on the survival of S*almonella* Enteritidis and S*almonella* Typhimurium in simulated gastric fluid and in intestine infection. J. Food Saf. 30, 398-414.

Pires, S. M. Vieira, A.R., Hald, T., Cole, D. 2014. Source Attribution of Human Salmonellosis: An Overview of Methods and Estimates. Foodborne Pathog. Dis. 9, 667-676.

Ron, E., Z., Rosenberg, E. 2001. Natural roles of biosurfactants. Environ. Microbiol. 3, 229-236.

RITTER, A.C. et al. Bacciu, D., Santi, L., Silva, W.O., Vaistein, M.H., Rubino, S. Uzzau, S., Tondo, E. C. 2012. Investigation of *rpoS* and *dps* Genes in Sodium Hypochlorite Resistance of *Salmonella* Enteritidis SE86 Isolated from Foodborne Illness Outbreaks in Southern Brazil. J. Food Prot., 75, 437-442.

Rufino, R. D., Luna, J., M., Sarubbo, L. A. 2011. Antimicrobial and anti-adhesive potential of a biosurfactant Rufisan produced by *Candida lipolytica* UCP 0988. Colloids Surf., B. 84, 1-5.

Rufino, R. D., De Luna, J. M. C., Takaki, G. M.C., Sarubbo, L.A. 2014. Characterisation and properties of the biosurfactant produced by *Candida lipolytica* UCP 0988. Electron J. Biotechno.11, 34-38.

Satpute, S.K., Banpurkar, A.G., Dhakephalkar, P.K., Banat, I.M., Chopade, B.A. 2010. Methods for investigating biosurfactants and bioemulsifiers: a review. Crit Rev Biotechnol. 30, 127-144.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Widdowson, M.A., Roy, S.L., Griffin, P.M. 2011. Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis.17, 7-15.

Secretaria da vigilância em Saúde (SVS) **Análise Epidemiológica dos Surtos de Doenças Transmitidas por Alimentos no Brasil, 2000 – 2014**. Disponível em http://www.anrbrasil.org.br/new/pdfs/2014/3_PAINEL_1_ApresentacaoRejaneAlvesVigilanciaEpidemiologica-VE-DTA-Agosto_2014_PDF.pdf Acesso em 10 de agosto de 2014.

Singh, P., Cameotra, S.S. 2004. Potential applications of microbial surfactants in biomedical sciences. Trends Biotechnol. 22, 142-146.

Singh, D.N., Tripathi, A.K. 2013. Coal induced production of a rhamnolipid biosurfactant by *Pseudomonas stutzeri*, isolated from the formation water of Jharia coalbed. Bioresour. Technol. 128, 215-221.

Spector, M. P., Kenyon, W. J. 2012. Resistance and survival strategies of *Salmonella* enterica to environmental stresses. Food Res. Int. 45, 455–481.

Sriran, M. I., Kalishwaral, K., Deepak, V., Gracerosepat, R., Srisakthi, K., Gurunathan. 2011. Biofilm inhibition and antimicrobial action of lipopeptide biosurfactant produced by heavy metal tolerant strain *Bacillus* cereus nk1. Colloids Surf., B**.** 85, 174-181.

Trugul, T., Cansunar, E. 2005. Detecting surfactant-producing microorganisms by the drop collapse test. World J. Microb. Biot. 21, 851-853.

Yin, H., Qiang, J., Jia, Y., Ye, J. Peng, J. Qin, H. Zhang, N. He, B. 2009. Characteristics of biosurfactant produced by *Pseudomonas aeruginosa* S6 isolated from oil-containing wastewater. Process Biochem. 44, 302-308.

Youssef, N. H., Duncan, K. E., Nagle, D. P., Savage, K. N., Knapp, R.M, Mcinerney, M. J. 2004. Comparison of methods to detect biosurfactant production by diverse microorganisms. J. Microbiol. Methods. 56, 339-347.

Zhang, X., Xu, D., Lundaa, T., Schererr, E. K. 2012.Isolation and identification of biosurfactant producing and crude oil degrading *Pseudomonas* aeruginosa strains. Chem. Eng. J. 28, 145-146.

3.2 Artigo 2

Biosurfactant produced by *Salmonella* Enteritidis SE86 can increase adherence and resistance to sanitizers on lettuce leaves (*Lactuca sativa* L., *cichoraceae*)

Eliandra Mirlei Rossi;[a,b]* Luniele Beilke;[a] Marília Kochhann;[a] Diana Helena Sarzi;[a] Eduardo César Tondo[b]

[a] Laboratório de Microbiologia, Departamento de Ciências Biológicas e da Saúde, Universidade do Oeste de Santa Catarina (UNOESC), Campus de São Miguel do Oeste-SC, Brazil.

[b] Laboratório de Microbiologia e Controle de Alimentos, Instituto de Ciência e Tecnologia de Alimentos, Universidade Federal do Rio Grande do Sul (ICTA/UFRGS), Av. Bento Gonçalves 9500, Prédio 43212, Campus do Vale, Agronomia, Porto Alegre/RS, Brazil
*Corresponding author:
Tel.: 055 51 3308 6677
E-mail address: eliandra_bio@yahoo.com.br

Abstract: *Salmonella* Enteritidis SE86 is an important foodborne pathogen in Southern Brazil and it is able to produce a biosurfactant. However, the importance of this compound for the microorganism is still unknown. This study aimed to investigate the influence of the biosurfactant produced by *S*. Enteritidis SE86 on adherence to slices of lettuce leaves and on resistance to sanitizers. First, lettuce leaves were inoculated with *S*. Enteritidis SE86 in order to determine the amount of biosurfactant produced. Subsequently, lettuce leaves were inoculated with *S*. Enteritidis SE86 with and without the biosurfactant, and the adherence and bacterial resistance to different sanitization methods were evaluated. *S*. Enteritidis SE86 produced biosurfactant after 16 hours (emulsification index of 11 to 52.15 percent, $P < 0.05$) and showed greater adherence capability and resistance to sanitization methods when the compound was present. The scanning electron microscopy demonstrated that *S*. Enteritidis was able to adhere, form lumps, and invade the lettuce leaves' stomata in the presence of the biosurfactant. Results indicated that the biosurfactant produced by *S*. Enteritidis SE86 contributed to

adherence and increased resistance to sanitizers when the microorganism was present on lettuce leaves.

Keywords: Lettuce, microbial adherence and resistance, *Salmonella* Enteritidis SE86, biosurfactant, disinfection

1. INTRODUCTION

Salmonella Enteritidis SE86 is a recognized food pathogen responsible for several foodborne disease (FBD) outbreaks in Southern Brazil (Geimba et al., 2004; Oliveira et al., 2009; Tondo and Ritter, 2012; Capalonga et al., 2014; Tondo et al., 2015). Several studies have been carried out taking into account the importance of this pathogen with the aim of understanding the reasons that it continues to be an important foodborne pathogen in this region since 1999 (Geimba et al., 2004; Capalonga et al., 2014; Tondo et al., 2015). Among all the characteristics that may contribute to that, we may highlight its great acid adaptation capability when the pathogen is exposed to acidic environments and, as a consequence, an increase in virulence (Perez et al., 2012) and ability to survive in simulated gastric fluid, (pH 1.5; 2), besides its resistance to sodium hypochlorite at 200 and 400 ppm (Machado et al., 2010). A previous study (Machado, 2007) demonstrated that *S.* Enteritidis SE86 was able to produce expressive amounts of biosurfactant during its growth in brain heart infusion broth (BHI). Nevertheless, the characteristics and functions of this compound have not been studied yet.

The term biosurfactant is described as a "surface active agent" produced by microorganisms (Marchant and Banat, 2012). These are amphiphilic compounds used as detergents or wetting, emulsifying, dispersing and foaming agents in many industrial formulations (Nitschke and Pastore, 2002). Even though they have been highly used, the physiological function of biosurfactants for microbial cells is still not completely understood, and the way these compounds rule food microorganisms is practically unknown. One of the few studies concerning biosurfactants in foods was published by Mellor et al. (2011), who reported that a biosurfactant produced by *Pseudomonas fluorescens* was able to alter the characteristics of chilled chicken meat (increased decomposition) and the compound facilitated the survival of the bacterium.

The production of biosurfactants is usually associated with the presence of large amounts of microorganisms (Ron and Rosenberg, 2001) and this factor can contribute to increased pathogenicity. Also, several researchers have reported that biosurfactants can contribute to the adherence of pathogens to surfaces and the formation of biofilms (Ron and Rosenberg, 1999; Ron and Rosenberg, 2001; Nitschke and Pastore, 2002). Furthermore, the ability of microorganisms to produce biosurfactants can also be linked with their resistance to sanitizers, because generally they present organic compounds that can protect bacterial cells.

Recently, it was stated that bacteria such as *S*. Enteritidis have a natural tendency to stick to surfaces, which includes lettuce leaves (Lima et al., 2013).

Lettuce (*Lactuca sativa* L., *cichoraceae*) is the most consumed green leaf in the world; this is a plant of easy acquisition, standing out due to its nutritional quality and because it is considered a low cost leafy vegetable (Abreu et al., 2010; Lima et al., 2013). During their growing cycle, lettuces can be contaminated by *Salmonella* and, as a consequence, several cases of salmonellosis have been related to the consumption of lettuces (Horby et al., 2003; Sagoo et al., 2003; Takkinen et al., 2005; Nygard et al., 2008; Irvine et al., 2009). In order to avoid contamination, lettuce leaves must be washed and sanitized before going to the table. However, if a biosurfactant is produced by *Salmonella*, microbial cells can easily adhere to the leaves and be protected against inactivation.

The aim of this study was to investigate the influence of the biosurfactant on adherence and resistance of *S*. Enteritidis SE86 to sanitizers on lettuce leaves.

2. MATERIALS AND METHODS

2.1. Lettuce samples

All lettuces used in this study were purchased in a supermarket in Porto Alegre, Capital of Rio Grande do Sul, Southern state of Brazil. Before the experiments started, lettuces were transported to the laboratory, inside thermal boxes, at 4°C for a maximum period of one hour. Before experiments, injured leaves were removed and the remaining ones were washed with potable water. Whole lettuce leaves were used for the experiments on resistance to sanitizers described in section 2.7. Slices of lettuce leaves with sizes of 10x10cm were used

for the experiments of adherence, according to Sagong et al. (2011). This was done in order to express results as number of CFU/cm^2. All sliced leaves were cut similarly, aiming to avoid interference in the results.

Before experiments, whole lettuce leaves and sliced leaves were washed and sanitized with potable water with 200 ppm sodium hypochlorite added, for 15 minutes (Antunes, 2009). After that, leaves were rinsed with sterile distilled water with 0.5% sodium thiosulfate added (Synth, Diadema-SP).

2.2. Microorganism

In this study, we used the *S.* Enteritidis SE86 strain, which was isolated from a cabbage involved with a salmonellosis outbreak in the State of Rio Grande do Sul, Brazil, in 1999. This strain was characterized by Geimba et al. (2004) and presents the same profile and genotypic characteristics of *S.* Enteritidis responsible for several cases of salmonellosis that occurred from 1999 to 2012 in Rio Grande do Sul (Capalonga et al., 2014; Tondo et al., 2015). For the tests, the strain was cultivated in brain heart infusion broth (BHI, Oxoid, Basingstoke, England) at 36±1°C, for approximately 18 hours.

2.3. Biosurfactant production on lettuce leaves

Four whole lettuce leaves were submerged in 100ml of minimal medium containing 4.4 log CFU/ml of *S.* Enteritidis SE86 and incubated at 36±1°C for 120 hours.

Aliquots of 6ml were removed every two hours for up to 60 and, after each time period, aliquots were withdrawn every 24 hours for up to 120 hours of culture in order to determine the emulsification index (IE24), pH, and bacterial count. Bacterial counts were performed in triplicate by seeding the samples onto plates containing xylose lysine deoxycholate agar (XLD: Merck, Darmstadt, Germany) and incubated at 36±1°C for 24 hours. The pH was evaluated by aliquots (10ml) of the samples and then analyzed with a pH meter (PHTECK). The emulsification index (IE24) was assessed using the method described by Cooper and Goldenberg (1987).

All experiments were repeated three times and the averages were subsequently expressed as the final result.

2.4. Preparation of the inoculum of *S.* Enteritidis SE86 with and without biosurfactant

The inoculum of *S.* Enteritidis SE86 without biosurfactant was prepared using 40ml of brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) incubated at 36±1°C for 72 hours. After incubation, the culture was centrifuged at 3500rpm for 15 minutes and washed with phosphate buffered saline (PBS) three times. Then, the washed cells were inoculated in 100ml minimal medium until they reached a concentration of approximately 8.0 log CFU/ml.

In order to prepare the inoculum of *S.* Enteritidis SE86 with biosurfactant, the compound was partially purified, according to the following procedures. The

biosurfactant recovery was prepared by centrifuging at 3500rpm for 15 minutes inoculum *S.* Enteritidis SE86 in BHI broth incubated at 36±1°C for 72 hours. The supernatant was homogenized with ethanol (-4°C) at 95% concentration 4:1 and stored at 4°C for 24 hours. Subsequently, the precipitate (biosurfactant) was recovered by centrifugation at 3500rpm for 15 minutes and the supernatant was discarded. After the alcohol had evaporated completely, the pellet was resuspended in sterile distilled water and dialyzed. The dialysis was done using a membrane tube (SIGMA) submitted to constant agitation in distilled water for 24 hours (Kumar et al., 2004; Ciapina, 2008; Pacheco et al., 2010). 100ml of solution with partially purified biosurfactant and 8.0 log CFU/ml of *S.* Enteritidis SE86 were used.

2.5 Resistance to sanitizers of *S.* Enteritidis SE86 with and without biosurfactant *in vitro*

The susceptibility of *S.* Enteritidis SE86 with and without biosurfactant to sanitizers *in vitro* was evaluated using sodium hypochlorite (50 and 200ppm) and vinegar (2% and 20%). The test was performed according to the methodology recommended by Ordinance 101/93 published by the Brazilian Ministry of Agriculture and Food Supply (Brasil, 1993).

Initially, the concentrations of sodium hypochlorite (Q. Boa®) and vinegar (fermented acetic acid from red wine and alcohol—koller®) were prepared in sterile distilled water. Nine mL of each sanitizer were aseptically placed into sterile vials,

to which was added one ml of bovine serum albumin solution (1%). After that, 0.1 ml inoculum (*S.* Enteritidis SE86 with and without the biosurfactant) was added separately to each tube containing sanitizers and the exposure time was measured. After 5, 10, 15, 20, and 30 minutes of exposure, an aliquot of 0.01 ml of suspension was transferred into tubes containing BHI broth. The tubes were incubated for 96 hours at 36±1°C, and the bacterial growth was checked every 24 hours. In the case of bacterial growth, the test was considered positive (resistant). The negative confirmation of results (tubes without growth) was performed through inoculation on trypticase soy agar (TSA agar, Merck, Darmstadt, Germany) incubated at 36±1 ° C for 24 hours.

Each experiment was performed in triplicate on different days.

2.6. Adherence of *S.* Enteritidis SE86 to slices of lettuce leaves (*Lactuca sativa L., cichoraceae*)

Adherence of *S.* Enteritidis SE86 to slices of lettuce leaves was assessed using the methods proposed by Lima et al. (2013) with the following adaptation: the slices of lettuce leaves were cleaned, as described in section 2.1.

Before each treatment, three slices of lettuce leaf were immersed in 100ml of minimal medium containing *S.* Enteritidis SE86 at a concentration of approximately 8.0 log CFU/ml, with and without the biosurfactant, for 15, 30, and 60 minutes at room temperature (25°C). The preparation of the inoculum of *S.* Enteritidis SE86 with and without biosurfactant is described in section 2.4. After

that, slices of lettuce leaf were submerged in 100ml of PBS and immediately sonicated for five minutes, using ultrasonic equipment (LF Equipamentos, Anhangaba SP) with intensity of 40kHz. Sonication was used in order to remove adhered cells following the methods described by Sinde and Carballo (2000). This method was used because it does not damage cells and is considered very efficient in removing bacteria from biomaterials, especially from rough or irregular surfaces (An and Skowronski, 2000).

The counting of *S*. Enteritidis SE86 was performed on XLD agar incubated at 36±1°C for 24 hours. Counts were done in triplicate and each experiment was repeated five times.

2.7. Influence of biosurfactant on the efficiency of sanitation methods used for disinfection of whole lettuce leaves contaminated with *S.* Enteritidis SE86

First of all, 250g of whole lettuce leaves were immersed into 500ml of the *S.* Enteritidis SE86 inoculum with and without biosurfactant (prepared as described in section 2.4) for 60 minutes.

Sanitation treatments were performed by immersing artificially contaminated lettuce leaves (25g) in 500ml of each treatment solution (i.e. potable water for 30 minutes; 50ppm and 200ppm sodium hypochlorite for 15 and 30 minutes; 2% and 20% vinegar aqueous solution for 15 minutes). At the end of the contact time, each treatment solution was drained off, and leaves were rinsed with 200ml of neutralizing buffer solution (0.5% thiosulfate sodium, Synth, Diadema SP)

for 30 seconds, as recommended by Abadias et al. (2008), and then rinsed with potable water.

The negative and positive controls were non-contaminated lettuce leaves and lettuce leaves artificially contaminated with *S*. Enteritidis SE86, respectively. Washing was carried out only with potable water in order to evaluate of bacterial removal.

After treatments, lettuce leaves (25g) were blended in a Stomacher bag containing 225mL 0.1% peptone water (Merck, Darmstadt, Germany) for 60 seconds. *S*. Enteritidis SE86 counting was carried out on XLD agar after incubation at 36±1°C for 24 hours. Typical colonies (black) were counted in triplicate and the identity of the microorganism confirmed by biochemical tests.

All treatments were performed ten times on different days and the measurement of free chlorine in solutions was done using a Spectroquant® Kit (Merck).

2.8. Scanning electron microscopy of *S.* Enteritidis SE86 on surface of lettuce leaves with and without biosurfactant

Lettuce slices (1cm x 1cm) were prepared using the central region of lettuce leaves (washed and disinfected as described in section 2.1).

Three artificially contaminated lettuce slices with and without biosurfactant were let for one hour at room temperature (25ºC). After that, leaves were gently washed twice using 0.1% peptone water and fixed with 3.0% glutaraldehyde and

0.05M phosphate buffer, pH 7.0, for one hour. The slices were washed four times (15 minutes each) with phosphate-buffered saline. After that, samples were dehydrated by increasing concentrations of ethanol solution (30%, 50%, 70%, 80%, 95%, and 100%), with 15 minutes of contact each, and finally acetone PA for 30 minutes. The slices were dried with CO_2 in a critical-point drier (CPD 030; Bal-Tec), coated with gold (BAL-TEC SCD 050), and taken for observation on a JSM 5800 scanning electron microscope (SEM). Three lettuce slices submerged only in sterile distilled water were used as negative controls.

2.9. Statistical analysis

The ANOVA test was applied (Assistat 7.7 Beta) with $P<0.05$, in order to assess significant differences in the adherence of *S*. Enteritidis SE86 and its resistance to washing and disinfecting methods on lettuce leaves.

3. RESULTS

3.1. Production of biosurfactant on lettuce leaves

The results showed that *S*. Enteritidis SE86 produced biosurfactant when in contact with the lettuce leaves for more than 16 hours, presenting an emulsification index (IE_{24}) of 11% when bacterial population reached 7.11 log CFU/ml (Figure 1). The greater emulsification index was 52.15%, after 120 hours of contact with lettuce leaves and when the population was 9.8 log CFU/ml and the pH remained at 7.0 during all experiments. During the preparation of the inoculum in minimal

medium, SE86 also produced emulsifier (EI24 46%); however, after 120 hours, the IE24 decreased to 3% (results not shown), probably because the energy sources were depleted.

3.2. *In vitro* resistance to sanitizers

The *in vitro* testing of the susceptibility to sanitizers revealed that the biosurfactant was responsible for increasing the survival of *S*. Enteritidis SE86 in 50ppm sodium hypochlorite and 2% and 20% vinegar solution. As an example, SE86 without biosurfactant was completely inactivated by 50ppm sodium hypochlorite in 15 minutes, while with biosurfactant, the microorganism survived for 30 minutes. Similarly, SE86 without surfactant was eliminated by 2% and 20% vinegar after 15 minutes and zero minutes of exposure, respectively. Nevertheless, the presence of surfactant made SE86 survive for 20 and 5 minutes, respectively (Table 1). The biosurfactant did not influence the survival of SE86 exposed to 200ppm sodium hypochlorite.

3.3 Influence of surfactant on the adherence of *S*. Enteritidis SE86 to slices of lettuce leaf

The results of this study showed that there were significant differences (*P*<0.05) in the adherence of *S*. Enteritidis SE86 to slices of lettuce leaf when biosurfactant was present (Table 2). The highest counts of adhered SE86 were observed after 60 minutes of contact with slices of lettuce leaf. At that time,

average counts of 7.3 log CFU/cm^2 and 4.1 log CFU/cm^2 were obtained on lettuce leaves with and without biosurfactant, respectively.

Scanning electron microscopy demonstrated that *S*. Enteritidis SE86 was able to adhere to the slices of lettuce leaf, forms lumps, and enter the stomata when the biosurfactant was present (Figure 2).

3.4 Resistance to sanitation methods of lettuce leaves

It was observed that all treatments reduced the amount of *S*. Enteritidis SE86 on lettuces, but lettuces contaminated with *S*. Enteritidis SE86 and with biosurfactant demonstrated higher numbers of survival cells (significant difference $P<0.05$) than lettuces contaminated with *S*. Enteritidis SE86 without the surfactant (Figure 3). Reductions in counts of *S*. Enteritidis SE86 with biosurfactant ranged from 1.0 to 2.8 log CFU/g, whereas the reductions of *S*. Enteritidis SE86 without biosurfactant ranged from 1.3 to 3.3 log CFU/g ($P<0.05$) (Table 3). It was observed that the most effective treatment of lettuce contaminated with *S*. Enteritidis SE86 and biosurfactant was washing it with potable water and submerging it in 200ppm of sodium hypochlorite for 15 minutes. This showed a reduction of 2.8 log CFU/g. However, when lettuce leaves were contaminated only with *S*. Enteritidis SE86, the most effective reduction (3.3 log CFU/g) was obtained by the treatment that washed leaves with potable water and sanitized them with 50ppm sodium hypochlorite for 30 minutes. This result showed that *S*. Enteritidis SE86 without biosurfactant was inactivated by lower concentrations of sodium hypochlorite.

S. Enteritidis SE86 with biosurfactant was more resistant on lettuce leaves than in *in vitro* tests (Table 1 and Figure 3).

4. DISCUSSION

Microorganisms develop survival abilities in different environments and biosurfactant production can be an advantage to survive in foods (Mellor et al., 2011). However, the exact physiological function of biosurfactants is not yet completely elucidated (Nitschke and Pastore, 2002; Hamme et al., 2006; Abdel-Mawgoud et al., 2009; Jirku et al., 2015).

Results of the present study demonstrated that *S*. Enteritidis SE86 was able to produce biosurfactant on lettuce leaves and this ability may have facilitated the access to cutin on lettuce leaves, one functional component of the cuticle deposited on the surfaces and within the epidermal walls of aerial parts of plants. Cutin is composed of three dimensional polyesters of long fatty acid chains (Bacic et al., 1988) and the amphipathic property of biosurfactant may facilitate access to nutrients present on lettuce leaves, supplying energy for bacterial growth.

Several research groups have reported that environmental microorganisms are able to produce biosurfactants (Chen et al., 2012; Jain et al., 2013; Ayed et al., 2014; Rosa et al., 2015; Maoa et al., 2015); however, there are no scientific reports showing the production of surfactants by foodborne pathogens. To our knowledge, the present study is the first that demonstrates biosurfactant production by *Salmonella*. Other reports have demonstrated the production of biosurfactants by

degradative microorganisms on foods. For example, according to Mellor et al. (2011), the biosurfactant produced by *Pseudomonas fluorescens* contributed to increasing the total bacterial count on chicken stored aerobically for three days, suggesting that the biosurfactant contributed to the bioavailability of nutrients for the bacteria. These researchers suggested that the biosurfactant becomes a competitive advantage for the microorganism to maintain their survival, thereby enhancing the decomposition of chicken meat. Shaheen et al. (2010) have reported that a type of biosurfactant called surfactin may have contributed to the formation of biofilms by *Bacillus cereus* inside milk tanks.

The ability of *Salmonella* to adhere to lettuce leaves was reported by several studies (Lima et al., 2013; Patel and Sharma, 2010; Wei et al., 2006; Kroupitski et al., 2011). Results similar to the ones obtained in this study were found by Kroupitski et al. (2011), who found 7.0 log CFU of *S.* Typhimurium on the central region of lettuce leaves. Also, Lima et al. (2013) demonstrated that the count of *S.* Enteritidis cells that adhered to lettuce leaves differed *($P< 0.05$)* between the hydroponic and conventional systems, reaching 5.2±0.56 and 4.6±0.26, respectively.

The influence of biosurfactants on bacterial adherence to surfaces has been quite well studied, and the results are variable. Hassan et al. (2003) stated that Tween 85 surfactant reduced the adherence of *Escherichia coli* O157:H7 to lettuce leaves. Other researchers (Sotirova and Vasileva-Tonkova, 2009) reported that *Pseudomonas aeruginosa* NBIMCC 1390 with rhamnolipid biosurfactant increased

cell hydrophobicity to 31% adherence and that these compounds caused changes in the bacterial cell surface.

The results of our study suggest that the biosurfactant contributed to increase the survival of *S.* Enteritidis SE86 on lettuce leaves. According to Wei et al. (2006), high surface adherence of bacterial populations is a competitive tool against other microorganisms.

The results of *in vitro* resistance to sanitizers and resistance to sanitation methods of lettuce leaves showed that *S.* Enteritidis SE86 with biosurfactant is more resistant to antimicrobial activity of the compounds tested.

The bactericidal action of sodium hypochlorite is the result of microbial cell oxidation, after contact of sanitizer and cells (Watters et al., 2002; Møretrø et al., 2012; Bermúdez-Aguirre and Barbosa-Cánovas, 2013). According to Møretrø et al. (2012), pH and the presence of organic matter can affect the antimicrobial action of sodium hypochlorite. In our study, it was observed that biosurfactant decreased the antimicrobial action of sodium hypochlorite, probably because this organic compound linked to the sanitizer or avoided the contact of cells with the sanitizer.

Some studies have shown that acetic acid (vinegar) can reduce the amount of bacteria on foods and surfaces, including whole lettuce leaves (Karapinar and Gonul, 1992; Oliveira et al., 2012). Our study showed that *S.* Enteritidis SE86 in the presence of biosurfactant was more resistant to both vinegar concentrations (solution 2% and 20%). The counts of *S.* Enteritidis SE86 with biosurfactant on whole lettuce leaves sanitized with vinegar solution showed a reduction of 1.8 and

1.9 log CFU/g, whereas the reductions of *S*. Enteritidis SE86 without biosurfactant were 2.5 and 2.6 log CFU/g (Table 3).

Vinegars are able to decrease the external and internal pH of cells, inactivating microbial enzymes, and damaging membrane function and metabolic activities such as the transport of nutrients (Chang and Fang, 2007; Olmez and Kretzschmar, 2009). The less effective antimicrobial action of vinegar solution on lettuce contaminated with *S*. Enteritidis SE86 in the presence of biosurfactant suggested that biosurfactant protected SE86 from contact with the vinegar solution, or this compound was able to neutralize pH action.

The greater resistance of *S*. Enteritidis SE86 in the presence of biosurfactant on lettuce leaves suggests that the biosurfactant production may be a mechanism used by the bacterium to maintain its survival in different environments.

Thus this study demonstrated that *S*. Enteritidis SE86 can use the biosurfactant to increase its adhesion to the surface of lettuce leaves, form lumps, and also to penetrate the stomata of lettuce leaves. These effects may influence the increase of resistance to vinegar and sodium hypochlorite during lettuce sanitization. Furthermore, the surfactant production by adhered cells may protect them, avoiding contact with sanitizers.

5. CONCLUSION

Based on the results found during this study, it can be concluded that high counts of *S.* Enteritidis SE86 were able to produce biosurfactant on lettuce leaves. The presence of biosurfactant S. Enteritidis SE86 increased the adherence to slices of lettuce leaf and decreased the antimicrobial action of sanitizers (vinegar and sodium hypochlorite) used to sanitize whole lettuce leaves. In addition, when SE86 was added with biosurfactant and was analyzed by scanning electron microscopy, lumps of cells were observed and the bacterium was able to enter the stomata. The same results were not observed in the absence of biosurfactant.

New studies are necessary to investigate other probable functions of biosurfactant produced by SE86. As a perspective of the present study, we suggest investigating the influence of this biosurfactant on the microbial ecology of lettuce leaves, and on the multiplication and survival of SE86 in other foods.

Acknowledgements

This research received support from Universidade do Oeste de Santa Catarina, São Miguel do Oeste, Santa Catarina, Brazil, Universidade Federal do Rio Grande do Sul, Brazil and Support Fund for Maintenance and Development of Higher Education (FUMDES) of Santa Catarina State, Brazil.

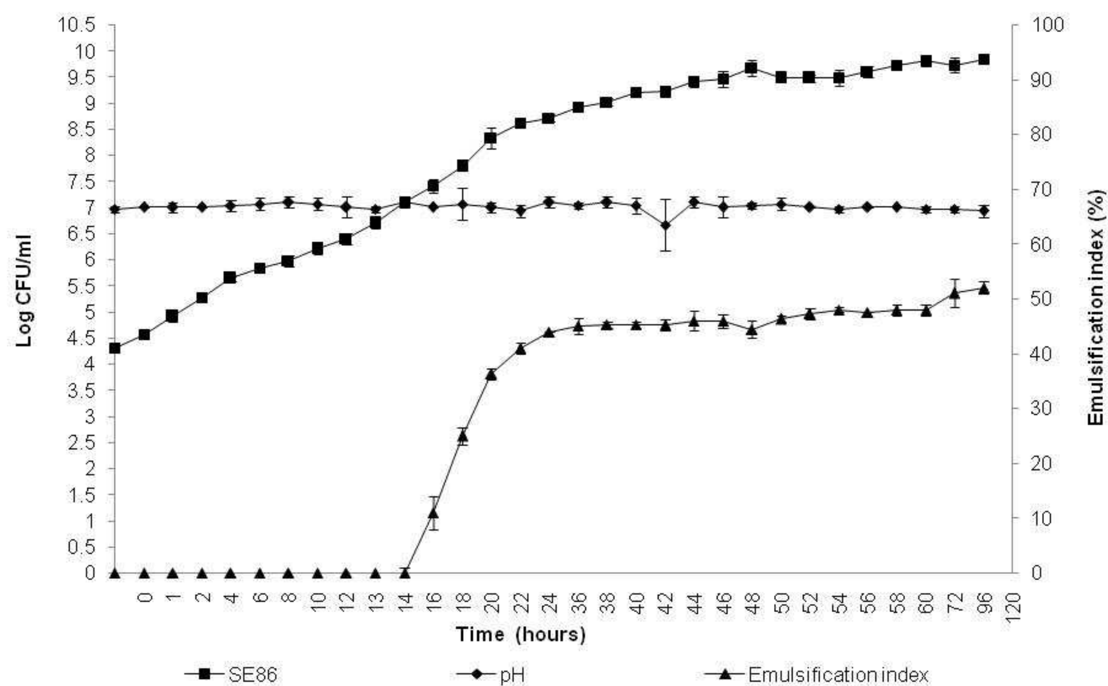

Figure 1: Growth, pH and emulsification index of *Salmonella* Enteritidis SE86 in minimal medium with whole lettuce leaves for 120 hours at 36±1°C.

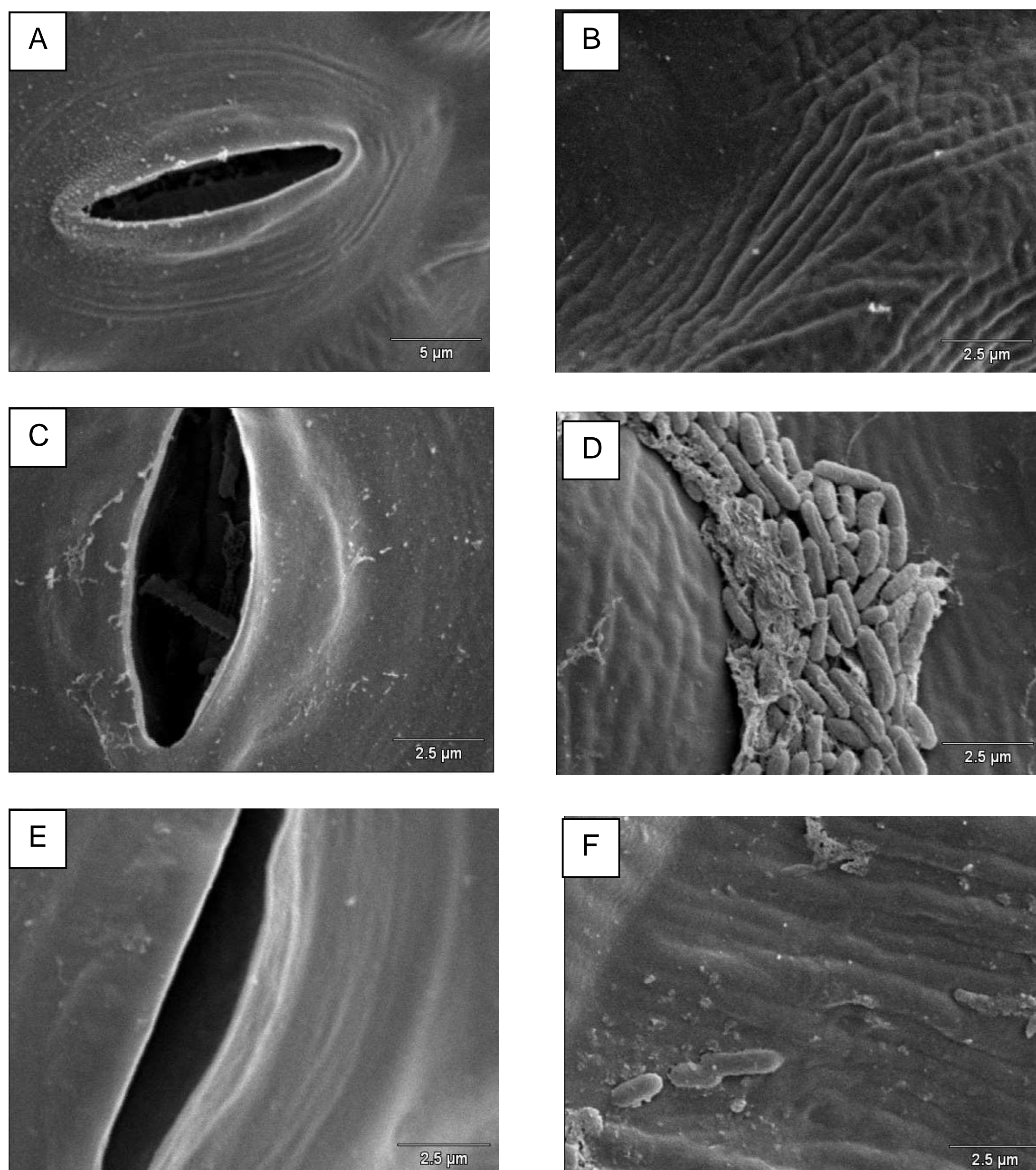

Figure 2: Scanning electron microscopy on lettuce leaf surface infected with *Salmonella* Enteritidis SE86 with and without biosurfactant.
Negative control: (A) stomata and (B) lettuce leaf surface.
S. Enteritidis SE86 with biosurfactant: (C) *S.* Enteritidis SE86 inside a stomata and (D) lumps of *S.* Enteritidis SE86 on lettuce leaf surface.

S. Enteritidis SE86 without biosurfactant: (E) stomata without *S*. Enteritidis SE86 and (F) lettuce leaf surface without formation lumps of *S*. Enteritidis SE86.

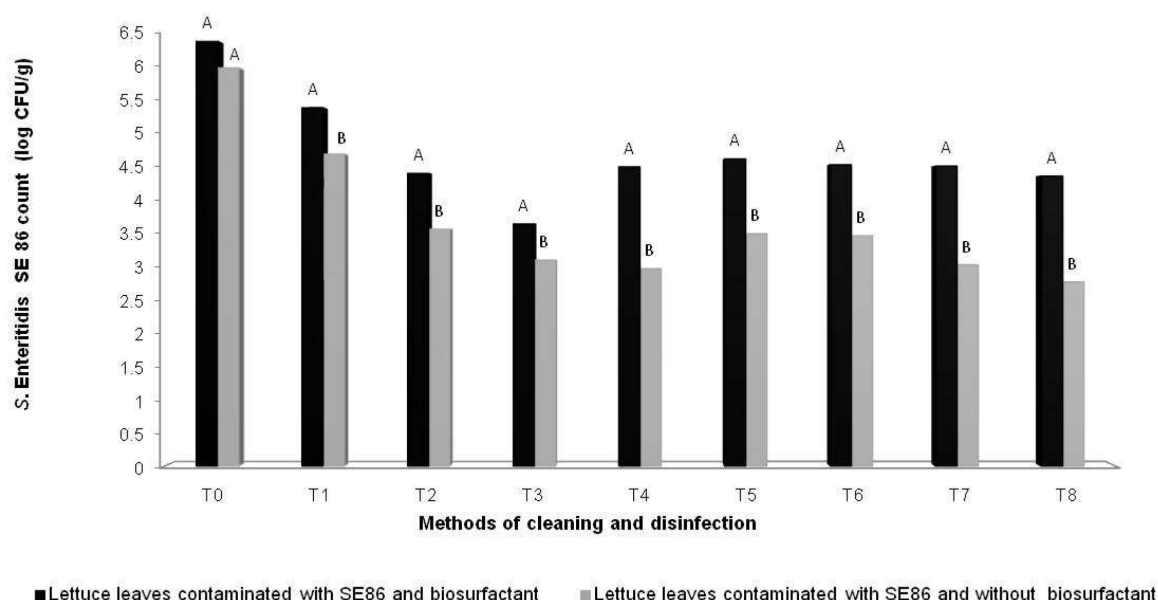

Figure 3: Mean (log CFU/g) of *S*. Enteritidis SE86 on whole lettuce leaves contaminated with *S*. Enteritidis SE86 and biosurfactant (Experiment 1) and *S*. Enteritidis SE86 without biosurfactant (Experiment 2).

* Statistical analysis between Experiments 1 and 2. Different letters indicate significant differences ($P<0.05$).

T0: Control positive: lettuce leaves contaminated with *S*. Enteritidis SE86; T1: washing lettuce with potable water; T2: immersion in potable water for 30 min; T3: immersion in 200ppm of sodium hypochlorite for 15 min; T4: immersion in 200ppm sodium hypochlorite for 30 min; T5: immersion in 2% vinegar solution for 15 min; T6: immersion in 20% vinegar solution for 15 min; T7: immersion in 50ppm of sodium hypochlorite for 15 min; T8: immersion in 50ppm of sodium hypochlorite for 30 min.

Table 1: *In vitro* susceptibility testing to disinfectants (200 and 50ppm sodium hypochlorite and 2% and 20% vinegar solution) of *S*. Enteritidis SE86 with and without biosurfactant.

Sanitizers	**Exposure time (minutes) of *S.* Enteritidis SE86 without biosurfactant**					**Exposure time (minutes) of *S.* Enteritidis SE86 with biosurfactant**				
	5	10	15	20	30	5	10	15	20	30
200 ppm sodium hypochlorite	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
50 ppm sodium hypochlorite	**R**	**R**	**R**	**S**	**S**	**R**	**R**	**R**	**R**	**R**
2 % vinegar solution	**R**	**R**	**R**	**S**	**S**	**R**	**R**	**R**	**R**	**S**
20% vinegar solution	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**S**	**S**	**S**
water	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**

R: resistant; S: sensitive

Table 2: Media and standard deviation about the adherence of *S*. Enteritidis SE86 with and without biosurfactant on slices of lettuce leaves at different times.

Time (minutes)	*S*. Enteritidis SE86 without biosurfactant (log CFU/cm^2)	*S*. Enteritidis SE86 with biosurfactant (log CFU/cm^2)
15	3.5±0.3 [c]	6.3± 0.2 [b]
30	3.5±0.3 [c]	6.0± 0.7 [b]
60	4.1±0.5 [c]	7.3± 0.3 [a]

Values represent the means of five replicates.
Different letters represent significant differences *($P<0.05$).*

Table 3: Reduction (log CFU/g) in counts of *S*. Enteritidis SE86 with and without biosurfactant on whole lettuce leaves after the treatments were performed.

Treatments	**Reduction in *S.* Enteritidis SE86 counts on lettuce (log CFU/g) with biosurfactant**	**Reductions in *S.* Enteritidis SE86 counts on lettuce (log CFU/g) without biosurfactant**
Washing with water	1.0 ^{b}	1.3 ^{b}
Water (30 min)*	2.0 ^{c}	2.5 ^{c}
200 ppm sodium hypochlorite (15 min)*	2.8 ^{d}	2.9 ^{cd}
200 ppm sodium hypochlorite (30 min)*	1.9 ^{c}	3.1 ^{cd}
2% vinegar solution (15 min)*	1.8 ^{c}	2.5 ^{cd}
20% vinegar solution (15 min)*	1.9 ^{c}	2.6 ^{cd}
50 ppm sodium hypochlorite (15 min)*	1.9 ^{c}	3.0 ^{cd}
50 ppm sodium hypochlorite (30 min)*	2.1 ^{c}	3.3 ^{d}

Log CFU/g: colony forming unit/g converted to log_{10}.
* The period of time the solutions spent submerged is shown in parenthesis next to each treatment used.
Statistical analysis between treatments. Different letters indicate statistically significant differences between treatments ($P<0.05$).

References

Abadias M., Ussal J., Oliveira M., Alegre I., and Vinãs I. (2008). Efficacy of neutral electrolyzed water (NEW) for reducing microbial contamination on minimally-processed vegetables. *International Journal of Food Microbiology*, 123, 151–158.

Abdel-Mawgoud A.M., Aboulwafa M.M., and Hassouna N.A.H. (2009). Characterization of rhamnolipid produced by *Pseudomonas aeruginosa* isolate Bs20. *Applied Biochemistry and Biotechnology*, 157, 329–345.

Abreu I.M.O., Junqueira A.M.R., Peixoto J.R., and Oliveira S.A. (2010). Qualidade microbiológica e produtividade de alface sob adubação química e orgânica. *Ciência e Tecnologia de Alimentos*, 30, 108–118.

An Y.H., and Skowronski P. (2000). “General considerations for studying bacterial adhesion to biomaterials,” in *Handbook of bacterial adhesion: Principles, methods and applications*, ed. H. H. An and R. J. Friedman (Springer Science and Business Media: LLC) 121–132.

Antunes M.A. (2009). Contaminação crescimento e inativação de microrganismos na cadeia de produção da alface (*Lactuca sativa*) Propriedade de Santo Antão, Tese, Universidade Federal de Viçosa, Minas Gerais.

Ayed H.B., Hmidet N., Béchet M., Chollet M., Chataigné G., Jacques P., and Nasri M. (2014). Identification and biochemical characteristics of lipopeptides from *Bacillus mojavensis* A21. *Process Biochemistry*, 10, 1699–1707.

Bacic A., Harris P.J., and Stone B.A. (1988). “Structure and function of plant cell walls,” in *The Biochemistry of Plants*, ed. J. Preiss, W. Stumpf, P. Michael Conn (Academic Press) 297–373.

Bermúdez-Aguirre D., and Barbosa-Cánovas G.V. (2013). Disinfection of selected vegetables under non thermal treatments: Chlorine, acid citric, ultraviolet light and ozone. *Food Control*, 29, 82–90.

Brasil. Ministério da Agricultura, Pecuária e Abastecimento. Portaria n. 101, de 17 de agosto de 1993. Métodos de Análise Microbiológica para Alimentos. Diário Oficial [da] República Federativa do Brasil. Brasília, DF, 17 ago. 1993, Seção I, pp. 11937–45.

Capalonga R., Ramos R.C., Both J.M.C., Soeiro M.L.T., Longaray S.M., Haas S., and Tondo, E.C. (2014). Characterization of *Salmonella* Enteritidis involved in

foodborne outbreaks in Southern Brazil from 2007 to 2013. *The Journal of Infection in Developing Countrie*s (submitted).

Chang J., and Fang T.J. (2007). Survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovars Typhimurium in iceberg lettuce and the antimicrobial effect of rice vinegar against E. coli O157:H7. *Food Microbiology*, 24, 745–751.

Chen J., Huang P.T., Zhang K.Y., and Ding F.R. (2012). Isolation of biosurfactant producers, optimization and properties of biosurfactant produced by *Acinetobacter* sp. from petroleum-contaminated soil. *Journal of Applied Microbiology*, 112 (4), 660–671.

Ciapina E.M.P. (2008). Produção de Biossurfactante por *Rhodococcus erythropolis* em Biorreator de Bancada e Avaliação do seu Efeito na Biodegradação de Borra Oleosa da Indústria do Petróleo/ Elisa Mara Prioli Ciapina – Rio de Janeiro.

Cooper D.G., and Goldenberg A.B.G. (1987). Surface-active agents from two *Bacillus* species. *Applied and Environmental Microbiology*, 53, 224–229.

Geimba M.P., Tondo E.C., de Oliveira F.A., Canal C.W., and Brandelli, A. (2004). Serological characterization and prevalence of spvR genes in *Salmonella* isolated from foods involved in outbreaks in Brazil. *Journal of Food Protection,* 67, 1229–1233.

Hamme J.D.V, Singh A., and Ward O.P. (2006). Physiological aspects: Part 1 in a series of papers devoted to surfactants in microbiology and biotechnology. *Biotechnology Advances*, 24, 604–620.

Hassan A.N., and Frank J.F. (2003). Influence of surfactant hydrophobicity on the detachment of *Escherichia coli* O157:H7 from lettuce. *International Journal of Food Microbiology,* 87, 145–152.

Horby P.W., O'Brien S.J., Adak G.K., Graham C., Hawker J.I., Hunter P., et al. (2003). A national outbreak of multi-resistant *Salmonella enterica* serovar Typhimurium definitive phage type (DT) 104 associated with consumption of lettuce. *Epidemiology and Infection,* 130, 169–178.

Irvine W.N., Gillespie I.A., Smyth F.B., Rooney P.J., McClenaghan A., Devine M.J., and Tohani V.K. (2009). Investigation of an outbreak of *Salmonella enterica* serovar Newport infection. *Epidemiology and Infection*, 137, 1449–1456.

Jain R.M., Mody K., Joshi N., Mishra A., and Jha B. (2013). Production and structural characterization of biosurfactant produced by an alkaphilic bacterium,

Klebsiella sp.: Evaluation of different carbon sources. *Colloids and Surfaces B: Biointerfaces*, 108, 199–204.

Jirků V., Čejková A., Schreiberová O., Ješdík R., and Masák J. (2015). Multicomponent biosurfactants: A “Green Toolbox” extension. *Biotechnology Advances*, 33, 1272-1276.

Karapinar M., and Gonul S.A. (1992). Effects of sodium bicarbonate, vinegar, acetic and citric acids on growth and survival of *Yersinia enterocolitica*. *International Journal of Food Microbiology*, 16, 343–347.

Kroupitski Y., Pinto R., Belausov E., and Sela S. (2011). Distribution of *Salmonella* typhimurium in romaine lettuce leaves. *Food Microbiology*, 28, 990–997.

Kumar C.G., Joo S.H., Choi J.W., Koo Y.M., and Chang C.S. (2004). Purification and characterization of an extracellular polysaccharide from haloakalophilic *Bacillus* sp. I-450. *Enzyme Microbial Technology*, 34, 673–681.

Lima P.M., São José J.P.M., Andrade N.J., Pires A.C., and Ferreira S.O. (2013). Interaction between natural microbiota and physicochemical characteristics of lettuce surfaces can influence the attachment of *Salmonella* Enteritidis. *Food Control*, 30, 157–161.

Machado T.R.M. (2007). Avaliação da aderência ao aço inoxidável e ao polietileno por três sorovares de *Salmonella* e da capacidade de desinfecção dessas superfícies. Dissertação (Mestrado em Microbiologia Agrícola e do Ambiente). Universidade Federal do Rio Grande do Sul (UFRGS): Porto Alegre, 143 f.

Machado T.R.M., Malheiros P.S., Brandelli A., and Tondo E.C. (2010). Avaliação da resistência de *Salmonella* à ação de desinfetantes ácido peracético, quaternário de amônio e hipoclorito de sódio. *Revista Instituto Adolfo Lutz*, 69, 475–481.

Maoa X., Jianga R., Xiaoa W., and Yu J. (2015). Use of surfactants for the remediation of contaminated soils: A review. *Journal of Hazardous Materials*, 285, 419–435.

Marchant R., and Banat I.M. (2012). Biosurfactants: A sustainable replacement for chemical surfactants? *Biotechnology Letters*, 34, 1597–1605.

Mellor G.E., Bentley J.A., and Dykes G.A. (2011). Evidence for a role of biosurfactants produced by *Pseudomonas fluorescens* in the spoilage of fresh aerobically stored chicken meat. *Food Microbiology*, 28, 1101–1104.

Møretrø H.E., Live L., Nesse, L.L., Vestby L.K., and Langsruda S. (2012). Control of *Salmonella* in food related environments by chemical disinfection. *Food Research International*, 45, 532–544.

Nitschke M., and Pastore G.M. (2002). Biossurfactantes: Propriedades e aplicações. *Química Nova*, 25, 772–776.

Nygard K., Lassen J., Vold L., Andersson Y., Fisher I., Lãfdahl S., Threlfall J., et al. (2008). Outbreak of *Salmonella* Thompson infections linked to imported rucola lettuce. *Foodborne Pathogens and Disease*, 5, 165–173.

Oliveira F.A., Geimba M.P., Pasqualotto A.P., Brandelli A., Pasquali G., Da Silva, W.P., and Tondo E.C. (2009). Clonal relationship among S*almonella enterica* serovar Enteritidis involved in foodborne outbreaks in Southern Brazil. *Food Control,* 20, 606–610.

Oliveira A.B.A., Ritter A.C., Tondo E.C., and Cardoso M. I. (2012). Comparison of different washing and disinfection protocols used by food services in Southern Brazil for lettuce (*Lactuca sativa*). *Food and Nutrition Sciences*, 3, 28–33.

Ölmez H., and Kretzschmar U. (2009). Potential alternative disinfection methods for organic fresh-cut industry for minimizing water consumption and environmental impact. *Food Science and Technology*, 42, 686–693.

Pacheco G.J., Ciapina E.M.P., Gomes E.B., and Pereira J.N. (2010). Biosurfactant production by *Rhodococcus erythropolis* and its application to oil removal. *Brazilian Journal of Microbiology*, 41, 685–693.

Patel J., and Sharma M. (2010). Differences in attachment of *Salmonella* enterica serovars to cabbage and lettuce leaves. *International Journal of Food Microbiology*, 139, 41–47.

Perez K. J., Martins F. S., Cara D.C.M., Nicoli J.R., and Tondo E.C. (2012). Evaluation of intestinal invasion in germ-free mice challenged with acid-adapted and nonacid-adapted *Salmonella* Enteritidis SE 86 an *Salmonella* Typhimurium ST99. *Journal of Food Safety.* 32, 108–114.

Ron E.Z., and Rosenberg E. (1999). High- and low-molecular-mass microbial surfactants *.Applied Microbiology Biotechnology*, 52, 154–162.

Ron E.Z., and Rosenberg E. (2001). Natural roles of biosurfactants. *Environmental Microbiology*, 3, 229–236.

Rosa C.F.C., Freire D.M.G., and Ferraz H.C. (2015). Biosurfactant microfoam: Application in the removal of pollutants from soil. *Journal of Environmental Chemical Engineering*, 3, 89–94.

Sagong H.G., Lee S.Y., Chang, P.S., Heu S., Ryu, S., Choi Y.J., and Kang D.H. (2011). Combined effect of ultrasound and organic acids to reduce *Escherichia coli* O157:H7, *Salmonella* Typhimurium and *Listeria monocytogenes* on organic fresh lettuce. *International Journal of Food Microbiology*, 145, 287–292.

Sagoo S.K., Little C.L., Ward L., Gillespie I.A., and Mitchell R.T. (2003). Microbiological study of ready-to-eat salad vegetables from retail establishments uncovers a national outbreak of salmonellosis. *Journal of Food Protection*, 66, 403–409.

Shaheen R., Svensson B., Andersson M.A., Cristiansson A., and Salkinoja-Salomen, M. (2010). Persistence strategies of *Bacillus cereus* spores isolated from dairy silo tanks. *Food Microbiology*, 27, 347–355.

Sinde E., and Carballo J. (2000). Attachment of *Salmonella* sp. and *Listeria monocytogenes* to stainless steel, rubber and polytetrafluorethylene: The influence of free energy and the effect of commercial sanitizers. *Food Microbiology*, 17, 439–447.

Sotirova A., and Vasileva-Tonkova E. (2009). Effects of rhamnolipid-biosurfactant on cell surface of *Pseudomonas aeruginosa*. *Microbiological Research*, 164, 297–303.

Takkinen J., Nakari U., Johansson T., Niskanen T., Siitonen A., and Kuusi M.A. (2005). Nationwide outbreak of multiresistant *Salmonella* Typhimurium var Copenhagen DT104B infection in Finland due to contaminated lettuce from Spain, May 2005. *Euro Surveillance,* 10.

Tondo E.C., and Ritter A.C. (2012). *Salmonella* and Salmonellosis in Southern Brazil: A review of last decade *Salmonella*: Classification, genetics and disease outbreaks. *Nova Science Publishers*, New York, USA.

Tondo E.C., Ritter A.C., and Casarin L.S. (2015). Involvement in foodborne outbreaks, risk factors and options to control *Salmonella* Enteritidis SE86: An important food pathogen in Southern Brazil. In: HACKETT C.B. *Salmonella* prevalence, risk factors and treatment options. *Nova Publishers,* 65–77.

Watters L.H., Chinnan M.S., Walker S.L., Doyle M.P. and Lin C.M. (2002). Consumer acceptance of fresh-cut iceberg lettuce treated with 2% hydrogen peroxide and mild heat. *Journal of Food Protection*, 65, 1221–1226.

Wei H., Wolf G., and Hammes W.P. (2006). Indigenous microorganisms from iceberg lettuce with adherence and antagonistic potential for use as protective culture. *Innovative Food Science and Emerging Technologies*, 7, 294–301.

4 DISCUSSÃO GERAL

Micro-organismos desenvolvem habilidades para sobreviver em diferentes ambientes e a produção de biossurfactantes pode ser uma vantagem desenvolvida para sobreviver em alimentos (Mellor et al., 2011). Apesar disso, a exata função fisiológica dos biossurfactantes ainda não é completamente elucidada (Nitschke & Pastore, 2002; Hamme et al. 2006; Abdel-Mawgoud et al., 2010. Jirků et al., 2015).

Os biossurfactantes constituem uma das principais classes dos surfactantes. Esses compostos são produzidos por vários micro-organismos, classificados de acordo com sua composição química (Luna et al., 2014). Nos últimos anos, os biossurfactantes estão sendo amplamente estudados, pois podem possuir diferentes aplicações (Youssef et al., 2004; Hamme et al., 2006; Nitschke & Costa, 2007; Mellor et al., 2011; Ismail et al., 2012; Chen et al., 2012Jain et al., 2013; Oliveira et al., 2013, Ayed et al. 2014;, Mao et al., 2015; Rosa et al., 2015). A maioria dos estudos geralmente envolve micro-organismos de origem ambiental, mas bactérias deteriorantes de alimentos com capacidade de produzir biossurfactante têm despertado interesse (Mellor et al., 2011).

Atualmente não há relatos científicos que discutam a produção de biossurfactantes por patógenos alimentares, o que destaca a importância desse trabalho, uma vez que os resultados demonstraram que *S.* Enteritidis SE86 foi capaz de produzir biossurfactante, tornando-se o primeiro relato cientifico que evidencia a caracterização do biossurfactante produzido por um patógeno alimentar.

Os resultados revelaram que a produção de biossurfactante ocorreu tanto em meio mínimo como em caldo BHI. No caldo BHI a produção iniciou após 6 horas de incubação (índice de emulsificação - IE_{24}: 18,1%) e o maior IE_{24} (62,25%) ocorreu após 72 h de incubação. Já no meio mínimo, a produção de biossurfactante foi detectada somente após 18 h de incubação com IE_{24} de 12%, sendo que o maior IE_{24} observado foi de 46%, somente após 46 h. Desse modo, observou-se que em ambos os meios houve produção de biossurfactante, mas em caldo BHI a produção foi mais rápida (após 6 h), pois o crescimento de *S.* Enteridis SE86 nesse meio foi mais rápido (7,78 Log UFC/mL). Logo, essa diferença no tempo de produção e quantidade de biossurfactante entre os dois meios de cultura (Caldo BHI e meio mínimo) pode ter ocorrido devido a velocidade do metabolismo microbiano ser maior no caldo BHI, tendo em vista que é um meio rico em nutrientes, diferente do meio mínimo que apresenta na sua composição somente cloreto de sódio, fosfato dissódico hidrogênio e fosfato de potássio dihidrogênio.

Neste trabalho verificou-se que o biossurfactante foi produzido somente quando a contagem de *S*. Enteritidis SE86 nos meios testados (caldo BHI e meio mínimo) foi acima de 7,7 Log UFC/mL. Vários pesquisadores têm demonstrado que a produção de biossurfactante por micro-organismos acontece somente quando as concentrações celulares são elevadas (Ron & Rosenberg, 2001; Hamme et al., 2006 ; Mellor et al., 2011)

No meio mínimo foi observado que somente houve multiplicação de *S*. Enteritidis SE86 e consequentemente produção do biossurfactante, quando a concentração inicial de *S.* Enteritidis SE86 na curva de crescimento foi de no mínimo 4,5 Log UFC/mL (dados não mostrados). Esses resultados sugerem que as deficiências nutricionais do meio mínimo interferem na multiplicação de *S*. Enteritidis SE86 e, consequentemente, na produção de biossurfactante.

A multiplicação da *S.* Enteritidis SE86 de 4,5 LogUFC/mL até 8,4 Log UFC/mL em meio mínimo pode ser explicada conforme o trabalho de McMeechan et al. (2005) no qual afirmam que o glicogênio acumulado na célula pode fornecer um constante fornecimento de carbono para o metabolismo bacteriano. Esses pesquisadores concluem que glicogênio tem um papel no crescimento das bactérias do gênero *Salmonella* no ambiente extra intestinal (como água, meio mínimo e fezes), pois em seu estudo verificaram que mesmo em condições nutricionais mínimas, cepas de *Salmonella* mantiveram sua sobrevivência ativando genes (*glgC*) responsáveis pela biossíntese de glicogênio e, consequentemente, pelo fornecimento do carbono.

Segundo Spector & Kenyon (2012), em ambientes sem carbono, bactérias do gênero *Salmonella* crescem em densidades menores, pois dependem de diferentes tipos de respostas fisiológicas para manter sua sobrevivência. Essas bactérias sofrem uma reprogramação celular gerando células menores, mais resistentes e fisiologicamente mais eficientes para: (a) sistema de aproveitamento de substratos, produção de enzimas para “canibalismo” de componentes celulares desnecessários, como por exemplo, RNA e proteínas a partir de ribossomos, bem como de lipídios e peptidioglicano da parede celular; (b) produção de enzimas que causam condensação do cromossomo para protegê-lo dos danos; (c) enzimas que modificam os componentes da membrana como os tipos e quantidades de ácidos graxos na membrana lipídica ou lipopolissacarídica (LPS) da membrana externa, e (d) produção de enzimas para evitar ou reparar danos celulares (Spector & Kenyon, 2012).

Outra condição observada para produção do biossurfactante foi o pH do meio. A bactéria somente produziu o composto quando o pH foi ≥6,0, embora os testes de estabilidade demonstraram que o biossurfactante produzido por *S.* Enteritidis SE86 mantém sua estabilidade em diferentes pH (3, 4, 5, 6, 7, 8, 9 e 10), elevadas temperaturas (30, 40, 50, 60, 70, 80, 90 e 100 ºC) e diferentes concentrações de NaCl (2, 3, 4, 5, 6, 7, 8, 9 e 10%). Essa estabilidade dos biossurfactantes já foi relatada em outras pesquisas (Ayed et al., 2014; Barros et al., 2008) e assumem importância nesse trabalho, visto que essas condições podem ser normalmente encontradas em alimentos, seja devido ao

processamento ou colocadas propositalmente como barreiras para o controle microbiano. Esses resultados sugerem que se a *S.* Enteritidis SE86 possui a capacidade de produzir biossurfactante nos alimentos, esses compostos podem permanecer nos alimentos processados, sem serem destruídos.

De acordo com Bento et al. (2008), os biossurfactantes podem ser quantificados através de medidas de tensão superficial e interfacial, constituindo uma ferramenta comum para monitorar as modificações do meio durante a multiplicação microbiana. Diversos pesquisadores (Youssef et al., 2004; Mellor et al., 2011; Darvishi et al., 2011; Aparna et al., 2012; Ismail et al., 2013) têm demonstrado que a produção de biossurfactante está relacionada com a diminuição da tensão superficial do meio e que esses valores estão associados, na maioria das vezes, com o tipo de biossurfactante. Neste trabalho, observou-se que após 48 horas *S.* Enteritidis SE86 incubada à 36 ± 1 °C nos meios de cultura testados (caldo BHI e meio mínimo) foi capaz de reduzir a tensão superficial em 55,8 mN/m em meio mínimo e 44,5 mN/m em caldo BHI. Os ramnolipídeos e os lipopeptídeos normalmente diminuem a tensão superficial para valores próximos de 30 mN/m (Bento et al., 2008; Aparna et al., 2012; Ismail et al., 2013), enquanto que os biossurfactantes com alta massa molecular (emulsan, alasan e liposan) do tipo polissacarídeos são emulsificantes eficazes (bioemulsificantes) sem promover, no entanto, reduções acentuadas nos valores de tensão superficial (Rosenberg & Ron, 1999; Bento et al., 2008).

As características do biossurfactante produzido por *S.*Enteritidis SE86 encontrados neste estudo (composto neutro, com baixa capacidade de reduzir a tensão superficial, negativo para o teste azul de metileno (CTAB), presença de proteínas e carboidratos na composição), sugerem que *S.* Enteritidis SE86 seja produtora de um biossurfactante polimérico, uma vez que esses compostos apresentam alta atividade emulsificante e, na maioria das vezes, não reduzem significativamente a tensão superficial, além de não emulsificar quando expostos ao aquecimento com proteinase (Rosenberg & Ron, 1999; Bento et al., 2008; Heyd et al., 2008; Araújo et al., 2011).

Além disso, verificou-se que essa bactéria também produziu biossurfactante em folhas de alface, com IE_{24} de 11%, após 16 h de contato, e, após 120 h, observou-se o maior IE_{24} (52,15%) e a maior contagem de *S.* Enteritidis SE86 (9,8 Log UFC/mL), bem como a decomposição completa das folhas de alface. As razões para a produção de biossurfactantes em alimentos ainda não são claramente explicadas. Alguns trabalhos (Shaheen et al., 2010; Peng et al., 2002; Mellor et al., 2011) discutem que é possível que esses compostos confiram vantagem competitiva aos micro-organismos para manter a sua sobrevivência nos alimentos. De acordo com Mellor et al. (2011) o biossufactante de *Pseudomonas fluorescens* (isolada de uma coxa de frango) na carne de frango refrigerada facilitou o acesso a fontes de carbono (gordura da carne), consequentemente, possibilitou o aumento da multiplicação dos microrganismos (*P. fluorescens* e contagem total de bactérias heterotróficas) e

contribui para o aumento na decomposição da carne de frango refrigerada. Esses pesquisadores (Mellor et al., 2011), além de terem sugerido algumas razões para os micro-organismos produzirem biossurfactante na carne de frango refrigerada, afirmam que são necessárias novas abordagens sobre o efeito da produção dos biossurfactantes nos alimentos.

Os resultados encontrados no presente estudo demonstraram que *S.* Enteritidis SE86 com biossurfactante aumentou a sua aderência nas folhas de alface em todos os tempos testados (15, 30 e 60 minutos). A maior quantidade de S. Enteritidis SE86 aderida (7,3 Log UFC/cm^2) foi observada após 60 minutos de contato nas folhas de alface com biossurfactante, enquanto que nas folhas de alface contaminadas somente com *S.* Enteritidis SE86 a quantidade de células aderidas foi de 4,1 Log UFC/cm^2.

A capacidade de micro-organismos do gênero *Salmonella d*e aderir nas folhas de alface foi relatada por diversos estudos (Lima et al., 2013; Patel & Sharma, 2010; Wei et al., 2006; Kroupitski et al., 2011), mas não há relatos científicos correlacionando com a presença de biossurfactantes e *Salmonella* sp. Sotirova et al. (2009) relataram que *Pseudomonas aeruginosa* NBIMCC 1390 (cepa ambiental) na presença de ramnolipídeo aumentou a hidrofobicidade da célula e, consequentemente, a sua aderência.

De acordo com Patel & Sharma (2010) e Yaron (2014) a capacidade de bactérias do gênero *Salmonella* se aderir em folhas de alface proporciona diversas vantagens para o micro-organismo como: facilitar a disponibilidade de nutrientes,

facilitar a formação de biofilmes e consequentemente dificultar a eliminação da bactéria da superfície desses vegetais folhosos.

Os testes *in vitro* de suscetibilidade ao hipoclorito de sódio e vinagre demonstraram que o biossurfactante aumentou a sobrevivência de *S.* Enteritidis SE86 ao hipoclorito de sódio 50 ppm e vinagre 2% e 20%. Observou-se que *S.* Enteritidis SE86 sem biossurfactante foi completamente inativada por hipoclorito de sódio 50 ppm, em 15 minutos, enquanto que com o biossurfactante o micro-organismo sobreviveu por 30 minutos. Em vinagre 2% e 20%, *S*. Enteritidis SE86 sem o biossurfactante foi eliminada em 15 e 0 minutos respectivamente, entretanto na presença do biossurfactante ela sobreviveu por 20 (vinagre 2%) e 5 minutos (vinagre 20%).

Diversos trabalhos têm demonstrado que o ácido acético é capaz de diminuir a quantidade de bactérias em alimentos e superfícies, incluindo os vegetais. (Karapinar; Gonul, 1992, Torriani, Orsi; Vescovo, 1997), pois possui como mecanismo de ação a diminuição do pH e consequente inativação de enzimas e funções na membrana (Chang; Fang, 2007; Olmez, Kretzschmar, 2009). Desse modo, sugere-se que o biossurfactante produzido por *S*. Enteritidis SE86 possa desempenhar a função de proteção da célula, impedindo o contato com o ácido.

Já a ação antimicrobiana do hipoclorito de sódio (agente bactericida amplamente utilizado na indústria e no ambiente doméstico) é resultante da oxidação das células microbianas provocando a morte do microrganismo, mas

diversos fatores como pH e presença de matéria orgânica podem afetar sua ação antimicrobiana (MØRETRØ et al., 2012). Assim, sugere-se que atividade antimicrobiana do hipoclorito de sódio nas folhas de alface com *S.* Enteritidis SE86 e biossurfactante pode ter sido afetada pelo biossurfactante por ser um composto orgânico.

Em folhas de alface, o presente trabalho demonstrou que todos os tratamentos utilizados para sanitização reduziram as contagens de *S.* Enteritidis SE86, mas quando as superfícies das folhas de alface foram contaminadas com *S.* Enteritidis SE86 e o biossurfactante, observou-se maior quantidade de bactérias sobreviventes (diferenças significativas $p<0,05$). As reduções na contagem de *S*. Enteritidis SE86 com o biossurfactante variaram entre 1,0 e 2,8 Log UFC/g, enquanto que as reduções de *S.* Enteritidis SE86 sem biossurfactante variaram entre 1,3 para 3,3 Log UFC/g.

As análises estatísticas demonstraram que houve diferenças significativas maiores entre as quantidades restantes de *S.* Enteritidis SE86 em todos tratamentos, nos quais as folhas de alface foram contaminadas com *S.* Enteritidis SE86 e o biossurfactante. Esses resultados indicam que o biossurfactante produzido por *S*. Enteritidis SE86 interferiu na ação antimicrobiana dos sanitizantes testados (hipoclorito de sódio e vinagre).

Este estudo demonstrou que *S*. Enteritidis SE86 pode utilizar o biossurfactante para aumentar sua aderência na superfície de folhas de alface e penetrar nos estômatos da célula vegetal, consequentemente, tal aderência

associada ao biossurfactante pode diminuir a ação antimicrobiana do vinagre e hipoclorito de sódio, utilizados para sanitização de folhas de alface.

5 CONCLUSÃO

De acordo com os resultados encontrados no presente estudo, é possível concluir que:

- *S*. Enteritidis SE86 possui a capacidade de produzir biossurfactante, em caldo BHI, meio mínimo e em folhas de alface.

- As características do biossurfactante sugerem um composto polimérico.

- Para produzir biossurfactante *S*. Enteritidis SE86 depende de alguns fatores como por exemplo, pH $\geq$6,0 e concentração de células elevada (acima de 7,1 Log UFC/mL).

- O biossurfactante produzido por *S*. Enteritidis SE86 é resistente a diferentes temperaturas, pH e NaCl, o que pode ser considerado uma característica importante, uma vez que *S.* Enteritidis SE86 é um patógeno alimentar e fatores como pH, temperatura e pressão osmótica podem ser utilizados para controlar a multiplicação dos microrganismos nos alimentos.

- O biossurfactante produzido por *S.* Enteritidis SE86 aumenta sobrevivência da bactéria em hipoclorito de sódio 50 ppm e solução de vinagre 2% e 20%.

- *S*. Enteritidis SE86 na presença do biossurfactante aumenta sua aderência na superfície de folhas de alface e a presença desse composto diminui a ação antimicrobiana do vinagre e hipoclorito usados na desinfecção desta hortaliça.

REFERÊNCIAS

ABREU, I., M., O.; JUNQUEIRA, A. M. R.; PEIXOTO, J. R.; OLIVEIRA, S. A. Qualidade microbiológica e produtividade de alface sob adubação química e orgânica. **Ciência e tecnologia de alimentos**, v. 30, p. 108-118, maio 2010.

ADAK, G.K.; MEAKINS, S.M.; YIP´, H. Disease risk from foods, England and Wales, 1996 – 2000. **Emerging Infectious Diseases**, v.11, n. 3, p. 365 – 372, 2005.

ANDERSON, M., JAYKUS, L.A., BEAULIEU, S., DENNIS, S., Pathogen–produce pair attribution risk ranking tool to prioritize fresh produce commodity and pathogen combinations for further evaluation (P3ARRT). **Food Control** v. 22, 1865–1872 2011.

ARAUJO, E. A., ANDRADE, N. J., SILVA, L. H. M., CARVALHO, A. F., SA, S. A. C., RAMOS, A. M. Control of microbial adhesion as a strategy for food and bioprocess technology. **Food and Bioprocess Technology**, v. 3, p. 321-332, 2010.

ARAUJO, L. V., ABREU, F., LINS, U., ANNA, L. M. M. S. NITSCHKE, M. FREIRE, D.M.G. Rhamnolipid and a surfactin inhibit *Listeria monocytogenes* adhesion. **Food Resource International**, v. 44, p. 481-488, 2011.

ALSAYEQH, A. F. Foodborne disease risk factors among women in Riyadh, Saudi Arabia. **Food Control**, v. 50, p. 85–91, April, 2015.

APARNA, A.; SRINIKETHAN, G.; SMITHA, H.; Production and caracterization of biosurfactant produced by a novel pseudomonas sp. 2b. **Colloids and surfaces B: Biointerfaces**, v.95, p. 23-29, 2012.

ARAUJO, L.V. de; ABREU, F.; LINS, U.; ANNA, L.M. De M.S. Rhamnolipid and a surfactin *inhibit Listeria monocytogenes* adhesion. **Foood Research International,** v. 44,p. 481-488, 2011.

AYED, H.B. , HIMET, N., BÉCHET, M., CHOLLET, M., CHATAIGNÉ, G., LECLÉRE, V., JACQUES, P., NASRI, M. Identification and biochemical characteristics of lipopeptides from *Bacillus mojavensis* A21. **Process Biochemistry**. v. 49, 1699–1707, 2014.

BARBOSA, L. N.; RALL, V. L. M.; FERNANDES, A. A. H.; USHIMARU, P. I.; PROBST, I. S., FERNANDES Jr, A. **Foodborne Pathogens and Disease**. v. 6, n.6, p. 725-728, July/August 2009.

BARRERA, M..J.; BLENKINSOP, R.; WARRINER, K.The effect of different processing parameters on the efficacy of commercial post-harvest washing of minimally processed spinach and shredded lettuce. **FoodControl**, v. 25, p. 745-751, 2012.

BARROS, F. F. C.; PONEZI, A. N.; PASTORE, G. M. Production of biosurfactant by *Bacillus subtilis* LB5a on a pilot scale using cassava wastewater as substrate. **Journal of Industrial Microbiology & Biotechnology**. n. 9, v. 35, p. 1071-1078, 2008.

BENTO, F. M.; CAMARGO, F. A. O.; GAYLARDE, C. C. Biossurfactantes. In: DE MELO, I. S. ; DE AZEVEDO, J. L. [Org.] **Microbiologia Ambiental**. 2. Ed. Jaguariúna, Embrapa Meio Ambiente, 2008. Cap.7. p. 152-184.

BERMÚDEZ-AGUIRRE D., BARBOSA-CÁNOVAS GV. Disinfection of selected vegetables under non-thermal treatments: Chlorine, acid citric, ultraviolet light and ozone. **Food Control,** v.29, p. 82-90, 2013.

BRADEN, C. R.; TAUXE, R. V. Emerging Trends in Foodborne Diseases. **Infectious Disease Clinics of North America.** v. 27, p. 517–533, 2013.

CAPALONGA, ROBERTA.; RAMOS, R. C.; BOTH, J. M. C.; SOEIRO, M. L. T., LONGARAY S. M.; HAAS, S.; TONDO, E. C. *Salmonella* serotypes, resistance patterns, and food vehicles of salmonellosis in southern Brazil between 2007 and 2012. **The Journal of Infection in Developing Countries**, v. 8, n.7, p. 811-817, 2014.

CASARIN, L. S.; TONDO, E. C.; KLEIN, M. P.; BRANDELLI, A. Survival of *Escherichia coli*, *Staphylococcus aureus* and *Salmonella* Enteritidis in frozen chicken hamburger. **Journal of Muscle Foods**. v. 20, p. 478–488, 2009.

CASARIN, L. S. ; BRANDELLI, A. ; CASARIN, F. O.; SOAVE, P. A. ; WANKE, C,H, ; TONDO, E. C. Adhesion of *Salmonella* Enteritidis and Listeria monocytogenes on stainless steel welds. **International Journal of Food Microbiology.** v. 19, p. 103–108, 2014.

Chang J., Fang T.J. Survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovars Typhimurium in iceberg lettuce and the antimicrobial effect of rice vinegar against E. coli O157:H7. **Food Microbiology**, v. 24, p. 745–751, 2007.

CHEN J., HUANG P.T., ZHANG K.Y., DING F.R. Isolation of biosurfactant producers, optimization and properties of biosurfactant produced by *Acinetobacter* sp. from petroleum-contaminated soil. **Journal of Applied Microbiology** . v. 112, n. 4, p. 660–671, 2012.

CRITZER, F. J; DOYLE, M. P. Microbial ecology of foodborne pathogens associated with produce. **Current Opinion in Biotechnology,** v. 21, p. 125–130, 2010.

DARVISHI, P., AYATOLLAHI, S., MOWLA, D., NIAZI, A. Biosurfactant production under extreme environmental conditions by an efficient microbial consortium, ERCPPI-2. **Colloids Surf., B.** v.84, p. 292-300, 2011.

DE PAULA, C. M. D., GEIMBA, M. P., DO AMARAL, P. H., TONDO, E. C. Antimicrobial resistance and PCR-ribotyping of *Shigella* responsible for foodborne outbreaks occurred in southern Brazil. **Brazilian Journal of Microbiology**. v. 41, p. 966–977. 2010.

DELEU, M.; PAQUOT, M. From renewable vegetables resources to microorganisms: new trends in surfactants. **Comptes Rendums Chimie**. v. 7, p. 641–646, 2004.

ERICKSON, M. C. LIAO, J.; PAYTON, A. S.; RILEY, D.G.; WEBB, C.C.; DAVEY, L. E.; KIMBREL, S.; MA, L.; ZHANG, G.; FLITCROFT, I.; DOYLE, M.P.; BEUCHAT, L. R. Preharvest Internalization of *Escherichia coli* O157:H7 into Lettuce Leaves, as affected by insect and Physical Damage. **Journal of Food Protection,** v. 73, n. 10, p. 1809-1816, 2010.

FONTES, G. C.; AMARAL, P. F. F; COELHO, M. A. Z. Produção de biossurfactante por levedura. **Química Nova [online].** v.31, n.8, p. 2091-2099, 2008.

FRANCY, D. S.; THOMAS J. M.; RAYMOND R. L.; WARD C. H. Emulsification of hydrocarbons by subsurface bacteria. **Journal of Industrial Microbiolog**y. v. 8, n. 4, p. 237-245, 1991.

GEIMBA, M. P. TONDO, E. C.; DE OLIVEIRA, F. A.; CANAL, C. W.; BRANDELLI, A. Serological Characterization and prevalence of *spv*R genes in *Salmonella* isolated from foods involved in outbreaks in Brazil. **Journal of Food Protection**, Brasil, v. 67, n. 6, p. 1229-1233, 2004.

GREIG, J.D.; RAVEL, A. Analysis of food borne outbreak data reported internationally for source attribution. **International Journal of Food Microbiology,** v**.** 130, p. 77-87, 2009.

GUDIÑA, E.J.; PEREIRA, J.F.B.; COSTA, R.; COUTINHO, J.A.P.; TEIXEIRA, J.A.; RODRIGUES, L.R. Biosurfactant- producing and oil- degracing Bacillus subtilis strains enhance oil recovery in laboratory sand – packed columns. **Journal of Hazardous Materials**, v. 216, p.106-113, 2013.

GOMES- NETO N.J., PESSOA R.M.L., QUEIROGA M.I.B.N., MAGNANI M., FREITAS F.I.S., SOUZA E.L., MACIEL J.F. Bacterial counts and the occurrence of parasites in lettuce (*Lactuca sativa*) from different cropping systems in Brazil . **Food Control**, v. 28, p. 47-51, 2012.

HAVELAAR, A. H.; BRUL, S.; DE JONG, A.; DE JONGE, R.; ZWIETERING, M. H., KUILE, B. H. Future challenges to microbial food safety. **International Journal of Food Microbiology,** p. S79-S74, 2009.

HAMME, J. D. V; SINGH, A.; WARD, O. P. Physiological aspects: Part 1 in a series of papers devoted to surfactants in microbiology and biotechnology. **Biotechnology Advances.** v. 24, n.6, p. 604–620, 2006.

HEYD, M. KOHNERT, A., TAN, T. H., NUSSER, M., KIRSCHHÖFER, F., BRENNER-WEISS, G., FRANZREB, M., BERENSMEIER, S. Development and trends of biosurfactant analysis and purification using rhamnolipids as an example. **Analytical and Bioanalytical Chemistry.** v. 391, n. 5, p. 1579-1590, 2008.

HORBY, P.W.; O'BRIEN, S.J.; ADAK, G.K.; GRAHAM, C.; HAWKER, J.I.; HUNTER, P.; LANE, C.; LAWSON, A.J.; MITCHELL, R.T.; REACHER, M.H.; THRELFALL, E.J.; WARD, L.R.; AGUILERA, J.F.; CATCHPOLE, M.A.; GILL, O.N.; GILLESPIE, I.A.; GRAHAM, C.; GREGOR, S.; HOLLYOAK, V.A.; HORBY, P.W.; LANE, C.; LAMAGNI, T.; BAIGUE, S.; LE LONG S.M.; MCCARTNEY A.C.; ROONEY R.; SAID, B.; SMERDON W.J.; SYED Q.; TWISSELMAN B. A national outbreak of multi-resistant*Salmonella enterica* serovar Typhimurium definitive phage type (DT) 104 associated with consumption of lettuce**. Epidemiology and Infection,** v. 130, p. 169–178, 2003.

IRVINE, W.N.; GILLESPIE, I.A.; SMYTH, F.B.; ROONEY, P.J.; MCCLENAGHAN, A; DEVINE M.J.; TOHANI, V.K. Investigation of an outbreak of *Salmonella enterica* serovar Newport infection. **Epidemiology and Infection**, v. 137, p. 1449–1456, 2009.

ISMAIL, W., AL-ROWAIHI, I. S., AL-HUMAN, A., HAMZA, R. Y., NAYAL, A. M. E., BOUOUDINA, M. Characterisation of a lipopeptide biosurfactant produced by a crude-oil-emulsifying *Bacillus* sp. I-15. **Int. Biodeterior. Biodegrad. v.** 84, p. 168-178, 2013.

JAIN, R. M., MODY, K., JOSHI, N., MISHRA, A., JHA, B. Production and structural characterisation of biosurfactant produced by an alkaphilic bacterium, *Klebsiella* sp.: Evaluation of different carbon sources. **Colloids Surf.,** B**.** v. 108, p. 199-204. 2013.

Jirků, V.; Čejková, A.; Schreiberová, O.; Ježdík, R.; Masák, J. Multicomponent biosurfactants — A "Green Toolbox" extension . **Biotechnology Advances** , 2015.

JOSÉ, J. F. B. S.; VANETTI, M. C. D. Effect of ultrasound and commercial sanitizers in removing natural contaminants and *Salmonella* enterica Typhimurium on cherry tomatoes. **Food Control**. v. 24, n. 1-2, p. 95-99, March-April 2012.

KIM, Y. S.; LEE, S. H.; KIM, S. H.;, JOO, Y.; BAHK , G. J. Investigation of the experience of foodborne illness and estimation of the incidence of foodborne disease in South Korea. **Food Control**. v. 47, p. 226-230, 2015.

KOSEKI, S., ISOBE, S. Prediction of pathogengrowth on iceberg lettuce under real temperature history duringdistribution from farm to table. **Int. Journal of Food Microbiology.** v.104, p. 239-248, 2005.

KROUPITSKI Y., PINTOA R., BELAUSOVB E., SELAA E. Persistence strategies of *Bacillus cereus* spores isolated from dairy silo tanks. **Food Microbiology**, v. 28, p. 990-997, 2011.

LIMA, P. M.; JOSÉ, J. F.B.S; ANDRADE, N. J.; PIRES, A. C. S.; FERREIRA, S. O. Interaction between natural microbiota and physicochemical characteristics of lettuce surfaces can influence the attachment of *Salmonella* Enteritidis**. Food Control.** v. 30, n. 1, p. 157–161, March 2013.

LUNA, J. M.; RUFINO, R.D.; JARA, A.M. A.T.; BRASILEIRO, P.P.F.; SARUBBO, L. A. Environmental applications of the biosurfactant produced by Candida sphaerica cultivated in low-cost substrates. **Colloids and Surfaces A: Physicochem. Eng. Aspects** (2014) In Press.

MACHADO, T., R., M.; MALHEIROS, P., S.; BRANDELLI, A.; TONDO, E., C. Avaliação da resistência de *Salmonella* à ação de desinfetantes ácido peracético, quaternário de amônio e hipoclorito de sódio. **Rev. Instituto Adolfo Lutz. São Paulo,** v. 69, 2010.

MACHADO, T. R. M. **Avaliação da aderência ao aço inoxidável e ao polietileno por três sorovares de *Salmonella* e da capacidade de desinfecção dessas superfícies.** Dissertação (Mestrado em Microbiologia Agrícola e do

Ambiente)- Universidade Federal do Rio Grande do Sul- UFRGS, Porto Alegre, 143 f, 2007.

MAGALHÃES, L.; NITSCHKE, M. Antimicrobial activity of rhamnolipids against Listeria monocytogenes and their synergistic interaction with nisina. **Food Control**. v.29, n. 1, p. 138-142, 2013.

MALHEIROS, P. S.; DE PAULA, C. M. D. ; TONDO, E. C. Cinética de crescimento de *Salmonella* Enteritidis envolvida em surtos alimentares no RS: uma comparação com linhagens de outros sorovares. **Ciência e Tecnologia de Alimentos [online].** v. 27, n.4, p. 751-755, 2007.

MAO A. X.,JIANGA R., XIAOA W., YUJ. Use of surfactants for the remediation of contaminated soils: A review. **Journal of Hazardous Materials**. v. 285, p. 419–435, 2015.

MARCHANT, R.; BANAT, I. M. Biosurfactants: a sustainable replacement for chemical surfactants?. **Biotechnology Letters.** v. 34, n. 9, p. 1597–1605, 2012.

MARTIA, M.E.; COLONNAA, W.J.; PATRA, P.; ZHANG, H.; GREEN, C.; REZNIK, G.; PYNN, M.; JARRELL, K.; NYMAN, J.A.; SOMASUNDARAN, P.; GLATZ, C.E.; LAMSAL, B.P. Production and characterization of microbial biosurfactants for potential use in oil-spill remediation. **Enzyme and Microbial Technology, v.** 55 p. 31–39, 2014.

MAWGOUD , A.; LÉPINE, F. ; DÉZIE, E. Rhamnolipids: diversity of structures, microbial origins and roles Ahmad Mohammad. **Journal Applied Microbiology Biotechnology**, 86, p. 1323–1336, 2010.

McENTIRE, J. Foodborne Disease: The Global Movement of Food and People **Infectious Disease Clinics of North America**. Am 27, p. 687-693, 2013.

MCMEECHAN, A., LOVELL, M. A., COGAN, T. A., MARSTON, K. L., HUMPHREY, T. J., BARROW, P. A. Glycogen production by different *Salmonella enterica* serotypes: contribution of functional *glgC* to virulence, intestinal colonisation and environmental survival. **Microbiology**. v.151, p. 3969–3977, 2005.

MELLOR , G.E.; BENTLEY, J.A.; DYKES, G.A. Evidence for a role of biosurfactants produced by Pseudomonas Fluorescens in the spoilage of fresh aerobically store chicken meat. **Food Microbiology,** v. 28, p. 1101-1104, 2011.

MONTEIRO, A. S. A.S.; BONFIM,V.S.; DOMINGUES,A. CORRÊA JR.,E.P.; SIQUEIRA,C.L.; ZANI, V.L. Identification and characterization of bioemulsifier-

producing yeasts isolated from effluents of a dairy industry. **Bioresource Technology**. v. 101, p. 5186-5193, 2010.

MÜRMANN, L., SANTOS, M. C. dos; LONGARAY, S.M., BOTH, J.M.C; CARDOSO, M. Quantification and molecular characterization of *Salmonella* isolated from food samples involved in salmonellosis outbreaks in Rio Grande do Sul, Brazil. **Brazilian Journal of Microbiology** v.39, n.3, p. 529-534, 2008.

NITSCHKE, M.; COSTA, S. G. V. A. O. Biosurfactants in food industry. **Trends in Food Science & Technology**. v. 18, n. 5, p. 252-259, 2007.

NITSCHKE, M.; PASTORE, G. M. .Biossurfactantes: propriedades e aplicações. ***Química Nova*** **[online].** v. 25, n. 5, p. 772-776, 2002.

NITSCHKE, M.; ARAÚJO, L.V.; COSTA, S.G.; PIRES, R.C.; ZERAIK, A.E; FERNANDES A.C.; FREIRE, D.M.; CONTIERO, J. Surfactin reduces the adhesion of food-borne pathogenic bacteria to solid surfaces**. Letters in Applied Microbiology. V.** 49, p.241–247, 2009.

NYGARD, K.; LASSEN, J.; VOLD, L.; ANDERSSON, Y.; FISHER, I.; LÃFDAHL, S.; THRELFALL, J.; LUZZI, I.; PETERS T.; HAMPTON M.; TORPDAHL, M.; KAPPERUD, G.; AAVITSLAND P. Outbreak of *Salmonella* Thompson infections linked to imported rucola lettuce. **Foodborne Pathogens and Disease,** v.5, p. 165–173, 2008.

OLIVEIRA F.A; FRAZZON, A.P.G; BRANDELLI, A.; TONDO, E.C. Use of PCR-ribotyping, RAPD, and antimicrobial resistance for typing of Salmonellaenteritidis involved in food-borne outbreaks in Southern Brazil. **Journal Infection Developing Countries**, v. 1, n. 2, p. 170-176, 2007.

OLIVEIRA, A. B. A.; RITTER, A. C.; TONDO, E. C.; CARDOSO, M. I. Comparison of Different Washing and Disinfection Protocols Used by Food Services in Southern Brazil for Lettuce (*Lactuca sativa*). **Food and Nutrition Sciences**, v. 3, n. 1, p. 28-33, 2012.

OLIVEIRA, F., A.; GEIMBA, M. P.; PASQUALOTTO, A. P.; BRANDELLI, A.; PASQUALI, G.; DA SILVA, W. P.; TONDO, E. C. Clonal relationship among S*almonella enterica* serovar Enteritidis involved in foodborne outbreaks in Southern Brazil. **Food Control**. v. 20, n. 6, p. 606–610, 2009.

OLIVEIRA, F.A., PASQUALOTTO, A.P., PADILHA, W.; TONDO, E.C. Characterization of *Salmonella* Enteritidis isolated from human samples. **Food Research International**, 2010.

OLIVEIRA, M; WIJNANDS, L.; ABADIAS, M.; AARTS, H.; FRANZ, E. Pathogenic potential of *Salmonella* Typhimurium DT104 following sequential passage through soil, packaged fresh-cut lettuce and a model gastrointestinal tract. **International Journal of Food Microbiology.**v. 148, n. 3, p. 149–155, August 2011.

OLIVEIRA, D. W. F.;FRANÇA, I. W. L.; FÉLIX, A. K. N.; MARTINS, J. J. L.; GIRO, M. E. A.; MELO, V. M. M.; GONÇALVES, L. R. B. Kinetic study of biosurfactant production by *Bacillus subtilis* LAMI005 grown in clarified cashew apple juice. **Colloids and Surfaces B: Biointerfaces**. v. 101, n.1, p. 34–43, January 2013.

PATEL, J.; SHARMA, M. Differences in attachment of *Salmonella* enterica serovars to cabbage and lettuce leaves. **International Journal of Food Microbiology**, v. 139, p. 41-47, 2010

PENG, J.S., TSAI, W.C., CHOU, C.C., Inactivation and removal of *Bacillus cereus* by sanitizer and detergent. **International Journal of Food Microbiology**. v. 77, p. 11-18, 2002.

PEREZ, K. J. ; CECCON, R. V.; MALHEIROS, P. S.; JONG, E. V.; TONDO, E. C. Influence of acid adaptation on the survival of S*almonella* Enteritidis and S*almonella* Typhimurium in simulated gastric fluid and in intestine infection. **Journal of Food Safety.** v. 30, n. 2. p. 398 – 414, 2010.

PEREZ, K. J.; MARTINS, F. S.; CARA, D. C.M.; NICOLI, J. R.; TONDO, E. C. Evaluation of intestinal invasion in germ-free mice challenged with acid-adapted and nonacid-adapted *Salmonella* Enteritidis SE 86 an *Salmonella* Typhimurium ST99. **Journal of Food Safety**. v. 32, n. 1. p. 108–114, 2012

PIGGOT, D. C. Foodborne Illness Emergency **Medicine Clinical of North América,** v. 26, pág 475–497, 2008.

PIRES, S. M.; VIEIRA, A. R.; HALD, T.; COLE, D. Source Attribution of Human Salmonellosis: An Overview of Methods and Estimates. **Foodborne Pathogens and Disease**., v. 11, n. 9, p. 667-676, September 2014.

RAVISHANKAR, S.; ZHU, L.; JARONI, D. Assessing the cross contamination and transfer rates of *Salmonella enterica* from chicken to lettuce under different food-handling scenarios. **Food Microbiology**, v. 27, p. 791-794, 2010.

RITTER, A.C.; BACCIU, D.; SANTI, L.; SILVA, W.O; VAINSTEIN, M.H.; RUBINO S; UZZAU, S.; TONDO, E.C**.** Investigation of *rpoS* and *dps* Genes in Sodium Hypochlorite Resistance of *Salmonella* Enteritidis SE86 Isolated from Foodborne

Illness Outbreaks in Southern Brazil. **Journal of Food Protection**, n. 3, p. 437-442, 2012.

RIVARDO F. et al. Synergistic effect of lipopeptide biosurfactant with antibiotics against *Escherichia coli* CFT073 biofilm. **International Journal of Antimicrobial Agents.** v.37. n. 4, p. 324-331, 2011.

RON, E., Z.; ROSENBERG, E. Natural roles of biosurfactants. **Environmental Microbiology,** Israel, v. 3, n. 4, p. 229-236, 2001.

ROSENBERG, E.; RON, E. Z. High- and low-molecular-mass microbial surfactants. **Applied Microbiology and Biotechnology**, v. 52, n. 2, p. 154-162 August 1999.

SAGOO, S.K.; LITTLE C.L.; WARD L.; GILLESPIE I.A.; MITCHELL. R.T. Microbiological study of ready-to-eat salad vegetables from retail establishments uncovers a national outbreak of salmonellosis. **Journal of Food Protection**, v. 66 p. 403–409, 2003.

SANTANA, L., R., R.; CARVALHO, R. D.S.; LEITE, C. C.; ALCÂNTARA, L. M.; OLIVEIRA, T. W. S.; RODRIGUES, B. M. Qualidade Física, Microbiológica e Parasitológica de Alfaces (*Lactuca sativa*) de Diferentes Sistemas de Cultivo. **Ciência e Tecnologia de Alimentos**, v. 26, n. 2, p. 264-269. 2006.

SARAVANAKUMARI, P.; MANI, K.· Structural characterization of a novel xylolipid biosurfactant from *Lactococcus lactis* and analysis of antibacterial activity against multi-drug resistant pathogens. **Bioresource Technology**. v. 101, n. 22, p. 8851–8854, 2010.

SATPUTE, S.K. et al. Methods for investigating biosurfactants and bioemulsifiers: a review. **Critical Reviews in Biotechnology**. v. 30, n. 2, p. 127-144, 2010.
Merchant

SARTER, S.; SARTER.G.; GILABERT, P. A. Swot analysis of HACCP implementation in Madagascar. **Food Control.** v.21 p. 253–259, 2010.

SCALLAN, E. HOEKSTRA, R. M.; ANGULO, F.J.; TAUXE, R. V.; WIDDOWSON, M. A.; ROY, S. L.; JONES, J.L.; GRIFFIN, P. M. Foodborne illness acquired in the United States--major pathogens. **Emerging Infectious Diseases.** v.17, n. 1, p. 7-15, 2011.

SCALLAN, E.; GRIFFIN,P.M.;ÂNGULO, F.J.; TAUXE, R.V.; HOEKSTRA, R.M. Foodborne illness acquired in the United States- Unspecified Agents. **Emerging Infectious** Disease. v.17, n. 1, p 16–22, 2011.

SPECTOR, M. P., KENYON, W. J. Resistance and survival strategies of *Salmonella* enterica to environmental stresses. **Food Resource International**, v. 45, p. 455–481, 2012.

Secretaria da vigilância em Saúde (SVS) **Análise Epidemiológica dos Surtos de Doenças Transmitidas por Alimentos no Brasil, 2000 – 2014**. Disponível em http://www.anrbrasil.org.br/new/pdfs/2014/3_PAINEL_1_ApresentacaoRejaneAlvesVigilanciaEpidemiologica-VE-DTA-Agosto_2014_PDF.pdf Acesso em 10 de agosto de 2014.

SHAHEEN, R. SVENSSON, B.; ANDERSSON, M. A. ; CHRISTIANSSON, A. ; SALKINOJA-SALONEN , M. Persistence strategies of *Bacillus cereus* spores isolated from dairy silo tanks. **Food Microbiology.** v.27, n.3, p. 347-355, 2010**.**

SHINOHARA, N. K.S.; DE BARROS, V.B.; JIMENEZ, S. M. C.; MACHADO, E. .C. L.; DUTRA, R. A. F.; FILHO, J. L. .L. **Ciência & Saúde Coletiva**, v. 13, n. 5, p. 1669-1674, setembro-outubro, 2008.

SINGH, P.; CAMEOTRA, S. S. Potential applications of microbial surfactants in biomedical sciences. **Trends in Biotechnology**. v.22, n. 3, p. 142-146, 2004.

SINGH, A.; HAMME, J., D., V.; WARD, O., P. Surfactants in microbiology and biotechnology: Part 2. **Aplication aspects. Biotechnology Advances**. v. 25, p. 99-121, 2007.

SRIRAN, M.I.; KALISHWARAL, K.; DEEPAK, V.; GRACEROSEPAT, R.; SRISAKTHI, K.; GURUNATHAN. Biofilm inhibition and antimicrobial action of lipopeptide biosurfactant produced by heavy metal tolerant strain *Bacillus* cereus nk1. **Colloids and surfaces B: biointerfaces,** v.85, p.174-181, 2011.

SOTIROVA A., VASILEVA-TONKOVA E. Effects of rhamnolipid-biosurfactant on cell surface of *Pseudomonas aeruginosa*. **Microbiological Research**, v. 164, pp. 297–303, 2009.

TAKKINEN, J.; NAKARI, U.; JOHANSSON, T.; NISKANEN, T.; SIITONEN, A.; KUUSI, M. A nationwide outbreak of multiresistant *Salmonella* Typhimurium var Copenhagen DT104B infection in Finland due to contaminated lettuce from Spain, May 2005. **Euro surveillance** 10, 2005.

TORGERSON, P. R.; DE SILVA, N. R.; FÈVRE, E. M.; KASUGA, F.; ROKNI, M.B.; ZHOU, XIAO-NONG; SRIPA, B.; GARGOURI, N.; WILLINGHAM, L. A.; STEIN, CLAUDIA. The global burden of foodborne parasitic diseases: an update. **Trends in Parasitology**,v. 30, n. 1, p. 20-26, January 2014.

SCALLAN, E.; MAHON, B. E. Foodborne Diseases Active Surveillance Network (FoodNet) in 2012: A Foundation for Food Safety in the United States. **FoodNet in CID** v.54 , Suppl 5, p. S381, 2012.

THAVASI, R.; JAYALAKSHMI, S.; BANAT, I.M. Biosurfactants from marine bacterial isolates. **Journal of Petroleum and Environmental Biotechnology**,v. 01, p.S1:1-6. 2011.

TONDO, E.C.; RITTER, A. C.; CASARIN, L. S. Involvement in foodborne outbreaks, risk factors and options to control *Salmonella* Enteritidis SE86: an important food pathogen in Southern Brazil. In: HACKETT C.B. *Salmonella* **prevalence, risk factors and treatment options.** Nova publishers, 2015. p.65-77.

WEI H., WOLF G., HAMMES W.P. Indigenous microorganisms from iceberg lettuce with adherence and antagonistic potential for use as protective culture. **Innovative Food Science and Emerging Technologies**, v.7, p. 294-301, 2006.

YARON, S. Microbial Attachment and persistence on plants. In: Matthews, K. R. ; Sapers, G. Gerba, C. **The produce contamination problem: Causes and Solutions**. 2.ed. Elsevier , 2014. p. 21-57.

YOUSSEF, N.H.; DUNCAN, K.E.; NAGLE, D.P.; SAVAGE, K.N.; KNAPP, R.M.; McINERNEY, M.J. Comparison of methods to detect biosurfactant production by diverse microrganisms. **Journal of Microbiological Methods**, v.56,p.339-347, 2004.