UNIVERSIDADE FEDERAL DA BAHIA
FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA DE ALIMENTOS

ANISIO IURI LIMA DOS SANTOS ROSÁRIO

OCORRÊNCIA E CARACTERIZAÇÃO DE *Escherichia coli* PRODUTORA DE SHIGA-TOXINA ISOLADAS DE PRODUTOS LÁCTEOS NO ESTADO DA BAHIA

Salvador
2019

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Dissertação apresentada ao Programa de Pós-Graduação em Ciência de Alimentos da Faculdade de Farmácia da Universidade Federal da Bahia, como requisito para obtenção do título de Mestre.

Orientadora:
Profª. Drª. Marion Pereira da Costa.
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Prof. Dr. Maurício Costa Alves da Silva.
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ANÍSIO IURI LIMA DOS SANTOS ROSÁRIO

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Aprovada em 29 de abril de 2019.

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que me fazia repetir diversas vezes que
um dia teria um neto cientista.

“A ciência nunca resolve um problema sem criar pelo menos outros dez.”

(George Bernard Shaw)

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NOTA DE ESCLARECIMENTO

Neste trabalho, os capítulos apresentam formatação própria. Isso se deve ao fato de que cada um dos periódicos para os quais foram submetidos os conteúdos aqui presentes, apresenta exigências diferentes com relação à formatação.

O Capítulo I segue as normas da ABNT, pois a dissertação encontra-se à disposição na Biblioteca Universitária Reitor Macedo Costa, da Universidade Federal da Bahia.

O Capítulo II segue as normas do periódico *Critical Reviews in Food Science and Nutrition*.

O Capítulo III segue as normas do periódico *Food Microbiology*.

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LISTA DE ABREVIATURAS E SÍMBOLOS

% - Porcentagem

≥ - maior ou igual

°C – Graus Celcius

µg – micrograma

AH – *acid habituation*

AIEC - *E. coli* aderente-invasiva

AR – *acid resistance*

a_w – *water activity*

BHI – *brain and heart infusion*

Ca – cálcio

CA – *colanic acid*

CDC – *Centers for disease control and prevention*

CH – Colite hemorrágica

CLSI – *Clinical and laboratory standards institute*

DAEC - *E. coli* difusamente aderente

DNA – Ácido desoxirribonucleico

DVA – Doença veiculada por alimentos

D-value – *decimal reduction time*

E. coli – *Escherichia coli*

EAEC ou EAggEC - *E. coli* entero-agregativa

EHEC - *E. coli* enterohemorrágica

EIEC - *E. coli* enteroinvasora

EMB – Eosina Azul de Metileno

EPEC - *E. coli* enteropatogênica

ETEC - *E. coli* enterotoxigência

g – grama

GABA - *gamma-amino butyric acid*

Gb3 – *Globotriaosylceramide*

GMP - *Good Manufacturing Practices*

h – Horas

H^+ - proton

HACCP – *Hazard Analysis and Critical Control Point*

HTST - *High-temperature-short-time*

IMViC – Acrônimo para *indole test, methyl red test, Voges-Proskauer, citrate test*

L – Litros

LEE – *Locus of enterocyte effacement*

LHR - *locus of heat resistance*

LST – *Lauryl Sulfate Tryptose*

LTLT - *Low-temperature-long-time*

min – minutos

mL – Mililitros

m-PCR – multiplex PCR

MPN – *Most probable number*

mRNA – RNA mensageiro

mv - *millivolt*

NaCl – *sodium chloride*

nº - número

P – fósforo

PCR – *polymerase chain reaction*

PFGE – *Pulse-field gel electrophoresis*

pH – potencial hidrogeniônico

pH_i – *internal* pH

PS – *potassium sorbate*

rRNA – RNA ribossomal

s – segundos

SHU ou HUS – Síndrome hemolítica urêmica ou *hemolytic uremic syndrome*

SOP – *Standard Operating Procedures*

STEC – *Shiga toxin-producing E. coli* ou *E. coli* produtora de Shiga toxina

t - Toneladas

T3SS – *Type* 3 *secretory system*

THG – Transferencia horizontal de genes

UFC ou CFU – Unidade formadora de colônia ou *colony forming unit*

β – beta

ROSÁRIO, Anisio Iuri Lima dos Santos. *Ocorrência e caracterização de Escherichia coli produtora de Shiga-toxina isoladas de produtos lácteos no Estado da Bahia*. 137f. Dissertação (Mestrado) – Faculdade de Farmácia, Universidade Federal da Bahia, Salvador, 2019.

RESUMO

Escherichia coli é uma bactéria comensal do trato gastrintestinal de humanos e animais de sangue quente, pertencente ao grupo dos coliformes. Sua utilização na indústria de lácteos se dá na avaliação da qualidade do produto final, uma vez que a presença desta bactéria em alimentos é indicativa de contaminação fecal e falhas higiênico-sanitárias. De fato, leites e derivados estão entre os principais alimentos relacionados com casos de doenças veiculadas por alimentos (DVA) no Brasil e em outros países. Atrelado a isso, estirpes diarreiogênicas de *E. coli* estão entre os microrganismos mais frequentemente envolvidos nesses casos. Entre elas, a *E. coli* produtora de *Shiga-toxina* (STEC) se destaca pela capacidade de produção de potentes toxinas relacionadas com casos graves de diarreia sanguinolenta, síndrome hemolítico urêmica e morte. Entretanto, a ocorrência dessas bactérias ainda é subnotificada no país. O objetivo desse estudo foi verificar a ocorrência de coliformes a 45 °C, *E. coli* e STEC em leites e derivados produzidos no Estado da Bahia, Brasil, assim como caracterizar e analisar o perfil de susceptibilidade dos isolados de STEC frente a antimicrobianos. Além disso, descrever os mecanismos de reposta ao estresse causado pelo calor, ambiente ácido e osmótico, que permitem a sobrevivência de STEC em queijos. Dessa forma, foi relatado como cepas de STEC conseguem sobreviver a altas temperaturas, condições hiperosmóticas, exposição a ácidos orgânicos fracos e diminuição de pH relacionados à fabricação de queijo, armazenamento e à própria matriz do queijo. Além disso, discutimos como essas respostas de estresse interagem entre si, melhorando a adaptação e, consequentemente, a persistência de STEC no queijo. Ademais, mostramos como os genes de virulência podem ser afetados por mecanismos de resposta ao estresse, aumentando a adesão celular ou a produção de fatores de virulência, o que acaba levando a uma seleção de patógenos mais resistentes e virulentos dentro da indústria de alimentos. Em outro trabalho, foram analisadas 123 amostras entre março de 2017 a setembro de 2018. Isolados de *E. coli* foram submetidos à pesquisa para presença de STEC, e os isolados confirmados foram submetidos à sorotipagem para cepas não-O157 (*big six*), análise por PFGE, e avaliação do perfil de resistência a 12 antimicrobianos. Como resultado, 38 (31%) amostras estavam contaminadas por coliformes termotolerantes. Dessas, 21 (17%) apresentaram contaminação acima do previsto em legislação. *E. coli* foi isolada em nove (7%) amostras. A presença de STEC foi confirmada em cinco (4%) amostras, originando nove isolados: creme de

leite pasteurizado (2/9), queijo minas padrão (2/9), queijo minas frescal (4/9) e ricota (1/9). Todas as amostras foram negativas para a pesquisa de sorotipos não-O157 (“bix six”). A técnica de PFGE revelou três isolados 100% similares, provenientes de dois produtos diferentes e fabricados no mesmo local, sugerindo contaminação cruzada. Duas cepas de STEC apresentaram resistência à estreptomicina, e cinco apresentaram resistência intermediária à ampicilina, incluindo as duas citadas anteriormente. A ocorrência de STEC em leites e derivados, representa um risco para a saúde do consumidor, especialmente os imunocomprometidos e de faixa etárias como idosos e crianças. Embora o estudo não tenha revelado cepas multidroga resistentes, o risco de ingestão de produtos prontos para o consumo, como leites e derivados, deve ser considerado, visto a gravidade das doenças causadas pela bactéria. Esse é o primeiro relato da presença de STEC em creme de leite pasteurizado, queijo minas padrão e ricota produzidos a partir de leite pasteurizado, em todo o mundo.

Palavras chave: STEC. genes *stx1* e *stx2*. doença veiculada por alimento. saúde pública.

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ABSTRACT

Escherichia coli lives as a commensal bacterium inside the gastrointestinal tract of humans, and warm-blooded animals, belonging to the coliform group. Its importance in the dairy industry is given as a quality indicator, since the presence of *E. coli* in food is an indicative of fecal contamination and hygienic-sanitary failures. In fact, milk and dairy products are one of the main causes of foodborne diseases in Brazil and other countries. In addition, diarrheogenic *E. coli* strains are often related to foodborne disease cases. In this case, Shiga toxin-producing *E. coli* (STEC) is known for its ability to produce potent toxins related to severe cases of bloody diarrhea, hemolytic uremic syndrome, and death. However, notification and occurrence status of STEC in food products is still underreported in Brazil. The objective of this study was to verify the occurrence of coliforms, *E. coli* and STEC in milk and dairy products produced in the state of Bahia, Brazil, as well as characterizing and analyzing the antimicrobial susceptibility profile of STEC isolates. In addition, to describe the mechanisms of response to stress caused by heat, acid and osmotic environment, which allow the survival of STEC in cheese. Thus, it was described how STEC strains can survive to high temperatures, hyperosmotic conditions, exposure to weak organic acids and pH decreased related to cheese manufacturing, storage and the cheese matrix itself. In addition, we discuss how these stress responses interact with each other, improving the adaptation and, consequently, the persistence of STEC in cheese. Also, we described how virulence genes can be affected by mechanisms of stress response, increasing cell adhesion and the production of virulence factors, which leads to the selection of more resistant and virulent pathogens in the food industry. In another work, we analyzed 123 samples acquired between March 2017 and September 2018. *E. coli* isolates were tested for STEC presence, and confirmed isolates were submitted to serotyping for non-O157 ("big six") strains, and also PFGE analysis. As a result, 38 (31%) samples had the presence of thermotolerant coliform. Also, 21 (17%) samples were unsatisfactory for consumption, according to Brazilian standards. *E. coli* was isolated from nine (7%) samples. The presence of STEC was confirmed in five (4%) samples, totalizing nine isolates: pasteurized cream (2/9), minas padrão cheese (2/9), minas frescal cheese (4/9) and ricotta (1/9). All samples were negative for non-O157 serotypes. Isolated were not positive for "big-six" assay. PFGE

technique revealed three 100% similar isolates coming from two different products, altough produced in the same industry, suggesting a cross-contamination situation. Two STEC strains showed resistance to streptomycin, and five showed intermediate resistance to ampicillin, including the two strains previously mentioned. The occurrence of STEC in milk and dairy products represents a risk to consumer health, especially immunocompromised people. Although the study did not reveal multidrug resistant strains, the risk of eating STEC contaminated ready-to-eat products, such as milk and dairy should be considered given the severity of the diseases caused by this bacteria. This is the first report of the STEC presence in pasteurized cream, minas padrão cheese and ricotta cheese made from pasteurized milk worldwide.

Key words: STEC. *stx1* and *stx2* genes. foodborne disease. public health.

INTRODUÇÃO GERAL

O leite representa uma das principais fontes de proteína animal consumida no mundo, sendo o Brasil o terceiro maior consumidor e o quinto maior produtor mundial (USDA, 2018). A Bahia possui o terceiro maior rebanho leiteiro do Brasil (SEBRAE, 2017). Considerando o alto potencial de produção e consumo de leite e derivados na Bahia e no Brasil, o controle microbiológico desses produtos é necessário para garantir uma produção livre de patógenos. Neste contexto, a contaminação de produtos lácteos por bactérias do grupo coliforme pode interferir na qualidade geral do produto, causando perda de suas características físico-químicas, com consequente redução da validade comercial, além de indicar falhas higiênico-sanitárias. (TRMCIC et al., 2016). Além disso, dentro desse grupo, microrganismos como *Escherichia coli*, bactéria comensal do trato gastrintestinal de homens e animais, são conhecidos por serem indicadores de contaminação fecal do alimento (CONWAY; COHEN, 2015). A ocorrência de *E. coli* em alimentos prontos para o consumo indica contaminação durante alguma etapa do processamento, podendo ainda estar relacionado com contaminação cruzada ou falha nos processos térmicos (VAN ASSELT et al., 2017).

Embora a maioria dos isolados de *E. coli* seja incapaz de causar doenças no hospedeiro, estirpes diarreiogênicas podem ser encontradas nos mais diversos tipos de alimentos (GOMES et al., 2016). Os sintomas e mecanismos de patogenicidade das estirpes diarreiogênicas estão relacionados a características próprias do grupo em que são classificadas: *E. coli* enterotoxigência (ETEC), *E. coli* enteropatogênica (EPEC), *E. coli* enteroinvasora (EIEC), *E. coli* entero-agregativa (EAEC), *E. coli* difusamente aderente (DAEC), *E. coli* aderente-invasiva (AIEC), *E. coli* Shiga-toxigênica (STEC) e, dentro dessa última categoria, *E. coli* enterohemorrágica (EHEC) (ROBINS-BROWNE et al., 2016). Na cadeia produtiva leiteira, os bovinos são considerados como uma das principais fontes de veiculação de STEC (BARTH et al., 2016). Por conta disso, o leite pode ser exposto a esse patógeno por contaminação fecal durante as etapas de ordenha. Neste caso, a pasteurização do leite é altamente eficiente na destruição das células destas bactérias, embora não resolva os casos de contaminação pós-processamento (CARDOSO; MARIN, 2016; AHMED; SAMER, 2017). A persistência de cepas STEC em leite e derivados é alcançada através de mecanismos intrínsecos de resposta celular, relacionados à resistência térmica, presença de ácidos e osmolaridade. Essas respostas permitem ao patógeno superar as condições adversas encontradas nesses produtos, podendo permanecer viável no produto final (PENG et al., 2011).

O quadro clínico causado pela infecção por STEC deriva da ação de potentes toxinas produzidas por essas bactérias, capazes de causar intenso dano às células intestinais, além de usualmente afetar outros tecidos, causando doenças como diarreia sanguinolenta (colite hemorrágica), síndrome hemolítico urêmica, insuficiência renal aguda, sintomas neurológicos e morte (LEE et al., 2016; CASTRO et al., 2017). De fato, a *E. coli* foi citada como a bactéria mais frequentemente isolada em casos de surtos veiculados por alimentos no Brasil nos últimos dois anos (BRASIL, 2019). Além disso, leites e derivados estão entre as causas mais comuns de surtos de doenças veiculadas por alimentos no Brasil e em outros países, como os Estados Unidos (CDC, 2015; BRASIL, 2019).

Estudos demonstram que cepas bacterianas patogênicas, incluindo casos de STEC, estão adquirindo resistência aos principais antimicrobianos utilizados na medicina humana e veterinária (VON WINTERSDORFF et al., 2016). A transferência desses genes de resistência é de grande preocupação para a saúde pública porque dificulta o tratamento de doenças infecciosas comumente tratadas anteriormente (MARTÍNEZ; BAQUERO, 2015).

Desta forma, por se tratarem de alimentos prontos para o consumo, a presença de STEC em leites e derivados constitui um sério risco para a saúde pública, sob a prerrogativa de causar doenças em consumidores de todas as idades, incluindo a morte. Além disso, a presença de isolados que apresentem resistência a antimicrobianos amplamente utilizados na medicina moderna aumenta a probabilidade de casos mais graves de doenças. Nesse sentido, justifica-se a necessidade de pesquisas que determinem a ocorrência, as rotas de contaminação e os mecanismos bacterianos de sobrevivência nos principais produtos acometidos, além de mecanismos de controle que sejam eficazes para a terapêutica de pacientes infectados.

OBJETIVO GERAL

Determinar a ocorrência de coliformes termotolerantes e *Escherichia coli* em leites e derivados produzidos no Estado da Bahia, bem como verificar a ocorrência de *E. coli* produtora de Shiga-toxina. Além disso os isolados foram caracterizados quanto ao perfil de susceptibilidade a antimicrobianos. Ademais, descrever os mecanismos de sobrevivência desse patógeno em queijos.

OBJETIVOS ESPECÍFICOS

a) Investigar a ocorrência de coliformes a 45°C e *Escherichia coli* em leites e derivados produzidos no Estado da Bahia;

b) Identificar a presença de isolados produtores de Shiga toxina;

c) Detectar a presença ou ausência de genes associados a alguns dos principais sorogrupos (O26, O45, O103, O111, O121 e O145) envolvidos em casos de doenças veiculadas por alimentos;

d) Verificar a semelhança filogenética entre os isolados de *E. coli* produtora de Shiga toxina;

e) Determinar o perfil de susceptibilidade a antimicrobianos de interesse médico e veterinário entre os isolados de *E. coli* produtora de Shiga toxina;

f) Descrever os mecanismos de sobrevivência da STEC ao estresse térmico, ácido e osmótico;

g) Descrever a relação entre mecanismos de sobrevivência ao estresse e virulência.

CAPITULO I

REFERENCIAL TEÓRICO

1 PRODUÇÃO E O COMÉRCIO DE LEITE NO BRASIL E NO MUNDO

Nas últimas décadas, tem-se observado um crescente aumento na produção mundial de leite (FAO, 2019). Fatores como a mecanização e a adoção de novas tecnologias nas fazendas, mudanças demográficas, pressões econômicas e uma maior exigência dos consumidores são alguns dos responsáveis por esse crescimento (BARKEMA et al., 2015). A adoção de ordenha robótica em fazendas leiteiras, por exemplo, influencia no aumento da frequência e da produção de leite, inclusive reduzindo a incidência de algumas doenças (RODENBURG, 2016). Segundo Noordhuizen et al. (2008), uma das principais diferenças entre fazendas de leite em países desenvolvidos e subdesenvolvidos é o maior nível de instrução do proprietário, o conhecimento de novas tecnologias e um perfil mais empreendedor, o que faz com que esses estabelecimentos apresentem melhor produtividade em relação a outros. Além disso, Barkema et al. (2015) pontuam como fator diferencial o crescente aumento do rebanho associado ao menor envolvimento de participantes da família nos cuidados da fazenda, o que resulta na necessidade de empregar mão de obra mais especializada, aumentando assim a produção da propriedade.

A Europa foi responsável pela maior parte da produção global de leite de vaca em 2017, com mais de 221,3 milhões de toneladas (t) de leite produzido (Tabela 1).

Tabela 1 - Produção mundial de leite bovino e número de animais produzidos em 2017, em toneladas.

Continente	Produção (t)	Número de animais
África	35 376 022	71 185 293
Américas	184 552 255	49 846 119
Ásia	204 089 340	114 404 216
Europa	221 362 061	35 959 334
Oceania	30 241 371	6 619 179
Total	675 621 019	278 014 142

Fonte: FAO/FAOSTAT, 2019.

Dentro do continente, a Rússia manteve-se no topo da lista, apresentando quase 32 milhões t produzidas no mesmo ano. Apesar disso, a produção russa vem diminuindo com o passar dos anos devido a pagamentos não liquidados de subsídios fornecidos por programas estatais (DAVIS; HAHN, 2016). Entre os países participantes da União Europeia, destacaram-se Alemanha (14,7%), França (11%), Reino Unido (6,9%) e Holanda (6,4%) (FAO/FAOSTAT, 2019). Segundo van Asselt et al. (2017), a liderança da união europeia no *ranking* mundial está relacionada à sua intensa produção e exportação de diferentes produtos lácteos. Durante muitas décadas, a União Europeia optou por exportar sua produção excedente de leite, com um papel menor atribuído à intervenção nacional e ao armazenamento de produtos excedentes. Como resultado, as empresas de lácteos europeias venderam volumes relativamente grandes de lácteos para outros países, e no processo adquiriram ampla experiência na exportação desses produtos, construindo, assim, extensas redes de *marketing* de exportação, o que ajudou o grupo a chegar no atual patamar (VITALIANO, 2016). Assim, Davis e Hahn (2016) pontuam que a União Europeia manteve-se no topo dos exportadores globais de lácteos, seguido da Nova Zelândia, Estados Unidos e Austrália. Ademais, entidades europeias como o *Belgian Bone Club* e a *European Society for Clinical and Economic Aspects of Osteoporosis, Osteoarthritis and Musculoskeletal Diseases*, continuam a estimular a ingestão de derivados lácteos por trazerem benefícios para o funcionamento do organismo e dos ossos, além de não causarem risco de doença cardiovascular (ROZENBERG et al., 2015).

A Ásia é detentora do maior rebanho bovino (Tabela 1) e apresenta a segunda maior produção global. A Índia continua sendo o maior produtor mundial de leite, possuindo também o maior rebanho leiteiro do mundo (incluindo animais de outras espécies) (FAO/FAOSTAT, 2019). A grande produção da Índia se justifica não só pelo tamanho do rebanho, mas também por fatores como: a melhoria da atividade veterinária, uso de inseminação artificial, melhoria na alimentação e instrução dos fazendeiros, além do aumento da demanda de produtos lácteos (DAVIS; HAHN, 2016). Atrás do Paquistão, a China é o terceiro maior produtor asiático de leite, com cerca de 34,4 milhões t de leite produzido em 2017, sendo 30,3 milhões t de leite bovino (FAO/FAOSTAT, 2019). Salois (2016) pontua que a demanda do consumidor e o apoio governamental ajudaram a expandir a produção leiteira chinesa na última década. Além disso, o autor declara que a indústria de lácteos chinesa está passando por uma rápida consolidação da cadeia de suprimentos, onde a produção baseada em pequenas propriedades está dando lugar a grandes fazendas, cada vez mais eficientes. Essa realidade é diferente da africana, onde a produção de leite está crescendo mais vagarosamente em relação ao restante do mundo, por conta de questões relacionadas à pobreza e a condições climáticas não favoráveis (FAO, 2019).

Um estudo conduzido em 200 propriedades produtoras de leite na Etiópia, demonstrou que a média da produção de leite/dia/vaca foi de 13,89L, e a venda do leite era responsável por cerca de 77% e 20% do lucro total dos produtores de áreas urbanas e semiurbanas, respectivamente. Cerca de 94% do leite produzido era vendido e o lucro era utilizado principalmente para manutenção da produção a partir da alimentação dos animais, sobrando pouco dinheiro para outros usos, o que acaba diminuindo a potencialidade do aumento de produção (LEMMA et al., 2017).

Contudo, os Estados Unidos foi o país que mais produziu leite bovino em 2017, apresentando cerca de 97,7 milhões t, com expetativa de aumento para 100,1 milhões t em 2019 (USDA, 2018). Segundo Vitaliano (2016), através de mudanças nas políticas e ajustes nos programas de *marketing*, os Estados Unidos está se tornando uma poderosa força na indústria mundial de laticínios, pela alta qualidade dos produtos produzidos. Apesar disso, por conta do aumento crescente da competição global, a USDA estima que a exportação de derivados como queijo e manteiga em 2019 caia 4% e 12%, mantendo-se em 334 e 54 mil t, respectivamente. No ano de 2017, o Brasil ficou em terceiro lugar no *ranking* mundial de produção de leite bovino, atrás apenas dos Estados Unidos e da Índia (83,6 milhões t) (FAO/FAOSTAT, 2019).

A Tabela 1 traz um panorama da produção mundial de leite bovino e do número de animais produzidos no ano de 2017.A produção nacional de leite bovino foi superior a 33,4 milhões t no ano de 2017 (FAO/FAOSTAT, 2019), fazendo com que o Brasil ocupasse lugar de destaque na produção leiteira no continente americano (Tabela 2). Segundo David e Hahn (2016), o aumento da produção leiteira brasileira com o passar dos anos se deve ao fato dos inúmeros programas governamentais criados com o intuito de aumentar a produtividade dos animais, através da melhoria das pastagens e de genética animal. Os autores também pontuam o fomento da produção causado pela isenção de impostos estatais com relação à venda de leite pelos produtores e cooperativas.

Tabela 2 - Relação dos 10 maiores produtores de leite bovino do continente americano no ano de 2017.

País	**Produção (t)**
Estados Unidos	97 734 736
Brasil	33 490 810

México	11 767 556
Argentina	10 097 500
Canadá	8 100 000
Colômbia	7 100 000
Uruguai	2 049 000
Peru	2 010 985
Chile	1 990 519
Equador	1 850 000
Total	184 552 225

Fonte: FAO/FAOSTAT, 2019.

Entre as regiões brasileiras, a Região Sul foi responsável por 35,7% da produção leiteira no mesmo ano, 1,3% a menos do que o volume produzido em 2016. A Região Sudeste aparece em segundo lugar, com 34,2% e a Centro-Oeste em terceiro, com 11,9% da participação na produção nacional. Apresentando um rebanho de 5,8 milhões de vacas, 223 mil produtores e 771 laticínios (EMBRAPA, 2018), Minas Gerais se destacou como o principal produtor de leite, produzindo cerca de 8,9 milhões t em 2017, acompanhado por Rio Grande do Sul (4,5 milhões t) e Paraná (4,4 milhões t) (IBGE, 2018). Dessa forma, entendendo a importância da participação de Minas Gerais na produção nacional de leite, Bruhn et al. (2016) objetivaram caracterizar 40 propriedades produtoras de leite na região sul do estado, onde se localiza a maior parte da produção. Entre as propriedades estudadas, eles observaram produção diária média de 20 ± 7,4L/vaca e produção média de 1350L de leite/propriedade. Além disso, o sistema de criação predominante foi o semi-intensivo (60%), com duas ordenhas por dia (87,5%), em sistema mecanizado (72,5%), revelando uma heterogeneidade no processo produtivo do estado.

Apesar da intensa produção, o Brasil ainda precisa importar produtos lácteos para suprir a demanda da população. Segundo a EMPRABA (2018), em 2017, o volume de importação de lácteos superou 169 mil t, ao passo que se observou um volume de exportação em torno de 38 mil t, gerando um déficit maior que 130 mil t. Dentre os produtos importados em 2017, destacam-se o leite em pó (61,5%), queijos (18,8%) e o soro de leite em pó (13,9%), tendo como principais fornecedores, Argentina e Uruguai. Dessa forma, o Brasil representa um terço

das exportações argentinas e 40% das compras internacionais de leite do Uruguai. De forma semelhante, os principais produtos exportados foram leite em pó (62,2%), leite UHT (18,7%) e queijos (9,1%).

2 *Escherichia coli* PRODUTORA DE SHIGA-TOXINA

Os microrganismos denominados coliformes pertencem à família Enterobacteriaceae e, de uma forma geral, são definidos como bastonetes aeróbicos ou anaeróbicos facultativos, Gram negativos, não formadores de esporos, e capazes de fermentar a lactose com produção de gás e ácido, quando encubados em 32°C ou 35°C por até 48h (HERVERT et al., 2017). Há muito tempo, os coliformes vêm sendo utilizados como microrganismos indicadores, avaliando, principalmente, a contaminação da água por fezes e identificando falhas higiênico-sanitárias na produção de lácteos pasteurizados e outros produtos (MARTIN et al., 2016). Os coliformes podem ainda ser divididos em coliformes totais e coliformes termotolerantes (antigamente denominados coliformes fecais). Assim, os coliformes totais fermentam lactose e produzem gás a 35°C, e os termotolerantes, em temperatura de 44-45°C (FRANCO; LANDGRAF, 2003). Os coliformes são representados por quatro gêneros principais: *Escherichia*, *Klebsiella*, *Citrobacter* e *Enterobacter*, embora seja sabido que mais de 20 gêneros compõem esse grupo em menor número (IMHOFF, 2005; MASIELLO et al., 2016).

Nos Estados Unidos, a *Food and Drug Administration* estabelece o limite máximo de coliformes em leite pasteurizado tipo "A" até 10 UFC/mL (FDA, 2015). No Brasil, o Ministério da Agricultura, Pecuária e Meio ambiente determina o limite máximo de 5 UFC/mL de Enterobacteriaceae (família a qual pertence as bactérias do grupo coliforme) para o produto ser considerado apto à alimentação (BRASIL, 2018). Os coliformes totais possuem uma distribuição ubíqua, onde os membros do grupo podem ocorrer tanto nas fezes como em *habitats* naturais, no solo, nas plantas, nos animais, etc. Portanto, a presença de coliformes totais em alimentos remete apenas a falhas de higiene durante o processo produtivo, não remetendo à contaminação por fezes. Nayma et al. (2017) pontuam que mesmo dentro do grupo dos coliformes termotolerantes, existem algumas bactérias, como por exemplo espécies de *Klebsiella*, que não possuem origem fecal. Contudo, uma vez que a espécie *Escherichia coli* tem como *habitat* primário o trato gastrintestinal de animais de sangue quente, torna-se, portanto, o único coliforme cuja presença em alimentos pode indicar contaminação fecal,

humana ou animal, em alguma etapa do seu processo produtivo (VAN ASSELT et al., 2016; ERCUMEN et al., 2017).

E. coli pode ser definida como Gram negativa, aeróbia facultativa, oxidase negativa e não formadora de esporos (GOMES et al., 2016). Bioquimicamente, pode ser diferenciada dos demais coliformes através do crescimento em ágar Eosina Azul de Metileno (EMB), onde apresenta coloração brilhosa, de tom verde-metálico característico, causado pela rápida fermentação da lactose (Figura 1). Ainda, pode ser diferenciada dos outros coliformes termotolerantes através da produção de indol, reação positiva ao vermelho de metila, reação negativa ao teste de Voges-Proskauer e a não utilização de citrato (IMViC) (KONEMAN et al., 2010; HEMRAJ; DIKSHA; AVNEET, 2013).

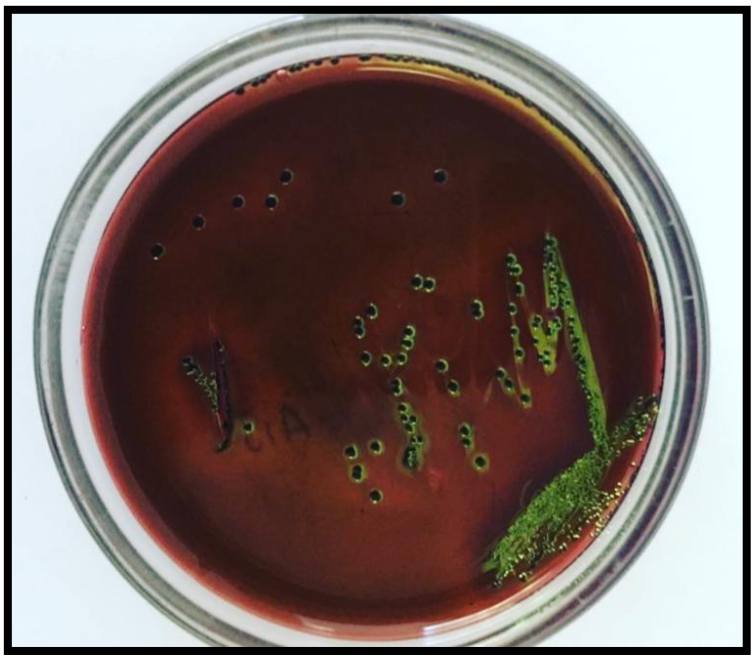

Figura 1 - Colônias características de *Escherichia coli* em ágar Eosina Azul de Metileno (EMB). Fonte: Arquivo pessoal.

As cepas de *E. coli* são classificadas baseado na presença de antígenos encontrados na célula bacteriana. O antígeno O faz parte da camada de lipopolissacarídeo celular, sendo o mais importante para classificação do sorogrupo. Por serem conhecidos fatores de virulência, é de importância para medicina humana e veterinária (DEBROY et al., 2016). Os antígenos H e K são relacionados às proteínas flagelares e a polissacarídeos capsulares, respectivamente (FRANCO; LANDGRAF, 1996). Como desde muito tempo atrás, poucos laboratórios eram capazes de tipificar os antígenos K, a tipificação baseada nos antígenos O e H se tornaram "padrão ouro" para *E. coli* (DEBROY et al., 2016).

A maioria das cepas de *E. coli* não é patogênica, agindo apenas como comensais da microbiota intestinal (CONWAY; COHEN, 2015). Apesar disso, algumas cepas patogênicas podem causar transtornos como diarreia, além de afetarem outros sistemas, causando infecções urinárias, pneumonia, problemas respiratórios em geral, meningites e até a morte (VILA et al., 2016). As chamadas *E. coli* diarreiogênicas são divididas em grupos, de acordo com os mecanismos de patogenicidade e, consequentemente, com os sintomas causados.

A *E. coli* enteropatogênica (EPEC) causa vômito, febre, e diarreia moderada a grave, podendo ser fatal, em especial para crianças em países menos desenvolvidos. Após colonizar o indivíduo, a bactéria provoca uma lesão conhecida como *attaching and effacing* nas vilosidades intestinais (HAZEN et al., 2016; KALLONEN; BOINETT, 2016).

E. coli enterotoxigênica (ETEC) é capaz de produzir duas toxinas, uma termoestável e outra termolábil. A bactéria é responsável pela doença conhecida como diarreia do viajante, afetando pessoas que viajam para países subdesenvolvidos, com situações insatisfatórias de higiene e tratamento insuficiente de água (SHEIKH, 2019).

A *E. coli* enteroinvasiva (EIEC) é bastante relacionada com espécies de *Shigella* por apresentarem aspectos semelhantes com respeito à virulência, aspectos bioquímicos, genéticos e patogênicos. Esse grupo é conhecido por causar uma colite inflamatória invasiva no hospedeiro, culminando em disenteria e febre, além da presença de sangue e muco nas fezes (NAVE et al., 2016).

Denominada de *E. coli* entero-agregativa (EAggEC ou EAEC) por apresentar um comportamento especial na colonização descrito como “empilhamento de tijolos”, esse grupo causa diarreia aquosa persistente, chegando a durar mais de 14 dias. São produtoras de citotoxinas e enterotoxinas, e muitas vezes estão associadas à má nutrição e retardo de crescimento (KNEIFEL; FORSYTHE, 2017).

E. coli difusamente aderente (DAEC) tem um padrão de aderência distinto das outras categorias descritas anteriormente. Pode expressar diferentes fatores de virulência, podendo ser considerada ETEC ou EPEC, segundo algumas análises de bioinformática. Além disso, algumas cepas causam infecções extraintestinais e diarreia em crianças. Sua patogenicidade ainda não está completamente esclarecida (DUBREUIL, 2010; SERVIN, 2014; ROBINS-BROWNE et al., 2016).

E. coli aderente-invasiva (AIEC) não costuma causar diarreia, mas acredita-se que seja associada ao aparecimento da doença de Crohn. Além disso, tem a capacidade de se aderir e invadir o tecido epitelial, se multiplicando dentro de macrófagos (ALHAGAMHMAD et al., 2016; ROBINS-BROWNE et al., 2016).

A *E. coli* produtora de toxina Shiga (STEC) é relatada por ser um importante patógeno de origem alimentar, frequentemente relacionado a vários surtos devido à ação de potentes toxinas que podem causar danos às células intestinais (FRANZ et al., 2015). A patogênese da STEC está primariamente associada à toxina Shiga, codificada pelos genes *stx*, presentes na ilha de patogenicidade do LEE (*locus of enterocyte effacement*) (CASTRO et al., 2017). Duas variantes de toxina são conhecidas: Stx1, descrita pela primeira vez na *Shigella dysenteriae*, mas também produzida por cepas STEC; e Stx2, que é imunologicamente distinta, mas muito semelhante à Stx1 no seu modo de ação. As cepas de STEC podem produzir uma ou ambas as toxinas (MELTON-CELSA, 2014). No entanto, as produtoras de Stx2 são frequentemente relacionados com casos mais graves de infecção (STEINER, 2016). A *E. coli* enterohemorrágica (EHEC) está dentro do grupo da STEC. Como tal, é capaz de codificar a Shiga toxina, sendo caracterizada por causar infecções mais intensas, como a colite hemorrágica (CH) e a síndrome hemolítico-urêmica (SHU), podendo levar o hospedeiro à morte (ROBINS-BROWNE et al., 2016). O sorotipo mais conhecido envolvido em casos de doenças causadas por EHEC é o O157:H7, que costuma produzir diversos fatores de virulência, como enterohemolisina e fatores de agregação celular, (FELDISINE et al., 2016).

Além da produção de Shiga toxina, podem ser citados outros mecanismos de virulência relatados de STEC, como por exemplo outros genes (*eae*, *tir*, *espA*, *espB*, *espC* e *espD*) presentes na ilha de patogenicidade do LEE, que são genes relacionados com aderência, iniciação da transdução do sinal do hospedeiro e lesões de fixação e apagamento (BOLTON, 2011). Segundo Castro et al. (2017), o LEE é divido em cinco operons, indo de LEE-1 ao LEE-5. As regiões 1, 2 e 3 codificam o sistema de secreção do tipo III (T3SS), responsável por carrear as proteínas bacterianas até o enterócito. Já a região 4 forma uma espécie de canal por onde o T3SS consegue transportar as proteínas efetoras. Há ainda a região 5, onde estão localizados os genes de adesão, incluindo o receptor de Intimina (TIR), o gene *eaeA* para Intimina e o *cesT*, para a chaperona TIR.

Após o contato com o enterócito, a STEC injeta proteínas de ação citotóxica, se ligando ao T3SS, que funciona como uma ponte entre a bactéria e a célula. A infecção continua com a ligação da Tir com a Intimina, formando um vínculo resistente entre a bactéria e a célula infectada, mediante a formação de um pedestal formado pela acumulação de actina, resultado da alteração do citoesqueleto celular (LARA-OCHOA et al., 2010; CASTRO et al., 2017). Nesse momento, a STEC inicia a produção de Shiga toxina, constituída da subunidade A, que apresenta ação tóxica e inibitória da síntese proteica das células, e de cinco subunidades B, responsáveis pela ligação da toxina no receptor celular Gb3 para internalização da toxina na

célula. A toxina é então transportada para o complexo de Golgi e retículo endoplasmático, onde a subunidade A se liga à subunidade *N-glycosidase* da célula (CASTRO et al., 2017). Essa ligação atua destruindo a porção 28S rRNA, interrompendo a síntese de proteínas, o que leva à morte celular (MELTON-CELSA et al., 2011).

Além dos danos causados às células intestinais, a STEC é capaz de causar outras lesões sistêmicas, incluindo diarreia sanguinolenta (colite hemorrágica), e mais seriamente, síndrome urêmica hemolítica (SHU) (CASTRO et al., 2017). Jovens e idosos são mais propensos a desenvolver complicações perigosas, como SHU, insuficiência renal aguda e sintomas neurológicos como sequelas (LEE et al., 2016). Não existe um tratamento estabelecido para infecção por STEC, sendo recomendado ao paciente hidratação e terapia suporte (RAHAL et al., 2015). Isso acontece porque o uso de antibióticos pode propiciar o desenvolvimento de SHU em alguns pacientes por induzir a produção e liberação intracelular de Shiga toxina, sendo, então, contraindicado (HOWARD-VARONA et al., 2018).

3 *Escherichia coli* E STEC EM LEITES E DERIVADOS

Surtos causados por *E. coli* e STEC estão sendo relatados com mais frequência, pois os laboratórios de vigilância estão aprimorando técnicas de detecção desses surtos e implementando técnicas para isolar o agente etiológico. Dados do Ministério da Saúde revelam que, no Brasil, de 2009 a 2018 foram contabilizados 6.809 surtos de doenças veiculadas por alimentos (DVA), com mais de 120 mil pessoas doentes durante esses anos, e 9 óbitos relacionados em 2018. Ainda, a região Sudeste aparece com mais ocorrências, seguida das regiões Nordeste e Sul. A *Escherichia coli* aparece no topo dos agentes etiológicos envolvidos nesses surtos, sendo, inclusive, o agente etiológico mais identificado laboratorialmente nos anos de 2017 e 2018. Leite e derivados são a quarta maior causa dos surtos (7,8%), atrás apenas de surtos causados por alimentos mistos (25,5%), água (21,1%) e múltiplos alimentos (10,7%), revelando a importância da inocuidade desse tipo de produto para a saúde pública (BRASIL, 2019). Em outros países onde a vigilância é mais apurada, como nos Estados Unidos, dados mais recentes de 2015 informam que 4.831 casos de infecções por STEC foram confirmados no país, o que representa um aumento de 9% em relação ao ano de 2014 (CDC, 2017).

Como a principal forma de infecção por *E. coli* e STEC se dá pela via fecal-oral, as fontes mais comuns de contaminação são a água e os alimentos contaminados, principalmente pelo contato direto dos alimentos com fezes (KOUDELKA; ARNOLD; CHAKRABORTY, 2018). Além disso, as infecções também podem ocorrer por contato direto com pessoas infectadas e

animais de produção ou companhia que albergam a bactéria (KINTZ et al., 2017). De fato, como o bovino é reconhecido como uma das principais fontes de eliminação de STEC no ambiente, o leite pode ser exposto a esse patógeno por contaminação fecal durante as etapas de obtenção do leite (BARTH et al., 2016).

Tanto para leite quanto para derivados lácteos, o risco de contaminação por STEC começa na obtenção da matéria-prima. Como evidência, STEC já foi relatada no leite cru de diferentes espécies zootécnicas, como vaca, ovelha e cabra (BANDYOPADHYAY et al., 2012; NOBILI et al., 2016; GONZALES-BARRON et al., 2017; OTERO et al., 2017). Por conta disso, a utilização da pasteurização para o consumo e produção de derivados é altamente indicada, uma vez que é sabido que essas bactérias não conseguem sobrevier a esse processo térmico (AHMED; SAMER, 2017). Assim, a Instrução Normativa nº 76, de 30 de novembro de 2018, sugere a utilização do binômio tempo/temperatura de 72°C a 75°C por 15 a 20s para a produção de leite pasteurizado (BRASIL, 2018). Além disso, diferentes binômios podem ser utilizados para a produção de outros produtos derivados. No entanto, STEC também pode contaminar leite e derivados durante as etapas subsequentes à pasteurização, podendo persistir no produto final (CARDOSO; MARIN, 2016).

Diversos estudos mostram a presença de STEC em leites e derivados em diferentes etapas do seu processo de fabricação. Por exemplo, Rios et al. (2019) relataram a presença de STEC em amostras de leite bovino coletadas na região de Castilla y León, na Espanha. De um total de 214 amostras, 5 (2,3%) foram positivas para o microrganismo procurado, eram produtoras de *stx1* e *stx2*, além de duas delas serem produtoras de enterohemolisina. Num outro estudo realizado na mesma localidade, Álvarez-Suárez et al. (2016) analisaram seis isolados de STEC provenientes de leite caprino. Como resultado, uma cepa foi positiva para *stx1*, uma para *stx2* e quatro codificavam as duas toxinas, além de observarem isolados positivos para *eae*, indicando alto potencial virulento dessas cepas. Outro estudo realizado no Egito com leite cru de búfala e leite pasteurizado de vaca revelou a presença de duas cepas STEC positivas para *stx1*, uma positiva para *stx2* e quatro positivas para os dois genes, sendo que duas eram também positivas para o fator de virulência *eaeA* (AHMED; SAMER, 2017).

Os principais casos relacionados à presença de STEC em derivados lácteos vêm do consumo de queijo feito a partir de leite cru. Esse produto está relacionado com aspectos sensoriais mais intensos, em comparação com o queijo elaborado com leite pasteurizado (YOON; LEE; CHOI, 2016). No entanto, por questões de segurança do consumidor e inocuidade do produto, a produção de queijo a partir de leite não pasteurizado só é permitida se o queijo passar por um processo de maturação de no mínimo 60 dias, a temperaturas elevadas

(≥ 2°C) (FDA, 2017). Contudo, diversos estudos realizados em diferentes partes do mundo já relataram que o queijo feito de leite cru pode abrigar cepas de STEC, independente do período de maturação. Por exemplo, em um trabalho realizado em Lima, no Peru, Mora et al. (2007) detectaram oito cepas STEC pertencente ao sorogrupo O157:H7 em 102 amostras de queijo feito a partir de leite cru comercializados em diferentes mercados na cidade. Um estudo realizado na Turquia revelou a presença de duas cepas de *E. coli* O157:H7 em 50 amostras de queijo branco em conserva feito a partir de leite cru (ÖKSÜZ et al., 2004). Em trabalho realizado na Suíça, cepas de STEC foram isoladas de queijo semiduro maturado de vaca e de cabra, onde algumas cepas foram também positivas para a presença de produção de hemolisina, caracterizando perigo para o consumo (STEPHAN et al., 2008). Além disso, cepas de STEC foram isoladas de queijo de leite de ovelha cru espanhol amadurecido por dois meses e meio, e também por 12 meses em fábricas de queijos, mostrando que cepas de STEC são capazes de sobreviver ao processo de maturação (CARO; GARCÍA-ARMESTO, 2007). Na Itália, Nobili et al. (2016) reportaram a presença de três cepas STEC positivas para *stx2* provenientes de duas amostras de leite cru e uma amostra de queijo muçarela feito a partir de leite cru. Ainda, os autores ressaltaram que o queijo estudado foi produzido com leite originado da fazenda onde as duas amostras positivas foram coletadas, revelando uma possível fonte de contaminação.

Poucos relatos mostram que cepas STEC também podem ser isoladas de produtos lácteos pasteurizados, provavelmente devido a processos de pós-contaminação (FARROKH et al., 2013; FEREYDOUNI; DARBOUY, 2015; CALLON; ARLIGUIE; MONTEL 2016, CARDOSO; MARIN, 2017). Em um deles realizado no Brasil, Carvalho et al. (2014) relataram a presença de STEC em dois queijos minas frescal vendidos em feiras livres na cidade de Goiânia, Goiás.

4 SUSCEPTIBILIDADE E RESISTÊNCIA DE STEC A AGENTES ANTIMICROBIANOS

Durante muitos anos, os agentes antimicrobianos vêm sendo utilizados na medicina humana e veterinária com o objetivo de combater infecções causadas por microrganismos. A maioria dos medicamentos antimicrobianos é produzida a partir de compostos naturais de origem microbiana, além de versões sintéticas dos mesmos, com pouquíssimas drogas sendo totalmente sintéticas. Por conta disso, a resistência aos antimicrobianos pode ser considerada como uma representação da teoria darwiniana de competição a partir de moléculas antimicrobianas derivadas de microrganismos naturais (HOLMES et al., 2016). Além disso, o

uso generalizado desses medicamentos acaba gerando uma pressão de seleção, acelerando o desenvolvimento da resistência (MARSTON et al., 2016). De fato, os antimicrobianos estão entre as drogas mais comumente prescritas na medicina humana, sendo que cerca de 50% dos antibióticos prescritos são considerados desnecessários (CDC, 2013).

Na produção animal, o uso de antimicrobianos está relacionado com a manutenção da saúde e aumento de produção do rebanho. Nos Estados Unidos, cerca de 80% do consumo anual de antimicrobianos é utilizado na produção animal com finalidade terapêutica, profilática e como promotor de crescimento, sendo que uma parte significante desses mesmos medicamentos é também utilizada na medicina humana para o tratamento de infecções e procedimentos médicos como cirurgias, transplantes de órgãos e quimioterapia. Essa prática contribui para a disseminação de patógenos resistentes a medicamentos, tanto em animais quanto em humanos, representando uma ameaça significativa à saúde pública (VAN BOECKEL et al., 2015).

Entendendo os riscos do uso extensivo de antimicrobianos na medicina veterinária, países europeus vêm reduzindo ou suspendendo o uso dessas drogas como promotores de crescimento numa tentativa de diminuir a prevalência de bactérias resistentes que possam causar qualquer risco à saúde humana. Em 1986, a Suécia decidiu banir o uso de antimicrobianos como promotores de crescimento. Durante a década de 90, outros países como Dinamarca e Alemanha decidiram proibir o uso de avoparcina como promotor de crescimento, baseado na descoberta de cepas de *Enterococcus* resistentes à vancomicina em suínos e poedeiras suplementadas com avoparcina, sendo proibido também por toda União Europeia tempo depois. Em 2006, a UE decidiu banir completamente a utilização de antibióticos como promotores de crescimento em animais (SPEKSNIJDER et al., 2014). Em 2010, o Brasil esteve dentro dos cinco países com maior participação em consumo de antimicrobianos para produção de alimentos com 9% de participação, atrás apenas da China (23%) e Estados Unidos (13%). Estima-se que em 2030, o Brasil passe a participar em 8% no consumo desses medicamentos. Isso porque, apesar de estar entre os maiores consumidores de antibióticos no momento, o país já iniciou mudanças no sistema de produção animal para um uso mais responsável dos mesmos (VAN BOECKEL et al., 2015).

A resistência a antimicrobianos pode ocorrer por mutações espontâneas ou pela aquisição de genes que conferem essa característica, por meio da transferência horizontal de genes (THG), considerado o fator mais importante (VON WINTERSDORFF et al., 2016). Não apenas os genes de resistência a antibióticos encontrados em patógenos clínicos são relevantes, mas todas

as bactérias patogênicas, comensais e ambientais formam um reservatório desses genes, a partir do qual as bactérias patogênicas podem adquirir resistência via THG (GILLINGS, 2017).

Os mecanismos da THG são a transformação, transdução e conjugação, esse último sendo o mais importante. Na transformação, a bactéria é capaz de captar, integrar e expressar funcionalmente fragmentos de DNA extracelular (VON WINTERSDORFF et al., 2016). Para tal, é preciso que haja um fragmento de DNA no meio extracelular, a bactéria receptora precisa estar num estado de competência, e o DNA translocado deve ser estabilizado por integração no genoma receptor ou por recircularização (no caso de DNA plasmidial) (THOMAS; NIELSEN, 2005). O segundo mecanismo, a transdução, depende da ação de bacteriófagos. Nele, os bacteriófagos são capazes de transferir genes que são vantajosos para seus hospedeiros microbianos e, por sua vez, promovem sua própria sobrevivência e disseminação (BROWN-JAQUE; CALERO-CÁCERES; MUNIESA, 2015; VON WINTERSDORFF et al., 2016). Já a conjugação é a transferência de DNA através de um processo que requer o contato célula com célula, sendo uma doadora e uma receptora do material genético, por meio de pili ou adesinas (VON WINTERSDORFF et al., 2016). Embora a transferência de genes de resistência também possa ocorrer através da transformação ou da transdução, a conjugação é frequentemente considerada como o mecanismo responsável mais comum. Isso se deve ao fato do mecanismo fornecer uma melhor proteção ao ambiente extracelular, além de ser um meio mais eficiente do material genético entrar na célula hospedeira do que os outros mecanismos (WOZNIAK; WALDOR, 2010; VON WINTERSDORFF et al., 2016).

A resistência a antimicrobianos de STEC em animais de produção foi observada por Cabal et al. (2016), estudando bactérias isoladas de fezes e couro, em um rebanho bovino na Espanha. Os autores observaram cepas resistentes aos antimicrobianos testados, onde três isolados apresentaram resistência a nove diferentes antibióticos simultaneamente, revelando cepas multidroga resistentes. Os antibióticos que apresentaram resistência mais frequente foram: sulfametoxazol (62,5%), tetraciclina (57,8%), estreptomicina (57,8%) e trimetropina (50%). Os autores conseguiram ainda observar uma associação positiva entre a resistência desses compostos com a capacidade da cepa de codificar o gene *stx2*. Em outro estudo, Gupta, Sen e Das (2018) isolaram, de búfalos saudáveis criados em pequenas propriedades, 11 cepas STEC do total de 100 amostras de *swab* retal dos animais. Dessas, 10 isolados eram resistentes a tetraciclina, oito eram resistentes a sultametoxazol-trimetropina, seis foram resistentes a eritromicina, e seis a ampicilina, demonstrando que búfalos saudáveis também podem ser fonte de infecção para STEC. Ainda, Kennedy et al. (2017) analisaram 146 isolados de STEC não-O157 provenientes de fazendas e abatedouros irlandeses. Como resultado, 82% dos isolados

eram multirresistentes aos antimicrobianos testados, com perfis variando de três a 10 antibióticos. A resistência antimicrobiana foi significativamente maior nos isolados de abatedouro em comparação com os isolados provenientes da fazenda. Ainda, a resistência às sulfonamidas (99%), tetraciclina (82%), ampicilina (53%), cefalotina (31%), neomicina (26%), canamicina (21%), trimetoprim (17%) e cloranfenicol (5%), foi associada a transferência de plasmídeos.

Ntuli, Njage e Buys (2016) identificaram, em 258 amostras de leite cru e pasteurizado distribuídos a granel na África do Sul, 36% de amostras positivas para *E. coli*. Aproximadamente 60% (n=89) dos isolados foram considerados multidroga resistentes, com maior prevalência de resistência à cefalotina (51% dos isolados), neomicina (34%), ampicilina (24%), amoxicilina (23%) e oxitetraciclina (17%), sugerindo a exposição dessas bactérias a esses antibióticos em algum momento. Em outro estudo, Elmonir, Abo-Remela e Sobeih (2018) isolaram, em amostras de leite cru vendido em mercados informais no Egito, 22 cepas de *E. coli*, onde cinco foram confirmadas STEC. Os autores observaram que a maioria dos isolados de *E. coli* (87,5%) foi multidroga resistente, apresentando resistência a três ou mais classes de antibióticos. As maiores taxas de resistência foram para cefalotina (87,5%), seguida de ampicilina (68,8%), e tetraciclina (68,8%). Ahmed e Shimamoto (2015) isolaram STEC O157:H7 em 20 amostras de leite e nove de queijo vendidas no Egito. A incidência de STEC O157:H7 multidroga resistente foi de 58,6%, com uma cepa proveniente de leite e uma de queijo chegando a apresentar resistência a 18 antibióticos diferentes. Resistência a ampicilina e a amoxicilina + clavulanato foi observada em quase todas as cepas multidroga resistentes.

Ainda, Chaleshtori et al. (2017) detectaram 11 cepas STEC em iogurte, queijo e sorvete feitos a partir de leite cru, no Iran. Todas as amostras eram resistentes a um ou mais antibióticos, sendo que todas foram resistentes a amoxicilina + clavulanato, ampicilina, nitrofurantoína, tetraciclina, canamicina e gentamicina. Além disso, duas cepas foram resistentes a 12 antibióticos, quatro foram resistentes a 11, quatro resistentes a 10 e uma resistente a 9, corroborando com o estudo anterior. Os autores descreveram ainda uma associação positiva entre a presença de genes *stx1* e *stx2* com a resistência antimicrobiana. Em outro estudo, Rosa-Hernández et al. (2018) identificaram, em amostras de queijo frescal vendidas em mercados locais no México, 10 cepas STEC resistentes a pelo menos cinco antibióticos testados (amoxicilina + clavulanato, amicacina, eritromicina, gentamicina, colistina e canamicina). Uma das cepas foi resistente a 14 dos 16 antibióticos testados, outra cepa foi resistente a 12, duas resistentes a 11 e três resistentes a 10, revelando que os queijos, produzidos a partir de leite cru, são um risco para o consumidor.

Por conta do crescente número de casos de bactérias resistentes a antibióticos comumente utilizados, a descoberta de novos compostos bactericidas é importante. Os produtos naturais são uma das principais fontes de novos compostos que podem ser utilizados na fabricação de drogas atualmente. Esses compostos podem ser derivados de bactérias, microrganismos eucarióticos, plantas e vários organismos animais, embora os produtos microbianos e vegetais ocupem a maior parte dos compostos antimicrobianos descobertos até agora (BALOUIRI; SADIKI; IBNSOUDA, 2016).

Ribeiro et al. (2013) avaliaram o potencial antimicrobiano do óleo essencial de alecrim (*Rosmarinus officinalis* L.) inoculado em queijo coalho, contra cepas de *E. coli*. Os autores observaram um efeito inibitório após a adição do óleo, resultando em contagens negativas e/ou muito baixas da bactéria, podendo ser usado como aditivo para preservação do alimento. Amrutha, Sundar e Shetty (2017) desenvolveram uma nano emulsão a base de óleo essencial de cominho (*Cuminum cyminum*) e outra a base de óleo essencial de pimenta (*Piper nigrum*), que reduziram efetivamente a comunicação e a formação de biofilme, além de reduzir a produção de exopolisacarídeos, em bactérias como *E. coli* e *Salmonella*. A adição desses compostos em alimentos pode ajudar a reduzir naturalmente o número de bactérias patogênicas presentes. Em outro estudo, Marcial et al. (2016), demonstrou que a adição de óleo essencial de orégano (*Origanum vulgare* var *hirtum*) em queijo argentino evitou o crescimento de enterobactérias, sem mudar a aceitabilidade sensorial do produto, o que pode prolongar a validade comercial desse tipo produto.

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CAPITULO II

ARTIGO DE REVISÃO

Everybody loves cheese: the survival and persistence mechanisms of Shiga-toxin *E. coli* during cheese manufacturing

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LIST OF CHAPTER TITLES

ABSTRACT:

Cheese manufacturing involves high temperatures, fermentation and ripening steps that function as hurdles to microbial growth. On the other hand, the application of several different formulations and manufacturing techniques may create a bacterial protective environment. The persistent behavior of *Escherichia coli*, especially Shiga toxin-producing *E. coli* (STEC), in cheese relies in complex mechanisms that enable bacteria to respond to stressful conditions found in cheese matrix. In this review, we discuss how STEC manage to survive to high temperatures, hyperosmotic conditions, exposure to weak organic acids and pH decreasing related to cheese manufacturing, storage and the cheese matrix itself. Moreover, we discuss how these stress responses interact to each other by enhancing adaptation and consequently persistence of STEC in cheese. Further, we show how virulence genes can be affected by stress response mechanisms, inclusing the increasing of cell adherence and virulence factors production. These evidence show the ability of pre-adapted stressed STEC cells to persist in cheese matrix, which compromises the satefy of cheese products leading to the selection of even more resistant and virulent pathogens, affecting consumers of all ages.

Keywords: STEC, stress responses, cross protection, acid stress, osmotic stress, heat shock

1. Introduction

Milk and cheese consumption are dated from a long time ago, probably when humans understood domestication, following the nutritious and beneficial value such products can provide (Fox and McSweeney, 2004; Gross, 2018). Originally, cheese has been created in an attempt to concentrate and conserve milk in a stable state so it can be consumed posteriorly (Yoon, Lee and Choi, 2016). In fact, the development of techniques such as pasteurization, renneting and the utilization of starter cultures brought cheesemaking to another level of processing, thus enabling cheese diffusion around the world (Bennet and Johnston, 2004; McSweeney, Ottogalli and Fox, 2004; Johnson, 2017).

The cheesemaking process includes different manufacturing steps that work as hurdles, thus averting microbial proliferation. These steps include the filtration and clarification of milk, the high temperatures in pasteurization and contact with cooking processes, the addition of salt or contact with brine, fermentation processes, decreasing of water activity, addition or production of weak organic acids by lactic acid bacteria, among others. Cheese is considered a ready-to-eat food, not requiring further cooking or processing prior to its consumption (Moubarac et al., 2014). As the majority of the microorganisms are intended to be inactivated after proper milk pasteurization, the subsequent manufacturing steps plus the cheese intrinsic factors should be able to guarantee the safety of the final product, unless post contamination occurs (Fromm and Boor, 2004; Martin et al., 2016). Therefore, as raw milk cheese lacks the pasteurization process, it should be consumed carefully, especially those of soft and semisoft varieties, which are more prone to harbor foodborne pathogens than those of hard varieties (Leistner, 2000; Andreoletti et al., 2007).

Shiga toxin-producing *Escherichia coli* (STEC) is reported for being an important foodborne pathogen, often related to several outbreaks. STEC can produce toxins that may cause intestinal cells damage like bloody diarrhea (hemorrhagic colitis), and most seriously,

extracellular damage, leading to hemolytic uremic syndrome (HUS) (Castro et al., 2017). The main virulence factors associated with STEC are the Shiga toxins Stx1 and Stx2, whereas STEC strains can produce either, one or both toxins. Other virulence factors produce aggregation and hemorrhagic lesions (Steiner, 2016). Milk may be exposed to STEC by the contamination of bovine feces during milking steps, as bovine are recognized as one of the main sources of STEC shedding (Barth et al., 2016). To overcome this situation, the utilization of pasteurized milk for cheese production is highly indicated (Ahmed and Samer, 2017). However, STEC can also contaminate cheese during the manufacturing and post-processing steps, being which enables the microorganism to persist in the final product (Cardoso and Marin, 2016). Figure 1 shows the possible routes of contamination in a general pasteurized cheese making process (Modified from Peng et al., 2011). STEC persistence in cheese matrices is achieved through survival response mechanisms that enables the pathogen to surpass harsh conditions found in these products, becoming a risk for human consumption.

In this context, the aim of this review is (i) to considerate the cell response mechanisms promoted by *E. coli*, and more importantly Shiga toxin-producing *E. coli*, which enables the persistence of this pathogen in cheese products, and (ii) how they could enhance the pathogen virulence in cheese and cheese products. Discussion about heat shock, osmotic and acidic stress responses are pointed out, as well as the relation between stress response mechanisms and virulence traits.

2. Cheese manufacturing: an overview

Cheese is the main dairy product consumed around the world (Fox et al., 2017). It is produced from milk of different species in a variety of both taste and shapes, presenting distinct rheological characteristics (Silva and Costa, 2019). Although considered a very nourishing product, its nutritional composition can differ not only due to its raw material characteristics

(animal species, breed, lactation state and percentage of milk fat) but also according to the manufacturing steps it is submitted (Skeie, 2010).

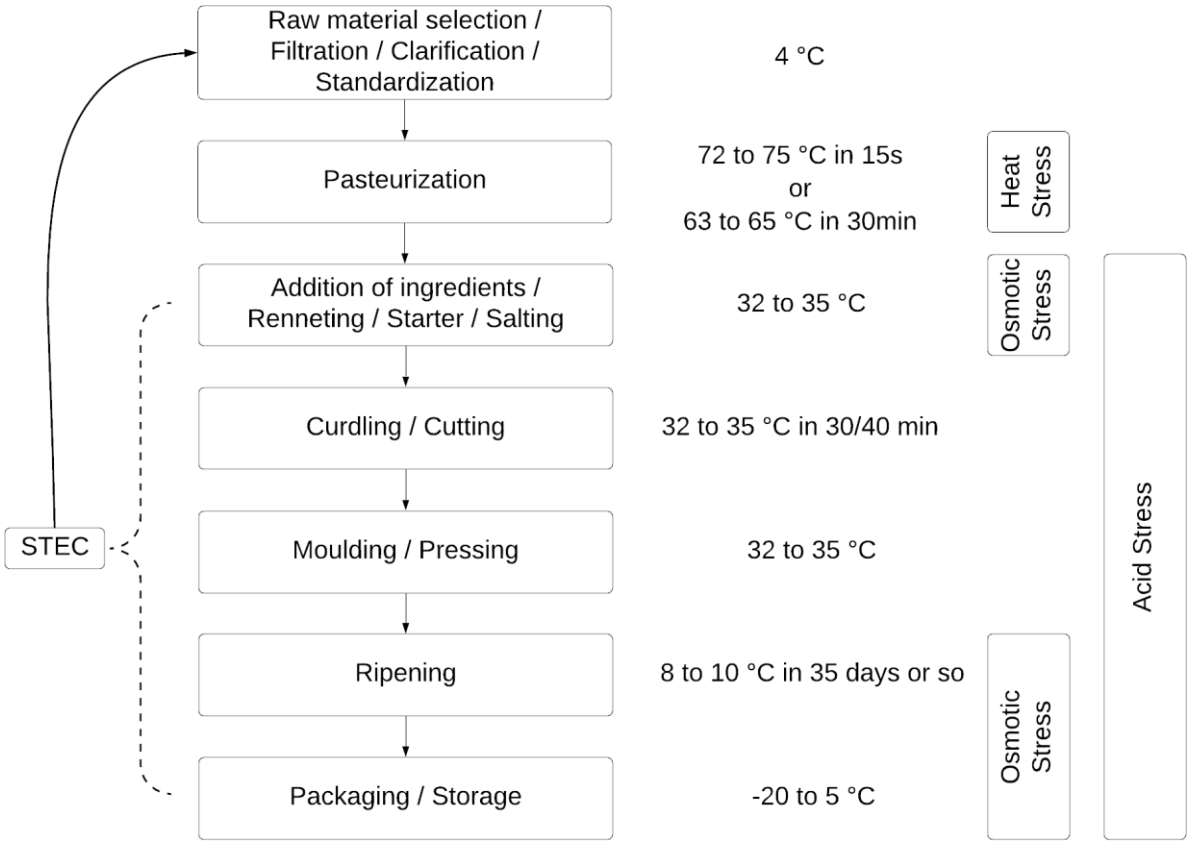

Figure 1: Flow chart of production of a general pasteurized cheese, including stresses conditions. Solid line represents standard contamination of STEC. Dashed line represents post-pasteurization contamination of STEC.

Immediately after milking, milk can be stored at the farm, generally at 4 °C, and then transported to the processing plant. In an optimum situation, transportation should be done as soon as possible. Once, the maintenance of raw milk at low temperatures for a long time can favor the psychrotrophic bacteria growth, which may affect the general quality of the final product due to the production of proteolytic and lipolytic enzymes (Oliveira et al., 2015). In a general cheese preparation, milk is usually filtered and clarified before cheesemaking. These

steps are responsible for the mechanical removal of milk impurities, such as hay, hair, or even clumps of somatic cells. The filtration step consists in using a cloth or a synthetic material filter to hold such undesirable solids. Similarly, the clarification step uses a two-phase centrifuge to remove materials heavier than milk proteins and fat. In addition, milk can undergo separation, which consists in utilizing a three-phase centrifuge that separates milk into cream, skim milk, and a third phase consisting of impurities, which improves milk quality. Further, milk can be standardized/homogenized regarding its fat content and then submitted to cheese manufacturing (Johnson, 2016).

The cheese production can be performed with pasteurized or unpasteurized milk. The milk pasteurization for cheesemaking can be made out of two processes: flash pasteurization (also called “high-temperature-short-time”, HTST), and low-temperature pasteurization (also called “low-temperature-long-time”, LTLT or vat/batch pasteurization). Such processes aim to improve cheese safety by elimination of pathogenic bacteria and decreasing spoilage bacteria in milk. The HTST is the most used process because it reduces costs and improves efficiency. In this process, the milk is heated at 72-75 °C for 15 seconds, usually in a plate heat exchanger pasteurizer. In this pasteurization, the cold milk is heated by the pasteurized milk warmness, whereas a partial cooling of this one is achieved, being 90% more efficient. Contrasting, the LTLT pasteurization consists of heating the milk at 63-65 °C for 30 minutes. It can be carried out in a double-jacketed tank, where immediately after the treatment, cold water is circulated for later cooling. Milk must be kept under mechanical agitation to facilitate the heat exchange and prevent the product from burning on the walls. This process is practically out of use in the industry due to economic and technological aspects. Thus,. Moreover, the milk should be rapidly cooled down to 32°C because not all microorganisms are destroyed and neither all enzymes are inactivated (Meunier-Goddik and Sandra, 2016).

On the other hand, cheese production from unpasteurized milk is only allowed for cheese with a maturation step of a minimum of 60 days at high temperatures (≥ 2 °C) (FDA, 2015). This regulation was created as a response of several foodborne outbreaks caused due to the consumption of raw milk cheese before the year of 1949 (Knoll, 2005). In fact, raw milk cheese consumption is associated with several foodborne outbreaks (Farrokh et al., 2013; Costard et al., 2017; Artursson et al., 2018).

The curdling step is present in all varieties of cheese, being the phase where the transformation of milk into curd occurs (Figure 1). The milk curd can be defined as a gel consisting mainly of the concentration of precipitated casein (milk protein) and fat, whereas the major amount of lactose, whey proteins, and soluble solids are removed together with whey. The casein precipitation can be achieved over acid coagulation, heat-acid coagulation, or enzymatic coagulation (renneting). Briefly, acid coagulation requires a low pH environment (pH ~4.6), achieved by accumulation or addition of organic acids. Because acid coagulation forms a fragile curd, this technique is used to produce soft cheese (Lucey, 2011). Contrasting, enzymatic coagulation requires the addition of a rennet, which breaks the κ-casein fraction in a specific spot, thus culminating in protein precipitation and curdling. This reaction does not need an acidic environment to happen, and it is the most used technique upon cheesemaking (Gregersen and Lucey, 2016).

Ripening (maturation) is the step where cheese is kept under controlled temperature and humidity, whereas numerous microbiological, biochemical, physical, and chemical reactions occur (Fox et al., 2017b). Ripening involves not only hydrolysis of protein and fat, and lactose fermentation, but also the synthesis of aromatic compounds formed due to protein and fat degradation (Diezhandino et al., 2015; McSweeney, 2017). In addition, the lactose fermentation is one of the most important reactions that occurs during cheesemaking. It can be done by a variety of lactic acid bacteria, generally mesophilic or thermophilic, via starter culture addition,

which will result in a decrease of pH caused by lactic acid production, thus affecting the final product characteristics and sensory features (Bekele et al., 2018). Moreover, organic acids production, in different types of cheese, may vary according to the ripening conditions and time, plus the chosen starter culture (Costa and Conte-Junior, 2015). Currently, the most used starter cultures in cheese are *Lactococcus lactis*, *Leuconostoc* sp., *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp., *lactis*, *Lactobacillus delbrueckii* subsp., *bulgaricus*, and *Lactobacillus. helveticus*, which should be used based on the type of cheese wanted (Engels, Dusterhoft and Huppertz, 2017). In addition, cheese is considered one of the best matrices for probiotic addition. Strains of *Lactobacillus* spp. and *Bifidobacterium* spp. are constantly being added to cheese in order to improve health quality of consumers, besides being suitable for industrial production (Blaiotta et al., 2017).

Moreover, cheese can be added with spices, extracts, herbs, and condiments to improve taste and aroma. Such addition is important because besides improving the product flavour, some of these ingredients show influence in the microbiological quality of the final product (Hayaloglu and Farkye, 2011). For example, the addition of oregano essential oil (*Origanum vulgare* var *hirtum*) in Argentinian cheese avoided the growth of enterobacteria without changing the cheese acceptance (Marcial et al., 2016). Likewise, Moreira et al. (2019) added a waste extract of pequi (*Caryocar brasiliense*), a fruit containing high phenolic compound concentrations, and observed no bacterial growth, such as *Staphylococcus*, coliforms and *E. coli*, and Enterobacteriaceae in goat cheese during a storage time of 21 days.

Finally, the storage step, when cheese is kept refrigerated prior or after selling, seems to affect cheese sensory characteristics: changes in pH, acidity, texture, elasticity, and color are frequently observed (Felicio et al., 2016). These effects are caused mainly due to proteolysis, residual lactose fermentation and also calcium dissociation. As storage time reduces the gradual

quality of cheeses, this product should not be kept under storage for extended periods (Fuentes et al., 2015; Delgado-Martínez et al., 2019).

3. STEC survival and persistence in cheese and cheese products

Outbreaks of STEC are becoming reported more frequently, as the surveillance laboratories are improving techniques for etiology agent detection. For instance, most recent data of 2015 stated that 4,831 cases of STEC infections were confirmed in United States, which is 9% more than in 2014 (CDC, 2017). As STEC are fecal-oral bacteria, the most common source of infection is contaminated food and water, mainly because of direct contact of food with cattle feces (Koudelka, Arnold and Chakraborty, 2018). Moreover, infections are also supposed to happen through direct contact with infected people, and STEC-hosting animals (Kintz et al., 2017). Regarding cheese products, the risk of STEC contamination starts at obtaining the raw material. As evidence, STEC has been isolated in raw milk from different zootechny species, such as cow, ewe, goat, and yak (Bandyopadhyay et al., 2012; Nobili et al., 2016; Gonzales-Barron et al., 2017; Otero et al., 2017). While the major related cases of STEC-containing cheese come from raw milk cheese production, a few reports had shown that STEC could be isolated from pasteurized cheese products, probably due to post contamination processes (Fereydouni and Darbouy, 2015; Callon, Arliguie and Montel, 2016; Cardoso and Marin, 2017).

As mentioned before, cheese has a great variety of formulations, culminating in several different types of products. Currently, there is no official classification on cheese based on its characteristics. However, a classic general classification (Davis, 1965) based on moisture property of cheese is still accepted nowadays. For instance, very hard cheeses like Parmigiano Reggiano present the lowest moisture percentage (less than 25%), moderate low pH (5.0-5.5), and lactic acid content between 0.8-1.2%, whereas hard cheeses, such as Emmental, show a

moisture percentage between 25 and 26% and moderate low pH (5.4-5.5). On the other hand, semihard cheeses, such as Cheddar possess an average of 36 to 40% of moisture, moderate low pH (5.0-5.4), and soft cheeses like Camembert usually have more than 40% of moisture content and lower pH (4.6-5.2) (Lawrence et al., 2004; Gobbetti, 2004; Deegan et al., 2013; Moller et al., 2013; Batty, Waite-Cusic and Meunier-Goddik, 2019). These intrinsic characteristics should function as hurdles to prevent bacterial growth (Leistner, 2000), although cheese from several varieties and types have been described as source of STEC contamination or outbreak, as cited previously.

Different developing and developed countries have created standards regarding the presence of commensal or pathogenic *E. coli* in food. For instance, the Hong Kong Centre for Food Safety (2007) establishes *E. coli* as the only indicator organism for ready-to-eat foods, considering the presence of *E. coli* O157 unacceptable for consumption. Further, the Food and Drug Administration (FDA, 2013) states that the presence of *E. coli* in cheese and cheese products should not surpass 110 MPN/g. In addition, processed cheese spread must be absent for *E. coli*. Similarly, the European Commission (2005) establishes that the presence of *E. coli* in heat-treated cheese should be lower than 1000 CFU/g. More recently, the Food Standards Australia New Zealand (2016) attributes the presence of STEC in ready-to-eat food as potentially hazardous. Besides that, some developing countries such as Brazil still lacks an actualized legislation where *E. coli* presence in food should be considered (Brasil, 2001).

Several studies have been shown the fate of STEC strains in cheese and cheese products regarding its manufacturing process. Raw milk cheese consumption is related to more intense sensory aspects compared to pasteurized milk cheese (Yoon, Lee and Choi, 2016). However, raw milk cheese has been reported to harbor STEC after long ripening periods. For instance, STEC strains were isolated from Spanish raw ewe's milk cheese ripened for two and a half months, and also for 12 months in cheese factories, showing STEC is able to survive aging

(Caro and García-Armesto, 2007). In addition, STEC was also isolated from cow's and goat's semihard ripened cheese in Switzerland, where some strains were positive for the presence of hemolysin production, revealing a threat to human consumption (Stephan et al., 2008). Moreover, in an attempt to challenge previously STEC isolates for ripening steps, Schlesser et al. (2006) inoculated acid-resistant *E. coli* O157 in raw milk designated for Cheddar cheese production, and after testing, samples were still positive for STEC after 360 days of ripening. Similarly, milk environment-adapted STEC strains were able to resist to production plus 16 days of ripening in semihard raw milk cheese, with some samples presenting counts higher than 10 CFU/g, which can be a concern because of the relatively low infectious dose of STEC (Peng et al., 2013). This suggests that the FDA (2017) standards previously mentioned of 60 days of maturation should be revised. In fact, controlling the raw material quality seems to be more effective to avoid STEC presence in raw milk cheese.

Miszczycha et al. (2012) evaluated the persistence of different STEC serotypes (O157:H7, O26:H11, O103:H2 and O145:H28) submitted to different manufacturing and ripening conditions, such as blue-type cheese (from sheep milk), uncooked pressed cheese with both long and short ripening periods (cow milk), cooked cheese (cow milk), and lactic cheese (goat milk). First, regarding blue cheese, the persistence of *E. coli* O157, O26:H11 and O103:H2 was tested. All strains decreased their concentrations during ripening, whereas after 240 days, only O26:H11 could be isolated after enrichment, besides showing a significantly higher growth and persistence than the others serotypes tested. Second, uncooked pressed cheese with seven months ripening was tested to *E. coli* O157 and O26:H11. Until day 60 of ripening, the levels of both strains remained constant. After, it was observed a concentration decrease during the ripening time and by day 210, the concentration levels decreased below detection levels for *E. coli* O157 in both core and rind, and for O26:H11 in the rind. At the 240th day, the concentration of O26:H11 in the core was significantly higher than *E. coli* O157 in

both core and rind. Third, regarding uncooked pressed cheese with short ripening of 20 days (plus 20 days wrapping), all types of strains were tested. In this case, concentrations of all strains remaining constant until the final day of 40, thus being a risk for consumption. Next, cooked cheese was tested for *E. coli* O157 and O26:H11. Although no STEC was recovered just after cheesemaking, strains could still be isolated at the end of the ripening period of 120 days after enrichment in the core (*E. coli* O157) and on the rind (both). Finally, lactic cheese was tested for all types of strains, and only strains belonging to the serotype O157 were not able to survive after day 60. This finding suggests that STEC cells are able to persist to different types of cheese manufacturing, with or without enrichment.

The fermentation step that most cheeses are submitted is an important preservation method to maintain cheese quality, and this can be made by the indigenous microbiota of raw milk, such as lactobacilli, streptococci, and lactococci, or by the addition of a starter culture (D'Amico and Donnelly, 2017). Although raw milk is not heat treated prior to cheese manufacturing, it is expected that its inherent microbiota and the starter culture together avoid pathogen survival by means of lowering pH and proper competition (Baylis, 2009). During the fermentation process, lactic acid bacteria hydrolyze lactose into glucose and galactose by the action of β-galactosidase (found in most species) or phospho-β-galactosidase (lactococci), producing lactate from glucose and exporting it, leading to a pH drop in cheese matrix (Poolman, 1993). While *E. coli* O157 strains present and ideal growth around pH 7, studies show that some O157 strains are able to survive to very acidic conditions, such as pH 4.0 and 3.0 (Dineen et al., 1998; Meira et al., 2017). This suggests that the acidification promoted by lactic acid bacteria is not very effective to avoid STEC persistence. However, lactic acid bacteria are known for producing natural antibacterial compounds that help to preserve fermented food, including cheese and cheese products (Portilla-Vázquez et al., 2016). Besides producing organic acids such as lactic, acetic and propionic acids, that decreases the matrix pH,

lactic acid bacteria also produce antimicrobial proteins called bacteriocins, such as nisin, pediocin, and lacticin, acting as a biopreservative (Favaro, Penna and Todorov, 2015). As evidence, the addition of an association of *Lactobacillus plantarum*, *Lactococcus lactis* and *Hafnia alvei* reduced the presence of STEC O26:H11 and O157:H7 in ripened cheese, without changing its sensory characteristics (Callon, Arliguie and Montel, 2016).

The fate of STEC during both ripening and storage has been described based on hurdles STEC can challenge in these manufacturing steps. In fact, STEC counts seems to reduce during cheese ripening in a variable way. Upon inoculation of *E. coli* O157 and non-O157 in white mold cheese, authors observed an increasing of STEC counts by 2 to 3 log CFU g^{-1} in the first 24-36h. During the ripening step of 14 days, the cheese core presented a pH between 5.7 and 6.1 and a_w was 0.98. Under this scenario, STEC O26:H11 kept a constant concentration, whereas a slightly decrease, of less than 1 log CFU^{-1}, were observed in STEC O103:H2 and O145:H28. Whereas, *E. coli* O157 presented a decrease of 1.3 log CFU^{-1}. The findings were similar regarding cheese rind, despite presenting a pH of 6.1 and a_w between 0.98 and 0.97. On the other hand, in a storage of 56 days, the concentration of all STEC decreased slowly in cheese core, but *E. coli* O157 concentrations reduced even more. In general, the rind of the cheese presented more concentration of STEC compared to the core, probably due to the higher pH in rind. This result suggests that *E. coli* O157 is less prone to survive to ripening and storage of white cheese mold than non-O157 strains (Miszczycha et al., 2016).

Moreover, the use of antimicrobial active packages, a packaging that provides both antimicrobial and physical barrier effects to food, may be used to reduce STEC numbers in cheese (Costa et al., 2018). Otero et al. (2014) investigated the action of two antimicrobial packaging films coated with oregano essential oil (*Origanum vulgare*) and Ethyl Lauroyl Arginate HCI (LAE) against *E. coli* O157 in raw milk sheep cheese. Upon 7 days of cold storage, the use of a polyethylene terephthalate film coated with a concentration of $\geq$ 6% LAE

significantly decreased the counts of STEC, without affecting cheese sensory characteristics, suggesting this cited film may be useful for reducing STEC counts in sheep cheese. In another study, Pérez et al. (2011) added potassium sorbate (PS) into whey protein concentrate edible film and tested against eight non-O157 strains, five of them isolated from soft and cottage cheese. After testing three different concentrations of PS (0.5%, 1% and 1.5%) under pH 5.0 and 6.0, they observed that increasing the concentration of PS increased bacterial inhibition. Also, inhibition was higher under pH 5.0 than 6.0, which could cause rejection of food consumption due to a denser appearance of the film under pH 5.0. Still, the film may be beneficial to avoid STEC post-processing contamination.

Furthermore, in order to infect an organism, STEC has to be able to survive stress conditions in the gastrointestinal tract, where STEC will be in contact with extreme low pH, acids formed by digestive processes or commensal bacterial metabolism, gastrointestinal secretions, adapted microbiota, peristalsis, among other conditions (Miszczycha et al., 2014; De Biase and Lund, 2015). In fact, Miszczycha et al. (2014) simulated human digestion of raw milk cheese experimentally contaminated with *E. coli* O157 and O26:H11, using a gastro-intestinal tract system model, to evaluate the survival of these bacteria during such stress conditions. In an environment that simulates the stomach, they observed that the survival of *E. coli* O157 was significantly affected after 60min. In contrast, O26:H11 concentration was significantly lowered only after 90 min, showing that the survival rate of O26:H11 was significantly higher than *E. coli* O157. Also, in the duodenum environment, *E. coli* O157 presented a significant decrease after 120min, while O26:H11 was not affected in the entire process, except for 60 min when the percentage of viable cells greatly increased for an unexpected reason. In the jejunum compartment, *E. coli* O157 showed no significant changes, whereas O26:H11 was able to grow in this environment. Finally, in the ileum environment, no significant changes were observed in *E. coli* O157, but O26:H11 showed much higher recovery

percentages. This data suggests that ecological differences between O157 and non-O157 strains may show superiority of non-O157 strains over O157 strains, while more studies still need to be done in order to make this statement an affirmation. Indeed, it has been observed an increasing number of infections caused by non-O157 strains in humans (Castro et al., 2017).

4. General STEC stress responses and tolerance in cheese matrix

Survival of bacteria, whether commensal or pathogenic, in any matrix, depends on its intrinsic and extrinsic factors. In regards to cheese, some of the most critical parameters that define bacterial persistence are pH, acidity, a_w, processing steps and storage temperature (D'amico and Donnelly, 2017). In addition, after the contact with the food matrix, the bacterial cell is exposed to several barriers and, in order to survive, it needs to surpass different types of challenges, being necessary that the bacteria have mechanisms to overcome theses hurdles. The physiological responses of STEC to stress condition are promoted by initiation factors that start a specific or a cascade of reactions that increase the resistance of the cell (Hengge-Aronis, 2002). Moreover, the general stress adaptive response is regulated by several genes responsible for maintaining the cell homeostasis (Hengge-Aronis, 2002).

Furthermore, different types of cheese have different production steps which are intimately related to different responses to stress. For instance, hard cheese usually has a low moisture content and low pH level, which contributes to an increasing of osmotic pressure, osmotic and acid responses, compared to soft cheese. Thus, hard cheese is less likely to host pathogens than soft cheese (Leistner, 2000). Pasteurized milk cheese is submitted to higher temperatures compared to raw milk cheese. Here, thermal technology makes it harder or even impossible for non-adapted bacteria to surpass such conditions (Baylis, 2009).

In addition, the majority of the adaptative response in STEC cells is promoted by sigma factor σ^S (RpoS or σ^{38}). This subunit is responsible for the expression of several genes,

regarding metabolism, transport and also general stress response (Hengge-Aronis, 2002). Sigma factors are subunits of RNA polymerase (RNAP) holoenzyme that acts as initiation factors, promoting an attachment of RNA portions to specific initiation subunits (Hengge-Aronis, 2002; McKenna et al., 2019). In non-stressing conditions, *rpoS* mRNA is verified at high levels and has little variation when comparing the stress response. However, during a stress condition, *rpoS* mRNA starts to rearrange from an inactive form to an active one, which causes an abrupt increasing of translations (Peng et al., 2011).

As cheese is exposed to many different antimicrobial stresses, each stress can trigger a specific or a conjugated response. A combined stress may generate a cross-protective response, which triggers different protective effects. For example, the sigma factor σ^S, that could be induced in response to acid stress, can also have effects on heat, cold, high-pressure, H_2O_2, and salt presence (Cheville et al., 1996; Robey et al., 2001; Mei et al., 2015; Li et al., 2018). Thus, cross-protection can generate certain adaptation when the bacteria have contact to stress found in the environment, leading to resistance to a previous or a distinct stress form (Riordan et al., 2000). For instance, acid adapted *E. coli* O157 exposed to pH 5.5 in HCl for 4 to 5 hours increased its resistance to lactic acid (pH 3.85), salt presence (10% NaCl) and a high temperature of 55 °C (Cheng, Yang and Chou, 2002). On the other hand, another study showed that when *E. coli* is exposed to a very aggressive stress and then it is submitted to a second lethal stress, the energy expenditure to maintain the homeostasis is so high that the cells are inactivated rapidly. However, when both stresses are applied at the same time, the bacteria are easily able to survive (Shadbolt, Ross and McMeekin, 2001). In cheese production, for example, STEC may contaminate milk in any step of manufacturing. If this contamination occurs directly in milk, STEC will face cold shock regarding milk cooling, and this may induce a general stress response in order to resist to subsequent stress. Thus, the surviving cells can overcome other challenges of inactivation, which may represent a risk to human health (Peng et al., 2011).

4.1 Heat stress in cheese

Although *E. coli* is known as a heat sensitive bacterium, some strains are considered to be among the most heat resistant foodborne pathogens (Li and Gänzle, 2016). In cheese manufacturing, STEC can be exposed to different sources of heat. First, the pasteurization process applied to raw milk in order to destroy pathogenic bacteria. Second, the different technological processes of cheese manufacturing where heat can be applied, e.g., ricotta and processed cheese are both manufactured at approximately 90 °C (Johnson, 2017; De Giorgi et al., 2018; Talbot-Walsh, Kannar and Selomulya, 2018). For instance, Fusco, Riccardi and Quero (2012) described that in pasta filata cheese (a variety where the curd is stretched and mold at high temperatures and then cooled), *E. coli* O157:H7 can also challenge a temperature as high as 90 °C and still persist in the final product. This occurs due to the temperature in the core of the curd that can stay around 55 °C. In fact, STEC are often isolated from pasteurized dairy products, which may be due to the fact that STEC encode the expression of genetic adaptations and may survive to thermal stress, mainly to inefficient pasteurization or sub-pasteurization processes (D'Aoust et al., 1988; Schlesser et al., 2006; Peng et al., 2011; Farrokh et al., 2013).

The main response of *E. coli* upon heat stress can be separated in induction period and adaptation period. The induction period occurs just after heat upshift, increasing the heat shock proteins (HSP) synthesis, reaching the highest after 5-10 minutes (Bukau, 1999). At the same time, two chaperones, Hsp70 (DnaK) and Hsp60 (GroEL), increase their synthesis at the rate of eight and 13-fold, respectively (Bukau, 1993). Further, the adaptation period occurs when the synthesis of HSP starts to decrease until it stabilizes. Consequently, an increasing of cellular HSP levels is observed (Bukau, 1993). The HSPs are able to mediate refolding, transporting,

repairing and degradation of proteins that were denatured by heat stress, thus guaranteeing the cell survival and persistence (Raivio and Silhavy, 2001).

In *E. coli*, the sigma factor σ^{32} is responsible for regulating the cytoplasmic general heat shock response, and also the normal expression level in cells that were not submitted to heat stress. The first mechanism regarding heat shock response is *rpoH* transcription. Four promoters are known to be responsible for *rpoH* transcription: while P1, P4, and P5 respond to σ^{70}, P3 is recognized by σ^{E}. While the most important promoters in heat shock response are P1 and P4, the activity of P5 requires a cAMP plus its receptor to function (Yura, 1996). The transcription of the cited promoters is influenced by the amount of heat stress applied to *E. coli*. For instance, when the cell is in a stable temperature of 30 °C, P1 is the major promoter of expression, together with P4. However, when the temperature increases to 42 °C, a little elevation in P1 and P4 transcription is observed, although P3 intensively increases its expression. Finally, when the cell is in contact with an extremely high temperature of 50 °C, expression of P1 and P4 starts to decrease until inactivation whereas P3 transcription continues to increase. (Gross et al., 1990; Bukau, 1993). The control of *rpoH* at basal expression levels are mainly due to σ^{70} and σ^{E}, while an increased σ^{32} synthesis relies on translational control (Yura, 1996).

The second mechanism of heat shock response is a *rpoH* translation. The importance of σ^{32} translational control can be explained because, upon heat shock, an increase of σ^{32} synthesis is observed, but not of *rpoH*. Besides that, *rpoH* mRNA levels increase its synthesis after the increase of σ^{32} synthesis (Nagai, Yuzawa and Yura, 1991; Yura, Nagai and Mori, 1993). When the bacterial cell is at a normal temperature, *rpoH* translation is repressed. However, under temperature upshift, there is a transient depression of translation, reaching a 12-fold rate. The control of rpoH translation is mediated by mechanisms that include three cis-acting *rpoH* mRNA regions, A, B and C regions (Nagai, Yuzawa and Yura, 1991; Arsène, Tomoyasu and

Bukau, 2000). The region A is known as a positive regulatory region on the 5' portion of *rpoH* mRNA. The also called "downstream box" is located close to the initiation codon and has 15 nucleotides. The region is a complementary part of 16S RNA, possessing a translation enhancing function (Morita et al., 1999). The region B, a negative regulatory region, is located within nucleotides 100 and 247, and seems to be involved in repressing translation under non-stress conditions (Morita et al., 1999; Arsène, Tomoyasu and Bukau, 2000). Base pairing between region A and part of region B seems to negatively modulate *rpoH* translation at steady-state conditions, by preventing translation initiation due to the formation of an internal loop that makes the Shine-Dalgarno (SD) sequence and initiation codon inaccessible (Morita et al., 1999; Arsène, Tomoyasu and Bukau, 2000; Peng et al., 2011). The induction of *rpoH* translation comes from the increasing of temperature that partially melts the mRNA structure, increasing the entry of ribosomes so that translation can occur (Morita et al., 1999). Moreover, the region C is also a negative regulatory region, that is located within nucleotides 364-433 of *rpoH*. It represses *rpoH* translation during the shut-off phase of the heat shock response. Also, region C seems to act in a protein level by mediating translational repression of rpoH (Nagai et al., 1994; Arsène, Tomoyasu and Bukau, 2000).

When stability to heat is achieved, there is a decreasing of σ^{32} level as well as repression of its activity (Connolly et al., 1999). This includes the modulation of HSPs, DnaK, DnaJ and GrpE, and also degradation of σ^{32} by the AAA-protease, FstH. FstH is a cytoplasmatic membrane protein that is believed to be the responsible for σ^{32} degradation, upon a destabilization caused by DnaK and DnaJ (Tatsuta et al., 1998; Arsène, Tomoyasu and Bukau, 2000; Rodriguez et al., 2008). DnaK chaperone system facilitates the inactivation of heat shock stress response and seems to indirectly participate of σ^{32} degradation and repression of *rpoH* translation during the ending phase of the heat shock response (Tatsuta et al., 1998; Connolly et al., 1999; Arsène, Tomoyasu and Bukau, 2000). When there is high availability of DnaK

substrates, they compete with σ^{32}, leading to a stabilization of σ^{32}. Further, the concentration of Dnark substrates start to decrease, whereas the degradation of σ^{32} increases, causing the ending of heat shock response (Peng et al., 2011). This occurs due to the fact that DnaJ acts by binding to a specific location of σ^{32}, destabilizing a region of σ^{32} that sits close the DnaK binding site. After that, DnaK destabilizes a region in N terminus, which seems to be the main target for FtsH, leading to degradation of σ^{32} (Rodriguez et al., 2008).

Regarding to chaperone actions, two chaperone system play a major role in *E. coli* heat shock response: DnaK and GroE systems. DnaK chaperones are known for refolding proteins and preventing aggregation (Georgopoulos and Welch, 1993). These actions are only possible because DnaK are associated with short hydrophobic segments of polypeptides substrates, avoiding the aggregation or folding of these substrates (Bukau and Horwich, 1998). DnaK system activity presents two conformations to DnaK that are related to ATP and ADP respectively. In fact, DnaK presents a N-terminal ATPase domain related to the nucleotide and another related to the substrate. When ATP binds to the nucleotide biding domain, it provokes a conformational change in the substrate biding domain, resulting in an open substrate cavity with high association (k_{on}) and dissociation (k_{off}) rate, leading to a faster exchange rate. However, if ADP attaches to the nucleotide biding domain, the substrate biding cavity closes, resulting in low k_{on} and k_{off}. Further, the DnaJ chaperone induces ATP hydrolyzes, leading to a shifting to the closed conformation, confining the substrate. GrpE replaces ADP with ATP, which will turn DnaK back to the open conformation, thus releasing the substrate (Mayer et al., 2000; Peng et al., 2011). Moreover, if a protein has more than one hydrophobic segment, DnaJ can bind to it forming a complex made of Dnak-substrate-DnaJ. Also, upon high temperature, GrpE suffers reversible conformational changes, thus decreasing its nucleotide exchange activity. Both of these mechanisms can lead to the closed conformation of DnaK under heat stress, which will result in many bound substrates (Grimshaw et al., 2001; Peng et al., 2011).

Equally important, the GroEL/GroES chaperonin system is important for basal temperature conditions as well as for heat shock response. The GroEL structure is a double-ring structure that forms a cylindric-like structure with a central cavity for folding proteins (Braig et al., 1994). Also, the GroES structure is a heptamer that resembles a dome, which is able to bind to GroEL central cavity, closing it (Hunt et al., 1996). In the GroEL/GroES reaction cycle, only one GroES ring is able to bind GroEL, thus forming the folding chamber. Seven ATP molecules are needed to provoke a conformational change, allowing GroES to bind to GroEL ring. This will cause another conformational change that will expand the folding chamber, shifting the cavity surface. Further, the cavity becomes hydrophilic, which leads to the liberation of the substrate to the *cis* chamber. Now that the cavity is hydrophilic, the non-native substrates start the folding process until the association of GroEL/GroES is broken, caused by ATP hydrolysis on the *cis* side. The detachment of GroES, the substrate, and ADP from the *cis* GroEL is activated by the biding of ATP plus the new substrate on the *trans* side. The other side becomes the new *cis* side, so the two GroEL rings shift between binding and folding, proceeding the continuation of the reaction cycle (Rye et al., 1997; Peng et al., 2011).

In a like manner, the sigma factor σ^E also plays an important role in heat stress response because it is responsible for maintaining the cell envelope integrity. Sigma factor σ^E is responsible for every reaction regarding cell envelope. The activation of σ^E in heat shock response is mediated upon protein unfolding in both cytoplasm and cell envelope, and also upon accumulation of unfolded proteins in the cell envelope. This last activation is modulated by specific inducers, like overexpression of porins (Mecsas et al., 1993; Ades et al., 1999).

Upon activation, σ^E transcribes genes intimately related to the cell envelope that acts by encoding chaperones and proteases that will remove or refold damaged or misfolded proteins. Because σ^E sites in the cytoplasm, it needs a mediator to sense the damage in the envelope. Signal of stress response is then transmitted from the envelope to σ^E through regulated

proteolysis of the inner membrane anti sigma factor RseA. Prior to the initiation phase, σ^E is inhibited by RseA because RseA blocks σ^E association with RNAP. According to Campbell et al. (2003), this association is about 300-fold greater than σ^E affinity to RNA polymerase. Because RNA cannot compete with the σ^E:RseA complex, the complex needs to be interrupted in order to σ^E interact to RNAP, activating the genes responsible for promoting the heat shock response. Thus, degradation of RseA is the main mechanism for this complex disruption (Ades, Grigorova and Gross, 2003). The proteolysis of RseA is associated with two proteases: DegS and YaeL. The process starts when DegS cleaves RseA in the periplasmic domain. Next, when the periplasmic domain is already removed, YaeL is able to degrade RseA (Alba et al., 2001; Kanehara, Ito and Akiyama., 2002). This entire process is induced by a signal from the disruption of outer membrane porins, a significant inducer of σ^E (Walsh et al., 2003). The cited reactions are autoregulatory because they are mediated by σ^E itself, which allows the cell to transcribe activation and inhibition in a same way. After RneA degradation, σ^E is free to bind to RNAP and start the transcription of genes of heat shock response, thus guaranteeing *E. coli* survival and persistence in food (Ades, Grigorova and Gross, 2003; Ades, 2004). The role of σ^E in stress response is not fully explored yet.

Recent bioinformatic studies correlate the presence of the genomic island called *locus of heat resistance* (LHR) to heat resistance in *E. coli* and other pathogens (Bojer et al., 2010; Gajdosova et al., 2011; Mercer et al., 2017). For instance, *E. coli* AW1.7, a non-pathogenic food isolate is capable of resisting to an elevated temperature of 71°C (Dlusskaya et al., 2011). If pathogenic strains, such as any STEC strain could acquire this attribute, they may become a threat to food safety and public health. The heat resistance of this isolate is not related to σ^{32} as previous stated, but to LHR. This region is responsible for regulating its heat chock response, whereas the activation of LHR gives *E. coli* AW1.7 an extreme heat resistance behaviour. LHR is composed of 16 open reading frames, where moderately resistant and sensitive strains possess

10 out of 16 orthologs. A full-length LHR provides a highly heat resistance behaviour and a shorten LHR provides moderate heat resistance. Thus, the heat resistance depends directly on the whole genomic island (Mercer et al., 2017). Yet, Liu et al. (2015) screened 100 STEC strains but did not find any highly heat resistant pathogen. While this is true, it is believed that 2% of all *E. coli* genome contains the LHR, making lateral gene transfer a possible danger do human health (Mercer et al., 2017).

4.2 Osmotic stress and water activity decrease in cheese

The use of sodium chloride (NaCl) in cheese is intimately related to food preservation. Besides its role in nutritional and flavour aspects, NaCl is important to control the microbiota growth because of its direct interaction with osmoregulation and also its direct effect in decreasing the moisture content of the final product, thus reducing water activity. In cheese production, NaCl can be added to cheese through direct addition or utilizing a brine (Velázquez-Varela et al., 2018). This increase of salt content will create an osmotic upshift that will require STEC to adapt to the new cited conditions. Upon direct addition of NaCl in the curd, STEC is exposed to salt as long as it diffuses in the cheese matrix. However, the utilization of brine can expose only the surface microbiota in a first place, and then diffuse to the core (Peng et al., 2011). It is important to mention that *E. coli* O157 has been described as able to resist for several weeks in cheese brines, thus representing a potential source of infection to cheese consumers (Ingham, Su and Spangenberg, 2000).

4.2.1 *E. coli* protective surface structures

The exopolysaccharides seem to play a role in cell desiccation, besides the formation of biofilm. For instance, colanic acid (CA), a viscous capsular polysaccharide produced by several *E. coli* variants, is credited to protect bacterial cell from desiccation. Ophir and Gutnick (1993)

found out that *E. coli* strains that were able to produce CA, showed much more resistance against water loss, protecting the colonies against desiccation in a better way than those colonies that were not able to produce CA. In addition, Chen et al. (2004) observed that CA producer *E. coli* O157 had a significant higher survival rate in the presence of 1.5M and 2.5M of NaCl compared to O157-strains that were unable to produce the saccharide, showing that CA protects the cell from osmotic stress. Another exopolysaccharide involved in protection is cellulose. A study presented that the population of STEC producing cellulose were significantly higher than their non-producing counterparts at 24- to 48-h intervals for 1M NaCl treatment and at 9- to 48h- intervals for 2M NaCl, explaining the role of cellulose in cell protection against osmotic stress (Yoo and Chen, 2010).

Moreover, in Mattick, Rowburry and Humphrey (2003) study, commensal *E. coli* and *E. coli* O157 strains were able to form protein elongated filaments upon exposure to low a_w of 0.96, which may be involved in osmotic stress response somehow. In fact, both commensal and pathogenic *E. coli* strains also demonstrated to form such filaments under different stress conditions, such as low and high temperature and low and high pH. The authors also stated that plate counting techniques for the tested strains failed to exibit precise results, compaired to measurements of protein concentration. This implies that, since the infectious dose of STEC strains are very low, commoly used plate counting techniques may underestimate the precise number of pathogens in a food outbreak.

4.2.2 Cheese salted with sodium chloride or brine

Upon exposure to osmotic stress, bacterial cell responds in two different ways: as an immediate response of seconds to minutes, on protein activity level by increasing or decreasing enzymes and transport systems; and as an extended response of minutes to hours, on gene transcription level, acting by means of *de novo* synthesis of enzymes, transport systems and

wall components (Wood, 1999). Because the long-term response usually occurs as a result of prolonged exposure to high external osmolarity, this response can recover the steady-state of the cell by restoring the intracellular concentration and solute composition, the energy for metabolism and also helps with growth and cell division (Wood, 1999; Krämer, 2010).

When an osmotic upshift happens, *E. coli* respond to it by uptaking potassium ions via K^+ uptake systems while synthesizing glutamate (which acts as a counter ion for K^+, avoiding depolarization) (Dinnbier et al., 1988; Burguess et al., 2016). *E. coli* possesses two potassium uptake systems: Kdp and Trk. The Kdp-ATPase system has a high affinity to K^+, being composed of four subunits: KdpA (K^+ binding and translocating), KdpB (energy supply), KdpC (complex gathering) and KdpF (stability). They are expressed under low potassium concentration and osmotic stress by the regulation of the KdpD-KdpE protein system. (Altendorf, Voelkner and Puppe, 1994). In contrast, the Trk system has a low affinity to K^+, but possesses a higher rate of transport. The system is composed of a translocating subunit, TrkH and TrkG (this last not found in all *E. coli* species), and a cytoplasmic regulatory subunit, TrkA and TrKE (SapD), that codes an ATP-binding cassette. The Trk system is presumed to symport 1 H^+ and 1 K^+ to the cytoplasm, somehow involving ATP (Corratge-Faillie et al., 2010).

Regarding long-term adaptation to osmotic stress, the cell replaces K^+ for compatible solutes because the high concentration of potassium affects metabolic functions overtime (Dinnbier et al., 1988). These selected solutes are mainly trehalose, proline and glycine-betaine that also act as protein stabilizers (Peng et al., 2011). Three different cytoplasmic transport systems are responsible for this process in *E. coli*, ProP, ProU and BetT (Krämer, 2010; Burguess et al., 2016). First, ProP is an osmoregulatory compatible solute-H^+ symporter activated upon an increase of extracellular osmolarity. Its activation is mediated by two promoters, P1 and P2, that are controlled by σ^S (Mellies et al., 1995). Second, ProU is a biding protein-dependent system that, like ProP, possesses a high affinity to import solutes such as

glycine-betaine, but a low, although functional, affinity to import proline, ectoine and others organic compounds (Lucht and Bremer, 1994).

Furthermore, *E. coli* is also able to synthetize or convert compatible solutes in order to maintain homeostasis. For instance, the *bet* genes have high affinity to import choline upon osmotic stress, which is realized by the osmosensing BetT transporter. This way, the cell has a substrate to convert choline to glycine-betaine, a potent osmoprotectant. This pathway is induced upon osmotic stress or addition of choline to the medium, happening under aerobic conditions (Eshoo, 1988). Similarly, *E. coli* is also able to synthetize and accumulate trehalose from glucose in an attempt to maintain cellular osmolarity. The pathway is dependent of two genes, *otsA* and *otsB* being induced by osmotic stress and by σ^S, when cells ate entering in stationary phase (Hengge-Aronis et al., 1991). This accumulation of trehalose is vital for the growth and survival of cells mainly in a moderate osmolarity environment (Giaver et al., 1988).

Moreover, *E. coli* can utilize other membrane proteins to deal with osmotic stress. As evidence, OmpC and OmpF are porins that are able to form channels in the external membrane, facilitating the passive diffusion of small hydrophilic molecules, which will help the cell to recover osmolyte level. Their expression is mediated by the signaling system EnvZ-OmpR (Cai and Inouye, 2002). OmpC and OmpF differ regarding structure and function: while OmpC possesses a smaller pore and slower flux, OmpF has a larger pore and consequently a faster flux. While in low osmolarity levels OmpF is the major porin, in a high osmolarity environment the high concentration of OmpR causes an up-regulation of *ompC* and consequent downregulation of *ompF*, which will turn OmpC the predominant porin (Feng et al., 2003).

4.3 Acid stress and pH homeostasis response in cheese

Acid resistant bacteria was first designated as those that were able to resist to pH 2.5 for 2h. In addition, if at least 10% of the inoculum submitted to these cited conditions were able to survive, the strain would be considered as acid resistant (Gorden and Small, 1993). For instance,

several studies had attested the acid resistance behavior of *E. coli* and STEC (Gorden and Small, 1993; Arnold and Kaspar, 1995; Leyer, Wang and Johnson, 1995). The acid resistance (AR) enables *E. coli* to survive harsh conditions like the presence of gastric acid. In fact, the low infectious dose related to STEC O157:H7 strains comes from its acid-resistant behaviour (Castanie-Cornet et al., 1999).

In cheese production, STEC can be exposed to acid stress depending on the employed technology. For example, in semihard Gouda cheese, the final product pH should be between 4.9 and 5.6. This happens mainly due to the fermentation processes of lactic acid bacteria used as starter culture (van den Berg et al., 2004). Also, in soft ripened cheese, the pH can be as low as 4.6, as a result of fermentation by mesophilic lactic acid bacteria (Shaw, 1981; Batty, Waite-Cusic and Meunier-Goddik, 2019). In addition, *E. coli* O157:H7 has been described to persist for 12 days in yogurt (pH 4.0) and 28 days in sour cream (pH 4.3) (Dineen et al., 1998).

In an acidic condition, STEC needs to adapt its internal pH in order to stay alive. If the intracellular pH drops too much, the cell is likely to die due to loss of function or denaturation of enzymes. To overcome these conditions, *E. coli* possesses distinct acid tolerance mechanisms related to its log and stationary phases. Such mechanisms involve σ^S and cAMP functions as well as decarboxylase enzymes that requires specific amino acids like glutamate, arginine, lysine, and ornithine (Foster, 1999; Foster, 2004).

4.3.1 Acid tolerance response in stationary phase cells and log-phase cells

Currently, five major mechanisms are accepted to be part of *E. coli* acid tolerance response in stationary phase cells. This includes four enzyme-based responses and an oxidative response. The oxidative system, also known as AR1, requires both the alternative σ^S and CRP (cAMP receptor protein) to mediate acid tolerance. It is repressed by glucose thus often cited as glucose-repressed acid resistance system. AR1 functions when cells grow in complex media

(Luria-Bertani broth) at pH 5.5 and then are challenged with a pH as low as 2.5, in minimal media, with no amino acids supply. However, when cells grow on the same complex media but at pH 8 and are submitted to low pH afterward, they are killed rapidly. This happens because pH 8-grown cells are likely to synthesise an inhibitor of AR1. Nevertheless, the role of AR1 components and mechanisms are still not clear (Castanie-Cornet et al., 1999; Foster, 2004).

The other four decarboxylase-based pathways are based on a decarboxylase enzyme induced by low pH, specific amino acids and antiporters. They work by exchanging α-carboxyl groups from the substrate for a proton from the cytoplasm, which will result in CO_2 and the end product. Then, the antiporters will transport the end product out of the cell and import another substrate at the same time. This will lead to an increasing of intracellular pH because of intracellular proton consumption (Peng et al., 2011). These enzyme-based pathways work upon specific conditions, where: AR2 (glutamate-dependent system) and AR3 (arginine-dependent system) allow *E. coli* to survive at extreme acidic conditions, like pH 2.5; AR4 (lysine-dependent system) and AR5 (ornithine-dependent system) enable *E. coli* to survive in less acidic condition, such as pH 4.5 (Zhao and Houry, 2010).

The AR2 is the most studied and efficient acid response system in *E. coli*. In this system, the decarboxylase enzymes GadA and GadB convert glutamate to gamma-amino butyric acid (GABA) and CO_2, thus consuming an intracellular proton. GABA is then transported out of the cell by antiporter GadC while more glutamate enters the cell. Similarly, in AR3, the decarboxylase enzyme AdiA converts arginine into agmatine and CO_2, consuming one proton. Agmatine in then taken out of the cell by antiporter AdiC in exchange for more arginine (Zhao and Houry, 2010). Because these systems work better at extreme pH (2.5), they are less likely to function in cheese manufacturing.

Furthermore, AR4 and AR5 work in a mild pH condition of 4.5-5, which is more likely to happen in cheese products. In AR4, the lysine decarboxylase Ldcl (CadA) converts lysine

into cadaverine and CO_2, consuming one proton. Similar to the others, the antiporter CadB takes out cadaverine in exchange for lysine. The same occurs to AR5, where the ornithine decarboxylase is SpeF and the ornithine-putrescine antiporter is PotE. Just like AR1 system, AR5 mechanisms are still not clear (Zhao and Houry, 2010).

Besides that, chloride and potassium also play a role in acid resistance response. When facing an extreme acidic condition e.g. pH 2.0, the transmembrane potential of *E. coli* is disrupted. *E. coli* can then revert the electrical membrane potential from -90mV to +30mV (in the presence of glutamate) or +80mV (in the presence of arginine). This may be due to the accumulation of protons in the cytoplasm and also due to the result of AR2 and AR3 pathways (Zhao and Houry, 2010). Thus, when exposed to elevated acid stress, *E. coli* ClC chloride channel imports Cl^- to the cytoplasm, bringing negative charge while removes excess protons, which helps to restore the membrane potential (Foster, 2004). Furthermore, the influx of potassium also regulates pH_i in *E. coli*. Symporters and K+ channels acts maintaining the membrane potential and also maintaining pH_i homeostasis (Epstein, 2003).

Another system involved in acid stress is the chaperone-based acid stress response. This system is related to the protection of periplasmic proteins. The HdeA and HdeB are the chaperones responsible for the response in the periplasm. In an acidic situation, they function by maintaining the substrates in a soluble state. Also, the chaperone system is able to aggregate themselves to proteins that failed to be solubilized. The optimal pH is below 3, so they function better under extreme acidic conditions (Zhao and Houry, 2010).

The *E. coli* acid tolerance response in log-phase are called acid habituation (AH). Although only a few studies had been done, it is known that AH occurs when *E. coli* is grown in nutrient broth at pH 5.0 and can survive the challenge of pH 3.5 or 3.0. The mechanisms are still unclear, but it seems to involve protein synthesis steps and mainly repair of DNA damage. It is proposed that PhoE, a phosphate-specific porin, works as a channel for the influx of H^+,

which culminates in periplasmic acidification and stimulation of a transmembrane sensory protein that could induce AH (Bearson, Bearson and Foster, 1997).

4.3.2 Acid tolerance response in acid coagulation, ripened cheese and cheese storage

As cited before, lactic acid bacteria are involved in many fermentation processes of dairy products, especially cheese. The fermentation process results in a drop of pH due to the production of different organic acids, such as lactic, acetic and propionic acids (Özcelik, Kuley and Özogul, 2016). Then, besides a drop in pH, STEC may also challenge the presence of organic acids in cheese. A weak organic acid acts by acidifying the internal pH by the entering of the protonated form in the cell, which will then deprotonate based on the internal pH plus the pK_a (acid dissociation constant) of the acid. This will allow the accumulation of the acid as an internal anion, increasing the cell turgor, which will affect cell growth (Foster, 1999; Peng et al., 2011).

Acidification of ripened cheese such as camembert, parmesan and cheddar are mainly caused due to the accumulation of organic acids produced by starter cultures. This is also true regarding cheese storage (Todaro et al., 2017; Batty, Waite-Cusic and Meunier-Goddik, 2019). However, in acid coagulated cheese such as petit suisse, cream cheese or cottage cheese, there is an acidification step. It consists in a drop of the pH by the direct addition of acids such as citric, acetic or lactic, or due to starter culture fermentation. Upon acidification, the calcium salts of the casein micelles are solubilized, producing progressive migration of calcium and phosphates to the aqueous phase, promoting casein demineralization at pH 4.5-4.8 (isoelectric point of casein). The solubility of Ca and P inside the micelle in the aqueous phase leaves the micelle lacking its minerals, so the micelle disintegrates and precipitates (Hofland et al., 1999; Lucey, 2011). In bacteria, the presence of such organic acids can disrupt the membrane integrity due to the lipophilic behavior of weak organic acids. This accumulation can also cause osmotic

stress, as well as interaction with enzymes and proteins, impairing some metabolic functions (Roe et al., 1998; Peng et al., 2011).

4.4 Cross protection stress

The cheesemaking process and the cheese matrix itself constitute of several antimicrobial hurdles that help preventing bacterial growth in different and sometimes simultaneous ways. In this case, when STEC are exposed to more than one stress condition, up- or downregulation of different stress responses might occur, originating what we call as cross-protection effects (RIVERA-REYES; CAMPBELL; CUTTER, 2019).

Trying to understand the cross-protection effect between acid stress adaptation with following thermal inactivation of *E. coli*, Haberbeck et al. (2017) chose 48 *E. coli* strains that presented more acid resistance than *E. coli* O157. Thus, they adapted *E. coli* strains to acid resistance letting them grow overnight in pH 5.5. Strains were submitted to inactivation at both pH 7.0 and 6.2. Authors found out that acid adaptation to pH 5.5 improved acid resistance of all 48 the strains, compared to the treatment control (pH 7.0). For instance, the average regarding acid adapted cells was resisting to 58 °C for 6 min, whereas the control group resisted for 4 min. Authors also described that strains presented more thermal resistance when inactivated at pH 6.2, rather than 7.0. Interestingly, they also described three highly heat resistant strains (not food related) with D_{58}-values ranging from 17.6 to 69 min. In comparison, Gabriel and Nakano (2011) demonstrated that upon incubation in slowly acidified nutrient broth supplemented with 1% glucose, *E. coli* O157 presented an increase in thermal resistance, by enhancing its D_{55}-values from 51.39s (control group) to 250s (glucose supplemented), which can be applied to cheese matrixes containing glucose. Indeed, this result demonstrates that acid adaptation promoted a cross-protection behavior regarding thermal inactivation. While this is

true, more studies are needed, especially testing higher temperatures, such as used in the pasteurization processes.

Regarding the cross-protection effect between salt and thermal resistance, Lee et al. (2015) adapted three STEC strains, *E. coli* O157, *E. coli* O11 and *E. coli* O26 to salty environments containing 0%, 2% and 4% NaCl. After that, the authors challenged the strains to 50 °C for a heating time ranging from 0 to 120 minutes. As a result, *E. coli* O157 adapted to 4% NaCl presented significant less reduction than those adapted to 2% NaCl, or non-adapted (0%), showing an increasing of thermal resistance. In contrast, *E. coli* O11 and *E. coli* O26 did not show an increasing thermal resistance upon salt adaptation. This suggests that thermal resistance after salt adaptation is strain-dependent.

The combined effect following acid stress and salt addition in STEC was described by Bae and Lee (2017), where they submitted *E. coli* O157 strains to a combination of concentrations of acetic acid (0-2%) and NaCl (0 and 3%). Authors found out that although the levels of *E. coli* O157 decreased as the concentration of acetic acid increased, the survival of *E. coli* O157 in the presence of 3% NaCl was significantly higher than the survival in the absent of NaCl. In fact, this pattern occurred equally for all concentrations of acetic acid added, revealing that salt promotes a synergistic effect when combined to acetic acid. In a like manner, Lee and Kang (2016) submitted five *E. coli* O157 strains to a medium containing 1.5% acetic acid combined with concentrations of NaCl, ranging from 0 to 18%. As a result, four *E. coli* O157 strains were capable of increasing recover in a significantly way, upon exposure of up to 15% NaCl, compared to treatment control (1.5% acetic acid and 0% NaCl). However, only one *E. coli* O157 strain was able to survive in an environment of up to 9% NaCl. Again, results demonstrated that NaCl promotes increased resistance to the presence of acid, which can be applied to cheese matrix if we take in consideration that salt is added during cheesemaking as well as weak organic acids are produced during fermentation and ripening steps.

Moreover, Akhtar et al. (2016) tried to evaluate the correlation between antibiotic resistant strains and stress situations, such as exposure to lactic acid (2.5, 3.5, 5%), sodium hypochlorite (0.2, 0.5, 1ppm free chlorine), and heat treatment (60, 61, 62.5 °C). Authors selected six strains belonging to the serotypes O26 and O103, being two high antibiotic resistant strains, two low resistant strains, and two susceptible trains. In fact, reduced levels of bacteria were achieved with increased levels of lactic acid, sodium hypochlorite and temperature, as expected. All strains were reduced in 5 log CFU/mL after contact with 5% lactic acid for 10 minutes. Similar results were described for sodium hypochlorite assay. D-values for all O26 and O103 strains ranged from 0.37 to 2.09 and 0.37-1.71 minutes, respectively. In conclusion, the authors could not find a strong relation between antibiotic resistance and enhanced stress response mechanisms, although more studies are needed to surely confirm this statement. This result is very important regarding cheese production because multidrug resistant STEC, including *E. coli* O157, have been isolated from milk and cheese, as well as dairy cattle (Ahmed and Shimamoto, 2015; Iweriebor et al., 2015).

5.0 Relation between stress and virulence

Stress response genes activated when STEC is in contact with cheese matrix may be involved with virulence potential. As Mutz et al. (2019) suggest, the adaptation to certain type of stress during food processing is a concern due to the fact that it can lead bacteria to become adapted, and to produce more virulent cells, as they are able to overcome such conditions.

5.1 Virulence determinants: an overview

The pathogenesis of STEC is primarily associated with Shiga toxin, encoded by *stx* genes, present on the pathogenicity island of LEE (*locus of enterocyte effacement*) (Castro et al., 2017). Two toxin variants are known: Stx1, first described in *Shigella dysenteriae*, but also

produced by STEC strains; and Stx2, which is immunologically distinct yet very similar. Stx toxin is highly associated with the capacity of the bacteria to produce HUS. The toxin acts by destroying the 28S rRNA, stopping protein synthesis, which leads the cell death upon exposure to the toxin (Melton-Celsa et al., 2011). Further, Stx has been not only associated with *E. coli* and *Shigella*, but also with *Enterobacter cloacae*, *Citrobacter freundii* and *Enterococcus* (Koudelka, Arnold and Chakraborty, 2018).

Apart from shiga-toxin production, other reported virulence mechanisms of STEC are: LEE genes (*eae*, *tir*, *espA*, *espB*, *espC* and *espD*), related with adherence, initiation of host signal transduction, and attaching and effacing lesions; *ure* gene cluster (*ureA*, *ureB*, *ureC*, *ureD*, *ureE*, *ureF*, and *ureG*), associated with colonization and pathogenesis of *E. coli* O157; *E. coli* common pilus (ECP), associated with adherence and colonization; hemorrhagic coli pili (HCP), a pylus produced by *E. coli* O157 related to motility, invasion and biofilm formation; Efa1 and Cah, adhesins related to grave diseases, among others. Also, STEC produces many non-LEE encoded effectors (*nle* genes) that plays a role in invasion, cytotoxicity and attachment, as well as others (Bolton, 2011).

5.2 Virulence status of STEC cells exposed to sublethal stress in cheese

As stated before, the contact of STEC with sublethal conditions in cheese or other food matrixes may enhance STEC adaptability to survive stress conditions inside the human body, thus promoting infection by enhancing its virulence characteristics.

In order to characterize the heat shock response of STEC using a transcriptomic approach, Carruthers and Minion (2009) submitted an *E. coli* O157 strain to 50 °C for 15 min. Among other results, authors found out that there was no increase in *stx1* or *stx2* transcription, suggesting heat shock may not have a relation with virulence enhancement in broth growth. More recently, Singh and Jiang (2015) determined the expression of virulence genes in *E. coli*

O157 exposed to heat shock at 47.5 °C in tryptic soy broth. As a result, virulence genes such as *stx1*, *stx2*, *hlyA*, and *fliC* were down-regulated. On the other hand, the *eaeA* gene was up-regulated, although the result was non-significant. More studies are needed to understand the role of heat shock in virulence genes transcription.

In an attempt to evaluate gene expression in *E. coli* O157 isolated from both clinical and animal source foods, Bergholz, Vanaja and Whittam (2009) submitted three strains to osmotic and acidic environments. As expected, stationary-phase cells showed a significantly higher survival rate than log-phase cells under pH 3.5, and 37 °C. After 10 minutes in this acidic environment, authors observed the significant expression of genes known to be related to acid (*asr*, *osmY*, *gInK*, *adiY*) and osmotic (*bdm*, *proV*, *proW*, *osmC*, and *osmY*) responses, which can probably suggest a cross-protecting effect between these two responses. Also, several genes related to the envelope stress response were activated, suggesting damage in the cell membrane. Interestingly, genes related to the pathogenic island LEE such as *ler*, *orf4* and *orf5* were also induced, as well as those encoded by T3SS, such as *espJ*, *espB*, *espM2*, *espL3* and *espZ*. Besides, a gene present on the adherence island, *terZ*, was induced and highly expressed in exponential phase. This finding suggests adherence and virulence enhancement may be induced by acidic stress conditions, such as found in lactic cheese, acid coagulated cheese and some ripened cheese.

Trying to understand the fate of STEC in gastric passage, House et al. (2009) challenged three *E. coli O157* strains to pH 3.0 for 15 and 30 min, with and without an adaptation period in pH 5.0, also for 15 and 30 min. As expected, the adapted cells submitted to pH 5.0 prior to inoculation in pH 3.0 had a better survival rate, independent of the exposure time. In addition, authors also tested the adhesion of several acid-stressed *E. coli O157* strains to epithelial cell lines, HEp-2 and CaCo-2. The adhesion of such acid-adapted strains highly increased in both cell lines, compared to the unstressed control strains. As an example, adhesion of one STEC

strain in CaCo-2 cell line increased by 486% after 6h of adhesion. Similarly, other two STEC strains increased adhesion in 257% and 322% after 3h in HEp-2 cells. This is intimately related to another finding of this study, where it was observed that infected epithelial cells with acid-adapted strains increased apoptosis levels in 200-400%, compared to the infected cells by unstressed strains. Moreover, the authors tested the relation between the production of Shiga toxin and acid stress in Vero cells. None of the treatment resulted in significantly results regarding the amount of toxin production, either from periplasmic or secreted extracts, which makes toxin production unchanged regardless acid stress. Similarly, Leenanon, Elhanafi and Drake (2002) described an increasing of Shiga-toxin mRNA levels, whereas the production of the toxin did not increase subsequently in acid stress adapted *E. coli* O157. These results show that enhancement of stressed STEC cells virulence is not related to Shiga-toxin production, but to other virulence mechanisms.

Contrasting, Olesen and Jespersen (2010) investigated the fate of *E. coli* O157 virulence genes transcription upon long-term acid adaptation of 24h to pH 5.5, and exposure to salt (4.5% NaCl). After 24h in acidic pH, *stx2A* transcription was significantly induced in one strain, whereas significantly repressed in the other two tested strains. The same pattern happened upon 24h under salt exposure. Besides that, only one strain encoded Stx1, and *stx1A* transcription was significantly induced in a salty environment. Regarding *eae* and *tir*, intimin and translocated intimin receptor respectively, only a modest yet significant fold change occurred in both salt and acid environment, with exception of *tir* under salt stress where there was a significant transcription induction of one strain and a significant reduction of another one. Further, no significant changes were noted in *IpfA*, long polar fimbriae, in any tested strain under both challenges. Finally, all salt-adapted STEC strains presented an increasing of adherence in CaCo-2 cells. However, no significant changes were noted between acid adapted cells and control cells, in contrast to the previous cited study. This result suggests that the

addition of salt or brine to cheese may not avoid STEC persistence in this product, besides enhancing its virulence.

6. Concluding remarks

In general, cheese rather made by pasteurized or raw milk, is often considered as a safe food due to several hurdles its manufacturing processes present. While this is true, the presence of pathogenic strains of *E. coli*, such as STEC, besides indicating direct or indirect fecal contamination, present a risk to consumption due to the production of well-known toxins. STEC is able to infect humans upon oral intake of contaminated food, which is in agreement with several reports of occurrence and outbreaks caused by STEC in cheese. Although the pasteurization process is known to completely inactivate STEC strains in milk and cheese, consumption of raw milk cheese and post-pasteurization contamination are still a concern that can lead to the occurrence of the pathogen in such products. Undoubtedly, the certification of a good quality raw material free of pathogens together with the adoption of good practices of manufacturing is the best way to achieve a final product that is safe for consumption.

STEC is challenged by several stress situations during cheese production, including high temperatures due to pasteurization and different cooking processes, an osmotic upshift due to the addition of salt or brine, leading to a decreasing of a_w, and a decrease of pH due to the addition of organic acids in acid-coagulated cheese and the metabolism of lactic acid bacteria added as starter cultures. However, STEC developed stress response mechanisms to overcome these situations, such as the activation of sigma factor units, upregulation of genes related to stress responses, chaperone and chaperonin system mechanisms, membrane sensors and protective surface structures, enzymes and transport systems, among others. In addition, researches have reported the interaction between stress response and enhanced virulence. Thus, a better understanding of STEC adaptation to stress conditions and the role of virulence

enhancement of persistent STEC strains are crucial for the achievement of safe product production.

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CAPITULO III

ARTIGO EXPERIMENTAL

OCCURRENCE AND CHARACTERIZATION OF SHIGA TOXIN-PRODUCING *E. coli* ISOLATED FROM DAIRY PRODUCTS IN THE STATE OF BAHIA, BRAZIL.

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ABSTRACT

Coliform bacteria are a group of microorganisms which indicates poor hygiene practices when found in food. Within this group, *Escherichia coli* in food may indicate fecal contamination, besides representing the presence of potential pathogens that may cause human diseases. While most species are commensal, known pathogenic groups such as Shiga toxin-producing *E. coli* (STEC) represents a public health concern due to its ability to produce Stx1 and Stx2 toxins, which may cause intestinal and extraintestinal diseases. Therefore, monitoring milk production stages and checking the innocuity of dairy products are crucial for food safety. In this context, the aim of this study was to verify the occurrence of thermotolerant coliforms, *E. coli* and STEC strains, as well as evaluating the resistance profile to 12 antimicrobials used in both veterinary and human medicine. A total of 123 dairy samples were obtained from 15 different locations in the state of Bahia, Brazil. The samples were submitted to coliform assay and streak plating on Eosin Methylene Blue agar. Positive *E. coli* strains were submitted to duplex and multiplex polymerase chain reaction for STEC confirmation and serotyping of the "big six" non-O157, respectively. As a result, 38 (31%) samples had the presence of thermotolerant coliform bacteria. Unsatisfactory levels of thermotolerant coliform were observed in 21 (17%) of the tested samples, and a total of 9 (7%) samples had the presence of *E. coli*. In addition, 9 STEC strains were isolated from: pasteurized cream (2/9), minas padrão cheese (2/9), minas frescal cheese (4/9), and ricotta (1/9). Isolates were not positive for "big six" serotyping. PFGE revealed three 100% similar isolates coming from two different products, altough produced in the same industry, suggesting a cross-contamination situation. Moreover, two STEC strains exhibited resistance to streptomycin, whereas five strains presented an intermediary resistance to ampicillin, including the two aforecited. The presence of STEC strains in milk and milk products represents a risk to the consumer, especially the immunodepressive age group of children and older adults.

Key-words: STEC; *stx*$_1$ and *stx*$_2$ genes; foodborne disease; food safety; public health.

1. Introduction

Milk represents an excellent source of nutrients (protein, lipids, carbohydrates, vitamins and minerals), consumed worldwide by people of all age groups (Mitchell et al., 2015; Manary et al., 2016). However, microbiological contamination of dairy products can happen in any stage of food production (Rola et al., 2016). Indeed, the presence of coliforms in dairy can interfere in quality, causing food spoilage, lost of its physical and chemical characteristics, thus reducing its shelf life (Martin et al., 2016). In addition, microorganisms such as *Escherichia coli*, an intestinal commensal coliform, are known to be indicators of fecal contamination (Conway and Cohen, 2015; Van Asselt et al., 2016). Therefore, the presence of *E. coli* in food indicates a public health concern, since this bacterium has known pathogenic strains responsible for causing severe enteric disorders in consumers of all ages (FRATAMICO et al., 2016).

Shiga toxin-producing *E. coli* (STEC) is a pathogenic group of *E. coli* reported as an important foodborne pathogen. STEC is often related to several outbreaks due to the action of encoded potent toxins that can cause intestinal cells damage, as well as other systemic lesions, such as bloody diarrhea (hemorrhagic colitis), and most seriously, hemolytic uremic syndrome (HUS) (Castro et al., 2017). Furthermore, children and older adults are more prone to develop dangerous complications, such as HUS, acute renal failure, and neurological symptoms as sequelae, and even death (Lee et al., 2016). The main virulence factors associated with STEC are the Shiga toxins Stx1 and Stx2, whereas STEC strains can produce either, one or both toxins. However, Stx2-producer strains are often related to severe cases of infection, including HUS and death (Steiner, 2016). O157:H7 strains are the most associated serogroup with dangerous diseases (Castro et al., 2017). Nevertheless, other strains belonging to serogroups O26, O45, O103, O111, O121, and O145, the so-called “big six”, have been linked to severe cases in several different countries (Elder et al., 2016). According to the Foodborne

Outbreak Database (CDC, 2015), dairy and ready-to-eat products are listed as one of the major causes of multistate foodborne outbreaks. In fact, as bovine is recognized as one of the primary sources of STEC shedding (Barth et al., 2016), milk may be exposed to this pathogen by fecal contamination during milking steps. In this case, the milk pasteurization is highly indicated, as STEC is very likely to be inactivated in this process (Ahmed and Samer, 2017). However, STEC can also contaminate milk and milk products during manufacturing, being able to persist in the final product (Cardoso and Marin, 2016). STEC persistence in milk and milk products is achieved through survival response mechanisms that enable the pathogen to surpass harsh conditions found in these products, becoming a risk for human consumption (Peng et al., 2011).

Currently, Brazil is the third largest milk consumer and the fifth major milk producer country worldwide (USDA, 2018). Also, the Brazilian state of Bahia owns the third largest dairy cattle in Brazil (SEBRAE, 2017). While this is true, only very few studies have reported the occurrence of pathogenic strains of *E. coli* in dairy food produced in this region. Considering the importance of these microorganisms concerning the public health and food safety of animal origin products, the present study was aimed to report the prevalence of thermotolerant coliform and the occurrence and antimicrobial susceptibility of Shiga toxin-producing *Escherichia coli* in milk and dairy products produced in the state of Bahia, Northeast Brazil.

2. Material and Methods

2.1 Sampling procedures and microbiological analysis

A total of 123 dairy samples, including pasteurized milk (n = 31), pasteurized cream (n = 4), cheese (n = 52), fermented milk (n = 19) and butter (n= 17), were collected from selected

locations in the state of Bahia (Northeast Brazil): Baixa Grande, Cachoeira, Camaçari, Castro Alves, Feira de Santana, Ichú, Ipirá, Irecê, Mundo Novo, Pedrão, Riachão do Jacuípe, São Sebastião do Passé, Simões Filho, Teodoro Sampaio and Wanderley (Figure 1). The samples were acquired from Mars 2017 to September 2018 and transported to the laboratory at 5 ± 3 °C for microbiological procedures. All samples were produced under State Inspection Service (S.I.E.).

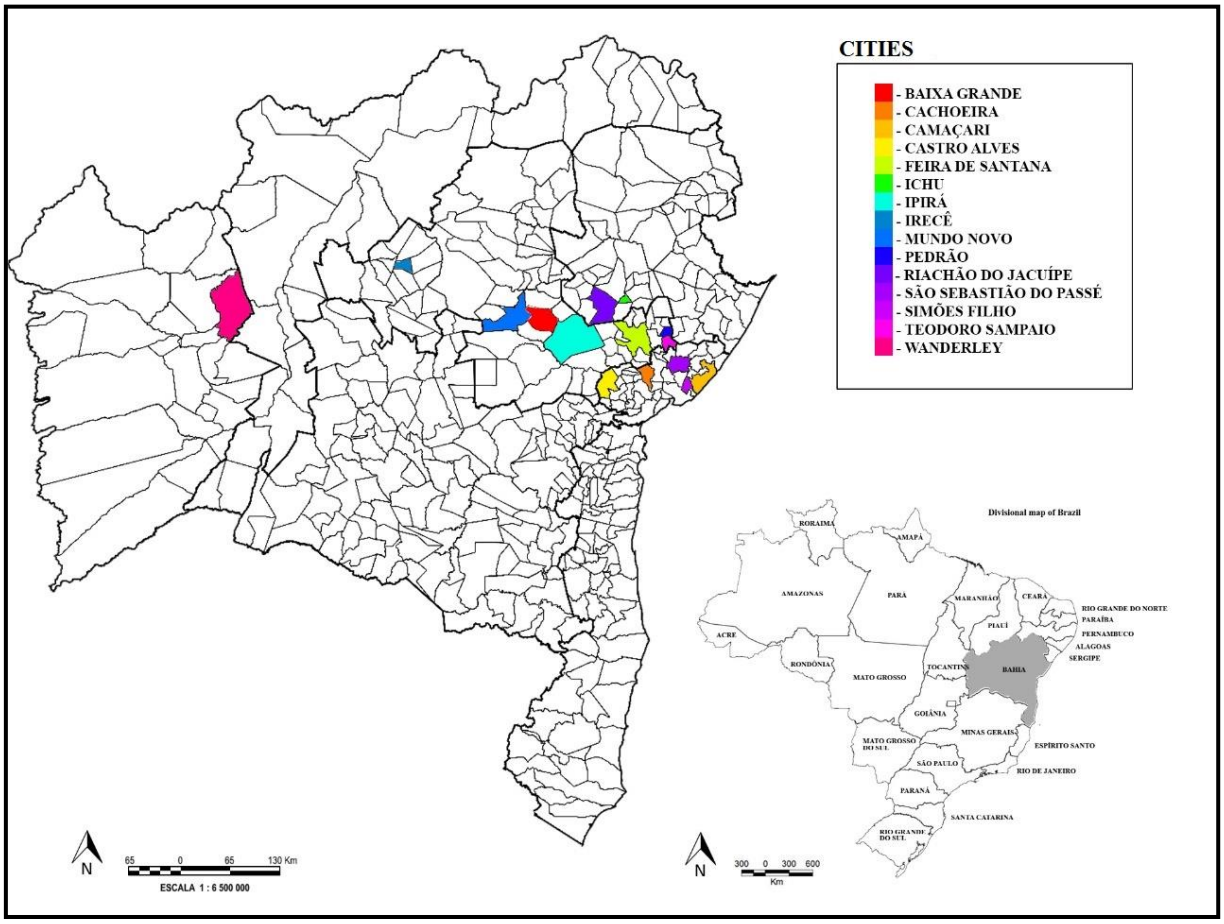


Fig. 1 In the left, divisional map of Bahia, showing the cities where dairy samples were obtained. In the right, divisional map of Brazil.

For the enumeration of bacteria, each dairy sample (25 g or 25 mL) were added to 225 mL of 0.1% buffered peptone water (Merck, Germany), achieving a dilution of 10^{-1}. The samples were then diluted serially to perform the microbiological analysis, according to The Compendium of Methods for the Microbiological Examination of Foods by Kornacki et al.

(2015). Thermotolerant coliforms were evaluated through the most probable number technique (MPN) (Kornacki et al., 2015). Thus, 1 mL aliquots from each dilution (10^{-1} to $10^{-3}/10^{-5}$) were incubated in tubes containing 10 mL of Lauryl Sulfate Tryptose (LST) broth (Kasvi, Italy) with inverted Durham tubes at 35 °C to 24-48 h. Then, aliquots from the positive LST containing tubes (growth and gas production) were added to tubes containing *Escherichia coli* (EC) broth (Himedia, India) and incubated at 44.5 °C for 24-48 h for thermotolerant coliform counts.

The presence of *Escherichia coli* was confirmed after bacterial isolation using the streak-plate technique in Eosin Methylene Blue agar (EMB) (Kasvi, India). Biochemical tests used to confirm *E. coli* isolation were: citrate, indole, methyl red, and Voges-Proskauer (IMViC) (Merck, Australia; Vetec, Brasil; Kasvi, India). *E. coli* strains were stored at -18 °C in brain heart infusion (BHI) broth (Kasvi, India) with 20% glycerol until further use.

2.2 Polymerase chain reaction assay (PCR) for STEC and the "big six" non-O157 strains

Fifty confirmed *E. coli* isolates were submitted to PCR technique in order to screen for Shiga toxin-producing *E. coli*. The DNA was obtained by thermal boiling at 100 °C for 10 minutes and posteriorly exposed to 4 °C for 2 minutes. DNA quantification was performed using the Qubit 2.0 kit (Invitrogen, United States). PCR reactions were carried out based on the primers described by FDA USDA (2011). Thus, a duplex-PCR reaction was performed to detect stx_1 and stx_2 genes. *E. coli* O157:H7 (ATCC 43895) and sterile ultrapure water (milli-Q) were used as positive and negative control respectively. In addition, two m-PCR reactions were performed to detect the six major serogroups involved in foodborne outbreaks, known as the "big-six". The following genes were targeted: *wzxO26*, *wzxO45*, *wzxO103*, *wzxO111*, *wbqEO121*, and *wzxO145*. Amplicons were visualized on a photodocumentator (MiniBis-Pro DNT; Bio-Imaging Systems, Israel). Primers used in all reactions are described in Table 1.

Table 1 - Set of primers composing the duplex and the m-PCR assays used in this study.

Target gene	Primer	Primer sequence (5'-3')	Amplicon size (bp)	Reference
stx$_1$	LP30	CAGTTAATGTGGTGGCGAAGG	348	USDA (2001)
	LP31	CACCAGACAATGTAACCGCTG		
stx$_2$	LP43	ATCCTATTCCCGGGAGTTTACG	584	USDA (2001)
	LP44	GCGTCATCGTATACACAGGAGC		
wzxO26	O26F4	AGGGTGCGAATGCCATATT	417	Bai et al. (2012)
	O26R4	GACATAATGACATACCACGAGCA		
wzxO45	O45-F	GGGCTGTCCAGACAGTTCAT	890	Bai et al. (2012)
	O45-R	TGTACTGCACCAATGCACCT		
wzxO103	O103F2	GCAGAAAATCAAGGTGATTACG	740	Bai et al. (2012)
	O103R2	GGTTAAAGCCATGCTCAACG		
wzxO111	O111F2	TGCATCTTCATTATCACACCAC	230	Bai et al. (2012)
	O111R2	ACCGCAAATGCGATAATAACA		
wbqEO121 *wbqFO121*	O121-F2	TCAGCAGAGTGGAACTAATTTTGT	587	Bai et al. (2012)
	O121-R2	TGAGCACTAGATGAAAAGTATGGCT		
wzxO145	O145F5	TCAAGTGTTGGATTAAGAGGGATT	523	Bai et al. (2012)
	O145R5	CACTCGCGGACACAGTACC		

2.3 Antimicrobial disk susceptibility test

Positive STEC strains were tested for antimicrobial susceptibility using the agar disk diffusion method described by the Clinical and Laboratory Standards Institute (CLSI, 2017). Bacteria were spread on Mueller-Hinton agar plates using sterile swabs (Himedia, India) and incubated at 37 ºC for 18 h. The antibiotics (Sensibiodisc, Brazil) used in this study were: ampicillin (10 µg), imipenem (10 µg), tetracycline (30 µg), gentamicin (10 µg), nitrofurantoin (300 µg), ciprofloxacin (5 µg), cefoxitin (30 µg), streptomycin (10 µg), chloramphenicol (30 µg), sulfamethoxazole-trimethoprim (25 µg), ceftazidime (30 µg), and nalidixic acid (30µg). Inhibition zones were then measured, and the strains were classified as susceptible,

intermediately resistant (reduced susceptibility), or resistant, according to CLSI guidelines (2017).

2.4 Pulse-field gel electrophoresis

Positive STEC stains were also submitted to pulse-field gel electrophoresis (PFGE) technique, using the CHEF-DR III (Bio-Rad, United States) equipment. According to the methods of CDC PulseNet protocol (RIBOT et al., 2006), using the *Xba*I enzyme to perform the digestion. Following the specifications, the initial switch time was 6.76 s, the final switch time was 35.38 s, with a total runtime of 18 h. Also, in order to perform the PFGE fingerprints analyses and comparisons, GelJ software was used (HERAS et al., 2015). Dice coefficient was used to the similarity analyses. CDC *Salmonella* ser. Branderup isolate H9812 were used as reference strain. Images were taken using DOC PRINT II (Vilber Lourmant, France).

3. Results

A total of 123 dairy products were collected from 15 different sampled locations (Figure 1). Thermotolerant coliform counting and *Escherichia coli* results from sampled locations are shown in Table 2. A total of 38 (31%) samples had the presence of coliform bacteria. Positive thermotolerant coliform results were found in 10 out of 15 cities, with the highest level being in Camaçari 100% (6/6). The thermotolerant coliform count ranged from > 3 (shown in table as blank spaces) to > 1.1 x 10^3 MPN/g or mL, with unsatisfactory levels in 20 (16%) of the tested samples (BRASIL, 2001; FDA, 2013). Also, the highest unsatisfactory levels of thermotolerant coliform were found in Cachoeira (31%). Moreover, *E. coli* positive

samples were detected in five out of 15 cities in the present study, with the highest level found in Ichú (25%).

Table 2 – Occurrence of thermotolerant coliform and *Escherichia coli* in dairy products from sampled locations in the state of Bahia, Brazil.

Location	Number of samples	Positive coliforms (%)	Unsatisfactory[a] (%)	Counting interval (MPN/g or mL)[b]	Positive *E. coli* (%)
Baixa Grande	1				
Cachoeira	13	5 (38)	4 (31)	23 – >1.1 x 10^3	2 (15)
Camaçari	6	6 (100)		23 – >1.1 x 10^3	
Castro Alves	8	2 (25)	1 (12.5)	>1.1 x 10^3	
Feira de Santana	5	1 (20)	1 (20)	93	1 (20)
Ichu	16	6 (37.5)	3 (19)	15 – >1.1 x 10^3	4 (25)
Ipirá	1				
Irecê	17	1 (6)	1 (6)	>1.1 x 10^3	
Mundo Novo	3				
Pedrão	1				
Riachão do Jacuípe	5	3 (60)	1 (20)	>1.1 x 10^3	
São Sebastião do Passé	28	10 (36)	7 (25)	23 – >1.1 x 10^3	1 (4)
Simões Filho	3	2 (67)		15 – 43	
Teodoro Sampaio	15	2 (13)	2 (13)	46	1 (7)
Wanderley	1				
Total	123	38 (31)	20 (16)		9 (7)

[a] = Unsatisfactory according to Brazilian regulation (BRASIL, 2001) and/or FDA Standards (FDA, 2013); [b] = Most Probable Number.

The occurrence of thermotolerant coliform bacteria and *Escherichia coli* by individual dairy samples were also analyzed and are shown in Table 3. Pasteurized milk, pasteurized cream, and butter showed a thermotolerant coliform prevalence of 19% (6/31), 25% (1/4) and 18% (3/17), respectively. Thus, all positive samples of pasteurized milk, pasteurized cream and butter were unsuitable for consumption according to Brazilian legislation (BRASIL, 2001). Fermented milk drink, yogurt, cottage, requeijão processed cheese, provolone, boursin, chèvre an l'huile and Saint-Paulin cheeses did not show any thermotolerant coliform count. Other cheeses presented the following thermotolerant coliform prevalence: minas frescal 80% (8/10), minas padrão 100% (1/1), coalho 80% (8/10), mozzarella 70% (7/10), and prato cheese

33% (1/3). Furthermore, *E. coli* was detected in 7% (9/123) of total samples (pasteurized milk, pasteurized cream, butter, ricotta, minas frescal cheese and minas padrão cheese).

Although Brazilian legislation (BRASIL, 2001) does not mention *E. coli* standards, all pasteurized milk and cream samples containing *E. coli* are unsatisfactory according to FDA Standards (FDA, 2013). Therefore, fifty isolates were confirmed as *Escherichia coli* and individual sampling data are also presented in Table 3.

Table 3 - Prevalence of thermotolerant coliform bacteria and *Escherichia coli* in dairy products collected in the state of Bahia, Brazil.

Sample			Coliforms			*E. coli*		
Type		Total	Positive Samples (%)	Unsatisfactory (%)	Counting interval (MPN/g or mL)[c]	Positive Samples (%)	Unsatisfactory (%)	Isolates
Pasteurized milk		31	6 (19)	6 (19)[a]	23 – >1.1 x 10^3	2 (6)	2 (6)[b]	6
Pasteurized cream		4	1 (25)	1 (25)[a]	460	1 (25)	1 (25)[b]	9
Butter		17	3 (18)	3 (18)[a]	43 – >1.1 x 10^3	1 (6)		1
Fermented milk	Fermented milk	1						
	Yogurt	18						
Cheese	Ricotta	5	2 (40)		23 - 43	1 (20)		2
	Cottage	1						
	Requeijão processed	4						
	Minas frescal	10	8 (80)	5 (50)[ab]	>1.1 x 10^3	3 (30)		19
	Minas padrão	1	1 (100)	1 (100)[ab]	>1.1 x 10^3	1 (100)		13
	Coalho	10	8 (80)	2 (20)[ab]	>1.1 x 10^3			
	Mozzarella	10	7 (70)	2 (20)[ab]	>1.1 x 10^3			
	Prato	3	1 (33)		43			
	Provolone	2						
	Boursin	2						
	Chévre a l'huile	2						
	Saint-Paulin	2						
Total		123	38 (31)	20 (16)				50

[a] = Unsatisfactory according to Brazilian regulation (BRASIL, 2001); [b] = Unsatisfactory according to FDA Standards (FDA, 2013); [c] = Most Probable Number per g or mL.

Amongst the fifty *E. coli* confirmed strains, none of them were positive for the stx_1 gene, whereas all 9 were positive for the stx_2 gene. Individual profile of *E. coli* strains virulence

genes is shown in Table 4. As the presence of the specific *stx* gene encodes the production of Shiga toxin, all nine confirmed *stx* isolates could be classified as STEC (CROXEN et al., 2013). However, none of the isolates was positive for the "big six" assay, infering they belong to other serogroups.

Table 4 - Source, virulence genes, and antimicrobial resistance of STEC strains isolated from dairy products collected in the state of Bahia, Brazil.

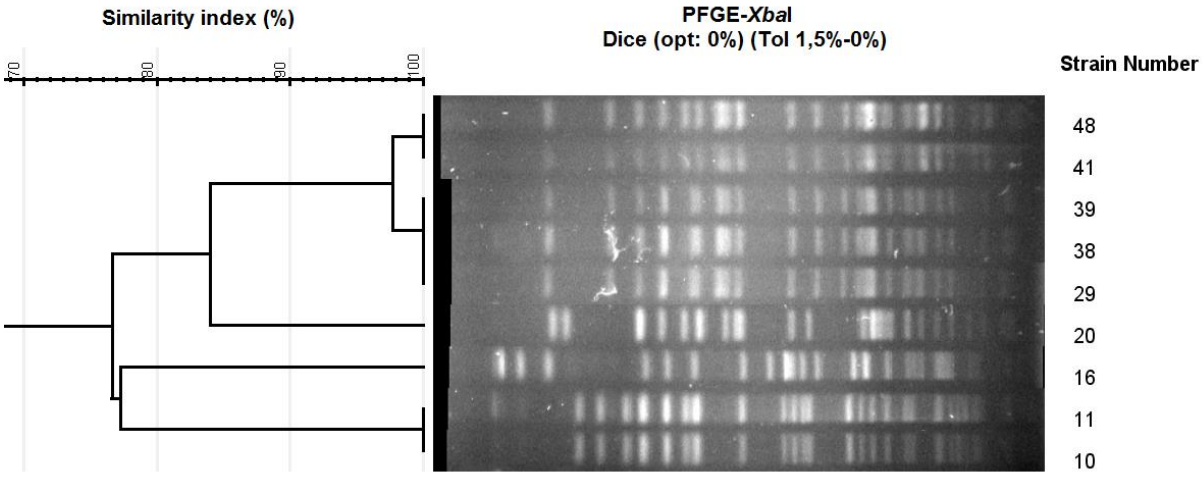
Strain	Sample	Source	Genes		Antimicrobial resistance
			stx$_1$	*stx*$_2$	
S10	23	Minas padrão cheese	-	+	Streptomycin; Intermediary ampicillin
S11	23		-	+	Streptomycin; Intermediary ampicillin
S16	24	Minas frescal cheese	-	+	Intermediary ampicillin
S20	24		-	+	Susceptible to all tested drugs
S29	76	Ricotta	-	+	Susceptible to all tested drugs
S38	77	Minas frescal cheese	-	+	Susceptible to all tested drugs
S39	77		-	+	Susceptible to all tested drugs
S41	78	Pasteurized cream	-	+	Intermediary ampicillin
S48	78		-	+	Intermediary ampicillin

S20, S29, S38 and S39 isolates were sensitive to all tested antibiotics. Resistance to streptomycin was detected in two STEC strains, S10 and S11. Also, these both STEC strains exhibited intermediate resistance to ampicillin. In addition, strains S16 and S41 and S48 also presented an intermediate resistance to ampicillin (Table 4).

PFGE profile with *Xba*I digestion demonstrated that S10 and S11 isolates were 100% similar, both obtained from the same sample. Strains S41 and S48 were also 100% similar and were obtained from sample 78. Interestingly, these strains presented the same antimicrobial resistance profile, which corroborates even more with PFGE similarity. Strains S29, S38 and S39 strains also presented 100% similarity but were isolated from other two different samples. All strains were pan-susceptible against the panel of antimicrobial tested. Also, these three strains showed 98% of similarity with S41 and S48 strains. Equally important, S16 and S20

strains were both isolated from the same sample, showing 77% similarity between them, whereas S20 strain presented 84% of similarity with S29, S38, S39, S41, and S48 strains. PFGE profile is shown in figure 2.

Fig. 2 *Xba*I PFGE profile for the nine isolated STEC strains.



4. Discussion

The occurrence of coliforms in dairy products was also described by Belli et al. (2013), who reported high coliform counts in mozzarella and soft cheese (mean value ≥ 2.7 Log CFU/g), but also low coliform counts for ricotta and yogurt (mean value ≥ 1.52 log CFU/g and ≥ 1.62 log CFU/g respectively). Similarly, thermotolerant coliform was detected by Melo et al. (2015) in four out of 38 (11%) dairy products samples originated from different countries. In the dairy industry, improving hygiene procedures are a fundamental step in the control of microorganisms, especially those capable of forming biofilms, whether spoiler or pathogenic. However, coliforms are widely found in bulk tanks in the United States as stated by van Kessel et al. (2004), that found 95% (818 out of 860) of bulk tank milk samples contaminated with

thermotolerant coliforms. Indeed, Azevedo et al. (2016) stated that prevalence of bateria in bulk tank milk if often related to milking mastitic cows, milking machine cleanliness, and poor hygiene practices during milking.

In this study, cheese presented the highest contamination level, even though all cheese samples were made out of pasteurized milk. However, for pasteurized milk, cream and cheese products, the pasteurization process should be able to destroy coliform bacteria. The presence of coliform bacteria in pasteurized products can be related to pasteurization failure or post contamination process caused mainly by intense manipulation during manufacture practices (Martin et al., 2016; Masiello et al., 2016). For instance, Trmčić et al. (2016) reported a prevalence of 42% and 21% for coliform in raw milk cheese and pasteurized milk cheese respectively, from market sources in New York city. Also, Islam et al. (2018) found only 2% of pasteurized milk contaminated with thermotolerant coliforms. In addition, de Deus et al. (2017) detected an average of 7.18 log CFU g^{-1} of coliform in raw *coalho* cheese and 4.48 log CFU g^{-1} in roasted *coalho* cheese sold in Itaparica Island, northeast Brazil. On the other hand, because of its specific technological process, fermented milk drink and yogurt have an elevated acidity, wich, in a general way, can control the growing of coliform bacteria and *E. coli* (HERVERT, 2017). As a matter of fact, none of these studied samples showed any thermotolerant coliform nor *E. coli* presence.

We have detected a considerable number of *E. coli* in pasteurized milk and cream, butter and cheese. Similarly, Belli et al. (2013) reported *E. coli* in cheese and ricotta. Likewise, in Trmčić et al. (2016) study, authors isolated *E. coli* from 23 (31%) cheese samples positive for coliforms. Because these bacteria live as commensals inside the gastrointestinal tract of warm-blooded animals, the presence of *E. coli* in food products is a reliable indicator of fecal contamination (Martin et al., 2016). Dairy products can also be contaminated trough contact with water, food manipulators and biofilms on equipment (Martin et al., 2016; Rola et al.,

2016). Therefore, the process of hygiene within the dairy industry is a fundamental step to control microorganisms, especially those capable of forming biofilms, whether spoiler or pathogenic. Thus, sanitary measures to increase food safety, such as the implementation of HACCP (Hazard Analysis and Critical Control Point), SOP (Standard Operating Procedures) and GMP (Good Manufacturing Practices) are characterized as good ways of preventing contamination and reducing the occurrence and pathogen dissemination (Nam, 2017; Aguiar, 2018).

In this study, all isolated STEC strains were positive for the *stx*$_2$ gene. In fact, the infection of strains-producing Stx2 is often related to more severe illnesses in human, such as HUS (Friedrich et al., 2002; Mellmann et al., 2008; Olavesen et al., 2016; Suardana et al., 2018). In addition, STEC was confirmed in pasteurized cream and cheese, with a prevalence of 18% (9/50). Two minas frescal cheese samples had the presence of STEC (2/10). Similarly, Carvalho et al. (2014) found two (6.7%) STEC strains in minas frescal cheese sold in street fairs in Goiânia, GO, Brazil. These results are higher than the one described by Leite Júnior et al. (2014), where they could not find any STEC in four minas frescal cheese samples. Minas padrão cheese (1/1), ricotta (1/5) and pasteurized cream (1/4) had the presence of STEC in one sample each. To our knowledge, this is probably the first report of STEC in these dairy products.

The isolation of STEC strains with 100% of similarity in two different samples produced in the same industry (S29, S38 and S39) suggested a cross-contamination situation that should be avoided during the processing steps. Interestingly, these three strains were 98% similar to other two strains (S41 and S48) that were isolated from a product produced in a different city, suggesting a possible mutual source of contamination.

In our study, none of the isolated STEC strains were multidrug resistant. Two strains (22%) showed resistance to streptomycin and intermediary resistance to ampicillin, whereas five (55.5%) only presented intermediary resistance to ampicillin. Resistance to

streptomycin and ampicillin was also observed by Rosa-Hernández at al. (2018) in STEC strains isolated of fresh cheese sold in Mexico. They reported seven multidrug resistant STEC strains resistant to up to 14 different antibiotics. In another study, Ahmed and Shimamoto (2015) demonstrated an incidence of 57.4% (31/54) of multidrug resistant STEC isolated from food samples produced in Egypt, including cheese and milk. The authors also reported two strains, one from cheese and another from milk that were resistant to 18 tested antibiotics.

The presence of STEC in food is generally related to bovine origin food, such as beef and milk products (Burgess et al., 2016). Contamination of pasteurized dairy products is usually related to post-processing contamination, once bacteria such as *E. coli* should not be able to survive the pasteurization process (Ahmed and Samer, 2017). On the other hand, some *E. coli* strains have been described to show D_{60}-values of more than 10 min or even resisting to an elevated temperature of 71 °C, which can become a health concern if a pathogenic strain acquires this characteristic (Dlusskaya et al., 2011; Li and Gänzle, 2016). In addition, because the shedding of STEC is commonly related to the presence of the bacteria in bovine feces, we can also infer that contamination of products may have occurred before milk processing, and that is because some STEC strains are capable of resisting processing temperatures, mainly sub-pasteurization treatment (Schlesser et al., 2006). Besides the fact that dairy products are often maintained in cold temperature in order to avoid spoilage, some STEC strains are able to resist cold temperature, persisting in low-temperature storage (Elhadidy and Álvarez-Ordóñes, 2016; Li et al., 2018).

5. Conclusion

E. coli and coliforms were detected in different dairy products from the state of Bahia, Brazil. Nine isolates were confirmed as Stx2-producer STEC, the most prevalent group

in outbreaks. The identification of STEC strains encoding *stx*$_2$ gene in dairy products can be a public health concern due to the risk of infection, including the development of HUS and HC in consumers of all age groups. In addition, the presence of STEC strains 100% similar within the PFGE profile in two different samples of cheese produced in the same industry suggested a cross-contamination situation that is unacceptable during the processing steps. These results highlight the persistence of pathogenic bacteria in the dairy production chain, evidencing a failure of hygiene maintenance inside the producing plant.

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