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CAMILA SAMPAIO CUTRIM

SOBREVIVÊNCIA DA *Escherichia coli* O157:H7 EM IOGURTE COM BAIXO TEOR DE LACTOSE

NITERÓI
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Dissertação apresentada ao Programa de Pós-Graduação em Medicina Veterinária da Universidade Federal Fluminense, como requisito parcial à obtenção do Grau de Mestre. Área de Concentração: Higiene Veterinária e Processamento Tecnológico de Produtos de Origem Animal.

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Aprovada em____de fevereiro de 2015

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Niterói, RJ

2015

"Crescer custa, demora, esfola, mas compensa. É uma vitória secreta, sem testemunhas. O adversário somos nós mesmos."

Martha Medeiros

AGRADECIMENTOS

Aos meus pais Marise Sampaio e Maximo Cutrim

“Quando alguém está em sua vida por uma "Razão" é, geralmente, para suprir uma necessidade que você demonstrou. Elas vêm para auxiliá-lo numa dificuldade, te fornecer orientação e apoio, ajudá-lo física, emocional ou espiritualmente. Elas poderão parecer como uma dádiva de Deus, e são! Relacionamentos de uma "Vida Inteira" te ensinam lições para a vida inteira: coisas que você deve construir para ter uma formação emocional sólida. Sua tarefa é aceitar a lição, amar a pessoa, e colocar o que você aprendeu em uso em todos os outros relacionamentos e áreas de sua vida. É dito que o amor é cego, mas a amizade é clarividente. Obrigado por ser parte da minha vida. ” (Martha Medeiros). Obrigada pelo amor incondicional dedicado a mim, vocês são o meu maior exemplo e a base de tudo!

Ao meu Orientador Marco Antonio Sloboda Cortez:

"Mas eu não quero me encontrar com gente louca", observou Alice.

" Você não pode evitar isso", replicou o gato.

"Todos nós aqui somos loucos. Eu sou louco, você é louca".

"Como você sabe que eu sou louca?" Indagou Alice.

"Deve ser", disse o gato, "Ou não estaria aqui" (Alice no país das maravilhas- Lewis Carroll). Obrigada por ter me ajudado a ingressar no “mundo dos loucos”.

Aos meus mestres.

“Em tempos em que quase ninguém se olha nos olhos, em que a maioria das pessoas pouco se interessa pelo que não lhe diz respeito, só mesmo agradecendo àqueles que percebem nossas descrenças, indecisões, suspeitas, tudo o que nos paralisa, e gastam um pouco da sua energia conosco, insistindo. ” Martha Medeiros

Aos meus amigos:

“A amizade é o conforto indescritível de nos sentirmos seguros com uma pessoa, sem ser preciso pesar o que se pensa, nem medir o que se diz” (George Eliot) Obrigada por fazerem parte desta jornada!

RESUMO

O mercado consumidor busca cada vez mais alimentos funcionais, com benefícios adicionais à nutrição. Os derivados lácteos são excelentes fontes desses benefícios visto que estão presentes na rotina alimentar da população, em variadas formas de consumo, abrangendo diferentes públicos-alvo e de variado poder aquisitivo. No entanto, alguns indivíduos são intolerantes à lactose, principal carboidrato do leite, que também está presente em produtos fermentados, o que leva a um grande prejuízo na comercialização dos produtos lácteos além de uma restrição alimentar para o consumidor. Dessa forma, estudos relacionados à incorporação da enzima β-galactosidase e ao comportamento das bactérias láticas em relação a ação inibidora frente aos microrganismos patogênicos devem ser realizados a fim de possibilitar a elaboração de uma classe de alimentos que atendam às premissas expostas, assim como às exigências das legislações e ainda, apresentem características microbiológicas, físico-químicas e sensoriais adequadas. Diante do exposto, objetiva-se com este trabalho verificar o efeito da redução da lactose sobre a viabilidade da *Escherichia coli* enterohemorrágica em iogurtes produzidos com leite bovino. A partir dos resultados do primeiro experimento (Artigo 1), foi observado que uma contaminação inicial de leite aumenta a contagem de *E. coli* O157: H7 durante a fermentação (4,34 log UFC.mL^{-1} para 6,13 log UFC.mL^{-1} no iogurte tradicional e 6,16 log UFC.mL^{-1}em baixa iogurte lactose) e altera as características dos iogurtes durante o processo de fermentação, com formação de grande quantidade de bolhas de ar e sinérese. Estes resultados foram decisivos para o desenvolvimento do segundo experimento (Artigo 2), em que foi utilizado um método de inoculação diferente e observou-se que uma contaminação inicial de leite por *E. coli* O157: H7 aumenta sua contagem durante a fermentação. Notou-se também que a *E. coli* foi capaz de sobreviver durante 10 dias em iogurte com leite tradicional e pré hidrolisado e durante 22 dias em iogurte preparado a partir de leite sem lactose.

Palavras-chave: Iogurte. Intolerância a lactose. β-galactosidase. *Escherichia coli*

ABSTRACT

The consumer market demands increasingly functional foods with additional benefits to nutrition. The dairy products are excellent sources of these benefits as they are present in the routine diet of the population in various forms of consumption, including different target audiences and different purchasing power. However, some individuals are intolerant to lactose, the main carbohydrate in milk, which is also present in fermented products, leading to a great loss to the dairy product's market and a food restriction to the consumer. In this way, studies related to the incorporation of the enzyme β-galactosidase and the behavior of lactic bacteria in relation to the inhibitory effect against pathogenic microorganisms should be performed to enable the development of a class of foods that meet the assumptions exposed, as well as the requirements of legislation and also suitable microbiological, physicochemical and sensory characteristics. Considering the above, the objective of this work was to verify the effect of the reduction of lactose on the viability of enterohaemorrhagic *Escherichia coli* in yogurts produced with cow's milk. From results of the first experiment (Article 1) it was observed that an initial contamination of milk enhance *E. coli* O157:H7 counts during fermentation (from 4.34 log cfu.mL^{-1} to 6.13 log cfu.mL^{-1} in traditional yogurt and 6.16 log cfu.mL^{-1}in low lactose yogurt) and changes the characteristics of yogurts during fermentation process, with a lot of air bubbles formation and syneresis.These results were decisive for the development of the second experiment (Article 2) in which it was used a different inoculation method and it was observed that an initial contamination of milk by *E. coli* O157:H7 enhance its counts during fermentation. It was also noted that *E. coli* was able to survive for 10 days in yogurt with whole and pre hydrolyzed milk and for 22 days in yogurt from free lactose milk.

Keywords: Yogurt. Lactose intolerance. β-galactosidase. *Escherichia coli*

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1 INTRODUÇÃO

O leite e seus derivados representam alguns dos produtos que mais movimentam o mercado mundial, sendo a produção brasileira de leite fluido em 2013 de aproximadamente 34 milhões de toneladas segundo dados da “Food and Agriculture Organization of the United Nations” (FAO) (FAO, 2015).

A industrialização do leite e a fabricação de derivados lácteos são formas de agregar valor à matéria prima, aumentar a validade comercial, além de impulsionar a cadeia leiteira através da geração de empregos sendo a fermentação uma das tecnologias mais utilizadas pela indústria. Em relação ao mercado lácteo, as vendas de derivados no Brasil vêm aumentando de forma significativa chegando a movimentar 235,6 milhões de dólares em 2014, representando aumento de 259,6% em relação a 2013 segundo dados da Companhia Nacional de Abastecimento (COMPANHIA NACIONAL DE ABASTECIMENTO, 2014)

Embora o mercado lácteo esteja crescendo consideravelmente, estima-se que 65% da população adulta mundial apresenta sinais e sintomas da má digestão da lactose (VUORISALO et al., 2012). A lactose é o principal carboidrato do leite e é digerida no intestino delgado pela enzima β–galactosidase mais conhecida como lactase. Em todos os mamíferos ocorre um declínio das quantidades desta enzima após o desmame, porém, os índices de pessoas lactase não persistente, que apresentam má digestão da lactose, na população mundial variam de acordo com características genéticas e culturais, como o hábito de consumo de leite e derivados.

O iogurte é uma alternativa de produto com teor reduzido de lactose, podendo ser consumido apenas por indivíduos com baixo índice de intolerância uma vez que a hidrólise do dissacarídeo é relativamente baixa durante o processo de fermentação (10 – 30%).

Contudo, a hidrólise da lactose pode ser inserida no processamento destes produtos, como solução para pessoas com má digestão da lactose, pois essa tecnologia envolve a diminuição significativa ou eliminação total da lactose nos alimentos através do uso de enzimas exógenas.

Embora o iogurte seja considerado seguro devido ao pH ácido, foram relatados surtos de *Escherichia coli* O157:H7 envolvendo este derivado lácteo (DE BUYSER et al., 2001; MORGAN et al., 1993). A *E. coli* é a espécie mais importante do gênero

Escherichia, e nos últimos anos a colite hemorrágica tem sido associada a uma cepa denominada *E. coli* O157: H7. Esta linhagem é conhecida como agente causador de diarreia sanguinolenta e causa predominante de síndrome urêmica hemolítica. Alguns pesquisadores já demonstraram que a *E. coli* O157:H7 é capaz de sobreviver às etapas de fermentação e estocagem dos iogurtes, sendo resistente ao ambiente ácido.

Diante do exposto, objetivou-se com este trabalho verificar o efeito da redução da lactose sobre a viabilidade da *Escherichia coli* enterohemorrágica em iogurtes tradicionais e com teor reduzido de lactose produzidos com leite bovino.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 LEITES FERMENTADOS

Após a secagem, a fermentação é o mais antigo método de conservação de alimentos, e esta técnica de preservação se tornou popular com o desenvolvimento das civilizações, porque não só preserva os alimentos, como também gera uma variedade de sabores, aromas e outras características sensoriais nos produtos (JASHBHAI; BABOO, 2003)

No Brasil, os leites fermentados são "produtos adicionados ou não de outras substâncias alimentícias, obtidas por coagulação e diminuição do pH do leite, ou leite reconstituído, adicionado ou não de outros produtos lácteos, por fermentação láctica mediante ação de cultivos de microrganismos específicos. Estes microrganismos específicos devem ser viáveis, ativos e abundantes no produto final durante seu prazo de validade" (BRASIL, 2007).

Os efeitos benéficos dos leites fermentados foram cientificamente confirmados pela primeira vez no início do século XX, com o microbiologista russo Ilya Ilyich Metchnikoff, que propôs uma teoria sobre o prolongamento da vida baseado no consumo diário de leites fermentados pelos búlgaros. Ele acreditava que a atividade metabólica das bactérias ácido-láticas inibiria as bactérias intestinais do mesmo modo que inibem a putrefação dos alimentos (METCHNIKOFF, 2004)

O iogurte é o principal representante dos leites fermentados e é um alimento tradicional nos Bálcãs e na Ásia Mediterrânea. A palavra “iogurte” é derivada da palavra turca “jugurt”, sendo conhecida por uma diversidade de nomes em diferentes países (TAMINE; ROBINSON, 2007).

O iogurte possui grande aceitação no mercado brasileiro e tem como vantagens, o baixo custo de produção, pois não necessita de equipamentos sofisticados para ser elaborado, ser de fácil preparo, e ser uma forma de aumentar a validade comercial do leite, permitindo assim aumento do valor agregado do produto (MARTINS et al., 2012). Além das vantagens de produção, o iogurte

conquistou uma grande importância econômica em razão do seu elevado valor nutricional, benefícios associados à saúde e pelo seu sabor atrativo (PENG et al., 2009)

Conforme o Regulamento Técnico de Identidade e Qualidade (RTIQ) de Leites Fermentados (BRASIL, 2007) entende-se por iogurte o produto cuja fermentação do leite se realiza com cultivos protossimbióticos de *Streptococcus thermophilus* e *Lactobacillus delbrueckii* subsp. *bulgaricus*.

Durante a fermentação, a proteína, a gordura e a lactose do leite sofrem hidrólise parcial, tornando o produto facilmente digerível, sendo assim considerado agente regulador das funções digestivas (RODAS et al., 2001; TEIXEIRA et al., 2000;).

O consumo de iogurte também é associado a diversos benefícios ao organismo tais como facilitar a absorção de cálcio, fosforo e ferro; ser fonte de galactose, que é um importante monossacarídeo envolvido na síntese do tecido nervoso em crianças (FERREIRA; TESHIMA, 2000) e apresentar a capacidade de inibir o crescimento de microrganismos causadores de infecções gastrointestinais (MIDOLO et al., 1995). Além disso, também já foram demonstradas propriedades anticarcinogênicas por meio da ligação das bactérias ácido láticas à compostos mutagênicos (ZSIVKOVITS et al., 2003)

2.2 LACTOSE

A lactose é um dissacarídeo composto por glicose e galactose unidas por uma ligação glicosídica β1-4. É o principal carboidrato presente no leite de todas as espécies mamíferas (Figura 1), sendo rara a existência em fontes não lácteas (HOLSINGER, 1988).

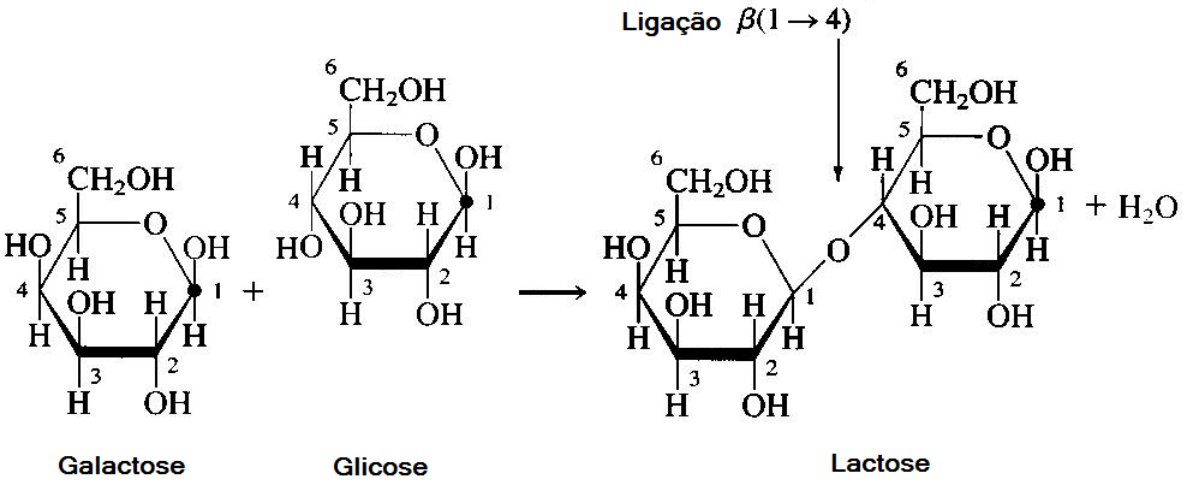

Figura 1: Esquema representativo da ligação glicosídica β1-4 da molécula de lactose

Fonte: http://lactosesintolerances.blogspot.com.br/

Este dissacarídeo é sintetizado nas glândulas mamárias a partir da glicose presente no sangue. Para a formação da galactose uma molécula de glicose é fosforilada, depois ligada a uridina trifosfato e isomerizada. Após, a molécula de galactose é ligada a outra molécula de glicose em uma reação catalisada pela enzima lactose sintetase (FOX; McSWEENEY, 1998).

Os mamíferos não são capazes de absorver a lactose diretamente, que deve ser primeiramente hidrolisada no intestino delgado, liberando os monossacáridos, os quais são absorvidos. A hidrólise é realizada pela enzima β-galactosidase, também conhecida como lactase, que é secretada pelos enterócitos (FOX, 2009).

2.3 INTOLERÂNCIA À LACTOSE

Intolerância à lactose é o termo comumente utilizado para descrever sintomas relatados por pessoas que apresentam má digestão da lactose após ingerirem leite e derivados (GRAND, 2010).

Quando a lactose não é absorvida, esta passa ao intestino grosso e a carga osmótica gerada pelo acúmulo da lactose faz com que ocorra a secreção de água e eletrólitos até que o equilíbrio osmótico seja atingido causando diarreia

(CHRISTOPHER; BAYLESS, 1971). A dilatação do intestino, causada pela osmose, induz uma aceleração do trânsito no intestino delgado.

O trânsito acelerado reduz ainda mais a hidrólise de lactose, porque o tempo de contato entre a lactose e a enzima residual é diminuída (LADAS; PAPANIKOS; ARAPAKIS, 1982).

Além disso, a lactose presente no lúmen intestinal é fermentada por microrganismos levando à formação de gases, que causam flatulências, cólicas e desconforto abdominal (FOX; McSWEENEY, 1998) (Figura 2).

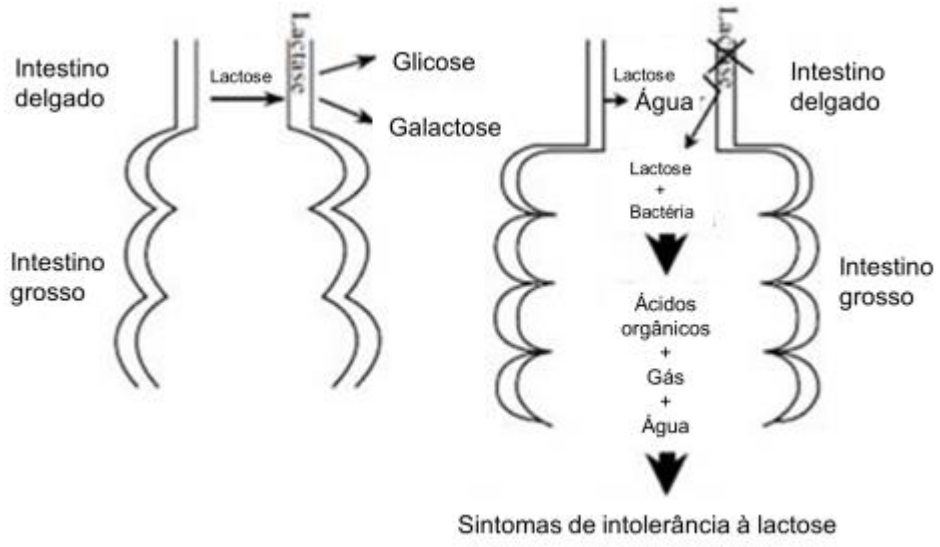

Figura 2: Esquema comparativo do efeito da presença e da ausência da lactase no intestino delgado

Fonte: http://saudedigestiva.blogspot.com.br/

A redução da quantidade de lactase (hipolactasia) pode ser primária (isto é, genética) ou secundária. Nos seres humanos a atividade máxima da lactase ocorre logo após o nascimento, mantendo-se alta durante o período de amamentação e algum tempo após o desmame, normalmente, entre 3 a 7 anos. Na idade adulta os níveis de lactase decaem, podendo chegar, aproximadamente a 10% da taxa existente no período da infância (FOX, 2009; GRAND, 2010) e esta situação é referida como hipolactasia do tipo adulto.

A deficiência de lactase primária, é congênita e consiste em uma forma grave de deficiência em lactase, na qual a atividade desta enzima é muito baixa ou

ausente no epitélio intestinal a partir do nascimento É uma condição extremamente rara sendo apenas algumas dezenas de casos documentados em todo o mundo, a maioria deles na Finlândia (SAVILAHTI; LAUNIALA;KUITUNEN, 1983).

Já a deficiência secundária pode resultar de pequenas ressecções intestinais, de doenças que danificam o epitélio intestinal, por exemplo, doença celíaca não tratada ou inflamação intestinal (BODÈ; GUDMAND-HØYER, 1988; PIRONI et al., 1988) e qualquer condição que aumente significativamente o tempo do trânsito gastrointestinal, dessa forma, é uma condição transitória e reversível (LABAYEN et al., 2001).

Segundo estudo desenvolvido por Mattar et al. (2009) a prevalência de indivíduos intolerantes à lactose no Brasil depende da atividade de genes específicos que variam conforme os diferentes grupos étnicos conforme ilustrado no gráfico 1

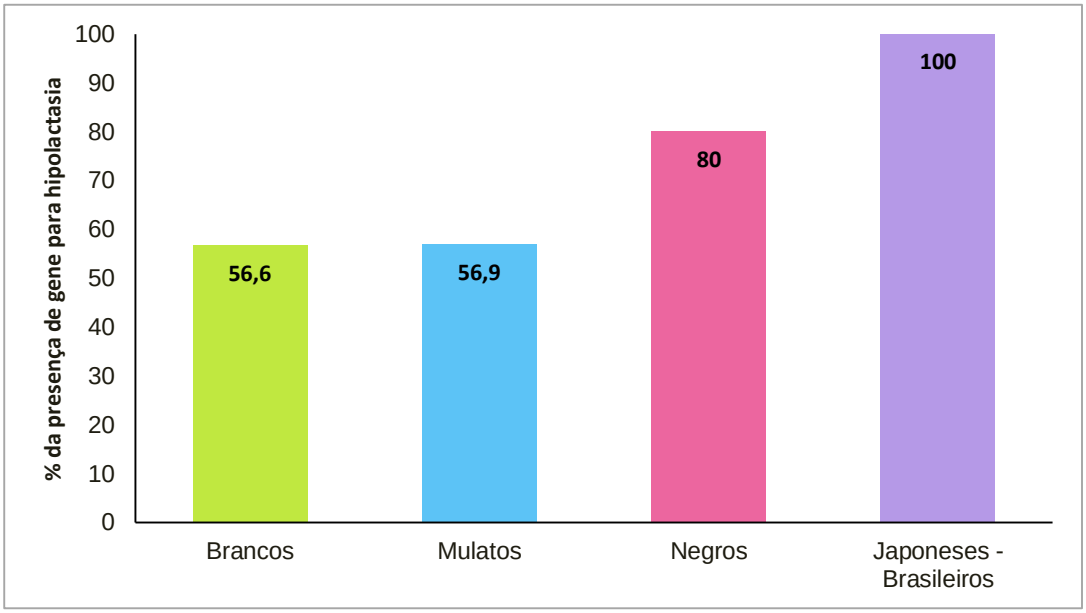

Gráfico 1: Percentual da presença de genes para hipolactasia nas diferentes etnias brasileiras

Adaptado de Mattar et al. 2009

Sabe-se que em leites fermentados, como o iogurte, o teor de lactose é cerca de um terço menor do que o presente no leite bovino, que é de aproximadamente

4,6 g/ 100 mL, e isso se deve ao fato de que durante a fermentação realizada pelas bactérias láticas, ocorre a conversão da lactose em ácido lático. (SCHAAFSMA, 2008). Dessa forma, muitos indivíduos intolerantes à lactose que são intolerantes ao leite podem tolerar a ingestão de iogurtes (BOUDRAA et al., 2001; KOLARS et al.,1984).

Embora alguns indivíduos não apresentem sintomas intestinais a partir do consumo de iogurtes, esta quantidade residual de lactose presente em derivados lácteos fermentados pode causar sintomas da má absorção da lactose em pessoas com elevado grau de intolerância, dessa maneira é indicado o consumo de produtos "lactose-free" ou com teor reduzido de lactose (HEYMAN, 2006).

Para a fabricação industrial de leite e derivados com teor reduzido de lactose são utilizadas basicamente duas técnicas, o tratamento com a enzima β-galactosidase e a utilização de sistemas de membranas filtrantes (REHMAN, 2009).

2.4 β-GALACTOSIDASE

A β-galactosidase ou lactase está amplamente distribuído na natureza e pode ser isolada a partir de diferentes fontes, como plantas (amêndoas, pêssegos, damascos, maçãs), animais, leveduras, bactérias e fungos (RICHMOND; GRAY; STINE, 1981).

A produção industrial de leite e produtos lácteos com teores reduzidos de lactose é amplamente realizada atualmente, este processo se iniciou no início dos anos 1970 com a disponibilidade comercial da lactase a partir de fontes microbianas (REHMAN, 2009)

A Agência Nacional de Vigilância Sanitária (ANVISA) especifica, por meio da Resolução da Diretoria Colegiada (RDC) nº 205 de 14 de novembro de 2006 que a enzima β-galactosidase para uso em indústrias de alimentos deve ser de origem microbiana, proveniente dos seguintes microrganismos: *Aspergillus niger, Aspergillus oryzae, Candida pseudotropicalis, Kluyveromyces fragilis, Kluyveromyces lactis, Kluyveromyces marxianus* e *Saccharomyces* sp (BRASIL, 2006). Tais espécies são classificadas como "Generally Recognized as Safe" pelo órgão norte americano "Food and Drug Administration", esta designação significa

que o aditivo alimentar foi testado e demonstrado ser seguro nas condições de uso pretendidas.

As condições ótimas para atividade das diferentes lactases dependem da sua origem. Em geral, as enzimas de origem fúngica apresentam pH ótimo entre 2,5 e 4,5, já as lactases oriundas de leveduras e bactérias atuam em condições ótimas em pH próximo a neutralidade (6 – 7,5) (GEKAS; LOPEZ-LEIVA, 1985).

A utilização da lactase em iogurtes têm sido foco de pesquisas atualmente assim como a determinação de condições ótimas para a aplicação desta enzima no processamento de derivados lácteos. Vinhal (2001) utilizou lactase proveniente de *Kluyveromyces fragilis* para reduzir o teor de lactose do leite. A enzima apresentou atividade ótima e boa estabilidade em pH entre 6,5 e 7,0, com temperatura ótima de 40ºC. Longo (2006) realizou a hidrólise enzimática para produção de iogurte e também determinou 40°C como a temperatura ótima da enzima.

2.5 *Escherichia coli*

A *Escherichia coli* é uma bactéria Gram negativa, anaeróbia facultativa, em formato de bastonete. Os microrganismos desta espécie são móveis, não formadores de esporos e são capazes de fermentar a lactose com produção de ácido e gás (SCHAECHTER, 2009).

A *E. coli* possui como habitat natural o trato intestinal de animais de sangue quente, sendo por isso considerada indicadora de contaminação fecal de alimentos (FRANCO; LANDGRAF, 2008). Esta espécie pertencente à família *Enterobacteriaceae*, que inclui um grande número de cepas que diferem entre si em relação ao potencial patogênico (SCHAECHTER,2009).

Diferentes cepas de *E. coli* podem ser sorologicamente diferenciadas com base em três antígenos de superfície principais: antígeno somático (O), antígeno flagelar (H) e antígeno capsular (K). Já foram descritos 173 antígenos O, 56 antígenos H e 103 antígenos K, porém, normalmente as cepas associadas a doenças diarreicas envolvem apenas os grupos O e H (MENG et al., 2007).

A doença diarreica é uma das principais formas de patogênese e pode ser causada por diversos mecanismos. São definidas seis estirpes diarreiogênicas: *E. coli* enterotoxigênica (ETEC), *E. coli* enteroinvasiva (EIEC), *E. coli* enterohemorrágica (EHEC), *E. coli* enteropatogênica (EPEC), *E. coli* enteroagregativa (EAggEC) e *E. coli* de adesão difusa (DAEC) (WILLEY; SHERWOOD; WOOLVERTON, 2008).

A EHEC carreia determinantes genéticos para produção de “Shiga-like” toxinas (Stx-1 e Stx-2), que são responsáveis pela destruição da borda em escova das microvilosidades do enterócitos causando colite hemorrágica com fortes dores abdominais e cólicas, seguida de diarreia sanguinolenta. As toxinas também levam a ocorrência da síndrome urêmica hemolítica, doença extraintestinal caracterizada por anemia hemolítica grave, que leva a posterior insuficiência renal (ibid).

2.5.1 *Escherichia coli* O157:H7

A principal estirpe da EHEC é *E. coli* O157:H7, que foi reconhecida pela primeira vez como um patógeno humano em 1982, quando foi associada a dois surtos de colite hemorrágica nos Estados Unidos da América (RILEY et al., 1983).

Diversos pesquisadores já demonstraram que os bovinos são os principais reservatórios para a *E. coli* O157:H7 (ELDER et al., 2000; HANCOCK et al., 1994; VAN DONKERSGOED; GRAHAM; GANNON, 1999; WELLS et al., 1991) porém, a eliminação fecal por outros animais domésticos e animais selvagens também tem sido descrita (FRANKLIN et al., 2013; JAY et al., 2007; RICE; HANCOCK; BESSER, 2003; SARGEANT et al., 1999)

A *E. coli* O157:H7 apresenta dose infectante consideravelmente baixa (cerca de 100 células) sendo a sua virulência decorrente de uma combinação de fatores (MENG et al., 2007). O principal fator de virulência da *E. coli* O157: H7 é a produção de Stx 1 e 2, que estão codificados em um bacteriófago inserido no cromossoma bacteriano e estas toxinas ao serem endocitadas pelas células bloqueiam a síntese proteica pelos ribossomos levando à morte celular (NATARO; KAPER, 1998) (Figura 3).

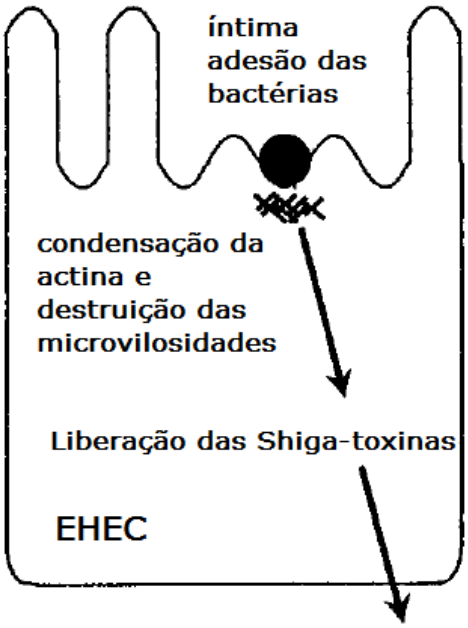

Figura 3: Esquema da destruição da borda em escova das microvilosidades dos enterócitos causada pelas toxinas Stx- 1 e Stx-2 da *Escherichia coli* enterohemorrágica.
Adaptado de Nataro e Kaper (1998)

Porém, existem outros fatores de virulência codificados em genes cromossomais e no plasmídeo de 60-MDa que são importantes na patogenicidade, como por exemplo, o mecanismo conhecido como “attaching and effacing” caracterizado pela íntima adesão das bactérias à membrana das células intestinais levando à destruição das microvilosidades. Além deste, também já foram identificados fatores de adesão às células intestinais como a intimina e adesinas além da capacidade de expressão de hemolisinas (MENG et al., 2007).

A *E. coli* O157:H7 usualmente não fermenta o D-sorbitol em 24h, embora cepas capazes de fermentar este composto já tenham sido identificadas (BOUVET et al., 1999; KARCH; BIELASZEWSKA, 2001). Este sorotipo também não apresenta a enzima β-glicuronidase, que hidrolisa composto 4-metilumbeliferil-ß-D-glucuronide (MUG) que em presença de luz UV emite fluorescência, e, também não é capaz de se multiplicar em temperaturas superiores a 44,5°C (DOYLE; SCHOENI, 1984; FENG; LUM; CHANG, 1991)

Uma das características distintas da *E. coli* O157: H7 é a sua capacidade de adaptação e sobrevivência em ambientes ácidos. Diversos pesquisadores já demonstraram a capacidade de sobrevivência das células da *E. coli* em produtos fermentados por um período considerável de tempo, destacando-se o iogurte (BACHROURI et al., 2002; DINEEN et al.,1998; GOVARIS et al., 2002; GULMEX, GUVEN, 2003; GURAYA et al., 1998; OGWARO et al., 2002;).

3 DESENVOLVIMENTO

3.1 ARTIGO 1: *Escherichia coli* O157:H7 SURVIVAL IN NATURAL AND LOW LACTOSE YOGURT DURING FERMENTATION AND STORAGE

Escherichia coli O157:H7 survival in natural and low lactose yogurt during fermentation and storage

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ABSTRACT

E. coli O157:H7 is able to survive during long periods in yogurt, a widely consumed dairy product, which presents a reduced content of lactose compared to milk. However, the decrease of lactose is not always sufficient to relieve the symptoms of malabsorption. This study aimed to evaluate the behavior of *E. coli* O157:H7 during lactose hydrolysis process and fermentation of traditional and low lactose yogurt. Also aimed verify *E. coli* O157:H7 survival after 12h of storage at 4±1°C. Four different yogurts were prepared with milk and pre hydrolyzed milk (β-galactosidase); two groups were inoculated with *E. coli* O157:H7 and two were controls. The survival of *E. coli* O157:H7 and pH of yogurts were determined during fermentation and storage. Results showed that an initial contamination of milk enhance *E. coli* O157:H7 counts during fermentation (from 4.34 to 6.13 in traditional yogurt and 6.16 in low lactose yogurt) and changes the characteristics of yogurts during fermentation process, with a lot of air bubbles formation and syneresis. The pH values of all yogurts after fermentation ranged from 4.53 to 4.67.Thus it is concluded that milk contamination by *E. coli* O157:H7 in addition to being a public health issue also affects the yogurt manufacturing technology.

Key words: *Escherichia coli* O157:H7, lactose hydrolysis, yogurt processing.

INTRODUCTION

Escherichia coli is a member of the *Enterobacteriaceae* family and it is one of the most prolific microorganisms in human intestinal tract. *E. coli* are normally harmless, but certain strains can be pathogenic (Tortora et al., 2012).

The enterohemorrhagic *E. coli* (EHEC) strains carry the genetic determinants for attaching-effacing lesions and Shiga-like toxin production. The attaching-effacing lesion causes hemorrhagic colitis with severe abdominal pain and cramps followed by bloody diarrhea. The Shiga-like toxins I and II (also called serotoxins 1 and 2) have also been implicated in two extra intestinal diseases; hemolytic uremic syndrome and thrombotic thrombocytopenic purpura (Willey et al., 2008).

A major form of EHEC is the *Escherichia coli* O157:H7 that was first recognized as a human pathogen in 1982 when was associated with two outbreaks of hemorrhagic colitis (HC) that affected at least 47 people in Oregon and Michigan (USA) (Riley et al., 1983). Since then many others foodborne outbreak cases have been reported involving different products like ground beef (Vogt et al., 2005; Centers for Disease Control and Prevention, 2014), fermented sausages (Sartz et al., 2008), salami (Centers for Disease Control and Prevention, 1995; Williams et al., 2000), unpasteurized cheese (Honish et al., 2005; Espié et al., 2006) and yogurt (Morgan et al., 1993).

Although yogurts are considered safe due to their low acidity, some authors have reported the survival of *E. coli* O157:H7 over considerable days and even weeks in yogurts (Bachrouri et al., 2002; Gulmex, Guven, 2003; Evrendilek, 2007). It has been shown that *E coli* O157:H7 cells have an effective mechanism to resist extreme acid stress situations and its resistance depends on the interaction with environmental compounds (Meng et al., 2007).

Fermented milks are widely consumed around the world and yogurt is the most popular fermented dairy product with high nutritional value including proteins, lipids and lactose, the principal carbohydrate of milk (Peng et al., 2009)

Approximately 75% of the world's population loses the ability to digest lactose into adulthood (Mattar, Mazo, Carrilho, 2012). It is known that in yogurt, the lactose content is about one third lower than in milk, due to fermentation conducted by lactic acid bacteria, converting lactose into lactic acid. (Schaafsma, 2008).However, in some cases, the

persistence of gastrointestinal malaise from the consumption of yogurt shows that the decrease of lactose content would not be sufficient to relieve the symptoms of indigestion. This led to the introduction of improvements to product production, such as reduced lactose content through exogenous lactases.

This study aimed to evaluate the behavior of *E. coli* O157:H7 during lactose hydrolysis and fermentation of traditional and low lactose yogurt. Also aimed verify *E. coli* O157:H7 survival after 12h of storage at 4±1°C.

MATERIALS AND METHODS

Microbial and physicochemical quality of commercial milk

Commercial (Ultra High Temperature) UHT whole milk (Ninho, Nestlé, Três Rios, Brazil) was analyzed to determine physicochemical quality by methods of Association of Official Analytical Chemists (AOAC). The pH determination was performed by standard method n° 973.41, titratable acidity (TA) by standard method n° 947.05, fat content by standard method n° 2000.18 , specific gravity of milk by standard method n° 925.22and freezing point by cryoscopic method n°990.22 (AOAC, 2012). Also was performed enumeration of *E. coli* O157:H7 (Merck, 2007) and lactic acid bacteria (LAB). (International Organization for Standardization - ISO, 2003).

Cultures

The *Escherichia coli* O157:H7 (CDC EDL - 933) strain was obtained from National Institute of Health Quality Control of Oswaldo Cruz Foundation (FIOCRUZ, Rio de Janeiro, Brazil). The organisms were inoculated in brain heart infusion (BHI) broth and incubated at 37° C for 24 hours (h). The bacterial cells were maintained on BHI and stored at 4°C. To activate the *E. coli* O157:H7 cells a loop-full of BHI stored was transferred to BHI an incubated at 37°C for 24h.

The lactic starter culture used was a thermophilic yoghurt culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus* (DVS YF-L812, Christian Hansen Laboratories, Denmark).

Before fermentation, the cultures were activated by adding the 50 U sachet to 500 mL of sterile 10% (w/v) reconstituted skim milk powder (Molico, Nestlé, São Paulo, Brazil) and stirred for 15 min, to achieve a homogenous culture (London et al., 2015). This volume was

distributed into test tubes and stored at -18°C.To perform the fermentation, the culture was thawed and then added to milk samples.

Milk experimental contamination

Commercial UHT whole milk was inoculated with *E. coli* O157:H7 to yield a final concentration of 4log Colony Forming Unit (CFU) per milliliter in the milk. This procedure was made before the lactose hydrolysis and yogurt production. Inoculations were done into preheated milk at 40°C.

Yogurt manufacture

There were prepared four different types of yogurt: traditional; traditional with *E. coli* O157:H7; low lactose; and, low lactose with *E. coli* O157:H7. For each treatment it was used 3L of UHT whole milk.

Traditional yogurt and low lactose yogurt with or without inoculation were produced using milk preheated at 40°C.The traditional yogurt was prepared from milk inoculated with starter culture. The low lactose yogurt was prepared from milk pretreated with β-galactosidase (450 mL/ 1000L of milk) (Maxilact LX 5000, DSM Food Specialties, Delft, Netherlands) for 1h at 40°C and inoculated with starter culture.

All yogurts types were incubated as 42°C ± 1°C until the final pH of 4.5–4.6 (Tamine, Robinson, 2007). After fermentation, inoculated yogurts and controls without *E. coli* O157:H7 were stored at 4°C.

Samples were taken for pH determination at each hour of fermentation. The enumeration of *E. coli* O157:H7 was determined at 0, 6 and 12h after inoculation and enumeration of lactic acid bacteria (LAB) was performed 12h after the end of fermentation.

Enumeration of microbial count

The microbial enumeration was performed with dilution of 25g of each sample into 225 mL of 0.1% peptone saline water and homogenization in Stomacher blender for 1 min. 1ml of each initial dilution was transferred into tubes with 9 mL of 0.1% peptone saline water and serially dilutions were made.

E. coli O157:H7 was determined by plating 1mL of appropriate dilutions in duplicate on Fluorocult *Escherichia coli* O157:H7 agar (Merck, Darmstadt, Germany). (Merck, 2007) Random isolates were confirmed by serology with *E. coli* O157 antiserum (Probac, São Paulo, Brazil).

For enumeration of lactic acid bacteria, 1 mL of determined dilutions were inoculated onto M17 Agar (Difco Laboratories, Michigan, USA) for the isolation of *S. thermophilus* incubated at 37°C for 48h and acidified (5.4). Man-Rogosa-Sharpe (MRS) agar (Difco Laboratories, Michigan, USA) was used to *L. bulgaricus* count, by anaerobically incubation in a GasPak™ container (Becton, Dickinson and Company, New Jersey, USA) at 37°C for 72h (ISO, 2003).

Determination of pH and Titratable acidity

The pH values of the samples were measured by immersing the electrode of a digital pHmeter (PG 1800, Cap Lab, São Paulo, Brazil) directly in the sample (AOAC, 2012) Buffer solution of pH 4 and 7 were used to calibrate the pHmeter.

TA in milk samples was determined by AOAC standard method 947.05 (AOAC, 2012).

Statistical Analysis

Data from physicochemical analysis and carbohydrates profile were subjected to one-way analysis of variance (ANOVA), testing the differences between the different types of yogurt at each sampling time. All ANOVA were subjected to Tukey's test at $P < 0.05$. Statistical analysis were performed using XLSTAT version 2013.2.03 (Addinsoft, Paris, France)

RESULTS AND DISCUSSION

Microbial and physicochemical quality of commercial milk

The means of pH, titratable acidity, specific gravity, freezing point and fat results of milk analysis were 6.79, 0.15 % lactic acid (v/v), 1.030, -0.557°H and 3.6 % (v/v), respectably. The microbial analysis of the commercial UHT showed that *E. coli* O157:H7 was not present in milk samples as well as LAB. These results are in accordance to Brazilian legislation

(Brasil, 1997) and guarantee that the results obtained in fermentation process are specifically from LAB and *E. coli* O157:H7 activity and not from possible previous contamination.

Yogurt pH

The pH of the yogurts inoculated with *E. coli* O157:H7 after 6h of fermentation were significantly different ($P < 0.05$) from the non-inoculated yogurts.

The pH dropped from 6.79 to 4.53, 4.54, 4.62 and 4.67 in traditional, low lactose, inoculated traditional and inoculated low lactose yogurts respectively after 6 h of fermentation (Figure 1).

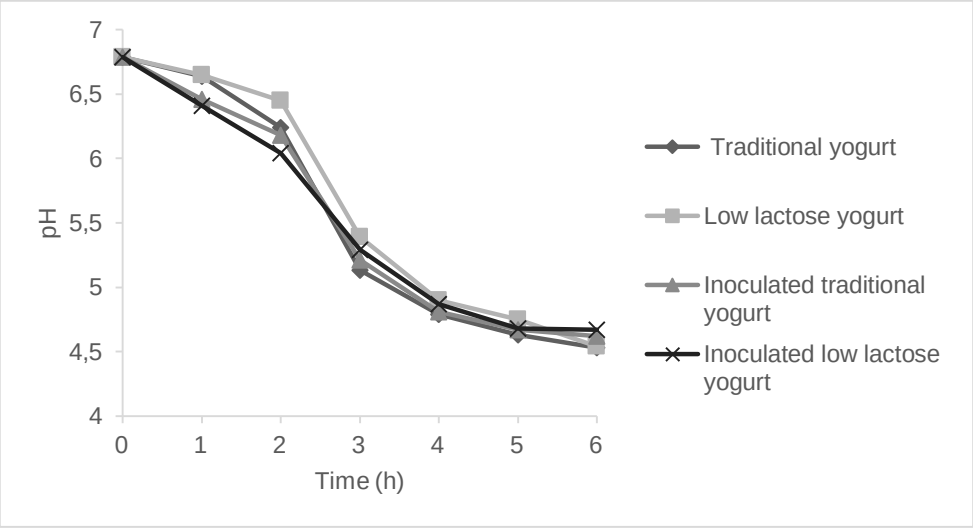

Figure 1: pH values during milk fermentation.

As it was expected LAB fermented lactose and glucose increasing the lactic acid content, and consequently lowering the pH. In accordance with our results, Rodrıguez et al. (2008), Vénica et al. (2013) and Wolf et al. (2015) showed that the lactose hydrolysis did not affect acidification process and no differences in pH values between yogurts prepared from milk with different lactose contents and control yogurts were found.

Fermentation process

At the third hour of fermentation in all inoculated yogurts began to show a formation of air bubbles in the coagulum, characterizing gas production from *E. coli* O157:H7 metabolism (Figure 2). Over the hours, these bubbles have become increasingly abundant and there was observed an intense syneresis (Figure 3). The final products after 6 hours of fermentation

presented visually changed, and it was not possible to observe homogeneous and firm coagulum as observed in not inoculated yogurts.

Figure2: Initial air bubbles of inoculated yogurts.

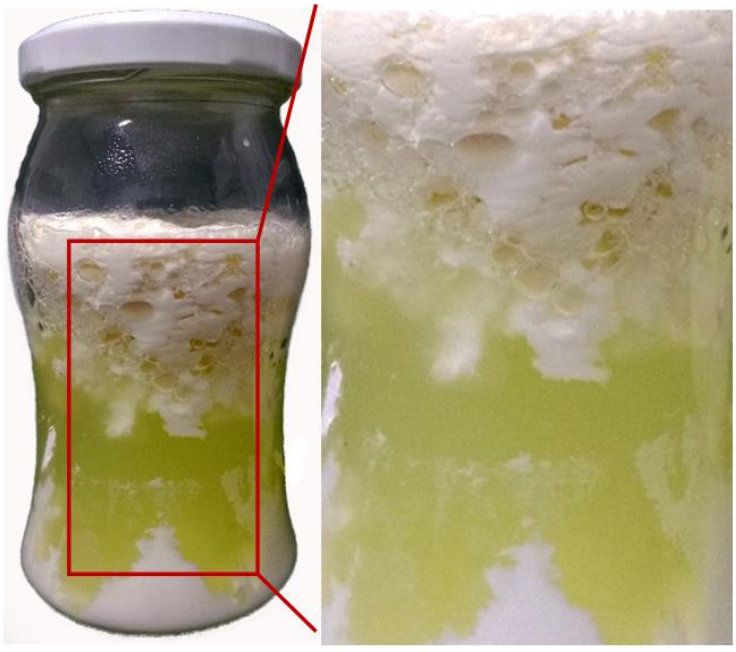

Figure 3: Final product after 6h of fermentation.

Xu et al. (1990) observed that different strains of *E. coli* O157:H7 are capable to rapidly ferment lactose and glucose with gas production. In this study the results showed that this fermentation occurred and the rapid growth of *E. coli* O157:H7 was facilitated by incubation at 40° C of all yogurts during one hour for lactose hydrolysis. Controls yogurts were also kept at this temperature to not generate variations when the starter cultures were added. Another factor that influenced on *E coli* growth was the absence of microbial competition and acidification from the starter cultures during the hydrolysis

Several researchers have studied the survival of *E. coli* O157:H7 in food systems, especially yogurt and related the survival of the cells during fermentation process (Massa et al., 1997;Lee, Chen, 2005; Bachrouri et al., 2006; Osaili et al., 2013). Our results suggest that even with inoculation pre fermentation *E. coli* O157:H7 was able to growth despite acidic environment developed and bacterial competition.

E coli O157:H7 enumeration

E. coli O157:H7 was not found in control yogurts not inoculated. Counts right after inoculation and 6 h later after fermentation increased from 4.34 to 6.13 and 6.16 log UFC mL^{-1}in tradition and low lactose inoculated yogurt respectively. This result is according to those of Kasımoğlu and Akgün (2004) and Bachrouri et al. (2006) that reported that cells of *E. coli* O157:H7 increased about a log CFU mL^{-1} from initial inoculum during the fermentation process. Osaili et al (2013) also reported a large increase of *E. coli* O157:H7 count of 3.05 log CFU mL^{-1} during fermentation.

After 12h of cooling at 4°C counts increase slightly to 6.87 and 6.75 log UFC mL^{-1}in tradition and low lactose inoculated yogurt respectively. The pH dropped to 4.44, 4.43, 4.46 and 4.59 in traditional, low lactose, traditional inoculated and low lactose inoculated yogurts

The results of present study showed that *E. coli* O157:H7 was able to adapt to acid environment and growth even after cooling and storage at low temperature. These findings were in agreement with Ogwaro et al., (2002) and Cirone et al. (2013) that reported increased of *E. coli* O157:H7 counts from 5 to 8–9 log CFU $mL^{-1}$and 5.3 to 6.4 after 24h of fermentation respectively.

In disagreement to our findings, has been suggested that *E. coli* O157:H7 not survive during fermentation process of yogurt and the presence of these bacteria in yogurt would

indicate the post-processing contamination (Dineen et al., 1998). However, Bachrouri et al. (2002) reported that *E. coli* O157:H7 was able to survive up to 20-21 days in pre-fermentation inoculated yogurts. Lee and Chen (2005) related *E. coli* O157:H7 survival in yogurt inoculated pre fermentation for 14-17 days. It was also reported that *E. coli* O157:H7 inoculated before yogurt fermentation was detected until 7 (Osaili et al., 2013) and 11days of storage (Tosun et al., 2006).

LAB enumeration

After 12h of cooling the counts of *S. thermophilus* were 9.83, 9.72, 8.31 and 6.85 log CFU mL^{-1} and *L. bulgaricus* counts were 10.35, 9.84, 8.36 and 5.85 log CFU mL^{-1} in traditional, low lactose, traditional inoculated and low lactose inoculated yogurts respectively as shown in Figure 4.

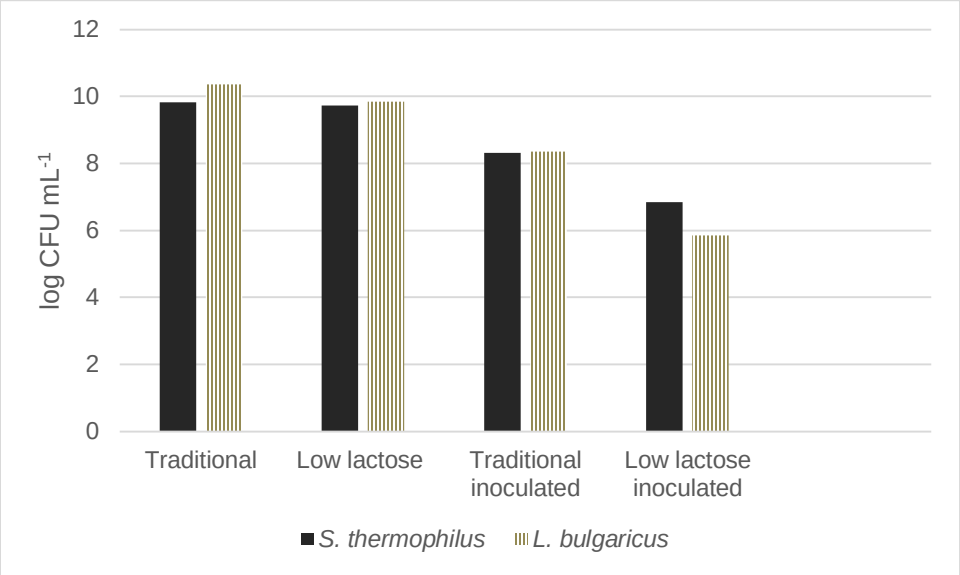

Figure 4: Counts of *S. thermophilus* and *L. bulgaricus* in yogurts after 12h of cooling and storage at 4°C

The symbiosis between *S. thermophilus* and *L. bulgaricus* is known for long time (Pette et al., 1950; Bautista, 1966). *S. thermophilus* and *L. bulgaricus* shows interaction mutually favorable. *L. bulgaricus* produces amino acids and small peptides that stimulate *S. thermophilus* growth by acting as an amino acid source (Accolas et al. 1971; Radke-Mitchell, Sandine 1984); *S. thermophilus* produces carbon dioxide (CO_2) and formic acid that stimulate *L. bulgaricus* (Suzuki et al. 1986).

Researchers have also reported that most strains of *S. thermophilus* are phenotypically galactose negative and does not contain genes necessary for galactose metabolism. They are able to metabolize only glucose portion of lactose and expel galactose into the medium (Mora et al., 2002; De Vin et al., 2005)

Our results shown increase of LAB counts during fermentation in control not inoculated yogurts confirming this symbiosis, however it was observed a decrease in counts in low lactose inoculated yogurts. This could be due the milk hydrolysis that reduced the lactose content restricting the growth of LAB and competition for residual lactose and glucose, used rapidly by *E. coli* O157:H7 (Xu et al., 1990). As most strains of *S. thermophilus* are unable to ferment galactose they did not growth and did not produce acids that stimulate *L. bulgaricus* growth either.

CONCLUSION

It is concluded that *E. coli* O157:H7 was able to survive in natural and low lactose yogurts and control measures (e.g. Good Manufacturing Practices and Hazard Analysis Critical Control Point) should be always improved to reduce the risk of contamination because even inoculation before fermentation allowed *E coli* O157:H7 growth.

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3.2 ARTIGO 2 SURVIVAL OF *Escherichia coli* O157:H7 DURING MANUFACTURE AND STORAGE OF TRADITIONAL AND LOW LACTOSE YOGURT.

Survival of *Escherichia coli* O157:H7 during manufacture and storage of traditional and low lactose yogurt

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Abstract

Yogurt is one of the most popular dairy product and its content of lactose is one third lower than milk, however some people presents symptoms of lactose malabsorption when consuming yogurt. To overcome this fact dairy industry starts to use β-galactosidase to obtain low lactose products. Yogurt have been considered safe because of their low pH, although recent researches had shown the survival of *Escherichia coli* O157:H7, which was associated with outbreaks involving dairy products. In this context, the aim of the study was to verify survival of *Escherichia coli* O157:H7 in yogurts with different lactose content. Six different yogurts were prepared with milk, pre hydrolyzed milk and lactose free milk. Three of them were inoculated with *E. coli* O157:H7. The survival of *E. coli* O157:H7, carbohydrates

profile and pH of yogurts were determined during fermentation and storage. Results showed that an initial contamination enhance *E. coli* O157:H7 counts during fermentation. *E. coli* was able to survive for 10 days in yogurt with whole and pre hydrolyzed milk and for 22 days in yogurt from free lactose milk. Thus, it is concluded that milk contamination by *E. coli* O157:H7 in free lactose milk increases its survival in 12 days.

Keywords: fermented milk, lactic acid bacteria, β-galactosidase, lactose hydrolysis, carbohydrates profile, HPLC

1. Introduction

The most popular fermented dairy product is the yogurt and its consumption is increasing worldwide (Shiby, Mishra, 2013). According to the Codex standard for fermented milks (Codex, 2003), yoghurt is a fermented milk characterized by fermentation by specific acid lactic cultures as *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus.* During fermentation, these bacteria perform three major biochemical conversions of milk components: conversion of lactose into lactic acid, proteolysis of caseins into peptides and free amino acids and lipolysis of milk fat into free fatty acids (Smit, Smit, Engels, 2005).

In the course of yogurt processing, lactic acid bacteria reduce the lactose content and this reduction is about one third in comparison with lactose present in milk, which is around 4.8% of milk content (Schaafsma, 2008). However, some people present deficiency in lactose absorption that requires β-galactosidase activity in the small intestinal brush border. Lactose malabsorption in general affects about two thirds of the world adult population (Vesa, Marteau, Korpela, 2000) and the persistent lactose is digested by colonic bacteria in the

intestinal lumen, which leads to production of short-chain fatty acids, hydrogen, carbon dioxide, and methane. These byproducts cause bloating, flatulence, and abdominal pain. Undigested lactose also increases the osmotic load, resulting in diarrhea (Lomer, Parkes, Sanderson, 2008). One of the ways to get around the negative effects of lactose inefficient digestion is the introduction of exogenous lactases in dairy production in order to obtain lactose free products (Rodriguez, Cravero, Alonso, 2008).

Although yogurts are considered safe due to their low acidity, some authors have reported the survival of *Escherichia coli* O157:H7 over considerable days and even weeks in yogurts (Bachrouri et al., 2002; Gulmex, Guven, 2003; Evrendilek, 2007). In addition, dairy products have been associated with *E. coli* O157:H7 outbreaks (Morgan et al., 1993; Alterkruse et al., 1998; De Buyser et al., 2001). It has been shown that *E coli* O157:H7 cells have an effective mechanism to resist extreme acid stress situations and its resistance depends on the interaction with environmental compounds (Meng et al., 2007).

There is not enough data regarding *E coli* O157:H7 survival in low lactose yogurts. Further, its behavior during fermentation process and storage is not properly clarified yet. Hence, the aim of this study was to evaluate the fate of *E coli* O157:H7 during fermentation and storage of yogurts with different lactose content.

2. Materials and Methods

2.1 Bacterial Cultures

The *Escherichia coli* O157:H7 (CDC EDL - 933) strain was obtained from National Institute of Health Quality Control of Oswaldo Cruz Foundation (FIOCRUZ) (Rio de Janeiro,

Brazil). The lyophilized strain was inoculated in brain heart infusion (BHI) broth and incubated at 37° C for 24 hours (h). The microorganisms were maintained on BHI and stored at 4°C. To activate the *E. coli* O157:H7 cells a loop-full of BHI stored was transferred to BHI an incubated at 37°C for 24h.

The lactic starter culture used was a thermophilic yoghurt culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus* (Christian Hansen Laboratories - DVS YF-L812) (Horsholm, Denmark). The lyophilized culture was reconstituted by adding a 50 U envelope (1 envelope/ 500L of yogurt) to 500 mL of sterile 10% (w/v) reconstituted skim milk powder (Molico, Nestlé, São Paulo , Brazil) and agitated for 15 min, to achieve a homogenous culture (London et al., 2015). This volume was distributed into test tubes and stored at -18°C. Before fermentation the culture was thawed and pre activated by inoculation of the necessary volume of culture (1mL/ 1L of yogurt) in 200 mL of whole milk and incubation at 42°C for 2 h (intermediate culture) (Bylund, 1995).

2.2 Milk Samples

The milk used for preparation of yogurts was whole milk treated by Ultra High Temperature and low lactose milk also treated by UHT (Ninho, Nestlé, Três Rios, Brazil), both obtained from local market, and with all packaging of the same type from the same batch.

2.3 Milk Hydrolysis

A part of whole milk was hydrolyzed with β-galactosidase (450 mL/ 1000L of milk) derived from dairy yeast *Kluyveromyces lactis* (Maxilact LX 5000, DSM Food Specialties,

Delft, Netherlands) for 1h at 40°C. Two of the six groups were pre hydrolyzed and other two were controls, the two remaining were prepared with lactose free milk.

2.4 Yogurt production

Yogurts were prepared from whole milk, pre hydrolyzed milk and lactose free milk preheated at 40 ° C. Six different treatments were prepared, three of them not inoculated and three inoculated with *E. coli* O157: H7: (A) Traditional yogurt; (B) Yoghurt with pre hydrolysis of lactose; (C) Yogurt lactose free; (D) Traditional yogurt inoculated; (E) Yoghurt with pre hydrolysis of lactose inoculated; and (F) Yogurt lactose free inoculated

In all treatments, the lactic culture was added and in inoculated groups the addition of *E. coli* O157: H7 occurred after the addition of starter culture. The treatments were incubated at 42 ± 1 ° C until pH 4.6 (Tamine, Robinson, 2007). Samples were taken for pH enumeration during fermentation.

2.5 Enumeration procedures

Enumeration of *E coli* O157: H7 was performed in the moment of inoculation, right after fermentation and during storage until organisms were undetectable. LAB enumeration was performed on 1st day, 7th day, 14th day, 28th day and 35th day.

The microbial enumeration was performed with dilution of 25g of each sample into 225 mL of 0.1% peptone saline water and homogenization for 1 min. After, 1ml of each initial dilution was transferred into tubes with 9 mL of 0.1% peptone saline water and serially dilutions were made (Swanson, Petran, Hanlin, 2001).

E. coli O157:H7was determined by plating 1mL of dilutions in duplicate on Fluorocult *Escherichia coli* O157:H7 agar (Merck, Darmstadt, Germany) (Merck, 2007). Random isolates were confirmed by serology with *E. coli* O157 antiserum (Probac, São Paulo, Brazil).

For enumeration of lactic acid bacteria, 1 mL of determined dilutions were inoculated in acidified (pH 5.4) Man-Rogosa-Sharpe (MRS) agar (Difco Laboratories, Michigan, USA) for isolation of *L. bulgaricus* incubated anaerobically in a GasPak container (Becton, Dickinson and Company, New Jersey, USA) at 37°C for 72h.; and in M17 Agar (Difco Laboratories, Michigan, USA) for the isolation of *S thermophilus* incubated at 37°C for 48h (International Organization For Standardization - ISO, 2003).

2.6 Determination of pH

The pH of samples was measured by submerging the probe of a digital pHmeter (PG 1800, Cap Lab, São Paulo, Brazil) directly into the yogurts samples.

2.7 Lactose, glucose, galactose and lactic acid analysis by HPLC

The carbohydrates and lactic acid were analyzed during fermentation and in day 1 of storage in order to characterize the yogurts carbohydrates profile. Analysis were performed in triplicate initiating with the extraction that was carried out using a modification of the method described by González de Llano, Rodriguez and Cuesta. (1996). Five milliliters of 45 mMol H_2SO_4 were added onto 1 mL of each sample and homogenized by vortexing for one minute. After that, all samples remained under agitation for one hour in a shaker table and then centrifuged at 5500*g* for 30 minutes at 4°C. Finally, the supernatant was filtered through Whatman® n. 1 filter paper (Sigma-Aldrich, St. Louis, MO, USA).

Filtered samples were injected (20 µL) in triplicate into an HPLC system consisted of a LC/20 AT pump integrated with CBM-20A and equipped with SPD-M20A diode array and refractive index RID-10A detectors (Shimadzu Corp., Tokyo, Japan). Carbohydrates and lactic acid separations were performed an HPX-87H 300 x 7,8 mm Aminex cation-exchange column (Bio-Rad, Hercules, CA, USA), maintained at 60 ºC was used.. The mobile phase used was 3 mM H_2SO_4 at isocratic flow rate at 0.5 $mL.min^{-1}$. Chromatograms from HPLC and compound quantification were obtained using the LC Solution software (Shimadzu Corp., Tokyo, Japan). Calibration curves were prepared from standard solutions prepared in Milli-Q water (Millipore, Billerica, MA, USA). Carbohydrates were identified by using a refractive index detector while lactic acid identification was performed by using a diode array detector model monitoring the absorbance at 210 nm. The interest peaks were identified by comparing retention times of the standards solutions with the samples. The quantitative analysis was carried out using an external standard curve.

2.8 Statistical Analysis

Data from physicochemical analysis and carbohydrates profile were subjected to one-way analysis of variance (ANOVA), testing the differences between the different types of yogurt at each sampling time. All ANOVA were subjected to Tukey's test at $P < 0.05$. Statistical analysis were performed using XLSTAT version 2013.2.03 (Addinsoft, Paris, France)

3. Results and Discussion

3.1 pH of Yogurt

During fermentation process, yogurts reached final pH (4.6 ±0.1) at different times. Group (D) natural inoculated took 4h, groups (B) control pre hydrolyzed and (E) pre hydrolyzed inoculated took 4.5h, group (F) lactose free milk inoculated took 5h, group (A) control natural took 5.5h and group (C) control lactose free milk took 6.5 h. Our results shown that in control not inoculated yogurts the pre hydrolysis of milk accelerates fermentation process in 1h; however, the fermentation of lactose free milk delays the fermentation in 1h in comparison with whole milk fermentation (group (A)). O'leary and Woychik (1976a) showed the same results for reduction in process time, in comparison to fermentation without enzyme. Nagaraj et al. (2009) found decrease in fermentation time of pre-hydrolyzed yogurts by 30-40 min and Martins et al. (2012) demonstrate that milk hydrolysis is involved in reduction of fermentation time from 4.55 to 3.68 h. These results could be explained by partial hydrolysis of lactose and release of free glucose and galactose, which are more readily used by LAB. In accordance to our results O'leary and Woychik (1976b) studied the utilization of glucose, galactose and lactose by yogurt culture in milk treated with lactase enzyme and showed that the acidity development is faster when the yogurt starter culture was grown in milk containing pre-hydrolyzed lactose. The delay observed in group (F) could be explained by partially inhibition of *lac*SZ operon in LAB due absence of lactose and presence of free glucose from hydrolyzed milk as shown by Lapierre, Mollet and Germond (2002). This operon consists of *lac*S gene that encode lactose permease that transports lactose into cells and *lac*Z gene, which encode β-galactosidase (Willem, 1996).

In the course of 28 days of storage, our results demonstrate that all yogurts showed acidification (Figure 1). In day 1, statistical difference was observed in groups (B) and (E), both with pre hydrolysis of lactose, which showed the lowest pH. It was also observed in

group (B), which had the milk pre hydrolyzed for 60 min, that was statistical difference in pH values during whole storage and they were the lowest pH values.

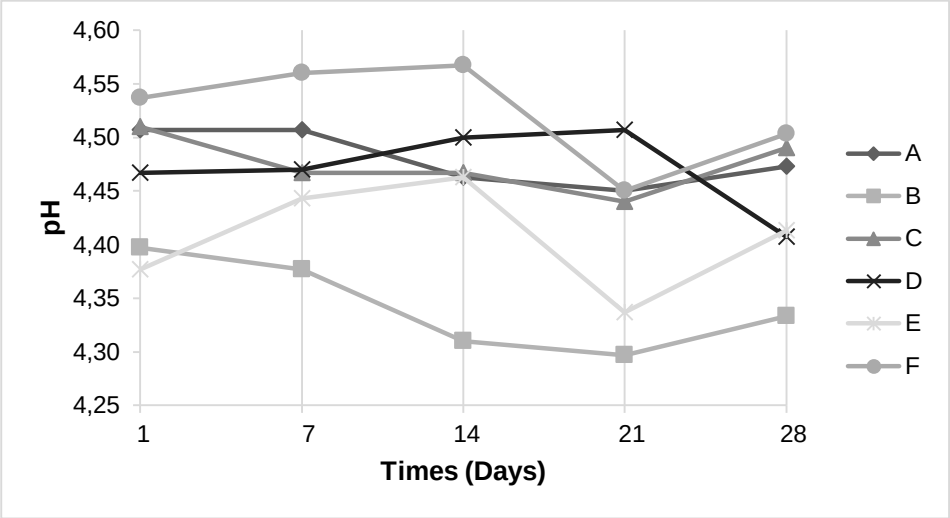

Figure 1: pH values during storage for 28 days

Yogurts are subjected to a pH decrease during refrigerated storage, and it is commonly called post-acidification (Kneifel, Jaros, Erhard, 1993). This decrease can be explained by *L. bulgaricus* production of lactic acid during fermentation and refrigerated storage as observed by Shah et al (1995) and Beal et al. (1999). Lapierre, Mollet, Germond, (2002) showed that in *L. bulgaricus*, the *lacR* gene, a repressor of *lac*SZ operon has lost regulatory function due to the insertion of some gene fragments, resulting in constitutive expression of *lacSZ* operon and consequently fermentation of carbohydrates and acid production.

In accordance with our results, O'leary and Woychik (1976a) showed that yogurts produced with pre hydrolyzed milk presented lower pH than control group. Wolf et al (2015) also showed a slow decrease in pH in yogurts with different lactose content, which ranged from 0.27 to 0.43 units. However, no statistical differences in pH values were detected among yogurt samples during fermentation and storage.

3.2 Enumeration of LAB

During storage time LAB were enumerated and in all groups both *L. bulgaricus* and *S.thermophilus* were in accordance to Codex (2011) determination with minimum of 10^7 cfu.g^{-1}. The count of both *L. bulgaricus* and *S.thermophilus* were between 7 and 10 log cfu mL^{-1}during whole storage. It was observed that inoculation of *E coli* O157:H7 did not affected LAB counts. In accordance with results obtained in the present study Canganella et al. (1998) found similar counts of *S.thermophilus* during storage, however they observed a smaller count of *L. bulgaricus* (5–6 log cfu g^{-1}) for 2–3 weeks in yogurts inoculated with undesirable micro-organisms. Bauchouri, Quinto and Mora (2006) also found similar counts, between 7 and 8 log cfu mL^{-1} in homemade yogurt inoculated with *E. coli* O157:H7.

3.3 Survival of *E coli* O157:H7

E. coli O157:H7 was not found in any not inoculated yogurt control sample, characterizing that counts obtained in inoculated groups were resulted from experimental contamination. The initial counts for *E coli* O157:H7, at the time of inoculation in milk were 5.8, 5.6 and 5.5 log cfu.ml^{-1}, right after fermentation counts reached their maximum counts, increasing to 6.3, 5.4 and 7.8 cfu.$ml^{-1}$for groups (D), (E) and (F) respectively.

The results of the present study demonstrate that *E coli* 017:H7 was able to grow despite acid environment developed during fermentation, also demonstrate that *E coli* O157:H7 grows faster in lactose free milk. Our findings are in accordance to Kasımoğlu and Akgün (2004) and Bachrouri, Quinto and Mora. (2006) that reported increase of *E. coli* O157:H7 counts during the fermentation process in comparison with initial inoculum. Osaili

et al (2013) also reported an increase of *E. coli* O157:H7 count higher than 2.75 log cfu mL^{-1} during fermentation. However, Massa et al (1997) inoculated 3 or 7 log cfu mL^{-1} of *E.coli* O157:H7 into milk and yoghurt and reported that its number did not change during the fermentation period at 42± 1°C.

The higher growth of *E. coli* O157:H7 observed in group (F) could be explained by fast fermentation of free glucose, resulting of industrial milk hydrolysis. Xu et al. (1990) observed that different strains of *E. coli* O157:H7 are capable to rapidly ferment lactose and glucose.

In our study populations of *E. coli* O157:H7 in groups (D) and (E) decreased to 0.7 and 1.8 log cfu.ml^{-1} respectively after 9 days of storage, and after 10 days the *E. coli* populations in these groups were completely inactivated. The pathogen persisted longer in group (F), in 10^{th} day count was 4.2 log cfu.ml^{-1}, becoming undetectable only after 22 days of storage (Figure 2).

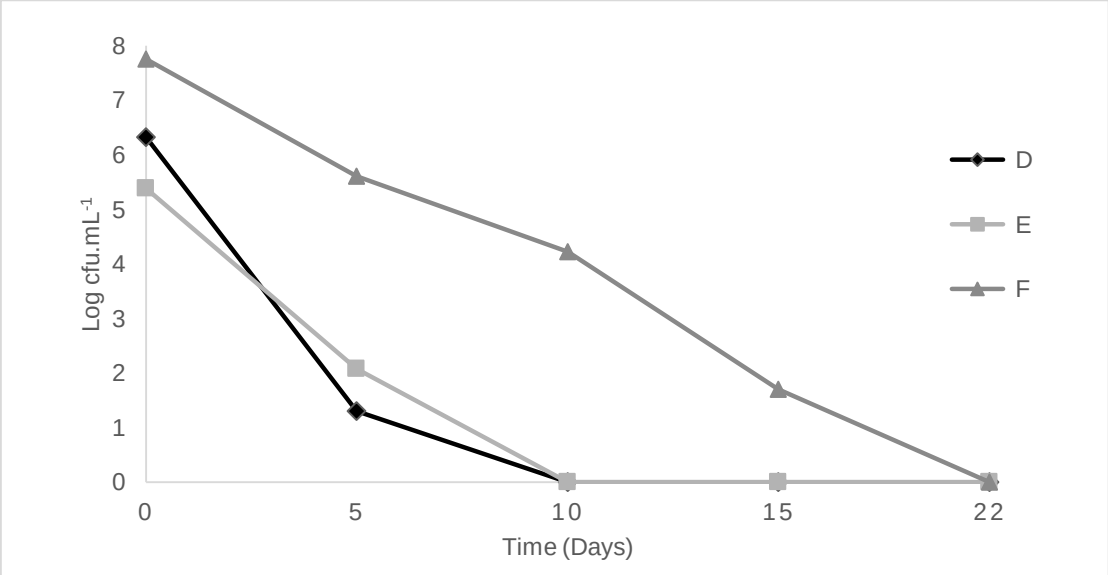

Figure 2: Survival of *E. coli* O157:H7 in yogurts (D), (E) and (F)

In accordance with results obtained in the present study for *E. coli* O157:H7 survival for considerable periods, Govaris et al (2002) reported that *E. coli* O157:H7 was no longer

detectable only after 7 days of storage at 4°C. Tosun, Seçkin and Aktuğ Gönül (2006) showed that in inoculated yogurts with *E. coli* O157:H7 the inactivation of the microorganisms occurred after 13 days and Bachrouri, Quinto and Mora. (2006) found similar results in yogurt stored at 8°C, with 10 days of *E. coli* O157:H7 survival. Lee and Chen (2005) observed a longer survival in yogurts with different strains of E. *coli* O157:H7, it was between 14 and 18 days. Cirone et al. (2013) presented similar results with extended persistence of E. *coli* O157:H7 in yogurt, up to 20 days of storage, revealing the capacity of these bacteria to tolerate acidity conditions.

3.4 Carbohydrates and lactic acid profile

In all six groups lactose decreased during fermentation process due LAB metabolism and *E coli* activity in inoculated groups (D, E, and F) Significant changes in lactose content occurred during the manufacture of hydrolyzed yogurts. During pre-hydrolysis of control group (B) and inoculated group (D), β-galactosidase was responsible for lactose cleavage and release of glucose and galactose, which significantly increased after 60 min of hydrolysis process in comparison with whole milk (Figure 3). The enzyme used in our research is a compound extracted from a milk yeast; thereby the optimum conditions for their activity are similar to the natural milk pH between 6.6 and 6.8 and presents optimum temperature between 35 – 40 °C. Therefore, during fermentation process, the acid production and consequent decrease of pH are responsible for exogenous enzyme inhibition, and the remainder of the lactose metabolism was performed by LAB and *E coli*.

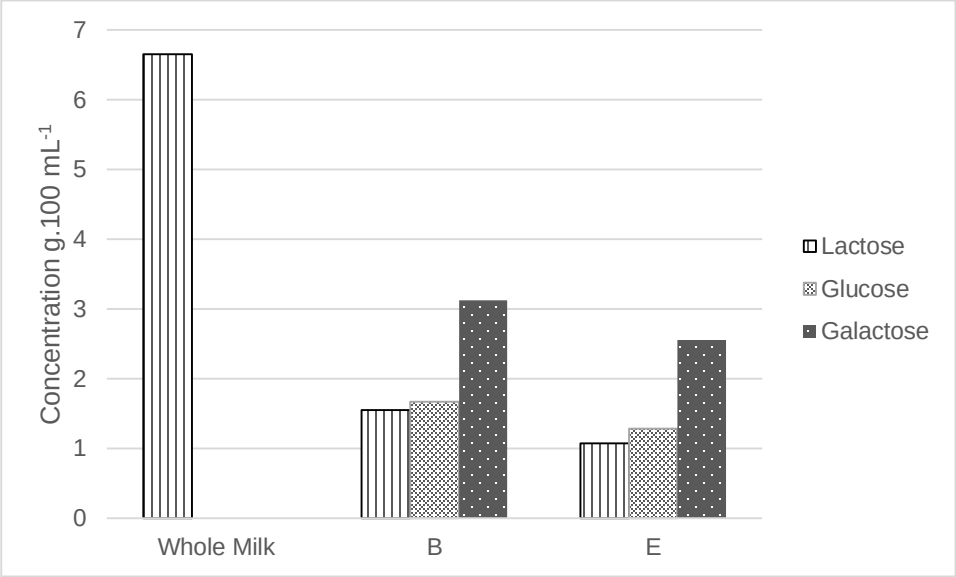

Figure 3: Values of lactose, glucose and galactose after 60 min of hydrolysis in groups (B) and (F) in comparison to lactose content of whole milk.

In control *E coli* not inoculated group (B), the values decreased approximately 87% at the end of the fermentation process, reaching mean contents of 0.88±0.01 g. $100mL^{-1}$. Already in inoculated and hydrolyzed group (E) lactose content decreased 94% reaching mean content of 0.22±0.01 $g.100mL^{-1}$. After 12 h of cooling storage when product are considered ready to eat, lactose content decreased even more in all groups and it was observed that groups (B) and (E) showed lower lactose values than those recommended by Brazilian legislation (Brasil, 1998) to consider a free lactose product. (0.47±0.02 and 0.22±0.01g. $100mL^{-1}$ respectively) (Tables 1, 2 and 3).

In accordance to results obtained from this study Vénica, Perotti and Bergamini (2014) observed important changes in the lactose content in hydrolyzed yogurts, with decrease of approximately 82% of lactose content at the end of the fermentation process. Wolf, Vénica and Perroti (2015) also find similar results with 75% to 78% lactose hydrolysis during fermentation of yogurt produced with simultaneous addiction on enzyme and starter culture. However, Martins et al. (2012) observed higher lactose conversion, of approximately 98% in

yogurts with simultaneous hydrolysis and fermentation, they showed a final lactose content of 0.19 g $100mL^{-1}$.

Regarding glucose content, it seems to be floating during fermentation in groups (A), (B), (D) and (E) with production by lactose hydrolysis and consumption by LAB in all groups and *E. coli* in groups (D) and (E). However, in groups (C) and (F) in which had almost complete hydrolysis of lactose from the beginning of process, glucose was only consumed during fermentation (Table 3). This situation changed after 24h of fermentation, when it was observed consumption of glucose in all groups, with final values of 0.00, 0.92±0.02, 1.91±0.04, 0.06, 0.99±0.02 and 1.84±0.01 g $100mL^{-1}$ for groups (A), (B), (C), (D), (E) and (F) respectively (Tables 1, 2 and 3).

Corroborating to our findings, Rodriguez, Cravero and Alonso (2008) also find that in control yogurts without enzyme the glucose content was smaller than in hydrolyzed yogurts with values approximately 0.2 g $100mL^{-1}$ for control yogurts and between 0.9 and 1.1 g $100mL^{-1}$ for hydrolyzed yogurts. Vénica, Perotti and Bergamini (2014) observed that glucose content of untreated yogurts was undetectable and the presence of glucose was only detectable in hydrolyzed groups, which present an overproduction of glucose by hydrolysis that LAB could not fully use during fermentation, the values ranged from 1.59 to 2.19 g $100mL^{-1}$.

In relation to galactose content we observed an increase in groups (A) and (D) from 0 to 0.44±0.01 and 0.12±0.03g $100mL^{-1}$, while in groups (B), (C), (E) and (F) was noted consumption of galactose during fermentation with final values of 4.92±0.09, 2.42±0.04, 1.49±0.03 and 2.71±0.06 g $100mL^{-1}$ (Tables 1, 2 and 3).

It has been demonstrate from long time that most strain of *S thermophilus* do not grow on galactose and ferment only the glucose portion of lactose, while the galactose is excreted into the medium in amounts stoichiometric with the uptake of lactose (Hutkins, Morris, 1987). However, studies had demonstrate that even galactose negative phenotypes (Gal^-) strains possesses the full complement of genes necessary for galactose metabolism and are able to fermented this carbohydrate (Vaughan et al., 2001, Erkus et al., 2014). Corroborating with our results, Anbukkarasi et al. (2014) used *S. thermophilus* Gal^+ to ferment yogurt and observed that galactose content after fermentation ranged from 0.38 to 0.98 depending on the *S thermophilus* strain utilized.

The catabolism of lactose by LAB results mainly in the production of lactic acid and this organic acid was also evaluated and as expect there was a sharp increased during fermentation, reaching its maximum values after 24h of storage in all groups (Tables 1, 2 and 3).

In accordance to our findings, Vénica, Perotti and Bergamini (2014) showed an acute increase in lactic acid content in natural and hydrolyzed yogurts nonetheless reported lower values with a mean value of 0.75 g $100mL^{-1}$.

Table 1: Carbohydrates and lactic acid quantified by HPLC analysis during fermentation and storage of not inoculated group (A) and. inoculated group (D).

Chemical compounds (g. 100 ml^{-1})	UHT milk	Fermentation Time (h)					Storage	
		0		4		5.5	24 h	
		A	D	A	D	A	A	D
Lactose	6.65±0.06	4.46^{aA}±0.12	6.82^{aB}±0.07	3.43^{bA}±0.04	4.73^{bB}±0.09	3.44 ^{bA}±0.02	3.13^{cA}±0.06	3.02^{cB}±0.07
Glucose	0.00	0.11^{aA}±0.00	0.00^{aB}	0.10 ^{bA}±0.00	0.00^{aB}	0.00^{Ca}	0.00^{cA}	0.06^{bB}±0.00
Galactose	0.00	0.10^{aA}±0.00	0.04^{aB}	0.44^{bA}±0.01	1.16^{bB}±0.03	0.56^{cA}±0.01	0.06^{dB}±0.01	1.08^{c} ±0.00
Lactic Acid	0.00	0.26^{aA}±0.02	0.15aB±0.00	0.67^{bA}±0.01	2.16^{bB}±0.04	2.83^{c}±0.14	3.37^{dA}±0.06	3.28^{cA}±0.09

$^{a-d}$ Letters indicate significant differences in the treatment, $P < 0.05$.

$^{A-D}$ Letters indicate significant differences among the different treatments, $P < 0.05$.

Table 2: Carbohydrates and lactic acid quantified by HPLC analysis during fermentation and storage of pre hydrolyzed not inoculated group (B) and pre hydrolyzed inoculated group (E).

Chemical compounds (g. 100 ml^{-1})	UHT milk	Fermentation Time (h)						Storage	
		0		4		4.5		24 h	
		B	E	B	E	B	E	B	E
Lactose	6.65±0.06	1.53^{aA}±0.01	0.65aB±0.01	1.13^{bA}±0.02	0.23bB±0.01	0.88^{Ac}±0.01	0.40cB±0.02	0.47^{dA}±0.02	0.22^{bB}±0.01
Glucose	0.00	3.06^{aA}±0.04	1.27aB±0.01	3.11^{aA}±0.07	1.17^{bB}±0.02	2.48^{bA}±0.05	1.01^{cB}±0.04	0.92^{cA}±0.02	0.99^{cB}±0.02
Galactose	0.00	4.6^{aA}±0.03	1.38aB±0.02	4.09^{bA}±0.11	1.24bB±0.02	4.92^{cA}±0.09	1.49ac±0.03	1.37^{dA}±0.02	1.57cB±0.03
Lactic Acid	0.00	0.06^{aA}±0.02	0.66aB±0.03	0.93^{bA}±0.04	0.83^{bB}±0.03	1.99^{cA}±0.03	1.31^{cB}±0.01	3.42^{dA}±0.07	2.64^{dB}±0.03

$^{a-d}$ Letters indicate significant differences in the treatment, $P < 0.05$.

$^{A-D}$ Letters indicate significant differences among the different treatments, $P < 0.05$.

Table 3: Carbohydrates and lactic acid quantified by HPLC analysis during fermentation and storage of lactose free not inoculated group (C) and lactose free inoculated group (F).

Chemical compounds (g. 100 ml^{-1})	UHT milk	Fermentation Time (h)						Storage	
		0		4		5	6.5	24 h	
		C	F	C	F	F	C	C	F
Lactose	0.45	0.60^{aA}±0.01	0.60^{aA}±0.01	0.35^{bA}±0.03	0.60^{aB}±0.01	0.59^{aB}±0.01	0.30^{cA}±0.01	0.27^{cA}±0.01	0.44^{bB}±0.01
Glucose	1.01±0.03	3.48^{aA}±0.03	3.50^{aA}±0.16	2.26^{bA}±0.04	2.21^{bB}±0.02	2.23^{bB}±0.04	1.78^{cA}±0.02	1.91^{dA}±0.04	1.84^{cB}±0.01
Galactose	1.92±0.04	5.29^{aA}±0.04	3.95^{aB}±0.27	2.85^{bA}±0.07	2.76^{bB}±0.03	2.71^{bB}±0.06	2.42^{cA}±0.04	2.44^{cA}±0.04	2.29cB±0.01
Lactic Acid	0.30	0.09^{aA}	0.13^{aB}±0.01	1.92^{bA}±0.03	1.80^{bB}±0.02	2.63^{cB}±0.02	1.96^{bA}±0.05	2.28^{cA}±0.06	0.32^{dB}±0.05

$^{a-d}$ Letters indicate significant differences in the treatment, $P < 0.05$.

$^{A-D}$ Letters indicate significant differences among the different treatments, $P < 0.05$.

4 Conclusion

E. coli O157:H7 was able to grow through fermentation process and survival during storage of yogurts with different lactose. The manufacture of yogurts with lactose free milk contaminated increased the survival time of microorganisms to double of days in comparison with whole milk and pre hydrolyzed milk. Thus, once these pathogen microorganisms are present in raw materials they could reach dairy products and consequently consumers. The severe control of good manufacturing practices during the production and storage of yogurt is emphasized.

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4CONSIDERAÇÕES FINAIS

Em relação aos resultados obtidos nesta dissertação pode-se concluir que a *Escherichia coli* O157:H7 foi capaz de sobreviver em iogurte com diferentes teores de lactose, apresentando crescimento durante o processo fermentativo e demonstrando ser capaz de causar alterações tecnológicas e sobreviver por considerável período de tempo.

Neste estudo foi comprovada a capacidade de adaptação da *E coli* ao ambiente ácido gerado durante a fermentação, assim como foram demonstradas as alterações tecnológicas ocorridas a partir da contaminação do leite antes do processo de pré hidrólise da lactose. No segundo experimento foi avaliado o período de sobrevivência da *E coli* desde a sua inoculação até a estocagem do produto pronto refrigerado após a fermentação. Foi observada a capacidade de sobrevivência por longo período de tempo, especialmente no iogurte preparado a partir de leite sem lactose.

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6. ANEXOS

6.1 COMPROVANTE DE SUBMISSÃO ARTIGO 1

Brazilian Journal of Microbiology

Escherichia coli O157:H7 survival in natural and low lactose yogurt during fermentation and storage

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Keyword:	Escherichia coli O157:H7, lactose hydrolysis, yogurt processing
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6.2 COMPROVANTE DE SUBMISSÃO ARTIGO 2

Elsevier Editorial System(tm) for Food Microbiology
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Manuscript Number:

Title: Survival of Escherichia coli O157:H7 during manufacture and storage of traditional and low lactose yogurt

Article Type: Research Paper

Keywords: fermented milk, lactic acid bacteria, β-galactosidase, lactose hydrolysis, carbohydrates profile, HPLC

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Abstract: Yogurt is one of the most popular dairy product and its content of lactose is one third lower than milk, however some people presents symptoms of lactose malabsorption when consuming yogurt. To overcome this fact dairy industry starts to use β-galactosidase to obtain low lactose products. Yogurt had been considered safe because of their low pH, although recent researches had shown the survival of Escherichia coli O157:H7 which have been associated with outbreaks involving dairy products. In this context, the aim of the study was to verify survival of Escherichia coli O157:H7 in yogurts with different lactose content. Six different yogurts were prepared with milk, pre hydrolyzed milk and lactose free milk. Three of them were inoculated with E. coli O157:H7. The survival of E. coli O157:H7, carbohydrates profile and pH of yogurts were determined during fermentation and storage. Results showed that an initial contamination enhance E. coli O157:H7 counts during fermentation. E. coli was able to survive for 10 days in yogurt with whole and pre hydrolyzed milk and for 22 days in yogurt from free lactose milk. Thus, it is concluded that milk contamination by E. coli O157:H7 in free lactose milk increases its survival in 12 days.