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RAPHAEL FERREIRA DE BARROS

ELABORAÇÃO E ESTOCAGEM DE IOGURTE COM TEORES REDUZIDOS DE LACTOSE POR MEIO DE HIDRÓLISE ENZIMÁTICA

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

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“Muitas das falhas da vida acontecem quando as pessoas não percebem o quão perto estão quando desistem”

Thomas Edison

RESUMO

Objetivou-se com o presente estudo, através da hidrólise enzimática da lactose, verificar a porcentagem de hidrólise do carboidrato e a influência da enzima β-galactosidase frente aos índices físico-químicos, ao comportamento dos microrganismos *Streptococcus thermophilus* e *Lactobacillus delbrueckii* subespécie *bulgaricus* utilizados no processamento e às condições de fermentação e armazenamento de iogurte com baixo teor de lactose. Os resultados do primeiro experimento (**Artigo 1**) demonstraram os valores de pH e acidez titulável ($P<0,05$) entre o tratamento sem pré-aquecimento, utilizando a temperatura inicial do leite igual a 25ºC, e, o tratamento com pré-aquecimento, utilizando a temperatura inicial do leite igual a ºC 40. O tratamento com pré-aquecimento atingiu valor mais elevado de acidez, assim como o valor do pH diminiu mais rapidamente do que o tratamento sem pré-aquecimento. Em ambos os tratamentos ($P>0,05$), as porcentagens de hidrólise da lactose foram suficientes para consumo da maioria das pessoas com má absorção de lactose. Foi demonstrado que a enzima contribuiu para reduzir a lactose, sem influenciar no processo de fermentação. No segundo experimento (**Artigo 2**), os resultados sugeriram que a pré-hidrólise enzimatica da lactose não influenciou no valore de pH, o qual se manteve constante (6,83). Durante a fermentação dos três tratamentos, os valores de pH dos tratamentos envolvidos foram, também determinados e comparados ($P<0,05$). No dia após a fermentação foram mensurados os valores de pH ($P>0,05$) entre o iogurte comum e o iogurte hidrolisado, e também comparado com iogurte pré-hidrolisado ($P<0,05$). Além disso, os microrganismos envolvidos na fermentação foram enumerados e observou-se uma queda na contagem das bacterias láticas específicas dos três tratamentos durante o armazenamento, porém, todos ainda assim permaneceram acima da contagem mínima exigida. O comportamento dos carboidratos e ácido lático foram obtidos por CLAE e demostraram principalmente uma queda de 93,66% na concentração de lactose do produto pré-hidrolisado. Iogurte tradicional e hidrolisado atingiram 59,87% e 85,42% de lactose hidrolisada, respectivamente. Assim, a redução de lactose, por alguns tratamentos, foram suficiente para favorecer o consumo do produto para seres humanos portadores da sindrome de má absorção da lactose, sem , portanto, influenciar na tecnologia de fabricação do Iogurte.

Palavras-chave: Leite Fermentado. β-galactosidase. Alimentos funcionais. CLAE. HPLC

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ABSTRACT

The aim of the study, through the lactose enzymatic hydrolysis, was to verify the influence of the β-galactosidase enzyme in the low-lactose yogurt process, compared to the behaviour of the microorganisms *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subspecies *bulgaricus* used during processing stages and the conditions of fermentation and storage. The results of the first experiment (**Article 1**) indicates that pH and titratable acidity values ($P<0,05$) among the non-preheated treatment (initial milk temperature equal 25 °C) and preheated treatment (initial milk temperature equal 40 °C). The preheat treatment reached higher acidity values and the pH dropped faster than non-preheat treatment. In both treatments, the percentages ($P>0,05$) of lactose hydrolysis were sufficient for consumption of most human with lactose malabsorption. It has been shown that the enzyme has contributed to reduce the lactose concentration without influencing the fermentation process. In the second work (**Article 2**), results suggest that, during lactose pre-hydrolyze there were not changes at pH values measured (6,83). During fermentation process, pH values between involved treatments were also determined and compared ($P <0.05$). On the first storage day, pH values were compared between the common yogurt and hydrolyzed yogurt ($P> 0.05$), and also compared with pre-hydrolyzed yogurt ($P <0.05$). Moreover, the microorganisms involved in the fermentation were enumerated and observed a decrease in the count of specific lactic acid bacteria of the three treatments during storage, but all still remained above the minimum required count.The behavior of carbohydrates and lactic acid were obtained by HPLC and demonstrated 93.66% less lactose in pre-hydrolyzate yogurt. Non hydrolyzed and hydrolyzed yogurt reached 59.87% and 85.42% hydrolyzed lactose, respectively. Hence, sufficient lactose reduction without fermentation influence was achieved, which favors the consumption for the most human, who has lactose malabsorption.

Key Words: Fermentado. β-galactosidase. Alimentos funcionais. CLAE. HPLC

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1 **INTRODUÇÃO**

Segundo a “Food and Agriculture Organization of the United Nations” – FAO, a produção de leite fluido atingiu 727 milhões de toneladas em 2011, incluindo leite de vaca, búfala, camelo, cabra e ovelha. O leite de vaca sozinho respondeu por aproximadamente 614,6 milhões de toneladas, o que representa incremento de 1,5% em relação a 2010. Em 2011, os países que tiveram os maiores aumentos, em termos absolutos, na produção de leite de vaca foram: Índia, Estados Unidos, Turquia e Brasil (EMBRAPA, 2013a; EMBRAPA, 2013b).

O leite fluido é a segunda maior “commodity” no “ranking” de movimentações financeiras no mundo (183.583.111.000 dólares por ano), sendo esta “commodity”, a quarta mais produzida mundialmente (614.578.723 milhões de toneladas). A principal mudança no “ranking” mundial de produção de leite foi a passagem do Brasil para a quarta posição, ultrapassando a Rússia, totalizando 34.255.236 milhões de toneladas de leite produzidos no ano (FAO, 2015).

Embora a produção de leite no país esteja crescendo, no Brasil não é observado um consumo *per capita* de leite muito elevado, quando comparado aos índices dos países desenvolvidos, como Estados Unidos e União Europeia. O consumo aparente refere-se ao total de leite e derivados consumidos pela população e também utilizados pelas indústrias. Enquanto no Brasil o consumo *per capita* gira em torno de 170 Kg, nos países desenvolvidos, este consumo ultrapassa os 270 Kg (IFCN, 2012).

Com os processos econômicos ocorridos no Brasil, o consumo de alimentos deverá sofrer variações e irá se elevar de maneira geral. Estima-se que o consumo de lácteos aumentará em torno de 3% ao ano e se as tendências continuarem, os maiores incrementos devem ocorrer nas categorias de leite fermentado, leite em pó e iogurte (EMBRAPA, 2013a; EMBRAPA, 2013b).

O leite é considerado um alimento completo, fonte de proteínas (3,2 a 3,5%), carboidratos – principalmente lactose (4,7 a 5,2%), gorduras (3,5 a 4,0%), minerais (0,6 a 0,9%) e vitaminas (PARK, 2007), o que faz o leite e os derivados lácteos serem considerados alimentos de alto valor nutritivo, não se limita apenas ao fato de possuírem nutrientes essenciais, mas também à forma e à distribuição equilibrada,

bem como a alta digestibilidade (biodisponibilidade) dos nutrientes (TRONCO, 2010).

A ingestão de leite e produtos lácteos conduz a diminuição do pH intestinal tanto pela própria acidez dos produtos como pela utilização da lactose pela microbiota intestinal existente, o que promove ao indivíduo um desenvolvimento favorável de microrganismos intestinais lactose tolerantes, inibindo o crescimento de bactérias putrefativas e patogênicas, além de melhorar a absorção do cálcio devido o mesmo se tornar mais disponível em pH reduzido (ibid).

Contudo, pesquisadores demonstraram que 65%, ou mais, da população mundial – principalmente adultos – possuem um determinado nível de intolerância ao principal carboidrato do leite, a lactose (RAITHEL, 2013; VUORISALO et al., 2012).

A intolerância à lactose – também conhecida como hipolactasia ou lactase não persistente – se deve à má digestão do carboidrato. Isto ocorre devido a um declínio na atividade enzimática, ou mesmo por insuficiência, ou ausência, na produção da enzima lactase (β-galactosidase) pelas células da mucosa do intestino delgado, de forma que a lactose ingerida e não completamente hidrolisada leva a ocorrência de processos gastrointestinais, dores abdominais, náuseas, flatulências, e outros sintomas, até mesmo a morte em casos extremos (MISSELWITZ, 2013).

A intolerância possui três diferentes origens: congênita, irreversível, que se não diagnosticada e controlada precocemente, pode levar o recém-nascido ao óbito; deficiência primária, responsável pela maioria dos casos, sofrendo variações de acordo com idade do indivíduo, quantidade de lactose consumida e fatores biológicos e/ou culturais, nos quais populações que não possuem o hábito do consumo de leite e derivados manifestam maior prevalência dos sintomas de intolerância à lactose; e por último, a deficiência de origem secundária, ou adquirida, reversível ou não, que é resultante de lesões causadas na mucosa do intestino delgado (VUORISALO et al, 2012).

Equívocos no diagnóstico, ou mesmo a falta de informações por parte de alguns médicos, fazem com que o consumo de leite e derivados lácteos seja abolido erroneamente das dietas de pessoas que manifestam sintomas de intolerância. Este episódio pode acarretar prejuízos nutricionais, visto o alto valor nutritivo dos produtos lácteos e sua importância na dieta dos indivíduos. Deve-se dar atenção a

este tipo de dieta, que se faz necessária à ingestão de alimentos com teor reduzido de lactose, razão pela qual, determinados produtos terem ganhado cada vez mais importância.

Os leites fermentados são alguns dos produtos derivados do leite produzidos pelas indústrias com o intuito de agregar valor na matéria prima, sendo o principal e o mais comercializado: o iogurte. Estes produtos podem ser consumidos por indivíduos pouco intolerantes, uma vez que a hidrólise do dissacarídeo é relativamente baixa (10 – 30%), porém presente. Em contrapartida, a hidrólise enzimática da lactose pode ser inserida no processamento destes produtos, diminuindo ainda mais o teor de lactose e favorecendo o consumo pelos intolerantes (SCHAAFSMA, 2008).

Alimentos com teor reduzido de lactose eram apenas um nicho de mercado para as indústrias. Todavia, muito se tem comentado a respeito deste assunto e com o aumento da demanda por produtos para os indivíduos com má digestão de lactose, este mercado se tornou uma necessidade real. Por ser muito pouco explorado, tem-se buscado quantificar os intolerantes no Brasil e no mundo, o que fez com que as indústrias vislumbrassem um grande mercado, surgindo à necessidade de se desenvolver novos produtos.

Objetivou-se com este estudo, verificar a influência da enzima β-galactosidase na elaboração de leites fermentados, em relação ao comportamento das bactérias: *Streptococcus thermophilus e Lactobacillus delbrueckii* subespécie *bulgaricus* nas condições de fermentação e armazenamento do iogurte. Também se avaliou a hidrólise da lactose em relação às características físico-químicas do leite e do produto final, assim como as características bacteriológicas intrínsecas necessárias. Para avaliar o comportamento da cultura acido lática, além da acidez, hidratos de carbono (lactose, glicose e galactose) e ácidos orgânicos foram quantificados por cromatografia líquida de alta eficiência.

2 **FUNDAMENTAÇÃO TEÓRICA**

2.1 LEITE FERMENTADO

O início do processo da elaboração de leite fermentado data de milhares de anos atrás, em torno de 10.000 aC, provavelmente na mesma época em que ocorreram mudanças nos hábitos dos indivíduos, os quais deixaram de simplesmente coletar e caçar os alimentos e começaram a também produzir o próprio alimento. Acredita-se que o processo de produção de leite fermentado ocorreu, acidentalmente, antes mesmo da domesticação de ovelhas, cabras e vacas. Esta arte de produzir leite fermentado era passada de geração em geração, o que contribuiu para a manutenção deste conhecimento até os dias atuais. Estima-se que até o século XIX se conheciam apenas os fundamentos básicos das fases de produção e esta se limitava em uma pequena escala de produção, porém com os descobrimentos e avanços dos conhecimentos em microbiologia, enzimologia, física, química, bioquímica e outros, incluindo a tecnologia industrial, o processo ganhou grande propulsão (FERREIRA, 2005; TAMINE, 2006; TAMINE; ROBINSON, 2007).

No Regulamento Técnico de Identidade e Qualidade (RTIQ) de Leites Fermentados está definido que:

> Leites Fermentados são os produtos adicionados ou não de outras substâncias alimentícias, obtidas por coagulação e diminuição do pH do leite, ou reconstituído, adicionado ou não de outros produtos lácteos, por fermentação láctica mediante ação de cultivos de microrganismos específicos (BRASIL, 2007).

No mesmo regulamento também são caracterizados os diferentes leites fermentados conforme a identidade de cada um, sendo a definição exata para Iogurte:

> Entende-se por Iogurte, Yougur ou Yoghurt [...] o produto [...] cuja fermentação se realiza com cultivos prosimbióticos de *Streptococcus salivarius* subsp. *thermophilus* e *Lactobacillus delbrueckii* subsp. *bulgaricus,* aos quais se podem acompanhar, de forma complementar, outras bactérias ácido-láticas que, por sua atividade, contribuem para a determinação das características do produto final (BRASIL, 2007).

Faz parte também do RTIQ, os requisitos físicos, químicos e microbiológicos que os diferentes leites fermentados devem atender (Quadro 1), além das características sensoriais de consistência firme, pastosa, semissólida ou líquida, sabor e odor característicos e cor branca, quando não adicionados nenhum corante e/ou outras substâncias alimentícias permitidas (BRASIL, 2007).

Quadro 1: Requisitos físicos, químicos e microbiológicos exigidos para leite fermentado, em especial, iogurte.

	Matéria Gorda Láctea ($g.100g^{-1}$)				Proteína láctea (g/100g)	Acidez (g. ác. Lático. $100g^{-1}$)	Contagem de Bactérias Lácteas Totais (ufc/g)
	Com Creme	Integral	Parcialmente desnatado	Desnatado			
IOGURTE	*Mín. 6,0*	*3,0 a 5,9*	*0,6 a 2,9*	*Máx. 0,5*	*Mín. 2,9*	*0,6 a 1,5*	*10^7*

Fonte: Adaptado de Brasil (2007).

Desde a antiguidade até os dias atuais, uma das principais razões para se fermentar o leite é estender a validade comercial do próprio leite. Porém, atualmente, se conhecem inúmeras outras vantagens de se produzir leite fermentado. Melhorar o sabor e aroma do leite, bem como melhorar a digestibilidade com os produtos lácteos fermentados são razões pela qual o referido produto tem ganhado importância. O fato de aumentar a variedade de produtos disponíveis no mercado, agregando valor à matéria prima, não deve ser negligenciado (CORTEZ; CORTEZ, 2010; ORDÓÑEZ, 2005; TAMINE. 2006;).

A fermentação está associada também com a segurança do alimento. Os leites fermentados são produzidos, principalmente, a partir do leite e por culturas láticas, sejam de origem bacteriana ou leveduras, dependendo do produto específico. Esses microrganismos utilizados não são patogênicos e, até o momento, não possuem riscos associados com a saúde dos indivíduos, portanto são considerados organismos “Geralmente Considerados Seguros” (do inglês “Generally Recognized as Safe” – GRAS). Em contrapartida, a produção de ácido lático e outros ácidos orgânicos (redução do pH), bem como fatores antimicrobianos como peróxido de hidrogênio, nisinas e outras bacteriocinas, atuam inibindo o crescimento de patógenos em grande parte dos casos, porém é importante salientar que a

tolerância destes patógenos frente a redução do pH, tem sido discutido, uma vez que a resistência pode ser aumentada durante o processo de acidificação do leite, devido às alterações bacterianas (FORSYTHE, 2002).

Os leites fermentados são produtos resultantes da fermentação do leite, por adição de fermentos láticos próprios, que devem ser viáveis, ativos e abundantes no produto final e durante a validade comercial. Os produtos lácteos com redução do teor de lactose, assim como os adicionados com probióticos, podem ainda ser incluídos na categoria de alimentos funcionais, que apresentam funções benéficas à saúde, além das vantagens nutricionais (PRADO, 2007).

O ácido produzido pelo inóculo durante a fermentação da lactose aumenta consequentemente a acidez do meio, o que contribui para a coagulação da caseína do leite, quando o ponto isoelétrico da mesma é alcançado (pH em torno de 4,5), sendo um efeito desejado nos produtos lácteos fermentados (TRONCO, 2010).

Lácteos fermentados estão associados a diversos benefícios. Além de facilitar a ação das proteínas e enzimas digestivas, facilitar a absorção de cálcio, fósforo e ferro, e ser fonte de galactose, que é um importante monossacarídeo envolvido na síntese do tecido nervoso em crianças (FERREIRA; TESHIMA, 2000), estão também envolvidos no controle de peso, efeitos imunológicos (ligados a diminuição de incidência de câncer, infecções, distúrbios gastrointestinais e alergias), na indução de produção de citocinas, produção e atividade de anticorpos, atividade fagocitária e atividades linfocitárias (MCKINLEY, 2005; SHAH, 2007).

2.2 BACTÉRIAS ÁCIDO-LÁTICAS

A nomenclatura “acido-lático” é dada a este grupo bacteriano por ter capacidade de fermentar a lactose presente no leite e formar como subproduto o ácido lático, principalmente. As principais bactérias ácido-láticas utilizadas no processamento dos leites fermentados são bactérias Gram-positivas, e em sua maioria homofermentativas, catalase e motilidade negativas. Dentre as quais destacam-se *Streptococcus thermophilus e Lactobacillus delbrueckii* subespécie *bulgaricus* (CORTEZ; CORTEZ, 2010; FERREIRA, 2005; ORDÓÑEZ, 2005).

São, em sua maioria, mesófilas, porém com algumas linhagens termófilas, capazes de crescer em temperaturas que variam de 5 ^{o}C a 45 ^{o}C e possuem a

capacidade de se desenvolver em pH baixo, em torno até de 3,8. Algumas espécies são também consideradas proteolíticas, contribuindo com sabor, aroma e possível simbiose e/ou inibição de outros microrganismos. Este grupo de microrganismo utiliza a lactose como principal fonte de energia e produz principalmente o ácido lático, responsável pela acidificação do produto (FORSYTHE, 2002).

De acordo com normas do Codex para Leite Fermentado (CODEX, 2011), o iogurte é fermentado por bactérias lácticas ácido homofermentativas.Os inóculos utilizados na fermentação do iogurte são considerados termofílicos, portanto a faixa de temperatura para que a fermentação ocorra de forma esperada se encontra entre 42 a 43 ^{o}C (ORDÓÑEZ , 2005).

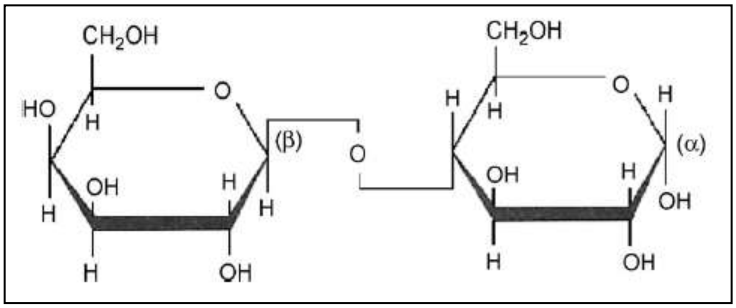
No processo de fermentação, a lactose do leite é fermentada por culturas lácticas específicas como *S. thermophilus* e *L. delbrueckii subsp. bulgaricus* e produz principalmente ácido láctico no final da cadeia do metabolismo (ADHIKARI et al, 2002; ANBUKKARASI et al, 2014). Inicialmente tem-se o crescimento do *Streptococcus* spp..Este microrganismo se desenvolve no pH ligeiramente ácido do leite e proporciona, além da queda parcial do pH, a formação de alguns peptídeos (a partir das proteínas do soro), os quais fornecem as condições ideais para o *Lactobacillus* spp. se desenvolver. O desenvolvimento do *Lactobacillus* spp. em contrapartida, faz com que seja disponibilizado aminoácidos, como glicina, histidina e valina, que favorecem (simbiose) o desenvolvimento do *S. thermophilus*, até que seja inibido pela acidez do produto. O *Lactobacillus delbrueckii ssp. bulgaricus*. é resistente a valores de pH inferiores ao *Streptococcus* spp e continua se desenvolvendo, produzindo compostos, incluindo ácido lático. Porém a inibição também ocorre, em pH 4,3 (FERREIRA, 2005; ORDÓÑEZ, 2005).

2.3 A INTOLERÂNCIA À LACTOSE ENVOLVIDA COM LEITE E DERIVADOS

A lactose é o principal carboidrato do leite proveniente da maioria dos mamíferos. O carboidrato, dissacarídeo, quase que exclusivo da matriz láctea, é formando por uma molécula de α-D-glicose e uma molécula de β-D-galactose, ou simplesmente glicose e galactose, unidas pela ligação glicosídica β1-4 (Figura 1). A concentração de lactose no leite bovino é em torno de 4,8%, 50 a 52% dos sólidos

não gordurosos, e é responsável por, além da pressão osmótica na galactopoiese, 30% do valor calórico fornecido pelo leite (FOX, 2009; HOLSINGER, 1988).

Figura 1: Desenho esquemático da molécula de lactose.



Fonte: Schaafsma (2008).

A lactose pode estar presente de duas formas diferentes (isoméricas), α e β-lactose, que quando ingeridas pelos mamíferos são, normalmente, hidrolisadas em seus componentes: glicose e galactose pela enzima lactase (β-galactosidase). Esta enzima está intimamente em contato com a membrana da mucosa do intestino delgado, onde ocorre a reação de hidrólise, e somente após estarem dissociados, os monossacarídeos estarão disponibilizados para serem absorvidos pelo intestino (FOX, 2009; SCHAAFSMA, 2008). No entanto, a percentagem de lactose pode ser prejudicial aos indivíduos com a atividade da lactase intestinal reduzida ou completamente ausente (MISSELWITZ, 2013).

A deficiência da enzima lactase – que pode ser causada pela insuficiência ou ausência da produção – por parte dos indivíduos, acarreta em um distúrbio metabólico denominado intolerância à lactose, conhecida também por hipolactasia ou lactase não persistente, que se deve a má digestão do carboidrato. Assim, a lactose ingerida e não hidrolisada, devido sua característica osmótica, ao atingir o intestino grosso, causa o influxo de água para dentro da luz intestinal, e concomitantemente pode ser fermentada por bactérias ali presentes, causando inchaço, cólicas, formação de gases (flatulência) e até mesmo diarréia (FOX, 2009; SCHAAFSMA, 2008).

A intolerância à lactose está associada a três diferentes tipos: o primeiro, hipolactasia primária, dos quais ocorrem em adultos, é a forma de hipolactasia mais encontrada em todo o mundo. O distúrbio é caracterizado pela diminuição programada geneticamente, no qual ocorre a redução da enzima e/ou da sua

atividade após o desmame, continuadamente durante curso de vida do indivíduo. No entanto, sofre variações conforme a idade do indivíduo, quantidade de lactose consumida e fatores biológicos e/ou culturais, os quais manifestam maior prevalência dos sintomas de intolerância à lactose aquelas populações que não possuem o hábito do consumo de leite e derivados (Quadro 2) (FOX, 2009; LLOYD;OLSEN[1], 1995 apud SWAGERTY; WALLING, 2002).

Quadro 2: Prevalência da hipolactasia tipo 1 em diferentes etnias.

GRUPOS	PREVALÊNCIA (%)
NORTE EUROPEUS	2 a 15
AMERICANOS BRANCOS	6 a 22
EUROPEUS (EUROPA CENTRAL)	9 a 23
INDIANOS (NORTE)	20 a 30
INDIANOS (SUL)	60 a 70
HISPÂNICOS	50 a 80
JUDEUS	60 a 80
NEGROS	60 a 80
ÍNDIOS NORTE AMERICANOS	80 a 100
ASIÁTICOS	95 a 100

Fonte: Adaptado de SWAGERTY; WALLING, 2002

Segundo Fox (2009), a lactase possui atividade máxima logo após o nascimento, mantendo-se elevada até pouco tempo pós desmame, normalmente, entre três a sete anos. Na idade adulta os níveis de lactase decrescem, podendo chegar, aproximadamente, a 10% da taxa existente no período da infância.

A hipolactasia secundária ou adquirida, reversível ou não, está acompanhada de qualquer doença ou afecção gastrointestinal que possam causar danos à mucosa do intestino delgado, mais precisamente na borda em escova dos enterócitos, ou aumentar signnificativamente o transito intestinal, de modo que possa acometar a produção da lactase ou prejudicar a interação entre o dissacarídeo e a enzima intestinal (FOX, 2009; LLOYD;OLSEN[1], 1995 apud SWAGERTY; WALLING, 2002).

[1] LLOYD, M. L.; OLSEN, W. A. *Disaccharide malabsorption*. In: HAUBRICH, W. S.; SCHAFFNER, F; BERK, J. E.; BOCKUS, H. L. BOCKUS Gastroenterology. 5. ed. Philadelphia: Saunders, p. 1087-1100, 1995:

O caso mais raro é a hipolactasia congênita (herança autossômica recessiva) responsável pela completa ausência de produção de lactase pelo indivíduo (desde recem-nascido) ao longo da vida (ibid).

Apesar de haver um consumo de lactose pela cultura “starter” do iogurte, a quantidade de lactose remanescente no leite fermentado é consideravelente alta (TAMINE; ROBINSON, 2007). Por tais razões, frente à intolerância à lactose do tipo 1 ou em alguns casos do tipo 2, as soluções se resumem a diminuição ou eliminação total da lactose dos alimentos, com destaque para a elaboração de leites fermentados e a adição da enzima β-galactosidase (CORTEZ; CORTEZ, 2010; WOLF; VÉNICA; PEROTTI, 2015). Somente com o processo de fermentação do leite pelos inóculos, ocorre a conversão da lactose em ácido lático, porém não são eficientes na maioria dos casos de intolerância, visto que em leites fermentados, por exemplo o iogurte, o teor de lactose é cerca de até 30% menor do que a concentração presente no leite (SCHAAFSMA, 2008), valor residual este relativamente alto.

2.4 ENZIMA β-GALACTOSIDASE E HIDRÓLISE DA LACTOSE

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 foi 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. Segundo Vinhal (2001), a enzima utilizada para reduzir o teor de lactose do leite, proveniente de *Kluyveromyces fragilis,* 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.

3 **DESENVOLVIMENTO**

3.1 ARTIGO 1: MILK PREHEAT INFLUENCE OVER LACTOSE HYDROLYSIS, COMPONENTS PRODUCTION AND YOGURT MANUFACTURE PROCESS

MILK PREHEAT INFLUENCE OVER LACTOSE HYDROLYSIS, COMPONENTS PRODUCTION AND YOGURT MANUFACTURE PROCESS

SHORT TITLE: Yogurt: Hydrolysis and Temperature Influence

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Abstract

The aim of the study was to verify the influence of different initial temperatures: room temperature (25°C± 1°C) and 41°C ± 1 °C over lactose hydrolysis, components production and yogurt manufacture process, and if it had obtained a low lactose concentration. Organic acids and carbohydrates were evaluated by HPLC. There were $P< 0.05$ on pH and titratable acidity analysis among the preheated and non-preheated treatments during whole fermentation process. The first one obtained higher pH and titratable acidity values as 4.6 ± 0.04 and 0.73 g lactic acid.100 mL^{-1} respectively, against 4.82 ± 0.01 and 0.64 g lactic acid. 100 mL^{-1} from second treatment. The lactose content was $P< 0.05$ between treatments until the last fermentation hour. However, at the end of fermentation lactose concentration reached 4.565±0.34 $mg.mL^{-1}$ and 4.398 ±0.18 $mg.mL^{-1}$ for Non-PHT and PHT ($P>0,05$), respectively. The glucose and galactose were remained buoyant during the fermentation period, which indicating the production and use of these. The lactic acid concentration achieved had corroborated with titratable acidity result, which lactic acid of non-preheat treatment was higher ($P<0,05$) -18.64±0.62 $mg.mL^{-1}$- than preheat treatment (17.56±0.53 $mg.mL^{-1}$). Thus, it can be concluded that the enzyme contributed to reduce the lactose content without influencing the fermentation process. In addition, both treatments obtained lower values of lactose, which is sufficient to consumption for the most human, who has lactose malabsorption.

Keywords. Fermented milk, β-galactosidase, Functional food, HPLC.

1. Introduction

Milk is considered a complete food source of protein (3.2 w/w), carbohydrates - particularly lactose (4.7 % w/w), fat (3.6 % w/w), minerals (0.6 w/w) and vitamins. The essential nutrients and the high digestibility (bioavailability) of nutrients make milk and dairy products being considered highly nutritious food (Park 2007). Lactose is the main carbohydrate in milk and is formed by one molecule of α-D-glucose and β-D-galactose. In cow's milk, the lactose concentration is around 4.8%, which means 50 to 52% of total solids (Fox 2009; Tamine and Robinson 2007). However, this lactose percentage can be detrimental to subjects with impaired or completely absent intestinal lactase activity (lactose malabsorption) (Misselwitz 2013).

Lactose malabsorption, also known as "lactose non-persistent", is due to poor digestion of this carbohydrate (Vuorisalo et al 2012) causing a gastrointestinal symptoms (lactose intolerance) (Misselwitz 2013). Researchers have shown that more than 65% of the world's population - mostly adults - has a certain level of this disorder. Raithel (2013) reported that lactose malabsorption has two different source: primary deficiency that include physiological lactase reduction and autosomal recessive familial lactase deficiency; as well as secondary deficiency featuring an inflammatory intestinal diseases . Therefore, various technologies are employed to development of reduced-lactose products, such as milk fermentation and enzymatic hydrolysis.

According to Codex Standards for Fermented Milk (Codex 2003), yogurt is a fermented milk by homofermentative acid lactic bacteria. In fermentation process, milk lactose are fermented by specific acid lactic cultures as *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* and produces mainly lactic acid on the end of metabolism chain (Adhikari et al. 2002; Anbukkarasi et al. 2014). Despite there is a

lactose consumption by yogurts starter culture, hence content a considerable amount of intact lactose in yogurt (Tamine and Robinson 2007).

The lactose residual in yogurts, is about 30% less than present in milk. However, it is still considered relatively high for some lactose malabsorption levels (Schaafsma, 2008). In these cases, only the milk fermentation process is not effective and for such reasons, one of the solutions to address who has some level of lactose intolerance is to elaborate a dairy product with lactose reduction or even lactose free, as obtained with fermented milk added of β-galactosidase enzyme. The use of enzyme in the manufacture of low-lactose dairy products is nowadays an usual practice (Wolf; Claudia and Perotti, 2015).

For these reasons, the hydrolysis conditions of milk lactose by the β-galactosidase enzyme action and the fermentation conditions by *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* were compared between two treatments with different initial temperature: room temperature (25°C) and 40°C. Therefore, the aim of the study was to verify the influence of the β-galactosidase enzyme into yogurt processes and components production, through the lactose enzymatic hydrolysis and milk fermentation, and if it had obtained a low lactose concentration required for humans with lactose malabsorption.

2. Materials and methods

2.1. Milk and its analysis

To elaborate the fermented milk was used commercial cow's whole UHT milk (Ninho®, Nestle, São Paulo, Brazil), acquired in the retail market, transported and stored

at room temperature (25°C), stemmed from dairy company with Brazilian Federal Inspection Service. To certificated the physicochemical quality was analyzed a sample of whole milk to determinate titratable acidity (Official Method 947.05), freezing point by cryoscopic method (Official Method 990.22) and pH (Official Method 973.41) (AOAC 2012). The pH was determined by inserting a pH probe into milk (pH meter PG1800, CapLab, São Paulo, Brazil) after calibrating with fresh pH 4.0 and 7.0 standard buffers.

2.2. Lactic acid culture and enzyme

In order to produce the yogurt was used the dosage suggested by the supplier which 50U of thermophilic lactic acid culture to 500 L of milk. The culture contains *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* strains (YF L-812 CHR HANSEN®).

The β-galactosidase enzyme used in this research to hydrolyze the lactose was Maxilact Lx 5000 (DSM Food Specialties, Delft, Netherlands) which is a refined lactase formulation derived from dairy yeast *Kluyveromyces lactis*; and the dosage used was 0.45 mL of enzyme per liter of milk, as suggested by the supplier, which was added into milk and stirring at the start of fermentation in both treatment. The lactose hydrolysis were performed at an oven at 41 ± 1 °C during fermentation time

2.3. Yogurt Manufacture

Yogurts were made applying the traditional method adapted to laboratory scale (Tamine and Robinson 2007) and prepared with UHT whole milk (Ninho® Nestlé, Três Rios - RJ, Brazil), into two separated groups, using 3 liters for each treatment. The first group,

non-preheat treatment (Non-PHT), was prepared at room initial temperature (25°C) and the second one, preheat treatment (PHT), was prepared with milk temperature at 41 ± 1 °C. In both groups occurred a single-stage process with the simultaneous addition of β-galactosidase enzyme and lactic acid culture, using the amount recommended by the respective manufacturer (as reported in item 2.2). The fermentation was performed at an oven at 41 ± 2 °C for 5 hours (300 minutes), so after the first hour both temperature were the same, and was interrupted when the pH reached 4.7 ± 0.1. Then, the yogurts were cooled immediately in a refrigerator at 4°C.

2.4. pH and titratable acidity analysis

During fermentation, the yogurts were analyzed every each hour to determinate pH and titratable acidity (AOAC 2012). In addition, the yogurts were also analyzed to determinate carbohydrates and lactic acid, which was extracted and carried out as following described.

2.5. Carbohydrates and lactic acid analysis by HPLC

The lactic acid and carbohydrates extraction was carried out using a modification of the method described by González de Llano et al. (1996) and performed in triplicate. 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 (TS – 2000 A VDRL shaker, Biomixer®) and then centrifugated at 5500*g* for 30 minutes at 4°C. Finally, the supernatant was filtered through Whatman no. 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). The interest peaks were identified by comparing retention times of the standards with the samples and the concentration of each component of the samples was determined from the area of individual peaks. Carbohydrates identification was performed by refractive index detector while lactic acid by using a diode array detector model monitoring the absorbance at 210 nm.

2.6. Statistical Analysis

The data were subjected to one-way analysis of variance (ANOVA). All ANOVA were subjected to Tukey's test at $P < 0.05$ using XLSTAT version 2013.2.03 (Addinsoft, Paris, France).

3. Results

Physicochemical analyses results are presented at Figure 1. The pH reached on the end of fermentation 4.82 ± 0.01 for Non-PHT and 4.6 ± 0.04 for PHT, which was

considered a $P< 0.05$ among the groups. Moreover, the titratable acidity had a similar result with PHT (0.73 g lactic acid.mL^{-1}) higher ($P<0,05$) than Non-PHT (0.64 g lactic acid.mL^{-1}), as visualized on Figure 2.

Lactose concentration started with 56.29±1.009 $mg.mL^{-1}$. On the first four hours, PHT kept the lactose content $P<0,05$ lower than Non-PHT. Nevertheless at the end of 300 minutes, the concentration reached were 4.565 ± 0.34 $mg.mL^{-1}$ and 4.398 ± 0.18 $mg.mL^{-1}$ of lactose for Non-PHT and PHT (Figures 3 and 4), respectively. Hence, with simultaneous fermentation process and lactose enzymatic hydrolysis, this compound decreased on both treatments, which there were $P>0,05$ at the end of fermentation.

In contrast, glucose and galactose concentration started with a very low value (0.16± 0.007 $mg.mL^{-1}$ and 0.89±0,044 $mg.mL^{-1}$, respectively) and increased during the first hour. Then, these compounds decreased slowly and slightly on both treatments. These behaviors can be seen in Figures 3 and 4. At the end of process, its glucose and galactose concentration were $P<0,05$. Non-PHT ended the process with glucose and galactose concentration equal 26.010 ± 1.13 $mg.mL^{-1}$ and 21.72 ± 0.75 $mg.mL^{-1}$, respectively. Likewise, on PHT the concentrate were 21.72 ± 0.75 $mg.mL^{-1}$ of glucose and 14.74 ± 0.25 $mg.mL^{-1}$ of galactose.

The lactic acid concentration in the beginning of fermentation was 0.152 ± 0.015 $mg.mL^{-1}$ and after 300 minutes of procedure, ended with a batch of lactic acid 18.644 ± 0.62$mg.mL^{-1}$ on Non-PHT and 17.557 ± 0.53 $mg.mL^{-1}$ on PHT. Since the beginning of procedure until the end, there was a significant difference between the treatments. However, either treatments have had an increase in lactic acid content. Regarding the acetic and citric acids content during fermentation, its amount remained constant around 13.52 ± 2.958 $mg.mL^{-1}$ and 14.24 ± 0.757 $mg.mL^{-1}$, respectively.

4. Discussion

The initial pH of milk decreased, while titratable acidity increased during fermentation, in both treatments (Non-PHT and PHT). These decrease and increased was evidently due the lactic acid production by lactose fermentation, as a microorganism metabolism resulted. One of the lactic acid bacteria feature are their capacity to ferment the mainly carbohydrate in milk and dairy products producing lactic acid as end product. The organic acids produced contribute not only to the flavor and aroma of fermented dairy products but also to their preservation.

Despite the expected, a lower level of lactose and consequently, a higher level of glucose and galactose, which usually happens in traditional yogurts. The addition of the enzyme showed a significantly lower lactose content, however, galactose and glucose values were not very high. The lactose hydrolysis by enzymatic via is one of the strategies more evaluated to achieve yogurts with low lactose content (Mlichova and Rosenberg, 2006). The addition of β-galactosidase changes the carbohydrate pattern, which may affect the sugar consumption by starter culture. Consequently, this fact could interfere the production of derived compounds as well as the physicochemical and sensory characteristics of products (Venica et al., 2013; Wolf, Claudia and Perotti 2015). Furthermore, lactic acid culture cleaved the lactose into its component monosaccharides and organic acids, being the predominant lactic acid as already reported by authors (O'leary and Woychik 1976; Dave and Shah 1997; Ordóñez (2005); Tamime and Robinson 2007; Walstra, Wouters and Geurts 2014)

According to Mora et al. (2002) and De Vin et al. (2005), the most of the lactic acid bacteria are efficiently capable of use glucose portion of lactose and release galactose into the medium. *S. thermophilus* is kind of one, able to metabolize lactose. In

addition, majority of this strains are galactose negative (Gal-). Even though, some strains are galactose positive (Gal+), which are able to metabolize galactose (Anbukkarasi et al., 2014). This fact may explain the galactose behavior after 60 minutes fermentation.

In preheat treatment, the greater hydrolysis of lactose, observed at the beginning of fermentation, as compared to Non-PHT treatment is related to β-galactosidase activity increased. Whilst, β -galactosidases are stable at temperatures between 20 and 37°C, these enzymes are found to have optimum temperatures around 40 and 50°C. In this condition, these retained 90–100% of their activity (Ustok 2010). As reported by Chandan and O'Rell (2013) that 40-45 °C is an optimum growth temperature to yogurt culture, helped PHT pH decrease quickly and ripping. For the Maxilact® manufacturer description, the enzyme used to hydrolyze the lactose is a compound extracted from a milk yeast, therefore the optimum conditions for their activity are similar to the natural milk pH (6.6) and temperature (35-40 °C).

5. Conclusion

Our findings confirmed that both treatments obtained a final lactose low concentration. Although there was P>0,05 between the different initial temperature (25 or 41°C±1°C), at the end of fermentation. Thus, the enzyme contributed to reduce the lactose until low-level providing for consumption by humans who have lactose malabsorption. The simultaneous enzymatic lactose hydrolysis and lactose-cleaving activity by yogurt cultures did not prevent the fermentation process, regardless of the

initial temperature, including proper yogurt fermentation and maintaining the physical and chemical characteristics.

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2. Conflict of interest

Raphael Ferreira de Barros, Camila Sampaio Cutrim, Marion Pereira da Costa, Carlos Conte Adam Junior and Marco Antonio Sloboda Cortez declare that they have no conflict of interest.

3. Statement of Human and Animal rights

This article does not contain any studies with human or animal subjects performed by any of the authors.

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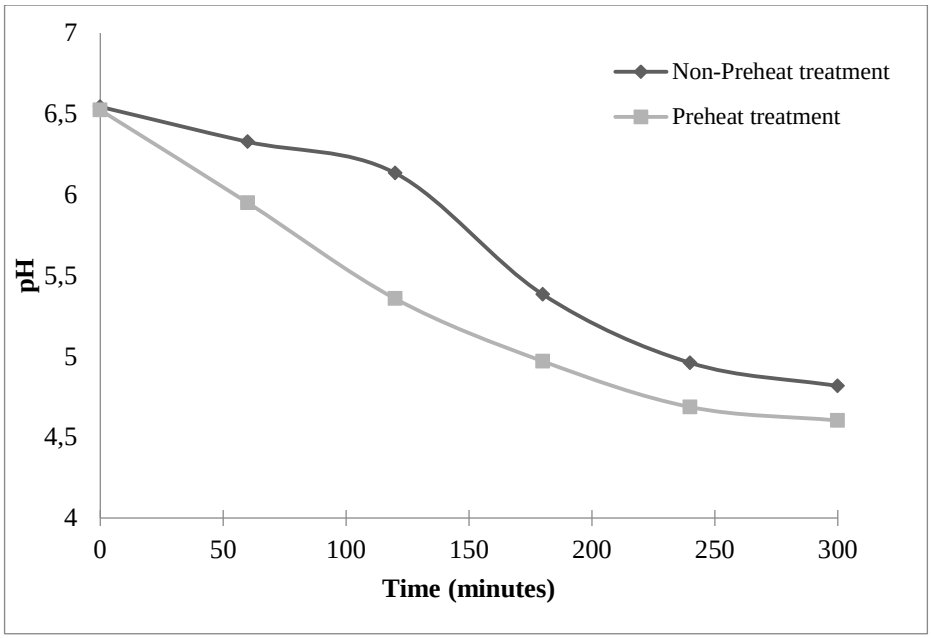

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324 Figure 1: pH determination values during the whole 5 hours (300

325 minutes) of milk fermentation process.

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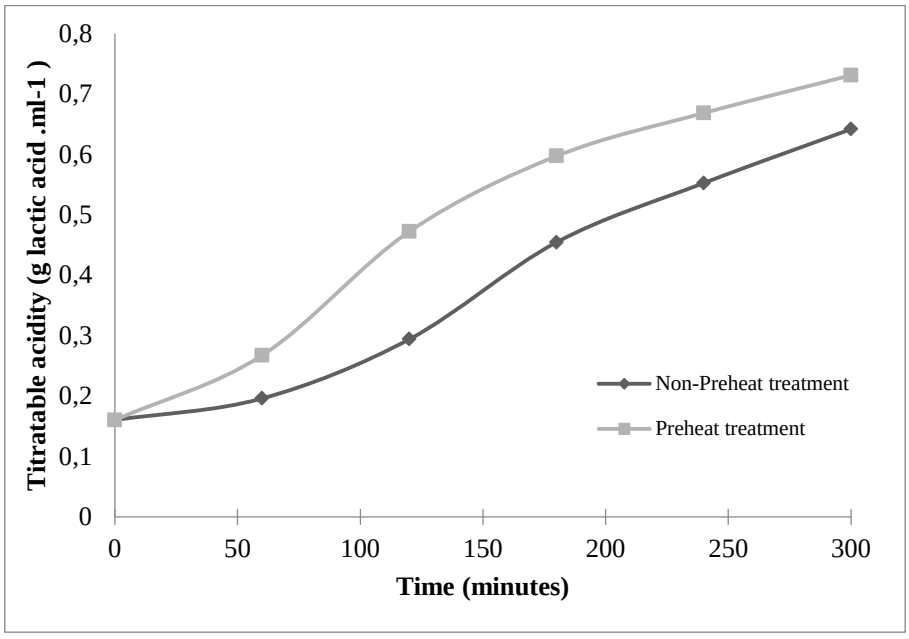

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328 Figure 2: Titratable acidity measured values during the whole 5 hours

329 (300 minutes) of milk fermentation process.

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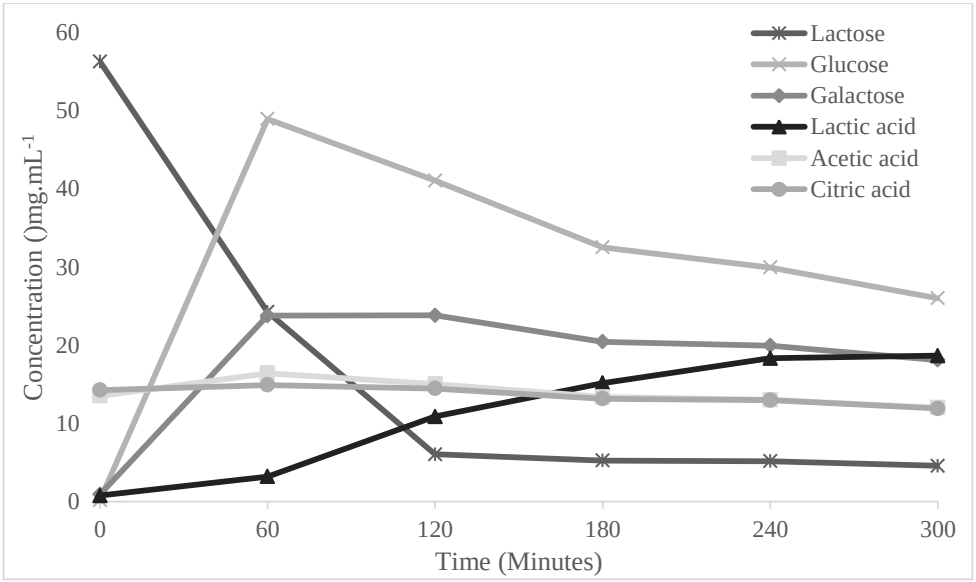

Figure 3: Carbohydrates and organic acids behavior found in non-preheat yogurt during milk fermentation process.

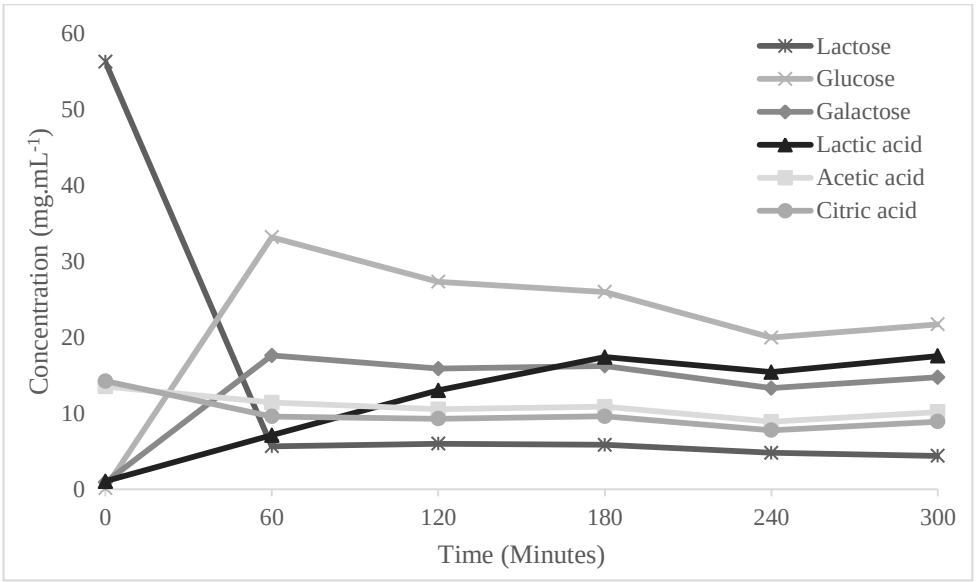

Figure 4: Carbohydrates and organic acids behavior found in preheat treatment yogurt during milk fermentation process.

339 Table 1: Characterization of UHT milk samples regarding pH, titratable acidity,
340 freezing point, carbohydrates and organic acids.

Analysis		Unit	Results
Carbohydrates (HPLC)	Lactose	(mg.mL^{-1})	56.29±1.009
	Glucose	(mg.mL^{-1})	0.16±0.007
	Galactose	(mg.mL^{-1})	0.89±0.044
Organic acids (HPLC)	Lactic acid	(mg.mL^{-1}**)**	0.15±0.158
	Acetic acid	(mg.mL^{-1}**)**	13.52±2.958
	Citric acid	(mg.mL^{-1}**)**	14.24±0.757
pH		-	6.58
Titratable acidity		(g lactic acid .100mL^{-1})	0.16
Freezing point		(° H)	0.5428

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3.2 ARTIGO 2: LACTOSE ENZYMATIC HYDROLYSIS BY B-GALACTOSIDASE: INFLUENCE ON YOGURT CULTURE PROFILE DURING MANUFACTURE AND STORAGE OF LOW-LACTOSE YOGURT

Interpretive Summary

Lactose Enzymatic Hydrolysis by β-Galactosidase: Influence on Yogurt Culture Profile During Manufacture and Storage of Low-Lactose Yogurt. By Barros et al. Milk and dairy products offer to consumers a high nutritional value food. However a considerable amount of the world's population has some intolerance to the main milk and dairy product's sugar (lactose). This study was determinate the effect of lactose hydrolysis (by enzymes) in milk on fermentation cultures during the manufacturing of yogurt and storage. Results showed that lactose reduction did not influence the milk fermentation process, which favors the consumption for the most human, who has lactose malabsorption.

RUNNING HEAD: LACTOSE HYDROLYSIS EFFECT ON YOGURT CULTURES

Lactose Enzymatic Hidrolysis by β-Galactosidase: Influence on Yogurt Culture Profile During Manufacture and Storage of Low-Lactose Yogurt

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ABSTRACT

The aim of this research was to determinate ideal fermentation and hydrolysis by β-galactosidase (EC 3.2.1.23) conditions as well as its influence on the behavior of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricu*s during fermentation and storage of three different treatments: (A) natural yogurt; (B) hydrolyzed-yogurt without enzymatic prior treated; and (C) hydrolyzed-yogurt with one hour of enzymatic prior treated. To assess the yogurt culture behavior, besides the acidity, carbohydrates (lactose, glucose and galactose) and lactic acid were quantified by HPLC. During lactose pre-hydrolysis there were not changes at pH or titratable acidity values measured ($P>0.05$). Fermentation started at pH 6.83 ± 0.00 and achieve a pH values ($P<0.05$) equal 4.46 ± 0,02; 4.63 ± 0.19; and 4.4 ± 0.02 for (A), (B) and (C) respectively, after four hour fermenting at 41 ± 1 °C. In a similar way, the titratable acidity resulted (C) (0.60g $LA.mL^{-1}$) was higher ($P<0.05$) than (B) (0.54 g $LA.mL^{-1}$) or (A) (0.58 g $LA.mL^{-1}$), after the same period and temperature. Cooled yogurts achieved, one day after milk fermentation, pH (A) 4.58 ± 0.01; pH (B) 4.59 ± 0,01; and pH (C) 4.62 ± 0,01. pH values had $P>0.05$ between (A) and (B), however (C) had the higher ($P>0.05$) pH. During refrigerated storage, changes occurred on pH values and acidity. pH determination demonstrated (C) different ($P<0.05$) than (A) and (B), as well as titratable acidity demonstrated (B) different ($P<0.05$) than (A) and (C). Both microorganisms enumerated decreased during storage, however remained above the required minimum count during storage days. In counts were found in $log.cfu.mL^{-1}$ (A) 9.04 *Streptococcus thermophilus* (ST) and 8.84 *Lactobacillus delbruekii* subsp. *bulgaricus* (LB); (B) 9.57 ST and 8.95 LB; and (C) 9.20 ST and 9.09 LB. Carbohydrates and lactic acid behavior

were observed in (C) after one-hour-pre-hydrolysis lactose concentration 18.02 ± 0.18 mg.mL^{-1} (73% less lactose) and one day after fermentation 4.23 ± 0.16 mg.mL^{-1} (93.66 % less lactose). (A) and (B) reached one day after fermentation, the lactose values: 26.76 ± 0.06 (59.87% less lactose) and 9.72 ±0.10 mg.mL^{-1} (85.42 % less lactose), respectively. Hence, lactose reduction did not influence the milk fermentation process, which favors the consumption for the most human, who has lactose malabsorption

Keywords. Fermented milk, β-galactosidase, lactic acid, HPLC.

INTRODUCTION

Nutritional quality of foods is one reason why consumers select healthy diets. Assigning foods into categories based on their nutrient composition will permit consumers to identify and select nutrient-dense foods (Drewnowski and Fulgoni III, 2008). Park et al. (2007) assert that milk is a complete food source of protein (3.2% w/w), carbohydrates – manly lactose (4.7 % w/w) –, fat (3.6 % w/w), minerals (0.6 w/w) and vitamins. The essential nutrients and the high digestibility of nutrients make milk and dairy products being considered highly nutritious food. On the basis of many researches, public and private health agencies are revising dietary recommendations to include dairy products as an important element of a health-promoting diet (Reusser and Miller, 2003).

Yogurt is a very popular cultured dairy product in which milk is fermented using *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* as the main

starter cultures. The yogurt culture uses the lactose as energy source and converts lactose into lactic acid lowering the pH, which allows gel formation (Adhikari et al., 2002; Anbukkarasi et al., 2014; Peng et al., 2009; Schaafsma, 2008).

Yogurt culture behavior during fermentation is briefly described by Tamine and Robinson (2007), as growth association between *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* involved, in which each organism provides compounds which benefit the other.

Initially, *S. thermophilus* ferments the lactose and produce lactic acid, favored by pH near neutrality. It develops growth providing pH lowering besides some aminated substances mainly from whey protein, which supply ideal conditions for *L. delbrueckii* ssp. *bulgaricus*. The *L.. bulgaricus* development, therefore, makes available small peptides and amino acids, such as glycine, histidine and valine, stimulating *S. thermophilus*. Symbiosis continue between both cocci and rods until *S. thermophilus* became inhibited by the acidity of product. *L.. bulgaricus* is more resistant to lower pH values than *S. thermophilus*, and continues its metabolisms, producing compounds including more lactic acid (inhibition occurs below tolerated acidity).

Codex (2011) determines that starter microorganisms shall be viable, active and abundant in the product to the date of minimum durability at a high number ($>10^7$ cfu. g^{-1}).

In spite of *S. thermophilus* and *L. bulgaricus* consume lactose to its metabolism; it remains at high levels, around 70% of milk lactose content (Harju et al., 2012; Schaafsma, 2008). Lactose is a disaccharide and the main carbohydrate in milk and most derivatives products. This carbohydrate is formed by one molecule of glucose and another one of galactose bonded by β1-4 glycosidic joint. The lactose content in cow's

461 milk is around 4.8%, which means 50 to 52% of non fat solids (Park et al., 2007;
462 Tamine and Robinson, 2007). However researchers have shown that more than 65% of
463 the world's population has a certain level of lactose malabsorption due to poor digestion
464 of the carbohydrate (Mattar et al., 2012; Vuorisalo et al. 2012) causing a gastrointestinal
465 symptoms (lactose intolerance) (Misselwitz et al., 2013).

466 Lactose malabsorption has two different sources: primary deficiency in which is
467 including physiological lactase reduction and autosomal recessive familial lactase
468 deficiency; and secondary deficiency in which is featured inflammatory intestinal
469 diseases (Raithel et al., 2013). Lactose maldigestion occurs when the disaccharides is
470 not absorbed in the small intestine due decline in enzyme activity, or even failure or
471 lack in the production of the enzyme lactase (β-galactosidase). It passes through the
472 gastrointestinal tract to the colon, where it then leads to symptoms of lactose
473 malabsorption (Matthews, et al., 2005).

474 The typical symptoms include abdominal pain, gut distension, borborygmi,
475 flatulence, diarrhea, nausea, vomiting, headache and light headedness, loss of
476 concentration and poor short term memory, long term severe tiredness and muscle pain
477 (ibid).

478 To avoid such malaise, signs and symptoms, once the lactose residual in yogurt
479 is not sufficiently hydrolyzed in such cases of lactose malabsorption levels (Schaafsma,
480 2008), one of the solutions to address who has some level of lactose intolerance is to
481 elaborate a dairy product with lactose reduction or even lactose free, as obtained with
482 fermented milk added of β-galactosidase enzyme. Richmond et al. (1981) reported a
483 review of research related to technological application, nutritional concerns and
484 immobilization β-galactosidase enzyme. However, currently, the use of enzyme in the

conduct of low-lactose dairy products is still usual practice (Harju et al., 2012; Wolf et al, 2015).

The aim of this research, in order to produce a lactose free or low lactose yogurt, was to determinate ideal fermentation and hydrolysis by β-galactosidase (EC 3.2.1.23) conditions as well as its influence on the behavior of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* during fermentation and storage of different treatments. To assess the yogurt culture behavior, besides the acidity, carbohydrates (lactose, glucose and galactose) and lactic acid were quantified by high performance liquid chromatography.

MATERIALS AND METHODS

Experimental Design

For this experiment, three different groups (or treatment) were elaborated. The first one was the control treatment (A) – TTA – which was the traditional yogurt. The second treatment (B) – TTB – was a hydrolyzed-yogurt with simultaneous addition of lactic acid (LA) cultures and β-galactosidase (β-GAL) – EC 3.2.1.23 – enzyme. Finally, the third and last treatment (C) – TTC – was a hydrolyzed-yogurt with one hour of milk pre-hydrolysis (prior treated).

During the pre-hydrolysis, a sample was taken at the beginning and at the end, from TTA and TTC to determinate pH, titratable acidity, carbohydrates and organic acids (by HPLC) behavior. Likewise, during fermentation procedure, a sample was taken from TTA, TTB and TTC every hour, including the start time, to determinate pH, titratable acidity carbohydrates and organic acid behavior.

In addition, estimation of lactose (LAC) hydrolysis from TTA (control) and TTC during one hour of milk LAC pre-hydrolyze by freezing point measurements were carried out.

Both, pre-hydrolysis, and after, fermentation procedure were performed at an oven at 41 ± 1 °C and treatments were interrupted when the pH reached 4.6. Then, the yogurts were cooled immediately in a refrigerator at 4°C and stored for 28 days.

During storage, physicochemical (pH and titratable acidity) and microbiological (counts of *Streptococcus thermophilus* [ST] and *Lactobacillus delbruekii* subsp. *bulgaricus* [LB]) analysis were performed at days 1, 7, 14, 21 and 28 of its storage period.

Milk and Milk Analysis

In order to elaborate the different treatments of fermented milk (including its variations), which were evaluated in this work, was used the cow's whole milk thermally treated by Ultra High Temperature – UHT – (Ninho®, Nestle, São Paulo, Brazil). It was acquired in the retail market, single lot, transported and stored at room temperature (25°C), stemmed from dairy company with Brazilian Federal Inspection Service.

To certificate the physicochemical quality was analyzed in triplicate a sample of whole milk to determine fat content (Official Method 2000.18), specific gravity at 15.0°C by termohigrometer method (Official Method 925.22), freezing point by cryoscopic method (Official Method 980.15), titratable acidity (Official Method 947.05) and pH (Official Method 973.41) (AOAC 2012). The pH was determined by inserting a

533 pH probe into milk (pH meter PG1800, CapLab, São Paulo, Brazil). Furthermore were
534 calculated an approximate total solids in milk with Ackerman disk (based on Richmond
535 formulae) and solids not fat in milk, subtracting the total fat (% w/w) from total solids
536 (% w/w). Also was researched fraud in milk including presence of chlorides and starch,
537 acidity neutralizing and conservatives (chlorine and hypochlorite) agents.
538 In the same way, to certificate the microbiological quality, UHT whole milk
539 used to product elaboration was analyzed in terms of aerobic plate count (Morton,
540 2001), total and faecal coliforms (Kornacki and Johnson, 2001), molds and yeasts
541 (Tournas et al., 2001), as well as lactic acid bacteria (LAB) (Richter and Vedamuthu,
542 2001).
543
544 ***Yogurt and Low-Lactose Yogurt Processing***
545
546 Yogurts were made applying the traditional method (Tamine and Robinson
547 2007) adapted to laboratory scale and prepared with preheated UHT whole milk at at an
548 oven at 41 ± 1 °C. The thermophilic LA cultures used to produce the yogurt were the
549 symbiotic culture of ST and LB (DVS YF-L812, Christian Hansen Laboratories,
550 Denmark).
551 In order to attempt the correct ratio between the strains and avoid contamination, a
552 reconstituted skim milk (10%; w/v) (Molico, Nestlé, São Paulo, Brazil) was made with
553 500 mL of sterile deionized water in controlled aseptic conditions. Thereafter, 50U
554 yogurt culture envelope was added into reconstituted milk, stirred until complete
555 lyophilized culture dissolution and was freeze after dilute in sterile glass tubes at -18°C

(London, 2015). Mother culture was prior enumerated to certificate the log.ml^{-1} it content.

To perform the fermentation, the culture (mother culture) was thawed overnight at 4°C, pre-activated in a small sample of milk at 41 ± 1 °C during two hours ; and then added to milk (0.1%; v/v) in a ratio (pre determinate) of 8 log.cfu.mL^{-1} (Bylund, 1995)

Three separated groups were formed: TTA: Traditional yogurt, with mixed starter culture (ST an LB) addition; TTB: hydrolyzed-yogurt with simultaneous addition of β-GAL and starter culture (without prior treated); and TTC: one hour milk pre-hydrolysis (prior treated) by β-GAL enzyme following starter culture addition.

The β-galactosidase enzyme used in this research to hydrolyze the LAC was Maxilact LX 5000 (DSM Food Specialties, Delft, Netherlands) which is a refined lactase formulation derived from dairy yeast *Kluyveromyces lactis*; and the dosage used was 0.45 mL of enzyme per liter of milk, as suggested by the supplier, which was added into milk and stirring at the start of fermentation in TTB and one hour before fermentation in TTC. Therefore, the lactose hydrolysis were performed at an oven at 41 ± 1 °C during fermentation time in TTB and one hour prior treated plus fermentation time in TTC.

To estimate (percentage) LAC pre-hydrolyze by freezing point, an equation described by Prozyn (2007) in which was taking in account the average depression of freezing point for each 1% lactose hydrolyzed, the initial (FPi) and the final (FPf) milk freezing point achieved results, was used (equation 1).

$$Lactose\ Hydrolysis = \left[350.877 \times (FPf) + \frac{(FPi)}{0.00285}\right] \qquad \text{(equation 1)}$$

Yogurt Analysis

Microbiological Analysis. The microbial enumeration was performed with dilution of 25g of each treatment sample into 225 mL of 0.1% peptone saline water in plastic bags and homogenization in Stomacher blender for 1 min. One milliliter of each initial dilution was transferred into tubes with 9 mL of 0.1% peptone saline water and serial dilutions were made (Swanson et al, 2001).

On day one, to guarantee the product quality and no interference from spoilage or pathogenic microorganisms, total and faecal coliforms; and molds and yeasts were enumerated as described by Kornacki and Johnson (2001) and Tournas et al. (2001) respectively.

Furthermore, on days 1, 7, 14, 21 and 28 to perform the count of ST was used a complete medium M17 Agar (Difco Laboratories, Michigan, USA) added of lactose, pour- plate method, followed by aerobic incubation at 37°C ± 1°C for 48 hours in an incubator. Hence, to perform the count of LB was used an acidified medium (pH = 5.4) Man-Rogosa-Sharpe (MRS) agar (Difco Laboratories, Michigan, USA), pour- plate method, followed by anaerobic incubation at 37°C ± 1°C for 72 hours in an incubator. To anaerobic condition was used the GasPak jar (Becton, Dickinson and Company, New Jersey, USA). Both LA bacteria from yogurt starter cultures were performed enumeration according to International Organization for Standardization and International Dairy Federation (ISO 7889 | IDF 117, 2003).

After specified period of incubation, count the colonies showed the features of each microorganism on plates which had between 15 and 300 colonies. Plates were else examined under subdue light to examine doubtful objects (ISO 7889 | IDF 117, 2003).

In addition, some colonies were stained using the Gram method to confirm that they are non-spore forming, Gram-positive, cocci or diplococci (in case of those grown on the M17 medium) or rods (in case of those grown on the acidified MRS medium) (Murano and Hudnall, 2001)

Physicochemical Analysis. Determination the pH (Official Method 973.41) and titratable acidity (Official Method 947.05) (AOAC, 2012) were performed, on the same storage days as reported on Microbiological Analysis (1, 7, 14, 21 and 28), besides carbohydrates and organics acids behavior, as described in HPLC analysis, which were performed one day after fermentation.

HPLC Analysis

The organic acid and carbohydrates extraction was carried out using a modification of the method described by González de Llano et al. (1996) and performed in triplicate. 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 (TS – 2000 A VDRL shaker, Biomixer®) and then centrifuged at 5500*g* for 30 minutes at 4°C. Finally, the supernatant was filtered through Whatman number 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 organic acids 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). Under these selected chromatographic conditions, we obtained the chromatogram of the standard mixture of all carbohydrates (LAC, GLU and GAL) and LA investigated in this study. The interest peaks were identified by comparing retention times of the standards with the samples and the concentration of each component of the samples was determined from the area of individual peaks. Carbohydrates identification was performed by refractive index detector while organic acid by using a diode array detector model monitoring the absorbance at 210 nm.

Statistical Analysis

One-way ANOVA with repeated measures was used to identify differences between pH values, titratable values, organic acids and carbohydrates content over the yogurt‘s fermentation and storage periods, as well as Student’s t test. Statistical significance was set at a 0.05 level of confidence. All analyses were performed using commercially available statistical package XLSTAT 2014.6.04 Excel add-in software (Microsoft, Redmond, USA)

RESULTS AND DISCUSSION

Milk Quality

In order to guarantee a reliable research results, UHT samples milk were analyzed to prove its quality and to certificate that all results came from own experiment. Microbiological analyses were performed and the results were absence of aerobic plate count; total and faecal coliforms; molds and yeast; and finally, absence of LAB. Still, physicochemical analyses resulted were expressed as mean, which were shown in Table 1. The results showed that acquired UHT milk was suitable for use and it was in accordance with "Reglamento Tecnico Mercosur de Identidad y Calidad de la Leche UHT" (Mercosur, 1995), as well as Brazilian legislation (Brasil, 1997) on Technical Regulation of Identity and Quality of UHT Milk from the Ministry of Agriculture, Livestock and Supply ("MAPA"), which establishes the minimum standards of product quality.

Pre-Hydrolysis

During LAC pre-hydrolysis (in TTC) there were not changes (P>0,05) at pH or titratable acidity values measured from TTA – treatment control unhydrolyzed – TTB and TTC (data not shown). Treatments were kept in the same temperature conditions (41 ± 1°C) and the results were stable, which indicate there was not β-GAL influence in pH or titratable acidity results.

Figure 1 represent the estimation of LAC pre-hydrolyze (percentage), obtained by freezing point and freezing point equation, during one hour which preceded the fermentation process. As observed, TTA and TTB were stable, as expected once these

676 treatments had no enzyme so far, with any lactose hydrolysis indeed. In opposition,
677 TTC (with pre-hydrolyze) achieved after 60 minutes 64.91 % of lactose-hydrolyzed
678 (Figure 2). Cunha et al. (2007) used the same equations to estimate their lactose-
679 hydrolyzed of active packaging incorporated with different enzyme content and storage
680 temperatures. Klein et al. (2010) also used the same estimative method to assess their
681 lactose concentration, before produce sweet milk, to avoid crystallization.

682 The pre-hydrolyze conditions were close to those recommended by Maxilact®
683 bulletin, in which reported the enzyme optimal conditions close to the natural milk pH
684 (6.5-6.8) and temperature around 35 -40°C.

685

686 ***pH and Titratable Acidity During Fermentation***

687

688 Regarding the results from pH determination during fermentation stage, data was
689 shown in Figure 3. It started at pH 6.83 ± 0.00 and decreased until pH values achieved,
690 on the end of stage, 4.46 ± 0,02 for TTA; 4.63 ± 0.19 for treatment B (TTB); and 4.4 ±
691 0.02 for TTC, so that were considered $P<0,05$.

692 Yogurt's pH values decreased during fermentation time due mainly LA
693 production as reported by some researchers (Anbukkarasi et al., 2014; Peng et al., 2009;
694 Schaafsma, 2008; Vénica, 2014). Its fermentation procedure is conducted by LAB,
695 specifically by ST and LB, which have capacity to ferment LAC and glucose (GLU)
696 producing LA. In all treatments, there were a sharp fall in pH values between the second
697 and the third hour, wherein the TTC`s pH dropped faster, as well as ended with $P<0.05$
698 value than other treatments, as found by Ismail et al. (1983). These results suggested a
699 greater drop in pH due partial cleaved LAC by exogenous β-galactosidase to its

constituent's monosaccharides GLU and galactose (GAL) and faster acid development on hydrolyzed-yogurt manufacture.

In the same way, TTA unhydrolyzed, and TTB, without LAC pre-hydrolyze, therefore higher amounts of LAC than TTC, did not achieved pH's value found in TTC. Similar to our study, O'leary and Woychik (1976a, 1976b) compared treatments with or without hydrolyzed-LAC and concluded that LAC treated by β-GAL made the process more efficient with significant pH drop. In accordance, Martins et al. (2012) used on their researches single-stage process with the simultaneous addition of β -GAL and lactic culture to evaluate the effects of initial LAC concentration, enzyme concentration and the time of addition of the enzyme comparing with a control and concluded it took less time to produce a yogurt with low LAC content. In the same way, Nagaraj et al. (2009) compared the unhydrolyzed and hydrolyzed-yogurt process and asserted that LAC hydrolysis reduced the yogurt setting time. However, in opposition, Vénica et al. (2014) reported that hydrolyzed yogurts showed lower levels of LA in comparison with yogurts, which made the pH correspondingly had stayed higher on the hydrolyzed one, suggesting an inhibition of starter culture activity caused by high concentration of GLU and GAL in the medium generate by the activity of exogenous β-GAL. Still, at odds with others researchers and our results, Wolf et al. (2015) did not found differences in pH values between yogurts prepared from milk with different LAC contents and control unhydrolyzed yogurts.

In a similar way, the titratable acidity had a similar result with TTC (0.60g $LA.100mL^{-1}$) higher ($P<0,05$) than TTB (0.54 g $LA.100mL^{-1}$) or TTA (0.58 g $LA.100mL^{-1}$), as visualized on Figure 4.

pH and Titratable Acidity During Refrigerate Storage

Once fermented, yogurts were cooled and achieved one day after fermentation, the following results from pH determination: 4.58 ± 0.01 to TTA; 4.59 ± 0,01 to TTB; and 4.62 ± 0,01 to TTC. The beginning of storage (day one) pH values had no significant difference between TTA and TTB, however TTOC was different ($P<0,05$). TTC had the higher pH value probably resulted of inhibition of the starter culture organisms due to the rapid production of large amounts of acid developed during fermentation process. O'Leary and Woychik (1976a) visualized the same behavior in their experiment in which pH values decreased about 0.2 pH units.

Take into account the titratable acidity analysis, TTA (0.74 g LA.100 mL^{-1}) TTB (0.74 g LA.100 mL^{-1}) and TTC (0.74g LA.100 mL^{-1}) on day one, after cooling, resulted acidity values significantly the same. The difference observed in the values of pH and acidity between the end of the production itself and the cold product ready for consumption (day one) is due LAB action while the temperature was favorable, until medium got cold and decrease LAB's metabolism.

During 28 days of yogurt refrigerated storage, changes occurred on pH values and acidity behavior (Figures 4 and 5, respectively). pH values decreased on the first seven days, stayed floating during 14 days (until day 21 of storage) and then continued decreasing until day 28. In the same way, however opposition behavior, titratable acidity increased on the first seven days, stayed floating during 14 days (until day 21 of storage), due yoghurt bacteria re-routing their metabolism towards the production of neutral compounds, and finally continued increasing until day 28. Results from pH determination demonstrated TTC different ($P<0,05$) than TTA and TTB and results

748 from titratable acidity demonstrated TTB different (P<0,05) than others. The results
749 achieved on the end of storage days (day 28), by each treatment, regarding pH and
750 titratable acidity were, respectively: 4.20 ± 0.01 and 0.84 g LA.100 mL^{-1} for TTA; 4.26
751 ± 0,01 and 0.79 g LA.100 mL^{-1} for TTB; and, 4.13 ± 0.02 and 0.79 g LA.100 mL^{-1} for
752 TTC. Even the yogurt being subjected to low temperature (6 ± 2°C), ST and LB
753 metabolisms reduced but did not completely stop, contributing indeed to post-
754 acidification. Beal et al. (2000), Ibarra et al. (2012), Kim et al. (2009), O'Leary and
755 Woychik (1976a), Vénica et al. (2013) also found similar results regarding the decline
756 in pH of yogurts during cold storage in which is reported the ability of the starter culture
757 organisms to carry out metabolic processes at cold storage temperatures. Our results
758 indicated lowering of the pH 0.28 units, 0.33 units and 0.49 units for TTA, TTB and
759 TTC, respectively. In the same way, the decrease in pH during storage ranged from 0.25
760 to 0.39 units was demonstrated by Vénica et al. (2013), equally as about 0.3
761 demonstrated by O'Leary and Woychik (1976a). Still, Beal et al. (2000) reported that
762 storage more pronounced lowering pH was in that which stopped fermentation with
763 higher pH values (4.8) than for those stopped lower (4.4). They assert, either, that
764 metabolic activity linked to post-acidification was pH dependent.
765
766 ***Lactic Acid Bacteria Behavior During Refrigerate Storage***
767
768 During refrigerated storage period, ST and LB enumerated from samples belongs to
769 TTA, TTB and TTC (during 28 days) were suitable, according to Codex (2011) in
770 which the sum of microorganisms constituting the defined starter culture (ST and LB)

771 must had a minimum of 10^7 cfu.g^{-1}. ST and LB enumeration from treatments sample
772 seeded on M17 agar and acidified MRS agar, respectively, were present in Table 2.
773 As seen in Figures 7 and 8, which demonstrate the ST and LB behavior, in
774 general both microorganisms count decreased from the first day until day 28. TTA,
775 unhydrolyzed yogurt, started and ended with lowest cfu count. ST cells declined
776 between day 1 and 7 of storage, when the yogurt acidity increased. Thereafter fluctuated
777 as the acidity (declining slowly) and finally, after day 21, continued to fall following the
778 acidity which further increased. LB cells remained stable with an acceptable variation,
779 declining slowly until day 28. TTB, hydrolyzed yogurt, Started with the largest count of
780 ST and LB cfu and ended with a count higher than TTA and lower than TTC. Initially it
781 had the same behavior than TTA. ST and LB cells declined between day 1 and 7 (during
782 acidity increasing), but thereafter, both remained floating, declining slowly until day 28.
783 TTC, pre-hydrolyzed yogurt, behaved differently, in which ST cells decreased since day
784 one, until day 28, and LB cell growth initially (between day 1 and 7), when the yogurt
785 acidity was increasing, and then declined slowly until day 28.
786 The initial cells decline during the first days (day 1 – 7), observed in TTA, TTB and
787 slightly for ST cells in TTC, was also reported by Kim et al. (2009), who demonstrated
788 that the cell number of lactic acid bacteria started to decrease at pH 4.7. Corroborating
789 with them and clarifying our results, Birollo et al (2000) founded viable counts of ST in
790 yogurt during storage changed from 8.33 log cfu.g^{-1} on day1 to 6.33 log cfu.g^{-1} on day
791 15 (values are lower than found in this experiment); and reported that counts of ST
792 slightly decrease during storage probably due to the gradual decrease in pH. Dave and
793 Shah (1996) and Serra et al. (2009) reported the decrease in LB count during the storage
794 period is due lower storage temperature and over acidification during storage of yogurt.

In TTC, the initial increase in LB count might be due by simultaneous higher acidity resistance from LB, higher pH than other treatments and the high concentration of glucose.

Carbohydrates and Lactic Acid Behavior

Starting from UHT whole milk previously analyzed, in which nothing has been consumed or formed by unknown accountable. It milk prossessed 66.68 ± 1.67 $mg.mL^{-1}$ of LAC, corroborating with approximate lactose content reported by Park et al. (2007), in addition, 0.06 ± 0.00 $mg.mL^{-1}$ of GLU and 0.12 ± 0.00 $mg.mL^{-1}$ of GAL also comprised the milk. LA content quantified by HPLC was 0.21 ± 0.03 $mg.mL^{-1}$, approaching the values determined by titratable acidity (0,15 $mg.mL^{-1}$) in accordance with "Reglamento Tecnico Mercosur de Identidad y Calidad de la Leche UHT" (Mercosur, 1995), as also the Brazilian legislation (Brasil, 1997).

Initially, the TTC was submitted to a one-hour-pre-hydrolysis preceding fermentation under stable temperature condition (41 ± 1°C). Ustok (2010) reported that β-galactosidase are stable in range of 20 to 37°C, although found optimum temperature in range of 40 to 50°C. A control group was also taken in account to compare both treatments. LAC content decreased significantly in TTC (Figure 9), during the one-hour-pre-hydrolysis, and achieved a concentration equal 18.02 ± 0.18 $mg.mL^{-1}$ (approximately 73% w/w of hydrolyzed-lactose) in which corresponded to a greater hydrolysis than estimated (64.91 %) by freezing point method (Prozyn, 2007). As already reported on this study, this optimum temperature and invariable milk pH ($P>0.05$) during pre-hydrolysis, favored the enzymatic activity of β-GAL. The same

behavior was not observed in control group – without β-GAL – in which LAC concentration kept unchanged ($P > 0.05$) whilst hydrolysis took place in TTC (data not shown). The reason to conducted the pre-hydrolysis, besides achieve a yogurts with low lactose content (Mlichova and 202 Rosenberg, 2006), it is decrease the fermentation time due change the carbohydrate pattern, increase in monosaccharides media, as reported by Nagaraj et al. (2009), O'Leary and Woychik (1976a).which may affect the sugar consumption by starter culture.

Data from LAC hydrolysis and GLU, GAL and LA behavior are described in Table 3. With fermentation procedure, significantly lactose amount decline in all treatments. The fermented milk took four hour to reach pH around 4.6 and it was enough to a significant difference among the treatments to be observed at the end of process. Chadan (2006) reported the normal fermentation period to achieved final pH 4.6 in natural yogurt is approximately 4 to 6 hours at 45 °C, however it was used on this experiment a little lower temperature and the same pH range was reached.

As expected, TTA had higher ($P<0.05$) final values of LAC content (24% w/w of hydrolyzed-lactose) than both hydrolyzed-yogurt. LAB cleaved the lactose into its component monosaccharides producing LA from GLU and GAL metabolism, lowering the pH. However, pH around 4.5, yoghurt bacteria decrease lactose utilization by re-routing their metabolism towards the production of more neutral compounds (O'leary and Woychik, 1976, Dave and Shah, 1997, Walstra, Wouters and Geurts, 2014) LAB and its own enzymes were the responsible for cleavage of LAC in unhydrolyzed-yogurt. On the other hand, there was no significant difference between TTB and TTC in which they reached 84.24 and 84.37 % (w/w) of hydrolyzed-lactose (as shown on table 3),

842 respectively. On these cases, it is important to know that LAB and exogenous β-
843 galactosidase enzyme acted together to achieved its hydrolysis degree.

844 Regarding GLU, different behaviors were observed in each experiment's
845 treatment. Its GLU (as well as GAL) was formed out lactose cleavage by LAB's
846 enzyme in TTA or simultaneously with exogenous enzyme in TTB and TTC'. However
847 may be also consumed together with LAC by LAB's metabolism as energy source.
848 Mora et al. (2002) and De Vin et al. (2005) reported that most of the LA bacteria use
849 glucose portion of lactose to its metabolism and release galactose into the medium.

850 In TTA, GLU started from 0.09 ±0.09 $mg.mL^{-1}$ and increased slowly until first
851 hour completed (P<0,05), in fermentation procedure, and ended after four hours since it
852 started with a significantly lower value among treatments. In the same way, TTB started
853 from very low GLU content but had a high significant production in the first hour due
854 simultaneous fermentation and hydrolysis. Thereafter it remained stable with an
855 acceptable variation until reach at the end an intermediate value (P<0.05) among
856 treatments. Finally, after pre-hydrolysis process, TTC already started with high amount
857 of GLU in medium, increased even more in the first hour, and then, probably due low
858 LAC concentration and poor GLU production, its values decrease achieved still
859 attaining significantly higher GLU content at the end of stage.

860 Although was expected to accumulate all GAL in medium, it was not observed
861 in this work. In accordance Anbukkarasi et al. (2014) reported the ST, which is able to
862 metabolize LAC. In addition, majority of this strains are galactose negative (GAL-).
863 Even though, some strains are galactose positive (GAL+), which are able to metabolize
864 GAL. In our study, GAL has had a similar behavior than GLU in which TTA increased
865 slowly with significant changes and stopped fermentation with lower values among

866 treatments; and TTB and TTC (saved the necessary differences among the initial values
867 due pre-hydrolyze in TTC) increased the GAL content followed by a decline likely due
868 to consumption by BAL. Nevertheless significant differences were observed in those
869 treatments and among those treatments.

870 LA started from very low concentration ($P > 0.05$) to sharp increase in all groups
871 achieving a range of 7.46 to 8.17 between the lower value (TTB) and higher value
872 (TTC) which there was not any significant difference after four hour fermenting. The
873 LA increasing came from the catabolism of LAC by ST and LB resulting mainly in the
874 production of lactic acid (Tamime and Robinson, 2007). In accordance with our results,
875 Vénica, Perotti and Bergamini (2014) reported the same sharp increase in LA content in
876 natural and hydrolyzed yogurts. This increasing order of values (TTB, TTA and TTC)
877 corroborated with the obtained results from titratable acidity analyses, however
878 titratable acidity method underestimated the results in 20 $mg.mL^{-1}$ for all treatments.
879 Measurement of pH and therefore in titrable acidity confirmed that the increase in lactic
880 acid content had effect on the pH of the yoghurts, also reported by Serra (2009).

881 The sharp increase of LA might be due LAB metabolism, nevertheless, these
882 microorganisms consumed GLU and GAL from LAC cleaved by exogenous β-GAL and
883 to a lesser extent, bacterial hydrolysis, as demonstrated by TTA against TTB and TTC
884 with β-GAL.

885 The storage first day, after yogurts have got cold (4°C) and the products
886 becomes ready to be consumed all the parameters were analyzed again and the results
887 indicated in table 3.

888 During the cooling period, LAC decrease even more in TTC ($P < 0.5$), achieving
889 4.23 ± 0.16 $mg.mL^{-1}$, this means 93.66 % (w/w) of lactose hydrolyzed. Hence, this

result is lower than those recommended by Brazilian legislation (Brasil, 1998) to consider a free lactose product (maximum of 0.5 g.LA.100 mL^{-1}). Nevertheless, GLU and GAL content became significantly higher than last hour of fermentation procedure ($P < 0.05$) and as consequence LA increased during this period.

In TTB, one day after fermentation, a significantly difference were observed in LAC decreasing and LA increasing, wherein GLU and GAL remained the same ($P>0.05$). In this case, LAC reached 9.72 ±0.10 $mg.mL^{-1}$, this means 85.42 % (w/w) of lactose hydrolyzed.

There were changes ($P<0,05$) at LAC, GLU and GAL in TTA during cooling period. While LAC decreased, GLU and GAL increased. The changes in this treatment were exclusively done by LAB, once did not have any exogenous enzyme added. LA achieved in control yorgurt (traditional yogurt) values equal 26.76 ± 0.06 (59.87% w/w of lactose hydrolyzed).

Vénica et al. (2013) reported hydrolysis percentages in their experimental yogurts ranged from 74 to 93% (w/w) in hydrolyzed yogurts against 15% (w/w) in traditional yogurt. Those result were lower than ours, however Rodriguez, et al. (2008) also found percentages (about 82.6%) in hydrolyzed yogurts close to ours results.

CONCLUSIONS

Results showed that fermentation occurred satisfactorily and microorganisms remain viable during storage. In addition it also showed sufficient lactose reduction without fermentation influence, which favors the consumption for the most human, which has lactose malabsorption.

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Table 1: Characterization of UHT milk samples regarding pH, titratable acidity, freezing point, fat content and specific gravity. In addition fraud researches

Analysis	Unit	Results**
pH	-	6.83
Titratable acidity	(g lactic acid .100mL^{-1})	0.15
Freezing point	(^{o} H)	-0.5458
Fat	(% ; w/w)	3.6
Specific gravity	-	1.030
Fraud*	-	negative

*Qualitative analysis: chlorides and starch, acidity neutralizing and conservatives (chlorine and hypochlorite) researches
**average values of triplicate analysis

Table 2: *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* enumeration from treatments sample seeded on M17 agar and acidified MRS agar, respectively, during 28 refrigerated days

	Streptococcus thermophilus **(log.CFU.mL^{-1})***			***Lactobacillus delbrueckii ssp. bulgaricus*** **(log.CFU.mL^{-1})***		
	TTA[1]	TTB[2]	TTC[3]	TTA[1]	TTB[2]	TTC[3]
Day 1st	10.02	10.42	10.22	9.32	10.14	9.53
Day 7th	9.74	9.69	10.14	9.30	9.25	10.20
Day 14th	9.79	9.68	9.64	9.18	9.40	9.78
Day 21st	9.84	9.50	9.50	9.15	9.25	9.53
Day 28th	9.04	9.57	9.20	8.84	8.95	9.09

*The results were expressed as values of logarithms of colony-forming units per milliliter (log CFU mL^{-1})

[1]Treatment A; [2]Treatment B; [3]Treatment C.

Table 3: Carbohydrates (lactose, glucose and galactose) and lactic acid quantified by HPLC analysis during fermentation and storage of treatments (A) natural yogurt; (B) hydrolyzed-yogurt; and (C) hydrolyzed-yogurt with one hour of milk pre-hydrolysis before fermentation

Chemical compounds (mg. ml^{-1})	Groups	Fermentation Time (h)			Storage
		0	1	4	24h
Lactose	A	65.26 ^{Aa}±0.33	65.27 ^{Aa}±0.51	50.68 ^{Ab}±1.59	26.76 ^{Ac}±1.06
	B	66.28 ^{Aa}±0.16	15.41 ^{Bb}±0.37	10.51 ^{Bb}±0.12	9.72 ^{Bc}±0.10
	C	18.02 ^{Ba}±0.18	16.66 ^{Ba}±2.02	10.42 ^{Bb}±0.56	4.23 ^{Cc}±0.16
Glucose	A	0.09 ^{Bd}±0.09	0.57 ^{Cc}±0.01	8.33 ^{Ca}±0.29	3.15 ^{Cb}±0.10
	B	0.06 ^{Bc}±0.20	21.95 ^{Ba}±0.47	19.55 ^{Bb}±0.33	19.72 ^{Ab}±0.64
	C	21.54 ^{Ab}±0.20	29.00 ^{Aa}±2.31	21.47 ^{Ab}±1.15	11.83 ^{Bc}±0.41
Galactose	A	2.69 ^{Bd}±0.18	3.17 ^{Cc}±0.10	6.08 ^{Ca}±0.24	4.77 ^{Cb}±0.12
	B	0.12 ^{Cc}±0.31	28.48 ^{Bc}±1.53	31.81 ^{Aa}±0.52	30.92 ^{Aa}±1.81
	C	31.75 ^{Ab}±0.29	39.19 ^{Aa}±2.12	25.96 ^{Bc}±3.83	28.92 ^{Bbc}±0.52
Lactic Acid	A	0.21 ^{Aa}±0.01	3.58 ^{Ab}±0.01	8.02 ^{Ac}±0.18	20.58 ^{Ad}±0.31
	B	0.21 ^{Aa}±0.01	3.55 ^{Ab}±0.04	7.46 ^{Bc}±0.07	19.50 ^{Bd}±0.17
	C	0.20 ^{Aa}±0.01	3.91 ^{Ab}±0.57	8.17 ^{Ac}±0.27	19.21 ^{Bd}±0.28

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

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

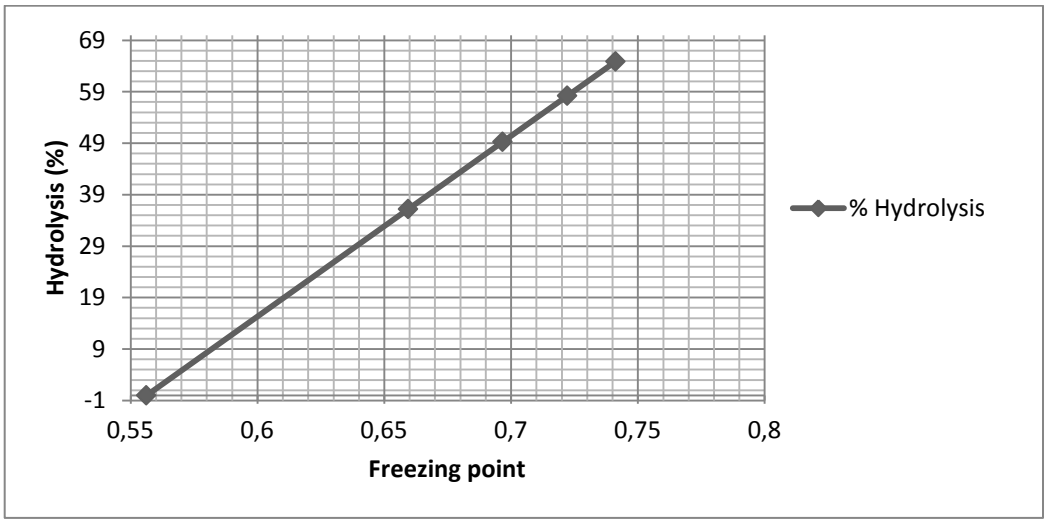

Figure 1: Estimation of milk-lactose pre-hydrolyze (percentage), obtained by freezing point* and freezing point equation**, during one hour

*The results are the mean of triplicate analysis

** $\% = \left[350.877 \times (final\ freezing\ point\ value) + \frac{(initial\ freezing\ point\ value)}{0.00285}\right]$

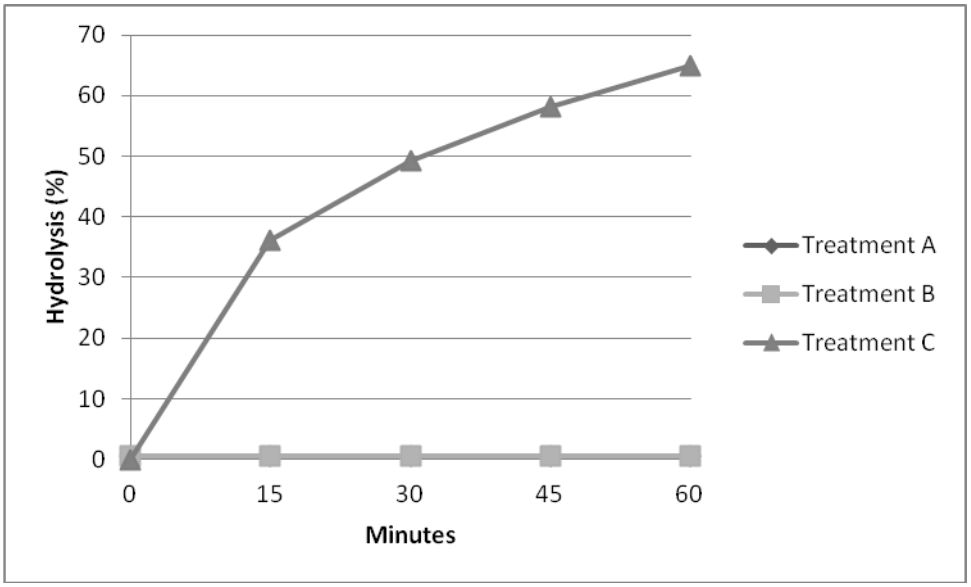

Figure 2: Percentage* of lactose-hydrolyzed in TTA, TTB (overlying TTA) and TTC during the lactose pre-hydrolyze in an oven at 41°C for 60 minutes before milk fermentation process

*The results are the mean of triplicate analysis

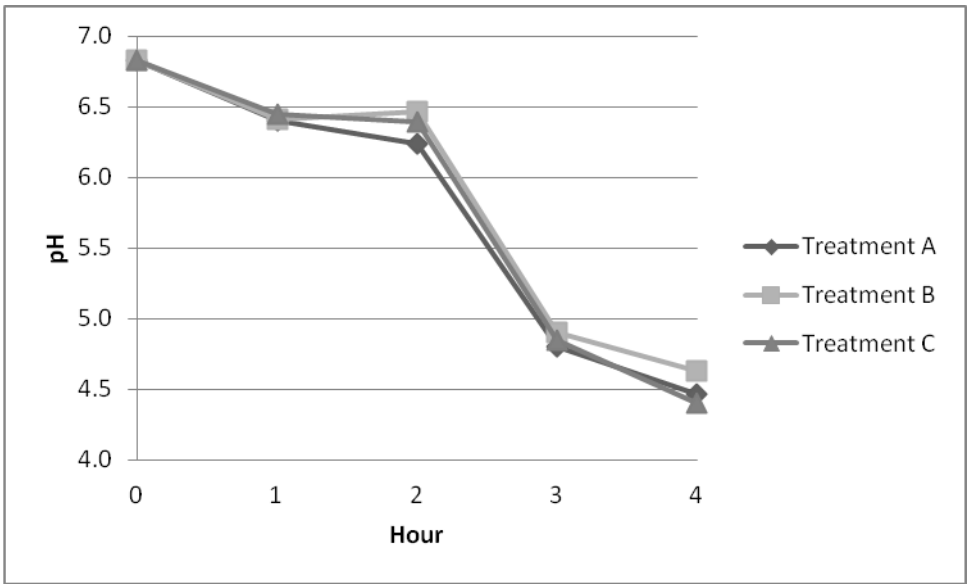

Figure 3: pH determination values* during the four hour of milk fermentation process

*The results are the mean of triplicate analysis

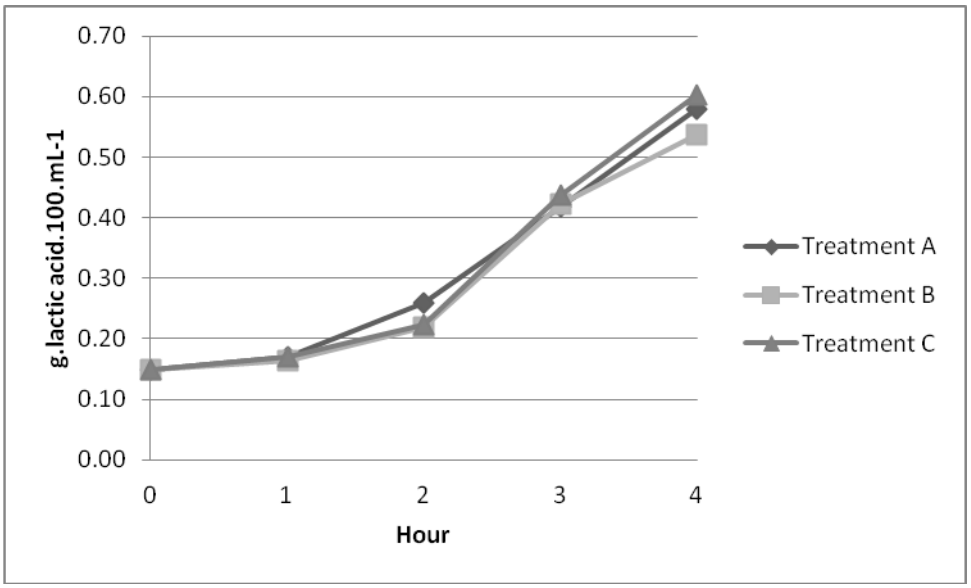

Figure 4: Titratable acidity measured values* during the four hour of milk fermentation process

*The results are the mean of triplicate analysis

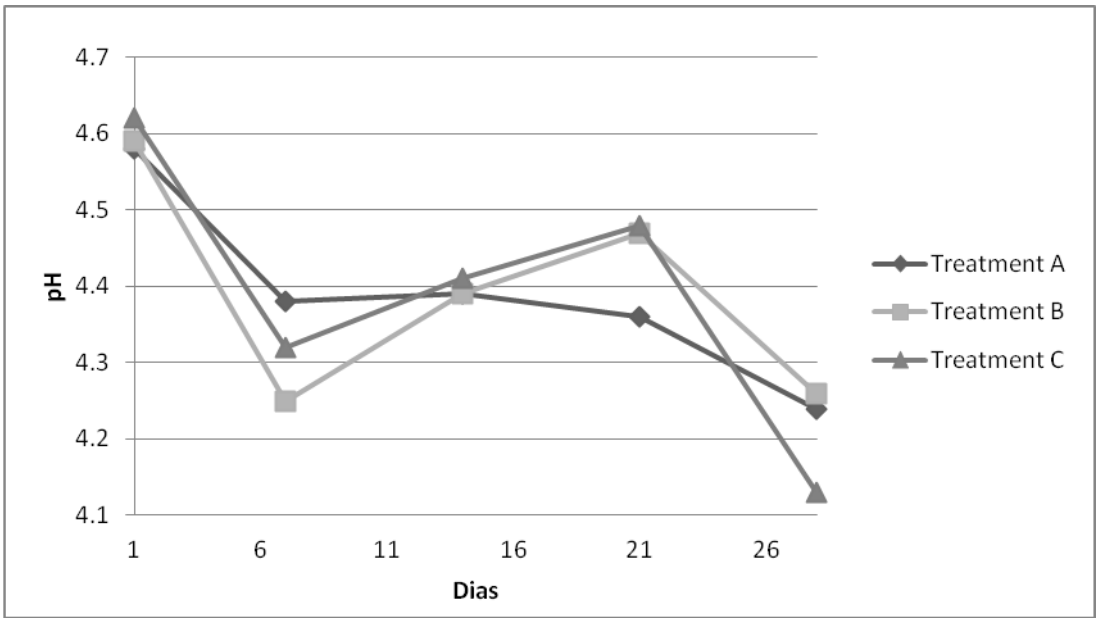

Figure 5: pH determination values* during 28 days of storage

*The results are the mean of triplicate analysis

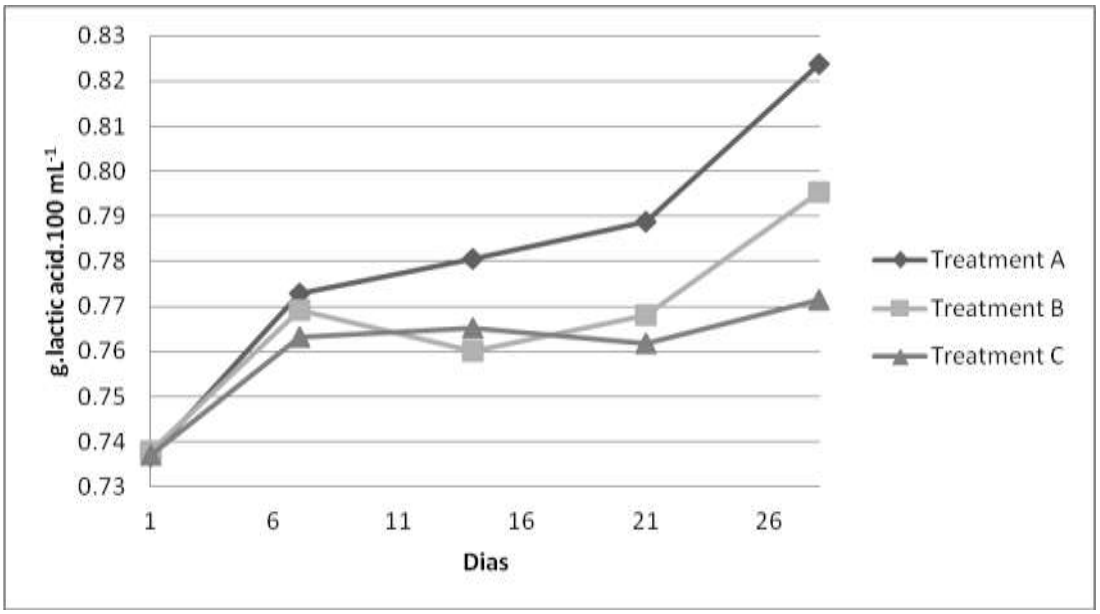

Figure 6: Titratable acidity measured values* during 28 day of storage

*The results are the mean of triplicate analysis

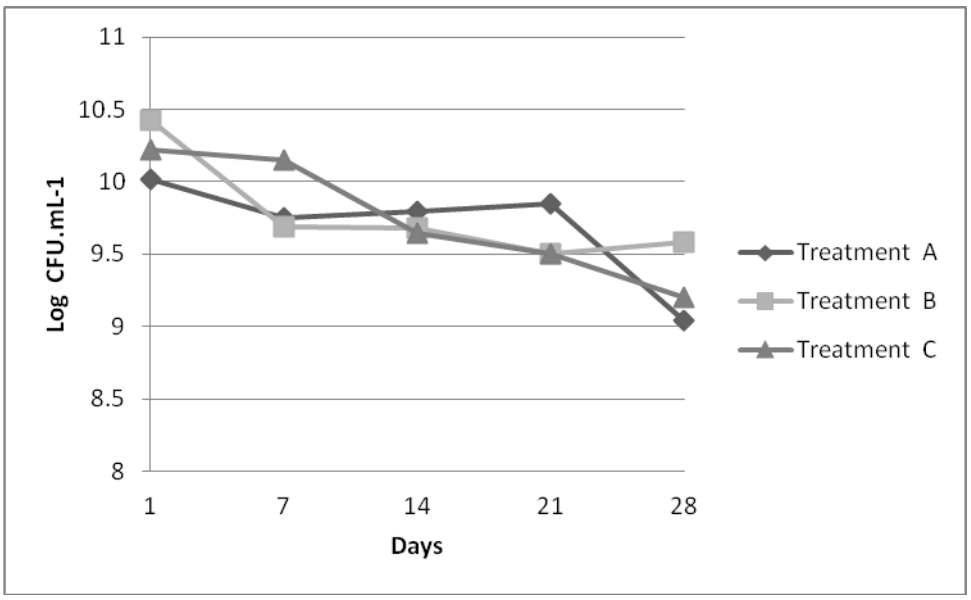

Figure 7: *Streptococcus thermophilus* counts isolated from TTA, TTB and TTC, during 28 days of storage under post-acidification conditions

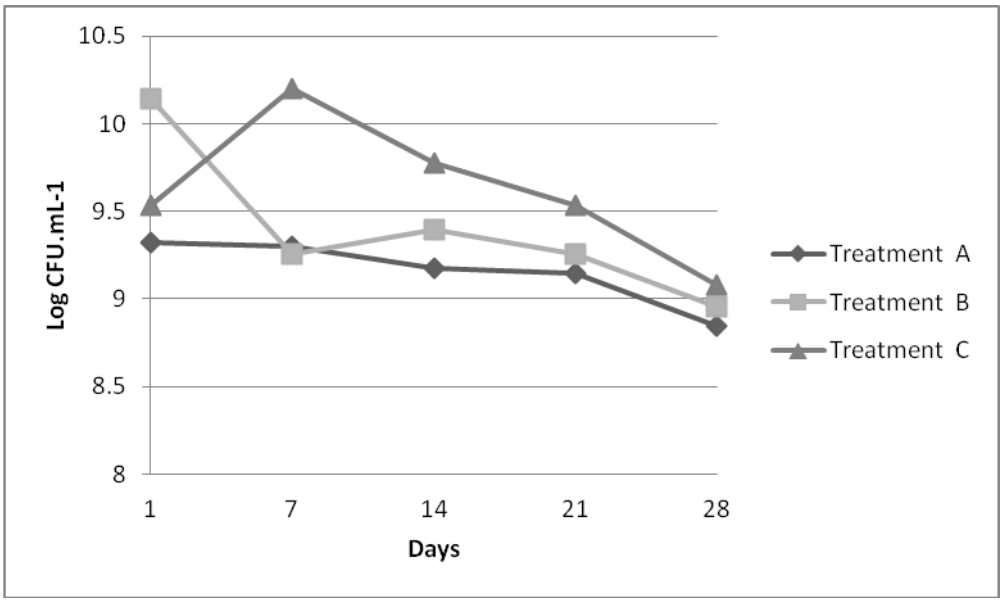

Figure 8: *Lactobacillus delbrueckii subsp. bulgaricu*s counts isolated from TTA, TTB and TTC, during 28 days of storage under post-acidification conditions

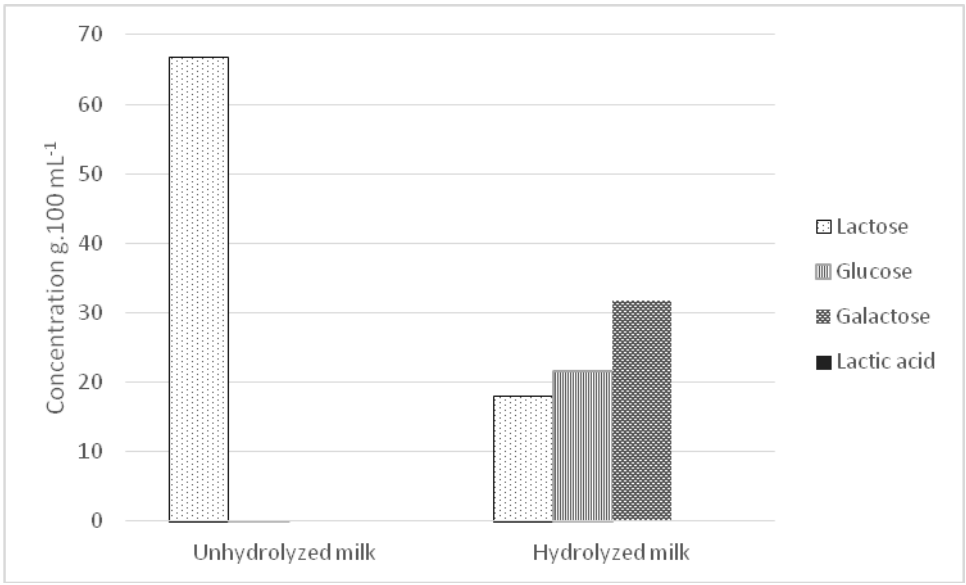

Figure 9: Milk carbohydrates and lactic acid content at the beginning (unhydrolyzed milk) and at end (hydrolyzed milk) of milk pre-hydrolysis step during one hour preceding fermentation

4 **CONSIDERAÇÕES FINAIS**

Pode-se concluir com os estudos realizados a viabilidade de se produzir iogurte com baixo teor, ou mesmo sem lactose. A redução da lactose, pelos tratamentos elaborados, foi suficiente para favorecer o consumo do produto por seres humanos com má absorção da lactose. A hidrólise enzimática do carboidrato não influenciou na tecnologia de fabricação do Iogurte, o qual atendeu durante todo periodo de estocagem (validade comercial) às exigências microbioógicas e físicoquímicas para os iogurtes.

Um novo nicho de mercado se abre para as indústrias lácteas de forma a atender a demanda de produtos sem lactose. Porém novas pesquisas devem ser realizadas para que os processos sejam cada vez mais otmizados, ou mesmo, novos produtos sejam elaborados.

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6 **OBRAS CONSULTADAS**

UNIVERSIDADE FEDERAL FLUMINENSE – UFF. *Apresentação de trabalhos monográficos de conclusão de curso*. 10. ed. rev. e atualizada por Estela dos Santos Abreu e José Carlos Abreu Teixeira. Niterói: EdUFF, 2012. 83 p.

7 **ANEXOS**

7.1 COMPROVANTE DE SUBMISSÃO: ARTIGO 1 – DAIRY SCIENCE AND TECHNOLOGY – MILK PREHEAT INFLUENCE OVER LACTOSE HYDROLYSIS AND YOGURT PRODUCTION PROCESS

Dairy Science & Technology

MILK PREHEAT INFLUENCE OVER LACTOSE HYDROLYSIS AND YOGURT PRODUCTION PROCESS

--Manuscript Draft--

Manuscript Number:	DSTE-D-15-00041
Full Title:	MILK PREHEAT INFLUENCE OVER LACTOSE HYDROLYSIS AND YOGURT PRODUCTION PROCESS
Short Title:	Yogurt: Hydrolysis and Temperature Influence
Article Type:	Original Article
Keywords:	Streptococcus thermophilus; Lactobacillus delbrueckii subsp. bulgaricus; β-galactosidase; lactic acid; HPLC.
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Abstract:	Dairy products are widely consumed worldwide, which offer to consumers a food with high nutritional value. However, a considerable amount of the world's population has some intolerance to lactose (lactose malabsorption). The aim of the study was to verify the influence of different initial temperatures: room temperature (25°C) and 40°C over β-galactosidase enzyme, in lactose enzymatic hydrolysis during milk fermentation. There were significant differences (P< 0.05) on pH and titratable acidity analysis among the preheated and non-preheated treatments during whole fermentation process. The first one obtained higher pH and titratable acidity values as 4.6 ± 0.04 and 0.73 g lactic acid.100 mL-1 respectively, against 4.82 ± 0.01 and 0.64 g lactic acid. 100 mL-1 from second treatment. Carbohydrates (lactose, glucose and galactose) and organic acids (lactic, acetic and citric acids) were quantified by high performance liquid chromatography. The lactose content was significant difference (P< 0.05) between treatments until the last fermentation hour. However, no significant difference was observed at the end of fermentation. The glucose and galactose were remained buoyant during the fermentation period, which indicating the production and use of these. The lactic acid concentration achieved had corroborated with titratable acidity result, which lactic acid of non-preheat treatment was significantly higher (18.644±0.62 mg.mL-1) than preheat treatment (17.557±0.53 mg.mL-1). Thus, it can be concluded that the enzyme contributed to reduce the lactose content without influencing the fermentation process. In addition, both treatments obtained lower values of lactose, which is sufficient to consumption for the most human, who has lactose malabsorption.
Suggested Reviewers:	Marco Furtado Universidade Federal de Juiz de Fora

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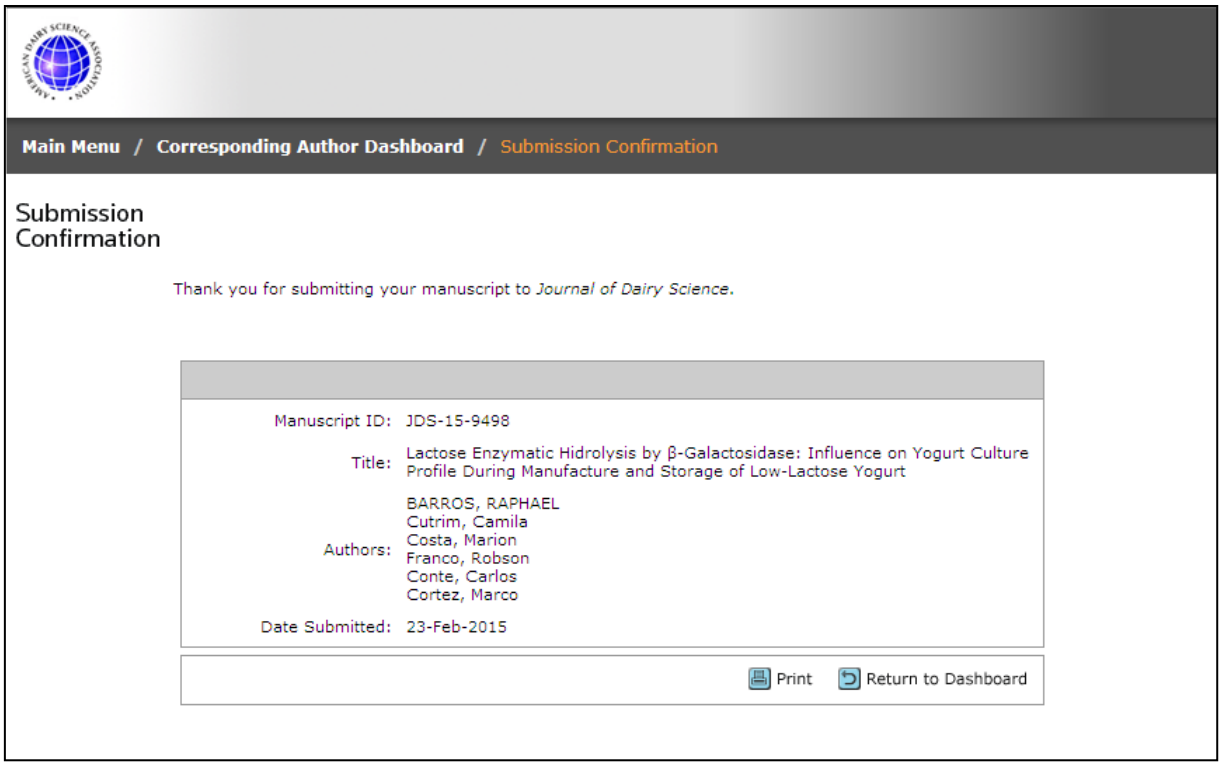
7.2 COMPROVANTE DE SUBMISSÃO: ARTIGO 2 – JOURNAL OF DAIRY SCIENCE – LACTOSE ENZYMATIC HIDROLYSIS BY B-GALACTOSIDASE: INFLUENCE ON YOGURT CULTURE PROFILE DURING MANUFACTURE AND STORAGE OF LOW-LACTOSE YOGURT



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Submission Confirmation

Thank you for submitting your manuscript to *Journal of Dairy Science*.

Manuscript ID:	JDS-15-9498
Title:	Lactose Enzymatic Hidrolysis by β-Galactosidase: Influence on Yogurt Culture Profile During Manufacture and Storage of Low-Lactose Yogurt
Authors:	BARROS, RAPHAEL Cutrim, Camila Costa, Marion Franco, Robson Conte, Carlos Cortez, Marco
Date Submitted:	23-Feb-2015

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