UNIVERSIDADE DE SÃO PAULO

FACULDADE DE CIÊNCIAS FARMACÊUTICAS

Programa de Pós-Graduação Tecnologia Bioquímico-Farmacêutica
Área de Tecnologia de Fermentações

Produção de bacteriocina por *Bifidobacterium lactis* a partir de soro de leite

Eduardo Marcos Balciunas

Dissertação para obtenção do grau de

MESTRE

Orientador:

Prof. Dr. Ricardo Pinheiro de Souza Oliveira

São Paulo

2013

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DEDICATÓRIA

Aos meus pais, que me trouxeram para este
mundo esempre apoiaram meus sonhos, não
importandoo rumo que estes me levassem.

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“The end will crush the light and sends a message,

it won't please the naked eye, without an end there is no light

to foretell, to blind you, the law of the claw reigns on and after still.”

(Philip Hansen Anselmo)

Balciunas, E. M. Produção de bacteriocina por *Bifidobacterium lactis* a partir de soro de leite. Dissertação (Mestrado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2013.

RESUMO

Objetivou-se a produção de bacteriocina de *Bifidobacterium animalis subsp. lactis*, comparando-se os meios de cultivo sintéticos BSM (*Bifidus Selective Medium*) e MRS (*Man Rogosa and Sharpe*) com o meio de cultivo natural (soro de leite). Inicialmente, foram determinadas curvas de crescimento e de pós-acidificação, consumo de glicose, lactose e produção de bacteriocina de *B.lactis* através de processos fermentativos utilizando os meios de cultivo BSM, MRS e soro de leite (SL). Os microrganismos indicadores utilizados no teste de sensibilidade à bacteriocina produzida por *B. lactis* foram *Lactobacillus sakei*, *Escherichia coli* e *Listeria monocytogenes*. Considerando a cepa *B. lactis* uma espécie de bactéria aerotolerante, foi realizado, em meio de cultura BSM, estudo prévio avaliando o seu crescimento, com a variação da agitação (25, 50 e 100 rpm) e com tempo de cultivo de 30 h, a 37°C de temperatura. Os melhores resultados de crescimento celular (9,4 log UFC/mL) foram obtidos na agitação de 50 rpm. Determinada a melhor condição de agitação (50 rpm) e temperatura (37°C), foi realizado, em soro de leite, estudo de crescimento, acidificação e consumo de lactose, variando a concentração de sólidos totais dissolvidos (5, 10, 15, 20 e 25% p/v), para se estabelecer a concentração de soro de leite ideal para os estudos de suplementação. A maior quantidade de biomassa produzida, aliada à menor pós-acidificação, foi encontrada em soro de leite a 10% (p/v) de sólidos totais, no qual o microrganismo apresentou, ao final do cultivo (30 horas), contagem de 9,13 log UFC/mL e valor de pH 4,29, respectivamente. Também se verificou a influência dos meios de cultivo no crescimento e na produção de bacteriocina de *B. lactis* em agitador rotativo (*shaker*), que consistiu na análise comparativa do efeito da suplementação de 1% dos seguintes ingredientes: extrato de levedura (EL), inulina (I), L-cisteína (Cl) e Tween 80 (T_{80}). As melhores condições de cultivo encontradas para a maior produção de biomassa e bacteriocina foram obtidas no soro de leite, à concentração de 10% (p/v) suplementado com 1% de extrato de levedura (9,9 log UFC/mL e 200 UA/mL). Na etapa final do trabalho, estas condições foram testadas em fermentador de bancada,

quando foi observado que o crescimento de *Bifidobacterium lactis* foi 10% maior em relação ao agitador rotativo. Quanto à atividade da bacteriocina produzida em fermentador de bancada, não houve diferença em relação ao agitador rotativo (200 UA/mL). Esta diferença no crescimento pode ser devido as melhores condições de anaerobiose oferecidas em fermentador de bancada, no qual houve a injeção de nitrogênio no meio de cultivo, sendo que, no agitador rotativo, a condição de anaerobiose foi gerada por um agente externo ao meio (uso de jarras de anaerobiose). Através do presente trabalho, pode-se concluir que a produção de bacteriocina por *B. lactis* é viável e apresenta resultados promissores quando utilizada a combinação soro de leite adicionado de extrato de levedura, o qual apresentou atividade antimicrobiana contra a cepa *Listeria monocytogenes.* A otimização do processo em fermentador de bancada demonstrou-se interessante quanto à produção de bacteriocina em nível industrial.

Palavras-chave: *Bifidobacterium lactis*, soro de leite, bacteriocina, anaerobiose, suplementação.

Balciunas, E. M. Production of bacteriocin by *Bifidobacterium lactis* from whey. Thesis (Master) – Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, 2013.

ABSTRACT

The objective was the production of bacteriocins by *Bifidobacterium animalis subsp. lactis*, comparing the synthetic culture medium BSM (*Bifidus Selective Medium*) and MRS (*Man Rogosa and Sharpe*) with the natural culture medium (whey). Initially, growth and post-acidification curves were determined, consumption of glucose, lactose and *B. lactis* bacteriocin production by fermentation processes using culture media BSM, MRS and milk whey (SL).The indicator organisms used in the test sensitivity to bacteriocin produced by *B. lactis* were *Lactobacillus sakei*, *Escherichia coli* and *Listeria monocytogenes*. Given the strain *B. lactis* one aerotolerant species of bacteria, it was conducted in culture medium BSM, a preliminary study assessing the growth, by varying the agitation (25, 50, and 100 rpm) with cultivation time of 30h at 37°C temperature. The best results of cell growth (9.4 log CFU / mL) were obtained at 50 rpm agitation. After the best condition of agitation (50 rpm) and temperature (37°C) determination, it was performed on whey, a study of growth, acidification and consumption of lactose, varying the concentration of total dissolved solids (5, 10, 15, 20 and 25% w/v), to settle the best concentration of whey for studies of supplementation. The highest amount of biomass produced, combined with the lowest post acidification was found in whey at 10% (w/v) of total solids, wherein the microorganism presented at the end of culture (30 hours) a counting of 9.13 log CFU/mL and pH 4.29, respectively. It was also verified the influence from the culture media on *B. lactis* growth and production of bacteriocin on a rotary shaker (shaker), which was the comparative analysis from the effect of supplementation by 1% of the following ingredients: yeast extract (EL), inulin (I), L-cysteine (IC) and Tween 80 (T_{80}). The best growing conditions found for higher biomass and bacteriocin production were obtained from the whey concentration of 10% (w/v) supplemented with 1% yeast extract (9.9 log CFU/ml to 200 AU/mL). In the final stage of the work, these conditions were tested in bench fermentor, where it was observed that the growth of *Bifidobacterium lactis* was 10% higher than in the rotary shaker. Regarding the activity of bacteriocin produced in fermenter bench, there was

no difference in the rotary shaker (200 AU / mL). This difference in growth may be due to the better anaerobic conditions offered in bench fermentor, which was the injection of nitrogen into the medium, and in a rotary shaker, the anaerobic condition was generated by an external agent to the medium (use of anaerobic jars). Through this study, it can be concluded that bacteriocin production by *B. lactis* is achievable and shows promising results when used the combination whey added yeast extract, which showed antimicrobial activity against the strain *Listeria monocytogenes*. The optimization process bench fermentor has been shown interesting as bacteriocin production at industrial level.

Keywords: *Bifidobacterium lactis*, whey, bacteriocin, anaerobic, supplementation.

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LISTA DE ABREVIATURAS E SIGLAS

AU	Unidades arbitrárias de atividade da bacteriocina ($mg.mL^{-1}$)
BSM	*Bifidus Selective Medium*
MRS	*Man Rogosa Sharpe*
BHI	*Brain Heart Infusion*
SL	Soro de Leite
LAB	Bactérias ácido-láticas
rpm	Rotações por minuto
S_i e S_f	Concentrações inicial e final de substrato ($g\ L^{-1}$)
GRAS	Geralmente Reconhecidas como Seguras (*Generally Recognized As Safe*)
t	Tempo de cultivo
t_g	Tempo de geração (h)
UFC	Unidade formadora de colônia (mL)
X	Concentração celular ($mg.L^{1}$)
μ_{max}	Velocidade específica de crescimento (h^{-})
T_{80}	Tween 80
I	Inulina
CI	L-Cisteína
EL	Extrato de Levedura
h	Hora
DBO	Demanda Biológica de Oxigênio
ppm	Partes por milhão

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1. INTRODUÇÃO E JUSTIFICATIVA

O soro de leite é considerado um subproduto de elevado valor nutricional, sendo constituído por aproximadamente 6,5% de sólidos totais; dentre os quais: 4,6% de lactose, 0,8% de proteína, 0,5% de ácidos graxos, de 0,5% a 0,8% de matéria mineral e de 0,2% de 0,8% de ácido lático (ANTUNES, 2003). Ressalta-se que as proteínas e os ácidos graxos, presentes no soro de leite, são as fontes de peptídeos bioativos e de ácido linoleico conjugado (CLA), respectivamente, as quais promovem efeitos benéficos à saúde do consumidor. Aproximadamente 85% a 95% do volume de leite manufaturado é descartado como soro, representando, desta forma, uma notável fonte de poluição, causada por sua alta demanda biológica e bioquímica de oxigênio e matéria orgânica (LIU et al., 2006, YOSHIDA; ANTUNES, 2009). Portanto, torna-se interessante o uso deste subproduto como substrato em fermentação na produção de biomoléculas com alto valor agregado, como compostos antimicrobianos produzidos por bactérias láticas.

As bactérias ácido-láticas são microrganismos que requerem meios sintéticos complexos, como caldos MRS® e M17®, para o crescimento microbiano. Apesar de comercialmente disponíveis, estes meios possuem elevado custo, o qual encarece e inviabiliza a produção em larga escala. Por isto, torna-se atrativa a pesquisa por novas formulações para a produção de novas bacteriocinas e até mesmo a utilização de diferentes compostos antimicrobianos, principalmente de classes diferentes, com o objetivo de aumentar a eficiência destes compostos na bioconservação de alimentos.

Ressalta-se a inovação científica e tecnológica do presente trabalho no que tange à utilização do soro de leite como meio de cultivo, valorizando o conceito da produção de uma biomolécula de alto valor agregado, pois o soro de leite é um subproduto gerado pelas indústrias de laticínios. Portanto, utilizando-se o soro de leite em pó como meio de cultura, inicia-se uma pesquisa direcionada a questões ambientais. Aponta-se que, no Brasil, 50% do soro de leite é descartado, sem tratamento, em rios, necessitando de cautela nesse descarte (BRIÃO, 2000). Considerando a produção de queijos, são 9 kg de soro de leite para 1 kg de massa de queijo produzido (ROOBBINS et al., 1996). O descarte incorreto do soro, em

cursos de água, pode causar sérios danos, devido à alta taxa de DBO (Demanda Biológica de Oxigênio) (PARKIN; MARSHALL, 1976). Neste caso, a produção de um antimicrobiano poderia ser associada com a reciclagem ou a reutilização de material de descarte industrial.

2. REVISÃO DA LITERATURA

O soro de leite é uma porção aquosa que se separa do coágulo durante a fabricação convencional do queijo e consiste em cerca de 85 a 90% do volume de leite utilizado para a transformação em queijo e contém 55% dos nutrientes contidos no leite (KOSIKOWSKI, 1979). As proteínas do soro estão em torno de 20% do total de proteínas contidas no leite, sendo 50% de β-lactoglobulina e 25% de α-lactoalbumina, podendo variar de acordo com a produção de soro e o tipo de queijo (CHEEKE; STANGEL, 1973). Principalmente na América do Norte e na Europa, grandes quantidades de soro de leite estão disponíveis, em 2008, os valores resultantes estimados relatados são de 4.10^7 toneladas para os EUA, e 5.10^7 toneladas para a Europa (GHALY; EL-TAWEEL, 1997). Soro mostra uma carência bioquímica de oxigénio (DBO, 40.000 - 60.000 ppm) e a demanda química de oxigénio (DQO, 50,000 - 80,000 ppm) elevadas, fazendo com que o escoamento do soro excedente seja bastante caro, ressaltando que a lactose, o composto predominanto no soro de leite, é o principal componente responsável por estes altos valores de BOD e COD (KIM et al., 1995, VIÑAS et al., 1994).

As propriedades antimicrobianas de bactérias ácido-láticas (LAB) permitiram o aumento da vidadeprateleira de muitos alimentos, através de processos de fermentação. A inibição de microrganismos deteriorantes dos alimentos pode ser atribuída à produção de compostos antimicrobianos, incluindo ácidos orgânicos, peróxido de hidrogênio, antibióticos e bacteriocinas (SARIKA; LIPTON; AISHWARYA, 2010). As bactérias ácido-láticas são um grupo diversificado e extremamente útil de microrganismos que, apesar de não pertencerem a um grupo taxonômico estrito, estão associados, já que possuem propriedades comuns (PFEILER; KLAENHAMMER, 2007). A característica comum é a produção de ácido láctico como produto principal ou único de uma fermentação. Devido a produção de

ácido láctico, as bactérias ácido-láticas têm sido historicamente associadas à fermentação de alimentos; como resultado, muitas delas, como *Lactococcus*, *Oenococcus*, *Lactobacillus*, *Leuconostoc* e *Pedicoccus* sp., são geralmente reconhecidas como seguras (GRAS) (MAYO et al., 2010), embora outras espécies, principalmente pertencentes aos gêneros *Enterococcus* e *Streptococcus*, são consideradas como patogênicas oportunistas na natureza (MADIGAN; MARTINKO; PARKER, 2003).

Algumas espécies de *Lactobacillus* e *Bifidobacterium*, utilizadas na fabricação de produtos lácteos fermentados, inibem o crescimento de outros microrganismos, incluindo os patogênicos intestinais e os deteriorantes, através da produção de compostos antibacterianos ou bacteriocinas (AHMAD et al., 2010). Além disso, essas espécies citadas, podem ser consideradas probióticas. Para isso, tem sido sugerido que uma das desejáveis propriedades das cepas probióticas seja a habilidade de produzir substâncias, como bacteriocinas, asquais oferecem o potencial de fornecer vantagem na colonização e na competição do trato gastrointestinal (TAMIME, 2005).

As bacteriocinas são geralmente definidas como peptídeos produzidos por bactérias que apresentam espectro de ação contra uma variedade de microrganismos, incluindo bactérias Gram-positivas e Gram-negativas, protozoários, fungos e vírus (REDDY; YEDERY; GUPTA, 2009). Bacteriocinas produzidas por bactérias Gram-positivas, particularmente bactérias ácido-láticas, apresentam amplo espectro como bioconservantes de alimentos e como agente terapêutico (GALVEZ et al., 2008; JACK; TAGG; RAY, 1995). O mais conhecido exemplo é a nisina, a qual éproduzida por muitas cepas de *Lactoccuslactis* subsp. *lactis*, sendo considerada o protótipo de bacteriocina de bactérias ácido-láticas (TAMIME, 2005, JOZALA et al., 2007). Novas bacteriocinas são identificadas por triagem de isolados bacterianos com atividade antimicrobiana, seguida de purificação e identificação de seus determinantes genéticos. Tais estratégias de triagem são fundamentais para a detecção e a identificação de bacteriocinas potentes de várias subclasses. Exemplos recentes incluem: uma bacteriocina de classe IIa, chamada avicina A, que foi identificada a partir de *Enterococcus avium*, estirpes isoladas de amostras de fezes de bebês humanos amamentados e saudáveis da Etiópia e da Noruega (BIRRI el al., 2010); uma bacteriocina de estrutura cíclica chamada garvicina ML, produzida por uma cepa de *Lactococcus garvieae*, isolado do pato-real (BORRERO et al., 2011);

uma bacteriocina de classe IIb, a enterocina X, de *Enterococcus faecalis*, isolada a partir de maçãs (HU et al., 2010), e uma bacteriocina glicolisada (glicocina F) de *Lb.* p*lantarum*, isolada de milho fermentado (KELLY; ASMUNDSON; HUANG, 1996; STEPPER et al., 2011).

A nisina foi descoberta em 1928, quando Rogers e Whittier observaram metabólitos inibitórios de LAB (Rogers e Whittier, 1928). A nisina tem sido extensivamente estudada e, em 1988, foi aprovada pela Food and Drug Administration (FDA), recebendo a denominação de *Generally Recognized as Safe* (GRAS) para ser usada como um conservante em muitos alimentos processados. Como resultado disso, pesquisas nesse campo começaram a crescer rapidamente, o que levou à descoberta de grande número de bacteriocinas produzidas por bactérias láticas (GALVEZ et al., 2008; REDDY; YEDERY; GUPTA, 2009).

Bacteriocinas são normalmente sintetizadas como pré-peptídios inativos que possuem uma sequência terminal N guia (XIE; VAN DER DONK, 2004). Este precursor é transportado para a superfície da célula durante a fase de crescimento exponencial e enzimaticamente convertido na sua forma ativa. O transportador contém uma porção terminal N peptídica responsável pela clivagem do peptídeo guia, bem como uma porção terminal C responsável pela hidrólise de ATP e fornecimento de energia (AUCHER et al., 2005). Para a classe II, proteínas acessórias são usadas para facilitar a translocação da membrana e/ou para clivar o peptídeo guia. O sistema responsável pela regulação da produção de bacteriocina é composto por três componentes: um peptídeo indutor (ou fator de ativação por realimentação), a histidina quinase transmembrana (receptor de realimentação) e um regulador de resposta (NES; EIJSINK, 1999). O peptídeo indutor é sintetizado no ribossomo em níveis baixos como um pré-peptídeo, que é clivado e segregado para o meio extracelular pelo sistema de transporte. Quando este composto atinge uma determinada concentração, ele ativa a histidina quinase transmembranar, o que leva à autofosforilação do resíduo de histidina, assim transferindo o fosfato a uma proteína reguladora de resposta. O regulador fosforilado ativa a transcrição da bacteriocina, além dos elementos que compõem o sistema de regulação, iniciando uma reação positiva (NES, EIJSINK, 1999). A regulação da produção de lantibióticos, tais como a nisina e a subtilina, é feita pela própria bacteriocina, que atua como um sistema de realimentação para estimular a sua produção em níveis elevados (KLEEREBEZEM; QUADRI, 2001). O mecanismo de imunidade de

bactérias produtoras de bacteriocinas é capaz de distinguir entre a bacteriocina produzida pela própria bactéria e a produzida por outras. A sua proteção pode ser promovida por uma classe específica de proteínas e/ou pelo sistema de transporte. O mecanismo pelo qual estes funcionam é semelhante, sequestrando a proteína estrutural ou competindo pelo receptor antagonista da bacteriocina (HOFFMANN et al., 2004).

Segundo Twomey et al. (2002), as bacteriocinas estão distribuídas em 3 classes. Em geral, a Classe I, ou Lantibióticos, representada pela nisina, é constituída por peptídeos termoestáveis de baixa massa molar (<10 kDa), diferenciados dos demais pela presença de lantionina e derivados. A Classe II é composta por pequenos peptídeos (<10 kDa) termoestáveis, divididos em três subclasses: IIa (pediocina e enterocina), IIb (lactocina G) e IIc (lactocina B). A Classe III é representada por peptídeos termolábeis de alta massa molar (>30 kDa), como helveticina J. É importante salientar que na Classe II encontra-se a bacteriocina produzida por *Bifidobacterium bifidum* (YILDIRIM; JOHNSON, 1998).

A atividade de bacteriocinas produzidas por bactérias ácido-láticas diferentes não é uniforme ou constante e depende da composição química e das condições físicas do alimento em que estão presentes. A atividade depende principalmente do pH e é reduzida pela afinidade dos compostos presentes no alimento, pela absorção celular, atividade de proteases e outras enzimas (SCHILLINGER; GEISEN; HOLZAPFEL, 1996). Uma correlação entre a degradação da nisina e extensão da proteólise em creme pasteurizado foi encontrada por Phillips, Griffths e Muir (1983). Buyong, Kok e Luchansky (1998) descreveram a redução da atividade da pediocina, de 64.000 para 2.000 UA/g, após seis meses de maturação do queijo tipo cheddar devido à ação de proteases e peptidases. O NaCl, a certas concentrações, pode reduzir o crescimento de culturas láticas e,consequentemente, a produção de bacteriocinas, além de proteger a bactéria alvo, como a *L. monocytogenes*, da ação das bacteriocinas (HUGAS et al., 2002). Sarantinopoulos et al. (2002) observaram a redução na atividade de bacteriocinas e o aumento na taxa de crescimento de *Enterococcus faecium* FAIR-E 198 após a adição de 2% de NaCl em meio MRS. Nilsen, Nes e Holo (1998) descreveram que a redução na produção de bacteriocina na presença do sal se deve à interferência do NaCl na produção do fator de ligação do receptor de indução.

A eficiência inibitória das bacteriocinas também está associada ao nível de contaminação do microrganismo alvo. Se a contaminação inicial é muito elevada, a atividade da bacteriocina será baixa e não será capaz de prevenir o desenvolvimento e a contaminação pelo microrganismo. Rilla, Martinez e Rodriguez (2004) investigaram a ação de *Lc. lactis* subsp. *lactis* IPLA 729 contra *S. aureus* em dois níveis diferentes de concentração, especificamente 1,8 . 10^4 e 7.2 . 10^6 UFC mL^{-1}, após 24h de incubação. Em particular, estes pesquisadores não detectaram *S. aureus* em várias amostras diluídas, enquanto que em algumas amostras mais concentradas foram encontradas altas concentrações desse microrganismo patogênico (5.0 . 10^4 UFC mL^{-1}).

As bifidobactérias foram primeiramente isoladas e descritas em 1899-1900 por Tissier. *Bifidobacterium* spp. são microrganismos Gram-positivos, anaeróbicos, sendo algumas espécies aerotolerantes, como no caso de *Bifidobacterium animalis* subsp. *lactis* (LI et al., 2010), esporulantes e não possuem motilidade. Além disso, esses microrganismos são catalase negativos (BIAVATI; MATTARELLI, 2005).

Até recentemente, mais de 34 espécies foram atribuídas ao gênero *Bifidobacterium* (VENTURA et al., 2007). As bifidobactérias têm sido isoladas de várias fontes, entre as quais a microbiota intestinal de seres humanos. São também consideradas como um dos gêneros predominantes do trato gastrointestinal (FALK et al., 1998). Essa predominância no trato gastrointestinal torna interessante o uso das bifidobactérias como probióticos, considerando que muitos efeitos positivos à saúde têm sido atribuídos a esses microrganismos (UELIVON, 2006). Um dos efeitos positivos das bifidobactérias no trato gastrointestinal de humanos poderia ser a produção de compostos antimicrobianos como, por exemplo, ácidos orgânicos, ácidos graxos (CLA – ácido linoleico conjugado) e bacteriocinas. Metabolicamente, as bifidobactérias produzem ácidos lático e acético sem produzir CO_2, exceto durante a degradação do gluconato. *Bifidobacterium* spp. de origem humana fermenta glicose, galactose, lactose e frutose como fontes de carbono (GOMES; MALCATA, 1999), utilizando uma via metabólica muito diferente da tradicional, que deveria ser levada em conta em qualquer estudo metabólico.

Diferentemente da situação dos *Lactobacillus*, somente número limitado de estudos tem sido realizado no que diz respeito à produção de compostos antimicrobianos ou bacteriocinas por cepas de bifidobactérias. Espécies de *Bifidobacterium* são geralmente inibidores de ampla gama de microrganismos,

devido à sua produção intensa de ácido lático e ácido acético como produtos do próprio metabolismo (EKLUND 1983). Gibson e Wang (1994) demonstraram que oito espécies de bifidobactérias apresentaram atividades antagônicas em relação aos patógenos Gram-positivos e Gram-negativos, não apenas devido à presença de ácidos, mas também devido às substâncias bactericidas ou bacteriostáticas excretadas por estas cepas de *Bifidobacterium*.

Yildirim e Johnson (1998) isolaram a primeira bacteriocina de *Bifidobacterium*, conhecida como Bifidocina B. Esta bacteriocina demonstrou-se muito eficaz contra microrganismos patogênicos de origem alimentar (YILDIRIM; WINTERS; JOHNSON, 1999). Estudos *in vitro* mostraram que bifidobactérias inibiram a ação da *Escherichiacoli* 0157:H7 entero-hemorrágica (GAGNON et al., 2004) e *L. monocytogenes*, devido à produção de compostos inibidores (TOURE et al., 2003).

Recentemente, Ahmad et al. (2010) descobriram uma nova bacteriocina produzida por *Bifidobacterium infantis* em meio sintético MRS (*Man Rogosa and Sharpe*) suplementado com L-cisteína. Essa nova bacteriocina, denominada Bifidina I, mostrou amplo espectro de ação, incluindo bactérias Gram-negativas e Gram-positivas, além de ter demonstrado clara inibição com relação à *L. monocytogenes*.

Produtos alimentares podem ser suplementados com bacteriocinas produzidas *ex situ*, obtidas de cultura da estirpe produtora em fermentador industrial, seguido pela recuperação adequada. Estas bacteriocinas podem ser adicionadas tanto parcialmente purificadas quanto purificadas em concentrados, o que exige a aprovação específica como conservantes, do ponto de vista legal. Até agora, a nisina e a pediocina PA-1 são as únicas bacteriocinas licenciadas como conservantes de alimentos (SIMHA et al., 2012). Muitos estudos preliminares sobre a atividade de bacteriocinas *in vitro* ou em sistemas alimentares estão sendo realizados com preparações parcialmente purificadas, obtidas a partir de caldos de fermentações de culturas, mas, na maioria dos casos, uma baixa concentração de bacteriocinas é recuperada (SCHILLINGER; GEISEN; HOLZAPFEL, 1996; STILES, 1996), o que demonstra a importância de se abordar esforços nas técnicas de purificação.

Os alimentos podem também ser suplementados com bacteriocinas produzidas *ex situ*, que podem ser adicionados sob a forma de concentrados brutos obtidos por cultura da estirpe produtora, quando produzidas em um substrato de grau alimentar (tais como o leite ou soro de leite). As preparações resultantes podem ser

consideradas como aditivos alimentares ou ingredientes, do ponto de vista legal, uma vez que alguns dos seus componentes podem desempenhar uma função reconhecida no alimento (tais como aumento do teor de proteínas ou espessantes). Eles também contêm metabólitos derivados de células antimicrobianas (tais como ácido láctico) e bacteriocinas, proporcionando a função bactericida adicional. Outras preparações lácteas têm sido descritas, além das já comercializadas, tais como os concentrados ALTA™ 2341 ou Microgard™, como lacticina 3147 (GUINANE et al., 2005) e variacina (O'MAHONY et al., 2001).

Bacteriocinas produzidas *ex situ* podem também ser aplicadas sob a forma de preparações imobilizadas, nas quais a bacteriocina parcialmente purificada é ligada a um transportador. O transportador atua como um reservatório e um difusor, das moléculas de bacteriocina concentrada, para a comida, assegurando o fornecimento de um gradiente contínuo de bacteriocina. O transportador também pode proteger a bacteriocina de inativação por interação com componentes alimentares e inativação enzimática. Além disso, a aplicação localizada precisa, de moléculas de bacteriocina sobre a superfície de alimentos, requer quantidades muito mais baixas de bacteriocina (em comparação com o volume de aplicação em alimentos integrais), diminuindo os custos de processamento. Na maioria dos casos, as preparações imobilizadas de bacteriocina são aplicadas sobre a superfície do alimento processado, evitando a contaminação pós-processo de proliferação de bactérias não desejadas na superfície do alimento. Um avanço recente nesta área é o uso de bacteriocinas imobilizadas no desenvolvimento de embalagens antimicrobianas (MAURIELLO et al., 2004, ERCOLINI et al., 2006).

A produção *in situ* de bacteriocinas oferece várias vantagens em relação à produção *ex situ*, sobre os aspectos legais e de custos. A redução dos custos dos processos de bioproteção pode ser altamente atraente, especialmente para pequenas indústrias e países em desenvolvimento, onde a segurança alimentar pode ser seriamente comprometida (HOLZAPFEL, 2002). Muitos estudos também têm focado na seleção e no desenvolvimento de culturas de proteção bacteriocinogênicas para aplicações alimentares (ROSS; MORGAN; HILL, 2002, PELÁEZ; REQUENA, 2005, MORENO et al., 2006, LEROY; VERLUYTEN; DE VUYST, 2006), tais como a inibição de bactérias deteriorantes e patogênicas durante o período de vida de prateleira de alimentos não-fermentados. Uma cultura de proteção pode crescer e produzir bacteriocina durante o armazenamento refrigerado

do alimento, o que deve ter impacto neutro sobre suas propriedades físico-químicas e organolépticas e/ou durante condições de abuso de temperatura, em que pode até mesmo agir como o microrganismo predominante, garantindo que bactérias patogênicas não proliferem e que alimentos deteriorados não sejam consumidos (HOLZAPFEL; GEISEN; SCHILLINGER, 1995).

Atualmente, a maioria das bactérias probióticas é utilizada na formulação de bioprodutos, como, por exemplo, iogurtes, leites fermentados, sorvetes e produtos farmacêuticos, visando promover efeitos positivos à saúde e prevenção de doenças (MATTILA-SANDHOLM et al., 2002). Por outro lado, é muito importante investigar e obter possíveis biomoléculas que sejam produzidas por estes microrganismos. Portanto, torna-se necessário produzir novos tipos de bacteriocinas, as quais têm sido muito usadas, como no caso da nisina, em produtos de interesse alimentar e farmacêutico.

3. OBJETIVOS

O objetivo deste trabalho foi produzir bacteriocina a partir de células de *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) por processo fermentativo, utilizando soro de leite como meio de cultivo natural suplementado ou não com inulina, Tween 80, L-cisteína e extrato de levedura nas concentrações de 1% (p/v), comparando-o com os meios sintéticos MRS Difco™ (*Man Rogosa and Sharpe*) e BSM Sigma™ *(Bifidus Selective Medium*).

4. MATERIAIS E MÉTODOS

4.1 Culturas microbianas

Neste trabalho, foram utilizados os seguintes microrganismos: *Bifidobacterium animalis* subsp. *lactis* HN019 (*B. lactis*), cultura comercial liofilizada (Danisco,

Sassenage, França), usada como produtora de bacteriocina, e *Bifidobacterium animalis* subsp. *animalis* ATCC 25527 (*B. animalis*), para fins comparativos; *Lactobacillus sakei* ATCC 15521, *Escherichia coli* ATCC 25922 e *Listeria monocytogenes* ATCC 13932, utilizados como biossensor da produção do peptídeo antimicrobiano.

4.2 Preparação dos meios de cultura

O soro de leite em pó (Cargill Agrícola S.A., Campinas, SP, Brasil) foi preparado utilizando diferentes concentrações de sólidos totais, sendo elas 5, 10, 15, 20 e 25 % (p/v). Baseado nos resultados de crescimento celular e acidificação do meio, a melhor concentração de sólidos totais foi 10% (p/v) (ver Figuras 5 e 6 e seção 6.4.1). A seguir, as bases de soro de leite foram suplementadas ou não com inulina, Tween 80, L-cisteína e extrato de levedura nas concentrações de 1% (p/v) para estudos comparativos com os meios de cultivo sintéticos.

Como meios de cultivo sintético, foram preparados o caldo MRS (*Man Rogosa and Sharpe* Difco™, Sparks, MD, USA) e o caldo BSM *(Bifidus Selective Medium*, Sigma™, St. Louis, MO, USA), suplementado ou não com 1% (p/v) de extrato de levedura (Difco™, Sparks, MD, USA), 1% (p/v) de Tween 80 (Alamar Tecno-Cientifica Ltda, Diadema, SP, Brasil), 1% (p/v) de inulina (Beneo-Orafti Corp.,Malvern, PA, USA) e 1% (p/v) de L-cisteína (Sigma Chemical Co., St. Louis, MO, USA), autoclavados em frascos SCHOTT® de 250 ml.

Todos os meios foram diluídos em água destilada estéril. As bases de soro de leite foram homogeneizadas com auxílio de agitador magnético, por 15 minutos, e tratadas termicamente a 90°C, durante 5 minutos (OLIVEIRA et al., 2011), em equipamento de banho termorregulável (Fisatom, Modelo 550 A). Posteriormente, as bases foram imediatamente resfriadas em banho de gelo e distribuídas em frascos SCHOTT® estéreis em câmara de fluxo laminar.

4.3 Suplementos

4.3.1 Inulina

A inulina foi escolhida como um dos ingredientes testados a adicionados aos meios de cultivo, pois em trabalhos recentes, a inulina foi usada como importante indutora de crescimento para bifidobactérias (OLIVEIRA et al., 2009a, 2009b, 2009c).

4.3.2 Tween 80

O Tween 80 ou polisorbato 80 é um tensioativo não ionico e agente emulsionante derivado do sorbitano polietoxilado e ácido oleico e é muitas vezes utilizado em alimentos. Ele é uma solução viscosa, liquida, amarela e solúvel em água. Na nomenclatura dos polissorbatos, a designação numérica polissorbato seguinte refere-se ao grupo lipofílico, neste caso, o ácido oleico. O Tween 80 é frequentemente usado em alimentos e outros produtos, como emulsionante (BUDAVAVI et al., 1996). O uso do Tween 80 como aditivo em meios de cultura se mostrou promissor em outros trabalhos de produção de bacteriocina encontrados na literatura (COLLADO; HERNÁNDEZ; SANZ, 2005), já que é um agente tensoativo, podendo aumentar na concentração de bacteriocinas, por facilitar a excreção desse peptídeo antimicrobiano.

4.3.3 Extrato de levedura

O extrato de levedura foi escolhido como um dos ingredientes a serem estudados na suplementação dos meios, pois em diversos estudos com o gênero *Bifidobacterium* e ouros gêneros de bactérias em diferentes meios de cultivo este ingrediente se mostrou promissor na influencia do crescimento. Loquasto et al.

(2011) comprovaram que o extrato de levedura adicionado ao soro de leite *B. lactis* aumenta o crescimento em relação ao meio não suplementado.

4.3.4 L-cisteína

A cisteína é um α-aminoácido com a fórmula química HO_2CCH (NH_2) CH_2SH. Mättö et al. (2006) estudaram o efeito da adição de L-cisteína no meio MRS no crescimento de *Bifidobacterium animalis* subsp. *lactis* E2010. Kiviharju, Leisola e Eerikäinen (2005) realizou um estudo de crescimento de *Bifidobacterium longum* ATCC 15707, adicionando L-cisteína ao meio. Nestes estudos, foi comprovado o efeito benéfico ao crescimento microbiano com a adição de L-cisteína ao meio de cultivo.

4.4 Preparação do inóculo em agitador rotativo

A pré-cultura foi preparada adicionando-se 45mg da cultura estoque liofilizada de *B. lactis* em 50 mL de caldo BSM (Sigma) em frascos SCHOTT® de 250 mL, nas seguintes condições de processo: agitação (50 rpm), temperatura (37°C) e tempo (24h) em anaerobiose.

A pré-cultura de *B. animalis* foi preparada adicionando-se 100µL da cultura estoque criopreservada em 50 mL de caldo BSM (Sigma) em frascos SCHOTT® de 250 mL nas seguintes condições de processo: agitação (50 rpm), temperatura (37°C) e tempo (24h) em anaerobiose.

A partir deste cultivo, uma alíquota contendo aproximadamente 10^8 UFC/mL foi transferida para frascos SCHOTT® de 250 mL contendo 100 mL de caldo MRS, caldo BSM ou soro de leite que, em seguida, foram incubados em agitador rotacional

(*shaker*) nas seguintes condições de processo: agitação (50 rpm), temperatura (37°C) e tempo de cultivo (30h).

4.5 Preparação do inóculo para fermentador de bancada

A pré-cultura de *B. lactis* foi preparada adicionando-se 145mg da cultura estoque liofilizada em 150 mL de caldo soro de leite em frascos SCHOTT® de 250 mL, nas seguintes condições de processo: agitação (50 rpm), temperatura (37°C) e tempo (24h) em jarras de anaerobiose. Após atingir a concentração de aproximadamente 10^8 UFC/mL (24h), o pré-inóculo foi transferido para o fermentador de bancada contendo 1.350 mL de caldo soro de leite suplementado com 1% de extrato de levedura.

4.6 Procedimentos de análises

Nos estudos realizados em agitador rotacional (*shaker*) e em fermentador de bancada, as análises foram realizadas a cada 3 horas, durante 30 horas de fermentação, sendo que as alíquotas foram coletadas assepticamente dos cultivos para análises de pH, consumo de glicose (para os meios sintéticos) e lactose (para o soro de leite), crescimento celular e determinação da atividade da bacteriocina. Cada análise foi realizada em triplicata.

4.7 Crescimento celular

O crescimento celular foi determinado através da diluição em série, ou seja, 0,1ml de amostra em tubos contendo 0,9ml de água peptonada esterilizada até atingir grau de diluição entre 10^6 e 10^9. A contagem foi realizada em placas de Petri

contendo meio de cultura BSM e ágar, expressa na forma de log UFC/mL, através do método de *pour plate*. As placas foram armazenadas em jarras de anaerobiose (BBL, GasPak® SystemBecton Dickinson Microbiological System, Cockeysville, Maryland, USA) e incubadas por 48 horas a 37°C.

4.8 Monitoramento de pH

A determinação do pH foi realizada através do uso de um pHmetro modelo Q-400M1 (Quimis, São Paulo, Brasil).

4.9 Determinação de glicose, lactose e ácido lático

A determinação das concentrações de lactose, glicose e de ácido lático foi realizada mediante cromatografia líquida de alta eficiência (CLAE), de acordo com o método descrito por Donkor et al. (2007). Previamente, cada amostra foi centrifugada a 15.000×g por 20 minutos, utilizando uma microcentrífuga U-32R (Boeckel, Hamburg, Germany) para remoção de microrganismos e separação do sobrenadante. Posteriormente, a amostra foi diluída para a concentração entre 0,5 e 2 g/L, filtrada, através de membrana de poro 0,45 µm (Millipore), e injetada em cromatógrafo líquido Ultimate 3000 (Dionex, Sunnyvale, CA, Estados Unidos) com detector por Índice de Refração (Shodex, Kawasaki, Kanagawa, Japan), a 35 °C, coluna HPX-87H (Bio-Rad, Hercules, CA, Estados Unidos), a 45 °C, fase móvel de H2SO4 (5 mM) com fluxo de 0,6 mL/min.Soluções de glicose, galactose, lactose e ácidos orgânicos (10 g/l cada) foram preparadas nas concentrações de 0,1; 0,2; 0,4; 0,8; 1,0 e 2,0 g/l e utilizadas como padrão para elaboração da curva de calibração. A quantificação dos açúcares foi executada através de curvas-padrão previamente obtidas com soluções-padrão de glicose e lactose (Sigma™, St. Louis, MO, USA) de alta pureza para uso em CLAE. Todas estas análises foram realizadas em triplicata.

4.10 Determinação da atividade da bacteriocina

Para a extração da bacteriocina, a amostra retirada do meio de cultivo foi centrifugada a 13.201g, a 10ºC, por 10 minutos, para se retirar a biomassa. O sobrenadante foi retirado, para as análises de atividade da bacteriocina, e o pH foi corrigido para 6,0, para que não houvesse interferência nos testes de atividade. A amostra foi então aquecida a 90ºC, por 10 minutos, para eliminação da influência de peróxidos produzidos pela *B. lactis* (ODAMAKI et al., 2011) e para eliminação de possíveis microrganismos contaminantes. Após homogeneização em vórtice, 40µl foram aplicados em poços no ágar de cultura BHI (DIFCO^{TM}, Sparks, MD, USA), que, por sua vez, estava pré-inoculado com o microrganismo indicador (*Lactobacillus sakei* ATCC 15521, *Escherichia coli* ATCC 25922 ou *Listeria monocytogenes* ATCC 13932).

4.11 Análises estatísticas

As análises estatísticas foram realizadas utilizando o teste de Tukey HSD. Para os resultados de determinação de velocidade de agitação do meio de cultivo, concentração de glicose, lactose e ácido lático, foi utilizada a One-way ANOVA. Para os resultados de estudo de atividade de bacteriocina, velocidade específica de crescimento e crescimento celular, foi utilizada a análise de variância fatorial (Factorial ANOVA).

5. RESULTADOS E DISCUSSÃO

5.1 Influência da velocidade de agitação dos meios de cultivo na contagem microbiana

O gênero *Bifidobacterium* é desprovido de catalase, uma enzima essencial para degradar H_2O_2 e, portanto, para tal, deve contar com enzimas como a NADH oxidase e a NADH peroxidase, para eliminar o oxigênio do meio de cultivo (ODAMAKI et al., 2011). Considerando a *B. lactis* uma espécie de bactéria aerotolerante (LI et al., 2010), foi realizado, em meio de cultura BSM, um estudo prévio de crescimento de *B. lactis* variando a agitação (25, 50 e 100 rpm) do caldo durante o processo fermentativo a 37ºC, por 30 horas. Como demonstrado na Tabela 1, a *B. lactis* apresentou melhor crescimento (13.10^8, ou seja, 9,13 log UFC/mL) a 50 rpm, ao final de 30 horas do cultivo fermentativo.O valor das contagens foi, em média, 108% superior ao alcançado a 25 rpm ($6,5.10^8$ ou 8,81 log UFC mL) e 182% superior em relação a 100 rpm ($4,8.10^8$ ou 8,68 log UFC/mL). Estes dados reforçam os estudos sobre a sensibilidade da *B. lactis*à presença de oxigênio, quando o estudo ocorre na agitação de 100 rpm. Porém, o crescimento também é defasado a baixas rotações (25 rpm), já que esta condição possivelmente dificulta a dispersão de nutrientes no meio de cultivo (ROSS; FIEGEL, 2012).

Tabela 1. Concentração celular de *Bifidobacterium lactis* após 30 horas de cultivo a diferentes agitações (*shaker*) utilizando meio BSM. Legenda: diferentes letras na mesma coluna significam que há diferença estatística entre os valores (P < 0,05).

Agitação (rpm)	Contagem (log UFC/mL)
25	$8,81 \pm 0,04^A$
50	$9,13 \pm 0,03^B$
100	$8,68 \pm 0,06^C$

5.2 Meio de cultivo BSM suplementado com L-cisteína, extrato de levedura, inulina e Tween 80

O meio BSM foi escolhido como meio de cultivo controle, pois é um meio sintético específico para o crescimento de bactérias do gênero *Bifidobacterium*, e sua suplementação foi feita para fins comparativos com os testes realizados tanto em soro de leite como em meio de cultura MRS. O meio de cultivo BSM geralmente é formulado a partir do MRS; porém, com a suplementação de antibióticos, como a “mupirocina” e a “nistatina” (SIMPSON et al., 2004, ASHRAF; SHAH, 2011). No entanto, o meio de cultivo utilizado no presente trabalho foi o BSM patenteado pela Sigma™, cuja formulação não é descrita.

Como pode ser observado na Figura 1, a *B. lactis* apresentou crescimento exponencial contínuo entre 6 e 24 horas de fermentação. A maior curva de crescimento foi observada no caldo BSM suplementado com extrato de levedura, apresentando contagem de 9,9 log UFC/mL. Este crescimento foi 108% maior em relação ao caldo BSM suplementado por cisteína, inulina ou Tween 80, após 27 horas de cultivo, no qual a contagem de *B. lactis* foi, em média, 9,5 log UFC/mL. Em caldo BSM sem adição de suplementos (controle), a contagem, após 27 horas de cultivo, foi de 9,4 Log UFC/mL. O meio de cultivo BSM suplementado com extrato de levedura apresentou crescimento 315% maior em relação ao meio BSM não suplementado. Uma hipótese para explicar esse comportamento seria que a concentração de proteínas presentes no caldo BSM foi o principal fator limitante, em relação aos outros nutrientes presentes, para o crescimento de *Bifidobacterium animalis* subsp. *lactis* HN019. Por outro lado, na Figura 2, pode-se observar que não houve variação expressiva do pH entre as diferentes suplementações em caldo BSM. Isso se deve,hipoteticamente, à presença de substâncias tamponantes na formulação do caldo BSM.

A concentração de glicose foi determinada no final da fase exponencial de crescimento, ou seja, após 24 horas do cultivo fermentativo. O meio BSM suplementado com extrato de levedura apresentou a menor concentração de glicose (0,027 g/L) em relação à concentração apresentada em presença de outros ingredientes estudados no caldo BSM, conforme apresentado na Tabela 2. A baixa concentração de glicose ao final do cultivo no meio BSM suplementado com extrato

de levedura pode estar relacionada à curva de crescimento apresentada na Figura 1, na qual este caldo apresentou a maior concentração celular no final da fase exponencial (9,8 log UFC/mL). O teor inicial de glicose no meio de cultura BSM foi de 4,2 g/L (resultados não apresentados).

Comparando-se o crescimento da cepa de *B. lactis* estudada no presente trabalho com outras espécies do gênero *Bifidobacterium* em meio de cultivo BSM, a *B. lactis* apresentou crescimento maior(9,8 log UFC/mL) em relação à *Bifidobacterium bifidum* NCIMB 700795 (8,86 log UFC/mL) e à *Bifidobacterium bifidum* NCIMB 702203 (7,36 Log UFC/mL) (SIMPSON et al., 2004). Já no caso de espécies, como *Bifidobacterium longum* NCIMB702259T (9,30 log UFC/mL) e *Bifidobacterium thermophilum* NCIMB 702254T (9,62 log UFC/mL) (SIMPSON et al., 2004), a *B. lactis* apresentou crescimento semelhante, porém ainda superior.

Quando o meio de cultivo BSM é utilizado para o crescimento de outros gêneros de bactérias, como *Bacillus*, *Lactobacillus* e *Streptococcus*, estes apresentam crescimento quase nulo, como pôde ser observado com*Bacillus cereus* ATCC 9139, *Lactobacillus bulgaricus* ATCC 11842T e *Streptococcus cremoris* ATCC 19257T, as quais apresentaram crescimento menor do que 2,0 log UFC/mL (SIMPSON et al., 2004). Esse baixo crescimento de outros gêneros pode ser explicado pela presença de antibióticos no meio de cultivo (SIMPSON et al., 2004).

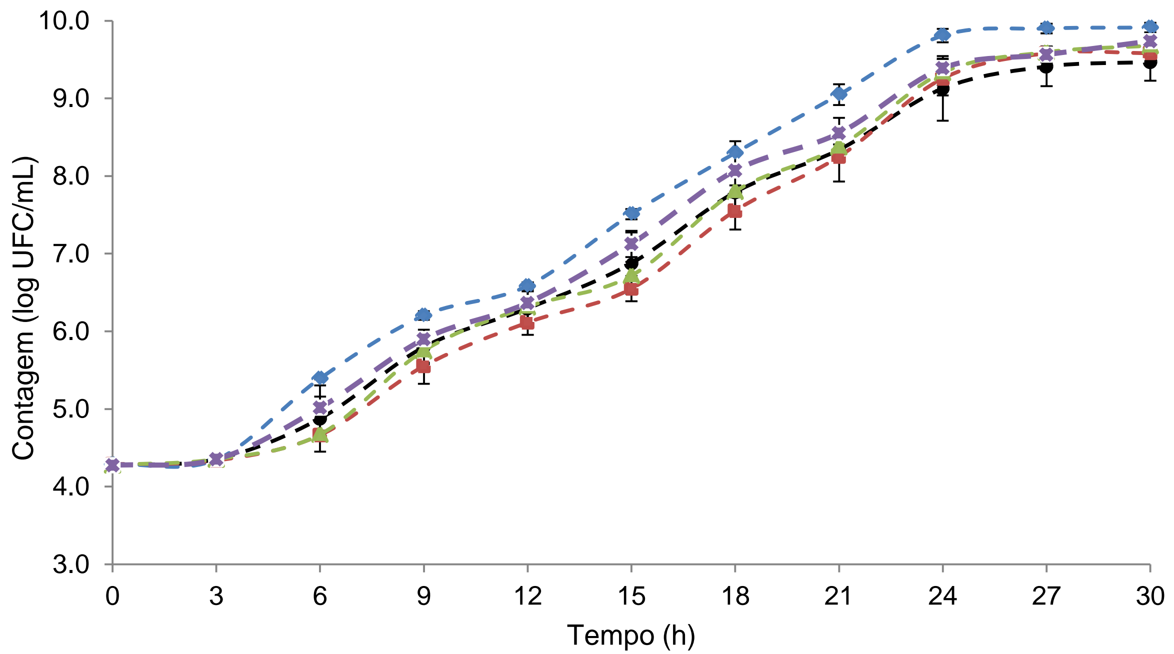

Figura 1. Curva de crescimento de *Bifidobacterium lactis* utilizando meio de cultivo BSM suplementado com extrato de levedura (EL), L-cisteína (Cl), Tween 80 (T_{80}) e inulina (I). Legendas: Controle (●); EL (♦); Cl (■); T_{80} (▲); I (X).

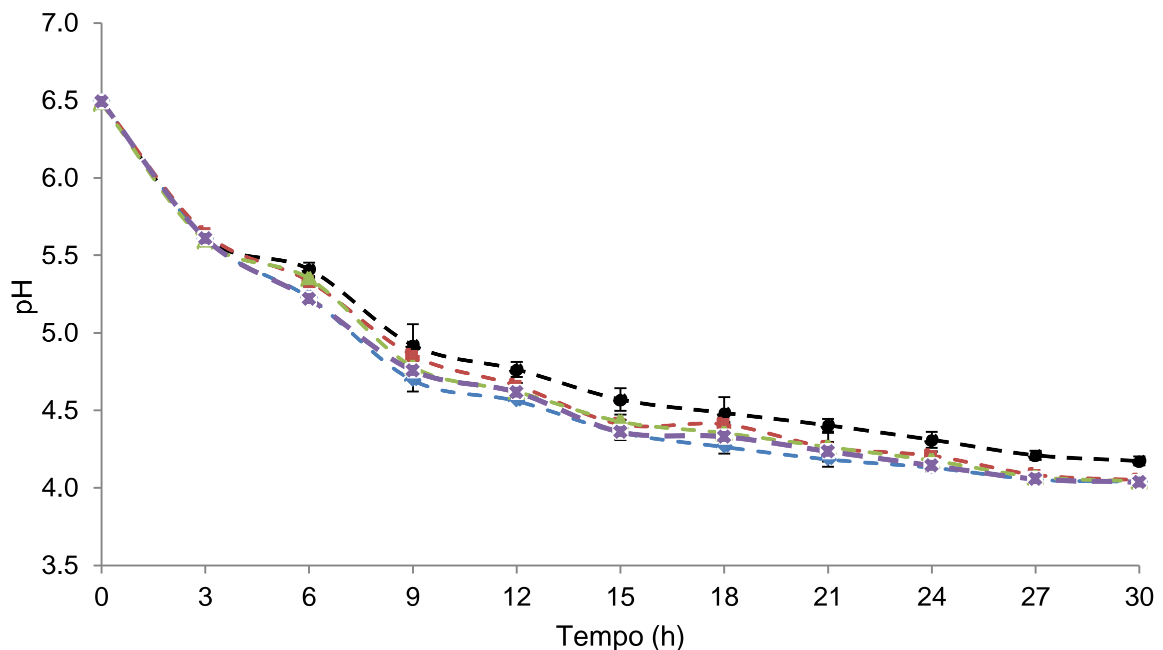

Figura 2. Curva de pH de *Bifidobacterium lactis* utilizando caldo BSM suplementado com extrato de levedura (EL), L-cisteína (Cl), Tween 80 (T_{80}) e inulina (I). Legendas: Controle (●); EL (♦); Cl (■); T_{80} (▲); I (X).

Tabela 2. Concentração de glicose (g/L) em meio de cultivo BSM suplementado com extrato de levedura (EL), Tween 80 (T_{80}), L-cisteína (CI) e inulina (I) após 27 horas de cultivo (início da fase estacionária).

Meio de cultura	Concentração de Glicose (g/L)
BSM	0,745 ± 0,031
BSM + T_{80}	0,428 ± 0,032
BSM + I	0,649 ± 0,015
BSM + CI	0,514 ± 0,021
BSM + EL	0,027 ± 0,029

5.3 Caldo MRS suplementado com L-cisteína, extrato de levedura, inulina ou Tween 80

O meio de cultura MRS é geralmente o mais utilizado para o crescimento do gênero *Bifidobacterium* em laboratórios de análise de controle de qualidade industrial, já que fornece condições ideais para o crescimento não seletivo de bifidobactérias (ROY, 2001). Logo, assim como o meio BSM, o meio MRS foi escolhido para os estudos de produção de biomassa, determinação de pH, consumo de glicose e produção de bacteriocina em relação ao soro de leite.

A determinação da curva de crescimento de *Bifidobacterium lactis* em caldo MRS, suplementado com L-cisteína, extrato de levedura, inulina, Tween 80 ou sem suplementação (controle), foi obtida nas condições de temperatura a 37°C, agitação de 50 rpm e tempo de cultivo de 30 horas. Na Figura 4, observa-se que houve crescimento celular durante todo o processo fermentativo, no qual a fase *Log* se encontra entre 6 e 27 horas de fermentação. A fase *Log*, quando utilizado o caldo MRS, foi 3 horas maior em relação ao meio de cultivo BSM; ou seja, o crescimento da *B. lactis* foi mais rápido em caldo BSM do que em MRS.

No tempo de 27 horas de cultivo, o valor de contagem das células de *B. lactis* foi 9,43 log UFC/mL em caldo suplementado com 1% de extrato de levedura, apresentando o melhor resultado, juntamente com o caldo suplementado por L-cisteína (9,32 log UFC/mL), comparado com inulina (8,8 log UFC/mL) e Tween 80 (8,6 log UFC/mL) (Figura 4). Assim como foi observado no caldo BSM (seção 6.2), o caldo MRS apresentou crescimento celular maior quando foi suplementado com extrato de levedura. Este resultado já era esperado, pois ambos os meios de cultivo possuem formulações semelhantes.

A Figura 4 representa a curva de pH durante o processo fermentativo, quando adicionados os ingredientes estudados ao caldo MRS. O valor de pH do meio suplementado com Tween 80 foi o mais elevado durante a maior parte do processo fermentativo. No final do processo fermentativo (após 30 horas de fermentação), o valor de pH no caldo MRS suplementado com inulina, L-cisteína e extrato de levedura foi de, em média, 4,2, sendo que, no caldo MRS suplementado com Tween 80, foi de 4,7.

Na Tabela 3, pode-se observar que o caldo MRS suplementado com extrato de levedura (0,425 g/L) apresentou menor concentração de glicose, juntamente com o caldo MRS suplementado com L-cisteína (0,430 g/L), em relação ao caldo MRS suplementado com Tween 80 (0,473 g/L) e ao caldo MRS suplementado com inulina (0,441 g/L). O teor de glicose, ao final da fase *Log*, apresenta diferença concomitantemente menor em proporcionalidade em relação aos resultados observados nas curvas de crescimento celular (Figura 3), com diferença deaproximadamente 10% entre a maior e a menor concentração de glicose. O caldo MRS suplementado com Tween 80 apresentou diferença estatística em relação à adição de L-cisteína e extrato de levedura; porém, não apresentou diferença significativa quando suplementado com inulina. Por outro lado, os meios de cultivo MRS, suplementados com inulina, L-cisteína e extrato de levedura, não apresentaram diferença significativa entre si. O teor inicial de glicose do meio MRS suplementado foi de 1,50 g/L (resultado não apresentado).

O crescimento da *Bifidobacterium animalis* subsp. *lactis* HN019 em meio de cultura MRS suplementado com L-cisteína se mostrou muito semelhante ao da cepa *Bifidobacterium animalis* subsp. *lactis* E2010 estudada por Mättö et al. (2006), no qual os autores utilizaram a mesma composição de meio de cultivo. Na *B. lactis*

E2010 estudada por Mättö et al. (2006), a concentração de biomassa encontrada foi de aproximadamente 8,8 log UFC/mL, após 24 horas de fermentação, enquanto que na *B. lactis* HN019, estudada no presente trabalho, o crescimento foi de 8,9 log UFC/mL.

O crescimento da cepa *B. lactis* em meio MRS suplementado com extrato de levedura (9,4 log UFC/mL) foi superior quando comparado com outras espécies do mesmo gênero cultivadas em meio BSM, como as cepas *Bifidobacterium bifidum* NCIMB 700795 (8,9 log UFC/mL) e *Bifidobacterium bifidum* NCIMB 702203 (7,34 log UFC/mL) (SIMPSON et al., 2004). Em relação às cepas *Bifidobacterium longum* NCIMB 702259T (9,30 log UFC/mL) e *Bifidobacterium thermophilum* NCIMB 702254T (9,62 log UFC/mL), o crescimento da cepa *B. lactis* foi semelhante (SIMPSON et al., 2004).

Quando o meio de cultivo MRS suplementado com L-cisteína foi utilizado para o crescimento de outros gêneros de bactérias, que não o *Bifidobacterium,* estas apresentam crescimento inferior ao da *B. lactis* (9,3 log UFC/mL), como pode ser observado em *Bacillus cereus* ATCC 9139 (7,19 log UFC/mL), *Lactobacillus bulgaricus* ATCC 11842T (8,30 log UFC/mL) e *Streptococcus cremoris* ATCC 19257T (7,09 log UFC/mL) (SIMPSON et al., 2004).

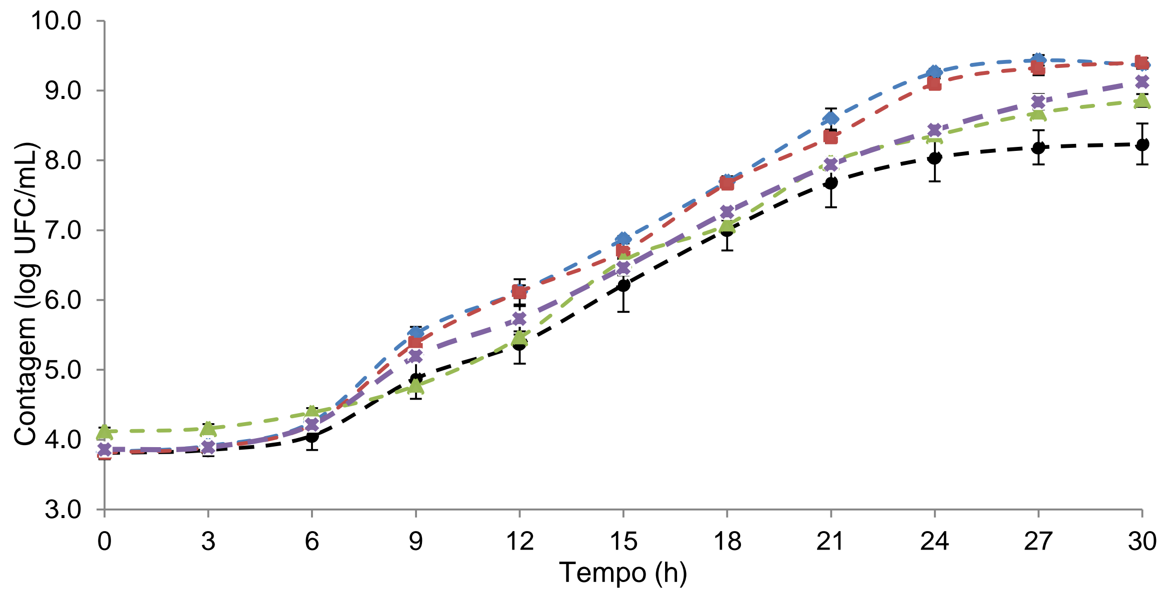

Figura 3. Curva de crescimento de *Bifidobacterium lactis* utilizando caldo MRS suplementado com extrato de levedura (EL), L-cisteína (CI), Tween 80 (T_{80}) e inulina (I). Legendas: Controle (●); EL (♦); CI (■); T_{80} (▲); I (X).

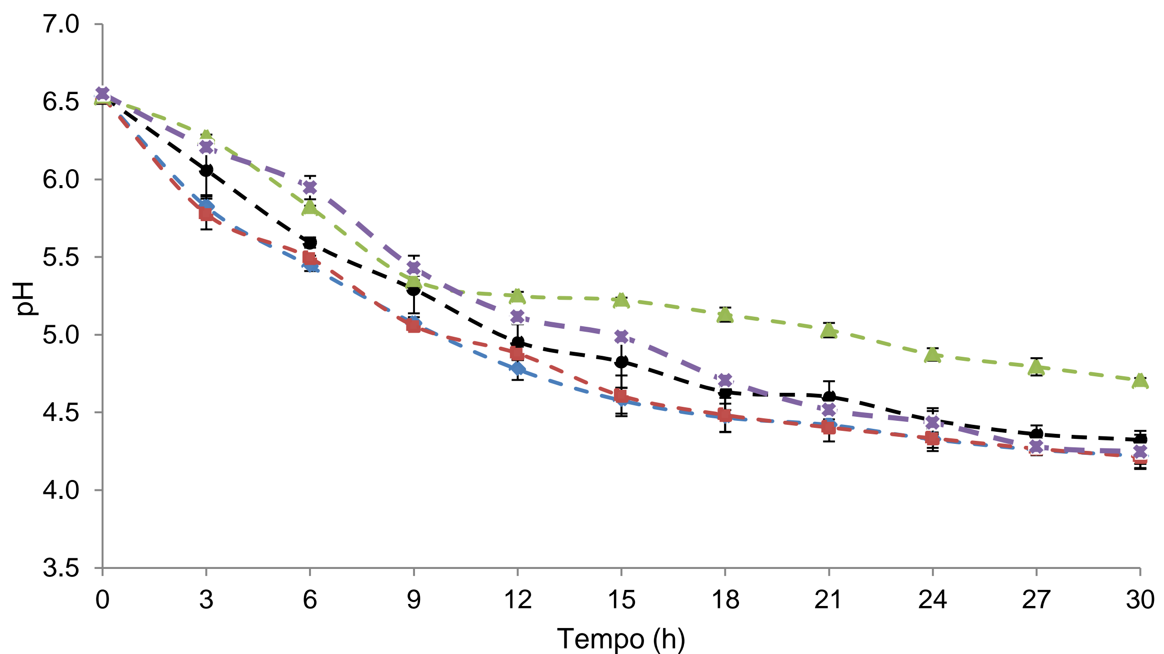

Figura 4. Curva de pH de *Bifidobacterium lactis* utilizando caldo MRS suplementado com extrato de levedura (EL), L-cisteína (CI), Tween 80 (T_{80}) e inulina (I). Legendas: Controle (●); EL (♦); CI (■); T_{80} (▲); I (X).

Tabela 3. Concentração de glicose (g/L) caldo MRS suplementado com extrato de levedura (EL), Tween 80 (T_{80}), L-cisteína (CI) e inulina (I) após 27 horas de cultivo (início da fase estacionária).

Meio de cultura	Concentração de Glicose (g/L)
MRS	0,478 ± 0,012
MRS + T_{80}	0,473 ± 0,014
MRS + I	0,441 ± 0,011
MRS + CI	0,430 ± 0,009
MRS + EL.	0,425 ± 0,017

5.4 Soro de leite suplementado com L-cisteína, extrato de levedura, inulina ou Tween 80

Os resultados obtidos dos estudos da determinação do teor de sólidos totais que proporcionou o melhor crescimento celular de *B. lactis* estão expostos na seção 5.4.1.

5.4.1 Determinação do teor de sólidos totais em soro de leite controle e crescimento celular

O soro de leite utilizado no presente trabalho foi constituído de aproximadamente 60% de lactose, 15% de proteína e 1% gordura, além de cálcio, sódio e ferro (ANTUNES, 2003). Portanto, visando a maior produção de biomassa de *B. lactis* e considerando o soro de leite um meio complexo, houve a necessidade de se

elaborar um estudo prévio, variando a sua concentração (5, 10, 15, 20 e 25 % p/v), para que, a seguir, fosse realizado estudo de suplementação com os ingredientes estudados no presente trabalho.

Na figura 5, no tempo de 27 horas de cultivo, o valor de contagem das células foi 8,87 log UFC/mL em soro de leite com concentração de 10% p/v, apresentando o melhor resultado, comparado a 5% (8,71 log UFC/mL), 15% (8,26 log UFC/mL), 20% (7,44 log UFC/mL) e 25% p/v (7,28 log UFC/mL).

Apesar de o soro de leite com concentração de 5% apresentar uma curva de crescimento muito próxima à curva do soro com concentração de 10% (Figura 5), o valor de pH do soro de leite a 5% de sólidos totais diminuiu e se estabilizou ao nível de acidez mais baixo (pH 4,19) (Figura 6), dentre as medições na amostra após 21 horas de cultivo, enquanto que o soro de leite com concentração 10% só atingiu este nível após 30 horas de cultivo. Deve ser lembrado que a acidificação do meio prejudica o crescimento celular da *B. lactis* (JALILI; RAZAVI; SAFARI, 2010).

Como pode ser observado na Tabela 4, houve maior consumo de lactose pela *B. lactis* no meio de cultura natural, composto por soro de leite, na concentração de 5 e 10%, no qual o consumo desse dissacarídeo, do início ao final da fermentação, foi de 49 e 37%, respectivamente, do total de lactose inicial. No soro de leite, em concentrações de 15, 20 e 25%, houve o consumo de apenas 19, 17 e 15%, respectivamente, do total de lactose inicial. Todos os resultados observados na Tabela 4 apresentam diferença estatística significativa entre si.

Através dos resultados discutidos acima, pode-se observar que o soro de leite, nas concentrações 15, 20 e 25% (p/v), apresenta teor de sólidos totais muito elevado para o crescimento adequado da *B. lactis*. Apesar de o soro de leite com concentração 5% apresentar bom consumo de lactose, as curvas de crescimento e pH observadas nas figuras 5 e 6 apresentam resultados inferiores, quando comparados aos apresentados nas curvas desse meio de cultivo natural na concentração de 10%, sendo, portanto, o meio de cultivo escolhido para os estudos nos quais foram adicionados os ingredientes em questão.

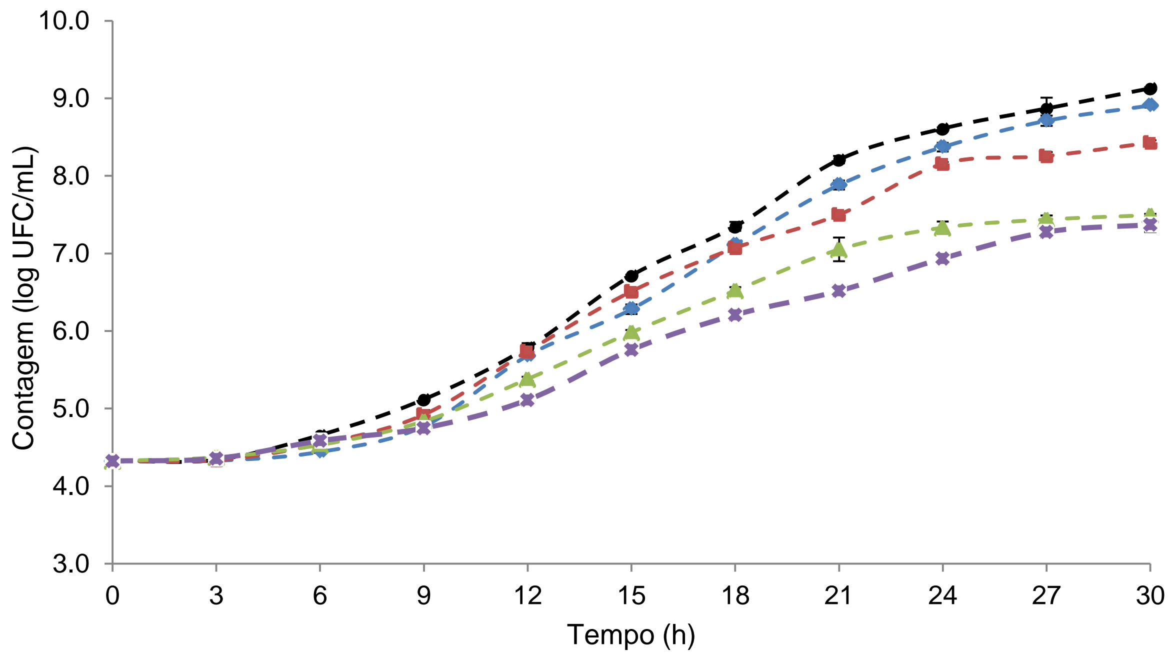

Figura 5. Curva de crescimento de *Bifidobacterium lactis* nas variações de concentração de soro de leite a 5, 10, 15, 20 e 25 % p/v. Legendas: 5% (♦); 10% (●); 15% (■); 20% (▲); 25% (X).

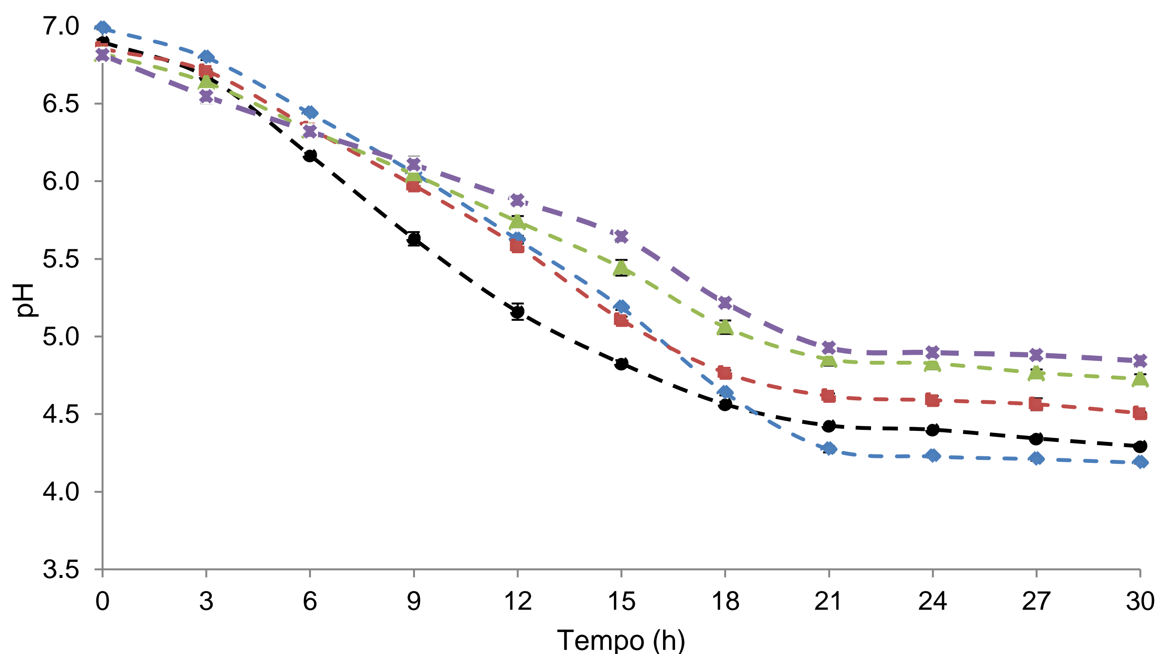

Figura 6. Curva de pH de *Bifidobacterium lactis* nas variações de concentração de soro de leite a 5, 10, 15, 20 e 25 % p/v. Legendas: 5% (♦); 10% (●); 15% (■); 20% (▲); 25% (X).

Tabela 4. Determinação da concentração de lactose no tempo inicial (T_0) e no tempo final de cultivo (**T_{30}**) de *Bifidobacterium lactis* utilizando soro de leite em diferentes concentrações.

Concentração do soro de leite no meio	**Concentração de lactose (g/L) em T_0***	**Concentração de lactose (g/L) em T_{30}****	**% de lactose consumida**
5%	24,9 ± 0,5	12,7 ± 0,3	49%
10%	54,3 ± 0,8	34,4 ± 0,3	37%
15%	76,1 ± 0,4	62,4 ± 0,7	18%
20%	109,1 ± 0,8	91,3 ± 1,2	17%
25%	129,7 ± 1,1	117,8 ± 0,9	9%

*T_0: tempo inicial da fermentação (0 h)

**T_{30}: Tempo final da fermentação (30 h)

5.4.2 Soro de leite a 10% de sólidos totais suplementado com L-cisteína, extrato de levedura, inulina ou Tween 80

Nesta etapa, foram realizados ensaios utilizando o soro de leite como meio de cultivo natural suplementado com extrato de levedura, L-cisteína, inulina ou Tween 80. As figuras 7 e 8 se referem ao crescimento celular e ao pH, respectivamente, durante o processo fermentativo (30 horas). Com relação ao crescimento celular (Figura 7), nota-se que, após 27 horas, as contagens de *B. lactis* foram maiores (9,8 log UFC/mL) no processo que forom adicionados inulina ou extrato de levedura, quando comparados com os valores obtidos nas amostras de soro de leite suplementado com L-cisteína (8,8 log UFC/mL) e com Tween 80 (9,2 log UFC/mL). Em trabalhos recentes, a inulina foi usada como importante indutora de crescimento para bifidobactérias (OLIVEIRA et al., 2009a, 2009b, 2009c).

A fase estacionária se iniciou após 24 horas de fermentação quando o soro de leite foi suplementado com extrato de levedura e inulina, enquanto que no soro de leite suplementado com L-cisteína, a fase estacionária se iniciou após 27 horas de

fermentação. Quando o soro de leite foi suplementado com Tween 80, a fase estacionária se iniciou após 30 horas de fermentação. Esta diferença de tempo que o microrganismo chega à fase estacionária se deve às diferentes velocidades de crescimento durante a fase exponencial; ou seja, quando o microrganismo apresenta velocidade de crescimento elevada, o mesmo atinge o limite de crescimento para as condições presentes no meio de cultivo, entrando, assim, em fase estacionária.

As curvas correspondentes aos valores de pH (Figura 8) referem-se aos resultados de crescimento da *B. lactis*, que são observados nas curvas de crescimento celular (Figura 7), ou seja, o soro de leite suplementado com inulina ou com extrato de levedura apresenta, respectivamente, pós-acidificação mais acentuada em relação aos meios de cultivo naturais suplementados com Tween 80 ou com L-cisteína.

O crescimento acentuado observado no meio soro de leite suplementado com extrato de levedura pode ser explicado devido à baixa concentração de peptídeos e aminoácidos presentes no meio, os quais são necessários para o crescimento celular (ANTUNES, 2003). A Tabela 5 mostra que houve pouca diferença na concentração de lactose no final da fase exponencial de crescimento celular (27 horas). A concentração inicial de lactose é de 53,0 g/L, para todas as suplementações estudadas no soro de leite (resultado não apresentado na Tabela 5). Através da análise estatística, pode-se observar que só houve diferença significativa no consumo de lactose através da suplementação do soro de leite com extrato de levedura a 1% v/v, já que esta suplementação apresentou o maior valor de concentração celular durante as 30 horas de cultivo e, portanto, o maior consumo de nutrientes.

Na Tabela 5, na qual é apresentada a concentração de ácido lático, pode-se observar que, no soro de leite suplementado com inulina (4,55 g/L), houve concentração desse ácido orgânico maior do que em relação ao soro de leite suplementado com extrato de levedura (3,23 g/L), havendo diferença estatística entre os valores. A produção de ácido lático durante ocultivo em soro de leite é observada em diversos gêneros de bactérias (TANG; YANG; OKOS, 1988).

O extrato de levedura se mostrou um excelente suplemento para o crescimento de *B. lactis* em soro de leite, pois este meio de cultivo natural é carente de peptídeos pequenos e aminoácidos livres (GOMES et al., 1998). Como já foi observado por Gomes et al. (1998), a *B. lactis* apresenta crescimento superior em soro de leite

hidrolisado suplementado com aminoácidos em relação ao soro de leite não suplementado. Em outros estudos, Loquasto et al. (2011) comprovaram que o soro de leite a 10% v/v suplementado com 1% de extrato de levedura é um bom meio de cultivo para *B. lactis*.

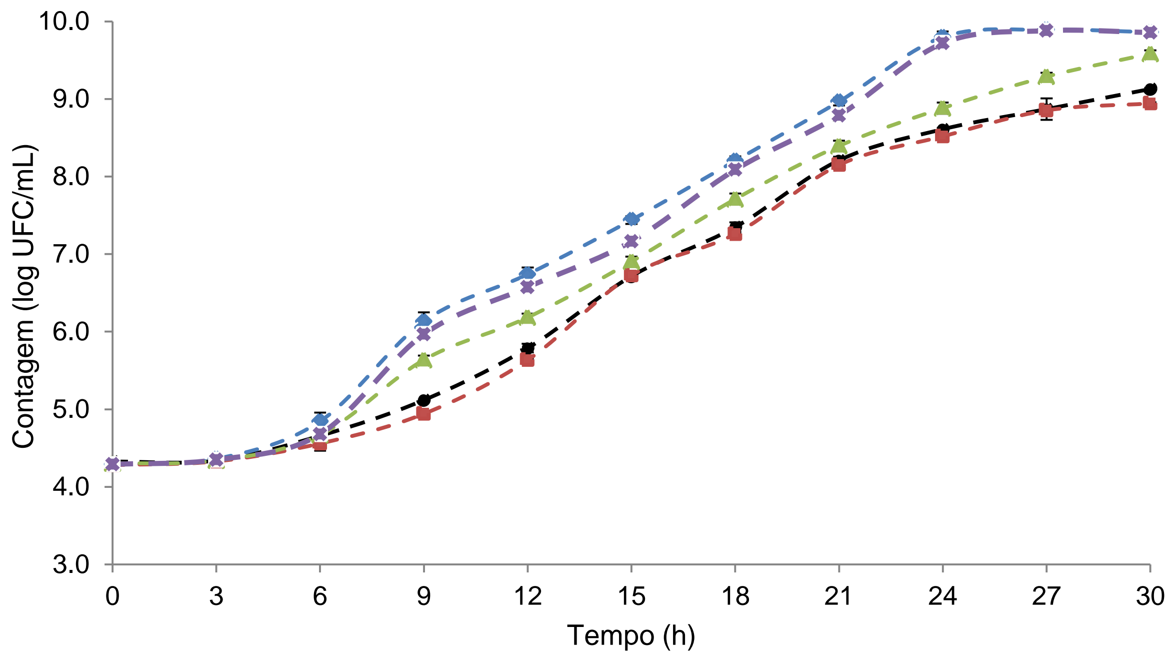

Figura 7. Curva de crescimento de *Bifidobacterium lactis* utilizando soro de leite suplementado com extrato de levedura (EL), L-cisteína (CI), Tween 80 (T_{80}) e inulina (I). Legendas: Controle (●); EL (♦); CI (■); T_{80} (▲); I (X).

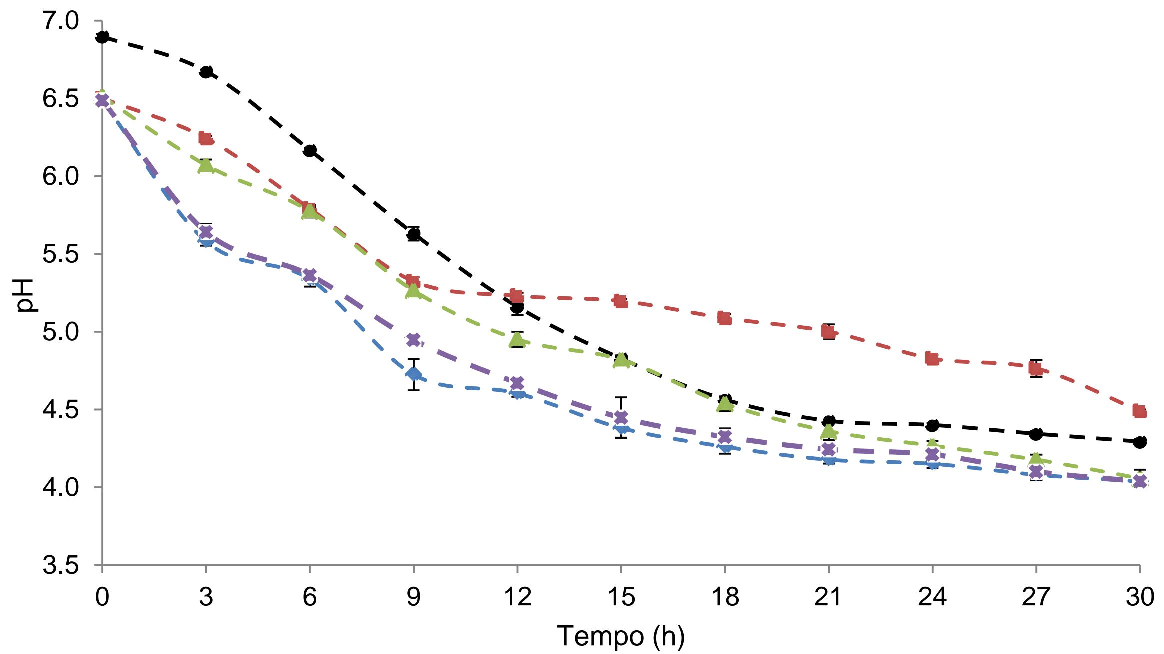

Figura 8. Curva de pH de *Bifidobacterium lactis* utilizando soro de leite suplementado com extrato de levedura (EL), L-cisteína (CI), Tween 80 (T_{80}) e inulina (I). Legendas: Controle (●); EL (♦); CI (■); T_{80} (▲); I (X).

Tabela 5. Concentração de lactose e ácido lático (g/L) em caldo soro de leite (SL) suplementado com extrato de levedura (EL), Tween 80 (T_{80}), L-cisteína (CI) e inulina (I) após 27 horas de cultivo (início da fase estacionária). Legenda:diferentes letras na mesma coluna significam que há diferença estatística entre os valores (P < 0,05).

Meio de cultura	Lactose (g/L)	Ácido Lático (g/L)
SL + T_{80}	34,6± 0,3^{B}	4,63± 0,21^{C}
SL + I	33,9± 0,4^{B}	4,55± 0,14^{C}
SL + CI	34,2± 0,3^{B}	4,37± 0,18^{C}
SL + EL	32,5± 0,6^{A}	3,23± 0,12^{D}

5.5 Estudo comparativo entre *Bifidobacterium animalis* e *Bifidobacterium lactis*

O estudo comparativo da *Bifidobacterium animalis* subsp. *lactis* HN019 com outra cepa de mesmo gênero foi necessário devido a atividade insatisfatória da bacteriocina produzida por esta cepa. A cepa *Bifidobacterium animalis* subsp. *animalis* ATCC 25527 foi escolhida, pois pertence à mesma espécie que *B. lactis* e apresenta bons resultados de atividade, como observado por Martinez et al. (2013).

Tanto a *B. lactis* como a *B. animalis* apresentaram crescimento maior, quando cultivadas em meio soro de leite suplementado com extrato de levedura em relação ao meio não suplementado, em estudos realizados por Loquasto et al. (2011). Porém, no presente estudo, o crescimento e a atividade da bacteriocina produzida por *B. animalis*foram inferiores aos de *B. lactis*, quando esta foi cultivadaneste meio. Na Figura 8, é apresentada a curva de crescimento celular de *B. lactis* e *B. animalis*. Em particular, no final da fase exponencial de *B. lactis* (24 horas de cultivo), a contagem foi de 9,79 log UFC/mL, sendo que, quando utilizado o *B. animalis*, o valor da contagem foi de 8,89 log UFC/mL, ou seja, uma concentração celular 8 vezes maior de *B. lactis* em relação à *B. animalis*.

Quando a curva de pós-acidificação de *B. lactis* é comparada com a curva obtida por *B. animalis*, pode-se observar que o valor de pH encontrado foi, em média, de 0,5 a 1 pH menor durante as 30 horas do processo fermentativo (Figura 9). Isso se deve à diferença no crescimento entre estas duas cepas, sendo que *B. lactis* apresenta crescimento e consequente acidificação do meio, através da produção e da excreção de ácido lático, maiores em relação à *B. animalis.* O pH encontrado por Loquasto et al. (2011), ao final de 24 horas de fermentação em meio soro de leite suplementado em ambos microrganismos, foi de 4,3, semelhante ao encontrado em *B. lactis* (pH 4,2) no presente estudo.

Loquasto et al. (2011) observaram que o crescimento da *B. animalis* e da *B. lactis* não foi diferente entre si em meio soro de leite, porém seu estudo foi realizado em anaerobiose completa e não em microaerofilia. Quando a *B. animalis* é cultivada em aerobiose, não há crescimento celular (LOQUASTO et al., 2011), porém, em meio MRS em anaerobiose, a concentração celular encontrada por Lima et al. (2009) foi

de 10,13 log UFC, evidenciando a sensibilidade do microrganismo à presença de oxigênio.

Em relação à atividade antimicrobiana frente à *L. monocytogenes* ATCC 13932, não houve diferença significativa entre as cepas *B. animalis* (190 AU/mL) e *B. lactis* (200 AU/mL). Estes resultados não justificariam o estudo da cepa *B. animalis* ATCC 25527 (como cepa comparada à *B. lactis*) em fermentador de bancada. O baixo desempenho da cepa *B. animalis* nas condições ótimas encontradas para *B. lactis* pode ser explicado devido a primeira ser um microrganismo anaeróbio estrito (LOQUASTO et al., 2011), diferentemente da *B. lactis*, que é um microrganismo anaeróbio aerotolerante (LI et al., 2010).

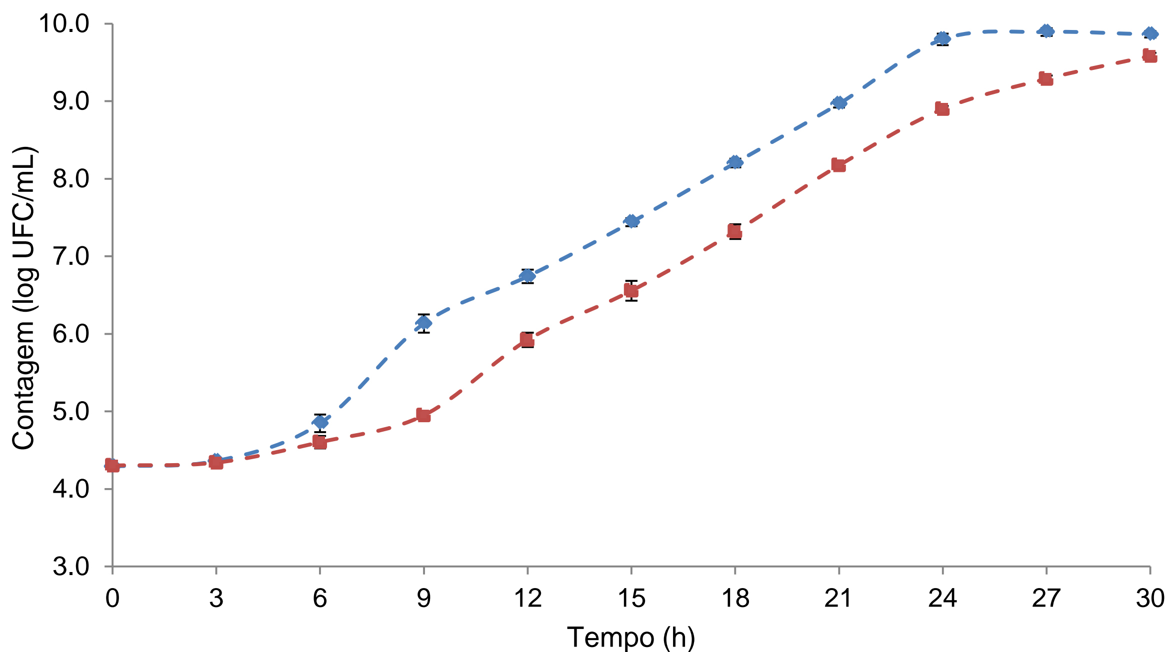

Figura 9. Curva de crescimento das cepas *Bifidobacterium lactis* (♦) e *Bifidobacterium animalis* (■) utilizando soro de leite suplementado com 1% de extrato de levedura.

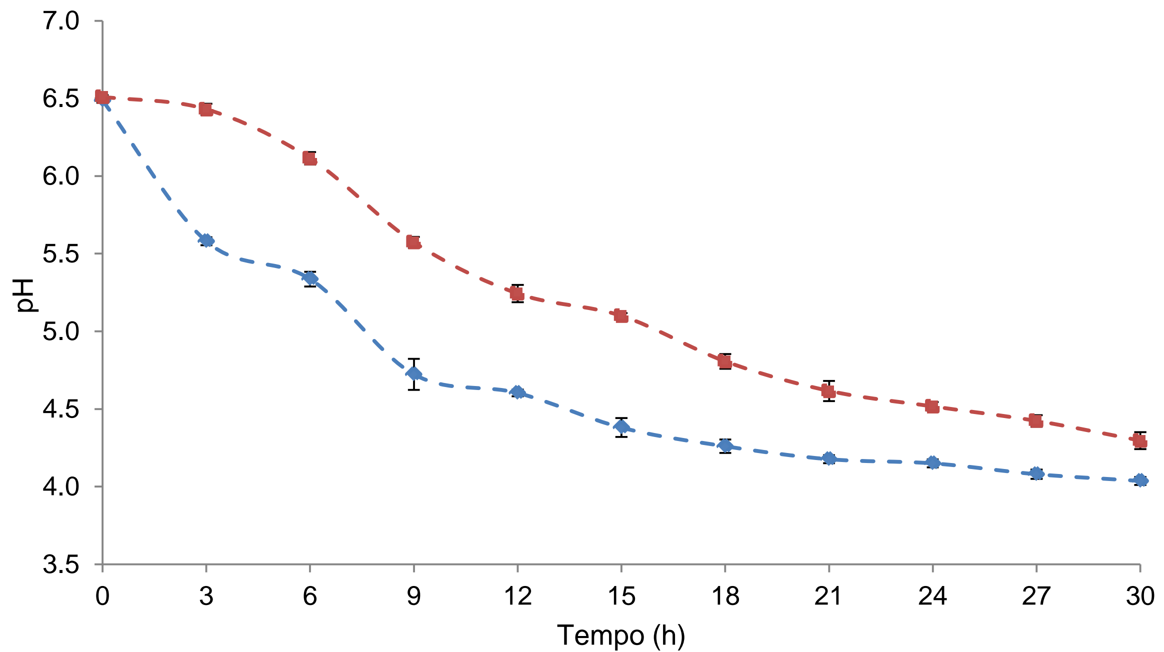

Figura 10. Curva de pH das cepas *Bifidobacterium lactis* (♦) e *Bifidobacterium animalis* (■) utilizando soro de leite suplementado com 1% de extrato de levedura.

5.6 Fermentador de bancada

Para os testes em fermentador de bancada, foi escolhido o meio de cultivo que proporcionou maior concentração de biomassa ao final da fase exponencial de crescimento de *B. lactis* e, associado a este crescimento, a maior atividade de bacteriocina produzida. Buscando estes resultados, as melhores condições e o melhor meio de cultivo encontrados para os testes em fermentador de bancada foram: soro de leite no teor de 10% (p/v) de sólidos totais, 1% (p/v) de extrato de levedura, 50 rpm de agitação e 37ºC de temperatura.

Nestas condições, a *B. lactis* mostrou crescimento maior e acelerado em fermentador de bancada, quando comparado ao cultivo em *shaker*, como pode ser observado na Figura 10. O microrganismo atingiu a fase exponencial, em média, 3 horas mais rápido quando cultivado em fermentador de bancada, situação na qual apresentou concentração celular (9,57 log UFC/mL) 3 vezes maior em relação ao cultivo em *shaker* (8,96 log UFC/mL). Em relação à pós-acidificação (Figura 11), pode-se observar que o comportamento do pH foi semelhante, tanto em *shaker*

como em fermentador de bancada, não havendo diferenças significativas durante as 30 horas de cultivo.

O crescimento de *B. lactis* em meio soro de leite suplementado com extrato de levedura foi superior ao encontrado por Jalili, Razavi e Safari (2010), após 24 horas (7,16 Log UFC/mL), quando o microrganismo foi submetido à condições semelhantes de crescimento e meio de cultivo: soro de leite suplementado por 1% de extrato de levedura, fermentador de bancada, anaerobiose, 37 ºC, porém a 60 rpm. A fase *Log* encontrada no estudo feito por Jalili, Razavi e Safari (2010) durou apenas de 12 a 14 horas, enquanto que a encontrada no presente trabalho durou, em média, 18 horas. Apesar do presente trabalho estudar a mesma espécie de bactéria utilizada em Jalili, Razavi e Safari (2010), a diferença nos resultados de crescimento pode estar relacionada à cepa do presente trabalho ter sido modificada geneticamente pela DANISCO, melhorando portanto a sua capacidade de crescimento.

Como foi discutido na seção 5.1, o crescimento de *B. lactis* é melhor em condições de anaerobiose em relação à microaerofilia. Este comportamento pode ser observado quando essa cepa é cultivada em fermentador de bancada, no qual houve injeção constante de nitrogênio no meio de cultivo.

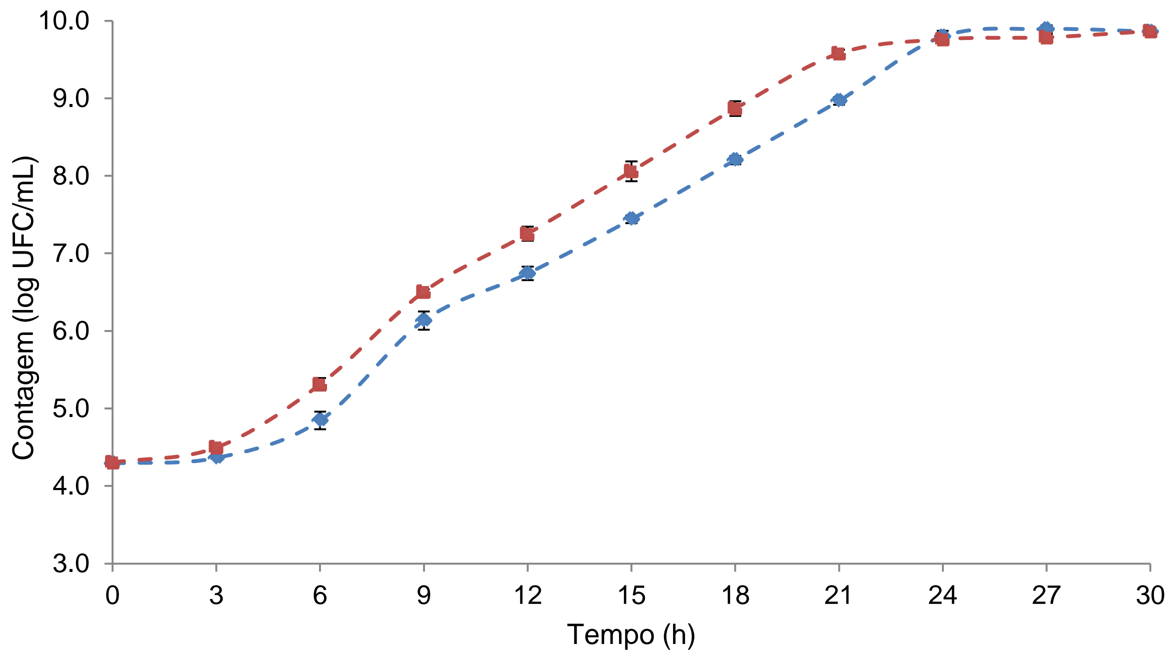

Figura 11. Curva de crescimento de *Bifidobacterium lactis* em fermentador de bancada (■) e em *shaker* (♦) utilizando soro de leite suplementado com 1% de extrato de levedura.

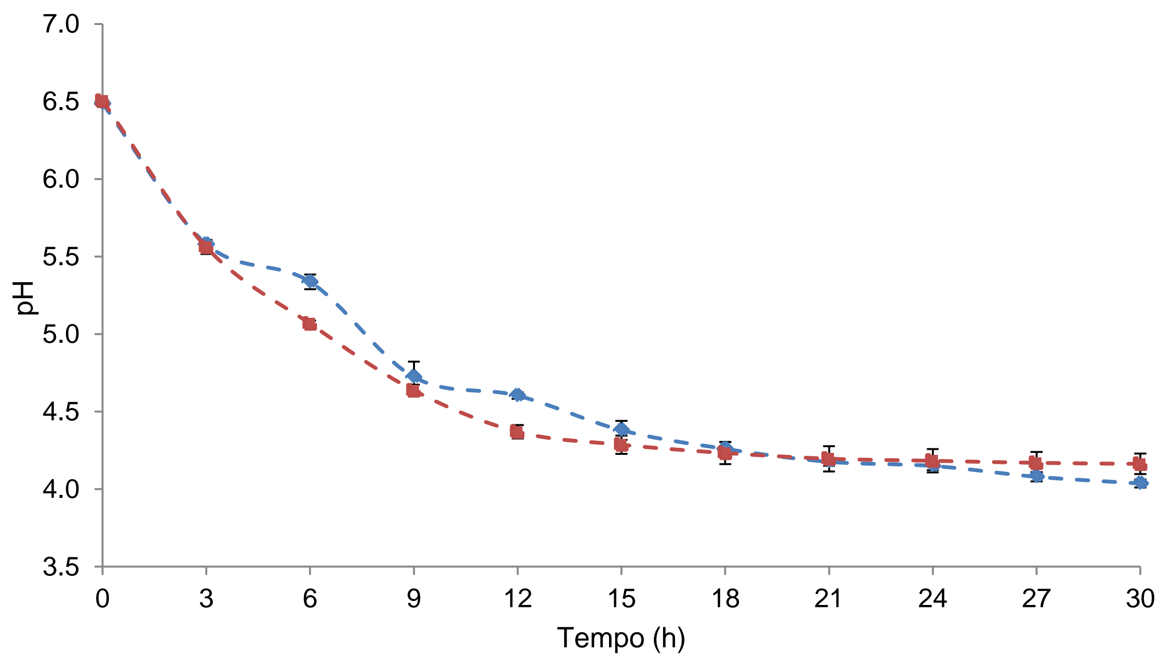

Figura 12. Curva de pH de *Bifidobacterium lactis* em fermentador de bancada (■) e em *shaker* (♦) utilizando soro de leite suplementado com 1% de extrato de levedura.

5.7 Velocidade específica de crescimento (μ_{max}) e tempo de geração (t_g)

Em particular, a velocidade específica de crescimento (μ_{max}) foi calculada para *B. lactis*, durante a sua fase de crescimento exponencial, segundo a equação de Monod (1942) à seguir:

$$\mu_{\max} = \frac{1}{(t_2 - t_1)} \ln \frac{X_2}{X_1}$$

em que X_2 e X_1 são as contagens (UFC/mL) nos tempos t_2 e t_1.

Posteriormente, o tempo de geração (t_g) foi calculado para cada cultura, a partir do valor correspondente de μ, através da equação (POWELL, 1956):

$$t_g = \frac{\ln 2}{\mu_{\max}}$$

Os dados de velocidades específicas de crescimento e tempo de geração estão apresentados na Tabela 6.

Tabela 6. Velocidades específicas de crescimento de *Bifidobacterium lactis* utilizando os meios de cultivo MRS, BSM e soro de leite (SL) em presença de inulina (I), L-cisteína (CI), Tween 80 (T_{80}), extrato de levedura (EL), BA (cultivo de *B. animalis* ATCC 25527 em *shaker* utilizando soro de leite suplementado com extrato de levedura) e F (cultivo de *B. lactis* em fermentador de bancada utilizando soro de leite suplementado com extrato de levedura).

Meio de cultura	**μ_{max} (h^{-1})**	**T_g (h)**
BSM	0,53	1,30
BSM + T_{80}	0,56	1,23
BSM + I	0,56	1,23
BSM + CI	0,57	1,21
BSM + EL	0,56	1,23
MRS	0,52	1,33
MRS + T_{80}	0,54	1,28
MRS + I	0,54	1,28
MRS + CI	0,60	1,15
MRS + EL	0,62	1,11
SL	0,54	1,28
SL + T_{80}	0,54	1,28
SL + I	0,61	1,13
SL + CI	0,54	1,28
SL + EL	0,61	1,13
BA	0,57	1,21
F	0,57	1,21

Na Tabela 6, observa-se que a velocidade específica de crescimento ($μ_{max}$), no meio de cultura MRS suplementado com extrato de levedura, apresentou maior valor (0,62 h^{-1}) em relação aos outros ingredientes adicionados. A menor velocidade específica de crescimento foi observada em caldo MRS sem adição dos suplementos estudados (controle) (0,52 h^{-1}). O efeito redutor do oxigênio no meio de cultivo causado pela adição de L-cisteína é benéfico para o crescimento de *B. lactis*, assim como também foi observado por Karna, Emata e Barraquio (2007), para a bactéria *Lactobacillus delbrueckii* ssp. *bulgaricus*. Como pode ser observado nos meios MRS (0,60 h^{-1}) e BSM (0,57 h^{-1}) suplementados com L-cisteína, houve um aumento da velocidade específica de crescimento em relação ao controle.

No entanto, quando utilizado o caldo MRS suplementado com Tween 80 ou inulina, o valor da velocidade específica de crescimento do *Bifidobacterium lactis* foi de 0.54 h^{-1}, ou seja, menor quando comparado com o valor de L-cisteína. Em particular, o caldo BSM suplementado com Tween 80, extrato de levedura e inulina, as velocidades específicas obtidas foram de 0.56 h^{-1}, ou seja, menores que BSM suplementado por L-cisteína.

Através das velocidades específicas observadas no meio de cultivo sintéticos MRS, pode-se concluir que o principal fator limitante na velocidade de crescimento de *B. lactis*, foi a concentração de proteínas; porém, em caldo BSM, o efeito redutor do oxigênio causado pela L-cisteína mostrou-se superior na influência da velocidade de crescimento em *B. lactis*. Isso se deve, como já foi discutido anteriormente (seção 6.2), à formulação específica do caldo BSM para o gênero *Bifidobacterium*. Portanto, em caldo BSM, a diminuição da concentração de oxigênio dissolvido no meio exerce maior influência na velocidade específica de crescimento de *B. lactis* em relação à suplementação com fontes de nutrientes diferentes.

Nota-se que quando o soro de leite foi utilizado como meio de cultura, a velocidade específica de crescimento do meio suplementado com L-cisteína ou Tween 80 foi inferior (0.54 h^{-1}), em relação com o que foi observado nos resultados com caldo BSM. No soro de leite suplementado com extrato de levedura e inulina, foi observada velocidade específica maior (0.61 h^{-1}) do que aquela obtida no soro de leite suplementado com Tween 80 (0.54 h^{-1}). Vale salientar que o soro de leite é um meio de cultivo natural, que contém substâncias nutricionais que favorecem o metabolismo do *B. lactis,* assim como outras bactérias ácido láticas, resultado já

observado em trabalhos de outros autores (MARTH, 1973, PENNA; THAMER, 2005).

O soro de leite suplementado com Tween 80, por sua vez, incrementou a velocidade específica de crescimento nos meios de cultivo, se comparado aos meios padrões, apesar de não ter gerado o melhor resultado. O uso do Tween 80 como aditivo em meios de cultura se mostrou promissor em outros trabalhos de produção de bacteriocina encontrados na literatura (COLLADO; HERNÁNDEZ; SANZ, 2005), já que é um agente tensoativo, podendo aumentar na concentração de bacteriocinas, por facilitar a excreção desse peptídeo antimicrobiano.

Por fim, pode-se observar que, no meio de cultivo contendo soro de leite suplementado com extrato de levedura ou inulina, houve maior velocidade de crescimento específica e, consequentemente, menor tempo de geração em relação ao caldo BSM suplementado com o mesmo composto. Somado a este rendimento, o custo do soro de leite como meio de cultura é aproximadamente 10 vezes inferior em relação ao custo do caldo MRS ou BSM. Ressalta-se que não há diferença estatística significativa entre os resultados de velocidade específica de crescimento observados nos caldos BSM e MRS suplementados, porém é observada diferença quando estes são comparados com o meio soro de leite suplementado por inulina ou extrato de levedura. Este comportamento da *B. lactis* já era esperado, pois os caldos BSM e MRS são meios de cultivo sintéticos com formulações semelhantes. De fato, o meio BSM é um meio de cultivo seletivo, ou seja, possui compostos que inibem o crescimento de outros microrganismos que não do gênero *Bifidobacterium*. Esse meio de cultivo apresenta uma substância chamada “mupirocina” que é um antibiótico e o seu uso implica em problemas éticos, já que bacteriocinas são geralmente aplicadas em alimentos (ASHRAF; SHAH, 2011).

5.8 Atividade da bacteriocina

Em relação à atividade antimicrobiana da bacteriocina, a cepa utilizada no presente estudo (*Bifidobacterium lactis*) expressou quantidade de bacteriocina insuficiente. Porém, esta bactéria probiótica mostrou-se ser bacteriocinogênica. A

atividade da bacteriocina foi de aproximadamente 120 UA/mL, quando utilizado o caldo MRS suplementado com L-cisteína; 60 UA/mL (caldo MRS suplementado com Tween 80); 90 UA/mL no caldo MRS suplementado com inulina; e 140 UA/mL (caldo MRS suplementado com extrato de levedura). Já no meio BSM, foi observada atividade aproximada de 130 UA/mL, quando adicionado L-cisteína; 130 UA/mL (Tween 80); 150 UA/mL (inulina); e 160 UA/mL, quando adicionado extrato de levedura.

Em soro de leite, os valores de atividade foram 100 UA/mL, com suplementação de L-cisteína; 110 UA/mL (Tween 80); 190 UA/mL (inulina); e 200 UA/mL (extrato de levedura). O único microrganismo bioindicador, no qual a *Bifidobacterium lactis* expressou essas atividades antimicrobianas, nos diferentes meios de cultura, foi a *Listeria monocytogenes*. Nos ensaios em *shaker*, os melhores resultados foram apresentados no soro de leite suplementado com inulina e com extrato de levedura, sendo que ambos não apresentaram diferenças significativas entre si.

A atividade da bacteriocina obtida em fermentador de bancada utilizando o soro de leite suplementado com extrato de levedura foi semelhante (200 AU/mL) à atividade encontrada na bacteriocina produzida no mesmo meio de cultivo em *shaker*, já que ambos cultivos apresentam a mesma formulação e crescimento celular semelhante. Através dos resultados apresentados na Figura 12, pode-se observar que a atividade da bacteriocina está relacionada principalmente com o crescimento celular, pois se trata de uma biomolécula de metabolismo primário (HUGAS et al., 2002).

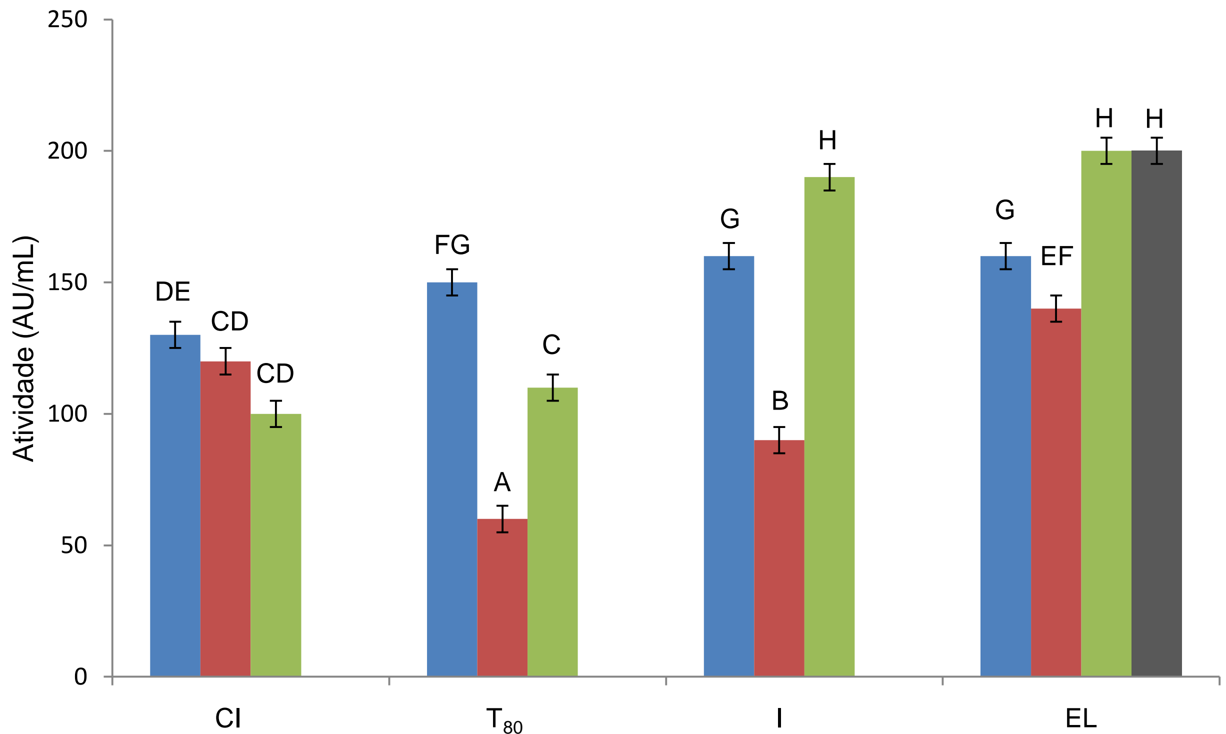

Figura 13. Atividade antimicrobiana da bacteriocina produzida por *Bifidobacterium lactis* utilizando os meios de cultivo: BSM (■), MRS (■) e soro de leite (■) e cultivo em fermentador de bancada utilizando o soro de leite (■) em presença de inulina (I), L-cisteína (CI), Tween 80 (T_{80}) e extrato de levedura (EL). Diferentes letras na mesma coluna significam que há diferença estatística entre os valores ($P < 0,05$).

6. CONCLUSÃO

O gênero *Bifidobacterium* é conhecido por possuir espécies anaeróbias estritas e anaeróbias aerotolerantes. A cepa *B. lactis* mostrou melhor crescimento celular e,consequentemente, expressão da bacteriocina em agitação baixa (50 rpm), quando utilizado agitador rotativo. Além disso, a cepa demonstrou atividade antimicrobiana contra a espécie patogênica *Listeria monocytogenes*.

O soro de leite suplementado com extrato de levedura ou inulina proporcionou o melhor desempenho de *B. lactis*, em relação ao crescimento celular, e se mostrou um meio de cultivo natural interessante para a produção de bacteriocinas. Com a adição de extrato de levedura, houve aumento significativo na produção desse

peptídeo antimicrobiano, o que torna este suplemento ideal para os ensaios em fermentador de bancada, nos quais a cepa *B. lactis* se mostrou muito promissora. De fato, o soro de leite, por se tratar de um meio de cultura mais barato que os meios sintéticos MRS e BSM, pode diminuir o custo de produção desta biomolécula de alto valor agregado, de modo que seu uso, portanto, se torna vantajoso.

7. SUGESTÕES PARA TRABALHOS FUTUROS

Os resultados do presente trabalho abrem perspectivas para novos projetos no uso da *B. lactis* estudada. Visando o emprego industrial da produção da bacteriocina propõe-se a purificação deste composto, assim como o seu sequenciamento para a identificação e classificação. Outro estudo sugerido é a ampliação da produção de bacteriocina em fermentador de bancada, testando-se novos ingredientes na suplementação do meio soro de leite. Outra proposta que pode ser desenvolvida em trabalhos futuros é o uso combinado de vários ingredientes visando à melhora na produção da bacteriocina.

8. ATIVIDADES ACADÊMICAS

8.1 Participação em Congresso

No período vigente do mestrado, houve a apresentação do pôster “Bacteriocin Production Using *Bifidobacterium animalis* in Milk Whey” na “XVII Semana Farmacêutica de Ciência e Tecnologia”, realizada na USP (Universidade de São Paulo), no período de 1 a 5 de outubro de 2012.

8.2 Publicação de Artigos

No período do presente trabalho (01/03/2012 a 15/07/2013), foram publicados três artigos em forma de *review*.

1. **BALCIUNAS**, E. M., MARTINEZ, F. A. C., TODOROV, S. D., FRANCO, B. D. G. M., CONVERTI, A., OLIVEIRA, R. P. S. Novel biotechnological applications of bacteriocins: A review. *Food Control,* v. 32, p. 134-142, 2013.
2. MARTINEZ, F. A. C., **BALCIUNAS**, E. M., CONVERTI, A., COTTER, P. D., OLIVEIRA, R. P. S. Bacteriocin production by *Bifidobacterium spp.* A review. *Biotechnology Advances,* v. 31, p. 482-488, 2013.
3. MARTINEZ, F. A. C., **BALCIUNAS**, E. M., SALGADO, M., DOMÍNGUEZ, J., CONVERTI, A., OLIVEIRA, R. P. S. Lactic acid properties, applications and production: A review. *Trends in Food Science & Technology*, v. 30, p. 70-83, 2013.
4. SABO, S. S., **BALCIUNAS**, E. M., VITOLO, M., OLIVEIRA, R. P. S. *Lactobacillus plantarum*: a versatile lactic acid bactéria. *Food Science and Technology*, Artigo submetido.

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ANEXOS

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Review

Novel biotechnological applications of bacteriocins: A review

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ABSTRACT

Nowadays, consumers are aware of the health concerns regarding food additives; the health benefits of "natural" and "traditional" foods, processed without any addition of chemical preservatives, are becoming more attractive. One of the alternatives to satisfy this request are bacteriocins, which are antimicrobial peptides produced by a large number of bacteria, including lactic acid bacteria, normally acting against closely related and some spoilage and disease-causing Gram-positive pathogens. For this reason they are used in several applications, among which are biopreservation, shelf-life extension, clinical antimicrobial action and control of fermentation microflora. Toxicological studies showed that nisin intake does not cause any toxic effect to humans having an estimated lethal dose of 6950 mg/kg; thus, it is one of the bacteriocins mostly applied in the food industry as antibotulinic agent in cheese and liquid eggs, sauces and canned foods. It exhibits a wide-spectrum antimicrobial action against *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and other pathogens. Food-grade substrates such as milk or whey can be supplemented with *ex situ* produced bacteriocin preparations obtained by fermentation. Preparations can be added as partially purified or purified concentrates requiring specific approval as preservatives from the legislative viewpoint. Demand for new antibacterial compounds has brought great interest for new technologies able to enhance food microbiological safety. Also the dramatic rise in antibiotic-resistant pathogens has stimulated renewed efforts to identify, develop or redesign antibiotics active against multi-resistant bacteria. Numerous antibacterial agents are now being re-considered for application, among others are bacteriophages, probiotics, antimicrobial peptides and bacteriocins. To optimally exploit their desired activities, chemical or genetic engineering methods are often employed. In this review we focus on recent classification of bacteriocins, their mode of action, biotechnological applications in food and pharmaceutical industries, purification techniques and biosafety, as well as recent attempts to generate custom-designed bacteriocins using genetic engineering techniques.

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1. Introduction

Lactic acid bacteria (LAB) are a diverse and very useful group of bacteria that, while not adhering to a strict taxonomic group, are gathered on the basis of shared properties (Oguntoyinbo & Narbad, 2012) and have the common trait of producing lactic acid (LA) as a major or sole fermentation product. For these reasons, LAB have historically been associated with the fermentation of foods, and as a result many LAB, like *Lactococcus*, *Oenococcus*, *Lactobacillus, Leuconostoc*, *Pedicoccus* and *Streptococcus* sp., are generally recognized as safe (GRAS) and/or probiotics (Mayo et al., 2010).

The desirable property of a probiotic strain is the ability to produce antimicrobial substances such as bacteriocins that offer the potential to provide an advantage in competition and colonization of the gastrointestinal tract. Bacteriocins are generally defined as peptides produced by bacteria that inhibit or kill other related and unrelated microorganisms. Bacteriocin was firstly identified by Gratia (1925) as an antimicrobial protein produced by *Escherichia coli* and named colicin. The interest in bacteriocins produced by GRAS microorganisms has been leading to considerable interest for nisin, being the first bacteriocin to gain widespread commercial application since 1969. As a result, the field has developed increasingly, resulting in the discovery and detailed characterization of a great number of bacteriocins from LAB in the last few decades (Collins, Cotter, Hill, & Ross, 2010).

Nowadays, consumers are aware of the health concerns regarding food additives; the health benefits of "natural" and "traditional" foods, processed without any addition of chemical preservatives, are becoming more attractive. Thus, because of recent consumer demand for higher quality and natural foods, as well as of strict government requirements to guarantee food safety, food producers have faced conflicting challenges (Franz, Cho, Holzapfel, & Gálvez, 2010). Chemical additives have generally been used to combat specific microorganisms. The application of bacteriocins as biopreservatives for vegetable food matrices started approximately 25 years ago. In these years, a lot of studies have focused on the inhibition of spoilage and/or human pathogens associated with vegetable foods and beverages by bacteriocins, and their application appeared as a good alternative to chemical compounds and antibiotics. When deliberately added or produced *in situ*, bacteriocins have been found to play a fundamental role in the control of pathogenic and undesirable flora, as well as in the establishment of beneficial bacterial populations (Collins et al., 2010).

Traditionally, new bacteriocins have been identified by screening bacterial isolates for antimicrobial activity followed by purification and identification of the bacteriocin and its genetic determinants. Such a strategy is still fundamental for detection and identification of powerful bacteriocins of various subclasses, and recent examples of this include a) a class IIa bacteriocin named avicin A that was identified from *Enterococcus avium* strains isolated from faecal samples of healthy human infants from both Ethiopia and Norway (Birri, Brede, Forberg, Holo, & Nes, 2010), b) a circular bacteriocin named garvicin ML produced by a *Lactococcus garvieae* strain isolated from mallard duck (Borrero et al., 2011), c) a class IIb bacteriocin named enterocin X isolated from an *Enterococcus faecium* strain from sugar apples (Hu, Malaphan, Zendo, Nakayama, & Sonomoto, 2010) and d) a glycosylated bacteriocin (glycocin F) from *Lactobacillus plantarum* isolated from fermented corn (Kelly, Asmundson, & Huang, 1996).

In the next sections, we will present bacteriocin classification, their mode of action and structure, biotechnological applications in food and pharmaceutical industries and problems associated with resistance and purification.

2. Classification

According to Klaenhammer (1993), bacteriocins can be divided into four classes. The class I of lantibiotics, represented by nisin, gathers very low molecular weight (<5 kDa) thermostable peptides characterized by the presence of lanthionine and derivatives. The class II is composed of small thermostable peptides (<10 kDa) divided into three subclasses: IIa (pediocin and enterocin), IIb (lactocin G) and IIc (lactocin B). The class III is represented by high molecular weight (>30 kDa) thermolabile peptides such as the helveticin J, while in the class IV we can find large peptides complexed with carbohydrates or lipids. However, Cleveland, Montville, Nes, and Chikindas (2001) believe that these structures are artifacts of partial purification and not a new class of bacteriocins.

Cotter, Hill, and Ross (2005) suggested a new classification where bacteriocins are divided into two categories: lantibiotics (class I) and not containing lanthionine lantibiotics (class II), while high molecular weight thermolabile peptides, which are formally components of the above class III, would be separately designated as "bacteriolysins". These authors also suggested that the above class IV should be extinguished. Finally, Drider, Fimland, Hechard, Mcmullen, and Prevost (2006) divided bacteriocins into three major classes according to their genetic and biochemical characteristics (Table 1), and we will refer to such a classification in the following.

2.1. Class I or lantibiotics

Lantibiotics are small peptides (19–38 amino acid residues) with rare thermostable amino acids in their composition, which may result from the combination of two alanine linked by a disulfide bond as for lanthionine, or from an amino butyric acid linked to an alanine by a disulfide bond as for β-methyl-lanthionine (Jarvis, Jeffcoat, & Cheeseman, 1968).

The main representative of this class is nisin, which is produced by some strains of *Lactococcus lactis* subsp. *lactis* and is composed of 34 amino acid residues. Two variants of nisin are nisin A and nisin Z, which differ structurally in only one amino acid, but have similar

Table 1
Classification of bacteriocins.

Classification	Features	Subcategories	Examples
Class I or lantibiotics	Lantionine or peptides containing β-lantionine	Type A (linear molecules)	Nisin, subtilin, epidermine
		Type B (globular molecule)	Mersacidin
Class II	Heterogeneous class of small thermostable peptides	Subclass IIa (antilisterial-pediocine bacteriocins type)	Pediocin, enterocin, sakacin
		Subclass IIb (composed of two peptides)	Plantaricin, lactacin F
		Subclass IIc (other bacteriocins)	Lactococcin
Class III	Large thermolabile peptides		Helveticin J, millericin B

Source: Adapted from Drider et al. (2006).

activity (Mulders, Boerrigter, Rollema, Siezen, & Vos, 1991). Due to the acidic nature of its molecule, nisin is completely stable in solution at pH 2.0 and can be stored for long time in the temperature range of 2–7 °C, while above pH 7.0 inactivation occurs even at room temperature (Delves-Broughton, 1990).

Toxicological studies showed that nisin intake does not cause any toxic effect to humans with an estimated lethal dose (LD_{50}) as high as 6950 mg/kg (close to that of salt) when administered orally (Jozala, Andrade, Arauz, Pessoa Jr., & Vessoni-Penna, 2007). In general, some authors have ascribed the high LD_{50} values of bacteriocins to digestive enzymes capable of rapidly inactivating trypsin and chymotrypsin produced in the pancreas (Vaucher et al., 2011).

Nisin has been largely using in the food industry as antibotulinic agent in cheese and liquid eggs, sauces and canned foods. It exhibits a wide-spectrum antimicrobial action against *L. monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and other pathogens and LAB species (Rilla, Martinez, & Rodriguez, 2004), which is mediated by a dual action mechanism encompassing interference with cell wall synthesis and promotion of pore formation in cell membrane. The resulting changes in permeability, with outflow of essential compounds (K^+ ion, amino acids and ATP) through the pores, are responsible for cell death (Breukink et al., 1999).

Nisin is the only bacteriocin approved for food applications being considered to be safe by the Food and Agriculture Organization/World Health Organization (FAO/WHO) in 1969. According to Ross, Morgan, and Hill (2002), dairy products can contain nisin as a food additive for processed cheese at concentration up to 12.5 mg/kg pure nisin. In addition, it was also included as biopreservative ingredient in the European food additive list, where it was assigned the number E234.

2.2. Class II

This subclass is composed of small thermostable peptides (<10 kDa) with an amphiphilic helical structure that allows for their insertion in the cytoplasmic membrane of the target cell, thereby promoting membrane depolarization and cell death. Three subdivisions are proposed for this class, according to Drider et al. (2006).

2.2.1. Subclass IIA

The subclass IIa is composed of bacteriocins showing high specificity against *L. monocytogenes*. Its representatives have 37–48 amino acid residues with an N-terminal portion with pleated sheet configuration and a C terminus containing one or two α-helices (Fimland, Johnsen, Dalhus, & Nissen-Meyer, 2005). The bacteriocins of this class fall into the cell membrane of the target microorganism by the C terminus, promoting the formation of pores and consequent dissipation of proton motive force (Kaiser & Montville, 1996). In the attempt to maintain or restore the proton motive force, there is acceleration in the consumption of ATP and consequently cell death.

Pediocin PA-1, which is composed of 44 amino acid residues, is the only bacteriocin belonging to the subclass IIa that is synthesized not only by different species, but also by different genera of LAB. It was initially detected in *Pediococcus acidilactici* (Bhunia, Johnson, & Ray, 1987). Since then, other strains and species of pediococci were described as producers of pediocin (Díez et al., 2012). Ennahar et al. (1996) isolated a strain of *L. plantarum* in Munster cheese able to produce pediocin AcH, a bacteriocin with an antagonistic effect on pathogenic and deteriorating microorganisms, including *L. monocytogenes*, *S. aureus* and *Clostridium perfringens* (Bhunia et al., 1987; Loessner, Guenther, Steffan, & Scherer, 2003).

The first enterocin was identified by Kjems (1955) and subsequently classified as a member of the pediocin family. Since then, several enterocins have been described, that have representatives in more than one class of bacteriocins. Usually they are thermostable (121 °C/15 min) and resistant to lyophilization and storage at −20 °C for long periods. According to Cintas, Casaus, Havarstein, Hernandez, and Nes (1997), these compounds have selective antimicrobial activity, do not show antagonism with *Leuconostoc* and *Lactococcus*, but attack *C. perfringens*, *Clostridium botulinum*, *S. aureus* and especially species of the genus *Listeria*.

2.2.2. Subclass IIB

This subclass includes heterodimeric bacteriocins, i.e. bacteriocins that require the combined activity of two peptides. Normally, genes are located in the same operon and expressed simultaneously, and the two peptides act in combination frequently showing an important synergistic action. Their mechanism of action also involves the dissipation of membrane potential and a decrease in the intracellular ATP concentration. These peptides have very low activity when individually employed (Garneau, Martin, & Vederas, 2002).

2.2.3. Subclass IIC

Bacteriocins belonging to this subclass have a covalent bond between C and N terminals, resulting in a cyclic structure (Kawai et al., 2004). Enterocin AS-48, circularin A and reutericin 6 are representatives of this subclass.

2.3. Class III

This class gathers large thermolabile bacteriocins (>30 kDa) that have complex activity and protein structure. Their action mechanism is different from those of other bacteriocins, in that they promote lysis of the cell wall of the target microorganism. Their N-terminal portion is homologous to an endopeptidase involved in cell wall synthesis, while the C-terminal portion is responsible for recognition of the target cell (Lai, Tran, & Simmonds, 2002).

3. Mode of action and structure

Bacteriocins are usually synthesized as inactive pre-peptides that have an N-terminal sequence guide (Macwana & Muriana, 2012). These precursors are transported to the cell surface during the exponential growth phase and enzymatically converted into their active forms. The carriers contain an N-terminal peptidic portion responsible for the guide peptide cleavage as well as a C-terminal portion responsible for ATP hydrolysis and energy supply

(Aucher, Lacombe, Héquet, Frère, & Berjeaud, 2005). For class II, accessory proteins are used to facilitate the membrane translocation and/or cleave the peptide tab.

The system regulating the production of bacteriocins is composed of three components: an inducing peptide (or pheromone-activating factor), the transmembrane histidine kinase (pheromone receptor) and a response regulator (Nes & Eijsink, 1999). The peptide inducer is synthesized in the ribosome at low levels as a pre-peptide, which is cleaved and secreted in the outer environment by the carrier system. When this compound reaches a threshold concentration, it activates transmembrane histidine kinase, which leads to autophosphorylation of the histidine residue, thus transferring phosphate to a response regulator protein. The phosphorylated regulator activates the transcription of the bacteriocin in addition to the elements that make up the regulatory system, initiating a positive feedback (Nes & Eijsink, 1999). Regulation of the production of lantibiotics such as nisin and subtilin is done by the bacteriocin itself, which acts as a pheromone inducing their production at high levels (Kleerebezem & Quadri, 2001).

The mechanism of immunity of bacteriocin-producing bacteria makes distinction between bacteriocin produced by themselves and by other microorganisms. The protection can be promoted by a specific protein and/or the conveyor system. The mechanism by which they work is similar, by kidnapping the structural protein or by antagonistic competition for receptor of the bacteriocin (Hoffmann, Schneider, Pag, & Sahl, 2004).

3.1. Factors affecting bacteriocin efficiency

The activity of bacteriocins produced by different LAB is not uniform and constant and depends on the chemical composition and physical conditions of food; it mainly depends on pH and is reduced by bacteriocin binding to food components, adsorption to cell or protein, activity of proteases and other enzymes (Schillinger, Geisen, & Holzapfel, 1996). A correlation between nisin degradation and extent of proteolysis in pasteurized cream was found by Phillips, Griffths, and Muir (1983). Buyong, Kok, and Luchansky (1998) ascribed the reduction in pediocin activity from 64,000 to 2,000 U/g after six months of maturation of Cheddar cheese to the action of proteases and peptidases. NaCl at certain concentrations can reduce the growth of LAB and consequently the production of bacteriocins, besides protecting the target bacteria such as *L. monocytogenes* from their action (Hugas, Garriga, Pascual, Aymerich, & Monfort, 2002). Sarantinopoulos et al. (2002) observed reductions in bacteriocin activity and *E. faecium* FAIR-E 198 growth rate after addition of 2% NaCl to MRS broth. Nilsen, Nes, and Holo (1998) ascribed this phenomenon to the interference of NaCl in the production factor binding the inductor to the receptor.

Aside from interacting with food components, bacteriocins may be adversely affected by processing and storage conditions such as pH and temperature of the product. According Drosinos, Mataragas, Nasis, Galiotou, and Metaxopoulos (2005), the optimal pH for bacteriocin production (5.5) does not match that for microbial growth (6.5). Because of their maximum stability under acidic conditions, nisin activity is increased when used in acidic foods. Therefore, effective applications of nisin require that the pH of food is less than 7 to ensure satisfactory solubility, stability during processing and storage period (Hernandez et al., 1993). Leroy and De Vuyst (1999) reported that bacteriocin activity decreases with increasing temperature owing to increased activity of proteases.

The inhibitory efficiency of bacteriocins is also related to the level of food contamination by the target organism. If the initial contamination is too high, bacteriocin activity is low and unable to prevent the development of contaminating microorganisms. Rilla et al. (2004) investigated the action of *Lc. lactis* subsp. *lactis* IPLA 729 against *S. aureus* at two different concentrations, specifically 1.8×10^4 and 7.2×10^6 CFU mL^{-1}: after 24 h of incubation, they did not detect *S. aureus* in the more dilute sample, while the other showed a still high count (5.0×10^4 CFU mL^{-1}).

4. Biotechnological applications

There are potentially significant benefits to employing modern cutting-edge bioengineering to progress the traditional peptide discovery, description and production because of the gene-encoded nature of bacteriocins. One of the greatest advantages of bioengineering in the lantibiotic field involves the creation of strains producing larger amounts of lantibiotic peptides (Suda et al., 2010). Another strategy to improve lantibiotic-producing strains is to conjugate multiple large bacteriocin-encoding plasmids into a single strain (Collins et al., 2010), thereby making it able to kill the undesired target more effectively than the wild type (O'Sullivan, Ryan, Ross, & Hill, 2003). It is also possible to achieve this goal through the amplification and cloning of lantibiotic-encoding genes into shuttle vectors and heterologous production in other strains. Such an approach was used to improve the production of lacticin 3147 by an *Enterococcus* host (Ryan, Mcauliffe, Ross, & Hill, 2001).

Bioengineering of existing peptides could also lead to the creation of lantibiotics with improved power and/or suitable for specific applications (Collins et al., 2010). A number of studies allowed for better comprehension of the structure/function relationships of specific lantibiotics and pointed out the significance of nisin and related peptides within the hinge region, whose discrete alterations resulted in mutants with no mutacin II activity (Chen et al., 1998), or improved nisin Z activity, or even enhanced stability at high temperature and/or under neutral or alkaline conditions (Yuan, Zhang, Chen, Yang, & Huan, 2004). In addition, to improve the activity or inhibitory spectrum, peptides were developed with enhanced characteristics. For example, nisin Z studies that solubility and stability were significantly improved by peptide engineering without dramatically reducing specific activity (Rollema, Kuipers, Both, De Vos, & Siezen, 1995).

It is also possible to drastically alter lantibiotic and non-lantibiotic peptides by altering existing or introducing new post-translational modifications through the application of specific enzymes. To provide some examples, the cyclase of nisin (NisC) was utilized to cyclize and protect non-lantibiotic peptides against peptidases and proteases (Rink et al., 2007), a property which is particularly useful from a drug design standpoint, while the dehydratase of nisin (NisB) to introduce dehydro residues making the formation of thioether bridges into various peptides easier (Kluskens et al., 2005).

According to Mills, Stanton, Hill, and Ross (2011), bioengineering of bacteriocins is not limited to lantibiotics. Much effort has been devoted to the subclass IIA of bacteriocins to determine the structure–function relationships. Though variants generated in these types of studies are useful from an academic standpoint, none of them display increased activity against several microorganisms (Kazazic, Nissen-Meyer, & Fimland, 2002).

4.1. Applications in the food industry

Foods products can be supplemented with *ex situ* produced bacteriocin preparations obtained by cultivation of the producer strain in an industrial fermenter followed by adequate recovery. Bacteriocins can be added as partially purified or purified concentrates, which would require specific approval as preservatives from the legislative viewpoint. So far, nisin and pediocin PA-1 are bacteriocins licensed as food preservatives (Simha, Sood, Kumariya, & Garsa, 2012). Many preliminary studies on the activity of bacteriocins *in vitro* or in food

systems are carried out with partially-purified preparations obtained from culture broths, but in the most cases a low concentration of bacteriocin is often recovered (Schillinger et al., 1996; Stiles, 1996), which demonstrates the significance to address many efforts in this direction.

Foods can also be supplemented with bacteriocins *ex situ* produced that can be added in the form of raw concentrates obtained by cultivation of the producer strain in a food-grade substrate (such as milk or whey). The resulting preparations may be regarded as food additives or ingredients from the legal viewpoint, since some of their components may play a recognized function in the food (such as increase in protein content or thickening). They also contain the cell-derived antimicrobial metabolites (such as LA) and bacteriocins, affording an additional bioprotectant function. Other milk-based preparations have been described, in addition to already-marketed concentrates such as ALTA™ 2341 or Microgard™, such as lacticin 3147 (Guinane, Cotter, Hill, & Ross, 2005) and variacin (O'Mahony, Rekhif, Cavadini, & Fitzgerald, 2001). Bacteriocins *ex situ* produced can also be applied in the form of immobilized preparations, in which the partially-purified bacteriocin is bound to a carrier. The carrier acts as a reservoir and diffuser of the concentrated bacteriocin molecules to the food, guaranteeing a gradient-dependent continuous supply of bacteriocin. The carrier may also protect the bacteriocin from inactivation by interaction with food components and enzymatic inactivation. Moreover, the application of bacteriocin molecules on the food surface requires much lower amounts of bacteriocin (compared to application in the whole food volume), decreasing the processing costs. In most cases, immobilized bacteriocin preparations are applied on the surface of the processed food, avoiding post-process contamination and surface proliferation of unwanted bacteria. A recent advance in this field is the use of immobilized bacteriocins in the development of antimicrobial packaging (Ercolini, Storia, Villani, & Mauriello, 2006).

In situ, bacteriocin production offers several advantages compared to *ex situ* production, concerning both legal aspects and costs. Lowering the costs of biopreservation processes may be highly attractive, especially for small economies and developing countries, where food safety may be seriously compromised (Holzapfel, 2002). Several studies demonstrate the effectiveness of these compounds in food biopreservation, as shown in Table 2.

Many studies have also focused on the selection and development of protective bacteriocinogenic cultures for food applications (Leroy, Verluyten, & De Vuyst, 2006; Ross et al., 2002) such as inhibition of spoilage and pathogenic bacteria during the shelf life period of non-fermented foods. A protective culture may grow and produce bacteriocin during refrigerated storage of the food, which must have a neutral impact on its physicochemical and organoleptic properties, and/or during temperature abuse conditions, under which it may even act as the predominant spoiler, ensuring that pathogenic bacteria do not grow and that the spoiled food is not consumed (Holzapfel, Geisen, & Schillinger, 1995).

4.2. Applications in the pharmaceutical industry

With the availability of a powerful and effective arsenal of drugs, most pharmaceutical companies abandoned their antimicrobial drug development programs, as there seemed to be little need for new drug compounds (Knowles, 1997). Bacterial resistance to antimicrobials was observed right after their initial wide-scale use (Levin et al., 1998). Since then, the levels of resistance have continued to rise dramatically. It has reached the point that by 2000 the World Health Organization cautioned that infectious diseases might become untreatable as a result of high levels of multiply resistant pathogens. At first, antibiotic resistance was thought to be confined to hospital settings, where the use of antibiotics was most intensive; approximately one third of all hospitalized patients receive antibiotics with at least half of those prescriptions being unnecessary, poorly chosen or incorrectly administered (Van Houten, Luinge, Laseur, & Kimpen, 1998).

Compounding the problem further, an almost exclusive reliance on broad-spectrum antibiotic agents has contributed to a rapid emergence of multiresistant pathogens (Wester et al., 2002). The increasing threat of antibiotic resistance is also the result of antibiotic use in agricultural and food production settings. In the agricultural industry, the use of antibiotics for disease control, prophylactic agents and growth promotion, has contributed significantly to the emergence of resistant bacteria pathogenic to animals (Barton & Hart, 2001) and plants (Mcmanus, Stockwell, Sundin, & Jones, 2002). Additionally, bacteria isolated from animals in environments unrelated to clinical or agricultural management settings have been shown to naturally acquire high levels of antibiotic resistance (Sherley, Gordon, & Collignon, 2000). Ironically, it is likely that the extensive benefits of antibiotic use has contributed to the limited array of effective drugs available today for treating multi-resistant bacteria.

Only recently the alarming nature of this problem has motivated research efforts to find alternatives to our increasingly limited antibiotic resources. Numerous antibacterial agents are now being considered such as bacteriophages (Alisky, Iczkowski, Rapoport, & Troitsky, 1998), probiotic bacteria (Macfarlane & Cummings, 2002), antimicrobial peptides (Joerger, 2003), and bacteriocins (Twomey, Ross, Ryan, Meaney, & Hill, 2002). In order to optimally exploit the desired activities of these varied antimicrobial leads, researchers often employ chemical or genetic engineering methods (Lien & Lowman, 2003). Examples of some bacteriocins and their pharmaceutical applications are shown in Table 3.

The use of microcins is a possible alternative to control Gram-negative bacteria (Duquesne, Destoumieux-Garzón, Peduzzi, & Rebuffat, 2007). Similarly to pediocin-like bacteriocins, microcins belonging to class IIa such as microcin V are linear polypeptides, and the removal of the leader peptide is the unique post-translational modification that they undergo before being secreted by the cells (Duquesne et al., 2007; Pons, Lanneluc, Cottenceau, & Sable, 2002). Three different proteins may serve as

Table 2
Application of bacteriocins in food biopreservation.

Bacteriocin	Culture producer	Target microorganism	Food	Reduction (log CFU g^{-1})	References
Nisin	*Lactococcus lactis*	*Brochothrix thermosphacta*	Pork	3.5	Nattress, Yost, & Baker, 2001
Nisin	*L. lactis*	*Listeria monocytogenes*	Fermented milk	6.0	Benkerroum et al. (2002)
AcH Pediocin	*Lactobacillus plantarum*	*L. monocytogenes*	Cheese	1.0–2.0	Loessner et al., 2003
Enterocin	*Enterococcus faecium*	*L. monocytogenes*	Milk	2.0	Elotmani, Revol-Junelles, Assobhei, & Milliére, 2002
Enterocin	*Enterococcus faecalis*	*Staphylococcus aureus*	Sausage	5.3	Ananou, Maqueda, Martínéz-Bueno, Gálvéz, & Valdivia, 2005
Nisin Z	*Lactococcus lactis lactis*	*S. aureus*	*Afuega'l pitu* cheese	2.0	Rilla et al. (2004)

Source: Adapted from Nascimento, Moreno, and Kuaye (2008).

Table 3
Examples of some bacteriocins and their pharmaceutical applications.

Group of bacteriocins	Pharmaceutical applications
Lantibiotics	Blood pressure treatment Inflammations and allergies treatment Skin infections treatment Mastitis infections treatment Herpes treatment Dental carries treatment Peptic ulcer treatment
Colicins	Urinogenital infection Hemorrhagic colitis treatment Hemolytic uremic syndrome treatment
Microcins	Antibacterial agent Salmonellosis treatment

Source: Adapted from Gillor, Nigro, and Riley (2005).

a specific receptor for linear microcins, namely the membrane component F_0 of the ATP synthase, SdaC, and the mannose permease, required by MccH47, MccV, and MccE492, respectively (Biéler, Silva, & Belin, 2010; Gérard, Pradel, & Wu, 2005). Because of the Gram-negative envelope structure, an additional step is required by class IIa microcins, i.e. an OM transporter system is used for these peptides to reach the plasma membrane receptor. The enterocin CRL35, a pediocin-like bacteriocin isolated from Argentinean regional cheese, has a potent antilisterial activity but is inactive against Gram-negative bacteria (Farías, Farías, de Ruiz Holgado, & Sesma, 1996). On the other hand, microcin V, previously known as colicin V, is specifically active against Gram-negative bacteria (Gratia, 1925). In order to obtain a peptide with a broader antimicrobial spectrum, Acuña, Picariello, Sesma, Morero, and Bellomio (2012) fused by asymmetrical PCR the required portions of genes encoding enterocin CRL35 and microcin V, namely *munA* and *cvaC*. The hybrid bacteriocin purified from *E. coli* extracts, named Ent35–MccV, showed inhibitory activity against enterohemorrhagic *E. coli*, *L. monocytogenes*, and other pathogenic Gram-positive and Gram-negative bacteria (Acuña et al., 2012).

5. Differences between bacteriocins and antibiotics

In contrast to the currently used antibiotics, bacteriocins are often considered more natural because they are thought to have been present in many of the foods eaten since ancient times. Bacteriocins are inactivated by enzymes, such as trypsin and pepsin, found in the gastrointestinal tract and therefore, they do not alter the microbiota of the digestive tract (Cleveland et al., 2001). If bacteriocins are considered antibiotics, they may not be used in human food, since the use of antibiotics in food is illegal (Collins et al., 2010). Nisin is the only bacteriocin considered by the Codex Alimentarius committee FAO (Food and Agriculture Organization) as GRAS (Generally Regarded As Safe) and can be used as a food additive in the inhibition of post-germination spores and toxin formation by *C. botulinum* in pasteurized processed cheese. Antibiotics for use in animal feed have been first approved in 1951 by the U.S. Food and Drug Administration that now maintains a list of currently approved products. Over the years and especially more recently, a number of strategies for improvements in animal health, productivity, and microbial food safety that did not involve antibiotics have been explored, like probiotics and bacteriocins (Joerger, 2003).

Antibiotics and bacteriocins have different mechanisms of action. When nisin is combined with some antibiotics, antimicrobial synergy may occur. The mechanisms of resistance to nisin and antibiotics are different. Antibiotic-resistant cells are sensitive to nisin and nisin-resistant cells are sensitive to antibiotics (Cleveland et al., 2001; Fernández, Delgado, Herrero, Maldonado, & Rodríguez, 2008). More recently, microencapsulated nisin in nanovesicles prepared from partially purified soy lecithin was shown to be as effective as free nisin to inhibit *L. monocytogenes* growth in whole and skim milk at low temperatures over 14 days (da Silva-Malheiros, Daroit, da Silveira, & Brandelli, 2010). Naghmouchi, Le Lay, Baah, and Drider (2012) determined the synergistic effect of bacteriocins and antibiotics on sensitive and resistant variants of strains. In particular, a synergistic effect against *Pseudomonas fluorescens* was observed with 90% of the combinations of the class I or subclass IIa bacteriocins with antibiotics and 60% of the combinations of colistin with antibiotics. So, in the future, combination of antibiotics with antimicrobial peptides could allow for reduced use of antibiotics in medical applications and could help to prevent the emergence of bacteria resistant to antibiotics.

6. Resistance to bacteriocins

The resistance of spontaneous mutants to bacteriocins may be related to changes in membrane and cell wall, such as alterations in the electrical potential, fluidity, membrane lipid composition and load or cell wall thickness (Mantovani & Russel, 2001), or even a combination of all factors. According to Van Schaik, Gahan, and Hill (1999), these changes may occur following cell exposure to low concentrations of bacteriocins or as part of an adaptive response to some other stress. The mechanism of resistance of cells to nisin is not yet well understood. According to Abee (1995), the resistance of *L. monocytogenes* to nisin is related to variation in fatty acid composition of cell membranes, reducing the concentration of phospholipids, hindering the formation of pores.

Gravesen, Axelsen, Silva, Hansen, and Knochel (2002) reported that the frequency of resistance may vary between 10^{-2} and 10^{-7}, depending on the strain of *L. monocytogenes*. The mechanism of resistance to subclass IIa bacteriocins appears to be linked to reduced expression of mannose permease of the phosphotransferase system (Vadyvaloo, Hastings, Van Der Merwe, & Rautenbach, 2002).

7. Biosafety

Microorganisms like *Lactobacillus* spp., *Lactococcus* spp. and *Streptococcus thermophilus* have been used in food processing, and consumption of foods containing them or their metabolites has taken place for a long time (Ishibashi & Yamazaki, 2001). The safety of these microorganisms has not been questioned and reports of harmful effects of these bacteria have been very rare. Some LAB have proven to be associated with human infections, like endocarditis by *Lactobacillus fermentum* isolated in the mitral valve (Gallemore, Mohon, & Ferguson, 1995), pancreatitis by *Lactobacillus rhamnosus* isolated in the intra-abdomen and blood (Brahimi, Mathern, Fascia, Afchain, & Lucht, 2008), urinary tract infection by *P. acidilactici*, *Lactobacillus gasseri* and *Leuconostoc mesenteroides* (Taneja et al., 2005), and several other diseases. In addition, some LAB has been associated with resistance to antibiotics, but according to Songisepp et al. (2012), *L. plantarum* Tensia is not resistant to tetracycline.

However, various clinical studies have been conducted to assess the safety of probiotics in small groups of specific HIV infected patients, and the findings of these studies support the safety of probiotics consumed by such groups (Cunningham-Rundles et al., 2000).

8. Purification

Bacteriocin-producers are LAB that need complex nutritional exigencies to grow, and this not only increases the production cost, but also makes the purification of bacteriocins more difficult (Li, Bai, Cai, & Ouyang, 2002). Since bacteriocins form an extremely heterogeneous group of substances, specific purification protocols generally need to be designed for each of them, which may explain why only few bacteriocins have been purified to homogeneity like nisin. Three major methods for the purification of LAB bacteriocins can be distinguished according to their biochemical structure. First, purification can be done by a conventional method that is based on a rather laborious series of subsequent steps of ammonium sulfate precipitation, ion exchange, hydrophobic interaction, gel filtration, and reversed-phase high-pressure liquid chromatography (Parente & Ricciardi, 1999). Second, a simple three-step protocol has been developed, including (1) ammonium sulfate precipitation, (2) chloroform/methanol extraction/precipitation, and (3) reversed-phase high-pressure liquid chromatography, as the sole chromatographic step involved (Callewaert et al., 1999). Third, bacteriocins can be isolated through a unique unit operation, i.e. expanded bed adsorption, using a hydrophobic interaction gel, after maximizing the bioavailable bacteriocin titer through pH adjustment of the crude fermentation medium (Foulquié-Moreno, Callewaert, & De Vuyst, 2001).

Following the last two methods, which are more rapid and successful than the first conventional one, several bacteriocins with interesting industrial potential have been purified such as amylovorin L (produced by *Lactobacillus amylovorus* DCE 471 and belonging to the class II), several enterocins (produced by the *E. faecium* RZS C5, RZS C13 and FAIR-E 406 strains) and the lantibiotic macedocin (produced by *Streptococcus macedonicus* ACA-DC 198) (Callewaert et al., 1999; Georgalaki et al., 2002). Nisin, for example, has been purified using immunoaffinity chromatography (Prioult, Turcotte, Labarre, Lacroix, & Fliss, 2000), expanded bed ion exchange (Cheigh, Kook, Kim, Hong, & Pyun, 2004) and reversed-phase high-performance liquid chromatography (López et al., 2007). However, these methodologies greatly increase the cost of nisin, which is the most consumed bacteriocin in the world.

9. Conclusions

Bacteriocins have the potential to cover a very broad field of application, including both the food industry and the medical sector. They are a diverse group of antimicrobial proteins/peptides; therefore, they are expected to behave differently on different target bacteria and under different environmental conditions. Since the efficacy of bacteriocins is dictated by environmental factors, there is a need to determine more precisely the most effective conditions for application of each particular bacteriocin. For uses involving purified bacteriocins, cost of the compounds can become a significant barrier. Production of all but the smallest bacteriocins is currently only imaginable by culture of natural or genetically engineered producer organisms. Investments in research and development can be expected to be high, and the size of the market is difficult to predict, but the fact that nisin has found commercial uses indicates that economic aspects are not insurmountable barriers to bacteriocin applications.

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Research review paper

Bacteriocin production by *Bifidobacterium* spp. A review

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ABSTRACT

Bacteriocins are ribosomally-synthesized antibacterial peptides. These compounds are produced by a broad variety of different bacteria belonging mainly to the genus *Bifidobacterium*, to which health promoting properties have frequently been attributed. However, despite the fact that the identification of *Bifidobacterium*-associated bacteriocins was first reported in 1980 and that they exhibit antimicrobial activity against pathogenic microorganisms such as *Listeria monocytogenes*, *Clostridium perfringens*, and *Escherichia coli*, relatively little information is still available about the antimicrobial compounds produced by strains of this genus. More detailed understanding of the action mechanisms of these antimicrobials could allow us to determine the extent to which their production contributes to the probiotic properties of specific bifidobacteria strains and, potentially, be of crucial significance for ultimate preservation of functional foods or pharmaceutical applications. Here we review what is already known about their structure, classification, mode of action, functionality, immunity, production and purification.

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1. Introduction

Bifidobacteria are high GC, Gram-positive, non-spore-forming, non-motile and catalase-negative anaerobic bacteria belonging to the phylum of Actinobacteria (Ishibashi et al., 1997). They are able to ferment glucose to lactic and acetic acids via a metabolic pathway that is characterized by the presence of the enzyme fructose-6-phosphate phosphoketolase (F6PPK) (Ballongue, 2004; Gomes and Malcata, 1999). These microorganisms were first isolated by Tissier (1900), described as pleomorphic rods with different shapes, including curved, short and bifurcated Y shapes, and initially classified as *Bacillus bifidus communis*. Subsequently, they were renamed *Lactobacillus bifidus* before De Vries and Stouthamer (1967) suggested that they should be reclassified as a distinct genus (*Bifidobacterium*) because of the presence of F6PPK and the simultaneous absence of glucose-6-phosphatase dehydrogenase and aldolase, i.e. two enzymes present in lactobacilli (Ballongue, 2004; Cheikhyoussef et al., 2008; Ishibashi et al., 1997).

Bifidobacteria are an important group of human gut commensal bacteria, accounting for around 3–7% of the microbiota in adults and, according to some reports, up to 91% in newborns (Ballongue, 2004; Cheikhyoussef et al., 2009a). Some strains of *Bifidobacterium*

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possess traits that have resulted in them being employed as probiotics. According to the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) (FAO/WHO, 2001), probiotics are living microorganisms that, when ingested in sufficient quantities, exert health-promoting benefits to the host. Among the many probiotic traits that have been attributed to bifidobacteria are a) the induction of immunoglobulin production, b) improvement of food nutritional value by assimilation of substrates not metabolized by the host, c) anti-carcinogenic activity and d) folic acid synthesis (Bevilacqua et al., 2003; Cheikhyoussef et al., 2009a; Collado et al., 2005a; Gomes and Malcata, 1999; Touré et al., 2003). Interestingly for the purposes of this review, some bifidobacteria are also known to produce antimicrobials (Cheikhyoussef et al., 2009b; Gibson and Wang, 1994b; Gomes and Malcata, 1999; Ibrahim and Salameh, 2001) and, more specifically, bacteriocins (Anand et al., 1984, 1985; Cheikhyoussef et al., 2010; von Ah, 2006; Yildirim and Johnson, 1998; Yildirim et al., 1999).

Bacteriocins are ribosomally-synthesized antimicrobial peptides produced by bacteria that are active against other bacteria, either belonging to the same species (narrow spectrum) or even across genera (broad spectrum). Producing organisms are immune to their own bacteriocin(s), a property that is mediated by specific immunity proteins (Cotter et al., 2005b). Bacteriocin production takes place most frequently during the late exponential or early stationary phases of growth, is often influenced by quorum sensing and stress signaling (Klaenhammer, 1988; Kotelnikova and Gelfand, 2002; Riley and Chavan, 2007; Tagg et al., 1976), and is regarded as a probiotic trait (Dobson et al., 2012; O'Shea et al., 2012) contributing to the suppression of intestinal pathogens. In addition, the rise in demand for natural foods that do not contain chemical preservatives has increased the interest in their application as preservatives to ensure food quality and safety. Since the discovery of bacteriocins (Cascales et al., 2007; Cotter et al., 2005a), in-depth studies have been undertaken to get detailed information on their physicochemical properties, mechanisms of action and genetic determinants (Cotter et al., 2005a; Drider et al., 2006; Ennahar et al., 2000; Riley and Wertz, 2002; Tagg et al., 1976), all of which are of great significance for the ongoing attempts to commercialize them more extensively. A considerable part of research on bacteriocins has focused on the production and investigation of peptides from lactic acid bacteria (LAB) such as *Lactococcus* spp., *Leuconostoc* spp., *Enterococcus* spp., and *Pediococcus* spp., with a view to their potential application as natural preservatives of foods (Cheikhyoussef et al., 2009a; Deegan et al., 2006; Riley and Chavan, 2007). Despite the potential of bifidobacteria to suppress the growth of both Gram-negative and Gram-positive bacteria, their ability to produce bacteriocins has so far been underestimated, being their antimicrobial activity often ascribed to the inhibitory action of organic acids and the related pH decrease (Ballongue, 2004; Makras and De Vuyst, 2006; von Ah, 2006). However, exceptions exist.

Here we review the literature relating to bifidobacteria able to produce bacteriocins, with a focus on their distinctive features, factors influencing their production, purification, mechanisms of action and classification.

2. Antimicrobial compounds from *Bifidobacterium* spp.

Bifidobacteria have the capacity to synthesize organic acids and other antimicrobial compounds such as bacteriocins. Although some reports have suggested that the production of organic acids, via the heterofermentative pathways, is partially responsible for the inhibitory activity of bifidobacteria (Bruno and Shah, 2002; Ibrahim and Salameh, 2001), it is well accepted that at least some bifidobacteria also produce bacteriocins. In some cases, the antimicrobial activity was associated with the production of peptides, but the exact nature of the active substance was not determined (Anand et al., 1984, 1985; Bernet et al., 1993; Liévin et al., 2000; Meghrous et al., 1990); in other cases, the peptides involved were definitively identified.

Table 1 contains a list of known *Bifidobacterium*-associated bacteriocins and putative bacteriocins as well as their main characteristics. In general, it can be stated that research of *Bifidobacterium*-associated bacteriocins has been relatively unsatisfying and has provided more questions than answers. The following paragraphs provide information regarding a significant number of putative bacteriocins about which frustratingly little is known.

The first putative *Bifidobacterium*-associated bacteriocin found is bifidin produced by *Bifidobacterium bifidum* NCDC 1452. The antimicrobial activity of this strain was found to be the greatest when grown in skim milk, and from this medium it was extracted with methanol–acetone and partially purified by Sephadex G-15 chromatography. The purified product was refrigerated for 3 months or more without exhibiting any activity loss (Anand et al., 1984, 1985). Amino acid analysis of the peptide revealed high contents of phenylalanine and glutamic acid and, in less extent, threonine, aspartic acid, serine, glycine, proline, isoleucine and leucine. However, the study on bifidin did not progress since the mid-1980s.

A number of years later, Kang et al. (1989) described a *Bifidobacterium longum* strain that produced an uncharacterized antimicrobial, referred to as bifilong, that inhibited some Gram-negative and Gram-positive bacteria and was stable over a pH range of 2.5 to 5.0. Similarly, Meghrous et al. (1990) discovered thermoresistant proteinaceous compounds in the supernatant of *B. bifidum* cultures, which inhibited the growth of *Streptococcus*, *Lactococcus* and *Clostridium* spp. However, as the authors were specifically targeting antimicrobials able to inhibit Gram-negative bacteria, the active compounds were not isolated. Liévin et al. (2000) were successful in demonstrating the anti-*Salmonella typhimurium* activity of a highly lipophilic, low molecular weight (<3500 Da) compound produced by *Bifidobacterium* strains, which was precipitated with ammonium sulfate and partially purified by methanol–chloroform extraction and dialysis. However, once again, this compound was not further characterized. Following the same theme, Touré et al. (2003) isolated bifidobacteria strains from infants that displayed antagonistic activity against *Listeria monocytogenes*. Using methanol–acetone extraction, they purified the most hydrophilic proteinaceous antimicrobials, which were found to be resistant to high temperature (100 °C for 5 min) but sensitive to proteases. Saleh and El-Sayed (2004) provided a somewhat more detailed report on the production, in MRS broth with 0.05% L-cysteine. HCl, of putative bacteriocins, designated as bifilact Bb-12 and bifilong Bb-46, by *Bifidobacterium lactis* Bb-12 and *B. longum* Bb-46, respectively. These two bacteriocins were shown to exhibit strong activity against *Staphylococcus aureus*, *S. typhimurium*, *Bacillus cereus* and *Escherichia coli*. While the minimal inhibition concentrations (MICs) of partially purified bifilact Bb-12 and bifilong Bb-46 were found to be 40 and 20 mg/mL for *S. aureus* and 20 and 16 mg/mL for *E. coli*, respectively, one can expect that purified peptides, if obtained, would be even more active. Additional antimicrobials from six *Bifidobacterium* strains were found to exhibit broad inhibitory spectra against both Gram-negative and Gram-positive bacteria, namely *Clostridium difficile*, *Brochothrix thermosphacta*, *L. monocytogenes*, *S. aureus*, *Helicobacter pylori*, *S. typhimurium*, *Arcobacter butzleri*, and some pathogenic yeasts. These heat-stable compounds were sensitive to proteinases and resistant to pH in the range from 3 to 10 (Collado et al., 2005b), but were neither purified nor subject to further investigation. Finally, von Ah (2006) identified, recovered by methanol/acetone extraction and reversed-phase HPLC and partly characterized thermophilicin B67, a bacteriocin produced by *Bifidobacterium thermophilum* RBL67 that exhibited a narrow inhibition spectrum towards three *Listeria* strains and *Lactobacillus acidophilus*.

Ultimately, despite the many reports on *Bifidobacterium*-associated bacteriocins, bifidocin B from *B. bifidum* NCFB 1454 (Yildirim et al., 1999), bifidin I from *Bifidobacterium infantis* BCRC 14602 (partially sequenced) (Cheikhyoussef et al., 2010) and the lantibiotic bisin from *B. longum* DJO10A are the only bacteriocins that were in-depth

Table 1
Bacteriocins from *Bifidobacterium* spp. and their main characteristics.

Bacteriocin	Species and strain	Mol. wt. (kDa)	Heat range stability	pH range stability	Production phase	Optimal production	Inhibitory spectrum	Reference
Bifidin	*B. bifidum* NCDC 1452	(–)	(100 °C—30 min)	4.8–5.5	After 48 h	pH: 4.8	Gram-positive and Gram-negative bacteria	Anand et al. (1984, 1985)
Bifidocin B	*B. bifidum* NCFB 1454	3.3	(121 °C—15 min)	2–12	(12–18 h)	37 °C, pH 5.0–6.0	*Bacillus cereus*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Pediococcus acidolactici*, *Streptococcus faecalis*, etc.	Yildirim and Johnson (1998); Yildirim et al. (1999)
Bifilong	*B. longum*	120	(100 °C—30 min)	2.5–5.0	(−)	(−)	Gram-positive and Gram-negative bacteria	Kang et al. (1989)
Bifilact Bb-46	*B. longum* Bb-46	25–127	(121 °C—15 min)	4–7	(−)	(−)	*Staphylococcus aureus*, *Salmonella typhimurium*, *Bacillus cereus*, *E. coli*	Saleh and El-Sayed (2004)
Bifilact Bb-12	*B. lactis* Bb-12	25–89	Unstable for high temperatures	4–7	(−)	(−)	*Staphylococcus aureus*, *Salmonella typhimurium*, *Bacillus cereus*, *E. coli*	Saleh and El-Sayed (2004)
Thermophilicin B67	*B. thermophilum* RBL67	5–6	(100 °C—5 min)	2–10	24 h	pH 6 and 40 °C	*Listeria sp.*, *Lactobacillus acidophilus*	von Ah (2006)
Bifidin I	*B. infantis* BCRC 14602	3	(121 °C—15 min)	4–10	18 h	(−)	*LAB* strains, *Staphylococcus*, *Bacillus*, *Streptococcus*, *Salmonella*, *Shigella*, *E. coli.*	Cheikhyoussef et al. (2009a, 2010)
Lantibiotic (Bisin)	*B. longum* DJO10A	(−)	(−)	(−)	1–8 h	Auto-induction by crude lantibiotic	*Streptococcus thermophilus* ST403, *Clostridium perfringens*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Serratia marcescens*, *E. coli* DH5a.	Lee et al. (2011)

(−): not available.

characterized and whose amino acid sequence was at least partially elucidated. The (predicted) amino acid sequence of these bacteriocins is shown in Table 2. Bifidocin B and bifidin I show homology in the N-terminal region to Class IIa bacteriocins, also known as pediocin PA1-like bacteriocins (bacteriocin classification is detailed in Section 3), which contain a -Tyr-Gly-Asn-Gly-Val-Xaa-Cys (or YGNGV) consensus region and that are known for their potent antilisterial activity (Drider et al., 2006; Lozano et al., 1992). Bifidocin B is produced by *B. bifidum* NCFB 1454 and shows activity against *Listeria*, *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Staphylococcus*, *Clostridium*, *Leuconostoc* and *Bacillus* strains (Yildirim and Johnson, 1998). Its bactericidal activity brought about a 99% decrease in CFU/mL of all these sensitive indicator strains after only 30 min (Cheikhyoussef et al., 2009a; Yildirim et al., 1999). Bifidin I was purified in 2010 by Cheikhyoussef and coworkers from *B. infantis* BCRC 14602 I using a three-step purification procedure. Initial studies, using *Lactobacillus plantarum* BCRC 11697 as a target, established that bifidin I (1000 Activity Units [AU]/mL) brought about 93 and 95% growth inhibition after 2 and 6 h, respectively (Cheikhyoussef et al., 2009a). Further investigations established that bifidin I has a broad activity spectrum, including Gram-positive bacteria such as *Streptococcus*, *Staphylococcus* and *Clostridium*, and Gram-negative ones such as *Salmonella*, *Shigella* and *E. coli* (Cheikhyoussef et al., 2009b). Bisin was found to be effective against several indicator strains of *Streptococcus thermophilus*, *Bacillus subtilis*, *Serratia marcescens* and *S. aureus*, among others (Lee et al., 2008, 2011).

Therefore, since some of these bacteriocins may affect starter and probiotic cultures, care should be taken when selecting bacteriocin producing strains for inclusion in fermented foods, to make sure that pathogens rather than important LABs are the targets of their bacteriocins.

2.1. Antimicrobial compounds: production time and phases

The production of bacteriocins is generally associated with late logarithmic phase and early stationary phase of growth, but the concentrations obtained are often low, which makes their purification and subsequent application difficult. Therefore, a precise knowledge of the growth phase during which bacteriocin production is optimal can be critical. Unfortunately, since bacteriocin producing bifidobacteria can be grown in a variety of different growth media (the importance of which is highlighted below) and the activity of bacteriocins is measured in a number of different ways (different indicator microorganisms, assays etc.), a systematic comparison between different studies on bacteriocin production is quite difficult (Pongtharangkul and Demirci, 2004; von Ah, 2006). Although considerable variation exists, it is not possible to ascertain to what extent it is the result of different producing strains or assays employed. For instance, the antimicrobial production by *B. bifidum* NCDC 1452 appeared after 30 h of growth and reached a maximum after 48 h (Anand et al., 1985), whereas bifidocin B was produced by *B. bifidum* NCFB 1454 between the late logarithmic and early stationary phases (12–18 h) (Cheikhyoussef et al., 2008) and decreased in concentration along the stationary one (by 50 and 75% after 18 and 72 h, respectively) (Yildirim and Johnson, 1998). Furthermore, Collado et al. (2005a) observed that, in the presence of Tween 80, the antimicrobial activity of a variety of different *Bifidobacterium* strains reached a maximum in the early stationary phase (around 16 h of fermentation) but decreased or was absent thereafter (Deraz et al., 2005). Thus, while there is a general consensus with respect to the importance of the growth phase, the time lasted by a specific bacteriocin-producing strain to enter the idiophase can be quite variable.

2.2. Antimicrobial compounds: enzymes, pH and heat stability

The characteristics of *Bifidobacterium*-associated bacteriocins can vary considerably as shown in the following and more synthetically in Table 1. Bifidin from *B. bifidum* NCDC 1452 was optimally produced at pH 4.8, displayed maximal inhibitory potential between 4.8 and 5.5, and was stable even after exposure to 100 °C for 30 min

Table 2
The (predicted) amino acids sequence of *Bifidobacterium* spp. bacteriocins.

Bacteriocin	Amino acid sequence	Amino acid residues	References
Bifidin I	KYGDVPLY (partial sequence)	Unknown	Cheikhyoussef et al. (2010)
Bifidocin B	KYYGNGVTCGLHDCRVDRGKATCGIINNGGMWGDIG	36	Yildirim et al. (1999)
Bisin prepeptide	MSINEKSIVGESFEDLSAADMAMLTGRNDDGVAPASLSFAVSVLSVSFSACSVTVVTRLASCGNCK	66[a]	Lee et al. (2011)

[a] Prior to leader cleavage.

(Anand et al., 1984, 1985). Bifidocin B from *B. bifidum* NCFB 1454 retained its biological activity between pH 2 and 12 and was more stable under acidic than alkaline conditions. Whereas it retained full activity when stored at −20 °C or −70 °C for 1 or 3 months, a decrease from 51,200 to 30,000 AU/mL took place when samples were stored at −4 °C for 1–3 months, and 25 and 50% activity decreases were observed when crude extracts were heated at 90 °C for 30 and 60 min, respectively (Yildirim and Johnson, 1998; Yildirim et al., 1999). Bifidocin B was also found to be inactivated by proteases such as trypsin, α-chymotrypsin, papain, or pepsin, whereas lysozyme, ribonuclease A, glucose oxidase, lipase, amylase, dextranase and catalase had no effect (Yildirim and Johnson, 1998). Bifidin I from *B. infantis* BCRC 14602 was found to be stable over wide ranges of pH (from 4 to 10, maximum activity at pH 4.8) and temperature (30 min at 50 °C and 15 min at 121 °C), but was totally inactivated by protease and proteinase K, partially inactivated by alcalase 2.4 LFG (50% activity reduction) and almost not affected by α-amylase, lysozyme and lipase (Cheikhyoussef et al., 2009a). The activities of bifilact Bb-12 from *B. lactis* Bb-12 and bifilong Bb-46 from *B. longum* Bb-46 reached optimum values at pH 4 and 7 and decreased at pH ≤ 3 and ≥ 9, respectively, at −20 °C for 24 h or when sterilized at 121 °C for 15 min. In addition, although resistant to α-amylase or lipase, these antimicrobials were found to be sensitive to pepsin and trypsin (Saleh and El-Sayed, 2004). Finally, thermophilicin B67 from *B. thermophilum* RBL 67 showed activity over broad ranges of pH (4–8) and temperature (25–47 °C), with a maximum (256 AU/mL) at pH 5.5 and 35 °C (von Ah, 2006).

3. Classification of bacteriocins

Bacteriocins can be classified into two main classes (Cotter et al., 2005b). Class I (molecular weight < 5 kDa) includes the lantibiotics and are distinguished by the fact they undergo post-translational modification. Class II bacteriocins are unmodified peptides that can be divided into four subgroups, i.e. Class IIa–d. Class IIa (or pediocin PA1-like) peptides often exhibit potent antilisterial activity, consist of 37 to 48 amino acids and are positively charged (Drider et al., 2006). These bacteriocins share a conserved sequence motif in their N-terminal region consisting of Tyr-Gly-Asn-Gly-Val-X-Cys-XXXX-Val-X-Val (or YGNGVXCXXXXVXV, with X being any amino acid), which is stabilized by two cysteines forming a disulfide bridge, and a hydrophobic and/or amphiphilic C-terminal part consisting of one or two α-helices (Eijsink et al., 2002; von Ah, 2006). Bifidocin B from *B. bifidum* NCFB 1454 belongs to this class of bacteriocins having a N-terminal domain containing a Tyr-Gly-Asn-Gly-Val-X-Cys motif (Yildirim et al., 1999), while bifidin I from *B. infantis* BCRC 146, whose N-terminal domain although not completely sequenced contains a similar, but distinct Tyr-Gly-Asp-Val stretch (Cheikhyoussef et al., 2010), has its classification still under consideration (Table 2).

With regard to the N-terminal motif of the class II peptides, residues Lys-1, Lys-11 and His-12 (or their equivalents) have been reported as mediators of non-specific bacteriocin binding to target membranes, while residues Val-7, Cys-9, Cys-14, Val-16 and Trp-18 to be involved in membrane insertion processes (Eijsink et al., 2002; Ennahar et al., 2000). Indeed, modifications in the consensus sequence drastically reduce bacteriocin activity (Drider et al., 2006). On the other hand, it has been suggested that the C-terminal region of pediocin-like bacteriocins is responsible for target cell specificity (Johnsen et al., 2005).

Lee et al. (2008) described the production of the Class I lantibiotic bisin by *B. longum* DJO10A, which was found to be effective against both Gram-positive and Gram-negative bacteria. Bisin is encoded by a typical lantibiotic-associated gene cluster consisting of genes encoding, in two-component signal transduction system (*lanR2* and *lanK*), a lantibiotic prepeptide (*lanA*), a lantibiotic response regulator (*lanR1*), lantibiotic modification enzymes (*lanD* and *lanM*), a lantibiotic immunity protein (*lanI*) and a lantibiotic transporter with predicted protease activity (*lanT*) (Lee et al., 2008, 2011). Its production was detected when the producing strain was grown on agar, but not in broth due to repression of *lanA* transcription; however, the lantibiotic, when added to broth (at 160 AU antimicrobial activity), acted as an induction factor improving the production. Interestingly, the bacteriocin-producing phenotype of this strain is quite unstable as the 10.2 kb gene cluster, located between two IS30 elements, can be lost during serial subculturing (Lee et al., 2008).

Although the bifidocin B gene cluster has still to be identified, it has been established, through the use of acriflavin and the isolation of mutants unable to produce the bacteriocin, that its production by *B. bifidum* NCFB 1454 is associated with a plasmid of about 8 kb in size, whereas this plasmid is not required for immunity or sugar fermentation. The presence of a gene encoding bifidocin B on the 8 kb plasmid was confirmed by Southern blotting using an oligonucleotide based on the N-terminal amino acid sequence (Yildirim et al., 1999).

As noted above, there are several situations where further investigation is required to elucidate the nature of putative bacteriocins. In addition to peptides (< 10 kDa), there are some reports on larger antimicrobial proteins (bifilact Bb-46, bifilact Bb-12 and bifilong) (Cheikhyoussef et al., 2010; Collado et al., 2005a,b) that are potentially belonging to the bacteriolysin family of antimicrobials (Cotter et al., 2005b).

4. Influence of culture medium and bifidogenic factors

The availability of simple and inexpensive methods and reagents for the cultivation of *Bifidobacterium* spp. will be important for production of bacteriocins and their commercial applications. Most of *Bifidobacterium*-specific culture media have a complex composition, often containing antibiotics or induction factors, and imply long incubation times. Many nutritious culture media also negatively impact on the production of antimicrobials by the selected strains (Nebra and Blanch, 1999).

Bifidobacteria show high growth rates in rich synthetic media such as Trypticase–Peptone–Yeast extract (TPY) and MRS broths, but can also grow in simple media containing only lactose, free amino acids, mainly cysteine, glycine and tryptophan, and some nucleotides, vitamins and minerals (Gomes and Malcata, 1999). Ballongue (2004) highlighted the advantages of adding bifidogenic growth factors such as N-acetyl-D-glucosamine or cysteine, azide and China ink to MRS agar medium to differentiate bifidobacteria species, or vitamins such as pyridoxine (B6), thiamine (B1), cyanocobalamine (B12), folic acid (B9) and nicotinic acid (PP) or different selective agents such as polymyxin, propionate and linoleate. The control of magnesium, manganese and iron levels was shown to be essential for optimal growth of bifidobacteria or bacteriocin production, as a result of nutritional stress or induction, according to circumstances (Kang and Fung, 2000; O'Sullivan, 2001).

Most of bifidobacteria strains were also shown to grow effectively in milk-based media, which constitutes a great advantage taking in mind the high cost of synthetic media. So, several protein sources such as liver, meat or yeast extract, peptones, horse blood, tomato juice or human milk, along with antioxidant compounds such as cysteine, ascorbic acid, or sodium sulfite have also been successfully added to these media (Gomes and Malcata, 1999; Russell et al., 2011).

In addition to the components described above, many species of bifidobacteria can also utilize complex biopolymers able to improve cell growth and production of antimicrobials, including bifitose, fructooligosaccharides (FOS) and xylooligosaccharides, among others. These compounds, which are generally carbohydrates or their derivatives, are metabolized by bifidobacteria, but not by the host or the majority of other bacteria (Gomes and Malcata, 1999); among them, the different types of linear and branched FOS, oligofructose and

lactulose-based oligosaccharides show particularly high induction rates. Dietary fibers have also been found to be effective in promoting the intestinal growth of bifidobacteria (Dubey and Mistry, 1996; Ishibashi et al., 1997). However, not all strains have the same nutritional requirements.

There are also a number of nutritional and biological factors that influence antimicrobial production by bifidobacteria including, as mentioned above, nutrient shortage as well as the presence of a competing microbiota (O'Shea et al., 2012). Touré et al. (2003) reported that a co-culture of *L. monocytogenes* and *Bifidobacterium* spp. was able to stimulate bacteriocin production by the latter and, in some cases, the addition of surfactants such as Tween 80 increased the concentration of bacteriocins produced as a consequence of cell growth acceleration. Surfactants may also enhance the sensitivity of the indicator strain and form micelles with proteinaceous compounds, thus stabilizing the bacteriocins (Carolissen-Mackay et al., 1997; Cheikhyoussef et al., 2008; Collado et al., 2005b).

Finally, bifidobacteria are usually cultivated under anaerobic conditions. However, some studies have reported that some strains of *Bifidobacterium* spp. can decompose and detoxify oxygen metabolites by certain enzymes such as superoxide dismutase and catalase, and that oxygen sensitivity differs according to the species or strains. Thus, several oxygen-tolerant bifidobacteria have been reported by various authors (Chou and Hou, 2000; Li et al., 2010; Yang et al., 1998).

5. Purification and separation

Bacteriocin purification is usually difficult because these low molecular weight, hydrophobic peptides are often produced only in small amounts (Berjeaud and Cenatiempo, 2004). From an industrial point of view, several bacteriocins have been purified and characterized (De Vuyst and Leroy, 2007), but no bacteriocin from *Bifidobacterium* spp. has yet been prepared on an industrial scale. Table 3 gives a summary of the approaches used to date to (partially) purify *Bifidobacterium*-associated bacteriocins.

In many cases, the first step of the process is the precipitation of antimicrobials from culture supernatants usually adding ammonium sulfate. Such a salting out operation (60% saturation) followed by chloroform–methanol extraction was successfully employed by Cheikhyoussef et al. (2008) to concentrate the antimicrobials present in cell free supernatants of two strains of bifidobacteria (CA1 and F9), yielding a product effective against *S. typhimurium* SL1344 and *E. coli* C1845. Other alternatives include the use of acid or organic solvents as precipitating agents (Gibson and Wang, 1994a). Dialysis and ultrafiltration can also be used to further concentrate and purify bacteriocins, even though some *Bifidobacterium*-associated studies pointed out large product losses due to incomplete precipitation (Collado et al., 2005b; Liévin et al., 2000). The final purification step generally consists of reversed phase high-performance liquid chromatography (RP-HPLC) with acetonitrile gradient, which can be followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or mass spectrometry for molecular size determination.

Bifidocin B from *B. bifidum* NCFB 1454 was purified by precipitation with 70% ammonium sulfate followed by dialysis through a 1000 molecular weight cutoff dialysis membrane, which resulted in an 18-fold increase in antimicrobial activity of the dialysis product (58,200 AU/mL) compared to the cell-free culture supernatant (3200 AU/mL) (Yildirim and Johnson, 1998). Subsequently, Yildirim et al. (1999) developed a purification method based on a rapid and simple three-step process including freeze drying, Micro-Cel adsorption/desorption and cation exchange chromatography with a carboxymethyl cellulose column, which allowed increasing the specific activity from 285 AU/mg in the cell-free supernatant to 29,880 AU/mg after the Micro-Cel step, and to 540,000 AU/mg after the cation exchange chromatography, corresponding to a 1895-fold overall concentration effect (Table 3).

Bifidin I from *B. infantis* BCRC 14602 was partially purified by a two-step purification process (Table 3). Initial precipitation with ammonium sulfate, which resulted in an 80% yield and 4.56-fold concentration, was followed by dialysis using a 1000 Da molecular-weight cutoff and, finally, by freeze-drying. Such a process allowed obtaining a final preparation with specific activity of 31,605 AU/mg, corresponding to overall 120-fold purification and 64% yield (Cheikhyoussef et al., 2009a). A new method has recently been developed by Cheikhyoussef et al. (2010), whereby bifidin I was purified/concentrated by a three-step process. The purification protocol started with its recovery by adsorption/desorption onto/from silicic acid, which resulted in a preparation with specific activity of 67,696 AU/mg, corresponding to 257-fold concentration and 80% yield. This active preparation was then subject to cation exchange separation on SP-Sepharose at pH 7.6 and final purification by RP-HPLC (in the process establishing the cationic nature of bifidin I), which allowed increasing the specific activity up to 115,315 AU/mg (64% yield) and 36,571 AU/mg (25.6% yield), corresponding to 438- and 1390-fold concentrations, respectively.

Table 3
Summary of approaches taken to (partially) purify *Bifidobacterium* associated bacteriocins.

Bifidobacterium species and strain	Bacteriocin	Purification steps[a]	Volume (mL)	Total activity (AU)	Protein concentration (mg/mL)	Total protein (mg)	Specific activity (AU/mg)	Purification factor	Yield (%)	Reference
B. bifidum NCFB 1454	Bifidocin B	ADPC	(−)	(−)	(−)	(−)	58,200	18	(−)	Yildirim and Johnson (1998)
B. bifidum NCFB 1454	Bifidocin B	CFCs	1500	4,800,000	(−)	16,855	285	1	100	Yildirim et al. (1999)
		FD	150	4,704,000	(−)	16,774	281	0.99	98	
		MC	15	3,600,000	(−)	121	29,880	104	75	
		CMC	1.5	864,000	(−)	1.6	540,000	1895	18	
B. thermophilum RBL67	Thermophilicin B67	CFCs	2170	17,360	0.065	141.1	123	1	100	von Ah (2006)
		MEA	217	17,344	0.532	115.4	120	1	99.9	
		FD	7	5971	0.416	2912	2051	16.7	34.4	
B. infantis BCRC 14602	Bifidin I	CFCs	500	80×10^4	(−)	3040	263	1	100	Cheikhyoussef et al. (2009b)
		FD	50	64×10^4	(−)	2800	228	0.86	80	
		ADPC	5	51×10^4	(−)	16.2	31,605	120	64	
B. infantis BCRC 14602	Bifidin I	NCFS	500	800,000	(−)	3040	263	1	100	Cheikhyoussef et al. (2010)
		ADSA	50	640,000	(−)	9.44	67,696	257	80	
		SPSFF	5	512,000	(−)	4.44	115,315	438	64	
		RP-HPLC	1	204,800	(−)	0.56	365,714	1390	25.6	

[a] ADSA: adsorption and desorption onto/from silicic acid; ADPC: adsorption and desorption from producer cells; CFCs: cell-free supernatant; CMC: carboxymethyl cellulose (cation-exchange chromatography); FD: freeze-drying; MEA: methanol acetone extract; MC: micro-Cel; NCFS: neutralized cell free supernatant; RP-HPLC: reverse phase-high performance liquid chromatography; SPSFF: sulfopropyl sepharose fast flow; (−): not determined.

Bifidin, bifilong (Kang et al., 1989) and thermophilicin B67 (von Ah, 2006) were also purified/concentrated from the cell free supernatants of producing strains using methanol–acetone extraction followed by partial purification by chromatographic methods (Anand et al., 1985). In the case of thermophilicin B67, the specific activity of the methanol–acetone extract (853 AU/mL) pointed out a 17-fold purification compared with the freeze-dried supernatant (von Ah, 2006).

Finally, a crude lantibiotic preparation was collected from *B. longum* DJO10A agar cultures by methanol extraction and size fractionation. Although the lantibiotic activity (160 AU/mL) was low, it was sufficient for microtiter plate-based experiments (Lee et al., 2011).

Other nutritional factors can influence the purification process. For instance, high concentrations of peptides in the growth medium, resulting from the addition of beef or yeast extract, may interfere with various purification processes. Nonetheless, several investigations have demonstrated that the use of complex growth media with a high peptide content is necessary to ensure high bacteriocin productivity (Carolissen-Mackay et al., 1997), and thus a tradeoff is required. Similarly, if from one hand the addition of Tween 80 can enhance bacteriocin production, from the other it can affect the purification process through, for example, the formation of precipitates in the culture supernatants (Collado et al., 2005b).

6. Conclusions and future prospects

Despite the potential paramount importance and value of bacteriocin production by bifidobacteria, only a few of such bacteriocins have been purified and characterized. This review summarizes what is already known about *Bifidobacterium*-associated bacteriocins, with particular concern to those that have been explored in greatest depth, i.e. bifidocin B, bifidin I, thermophilicin B67 and bisin, thereby providing an overview of our understanding about their classification, mode of action, genetic determinants, spectra of activity and factors influencing their production. In addition, some bacteriocins from *Bifidobacterium* genus may affect starter and probiotic cultures; therefore, care must be taken when selecting bacteriocin producing strains for inclusion in fermented foods to make sure that important LABs are not the targets of their bacteriocins. The route of future application/commercialization of these *Bifidobacterium*-associated bacteriocins, as well as those still to be identified, will be dependent on whether the application will involve the use of the producing strain as a probiotic or will require the peptides in a (partially) purified or concentrated form. Therefore, it is important to emphasize that it is necessary to deepen the study of bacteriocins produced by *Bifidobacterium* spp., which proved to advantageously contribute to functional food properties and pharmaceutical applications.

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TRENDS IN FOOD SCIENCE & TECHNOLOGY

Review

Lactic acid properties, applications and production: A review

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Lactic acid was discovered in 1780 by C.W. Scheele in sour milk, and in 1881 Fermi obtained lactic acid by fermentation, resulting in its industrial production. The yearly world lactic acid production is expected to reach 259,000 metric tons by the year 2012. The interest in lactic acid is related to many aspects, among which is its relatively high added-value. In addition, such a chemical is GRAS (Generally Recognized As Safe), being recognized as harmless by the United States Food and Drug Administration, has a market with great growth potential, can be alternatively produced by fermentation or chemical synthesis and can employ a large variety of different waste materials as substrates. Lactic acid has many applications. Its existence in the form of two stereoisomers does in fact make the application of one of them or of the racemic mixture of great concern in different fields. In particular, the food and pharmaceutical industries have a preference for the isomer L(+), the only one that can be metabolized by the human body; however, the chemical industry requires one of the pure isomers or a mixture of both, according to the application. This review describes biotechnological processes to obtain lactic acid from polymeric substrates such as starchy and lignocellulosic materials. Open challenges are related to the technological optimization of the fermentation process and product purification and recovery. In addition, the opportunities and difficulties associated with using raw materials for lactic acid production are discussed.

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Introduction

Because of a number of different properties (Abdel-Rahman, Tashiro, & Sonomoto, 2011), lactic acid is an important industrial product that is used as a precursor of small (propylene glycol) or large (acrylic polymers) compounds (San-Martín, Pazos, & Coca, 1992). Their polymers are biodegradable, used as materials for packaging and labeling (San-Martín *et al.*, 1992), and biocompatible, being useful for the manufacture of prosthetic devices, sutures and internal drug dosing (Chahal, 2000, pp. 1–9). Among them, the polylactic acid (Boswell, 2001; Tsuji, Saeki, Tsukegi, Daimon, & Fujie, 2008) has several applications in the textile, medical and pharmaceutical industries (Singhvi, Joshi, Adsul, Varma, & Gokhale, 2010).

In the cosmetic industry, lactic acid is used in the manufacture of hygiene and esthetic products, owing to its moisturizing, antimicrobial and rejuvenating effects on the skin, as well as of oral hygiene products. Lactic acid derivatives such as lactate esters are widely used because of their hygroscopic and emulsifying properties (Gao, Ma, & Xu, 2011). In the pharmaceutical industry it is used as a supplement in the synthesis of dermatologic drugs and against osteoporosis (Bai, Zhao, Li, & Xu, 2004).

Approximately 70% of lactic acid produced is used in the food industry because of its role in the production of yogurt and cheese (Salminen, Ouwehand, Wright, & Daly, 1993). In the preparation of yogurts it is the main product of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*

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co-fermentation. In the manufacture of cheese, the pH decrease consequent to lactic acid release triggers the aggregation of casein micelles. Sometimes, depending on the sensory characteristics desired in the final product, direct acidification with lactic acid is exploited to avoid the risk of proliferation of undesirable microorganisms. In the field of grain production, lactic acid forms spontaneously because of the presence of microorganisms that carry out the lactic acid fermentation of the raw material (for example, wet processing of corn), leads to changes in the aroma and taste preparations and causes a decrease in pH that prevents the growth of pathogenic bacteria (Lee & Lee, 1993).

As far as the animal nutrition is concerned, controlled lactic fermentation increases the shelf life, palatability and nutritive value of silage. Ammonium lactate is an excellent non-protein nitrogen source, which is preferred in cattle to urea and ammonium citrate because it results in milk with higher nutritive value (Norton, Lacroix, & Vuillemard, 1994) and does not require any expensive purification.

Physico-chemical properties

Lactic acid (2-hydroxypropanoic acid) is an organic acid widely distributed in nature. It is the simplest 2-hydroxycarboxylic acid with a chiral carbon atom and exists in two enantiomeric forms (Fig. 1). The chemical behavior of lactic acid is determined by its physico-chemical properties, among which are a) acidic character in aqueous medium; b) bifunctional reactivity associated with the presence of a carboxyl and a hydroxyl group, which gives it great reaction versatility; and c) asymmetric optical activity of C2.

Production technologies and purification

The worldwide demand of lactic acid in 2007 was estimated to be 130,000–150,000 metric tons per year, with commercial prices of food-grade lactic acid ranging between 1.38 US$ kg^{-1} (50% of purity) and 1.54 US$ kg^{-1} (88% of purity) (John, Nampoothiri, & Pandey, 2007). According to forecasts, its production should increase significantly over the coming years mainly to provide the polylactic acid manufacturing sites, and is expected to reach 259,000 metric tons in 2012 (Mujtaba, Iqbal, Edreder, & Emtir, 2012). The Global Industry Analyst Inc. announced in January 2011 that the global market for lactic acid is forecast to reach approximately 329,000 metric tons by the year 2015.

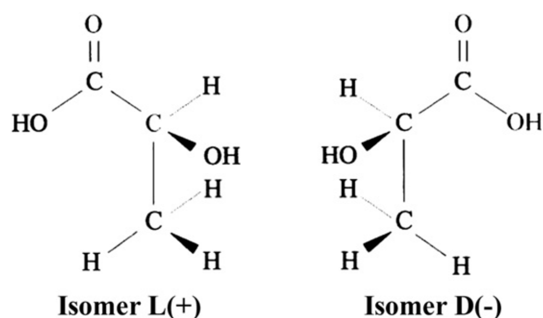

Fig. 1. Structure of D(−) and L(+) isomers of the lactic acid.

Commercial manufacturers

As regards the world production of lactic acid, several authors reported the most relevant commercial manufacturers (Datta & Henry, 2006; Datta, Tsai, Bonsignore, Moon, & Frank, 1995; John, Nampoothiri, *et al*., 2007). Currently, the major manufacturers of lactic acid include Archer Daniels Midland Company (USA), NatureWorks LLC (USA), Purac (The Netherlands), Galactic S.A. (Belgium) and several Chinese companies, among them are the CCA (Changzhou) Biochemical Co. Ltd., Henan Jindan Lactic Acid Co. Ltd., and Musashino Chemical Co. Ltd.

Chemical synthesis

For lactic acid chemical synthesis, acetaldehyde is let to react in liquid phase and under high pressure with hydrogen cyanide in the presence of a base to produce lactonitrile. After its recovery and purification by distillation, hydrochloric acid or sulfuric acid is added to hydrolyze lactonitrile to lactic acid, which is then esterified with methanol to produce methyl lactate, and this is recovered and purified by distillation. The purified methyl lactate is finally hydrolyzed in acidic aqueous solution to lactic acid and methanol, the latter being recycled in the same process (Dey & Pal, 2012; Narayanan, Roychoudhury, & Srivastava, 2004a). Other chemical routes for lactic acid synthesis include base-catalyzed degradation of sugars, oxidation of propylene glycol, carbon monoxide and water at high temperature and pressure, hydrolysis of chloropropionic acid, and nitric acid oxidation of propylene, among others (John, Sukumaran, Nampoothiri, & Pandey, 2007).

Fermentation

Lactic fermentation is relatively fast, has high yields and can lead, selectively, to one of the two stereoisomers of lactic acid or to their racemic mixture (Axelsson, 2004). After supplementation of nutrients, sugar solutions are inoculated with the selected microorganism, and the fermentation takes place. It is necessary to select the most favorable fermentation conditions, in terms of temperature, pH, aeration, agitation, and so on, which vary depending on the microorganism.

The search for low-cost raw materials to be used in the production of lactic acid by fermentation has been promoting the development of competitive processes. The materials most frequently used to this purpose can be classified into two groups, namely the monosaccharides and disaccharides and the polymeric substrates.

Monosaccharides and disaccharides

In theory, any carbohydrate source containing pentoses or hexoses could be used for the production of lactic

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acid. This category of carbon sources includes food industry byproducts such as molasses and whey. Molasses have high sucrose content and are cheap and plentiful (Kotzamanidis, Roukas, & Skaracis, 2002), while whey has high lactose content whose disposal constitutes a serious environmental challenge (Alvarez, Aguirre-Ezkauriatza, Ramírez-Medrano, & Rodríguez-Sánchez, 2010; Büyükkileci & Harsa, 2004). Another byproduct that was successfully used as substrate for lactic acid production is the date juice (Nancib *et al.*, 2001; Nancib, Nancib, & Boudrant, 2009).

Polymeric substrates

These substrates contain polysaccharides that, in most cases, cannot be directly assimilated by microorganisms, requiring an earlier stage of hydrolysis.

The so-called starchy materials contain starch, a biopolymer of glucose units linked *via* α(1−4) bonds forming chains of variable length, branched *via* α(1−6) bonds or not. Two different polysaccharide fractions are present in starch, namely the amylose that has a few branches and long linear chains and the amylopectin with opposite characteristics. Preparation of glucose solutions from starchy materials requires submitting the material to preliminary liquefaction by thermostable α-amylase and subsequent saccharification by α-amylase and amyloglucosidase, which prevents starch gelatinization (Massoud & El-razek, 2011; Palmarola-Adrados, Juhász, Galbe, & Zacchi, 2004). The resulting glucose solutions can be used directly as carbon source to produce lactic acid. These materials can also be fermented by some microorganisms directly without any preliminary hydrolysis stage because of their ability to release extracellular amylases.

On the other hand, lignocellulosic biomass represents the most abundant global source of biomass, and for this reason it has been largely utilized in many applications. It is mainly composed of cellulose, hemicellulose and lignin which form approximately 90% of the dry matter (Taherzadeh & Karimi, 2008). Lignocellulosic materials can be used to obtain sugar solutions that may be usefully exploited for the production of lactic acid through the following steps: a) pretreatment to break down the lignocellulosic structure, b) enzymatic hydrolysis to depolymerize lignocellulose to fermentative sugars, c) sugar fermentation to lactic acid by lactic acid bacteria and d) separation and purification of lactic acid (Abdel-Rahman *et al.*, 2011; Bustos, Moldes, Cruz, & Domínguez, 2005a; Chang, Lu, Yang, Zhao, & Zhang, 2010; Moldes, Alonso, & Parajó, 2001b; Parajó, Alonso, & Moldes, 1997; Yáñez, Alonso, & Parajó, 2004). In recent years, one of the most used processes to obtain lactic acid from lignocellulosic materials is the simultaneous saccharification and fermentation (Cui, Li, & Wan, 2011; Nakano, Ugwu, & Tokiwa, 2012; Ou, Ingram, & Shanmugam, 2011), which is able to prevent enzyme inhibition by the product (Romaní, Yáñez, Garrote, & Alonso, 2008).

Direct fermentation by fungi

Fungi and bacteria are the most widely employed microorganisms for lactic acid production. The main advantages of the use of fungi as fermenting agents are their ability to release extracellular amylases able to hydrolyze starchy materials, thus not requiring any prior stage of hydrolysis (Deng, Li, Xu, Gao, & Huang, 2012; Jin, Yin, Ma, & Zhao, 2005), and the easy separation of biomass because of mycelium formation. These fungi, which usually belong to the genus *Rhizopus* and produce especially the L(+) isomer (Wang, Sun, Wei, & Wang, 2005), have been employed with starches from corn (Bai *et al.*, 2004), rice (Fukushima, Sogo, Miura, & Kimura, 2004), potato, wheat and pineapple (Jin, Huang, & Lant, 2003; Jin *et al.*, 2005), and hydrolyzed corn cobs (Miura *et al.*, 2004), pine wood (Woiciechowski, Soccol, Ramos, & Pandey, 1999) and waste paper (Marques, Santos, Gírio, & Roseiro, 2008; Park, Anh, & Okuda, 2004).

Fermentation by bacteria

Lactic acid bacteria are named according to their ability to produce lactic acid as the major (and sometimes the sole) product of sugar fermentation. Many lactic acid bacteria also encode the enzymes required for aerobic respiration, but none synthesize heme (some lactic acid bacteria also lack menaquinones). Thus, the respiration chain is non-functional unless heme (and for some bacteria heme and menaquinones) are added to the culture medium (Pedersen, Gaudu, Lechardeur, Petit, & Gruss, 2012). Most lactic acid bacteria are catalase negative, immobile, do not form spores and have optimum growth temperature between 20 and 45 °C. In addition, they have high tolerance to acidic conditions (pH < 5), which confers them a competitive advantage over other bacteria. As shown in Table 1, the selection of a suitable microorganism enables one to ferment sugar solutions of different origin.

Lactic acid purification

Lactic acid purification is one of the most costly steps of the production process (Abdel-Rahman *et al.*, 2011; Tong *et al.*, 2004). Great attention should be paid to the addition of low-cost residues or other nutrients to the medium, because removal of impurities can significantly increase the costs of purification steps (Büyükkileci & Harsa, 2004). Methods to reduce impurities in the final product include extraction (Järvinen, Myllykoski, Keiski, & Sohlo, 2000), membrane separation (Persson, Jönsson, & Zacchi, 2001), ion exchange (Moldes, Alonso, & Parajó, 2001a), electrodialysis (Bailly, 2002) and distillation with chemical reaction (Choi & Hong, 1999; Edreder, Mujtaba, & Emtir, 2011).

According to Khunnonkwao, Boontawan, Haltrich, Maischberger, and Boontawan (2012), distillation is extremely difficult owing to the low volatility of lactic acid, and electrodialysis cannot separate charged components

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Table 1. Microorganisms and raw materials used in the production of lactic acid.

Material	Microorganisms	Carbon source	References
Monosaccharides and disaccharides			
Molasses	*L. casei*	Saccharose	Hofvendahl and Hähn-Hägerdal, 2000; Kotzamanidis *et al.*, 2002
	L. lactis	Saccharose	Milcent and Carrere, 2001
Pineapples syrup	*L. lactis*	Saccharose	Ueno, Ozawa, Ishikawa, Nakanishi, & Kimura, 2003
Camel milk	*L. delbrueckii*	Lactose	Gassem & Abu-Tarboush, 2000
Cow milk	*L. delbrueckii*	Lactose	Gassem & Abu-Tarboush, 2000
Whey	*L. acidophilus*	Lactose	Gupta & Gandhi, 1995; Kumar, Jha, & Chauhan, 2001
	L. bulgaricus	Lactose	Chakraborty & Dutta, 1999
	L. delbrueckii	Lactose	Chakraborty & Dutta, 1999
	L. casei	Lactose	Göksungur, Gündüz, & Harsa, 2005
	L. helveticus	Lactose	Amrane, 2001, 2003, 2005; Fitzpatrick and O'Keeffe, 2001;
	Lactococcus lactis	Lactose	Roukas & Kotzekidou, 1996, 1998
	S. thermophilus	Lactose	Liu, Liu, Liao, Wen, & Chen, 2004
Date juice	*L. rhamnosus*	Saccharose	Nancib *et al.*, 2001, 2005
Starchy materials			
Corn	*L. amylophilus*	Starch	Vishnu, Seenayya, & Reddy, 2002
Potato	*L. amylophilus*	Starch	Vishnu *et al.*, 2002
	L. delbrueckii	Glucose[a]	Ray, Mukherjee, & Majumdar, 1991
Wheat (bran) (flour)	*L. amylophilus*	Starch	Naveena, Altaf, Bhadrayya, Madhavendra, & Reddy, 2005
	L. bulgaricus	Glucose[a]	Hofvendahl and Hahn-Hägerdal, 1997
	L. casei	Glucose[a]	Hofvendahl and Hahn-Hägerdal, 1997
	L. lactis	Glucose[a]	Hofvendahl and Hahn-Hägerdal, 1997
Rice	*L. delbrueckii*	Glucose[a]	Fukushima *et al.*, 2004
Barley	*L. casei*	Glucose[a]	Linko and Javanainen, 1996
Yucca	*L. lactis*	Glucose[a]	Sirisansaneeyakul *et al.*, 2000
	L. plantarum	Starch	Shamala & Sreekantiah, 1988
	L. delbrueckii	Glucose[a]	John, Nampoothiri, *et al.*, 2007; John, Sukumaran, *et al.*, 2007
	L. casei	Glucose[a]	John, Nampoothiri, *et al.*, 2007; John, Sukumaran, *et al.*, 2007
Tapioca	*L. plantarum*	Glucose[a]	Shamala & Sreekantiah, 1988
Lignocellulosic hydrolyzates			
Bamboo	*L. plantarum*	Glucose	Asada, Nakamura, & Kobayashi, 2005
Corrugated	*L. coryniformis*	Glucose	Yáñez, Alonso, & Parajó, 2005
Alfalfa fifer	*L. delbrueckii*	Glucose	Sreenath, Moldes, Koegel, & Straub, 2001a, 2001b
	L. pentoaceticus	Glucose	Sreenath *et al.*, 2001b
	L. plantarum	Glucose	Sreenath *et al.*, 2001a,b
	L. xylosus	Glucose	Sreenath *et al.*, 2001b
Soy fiber	*L. delbrueckii*	Glucose	Sreenath *et al.*, 2001a
	L. plantarum	Glucose	Sreenath *et al.*, 2001a
Wood of eucalyptus	*L. delbrueckii*	Glucose	Parajó, Alonso, & Santos, 1996
Grape marc	*L. pentosus*	Xylose	Portilla, Moldes, Torrado, & Domínguez, 2007
Wheat straw	*L. pentosus*	Xylose	Garde *et al.*, 2002
	L. brevis	Xylose	Garde *et al.*, 2002
Waste paper	*L. rhamnosus*	G/X/C[c]	Marques *et al.*, 2008
Pulp	*L. delbrueckii*	Glucose	Roberto *et al.*, 2007
Cellulosic residue	*L. casei*	Glucose	Thomas, 2000
RSU[b]	*L. pentosus*	X/G/A[c]	McCaskey, Zhou, Britt, & Strickland, 1994
	L. plantarum	X/G/A[c]	McCaskey *et al.*, 1994
		Glucose	Bustos *et al.*, 2005b
Corn cobs	*L. delbrium*	Glucose	Luo, Xia, Lin, & Cen, 1997

[a] Starch hydrolyzates.
[b] Municipal waste.
[c] X = xylose/G = glucose/A = arabinose/C = cellobiose; *L.* = *Lactobacillus.*

especially contaminating amino acids and organic acids. On the other hand, nanofiltration combined with bipolar electrodialysis in downstream purification can replace multiple purification steps with only two steps, while yielding a monomer grade lactic acid from a mixture of unconverted sugars and lactic acid (Sikder, Chakraborty, Pala, Drioli, & Bhattacharjee, 2012).

Chromatography has been developed for many years as a very useful tool for pharmaceutical industry, biotechnology as well as in the production of fine chemicals (Tong *et al.*, 2004); in particular, the ion exchange technique is widely used in bioseparations, and several different ion exchangers have been successfully employed in the past few years to recover lactic acid (Thang & Novalin, 2008).

Fundamentals of biochemistry and metabolism of lactic acid bacteria

The largest and most diverse genus of lactic acid bacteria is *Lactobacillus*, which includes species with very different biochemical and physiological properties along with special resistance against acidic environment. Because of their high growth rate and productivity, microorganisms belonging to this genus are used in important industrial productions (Kylä-Nikkilä, Hujanen, Leisola, & Palva, 2000) and make use of two main routes to ferment glucose (Gao *et al.*, 2011; Mayo, Piekarczyk, Kowalczyk, Pablo, & Bardowski, 2010).

Lactic acid production from glucose and related fermentation pathways

Homolactic fermentation

This process takes place in two steps. In the former step, called glycolysis or Embden–Meyerhof–Parnas pathway, glucose is transformed into pyruvic acid, while in the latter this is reduced to lactic acid by the reducing power previously produced in the form of NADH. Thus, lactic acid is obtained from glucose as the sole product (Fig. 2) according to the overall equation:

$$\text{Glucose} \rightarrow 2\ \text{Lactic Acid} + 2\ \text{ATP} \quad (1)$$

Microorganisms that use only this route for the consumption of carbohydrates are called *Obligatory Homofermentative*, and these include, among others, *Lactobacillus acidophilus*, *Lactobacillus amylophilus*, *L. bulgaricus*, *Lactobacillus helveticus* and *L. salivarius* (Mayo *et al.*, 2010; Nigatu, 2000; Sanders & Klaenhammer, 2001).

Homolactic fermentation should theoretically yield 2 mol of lactic acid per mole of consumed glucose with a theoretical yield of 1 g of product per g of substrate, but the experimental yields are usually lower (0.74–0.99 g g^{-1}) because a portion of the carbon source is used for biomass production (0.07–0.22 g g^{-1}) (Bruno-Bárcena, Ragout, Córdoba, & Siñeriz, 1999; Burgos-Rubio, Okos, & Wankat, 2000; Hofvendahl & Hahn-Hägerda, 1997; Srivastava, Roychoudhury, & Sahai, 1992). Under stress conditions such as carbon source limitation, presence of different carbon sources other than glucose, high pH or low temperature, some homofermentative microorganisms can produce formic acid by mixed acid fermentation (Hofvendahl & Hahn-Hägerda, 2000) by the action of pyruvate-formate lyase (Gao *et al.*, 2011; Mayo *et al.*, 2010).

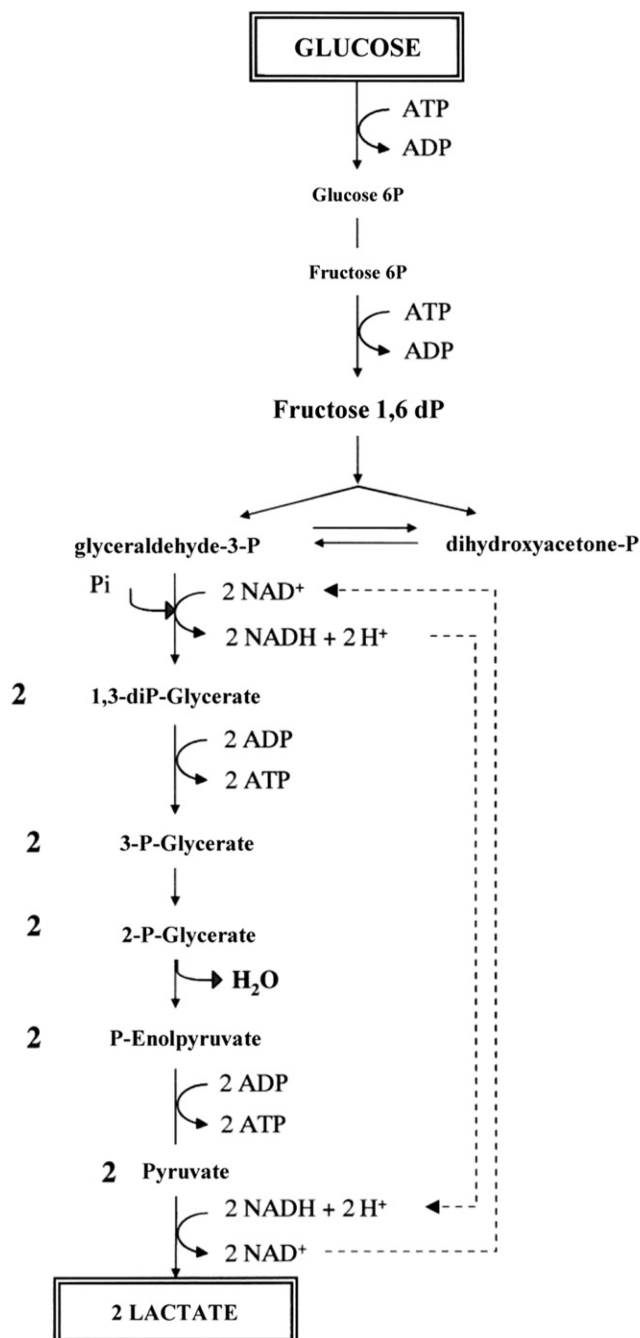
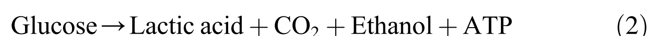


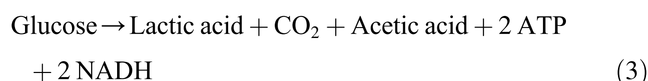
Fig. 2. Scheme of homofermentative pathway of glucose fermentation in lactic acid bacteria. Modified after Axelsson (2004) and Mayo *et al.* (2010).

Heterolactic fermentation

This process is characterized by the formation of co-products such as CO_2, ethanol and/or acetic acid in addition to lactic acid as the end product of fermentation (Fig. 3). The first step of glucose degradation, which is called pentose phosphate pathway, leads to glyceraldehyde 3-phosphate, acetyl-phosphate and CO_2. Glyceraldehyde 3-phosphate enters the glycolysis through which it is transformed into lactic acid, while acetyl-phosphate is converted into acetic acid and/or ethanol according to the overall equations:



$$\text{Glucose} \rightarrow \text{Lactic acid} + CO_2 + \text{Ethanol} + \text{ATP} \quad (2)$$



$$\text{Glucose} \rightarrow \text{Lactic acid} + CO_2 + \text{Acetic acid} + 2\ \text{ATP} + 2\ \text{NADH} \quad (3)$$

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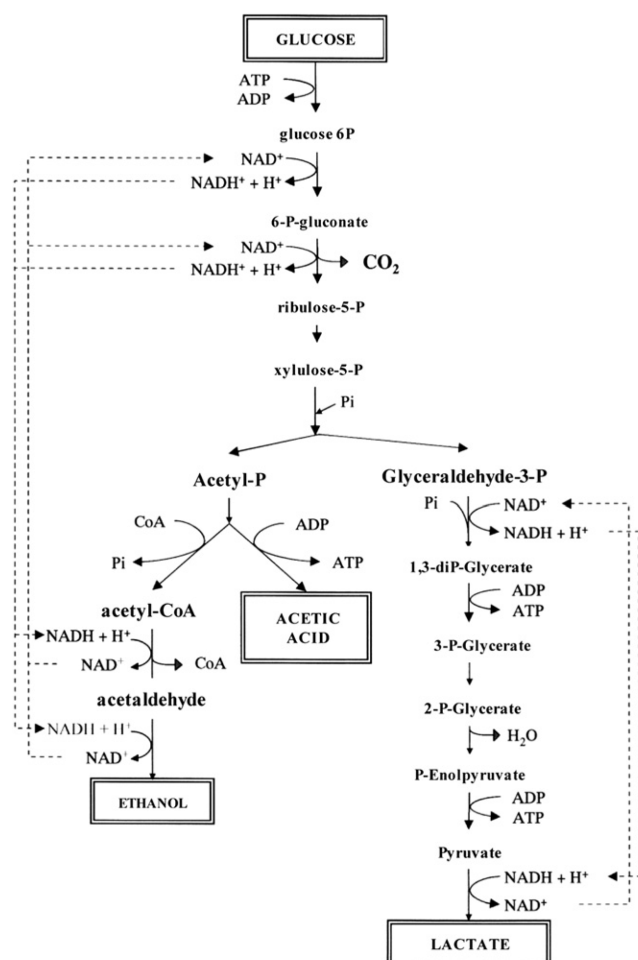

Fig. 3. Scheme of heterofermentative pathway of glucose fermentation in lactic acid bacteria. Modified after Axelsson (2004) and Mayo *et al.* (2010).

The relationship between the amounts of acetic acid and ethanol, which reduces the theoretical yield to 0.50 g g^{-1}, depends on the ability of the microorganism to reoxidize the NADH generated in the early stages of the process along with its energy requirements. Microorganisms that use only this metabolic pathway for the consumption of carbohydrates are called *Obligatory Heterofermentative*, among which are *Lactobacillus brevis*, *L. fermentum*, *L. parabuchneri* and *L. reuteri* (Mayo *et al.*, 2010; Nigatu, 2000; Sanders & Klaenhammer, 2001).

Lactic acid production from other carbon sources

In addition to glucose, there are other hexoses such as fructose, mannose or galactose, which can be consumed by lactic acid bacteria (Table 2). On the other hand, hexose-fermenting lactobacilli are unable to ferment pentoses. There are some species of this genus, classified as *Facultative Heterofermentative*, among which *L. alimentarius*, *Lactobacillus plantarum* (Gobbetti, Lavermicocca, Minervini, de Angelis, & Corsetti, 2000), *Lactobacillus casei*, *Lactobacillus rhamnosus* (Nigatu, 2000; Rivas, Torrado, Rivas, Moldes, & Domínguez, 2007; Romaní *et al.*, 2008), *Lactococcus lactis* (Ishizaki, Ueda, Tanaka, & Stanbury, 1992, 1993; Joshi, Singhvi, Khire, & Gokhale, 2010), *Lactobacillus pentosus* (Bustos *et al.*, 2005a; Moldes *et al.*, 2001a, 2001b) and *Lactobacillus xylosus* (Tyree, Clausen, & Gaddy, 1990), that perform both fermentations, consuming hexoses by the homolactic pathway and pentoses by the heterolactic one. The catabolism of pentoses requires additional conversion steps through which they are transformed into metabolic intermediates of the pentose phosphate pathway. By this way, as an instance, xylose is transformed into xylulose and then phosphorylated to xylulose 5-phosphate, arabinose into ribulose, and this in turn is phosphorylated to ribulose 5-phosphate (Gao *et al.*, 2011; Mayo *et al.*, 2010).

In recent years, the utilization of lignocellulosics as raw material for lactic acid production has required the development of methods for efficient utilization of xylose (Yoshida, Okano, Tanaka, Ogino, & Kondo, 2011). *L. xylosus* (Tyree *et al.*, 1990) and *L. rhamnosus* (Iyer, Thomas, & Lee, 2000) have been used in media containing a mixture of xylose and glucose and acidic hemicellulosic hydrolyzates of wood, respectively. *L. pentosus* allowed obtaining 33 g L^{-1} of lactic acid and 17 g L^{-1} of acetic acid from detoxified hemicellulosic liquor made from reeds (Perttunen, Myllykoski, & Keiski, 2002) and 44.8 g L^{-1} of lactic acid and 6.5 g L^{-1} of acetic acid from concentrated hemicellulosic hydrolyzates of trimming vine shoots (Bustos, Moldes, Cruz, & Domínguez, 2005b). In fermentations with *Bacillus coagulans* high levels of lactic acid were obtained from xylose and glucose (Ou *et al.*, 2011). Wang *et al.* (2009) reached 83 g L^{-1} of lactic acid from the co-fermentation of glucose and xylose by *Rhizopus oryzae* using low-energy ion beam irradiation. Mixed culture of lactic acid bacteria were also employed in the simultaneous fermentation of hexoses and pentoses, thereby allowing for efficient utilization of both cellulose- and hemicellulose-derived sugars (Cui *et al.*, 2011).

From the metabolic viewpoint, contrary to hexoses, the heterolactic fermentation of pentoses does not imply any excess of NADH; therefore, the only way to utilize acetyl-phosphate is its direct dephosphorylation to acetate with recovery of an additional mol of ATP:

$$\text{Pentose} \rightarrow \text{Lactic acid} + \text{Acetate} + 2\ \text{ATP} \qquad (4)$$

Lactic acid bacteria can also metabolize disaccharides such as lactose, maltose and sucrose, which are cleaved by the action of endocellular hydrolases. Additionally, certain species such as *L. rhamnosus* are able to consume cellobiose (Marques *et al.*, 2008), a disaccharide made up of two glucose units linked through β(1–4) bonds, which has special importance in processes employing cellulose hydrolyzates.

Stereospecific lactic acid production

Lactic acid bacteria may selectively produce one specific stereoisomer of lactic acid (D or L) or a mixture of them in various proportions. Such an ability is determined by the presence of the enzyme lactate dehydrogenase, which possesses

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Table 2. Microorganisms used in the production of lactic acid from synthetic media.[a]

Microorganism	Carbon source	References
L. amylophilus	Glucose	Mercier, Yerushalmi, Rouleau, & Dochain, 1992
	Starch	Vishnu *et al.*, 2002
L. bulgaricus	Fructose	Amoroso, Manca de Nadra, & Oliver, 1988
	Galactose	Burgos-Rubio *et al.*, 2000
	Glucose	Burgos-Rubio *et al.*, 2000; Chakraborty and Dutta, 1999
	Lactose	Burgos-Rubio *et al.*, 2000; Chakraborty and Dutta, 1999
L. casei	Glucose	Ha, Kim, Lee, Kim, & Kim, 2003; Kurbanoglu, 2004
	Lactose	Büyükkileci and Harsa, 2004; Göksungur *et al.*, 2005
L. coryniformis	Glucose	Yáñez *et al.*, 2005
	F/G/S	Zorba, Hancioglu, Genc, Karapinar, & Ova, 2003
L. delbrueckii	Glucose	Hofvendahl and Hähn-Hägerdal, 2000
	Fructose	Robison, 1988; Suskovic, Beluhan, Beluhan, & Kurtanjek, 1992
	Galactose	Kadam, Patil, Bastawde, Khire, & Gokhale, 2006
	Lactose	Hofvendahl and Hähn-Hägerdal, 2000; Welman & Maddox, 2003
	Maltose	Robison, 1988
	Saccharose	Kotzamanidis *et al.*, 2002; Srivastava *et al.*, 1992; Suskovic *et al.*, 1992; Vinderola, Costa, Regenhardt, & Reinheimer, 2002; Zlotkowska, 2000
L. helveticus	Lactose	Amrane, 2001, 2005
L. lactis	G/X/L	Bai *et al.*, 2003
	Saccharose	Milcent and Carrere, 2001; Ueno *et al.*, 2003
L. manihotivorans	Starch	Guyot, Calderon, & Morlon-Guyot, 2000
L. paracasei	Glucose	Xu *et al.*, 2006
L. pentosus	Glucose	Bustos, Moldes, Cruz, & Domínguez, 2004b
	Xylose	Portilla *et al.*, 2007
L. plantarum	Starch	Pintado, Guyot, & Raimbault, 1999; Shamala & Sreekantiah, 1988
L. rhamnosus	Gal/G/M/X	Iyer *et al.*, 2000; Romaní *et al.*, 2008
Lactococcus lactis	Glucose	Loubiere *et al.*, 1997; Sakai, 2004
	Xylose	Kanagachandran, Stanbury, Hall, & Ishizaki, 1997; Tanaka *et al.*, 2002
	Lactose	Hofvendahl and Hähn-Hägerdal, 2000
	Maltose	Sato, Tokuda, & Nakanishi, 2002
	Saccharose	Ueno *et al.*, 2003

[a]*L.* = *Lactobacillus*; G = glucose, X = xylose, L = lactose, Gal = galactose, M = mannose, F = fructose, S = saccharose.

stereospecific NAD^+-dependent activity (Hofvendahl & Hahn-Hägerda, 2000).

Among the bacteria that produce L(+) lactic acid are *L. amilophylus* (Yumoto & Ikeda, 1995), *L. brevis* and *L. buchneri* (Wu-Tai, Driehuis, & Van Wikselaar, 2003), *L. casei* (Büyükkileci & Harsa, 2004; Hujanen, Linko, Linko, & Leisola, 2001; John, Nampoothiri, *et al.*, 2007; Korbekandi, Abedi, Jalali, Fazeli, & Heidari, 2007), *Lactobacillus delbrueckii* (Hofvendahl & Hahn-Hägerda, 1997; John, Sukumaran, *et al.*, 2007; Thomas, 2000), *L. rhamnosus* (Lu, He, Shi, Lu, & Yu, 2010; Marques *et al.*, 2008; Narayanan, Roychoudhury, & Srivastava, 2004b), *L. lactis* (Bai *et al.*, 2003; Hofvendahl & Hahn-Hägerda, 1997) and *Streptococcus* sp. (Ishizaki & Ohta, 1989), whereas *Lactobacillus coryniformis* produces stereospecifically D(−)-lactic acid (Bustos, Alonso, & Vázquez, 2004; Yáñez, Moldes, Alonso, & Parajó, 2003), and *L. helveticus* (Kylä-Nikkilä *et al.*, 2000; Schepers, Thibault, & Lacroix, 2002), *L. plantarum* (Hofvendahl & Hahn-Hägerda, 2000; Yoshida *et al.*, 2011) and *L. pentosus* (Hammes & Vogel, 1995) mixtures of both isomers.

Factors affecting lactic fermentation by bacteria

Nutritional requirements of lactic acid bacteria

Several bottlenecks remain in lactic acid production processes, among which are meeting nutritional requirements of lactic acid bacteria, excess acidity, and substrate and product inhibition. To achieve good production, lactic acid bacteria need to be cultured under conditions that also ensure cell growth and viability, for which the necessary nutrients (carbon, nitrogen, minerals and vitamins) should be in directly available form (Roberto, Mussatto, Mancilha, & Fernandes, 2007).

Carbon can be present in the culture medium in the form of sugars, amino acids and organic acids that have high energy content (Cui *et al.*, 2011). Nitrogen, which is implied either in anabolic or catabolic processes, is available in the form of amino acids, peptides and inorganic compounds that can be added to the culture media as peptone, yeast extract, urea or ammonium sulfate (Nancib *et al.*, 2001). Mineral elements (Mg, Mn and Fe), which are provided in the medium in the form of salts ($MgSO_4$, $MnSO_4$ and $FeSO_4$) (Büyükkileci & Harsa, 2004; Fitzpatrick & O'Keeffe, 2001), and vitamins (mainly belonging to the B group) present in yeast extract are essential elements that act as cofactors in many enzymatic reactions.

Studies have been addressed to the optimization of nutrients (Fitzpatrick & O'Keeffe, 2001; Nancib *et al.*, 2001; Pauli & Fitzpatrick, 2002) as well as the utilization of corn steep liquor (Oh *et al.*, 2005; Wee, Yun, Lee, Zeng, & Ryu, 2005) and wastes from the winemaking process

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(Bustos, Alonso, & Vázquez, 2004; Bustos, Moldes, Cruz, & Domínguez, 2004a, 2005a, 2005b) as cheap sources of nitrogen, nutrients and minerals.

The cost of nutrients is one of the main drawbacks for the competitive biotechnological production of lactic acid. In an economic study carried out to produce lactic acid by fermentative means, it was found that yeast extract supplementation represented 38% of medium cost (Tejayadi & Cheryan, 1995). Consequently, it is economically interesting to find low-cost media to replace the traditional nutrients employed in these processes (Salgado, Rodríguez, Cortés, & Domínguez, 2009).

Acidity

Since lactic acid bacteria grow preferentially at pH between 5 and 7, the medium acidification associated with lactic acid production inhibits fermentation (Nomura, Iwahara, & Hongo, 1987; Roberto *et al.*, 2007). To minimize this occurrence, the pH can be maintained around 6 by addition of calcium carbonate at the beginning of batch fermentations, so that lactic acid can be neutralized at the same time it is formed. Hetényi, Németh, and Sevella (2011) tested five different compounds to control pH, namely ammonium hydroxide, sodium hydroxide, dimethylamine, trimethylamine and calcium carbonate. Trimethylamine proved to be the best neutralizing agent, even though the use of ammonium hydroxide would also be advisable from the technological viewpoint. Peeva and Peev (1997) used a combined method for lactic acid production by *L. casei*, where, in line with fermentation, enzymatic urea hydrolysis released the ammonium hydroxide required to neutralize lactic acid.

The use of mutant strains able to grow under low pH may be an alternative strategy to overwhelm inhibition by the acidic product. Several authors reported that the increase in acid resistance of lactic acid bacteria may be due to the restoration of the optimum intracellular pH through arginine utilization by arginine deiminase and NH_3 production (Araque, Bordons, & Reguant, 2012; Bourdineaud, 2006; Sanders, Vemena, & Kok, 1999). In addition, the use of strains able to tolerate acidic conditions would help to reduce the addition of buffering agents like calcium carbonate, thereby reducing the cost and pollution problems and making the recovery of free lactic acid from the fermentation broth easier (John & Nampoothiri, 2008).

Substrate inhibition

Substrate inhibition seems to depend on both the microorganism and the carbon source. Whereas an increase in the initial glucose concentration was shown in fact to delay the growth of *L. delbrueckii* and *L. bulgaricus* reducing both the specific productivity (Gonçalves, Xavier, Almeida, & Carrondo, 1991) and product yield (Burgos-Rubio *et al.*, 2000), such an inhibition was not observed using *L. casei* on sucrose up to 100 g L^{-1} (Büyükkileci & Harsa, 2004), *L. brevis* and *L. pentosus* on xylose up to 20 g L^{-1} (Garde, Jonsson, Schmidt, & Ahring, 2002) and *L. helveticus* on lactose up to 110 g L^{-1} (Schepers *et al.*, 2002). However, xylose inhibition of *L. lactis* fermentation was an order of magnitude stronger than that exerted by glucose (Ishizaki *et al.*, 1992, 1993). To minimize this inhibition, substrate can be added to the fermentation medium according to the fed-batch process (Roukas & Kotzekidou, 1998), but low initial substrate concentrations are required to obtain high lactic acid concentration (210 g L^{-1}), yield (0.97 g g^{-1}) and productivity (2.2 g L h^{-1}) (Bai *et al.*, 2003).

Product inhibition

Lactic acid was shown to exert an inhibitory effect on cell growth, which is stronger than that on fermentation activity (Madzingaidzo, Danner, & Braun, 2002; Milcent & Carrere, 2001). Loubiere, Cocaign-Bousquet, Matos, Goma, and Lindley (1997) suggested that lactic acid inhibition on cell proliferation and metabolism is possibly due to the increase in medium osmotic pressure, and that also some fermentation byproducts such as formic acid, acetic acid or sodium formate may exert individual inhibitory effects (Lin, Du, Koutinas, Wang, & Webb, 2008; Loubiere *et al.*, 1997). For example, Loubiere *et al.* (1997) observed a decrease of 50% on the growth of *Lc. lactis* in the presence of 76 and 187 mmol L^{-1} of formic acid and acetic acid, respectively. The concentration of the undissociated form of lactic acid plays a role in the inhibition (Bajpai & Ianotti, 1988) more important than that of lactate (Monteagudo, Rodríguez, Rinco, & Fuertes, 1997). To mitigate the effect of inhibition, various strategies have been proposed, among which are the use of fermentation technologies able to remove the product from the medium at the same time it is released (Kaufman, Cooper, Budner, & Richardson, 1996; Moldes *et al.*, 2001a); the neutralization of lactic acid to give its dissociated form that has a less inhibitory effect (Madzingaidzo *et al.*, 2002; Milcent & Carrere, 2001); and the microorganism adaptation and/or the use of mixed cultures (Cui *et al.*, 2011; Robison, 1988; Tsai, Coleman, Moon, Schneider, & Millard, 1993).

Fermentation technologies

Lactic acid production from sugar solutions

Even though only one type of microorganism is usually employed in the production of lactic acid, mixed cultures of various lactobacilli (Cui *et al.*, 2011; John, Sukumaran, *et al.*, 2007; Tsai *et al.*, 1993) or lactobacilli and *Kluyveromyces marxianus* (Plessas *et al.*, 2008) were shown to ensure better results compared to pure cultures. Other authors have used mixed cultures of two microorganisms, one of them to carry out the fermentation and the other to carry out the hydrolysis of a polymeric substrate (Ge, Qian, & Zhang, 2009; Kurosawa, Ishikawa, & Tanaka, 1988; Romaní *et al.*, 2008).

Suspended-cell systems

Most of the published work on fermentative production of lactic acid by free cells was carried out operating in

batch mode (Amrane, 2001; Büyükkileci & Harsa, 2004; Chen *et al.*, 2012; Korbekandi *et al.*, 2007), although there are examples of continuous (Dey & Pal, 2012; Lunelli *et al.*, 2011; Nishiwaki & Dunn, 2005; Salgado, Rodríguez, Cortés, & Domínguez, 2012; Xu *et al.*, 2006) and fed-batch (Bai *et al.*, 2003; Ge *et al.*, 2009; Zhang, Cong, & Shi, 2011) productions.

Ultrafiltration of effluents from continuous suspended-cell systems allows retaining and separating cells from the fermented medium and recirculating them to the bioreactor (Lu, Wei, & Yu, 2012; Richter & Nottelmann, 2004; Xu *et al.*, 2006), ensuring higher cell concentrations and productivities (33–57 g L^{-1} h) than batch systems with comparable yields (Dey & Pal, 2012; Ishizaki & Vonktaveesuk, 1996; Kwon, Yoo, Lee, Chang, & Chang, 2001). Dey and Pal (2012) obtained efficient production of lactic acid from sugarcane juice in a novel two stage membrane-integrated fermenter.

Immobilized-cell systems

Immobilization of lactic acid bacteria is able to remarkably increase yields and productivities compared with suspended-cell systems, because it allows preventing the limits related to washout. Support materials are usually alginate gel (Cortón, Piuri, Battaglini, & Ruzal, 2000; Voo, Ravindra, Tey, & Chan, 2011), *k*-carrageenan (Norton *et al.*, 1994) or agar (Zayed & Zahran, 1991). However, the entrapment within gel has some drawbacks such as the formation of pH gradients inside the particles, occlusions and preferential flow, loss of gel mechanical stability, reduction of cell activity along the time and occurrence of diffusion limitations (Elezi *et al.*, 2003).

Owing to these drawbacks, more stable immobilization supports have been proposed; among them are ceramic and porous glass particles (Bruno-Bárcena *et al.*, 1999) or gluten beads (Chronopoulos *et al.*, 2002), which, however, are relatively expensive. In other works, it was proposed the immobilization of *L. brevis* on delignified lignocellulosic materials (Elezi *et al.*, 2003), *L. plantarum* on polypropylene matrices treated with chitosan (Krishnan, Gowthaman, Misra, & Karanth, 2001) and *R. oryzae* on a fibrous matrix composed of stainless-steel mesh and cotton cloth (Chen *et al.*, 2012), which ensured high yields and productivities.

Lactic acid production by simultaneous saccharification and fermentation of polysaccharides

The aim of the "simultaneous saccharification and fermentation" (SSF) process is the one-step production of lactic acid from a polysaccharide material, consisting in the preliminary enzymatic hydrolysis of substrate to monosaccharides (saccharification) and their subsequent fermentation to lactic acid. This process has been studied using either starchy (Ge *et al.*, 2009; Linko & Javanainen, 1996) or lignocellulosic (Bustos *et al.*, 2005a; John, Nampoothiri, *et al.*, 2007; Marques *et al.*, 2008; Moldes *et al.*, 2001b; Romaní *et al.*, 2008; Yáñez *et al.*, 2003) waste materials.

There are some interesting advantages that make the SSF of great interest from an industrial point of view such as the cost reduction associated with the use of only one reactor for hydrolysis and fermentation (Bustos *et al.*, 2004a; Lee, Koo, & Lin, 2004). From the technological point of view, since the limiting step of SSF is the biopolymer enzymatic hydrolysis, the microorganism consumes glucose at the same rate it is formed, which allows reducing the substrate inhibition and, consequently, the enzyme loading and the risk of external contamination.

Using *Eucalyptus globulus* wood as raw material and *L. delbrueckii* NRRL-B445 as a fermenting agent, Moldes *et al.* (2001b) obtained interestingly 108 g L^{-1} of lactic acid after 115 h of SSF, corresponding to a yield of 0.94 g g^{-1}, by intermittent addition of substrate (after 8–75 h), cellulases and nutrients (48 h) and simultaneous elimination of produced lactic acid by ion exchange. Even higher lactic acid concentration (162 g L^{-1}) and excellent productivity (1.4 g L^{-1} h^{-1}) were reported by Lee *et al.* (2004) for similar exploitation of paper industry wastes. Lactic acid was also produced by SSF of broken rice, reaching a volumetric productivity of 3.59 g L^{-1} h^{-1} (Nakano *et al.*, 2012).

Conclusions

This review paper reports on the fermentative and biotechnology processes to produce lactic acid. Polymeric substrates cannot be directly assimilated by lactic acid bacteria; therefore, they require an earlier stage of hydrolysis prior to lactic acid fermentation. On the other hand, fungi as fermenting agents are able to release extracellular amylases and, consequently, to directly hydrolyze starchy materials, thus not requiring any prior stage of hydrolysis. In fact, the high cost of hydrolytic enzymes for the saccharification of hemicellulosic materials is a serious drawback lactic acid industry, but it is noteworthy that lignocellulosic biomass represents the most abundant global source of biomass, and for this reason it can be largely utilized to give bioproducts. Therefore, different technologies and microorganisms have to be developed with the aim to increase the fermentation yield and the volumetric productivity of lactic acid.

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