UNIVERSIDADE DE SÃO PAULO
FACULDADE DE CIÊNCIAS FARMACÊUTICAS
Programa de Pós-Graduação em Tecnologia Bioquímico-Farmacêutica
Área de Tecnologia de Fermentações

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

Fabio Andres Castillo Martinez

Dissertação para a obtenção do grau de
MESTRE

Orientador:
Prof. Dr. Ricardo Pinheiro de Souza Oliveira

São Paulo
2013

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partir de leite desnatado**

Comissão Julgadora da
Dissertação para obtenção do grau de Mestre

Prof. Dr. Ricardo Pinheiro de Souza Oliveira
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São Paulo, _____ de _____

DEDICATÓRIA

Dedico esta presentación para mis padres por su amor, confianza y apoyo incondicional, y por la idea de verlos siempre sonreír.

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RESUMO

Castillo, F. M. **Produção de bacteriocina por *Bifidobacterium lactis* a partir de leite desnatado. 2013. 107f. Dissertação (Mestrado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2013.**

Existe um número muito limitado de estudos referentes à produção de componentes antimicrobianos ou bacteriocinas produzidas por espécies de bifidobactérias. Nesse âmbito, o objetivo deste trabalho foi avaliar a produção de bifidobacteriocina em leite desnatado (LD). Para tanto, o estudo foi dividido em três etapas. A primeira etapa constituiu na preparação dos meios de cultura *Man, Rogosa e Sharpe* (MRS), *Bifidus Selective Medium* (BSM) e LD suplementado com 1% (p/v) de Tween 80 (T₈₀), Inulina (I) ou Extrato de levedura (YE). Nesta etapa, os processos fermentativos foram conduzidos em *shaker*, nas condições: 50 rpm/37°C/48h. Foram realizadas análises de pH, concentração de açúcares e ácidos, crescimento celular e determinação da atividade da bifidobacteriocina pelo método de difusão em ágar contra *L. monocytogenes*. Na segunda etapa, e baseado nos resultados obtidos, foi desenhado um delineamento composto central (CCD) construído a partir dos seguintes parâmetros: temperatura (34, 37, 40 °C) e concentração de YE (0,5; 1,0; 1,5 g/L). Na terceira etapa do trabalho, foram realizados os cultivos em biorreator de 2 L, contendo 10% de leite desnatado, nas seguintes condições: 200 rpm, 36°C, 2,0 g/L de YE, 48h de incubação em anaerobiose. Obteve-se em LD suplementado com YE (1%), combinado ao método de difusão em placa modificado (prévia refrigeração das placas por 12h), contra *L. monocytogenes* (2130 AU/mL), com uma fase exponencial de 24h, μ_m de 0,604/h. A otimização feita através do CCD permitiu atingir níveis de atividade de 3.000 AU/mL a 3.100 AU/mL (ensaios 7, 11 e 14, blocos 3 e 1) contra *L. monocytogenes*, em condições ótimas de crescimento de YE: 2,0 g/L¹ e T°C: 36°C. A análise de regressão mostrou ser estatisticamente significativa a relação entre as variáveis: “concentração de YE” e “temperatura”. Os resultados indicaram que o leite desnatado é um meio adequado para produção de bifidobacteriocina.

PALAVRAS-CHAVE: *Bifidobacterium lactis*, bacteriocina, leite desnatado, fermentação, extrato de levedura, delineamento composto central.

ABSTRACT

Castillo, F. M. **Bacteriocin production by *Bifidobacterium lactis* from skimmed milk. 2013. 107f. Dissertação (Mestrado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2013.**

There are few publications that have been reported about bacteriocin production by *Bifidobacterium* species. Therefore, the aim of this work was measure bacteriocin production in skim milk by *B. lactis*. Consequently, this work was divided in three stages. First, MRS, BSM and LD medium were tested with additives (Tween 80 (T_{80}), Inuline (I) or Yeast extract (YE)) for bacteriocin production and cellular growth. Fermentation processes were conducted in shaker under specific conditions: 50 rpm/37°C/48h. pH; sugars; acids; biomass, and bacteriocin activity against *L. monocytogenes*, *L. plantarum*, *E. coli*, *L. sakei* e *S. aureus* strains were analyzed. In the second stage, based on the obtained results, a central composite design (CCD) was created using the parameters: temperature (34, 37, 40 °C), and concentration of YE (0.5, 1.0, 1.5 g/L). After, the activity was measured by two methods of plates pre-diffusion (cooling and addition of Tween 20). Third step consisted of 2 L bioreactor cultivations containing 10% skim milk diluted in 1.5 L of water (6.5 pH), under 200 rpm, 36 °C, 2.0 g/L of YE, 48h, under anaerobic condition. Finally, the cultures supplemented with LD and YE (1%) with a modified plate diffusion method (cooling plates for 12 h) showed bacteriocin activity against *L. monocytogenes* (2130 AU/mL) with an exponential phase of 24 h, μ_m of 0.604/h. The optimization performed using CCD resulted in a higher level of activity 3000 AU/mL to 3100 AU/mL (Run 7, 11 and 14, blocks 3 and 1) against *L. monocytogenes*, also with ideal growth conditions of YE: 2,0 g/L¹ and T °C: 36 °C. The pH value varied between 6.4 and 4.0. Concentration of produced acid lactic varied from 3.03 to 4.72 g/L and biomass concentration from 3.4 to 11.1 Lg UFC/mL. Regression analysis was significant to the variables: YE concentration and temperature. Results indicated that skim milk is a proper medium for “Bifidobacteriocin” production.

KEY WORDS: *Bifidobacterium lactis*, bacteriocin, skim milk, fermentation, yeast extract, Central Composite Design.

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LISTA DE ABREVIATURAS E SIGLAS

AU	Unidades arbitrárias de atividade da bacteriocina (mg.mL^{-1})
BSM	<i>Bifidus Selective Medium</i>
BLIS	Substâncias semelhantes às bacteriocinas
CCD	<i>Central Composited Design</i>
CLA	Ácido linoleico conjugado
YE	Extrato de levedura
DO	Densidade óptica
FDA	<i>Food and Drug Administration</i>
GRAS	Geralmente Reconhecidas como Seguras (<i>Generally Recognized As Safe</i>)
HPLC	<i>High-Performance Liquid Chromatography</i>
I	Inulina
LAB	Baterias ácido-láticas
LD	Leite desnatado
MRS	<i>Man Rogosa Sharpe</i>
rpm	Rotações por minuto
SD	Soro de leite
Si e Sf	Concentrações inicial e final de substrato (g L^{-1})
t	Tempo de cultivo (horas)
Tg	Tempo de geração (h)
T ₈₀	Tween 80
UFC	Unidade formadora de colônia (mL^{-1})
X	Concentração celular (mg.L^{-1})
μ_m	Velocidade específica de crescimento (h^{-1})

LISTA DE ABREVIATURAS E SIGLAS

%	Porcentagem
°C	Graus Celsius
dt	Intervalo de tempo
dX	Concentração celular formada em dt (UFC/mL)
g	Grama
h	Hora
kDa	Quilodalton
Kg	Quilograma
L	Litro
M	Molar
min	Minuto
mg	Miligrama
mL	Mililitro
mm	Milímetro
P _{máx.}	Produção máxima (mg/L)
Prod	Produtividade (mg/L.h)
R ²	Coeficiente de correlação
X _{max}	Concentração celular máxima (UFC/mL)
X	Concentração celular (UFC/mL)
Y _{N/X}	Coeficiente angular de correlação entre atividade de BLIS e concentração celular
µL	Microlitro

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1. INTRODUÇÃO

O leite é considerado um produto de elevado valor nutricional. Os principais constituintes desse produto são água, lactose, proteínas, ácidos graxos e sais ou cinzas; o leite também apresenta, em sua composição, vitaminas e minerais (TAMIME, 2005). Por estas características, o leite é um produto muito utilizado na fermentação por bactérias lácticas para a produção de produtos lácteos. É importante ressaltar que o leite pode também ser utilizado como substrato em fermentação para a produção de biomoléculas de alto valor agregado, como compostos antimicrobianos (nisina) produzidos por bactérias lácticas (PENNA *et al.*, 2005).

As propriedades antimicrobianas de bactérias ácido-lácticas (LAB) permitiram o aumento da vida-de-prateleira 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 *et al.*, 2010).

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 (CASTILLO *et al.*, 2013^a; CHEIKHYOUSSEF *et al.*, 2010). Além disso, essas espécies podem ser consideradas probióticas. Para isso, tem sido sugerido que uma das desejáveis propriedades das cepas probióticas é a habilidade de produzir substâncias, como bacteriocinas, a qual oferece 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 *et al.*, 2004). Bacteriocinas produzidas por bactérias Gram-positivas, particularmente bactérias ácido-lácticas, apresentam amplo espectro como bioconservante de alimentos e como agente terapêutico (BALCIUNAS *et al.*, 2013;

GALVEZ *et al.*, 2008; JACK *et al.*, 1995). O mais conhecido exemplo é a nisina, a qual é bioproduzida por muitas cepas de *Lactococcus lactis* subsp. *lactis*, sendo considerada o protótipo de bacteriocina de bactérias ácido-láticas (JOZALA *et al.*, 2008).

A nisina foi descoberta em 1928, quando Rogers e Whittier observaram metabólitos inibitórios de LAB (ROGERS & WHITTIER, 1928). A nisina tem sido extensivamente estudada e, em 1988, foi aprovada pelo *Food and Drug Administration* (FDA), recebendo a denominação *Generally Recognized as Safe* (GRAS) para ser usada como um biopreservativo em muitos alimentos processados. Como resultado disso, pesquisas nesse campo começaram a crescer vertiginosamente, o que levou à descoberta de grande número de bacteriocinas produzidas por bactérias lácticas (GALVEZ *et al.*, 2008; REDDY *et al.*, 2004).

Geralmente, as bacteriocinas são sintetizadas no ribossomo, liberadas no meio extracelular e apresentam ação bactericida ou bacteriostática sobre bactérias Gram-positivas, dentre as quais importantes patógenos de veiculação alimentar como *Listeria monocytogenes*, *Clostridium botulinum*, *Bacillus cereus* e *Staphylococcus aureus* (HERNÁNDEZ *et al.*, 2005). Estruturalmente, são compostas de 20 a 60 aminoácidos e podem ser responsáveis pelas principais propriedades das bacteriocinas, que incluem a tolerância ácida, a termoestabilidade e a especificidade bactericida (CASTILLO *et al.*, 2013a; EIJSINK *et al.*, 2002; TAMIME, 2005).

Segundo Balciunas *et al.* (2013), 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). E a Classe III é representada por peptídeos termolábeis de alta massa molar (>30 kDa), como helveticina J. É importante salientar que na Classe IIa encontra-se a bacteriocina produzida por *Bifidobacterium bifidum* (YILDIRIM & JOHNSON, 1998; YILDIRIM *et al.*, 1999).

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 e possuem níveis de guanina e citosina elevados (BALLONGUE, 2004; GOMES & MALCATA, 1999). 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 flora 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 linoléico conjugado) e bacteriocinas. Metabolicamente, as bifidobactérias produzem ácido láctico e acético sem produzir CO₂, 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, o que precisa ser levado em conta em qualquer estudo metabólico. Segundo Itsaranuwat *et al.*, (2003), existem diferenças morfológicas, havendo o formato de “Y” ou a forma de cocos, dependendo da condição de crescimento e/ou das espécies. *B. longum* (BL) é encontrado em intestino grosso de humanos.

Diferentemente da situação dos *Lactobacillus*, somente número limitado de estudos tem sido realizado no que diz respeito à produção de compostos antimicrobianos ou de bacteriocinas por cepas de bifidobactérias. Espécies de *Bifidobacterium* são geralmente inibidores de ampla gama de microrganismos, devido à sua produção intensa de ácido láctico e ácido acético como produtos do próprio metabolismo (EKLUND, 1983). Gibson e Wang (1994a) demonstraram que 8 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 Bifidocin B. Esta bacteriocina demonstrou-se muito eficaz contra diferentes microrganismos patógenos (YILDIRIM *et al.*, 1999). Estudos *in vitro* mostraram que bifidobactérias inibiram a ação da *Escherichia coli* 0157:H7 enterohemorrágica (GAGNON *et al.*, 2004) e *L. monocytogenes*, devido à produção de compostos inibidores (TOURE *et al.*, 2003).

Recentemente, Cheikhyoussef *et al.*, (2009a; 2009b; 2010) descobriram uma nova bacteriocina produzida por *Bifidobacterium infantis* em meio sintético MRS suplementado com cisteína. Essa nova bacteriocina, denominada Bifidin I, mostrou amplo espectro de ação, incluindo bactérias Gram-negativas e Gram-positivas, além de clara inibição com relação à *L. monocytogenes*.

2. OBJETIVOS

2.1. Objetivo geral

O objetivo deste trabalho foi otimizar a produção de substâncias semelhantes às bacteriocinas (BLIS) a partir de células de *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*), em meio de cultivo de baixo custo (leite desnatado).

2.2. Objetivos específicos

O presente trabalho teve como objetivos específicos:

- Avaliar a influência dos aditivos (T_{80} , I e YE) e nas melhores proporções a serem utilizadas em meio de cultivo sintético (MRS e BSM) e natural (LD) no crescimento celular e na expressão de BLIS.
- Definir, a partir de modelos estatísticos (CCD e RSM), as melhores condições de crescimento e produção de BLIS para cultivos em fermentador de bancada.
- Analisar a atividade antimicrobiana das substâncias produzidas nas condições de fermentação ótimas, a partir de testes de antagonismo frente às bactérias Gram-positivas e Gram-negativas.

3. MATERIAL E MÉTODOS

3.1. Culturas microbianas, condições de crescimento e meios de cultura utilizados

Neste estudo, foram utilizados os seguintes microrganismos: *Bifidobacterium lactis* HN019 (*B. lactis*), cultura comercial liofilizada (Danisco, Sassenage, França) usada como produtora da bacteriocina. As linhagens usadas como microrganismos indicadores da atividade da bacteriocina estão apresentadas na Tabela 1.

Tabela 1. Microrganismos indicadores da atividade da BLIS.

Linhagem	Condições de cultivo*	Condições de preservação
<i>Escherichia coli</i> ATCC 25922	BHI/37°C/24h	BHI+40%Gli /-70°C
<i>Pseudomonas aeruginosa</i> ATCC 9721	BHI/37°C/24h	BHI+40%Gli /-70°C
<i>Staphylococcus aureus</i> ATCC 10390	BHI/37°C/24h	BHI+40%Gli /-70°C
<i>Lactobacillus sakei</i> ATCC 15521	MRS/30°C/24h	MRS+40%Gli /-70°C
<i>Listeria monocytogenes</i> ATCC 13932	BHI/37°C/24h	BHI+40%Gli /-70°C
<i>Lactobacillus plantarum</i> BAA-793	MRS/30°C/24h	BHI+40%Gli /-70°C

* BHI: meio de cultura *brain heart infusion*

3.2. Procedimento experimental

3.2.1 Preparação do inóculo de *B. lactis* em meio de cultivo

A pré-cultura foi preparada adicionando-se uma alçada da cultura estoque de *B. lactis*, em 50 mL de caldo MRS (DIFCO™, Sparks, MD, USA) suplementado com 1% (w/v) de L-cisteína (0,025 g/L) (Sigma Chemical Co., St. Louis, MO, USA), tioglicolato de sódio (100 µL/L) (INLAB, SP, Brasil) e azul de metileno (INLAB, SP, Brasil) em frascos de 50 mL, durante 24h, até se conseguir o valor de absorbância

previamente definido de $DO_{600nm}=0,9$ nas seguintes condições do processo: 25 rpm/37°C/anaerobiose. As condições de anaerobiose foram obtidas através do uso de jaras de anaerobiose (BBL Gaspak Anaerobic system, Becton Dickinson Microbiological System, Cockeysville, Maryland, USA). Posteriormente, o pré-inóculo foi centrifugado a 4.800 rpm/4°C/10min, a fim de separar o *pellet* do meio de cultivo, e, em seguida, foi lavado com solução salina 0,85% estéril. Finalmente, 25mL foram transferidos para 225mL da respectiva lavagem:

- caldo BSM (*Bifidus Selective Medium*, SIGMA, St. Louis, USA)
- caldo MRS (*Man, Rogosa and Sharpe*)
- LD (*Skim milk*, DIFCO, Sparks, MD, USA)

A composição dos meios de cultivo utilizados no presente estudo está apresentada nas Tabelas 2 e 3. A Figura 1 ilustra o procedimento previamente descrito, junto com as variáveis analisadas.

Tabela 2. Composição dos meios de cultivo utilizados: LD.

Meio de cultivo	Composição	Concentração
LD	Carboidratos	49,5%
	Proteínas	34-37%
	Ferro	0,4 mg/100g
	Cálcio	1248 mg/100g
	Colesterol	0,6-1,25%
	Sódio	494 mg/100g

Figura 1. Representação da técnica empregada para o crescimento e avaliação de *B. lactis* nos respectivos meios de cultivo.

Tabela 3. Composição dos meios de cultivo utilizados: Caldo MRS e BSM.

Caldo	Composição	g/L	Composição	g/L
Caldo MRS	Extrato de carne	10,0	Extrato de carne	NE*
	Extrato de levedura	5,0	Extrato de levedura	NE
	Glicose	20,0	Glicose	NE
	Polissorbato 80	1,0	Cloreto de sódio	NE
	Fosfato bi potássico	2,0	Compostos redutores e substancias tampão	NE
	Acetato de sódio	5,0	Sais inibitórios de bactérias Gram-negativas.	NE
	Citrato de amônio	2,0	Inibidores enzimáticos da gliceraldeído	NE
	Sulfato de magnésio	0,1	3 antibióticos	NE
	Sulfato de manganésio	0,05	Composto azo para pigmentação da bifidobactérias	NE
	Peptona No.3	10,0		

NE*: Não especificado.

3.2.2. Determinação da concentração adequada de leite desnatado

O leite desnatado (LD) foi preparado em cinco diferentes concentrações de sólidos totais, definidas como: 40, 35, 25, 20 e 15 g/L de leite desnatado em pó suplementados com 1% (p/v) de L-cisteína. Todos os meios de cultivo foram diluídos em água destilada estéril até completar o volume de 225 mL; posteriormente, foram homogeneizados, com auxílio de agitador magnético, por 15 min e tratados termicamente a 85°C, durante 10 minutos, em banho-maria (Nova Ética, Modelo 550 A). Posteriormente, os meios de cultivo foram imediatamente resfriados em banho de gelo e distribuídos em frascos Erlenmeyer estéreis de 500 mL em câmara de fluxo laminar. Finalmente, os meios de cultivo foram inoculados e incubados em agitador rotacional (*shaker*), nas mesmas condições de processo mencionadas anteriormente. Essas transferências, para o novo meio de cultivo (leite desnatado), foram repetidas por três vezes (1º, 2º e 3º repiques). A constante de saturação (K_s g/L) e a velocidade máxima (μ_{max}) foram calculadas a partir da linearização do comportamento enzimático que cumpre Monod pelo método de Lineweaver-Burk, como ilustrado na Figura 2.

Figura 2. Representação esquemática do ensaio para determinação da constante de saturação com lactose como fonte de carbono.

3.3. Processo fermentativo em *shaker* com adição de indutores de produção de bacteriocina

Os processos fermentativos em agitador rotacional (*shaker*) foram conduzidos nas seguintes condições: 25 rpm/37°C/48h/Anaerobiose, com a respectiva adição de Inulina (Beneo-Orafti Corp., Malvern, PA, USA), tween 80 (Alamar Tecno-Científica Ltda, Diadema, SP, Brasil) ou extrato de levedura (DIFCO™, Sparks, MD, USA), na concentração de 1% (p/v). Foram analisados os mesmos parâmetros em meios de cultura MRS, BSM e LD, a fim de oferecer medida comparativa entre meios de cultivo natural e sintético.

3.4. Procedimentos de análises

Nos estudos realizados em agitador rotacional (*shaker*), as amostras foram coletadas em intervalos de 3 horas até 48 h, totalizando 11 amostras, com suas respectivas análises de pH, crescimento celular, concentração de glicose ou lactose e determinação da atividade da bifidobacteriocina. Cada análise foi realizada em triplicata.

3.4.1. Crescimento celular

A determinação da concentração celular foi obtida através dos seguintes métodos, de acordo com o meio de cultura: (i) leitura de densidade óptica por espectrofotometria ao comprimento de onda de 600nm, para determinação de biomassa dos meios de cultura MRS e BSM; e (ii) por determinação de Unidades Formadoras de Colônia (UFC), através de diluições seriadas da suspensão bacteriana em solução peptonada 1%(p/v). Para o método de plaqueamento, a placa de *Petri* foi dividida em seis partes, aplicando 20µL da respectiva diluição na superfície do meio agarizado (*soft agar*, BSM) contendo 0,5% de L-cisteína, sem qualquer ajuste de pH (International Dairy Federation, 2003), e incubada a 37°C, por 24 h.

Estabeleceram-se, como contagem, as diluições com menos de 20 colônias vezes o fator de diluição, vezes o volume da amostra (0,02mL). O crescimento

celular foi representado através da curva de referência, associando DO_{600nm} às UFC da mesma suspensão, representada pela equação:

$$y = m (0,4156) - 1,7119$$

Equação 1

3.4.2. Monitoramento de pH

A acidificação foi avaliada utilizando um pHmetro modelo Q-400M1 (Quimis, São Paulo, Brasil). A variação de pH foi acompanhada durante todo o cultivo do volume de 250mL incubado em agitador rotativo ou em biorreator.

3.4.3. Determinação de ácidos e açúcares por HPLC

A concentração de açúcares (glicose, lactose e galactose) e ácidos orgânicos (ácido acético, ácido succínico, ácido láctico, ácido fórmico e ácido pirúvico) foi determinada por cromatografia líquida de alta eficiência (HPLC, do inglês *High-Performance Liquid Chromatography*). Previamente, cada amostra foi diluída para as concentrações 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 40 °C, coluna HPX-87H (Bio-Rad, Hercules, CA, Estados Unidos) a 45 °C, fase móvel de H_2SO_4 (5 mM), com fluxo de 0,6 mL/min e volume de injeção de 20 μL . 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.

3.4.4. Atividade antimicrobiana da bacteriocina

As cepas de *Lactobacillus sakei* ATCC 15521, *Listeria monocytogenes* ATCC 13932, *Staphylococcus aureus* ATCC 10390 e *Escherichia coli* ATCC 25922 foram utilizadas como microrganismos indicadores para determinação da atividade da bifidobacteriocina, através do método de difusão em placa (PENNA e MORAES, 2002; PENNA *et al.*, 2005). Elas foram inoculadas em caldo BHI (DIFCO™, Sparks, MD, USA) a 100 rpm e 37°C, durante 4-6h, até atingir o valor de absorbância previamente definido ($DO_{600nm} = 0,9$). Posteriormente, transferiu-se uma alíquota de 500 µL dessa suspensão para 49 mL de ágar BHI (40°C), que, finalmente, foram vertidos nas placas. Paralelamente, alíquotas da suspensão de células (para cada tempo de análise) foram centrifugadas a 4800 rpm, por 10 minutos a 10°C, e o sobrenadante foi coletado e filtrado em membrana com poros de diâmetro de 0,22 µm (Millipore®), sendo ajustado ao valor de pH aproximado de 6 a 6,5, com adição de hidróxido de sódio (NaOH 1M).

Posteriormente, a amostra foi aquecida em banho-maria, a 80 °C por 10min, com a finalidade de eliminar qualquer tipo de radicais livres, gerados por substâncias como peróxido de hidrogênio. Por fim, 20µL da suspensão foram inoculados na superfície do meio. Desta forma, verificou-se a capacidade da bacteriocina, a partir do teste de antagonismo, através da presença de halos de inibição causados pelo sobrenadante disposto sobre o microrganismo sensor (*spot on the lawn*), conforme ilustrado na Figura 3. A quantidade de bifidobacteriocina liberada nos meios de cultivo foi quantificada e expressa em unidades arbitrárias (AU/mL) pelo ensaio de difusão em ágar (PENNA, 2002; PENNA *et al.*, 2005), utilizando *L. sakei* ATCC 15521, *L. monocytogenes* ATCC 13932, *S. aureus* ATCC 10390 e *E. coli* ATCC 25922 como microrganismos indicadores (PONGTHARANGKUL & DEMIRCI, 2004).

Através de uma curva, associando concentração de bifidobacteriocina com unidades arbitrárias (AU) em relação ao diâmetro do halo de inibição, determinou-se a atividade de bifidobacteriocina formada (AU/mL). Para a obtenção dessa curva, foi realizada uma curva padrão, pela técnica de diluição crítica, já que não existe um padrão comercial de bifidobacteriocina, por se tratar de uma nova bacteriocina, referenciada nos trabalhos como bacteriocina de Classe IIa (CHEIKHYOUSSEF *et*

al., 2010; YILDIRIM *et al.*, 1999) ou como pertencente a um grupo ainda não definido (UELIVON, 2006) ou como lantibiótico (LEE *et al.*, 2011).

Figura 3. Representação do método *Spot on the lawn*, em ágar BHI. Fonte: Autor, baseado no esquema feito por Leite (2012)

3.4.5. Quantificação da atividade inibitória pela técnica de diluição crítica

A quantificação da atividade de *B. lactis* foi realizada através do ensaio de antagonismo em ágar, baseado na técnica de diluição crítica, descrita por Mayr-Harting *et al.* (1972). Para isso, foi realizado o mesmo procedimento descrito previamente no item 3.4.4 e na Figura 1, para o processamento da substância BLIS. Posteriormente, 100 µL do sobrenadante foram diluídos em solução tampão de fosfato de sódio 10mM, pH 7,0, nas proporções 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, etc.

Paralelamente, foram preparadas placas de ágar BHI inoculada com $10^5 - 10^6$ de *L. monocytogenes* ATCC 13932 como microrganismo indicador. Finalmente, sobre a placa, foram vertidos 20 µL do sobrenadante da substância BLIS sem diluição e 20 µL de cada uma das diluições, em pontos conforme ilustrado na Figura 4. As placas foram mantidas durante 12h a 4°C e, finalmente, foram incubadas por 24h a 37°C.

Conseguiu-se correlacionar a concentração de bifidobacteriocina com unidades arbitrárias (AU) em relação à última diluição em que havia halo de inibição, corrigida para 1 mL. A equação 1 expressa o valor de AU/mL, onde (P) significa o volume depositado na placa, (D) o número de diluições e (n) o número da diluição que não apresentou halo.

$$AU/mL = \frac{D^n \times 1000}{P} \quad (\text{Equação 1})$$

Figura 4. Representação esquemática do ensaio de diluição crítica para determinação da atividade antimicrobiana da BLIS contra *L. monocytogenes*. Fonte: Autor, baseado no esquema feito por Leite (2012).

3.5 Planejamento experimental

Este planejamento teve como objetivo otimizar a produção de BLIS, a partir da interação das seguintes variáveis: concentração de extrato de levedura (0,5 - 1,0 - 1,5 g/L) e temperatura (34 – 37 - 40°C). As variáveis foram avaliadas através de delineamento composto central (CCD), ou seja, 2^3 , incluindo 6 ensaios nas condições axiais e 3 repetições no ponto central, totalizando 17 ensaios, como ilustrado na matriz apresentada na Tabela 4.

Tabela 4. Matriz do delineamento composto central.

Ensaio	Variáveis	
	Temperatura °C	YE g/L
	(X ₁)	(X ₂)
1	37,0	0,15
2	40,0	0,5
3	42,0	1,0
4	40,0	1,5
5	34,0	0,5
6*	37,0	1,0
7	40,0	1,5
8	40,0	0,5
9	31,9	1,0
10*	37,0	1,0
11	34,0	1,5
12	34,0	1,5
13	37,0	1,0
14	37,0	1,8
15*	37,0	1,0
16	34,0	0,5
17	37,0	1,0

*: Pontos centrais do planejamento experimental.

Cada variável foi realizada em *shaker*, nas condições descritas no item 3.2.1 e na Figura 1, analisando a concentração de açúcares, pH, biomassa e atividade da bacteriocina, conforme descrito no item 3.4.

3.6. Cultivo em fermentador

A pré-cultura foi preparada adicionando-se uma alçada da cultura estoque de *B. lactis*, em 3 tubos com 50 mL de caldo MRS (DIFCO™, Sparks, MD, USA) (composição descrita na Tabela 3) suplementado com 1% (w/v) de L-cisteína em frascos de 50 mL, durante 24h, até alcançar o valor de absorbância previamente definido ($DO_{600nm}=0,9$), nas seguintes condições do processo: 25 rpm/37°C/Anaerobiose. Finalmente, foi separado o *pellet* e ressuspendido em 150mL de solução salina (0,85%) estéril (10% do volume final do meio de cultivo contido na dorna).

3.6.1 Descrição do fermentador de bancada

O fermentador de bancada ou biorreator modelo Bioflo 110 (*New Brunswick Scientific*, Hamburgo, Alemanha) possui capacidade nominal total de 2 L, sistema de agitação a partir de uma turbina de tipo *Rushton*, com injeção de nitrogênio. Apresenta um eletrodo de vidro esterilizável modelo 405-DPAS-SCK8S/225 (Mettler Toledo, Alphaville, Brasil), para medição de pH, e um eletrodo polarográfico esterilizável modelo inPro 6110/220 para medição da concentração de oxigênio (Mettler Toledo, Alphaville, Brasil). Tem uma sonda de temperatura ajustada internamente no biorreator, regulando o nível de temperatura fornecida pela manta de aquecimento elétrico, instalada ao redor da dorna do biorreator ou, pelo contrário, favorecendo o resfriamento pela injeção de água que flui pelo sistema de aço inox imerso no meio de cultura. A regulação dos dois sistemas mantém a temperatura no nível desejado (*set-point*). O condensador e o sistema de entrada de nitrogênio possuem, em cada extremo, filtros com porosidade de 0,22 μm , evitando a entrada de contaminantes do ar (Figuras 5 e 6).

A coleta da amostra é realizada por uma saída específica, composta por uma conexão entre mangueira, frasco amostrador de vidro estéril e uma seringa que induz a sucção da amostra. O volume total de amostras retiradas não supera 10%

do volume inicial do meio de cultivo, evitando, assim, qualquer alteração significativa nas condições do cultivo.

Figura 5. Biorreator *New Brunswick Scientific* (modelo Bioflo 110). Fonte: tese de doutorado de Arauz (2011).

Figura 6. Biorreator *New Brunswick Scientific* (modelo Bioflo 110). Fonte: tese de doutorado de Arauz (2011).

3.6.2 Preparo do meio de cultura no biorreator

O leite desnatado foi preparado adicionando-se 15 g num frasco *Schott* contendo 350 mL de água destilada estéril, com 125 µL de azul de metileno, como indicador redox, 350 mg de tioglicolato de sódio, como substância redutora de oxigênio, e 3 g de extrato de levedura. O concentrado foi pasteurizado a 85°C, durante 10min, com constante agitação e posterior exposição à luz UV durante 15min. A dorna, devidamente montada e com 1.000 mL de água destilada em seu interior, foi esterilizada em autoclave a 121°C e 1 atm durante 30 min. Depois do resfriamento, a dorna foi transferida para uma cabine de fluxo laminar, sendo transferidos, através de um funil estéril, 350 mL de meio pasteurizado e 150 mL do pré-inóculo descrito anteriormente, para volume final de 1.500 mL, sendo realizado esse estudo por triplicata. As condições de cultivo estão apresentadas na Tabela 5.

Tabela 5. Condições dos cultivos em fermentador.

Temperatura	36°C
Duração	48h
pH inicial	6,44
Volume do inóculo	150mL
Volume inicial do meio sem inóculo	1350mL
Volume final	1500mL
Agitação e vazão do nitrogênio	200 rpm / (0,5 L/min)
Volume das amostras	10mL

3.7. Metodologia de análise dos resultados

3.7.1. Fator de conversão de substrato em células ($Y_{X/S}$)

O fator de conversão de substrato (lactose) em células ($Y_{X/S}$) foi definido através das relações apresentadas na Equação 1, denominada como Método Convencional.

$$Y_{X/S} = \Delta X / -(\Delta S) = (X_f - X_i) / (S_i - S_f) \text{ (Equação 2)}$$

$Y_{X/S}$: fator de conversão de substrato em células (g/g)

ΔX : variação da concentração celular (g/L)

ΔS : variação da concentração de substrato (g/L)

X_i e X_f : concentrações inicial e final de células (g/L)

S_i e S_f : concentrações inicial e final de substrato (g/L)

3.7.2. Velocidade específica de crescimento (μ_m) e Tempo de geração (t_g)

Em particular, a taxa específica de crescimento (μ_m) foi calculada para *B. lactis*, durante a sua fase de crescimento exponencial, segundo a equação:

$$\mu_m = \frac{1}{(t_2 - t_1)} \ln \frac{X_2}{X_1} \quad (\text{Equação 3})$$

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

O tempo de geração (t_g) foi calculado para cada cultura a partir do valor correspondente de μ_m , através da equação:

$$t_g = \frac{\ln 2}{\mu_m} \quad (\text{Equação 4})$$

3.7.3. Velocidades instantâneas e específicas

A velocidade instantânea de crescimento (dX/dt) foi calculada pelo método proposto por Le Duy e Zajic (1973). A velocidade específica de crescimento (μ_x) foi obtida pela divisão da velocidade instantânea pela concentração celular nos pontos da curva nos quais foram calculadas as derivadas, como definidas nas equações abaixo:

$$\mu_x = 1/X \cdot dX/dt \quad (\text{Equação 5})$$

μ_x : velocidade específica de crescimento celular (1/h)

X : concentração celular (g/L)

3.8. Análise estatística

Os ensaios foram realizados em triplicata e os resultados foram avaliados através de análise de variância (ANOVA) usando o *software* Statistica 6.0. Os valores foram comparados usando o teste de Tukey (Sokal e Rohlf, 1979) a $P < 0,05$. Diferentes letras foram usadas para identificar as diferenças estatísticas entre eles.

4. RESULTADOS E DISCUSSÃO

4.1. Efeito da agitação sobre *B. lactis* em caldo MRS

Inicialmente, foram avaliadas as melhores condições de agitação para *B. lactis* em caldo MRS, a fim de aperfeiçoar o processo fermentativo e obter maior produção de biomassa. Desta forma, obteve-se maior concentração de biomassa com a menor velocidade de agitação (25 rpm). Este fato pode ser explicado devido a pouca interação entre as interfases ar-líquido, diminuindo, assim, a transferência de ar para meio e, portanto, favorecendo as condições de anaerobiose.

Tabela 6. Crescimento de *B. lactis* em *shaker* durante 24 horas a 37°C.

Agitação (rpm)	Peso seco(mg/mL)	pH Final
25	2.57±0.08	4.41±0.04
50	1.64±0.05	4.35±0.03
100	0.46±0.02	6.00±0.02

4.2. Processo fermentativo em agitador rotativo utilizando caldo MRS

Para esclarecer o comportamento cinético do microrganismo, os tempos de maior crescimento e as suas respectivas fases, foi realizada uma curva de crescimento durante 48 h de fermentação a 25rpm . A partir desta curva, pode-se definir claramente as fases de crescimento nas quais a cepa se encontrava. Apresentando fase inicial de adaptação durante as primeiras 2 horas, posteriormente, no intervalo de 4 a 24 horas, a cepa exibiu uma fase exponencial de crescimento, caracterizada, geralmente, pela maior formação de biomassa e pela alta velocidade de crescimento, seguindo, finalmente, à fase estacionária e de declínio. A Figura 7 mostra uma curva de crescimento da cepa *B. lactis* relacionada com o consumo de glicose e pH.

Pode-se observar que o maior valor de concentração de biomassa da cepa *B. lactis*, utilizando caldo MRS suplementado com L-cisteína (meio de cultivo sintético

padrão), foi 8,1 Log UFC/mL. De fato, este valor foi semelhante ao obtido por Janer *et al.*, (2004) (7,97 Log UFC/mL). No presente trabalho, esse alto crescimento celular foi obtido no pH 4,43, similar ao obtido por Florence *et al.*, (2012) e contrariamente aos trabalhos de Janer *et al.* (2004) e Chou & Hou (2000), que obtiveram maior crescimento das células com pH de 6,0 e 6,3, respectivamente. A capacidade heterofermentativa de *B. lactis*, e sua subsequente liberação de produtos como ácidos acético, láctico, fórmico ou succínico, dificulta a padronização dos valores de pH entre os diferentes experimentos, o que explica as diferenças de resultados obtidos entre experimentos (CASTILLO *et al.*, 2013; MEULEN *et al.*, 2006).

O consumo de glicose foi congruente com o crescimento microbiano, sendo inversamente proporcional, conforme *B. lactis* se desenvolve durante o cultivo. Não foi evidenciada a produção de bacteriocinas e tampouco algum tipo de atividade bactericida ou bacteriostática. De fato, mesmo se presente a BLIS, não foi suficiente para inibir o crescimento das cepas indicadoras.

4.3. Efeito de indutores no crescimento de *B. lactis* e na produção de bacteriocina em caldo MRS

Dado que a produção de bacteriocinas não foi visualizada nos experimentos iniciais e os que valores cinéticos reportaram velocidade específica (μ_m) de 0,35/h e tempo de geração (t_g) de 1,96/h, se priorizou a produção de bacteriocinas a partir da otimização de crescimento de *B. lactis*.

Figura 7. Curva de crescimento de *B. lactis* em caldo MRS.

Para atingir o objetivo principal do projeto, foram adicionados, no meio de cultura, inulina, Tween 80 e extrato de levedura na concentração de 1% (p/v). O uso da inulina é bem conhecido, por favorecer o crescimento de bifidobactérias pela sua capacidade de degradar fontes complexas de carbono, diferentemente de outros microrganismos (fatores bifidogênicos) (GIBSON & WANG, 1994b; GOMES & MALCATA, 1999). O efeito da inulina pode ser justificado pelo aumento dos monômeros de frutose e a subsequente assimilação pela via frutose-6-fosfato (OLIVEIRA *et al.*, 2012).

Entretanto, o extrato de levedura funciona como promotor de crescimento, ao fornecer fontes de nitrogênio e vitaminas facilmente metabolizáveis e de vital importância (BALLONGUE, 2004; SCHEPERS *et al.*, 2002). Já o Tween 80 estimula a produção da bacteriocina ou pode interagir com a membrana celular, facilitando os processos de adsorção ou remoção da camada lipídica, gerando, assim, maior ação bactericida (COLLADO *et al.*, 2005). A síntese de bacteriocinas é influenciada pelo

tipo e pelo nível de concentração de fontes de carbono, nitrogênio e fosfato e substâncias surfactantes.

Figura 8. Curva de crescimento de *B. lactis* em caldo MRS suplementado com extrato de levedura 1% (p/v). Representação da atividade da BLIS a partir dos histogramas.

A Figura 8 mostra o potencial efeito do extrato de levedura sobre o crescimento de *B. lactis*, reportando a velocidade específica (μ_m) de 0,46/h, com tempo de geração (t_g) de 1,51/h e produção máxima de biomassa, em 24 horas de cultivo, no valor de 9,3 Log (UFC/mL). Este resultado foi 20% maior que o obtido no caldo MRS sem nenhuma adição dos ingredientes. Estes resultados são similares aos obtidos por diferentes autores, que comprovaram como o extrato de levedura exerce estímulo o crescimento de bifidobactérias (BALLONGUE, 2004; KIVIHARJU *et al.*, 2005; UELIVON, 2006).

A produção de bacteriocina foi igualmente estimulada, gerando resultado de 1.200 AU/mL na inibição de *L. monocytogenes*. A partir dessa curva, pode-se observar que a maior produção de bacteriocina foi obtida entre 18 e 26h de cultivo, devido à influência da máxima taxa de crescimento. É importante ressaltar que as bacteriocinas são produtos associados ao crescimento e sua produção sofre forte influência do *quorum sensing*, que é mais ativo em maiores

concentrações de biomassa (EIJSINK *et al.*, 2002; ENNAHAR *et al.*, 2000; KOTELNIKOVA & GELFAND, 2002). De fato, bacteriocinas atuam como moléculas sinalizadoras para outras bactérias, sincronizando, dessa forma, os processos de produção de metabólitos (EIJSINK *et al.*, 2002).

A Figura 9 mostra os dados com a adição de Tween 80 e seu efeito positivo no crescimento microbiano. O maior valor de crescimento foi 8,01 Log UFC/mL, com velocidade específica (μ_m) de 0,35/h e t_g de 1,97/h. Estes valores corroboram os dados apresentados por Collado *et al.* (2005), pelos quais a adição de surfactantes favorece a produção de bacteriocinas, que, para este experimento, evidenciou o valor de 1.000 AU/mL.

Figura 9. Curva de crescimento de *B. lactis* em caldo MRS suplementado com Tween 80 1% (p/v).

A adição de inulina favoreceu a produção de biomassa (Figura 10), gerando a velocidade específica de crescimento de 0,39/h e o tempo de geração de 1,74/h; ou seja, valores melhores que os reportados pela adição de Tween 80. Em particular, apresentou maior produção de biomassa em relação ao meio de cultura suplementado com Tween 80, após 24 h de cultivo (8,9 Log UFC/mL). Em contrapartida, a atividade inibitória não mostrou aumento da ação bactericida, ao

contrário, apresentou diminuição dessa atividade (800 AU/mL). Isto se deve ao fato das condições de produção e crescimento de *B. lactis* carecerem de fatores de estresse (estresse térmico, oxidativo, osmótico ou mecânico) e, portanto, não induzirem nenhum processo de defesa ou resposta a condições adversas (IBRAHIM & SALAMEH, 2001). Dessa forma, seria um processo que levaria ao gasto desnecessário de energia para o microrganismo (RILEY & WERTZ, 2002).

Figura 10. Curva de crescimento de *B. lactis* em caldo MRS suplementado com Inulina 1% (p/v).

Tabela 7. Velocidades específicas de crescimento (μ_m) de *B. lactis* e tempo de geração (t_g) utilizando caldo MRS em presença de inulina, Tween 80 ou extrato de levedura.

Meio de cultura*	μ_m/h	Tg (h)
MRS + YE	0,465	1,505
MRS + I	0,399	1,734
MRS + T ₈₀	0,353	1,953

* MRS (caldo *Man, Rogosa and Sharpe*); YE (extrato de levedura); I (inulina) e T₈₀ (Tween 80)

4.4 Ensaios com caldo BSM

A fim de otimizar os parâmetros iniciais de crescimento em meio sintético, foi realizado um novo estudo com as variáveis e as condições previamente descritas no caldo BSM. Os resultados obtidos demonstraram que a adição de 1 % de extrato de levedura ou inulina favoreceu o crescimento e a formação de biomassa de *B. lactis*, enquanto que a adição de Tween 80 apresentou diminuição na formação de biomassa, com relação à curva controle, ou seja, na ausência desses ingredientes (Figura 11).

Figura 11. Curva de crescimento de *B. lactis* em caldo BSM.

A curva de crescimento controle apresentou fase exponencial entre 0 e 20 h, com posterior fase estacionária. Tanto o valor de pH, como o teor de glicose residual foram diminuindo com relação à formação de biomassa e metabólitos primários, destacando-se a formação de ácido acético com concentração final de 4,3 mM. Os resultados obtidos por Dubey e Mistry (1996) foram inferiores em relação ao presente trabalho, sendo que os autores obtiveram concentrações finais de ácido acético de 22,6 e 21mM com lactulose e fructooligosacarídeos (FOS) como fontes de

carbono (DUBEY & MISTRY, 1996). O valor de pH obtido no presente trabalho foi de 5,01, apresentando menor acidez que os obtidos com caldo MRS e leite desnatado (pH 4,41 e 4,5), respectivamente. A rápida acidificação no meio BSM provavelmente deve-se à alta formação de ácido lático, induzida pelo rápido consumo da fonte de carbono facilmente metabolizável (MEULEN *et al.*, 2006). O consumo de ácido pirúvico e a formação de ácido acético, observados nas figuras 11 – 14, devem-se, principalmente, à dissipação do piruvato, sendo que 50% dele são transformados em acetil-CoA e finalmente em ácido acético a partir das enzimas (1) piruvato formato liase (pfl); (2) fosfotranscetilase (pta) e acetato quinase (ack) e; (3) acetaldeído desidrogenase (adh) (MEULEN *et al.*, 2006). A preferência biológica do processo é devido à produção de ácido acético, a qual auxilia a manter a formação de NAD^+ , sendo este o caminho com menor perda de energia e maior estabilidade do sistema redox intracelular. Estudos prévios afirmam que o lento consumo da fonte de carbono leva à elevada formação de ácido acético, fórmico ou etanol e menos formação de ácido láctico (CASTILLO *et al.*, 2013; MEULEN *et al.*, 2006).

Figura 12. Curva de crescimento de *B. lactis* em caldo BSM com inulina 1% (p/v).

A Figura 12 representa o comportamento exercido pela adição de inulina na formação e no crescimento de *B. lactis*. Em particular, a curva de crescimento apresentou uma fase exponencial entre 0 até 24 h, com valor de pH de 4,94, sendo este maior que o resultado obtido em MRS (pH 4,3). A formação de ácido acético foi 10% maior com relação à curva controle (caldo BSM sem adição de ingredientes) e levemente maior com relação ao extrato de levedura no meio de cultivo BSM (Figura 13). Estudos prévios sustentam o correspondente incremento na formação de ácido acético a partir de oligossacarídeos, como inulina e lactulose (OLIVEIRA *et al.*, 2012). Nos resultados do presente trabalho, houve o incremento de 15% na formação de ácido acético a partir da suplementação do meio de cultura com inulina 1% (p/v). Outros estudos demonstram alta formação de ácido acético, na ordem de 36mM, como concentração máxima, e 7,7mM, como concentração mínima (OLIVEIRA *et al.*, 2012), por parte de *B. animalis* subsp. *lactis* com pH finais de 4,1 - 4,0. Já que a liberação de ácidos referenciada é consideravelmente maior que dos resultados obtidos neste trabalho (4,5mM com pH 4,9) podemos evidenciar uma alta divergência na produção de ácidos a partir de *B. lactis* (MAKRAS & DE VUYST, 2006).

Figura 13. Curva de crescimento de *B. lactis* em caldo BSM suplementado com extrato de levedura 1% (p/v).

O cultivo realizado no caldo BSM suplementado com extrato de levedura foi o que apresentou maior taxa de crescimento, com fase exponencial constante até a 24 h de cultivo e pico máximo de crescimento de 1,9 (600nm DO), sendo superior até em 20%, quando comparado com outras curvas obtidas com caldo BSM e seus respectivos suplementos (controle, inulina e Tween 80). A acidificação final em caldo BSM foi menor, sendo esta ao redor de pH 5,0, maior que as obtidas no caldo MRS (pH 4,2).

Figura 14. Curva de *B. lactis* em caldo BSM suplementado com Tween 80 1%(p/v).

A Figura 14 representa o comportamento obtido pela adição de Tween 80 ao meio BSM, na formação de biomassa e produtos primários. Neste caso, a formação de biomassa, como a dos ácidos acético e pirúvico, foi similar às obtidas na curva controle (meio BSM sem adição dos ingredientes). Contudo, apresentou menor formação de biomassa que as obtidas por inulina ou extrato de levedura, na ordem de 20%, em relação ao extrato de levedura, e 5%, em relação à inulina.

4.5 Determinação de concentração de lactose

Objetivando a produção de bacteriocina a partir de leite desnatado, estudaram-se diferentes concentrações deste substrato, avaliando a constante de saturação e a velocidade de crescimento nessas condições (Tabela 8), através da constante de saturação obtida da equação de Monod (equação 6).

$$\mu = \frac{\mu_{\max} \times S}{K_S + S} \quad \text{(Equação 6)}$$

Tabela 8. Valores de velocidades específicas de crescimento (μ_m), obtidas das fermentações com diferentes concentrações iniciais de sólidos totais de leite desnatado.

S_i (g/L)	μ_m /(h)
0	0
15,0	0,496
15,0	0,601
25,0	0,629
35,0	0,635
40,0	0,650

Figura 15. Efeito das diferentes concentrações de sólidos totais de leite desnatado (g/L) na velocidade específica de crescimento.

Para a determinação da constante de saturação e da velocidade máxima de crescimento, foram utilizados diferentes concentrações de leite desnatado e suas respectivas velocidades específicas de crescimento (Figura 16). Posteriormente, foi realizada a linearização de Lineweaver-Burk, para determinar os parâmetros cinéticos anteriormente descritos (Figuras 15 e 16).

Figura 16. Linearização de Lineweaver-Burk.

$$y = 10.54x + 1.23$$

$$R^2 = 0.844$$

(Equação 7)

A constante de saturação (K_s) é a concentração do nutriente limitante para o taxa de crescimento, que corresponde a metade do valor da taxa de crescimento máxima. De fato, essa constante representa a afinidade do organismo quanto ao nutriente. Os valores de μ_m e K_s dependem não só do organismo e do nutriente limitante no meio de fermentação, mas também de fatores como temperatura e

pH. Desta maneira, determinou-se o valor de velocidade máxima de crescimento de 0,81/h e uma constante de saturação de 8,6 - 10 g/L.

Esses resultados permitiram aperfeiçoar a concentração adequada de leite desnatado para os cultivos, tirando a perda de carga orgânica, ou seja, diminuindo o desperdício de substrato e obtendo valores maiores de crescimento.

4.6. Efeito de indutores no crescimento celular de *B. lactis* e na produção de bacteriocina em caldo leite desnatado (10 g/L)

De modo geral, os ensaios com leite desnatado apresentaram maiores velocidades específicas de crescimento, maior concentração máxima de biomassa e menores tempos de geração (Tabela 9), quando comparados com os valores obtidos nos ensaios com caldos MRS e BSM. Esses resultados podem ser explicados devido as moléculas de caseína, que funcionam como indutoras de crescimento para as cepas de *Bifidobacterium* spp. (DUBEY & MISTRY, 1996). Conforme relatado por Ballongue (2004), a presença de pontes bissulfeto nas moléculas de α -caseína exerce forte ação indutora no crescimento. Em contrapartida, outros autores sustentam que meios somente baseados em leite não oferecem condições ótimas de crescimento para *Bifidobacterium* spp. (GOMES & MALCATA, 1999; JANER *et al.*, 2004).

Tabela 9. Velocidades específicas de crescimento (μ_m) de *B. lactis* e tempo de geração (t_g) em leite desnatado em presença de inulina, Tween 80 ou extrato de levedura.

Meios de cultura*	μ_m/h	tg (h)
LD + YE	0,60	1,15
LD + I	0,49	1,41
LD + T ₈₀	0,44	1,56

* LD (leite desnatado); YE (extrato de levedura); I (inulina) e T80 (Tween 80)

Na figura 17, pode-se evidenciar que a adição de extrato de levedura induz à maior velocidade específica de crescimento e ao menor tempo de geração, sendo

estes valores, respectivamente, 0,60 h e 1,15 h. Estes resultados estão de acordo com os obtidos por vários autores, que afirmaram que houve forte indução no crescimento de bifidobactérias causado pelo extrato de levedura (BALLONGUE, 2004; KIVIHARJU *et al.*, 2005; UELIVON, 2006). Pode-se notar que a produção de bacteriocina foi igualmente estimulada, gerando o valor de 2.000 AU/mL na inibição de *L. monocytogenes*.

Figura 17. Curva de crescimento de *B. lactis* em leite desnatado suplementado com extrato de levedura.

Nos ensaios nos quais o leite desnatado foi suplementado com inulina (Figura 18), pode-se observar que μ_m foi igual a 0,49/h e t_g apresentou valor de 1,41 h (Tabela 9). Portanto, a adição de inulina aumentou, aproximadamente, 10% a velocidade específica de crescimento (0,44/h) em relação ao leite desnatado suplementado com Tween 80. Os resultados de atividade foram maiores em comparação às fermentações com caldo MRS, gerando valores de 1.800 AU/mL e 1.600 AU/mL para os meios com Tween 80 e inulina, respectivamente.

Nos valores de pH apresentados nas Figuras 17, 18 e 19, correspondentes aos dados com leite desnatado, pode-se observar a queda de pH de quase 2 unidades. Estes valores foram mantidos ao redor de (pH 4,5–5,0) valor correspondente à condição ótima de crescimento e estabilidade para as cepas de *Bifidobacterium* spp (variação de pH entre 4.5-5.0) (GOMES & MALCATA, 1999).

Figura 18. Curva de crescimento de *B. lactis* em leite desnatado com Inulina 1% (p/v).

A Figura 19 representa a influência de Tween 80 no crescimento de *B. lactis* e a concomitante produção de bacteriocina, exibindo um rápido consumo de glicose (na hora 10-15), sendo relativamente maior que os outros experimentos (Figura 17 e 18). Este efeito se deve, principalmente, ao fato de que o Tween 80 não representa nenhuma fonte de carbono metabolizável para *B. lactis*, diferentemente da inulina ou do extrato de levedura que apresentam além da glicose, outras fontes de carbono e nitrogênio.

Figura 19. Curva de crescimento de *B. lactis* em leite desnatado com Tween 80 1% (p/v).

4.7. O método de pré-difusão em placa

Visando à otimização da produção, da obtenção ou da identificação da bifidobacteriocina, foram realizados três diferentes testes. Os testes foram desenvolvidos com a melhor condição de crescimento (leite desnatado 10g/L com extrato de levedura a 1% (p/v)). A primeira condição estudada foi a modificação do protocolo de determinação da bacteriocina contra o microrganismo indicador, a partir da retenção do tempo de crescimento do microrganismo indicador (esfriamento), permitindo, assim, maior tempo de difusão para a bacteriocina. Este método mostrou ser efetivo, pois aumentou em até 10% o valor da atividade inibitória da bifidobacteriocina – resultados similares foram obtidos por Pongtharangkul & Demirci (2004a).

No segundo teste, no qual foi utilizado o Tween 20 (surfactante), foi sutilmente favorecida a ação bactericida da bifidobacteriocina, devido o aumento da difusão

em placa pela eliminação da tensão superficial, que, por outro lado, pode influenciar na remoção de camadas lipídicas interferentes na ação da bacteriocina (IBRAHIM & SALAMEH, 2001).

O terceiro e último teste objetivou testar as características antigênicas dos microrganismos competidores, verificando se poderiam causar algum efeito na produção de bacteriocina de *B. lactis*. O resultado do efeito obtido foi praticamente nulo (TOURE *et al.*, 2003).

Tabela 10. Métodos alternados na otimização da produção da BLIS.

Otimização da atividade microbiana da substância BLIS	Atividade inibitória da substância BLIS (AU/mL)
Controle. Convencional método de determinação enzimática. Descrito no item 3.4.5	2.000
Pre-difusão: Depois de aplicar os 20µL da suspensão da BLIS nas placas, estas foram armazenadas a 4°C por 12h e finalmente incubadas a 37°C por 24h.	2.130
Surfactantes: Adição de Tween 20 no BHI agar-soft	2.040
Condições de estresse: Adição de células de <i>L. monocytogenes</i> e <i>E. faecalis</i> lisadas pelo método de choque térmico às 12h de cultura	2.000

4.8. Delineamento central composto

Dado que o processo apresentou complicações iniciais na produção ou na determinação de BLIS, o trabalho foi encaminhado na otimização, a partir de suplementos e condições de crescimento de *B. lactis*. Portanto, foi esquematizado o processo a partir das condições encontradas na condução do trabalho (Figura 20).

Figura 20. Esquema do processo de otimização na produção de BLIS.

O delineamento central composto permitiu avaliar os efeitos lineares e quadráticos dos dois efeitos e a interação dos mesmos: Temperatura (34/ 37/ 40 °C) e Extrato de levedura (0,5/1,0/1,5 g/L). A superfície de resposta permitiu visualizar melhor a interação das variáveis e encontrar a condição ótima na produção de BLIS. O R^2 , o Valor- P e a falta de ajuste determinaram a significância do modelo, expresso na seguinte equação:

$$y = b_0 + b_1x_1 + b_2x_2 + b_{11}x_{11}^2 + b_{22}x_{22}^2 + b_{12}x_1x_2$$

Devido à grande variabilidade inerente na produção de bacteriocina, foram considerados significativos os parâmetros com p-valor menor de 5 % ($p < 0,05$) e com R^2 superior de 0,75 (indica idoneidade do modelo), o que permitiu determinar os fatores extrato de levedura e temperatura como estatisticamente significativos, com variação explicada (R^2) de 91,0; porém, a falta de ajuste foi significativa para o modelo, o que implica pouca assertividade das interações do modelo, como podemos observar na Tabela 11.

Tabela 11. Análise de variância

	df	Somas de quadrados	F	p-valor
Temperatura (L)	1	0,2929	0,879	0,447517
Temperatura (Q)	1	49,6898	149,069	0,006642
E. Levedura (L)	1	357,3943	1072,183	0,000931
E. Levedura (Q)	1	0,4448	1,335	0,367372
T°C x E. Lev	1	0,5000	1,500	0,345346
Falta de ajuste	5	8,2661	24,798	0,039214
Error Puro	2	0,3333		
Total SS	16			

Se o efeito das interações não for significativo, se pode interpretar isoladamente cada efeito principal (RODRIGUES & IEMMA, 2005). Portanto, se pode determinar a importância implícita da cada variável separadamente, como, por exemplo, a influência da adição de extrato de levedura e da temperatura na variável resposta (atividade da bacteriocina), conforme apresentado na Tabela 12 e na Figura 21.

Tabela 12. Planejamento experimental e resultados do delineamento composto central para a produção de bacteriocina.

Ensaio	Variáveis		pH	Atividade antimicrobiana (Zona de inibição em mm)	
	Temperatura °C	YE g/L			
	(X ₁)	(X ₂)			
1	37,0	0,15	5x10 ⁹	4,06	0
2	40,0	0,5	7x10 ¹⁰	4	0
3	42,0	1,0	1,5x10 ⁹	4,27	0
4	40,0	1,5	4,3x10 ¹⁰	3,98	10
5	34,0	0,5	2,3 x10 ⁸	4,6	0
6*	37,0	1,0	4,2 x10 ⁹	4,21	8
7	40,0	1,5	9 x10 ⁹	4,04	13
8	40,0	0,5	4,2 x10 ¹⁰	4,15	0
9	31,9	1,0	1 x10 ⁶	4,06	0
10*	37,0	1,0	5,1 x10 ⁹	4,14	8
11	34,0	1,5	1,5 x10 ⁹	4,27	14
12	34,0	1,5	2,3 x10 ⁹	4,2	11
13*	37,0	1,0	7,9 x10 ⁸	3,95	4
14	37,0	1,8	4,5 x10 ¹⁰	4,08	13
15*	37,0	1,0	3,9 x10 ⁹	4,14	7
16	34,0	0,5	7,2 x10 ⁸	5,05	0
17*	37,0	1,0	4,5 x10 ⁹	4,09	4

*: Pontos centrais do planejamento experimental.

A aplicação da RSM deu a seguinte equação de regressão, que é uma relação empírica entre a atividade da bacteriocina e as variáveis do experimento nas unidades codificadas, com condição ótima do modelo definida em 2 g/L de YE e 36°C de temperatura.

$$y = -437,06 + 17,38 T^{\circ}C + 32,89 YE - 0,233T^{\circ}C + 0,7595YE - 0,167T^{\circ}C.YE$$

Figura 21. Superfície de resposta relativa à atividade da bacteriocina estimada pela zona de inibição em função de concentração de YE e Temperatura °C.

Como se pode observar pelos resultados apresentados na Tabela 12 e na Figura 21, os melhores resultados da atividade antimicrobiana foram obtidos nos ensaios 7, 11 e 14, com intervalos de temperatura de 40°C, 34°C e 37°C, respectivamente, e com altas concentrações de YE (1,5 e 1,8 g/L), sendo a concentração de YE diretamente proporcional com a produção de bacteriocina. O aumento de biomassa obtido com a adição de extrato de levedura deve-se ao aumento da fonte de vitaminas, minerais, ácidos nucleicos e, principalmente, alguns peptídeos que aceleram a atividade da β -galactosidase até em 10%, o que levaria ao crescimento acelerado do microrganismo e ao aumento da população de *B. lactis* (AVONTS *et al.*, 2010; JALILI *et al.*, 2010). O presente trabalho demonstrou proporcionalidade entre o aumento do YE e o aumento da biomassa (Tabela 12) na ordem de $1,5 \times 10^9$ – $4,5 \times 10^{10}$ UFC/mL (Ensaio 11 e 14), onde pode-se observar uma maior produção de bacteriocina e na atividade da mesma, alcançando valores de 2.600 e 2.800 AU/mL.

A formação de biomassa apresentou tendência positiva com relação às altas temperaturas, alcançando seu ponto máximo em 40°C, na ordem de $4,2$ e $4,3 \times 10^{10}$ UFC/ML. Por outro lado se obteve $3,5 \times 10^{10}$ UFC/mL, sob 37°C com 1,8 g/L de YE – o que representa temperaturas dentro da faixa ótima de crescimento para *B. lactis* (GOMES & MALCATA, 1999). Contudo, houve atividade da

bacteriocina nos ensaios 11 e 12 (34°C) (Tabela 12), na ordem de 2.800 e 2.260 AU/mL, o que pode significar condições de estresse para *B. lactis*, repercutindo na produção da BLIS (condições não esclarecidas), mas que apresentam concordância com prévias investigações, nas quais baixas temperaturas induziram a produção de bacteriocinas para três diferentes cepas de *Enterococcus* (RAYKOVA *et al.*, 2008). As condições de temperatura ótimas para produção de bacteriocinas não necessariamente coincidem com as temperaturas ótimas de crescimento do microrganismo (LEROY *et al.*, 2003). Em termos moleculares, parece que a temperatura afeta o mecanismo de resposta da feromona sinalizadora na produção de bacteriocinas, repercutindo na biossíntese dos seus pré-peptídeos (DIEP *et al.*, 2000).

Os resultados obtidos no presente estudo corroboram os de diversos pesquisadores (AVONTS *et al.*, 2004; DIEP *et al.*, 2004; KIVIHARJU *et al.*, 2005; RAYKOVA *et al.*, 2008), os quais demonstraram forte influência do extrato de levedura e da temperatura na produção de bacteriocinas ou substâncias BLIS. O valor de atividade da bacteriocina, apresentado na Tabela 13, representou o aumento de 800 a 1.100 unidades arbitrárias nas condições ótimas de produção, representando assertividade do planejamento estatístico na otimização do processo, passando a se obter de 3.000 a 3.100 unidades arbitrárias. Observa-se também o incremento no rendimento (Tabela 13) e na produção de biomassa.

4.9. Cultivos em fermentador

A partir das condições delineadas pelo planejamento experimental, foi possível trabalhar com as condições ótimas para produção de BLIS, com maiores volumes de cultivo e condições com melhor controle operacional, através do uso do biorreator de bancada. O comportamento das fermentações está representado na Figura 22.

Figura 22. Curva de crescimento de *B. lactis* em leite desnatado utilizando fermentador de bancada. Biomassa (UFC/mL) (—●—), pH (—▲—), Lactose g/L (—■—) e ácidos orgânicos (—▼—). Representação da atividade da BLIS a partir dos histogramas.

Os estudos em fermentador permitiram obter um considerável aumento na formação de biomassa 10^{13} UFC/mL, de até três unidades logarítmicas, quando comparadas com as melhores condições em *shaker* (10^{10} UFC/mL). O consumo de lactose e a formação de ácidos orgânicos (lático, acético, succínico e pirúvico) foram inversamente proporcionais e refletem tanto a formação de biomassa como a queda de pH. Entre 12 e 24 h de cultivo, pode-se observar rápida formação de ácidos e acelerado consumo da lactose, enquanto que entre 27 e 48 (h) pode-se observar uma fase estacionária com pouca quantidade de lactose residual (1,5 g/L) e pouca formação de ácidos orgânicos (2,9 a 1,0 g/L). A produção de BLIS foi testada nos tempos determinados (22, 24 e 26 h) de cultivo e os valores obtidos de atividade antimicrobiana foram entre 3.000 e 3.100 AU/mL, correspondentes aos obtidos no plenajamento experimental.

Figura 23. Fotografia de placa utilizada no ensaio para determinação da atividade inibitória contra *L. monocytogenes* a partir da substância BLIS obtida no cultivo em biorreator.

4.10. Determinação da atividade da BLIS (AU/mL) pelo ensaio de diluição crítica

A determinação da atividade da BLIS foi realizada no final dos experimentos, com as condições de produção otimizadas. A presença de halos foi identificada até a quinta diluição (D), com fator de diluição de duas vezes (n) e com 20µL de volume depositado na placa (P) contra o microrganismo indicador (*L. monocytogenes*); a equação, apresentada previamente na seção 4.4.5, para determinação da atividade da bacteriocina foi expressa na seguinte forma:

$$\frac{AU}{mL} = \frac{D^n \times 1000}{P} = \frac{2^5 \times 1000}{20}$$

Portanto, o resultado de atividade foi 1.600 AU/mL de inibição máxima encontrada contra *L. monocytogenes* ATCC 13932.

Figura 24. Foto da atividade da BLIS contra *L. monocytogenes* no teste de diluição crítica.

4.11. Atividade da bacteriocina

Em relação à produção de BLIS, a cepa utilizada no presente estudo (*B. lactis* HN019) produziu a quantidade de substância foi significativa, quando comparada com outros estudos (CHEIKHYOUSSEF *et al.*, 2010; UELIVON, 2006; YILDIRIM & JOHNSON, 1998), portanto, esta bactéria láctica e probiótica mostrou-se ser bacteriocinogênica. A atividade da BLIS foi de aproximadamente 1.000 AU/mL, utilizando o caldo MRS suplementado com Tween 80, 800 AU/mL, quando utilizado caldo MRS suplementado com Inulina, e 1.200 AU/mL no caldo MRS com extrato de levedura. Quando utilizou-se o leite desnatado, como meio de cultivo natural, os resultados foram maiores quando comparados com o caldo MRS, ou seja, 1.600 AU/mL no leite desnatado suplementado com inulina, 1.800 AU/mL no leite desnatado suplementado com Tween 80 e 2.000 UA/mL no leite desnatado suplementado com extrato de levedura. De fato, a análise estatística ANOVA revelou diferenças significativas entre todos os resultados de atividade antibacterial (Tabela 11). Nota-se que os valores de atividades apresentados no presente estudo foram discrepantes quando comparados com outros estudos. Em particular, nos trabalhos de Yildirim *et al.* (1999) e Cheikhyyoussef *et al.* (2010), os valores de atividade de Bifidocin B variaram de 285 AU/mL a 540.000 AU/mL e os de Bifidin I de 263 a 365.714 AU/mL, respectivamente. Vale ressaltar que as espécies de bifidobactérias

usadas por esses pesquisadores foram diferentes, sem prévio conhecimento do *screening* feito na seleção dessas cepas (CHEIKHYOUSSEF *et al.*, 2010).

A partir do Test de Tukey, representado na Figura 25, pode-se distinguir os diferentes grupos avaliados a partir de sua atividade, ou seja, no primeiro grupo (A) encontra-se o produto obtido pela otimização do processo, o qual foi esquematizado na Figura 20. É importante salientar que o único microrganismo bioindicador, no qual o *B. lactis* expressou essas atividades antimicrobianas, nos diferentes meios de cultura utilizados no presente trabalho, foi a *L. monocytogenes*. Esse comportamento pode ser explicado porque esse peptídeo antimicrobiano (bacteriocina) apresenta, na sua estrutura, uma sequência homóloga às outras bacteriocinas de classe IIa, identificada na região N-terminal de Bifidocin B e Bifidin I, com a seguinte sequência de aminoácidos Lys-Tyr-Tyr-Gly-Asn-Gly (CHEIKHYOUSSEF *et al.*, 2010; YILDIRIM *et al.*, 1999). Essa região parece influir na ligação e no modo de ação da bacteriocina sobre *L. monocytogenes*, sendo sempre o microrganismo indicador para este tipo de bacteriocinas (DRIDER *et al.*, 2006).

Figura 25. Atividade antimicrobiana da bacteriocina produzida por *B. lactis* utilizando o caldo MRS suplementado com Tween 80 (MRS + T₈₀), inulina (MRS + I) e extrato de levedura (MRS + YE) e o leite desnatado suplementado com Tween 80 (LD + T₈₀), inulina (LD + I) e extrato de levedura (LD + YE). Diferentes letras nos histogramas significam que há diferença estatística entre os valores ($p < 0,05$).

Os resultados obtidos de velocidade específica também apresentaram diferenças estatísticas entre os diferentes meios de cultivo e os ingredientes adicionados, exceto os cultivos de leite desnatado utilizando tanto o método de pré-difusão em placa como o de adição de Tween 20. De acordo com Hopkins *et al* (1998), a velocidade específica de *B. lactis* atingiu valores entre 0,07 e 0,57 h⁻¹, quando utilizados diferentes tipos de açúcares, como galactooligosacarídeos e glicose, sendo portanto, similares ao obtidos no presente trabalho. Por outro lado, Rada *et al* (2002) reportaram valores de velocidades específicas de 0,58 até 0,80 h⁻¹, quando usados lactulose e glicose como suplementos.

Figura 26. Velocidade específica de *B. lactis* utilizando o caldo MRS suplementado com Tween 80 (MRS + T₈₀), inulina (MRS + I) e extrato de levedura (MRS + YE) e o leite desnatado suplementado com Tween 80 (LD + T₈₀), inulina (LD + I) e extrato de levedura (LD + YE). Diferentes letras nos histogramas significam que há diferença estatística entre os valores ($p < 0,05$).

4.12. Parâmetros cinéticos

A Tabela 13 resume os valores de rendimento, produção e velocidades de crescimento. Os resultados obtidos de velocidades específicas são similares aos reportados por Kiviharju *et al* (2005), Chou & Hou (2000) e Gibson & Wang (1994b), que fizeram determinações cinéticas no crescimento e na respectiva influência de diversas fontes nutricionais para *Bifidobacterium spp.*

Tabela 13. Parâmetros cinéticos de produção de bacteriocina e crescimento de *B. lactis*.

Condição de cultivo*	Fator de conversão de substrato em células ($Y_{x/s}$) (g/g)	Máxima produção da bacteriocina (AU/mL)	Velocidade específica de crescimento ($\mu\text{m/h}$)	Tempo de geração (tg (h))
MRS + YE	0,38	1200	0,465	1,953
MRS + T ₈₀	0,44	1000	0,353	1,505
MRS + I	0,39	800	0,399	1,734
LD + T ₈₀	0,13	1800	0,444	1,561
LD + I	0,24	1600	0,492	1,407
LD + YE	0,27	2000	0,604	1,146
LD + E ₇	0,71	2600	ND	ND
LD + E ₁₁	0,63	2800	ND	ND
LD + E ₁₄	0,75	2600	ND	ND
F	0,75	3000	1,128	0,62

* MRS (caldo Man, Rogosa and Sharpe); LD (leite desnatado); YE (extrato de levedura); I (inulina); T₈₀ (Tween 80); E₇, E₁₁ e E₁₄ (ensaios 7, 11 e 14) e F (cultivo em fermentador de bancada).

5. CONCLUSÃO

A condição que proporcionou maior crescimento celular e produção de bacteriocina foi o processo fermentativo no qual se utilizou leite desnatado (10 g/L de sólidos totais) suplementado com extrato de levedura 2% (p/v), utilizando temperatura ótima do intervalo 36 a 40°C, combinado com o método de pré-difusão em placa (resfriamento). O processo global permitiu constatar a presença de substâncias BLIS com valor de 3.000 AU/mL (Figura 23 e 24). Por outro lado, tanto a adição de inulina como a de Tween 80 (surfactante) favoreceram o crescimento celular e a expressão da bacteriocina (bifidobacteriocina).

De fato, a bacteriocina produzida por *B. lactis* mostrou-se eficaz contra *L. monocytogenes*, sendo este um patógeno de alta incidência no mundo e de difícil controle. Tanto a adição de extrato de levedura como a temperatura influenciaram estatisticamente na produção de substâncias BLIS. A partir da anterior formulação do meio de cultivo e das condições, poderão ser desenvolvidos, futuramente, estudos de determinação, separação, purificação e identificação da substância BLIS, com a finalidade de futura comercialização do produto.

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7. ANEXOS

7.1 - Artigos publicados

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; Cheikhyssef 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; Cheikhyssef 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; Cheikhoussef 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 (Cheikhoussef et al., 2009b; Gibson and Wang, 1994b; Gomes and Malcata, 1999; Ibrahim and Salameh, 2001) and, more specifically, bacteriocins (Anand et al., 1984, 1985; Cheikhoussef 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 (Cheikhoussef 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; Meghrou 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, Meghrou 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) (Cheikhoussef 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	<i>B. bifidum</i> 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	<i>B. bifidum</i> NCFB 1454	3.3	(121 °C–15 min)	2–12	(12–18 h)	37 °C, pH 5.0–6.0	<i>Bacillus cereus</i> , <i>Enterococcus faecalis</i> , <i>Listeria monocytogenes</i> , <i>Pediococcus acidolactici</i> , <i>Streptococcus faecalis</i> , etc.	Yildirim and Johnson (1998); Yildirim et al. (1999)
Bifilong	<i>B. longum</i>	120	(100 °C–30 min)	2.5–5.0	(–)	(–)	Gram-positive and Gram-negative bacteria	Kang et al. (1989)
Bifilact Bb-46	<i>B. longum</i> Bb-46	25–127	(121 °C–15 min)	4–7	(–)	(–)	<i>Staphylococcus aureus</i> , <i>Salmonella typhimurium</i> , <i>Bacillus cereus</i> , <i>E. coli</i>	Saleh and El-Sayed (2004)
Bifilact Bb-12	<i>B. lactis</i> Bb-12	25–89	Unstable for high temperatures	4–7	(–)	(–)	<i>Staphylococcus aureus</i> , <i>Salmonella typhimurium</i> , <i>Bacillus cereus</i> , <i>E. coli</i>	Saleh and El-Sayed (2004)
Thermophilicin B67	<i>B. thermophilum</i> RBL67	5–6	(100 °C–5 min)	2–10	24 h	pH 6 and 40 °C	<i>Listeria sp.</i> , <i>Lactobacillus acidophilus</i>	von Ah (2006)
Bifidin I	<i>B. infantis</i> BCRC 14602	3	(121 °C–15 min)	4–10	18 h	(–)	LAB strains, <i>Staphylococcus</i> , <i>Bacillus</i> , <i>Streptococcus</i> , <i>Salmonella</i> , <i>Shigella</i> , <i>E. coli</i> .	Cheikhoussef et al. (2009a, 2010)
Lantibiotic (Bisin)	<i>B. longum</i> DJO10A	(–)	(–)	(–)	1–8 h	Auto-induction by crude lantibiotic	<i>Streptococcus thermophilus</i> ST403, <i>Clostridium perfringens</i> , <i>Staphylococcus epidermidis</i> , <i>Bacillus subtilis</i> , <i>Serratia marcescens</i> , <i>E. coli</i> 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 (Driener 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 (Cheikhoussef et al., 2009a; Yildirim et al., 1999). Bifidin I was purified in 2010 by Cheikhoussef 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 (Cheikhoussef 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* (Cheikhoussef 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) (Cheikhoussef 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	Cheikhoussef et al. (2010)
Bifidocin B	KYYGNGVTCGLHDCRVDRGKATCGIINNGGMWGDIG	36	Yildirim et al. (1999)
Bisin prepeptide	MSINEKSIVGESFEDLSAADMAMLTGRNDDGVAPASLSFAVSVLSVSFACSQTVTVTRLASCGNCK	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 (Cheikhoussef 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 $\text{pH} \leq 3$ 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 (Eijssink 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 (Cheikhoussef 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 (Eijssink 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) (Cheikhoussef 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), cyanocobalamin (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 bifidose, 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; Cheikhoussef 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 Cheikhoussef 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 ultra-filtration 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 (Cheikhoussef et al., 2009a). A new method has recently been developed by Cheikhoussef 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.

<i>Bifidobacterium</i> 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
<i>B. bifidum</i> NCFB 1454	Bifidocin B	ADPC	(–)	(–)	(–)	(–)	58,200	18	(–)	Yildirim and Johnson (1998)
<i>B. bifidum</i> 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	
<i>B. thermophilum</i> RBL67	Thermophilicins 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	
<i>B. infantis</i> BCRC 14602	Bifidin I	CFCs	500	80 × 10 ⁴	(–)	3040	263	1	100	Cheikhoussef et al. (2009b)
		FD	50	64 × 10 ⁴	(–)	2800	228	0.86	80	
		ADPC	5	51 × 10 ⁴	(–)	16.2	31,605	120	64	
<i>B. infantis</i> BCRC 14602	Bifidin I	NCFs	500	800,000	(–)	3040	263	1	100	Cheikhoussef 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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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 antitoxigenic 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*, *Pedococcus* 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) Type B (globular molecule)	Nisin, subtilin, epidermine Mersacidin
Class II	Heterogeneous class of small thermostable peptides	Subclass IIa (antilisterial-pediocin bacteriocins type) Subclass IIb (composed of two peptides) Subclass IIc (other bacteriocins)	Pediocin, enterocin, sakacin Plantaricin, lactacin F Lactococcin Helveticin J, millericin B
Class III	Large thermolabile peptides		

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 (LD₅₀) 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 LD₅₀ 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 antitoxigenic 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 bio-preservative 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 reuterin 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, Griffiths, 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 lactacin 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 (Rollemma, 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 (Klusken 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, Stora, 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, Verluysen, & 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-Garzon, 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	<i>Lactococcus lactis</i>	<i>Brochothrix thermosphacta</i>	Pork	3.5	Nattress, Yost, & Baker, 2001
Nisin	<i>L. lactis</i>	<i>Listeria monocytogenes</i>	Fermented milk	6.0	Benkerroum et al. (2002)
AcH Pediocin	<i>Lactobacillus plantarum</i>	<i>L. monocytogenes</i>	Cheese	1.0–2.0	Loessner et al., 2003
Enterocin	<i>Enterococcus faecium</i>	<i>L. monocytogenes</i>	Milk	2.0	Elotmani, Revol-Junelles, Assobhei, & Millière, 2002
Enterocin	<i>Enterococcus faecalis</i>	<i>Staphylococcus aureus</i>	Sausage	5.3	Ananou, Maqueda, Martínez-Bueno, Gálvez, & Valdivia, 2005
Nisin Z	<i>Lactococcus lactis lactis</i>	<i>S. aureus</i>	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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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.

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, & Vuilleumard, 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.

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

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

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* $\alpha(1-4)$ bonds forming chains of variable length, branched *via* $\alpha(1-6)$ 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

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

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

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).

Heterolactic fermentation

This process is characterized by the formation of co-products such as CO₂, 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 CO₂. Glyceraldehyde 3-phosphate enters the glycolysis through which it is transformed into lactic acid, while acetyl-phosphate is converted

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

into acetic acid and/or ethanol according to the overall equations:

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; Romani 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:

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 $\beta(1-4)$ 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

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

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

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

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

Among the bacteria that produce L(+) lactic acid are *L. amylophilus* (Yumoto & Ikeda, 1995), *L. brevis* and *L. buchneri* (Wu-Tai, Driehuis, & Van Wijkelaar, 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-Hägerdal, 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-Hägerdal, 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-Hägerdal, 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 (MgSO₄, MnSO₄ and FeSO₄) (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 co-factors 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

(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, Coccagn-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; Romani *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 & Vonkavesuk, 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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